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Expression Profiling and Functional Validation of MicroRNAs Involved in Schizophrenia and Bipolar Disorder

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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"Don't measure yourself by what you have accomplished, but by what you should have accomplished with your ability." --John Wooden

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Abstract

EXPRESSION PROFILING AND FUNCTIONAL VALIDATION OF MICRORNAs INVOLVED IN SCHIZOPHRENIA AND BIPOLAR DISORDER

By Albert H. Kim, B.S., M.S.

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MicroRNAs (miRNAs) are a family of small non-coding RNAs that regulate gene expression at both the mRNA and protein levels. MiRNAs have been shown to affect neuronal differentiation, synaptosomal complex localization and synapse plasticity, all functions thought to be disrupted in schizophrenia. We investigated the expression of 667 miRNAs (miRBase v.13) in the prefrontal cortex of individuals with schizophrenia (SZ, N = 35) and bipolar disorder (BP, N =35) using a real-time PCR-based Taqman Low Density Array (TLDA). After extensive QC steps, 441 miRNAs were included in the final analyses. At a FDR of 10%, 22 miRNAs were identified as differentially expressed between cases and controls, 7 dysregulated in SZ and 15 in BP. Using *in silico* target gene prediction programs, the 22miRNAs were found to target brain-specific genes contained within networks overrepresented for neurodevelopment, behavior, and SZ and BP disease development.

Given that miRNAs can bind to their targets with imperfect complementarity, computational prediction of true miRNA:mRNA interactions has been difficult and therefore, functional validation of miRNA:mRNA interactions has been relatively sparse. Thus, it was the goal of this study to demonstrate biological functionality of miRNAs on their targets by evaluating transcriptional and translational levels of gene expression(real-time PCR, western blot) as well as determining miRNA target-site specificity (luciferase reporter gene assays). We investigated two miRNAs, miR-132 and miR-137, both of which have been shown to regulate neuronal function and development, and are believed to be associated with schizophrenia from two distinct avenues of research, miR-132 from expression studies and miR-137 from genetic studies. We demonstrated miR-132 down-regulates *NTF3*, *DISC1*, and *GRIK5* at the transcript level and down-regulates *GRIK5* at the protein level as well. Furthermore, we demonstrated miR-137 down-regulates *TCF4*, *CACNA1C*, *CDK6*, *ANK3*, and *ZNF804A* at the transcript level, and down-regulates *TCF4*, *CACNA1C*, and *CDK6* at the protein level. Going further, we also demonstrated miR-137 binds specifically to target sites in the 3'-UTR of *CACNA1C, TCF4,* and *CDK6*, suggesting repression of these genes is directly mediated by miR-137. In total, this study provides strong evidence that miRNA dysregulation may contribute to schizophrenia pathogenesis.

Introduction

Schizophrenia and Bipolar Disorder

Diagnosis

Both schizophrenia (SZ, MIM 181500) and bipolar disorder (BP, MIM 125480) are common (lifetime prevalence of 0.5-1% and 0.8–2.6% respectively) and debilitating psychiatric illnesses posing a major burden on public health due to their early onset, non-fatal course, and for many patients, need for long-term care. According to DSM-IV-TR, three diagnostic criteria must be met to be diagnosed with schizophrenia (American Psychiatric Association, 2000)**:** 1) The presence of two or more characteristic symptoms for a significant portion of a one-month period including delusions, hallucinations, disorganized speech, grossly disorganized or catatonic behavior , and negative symptoms (blunted affect, alogia, avolition); 2) Social or occupational dysfunction for a significant portion of time since the onset of the disturbance; 3) Continuous signs of the disturbance persist for at least six months, including at least one month of symptoms. Bipolar disorder on the other hand, is a mood disorder that is divided into three specific subtypes according to DSM-IV-TR: 1) Bipolar I, which requires one or more manic episodes, 2) Bipolar II, no manic episodes but one or more hypomanic episodes and one or more major depressive episode, and 3) Cyclothymia, a history of hypomanic episodes with periods of depression that do not meet criteria for major depressive episodes.

Etiology

The etiology of SZ and BP is currently unknown, however several major hypotheses have emerged. One of the most influential theories of schizophrenia etiology has been the dopamine

hypothesis, which holds that schizophrenia (or psychosis more generally) is the result of a functional excess of dopamine (DA) somewhere in the human CNS. It is derived from the evidence that antipsychotic drugs antagonize dopamine binding and reduce psychotic symptoms[1, 2]. Like the dopamine hypothesis, the glutamate hypothesis developed from observing the effects of mind-altering drugs. It was shown that recreational use or investigator administration of a single low dose of an NMDA receptor antagonist such as phencyclidine (PCP) or ketamine produces "schizophrenia-like" symptoms in healthy individuals and profoundly exacerbates pre-existing symptoms in patients with schizophrenia[3, 4]. Finally, the neurodevelopmental hypothesis posits that abnormalities during development can lead to the activation of pathological neural circuits during adolescence or young adulthood, resulting in the emergence of positive and/or negative symptoms[5].

While these hypotheses are generally accepted, in conjunction with environmental and developmental factors[6-15] consistent evidence for a substantial genetic component[16, 17] has been shown (heritability ~80%), with some shared between both diseases[18-20]. Protein-coding genes have long been the focus of research in disease genetics, but efforts to identify replicable protein-coding risk loci in SZ[21, 22] and BP[23] remain elusive. Current genome-wide association (GWA) studies of ever increasing size have finally begun to identify and consistently replicate schizophrenia candidate genes[19, 21, 22]. Yet, for several of the best supported SZ susceptibility loci such as the human leukocyte antigen (HLA) genes in the major histocompatibility (MHC) region, zinc-finger 804A (ZNF804A), and transcription factor 4 (TCF4), genetic association does not appear to reflect DNA variants with any obvious effect on protein structure.

MicroRNAs (miRNAs)

Background

MiRNAs are a large family of small non-coding RNAs that negatively regulate gene expression at both the mRNA and protein levels[24, 25]. MiRNAs were first discovered in *C.elegans* when Ambros and colleagues observed that small RNA products from the heterochronic *lin-4* gene regulated *lin-14* gene expression by means of an RNA-RNA antisense pairing[26]. However, not having homologs in other species, this RNA-based regulatory mechanism was thought to be unique to *C.elegans* and it was not until the *let-7* miRNA was discovered with homologs in humans and *Drosophila*, did this new class of RNA molecules gain acceptance[27]. Since then, miRNAs have been shown to play key roles in diverse regulatory pathways[28], including hematopoietic cell differentiation, apoptosis, cell proliferation, and organ development, as well as being linked to a number of human diseases ranging from cancers ("oncomirs")[29, 30] to autism[31].

Genomics

MiRNA genes are estimated to comprise approximately one percent of the predicted genes in worms, flies, and humans[32]. To date, 1100 human miRNA genes are annotated in the miRNA registry[33], with over one-third of human genes being predicted targets. It was initially thought that most human miRNAs came from regions of the genome quite distant from previously annotated genes, implying that they are generated as independent transcriptional units. However, recent analyses of miRNA gene locations showed that the large majority of miRNA genes are located within intronic regions of protein coding genes[34] (Figure 1A), the expression of which largely coincides with the transcription of the host gene[35]. In this way,

regulatory scenarios are easy to imagine in which such coordinated expression could be useful. The remaining miRNA genes are found in intronic regions of non-coding transcriptional units, ~10%, and exons of protein coding transcriptional units, the processing of which is dependent on alternative splicing, ~10%(Figure 1B).

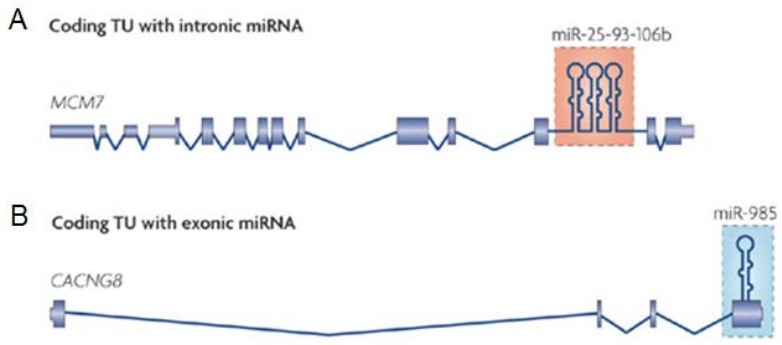


Figure 1: Genomic location of miRNAs. Adapted from Kim et al., *Nature Reviews Molecular Cell Biology,* 2009.

Processing

MiRNAs are initially transcribed by the normal cellular machinery to yield a large RNA transcript (~1000nt) containing an extended stem-loop structure called a primary miRNA or **pri-miRNA**. This pri-miRNA transcript can encode a single miRNA (monocistronic) or several miRNAs (polycistronic). Two subsequent processing events then lead to the functional, mature miRNA in animals. First, the RNase-III enzyme, Drosha, coupled with the dsRNA binding protein DGCR8 (DiGeorge Syndrome critical region 8), cleaves the **pri-miRNA** into a smaller (~70nt) hairpin precursor miRNA or **pre-miRNA**, which gets exported from the nucleus via a Ran-GTP dependent transporter. It is noteworthy that DGCR8 is one of several genes that is deleted in DiGeorge syndrome, the clinical manifestations of which range from schizophrenia to congenital heart defects[36]. It has also been shown that DGCR8 provides specificity for Drosha cleavage, without which non-specific cleavage and degradation of the pri-miRNA occurs[32]. Once in the cytoplasm, a second RNase-III enzyme, Dicer, cleaves the loop structure of the **pre-**

miRNA, resulting in a ~22bp double-stranded miRNA. Subsequent events concerning the dissociation of this duplex, degradation of the passenger strand, and incorporation of the active **mature** miRNA (~22nt) into the RNA-induced silencing complex (RISC) are largely unknown. However, studies suggest that the strand with the less thermodynamically stable 5' end gets incorporated into the RISC[37, 38]. This active RISC complex, consisting of the miRNA and Argonaute (Ago) proteins (Ago 1-4 in mammals), then directs either translational repression or site-specific cleavage of target mRNAs depending on the degree of complementarity to its target. Binding of miRNA–RISC to a partially complementary mRNA results in silencing through the inhibition of translation and subsequent degradation of mRNA by catabolism within processing bodies (P-bodies)[39]. On the other hand, direct cleavage or "slicing" of mRNA is solely catalyzed by Ago2[40] and requires perfect or near-perfect complementarity between the miRNA and the target[41]. (An overall schematic of miRNA processing is shown in Figure 2).

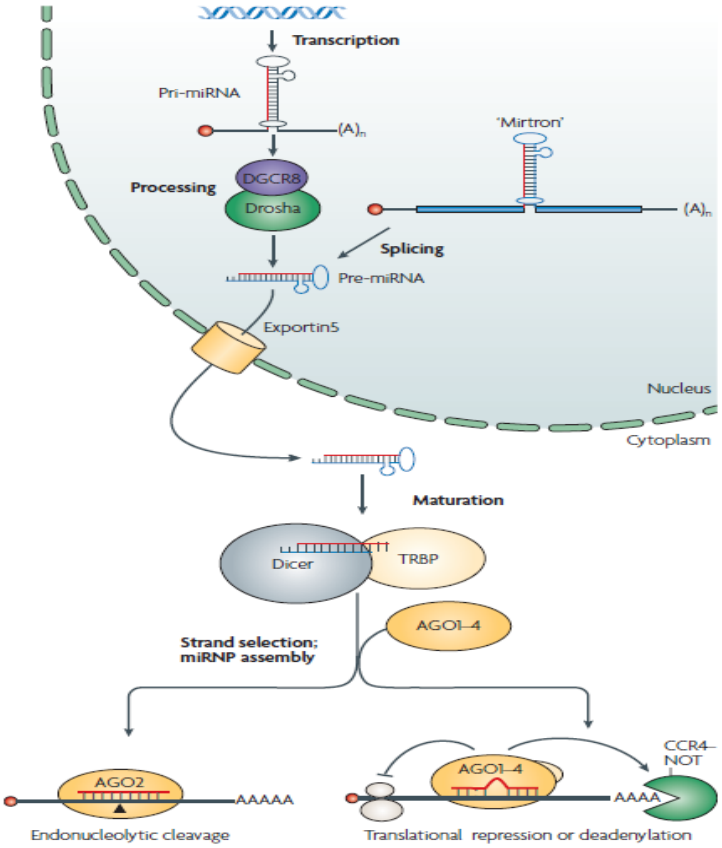


Figure 2: Schematic of miRNA processing. Adapted from Filipowicz et al., *Nature Reviews Genetics*, 2008.

Mechanism of miRNA binding

Most miRNAs in animals are thought to function through imperfect base-pairing to the 3'-untranslated region (3'-UTR) of the target mRNA[27, 42, 43]. The mismatch between a miRNA and its target site can take on different configurations, with a central unpaired bulge in either the miRNA or target mRNA strand, or both. Studies of how target site sequence and complementarity influence repression reveal that residues in the 5' portion of the miRNA (residues 2-8, the **"seed sequence"**) are most important[44, 45], with the degree of repression being dependent on the stability of pairing in this region. Other less common mechanisms that have been described include 1) **"canonical"** sites, which show good base-pairing at the 5' and 3' ends, and 2) **"3' compensatory"** sites which show weak 5' base-pairing and depend on a strong pairing at the 3' end. Given that miRNAs require only minimum binding by a short seed sequence, it is not surprising that microarray studies reveal that a single miRNA can target hundreds of messenger RNAs[46], suggesting that miRNAs can have pleiotropic effects on cellular processes. Most miRNAs control mRNA stability and translation by binding to sequence motifs in the 3'-UTR of target mRNAs[47, 48]. Although the majority of miRNA:mRNA interactions require perfect complementarity at the seed region, there are some exceptions to this rule, which complicates miRNA target predictions[49].

Principles of miRNA Target Prediction

Given that few miRNAs bind with perfect complementarity to their targets, much effort has been put toward devising a genome-wide computational search that captures most of the regulatory targets without bringing in too many false-positive predictions. Current prediction

methods are diverse, and all have room for improvement, however a general agreement has emerged on three important criteria[44, 50-52]. The first criterion is strong binding of the 5' seed sequence (nucleotides 2-7) of the mature miRNA to the 3'-UTR sequence of the target gene. The second criterion is assessing the thermodynamic properties of the miRNA:mRNA duplex by calculating the free-energy (ΔG) of the putative interaction, i.e. a lower ΔG indicating the binding of the miRNA to mRNA is stronger. Finally, the third criterion is conservation of the miRNA target site among different species. Several prediction programs are currently available that follow these major criteria, the more prominent or established being: miRanda[53], TargetScan[54], PicTar[55], PITA[56], RNAHybrid[57], RNA22[58], DIANA-MicroT[59], MicroInspector[60], miTarget[61], miRTarget2[62], and NBmiRTar[63]. All 11 of these algorithms has been compiled into a single database, miRecords[64], which allows the user to search all databases simultaneously.

miRNAs in Schizophrenia and Bipolar Disorder

A growing number of miRNAs are discovered every year and approximately two-thirds are expressed in brain[65] where they have been shown to be involved in maintaining brain function[66-69]. *In vitro* studies demonstrate that miRNAs are localized to the synaptosomal complex[70] where they affect neuronal differentiation[71] and modulate synapse plasticity[72], processes which have been implicated in SZ and BP[73, 74]. Thus, miRNAs are likely candidates in neurodegenerative disorders and studies have attempted to evaluate the impact of miRNAs in SZ etiology at both genetic[75-77] and expression[77-79] levels.

Expression Studies

Only a handful of miRNA expression studies in schizophrenia have been performed to date. Perkins[80] and co-workers were the first to broadly assess differential expression of

miRNAs in schizophrenia cases versus controls (Harvard Brain Bank). Using a custom miRNA microarray, they compared the expression of 264 human miRNAs in isolates from the post-mortem dorso-lateral prefrontal cortex (DLPFC) of individuals with schizophrenia (n=13), schizoaffective disorder (n=2), and healthy controls (n=21). The results showed 15 miRNAs were under-expressed (0.63-0.89-fold), and one over-expressed (1.77-fold) in cases compared to controls. Subsequent real-time PCR analysis confirmed 7 of the under-expressed miRNAs—miR-24, miR-26b, miR-29b, miR-30b, miR-30e, miR-92, and miR-195 ($p<0.05$).

Beveridge[81] and co-workers also used a custom microarray to analyze the expression of 76 miRNAs in the postmortem superior temporal gyrus (STG) of 21 matched pairs of schizophrenia and non-psychiatric controls. The results showed significant up-regulation of two miRNAs in cases, let-7g (1.8-fold) and miR-181b (2.8-fold). Taking this result forward, the authors used *in silico* prediction algorithms to predict gene targets for miR-181b and compared the list of putative targets to genes already shown in microarray experiments to be down-regulated in the same tissue. Two genes were of particular interest due to their previous implications in synaptic dysfunction—the calcium sensor gene visinin-like 1 (*VSNL1*) and the ionotropic AMPA glutamate receptor subunit (*GRIA2*). Both genes were shown to be suppressed with over-expression of miR-181b and were also shown to contain miR-181b target recognition elements in their 3'-UTRs via reporter gene assay. Furthermore, in a follow-up study[82], Beveridge and co-workers assessed miRNA expression in the STG and DLPFC of 21 matched cases and controls via microarray. The authors reported a schizophrenia-associated global elevation of miRNA expression which corresponded with an increase in the microprocessor component *DGCR8*. Several miRNAs were found to be up-regulated in both brain regions, the miR-15 family and miR-107 in the STG, and miR-181b and miR-16 in the DLPFC.

Genetic Studies

In contrast to miRNA expression studies, a number of genetic studies have been conducted looking at miRNA involvement in schizophrenia. Hansen[6] and co-workers studied the association between schizophrenia and genetic variants of miRNA genes using a case-control study design on three Scandinavian samples. Eighteen known SNPs within or near brain-expressed miRNAs in three samples (Danish, Swedish and Norwegian: 420/163/257 schizophrenia patients and 1006/177/293 control subjects), were analyzed. Subsequently, joint analysis of the three samples was performed on SNPs showing marginal association. Two SNPs rs17578796 in mir-206 and rs1700 in mir-198 showed nominal significant allelic association to schizophrenia in the Danish and Norwegian sample respectively ($p = 0.0021$ and $p = 0.038$), of which only rs17578796 was significant in the joint sample.

Feng[83] and co-workers examined hemizygous miRNAs on the X-chromosome in males so that the full phenotypic consequences of a variant could be assessed without the possible mitigating effects of a wild-type allele. 59 miRNA genes on the X-chromosome were amplified and sequenced in males with (193) and without (191) schizophrenia spectrum disorders to test the hypothesis that ultra-rare mutations in microRNAs collectively contribute to the risk of schizophrenia. Eight ultra-rare variants were identified in eight distinct miRNA genes (miR-18b, -505, -502, -188, -325, -660, -509, -510, and let-7f), six of the eight occurring in the mature sequence of the miRNA. *In vitro* testing of the variants via luciferase assay revealed a functional alteration of miR-18b, miR-188, miR-502, and let-7f.

Xu[84] and coworkers investigated eight miRNAs identified to be differentially expressed in schizophrenia from previous studies and 15 of their experimentally validated target sites for

mutation screening and association studies in a Chinese case–control sample (456 cases/453 controls). The authors identified a new, potentially functional variant, ss178077483, located in the terminal loop of pre-mir-30e to be strongly associated with schizophrenia (P=0.00017, OR=4.952). In addition, expression of mir-30e in peripheral leukocytes from cases and controls was significantly higher in cases (p=6.79e–7).

Recently, in what is regarded as one of the largest genetic studies in schizophrenia to date, the Schizophrenia Psychiatric GWAS Consortium(PGC) examined the role of common genetic variation in schizophrenia based upon a combined GWAS broken into 2 stages: Stage 1, a discovery sample of 21,856 individuals of European ancestry (EA; 9,394 cases and 12,462 controls) and Stage 2, a replication sample of 29,839 independent subjects (8,442 cases and 21,397 controls) (Gejman et al. in press). After combining Stage 1 and 2 samples, the authors obtained genome-wide significant associations with schizophrenia for seven loci, five novel (1p21.3, 2q32.3, 8p23.2, 8q21.3, and 10q24.32-q24.33) and two (6p21.32-p22.1 and 18q21.2) previously implicated. The strongest novel finding ($p=1.6\times10^{-11}$) for association to schizophrenia was to a genetic variant within the precursor of miR-137, a miRNA previously reported to regulate neuronal development[85]. Moreover, four other schizophrenia loci achieving genome-wide significance in the study are predicted to be targeted by miR-137, suggesting miR-137 gene dysregulation as a novel etiologic mechanism in schizophrenia.

Significance

Given their abundance in the brain and implicated effects on brain development and function, miRNAs make particularly excellent candidates for studying psychiatric illnesses. To date, the few studies that have attempted to profile miRNAs differentially expressed in

schizophrenia have used relatively small sample sizes (n<21), and have been limited to miRNAs believed to be expressed in the brain only (<264). In contrast, our study included 35 patients with schizophrenia, 35 with bipolar disorder, and 35 controls, which is the largest sample profiled to date as well as being the first to profile miRNAs in bipolar disorder. In addition, all 667 known human miRNAs (at the time) were assayed, giving us an unbiased view of miRNAs dysregulated in disease. Overall, studying the miRNA expression profiles in schizophrenia and bipolar disorder provides a foundation for understanding the complex etiology of each disease, helps to define and elucidate the genetic risks, and may ultimately serve as a reliable biomarker for disease progression and/or prognosis. Our study may also clarify the discrepancies seen in the existing association data by assigning new functional roles for variants found in previous studies. Finally, because miRNAs have the ability to regulate many genes at the same time, our study can also potentially bring together the numerous candidate genes reported to be associated with disease, ultimately leading to a unified biological explanation for the pathological processes underlying schizophrenia and bipolar disorder.

Due to the fact that miRNAs can bind to their targets with imperfect complementarity, computational prediction of miRNA:mRNA interactions has been plagued with false-positives. Moreover, current widely accepted approaches for experimental validation of predicted miRNA:mRNA interactions are low-throughput and time and labor intensive. Therefore, only a limited number of miRNA:mRNA interactions have been validated so far. Thus, another goal of this study was to demonstrate biological functionality of miRNAs on their targets through a series of molecular-based approaches evaluating transcriptional and translational levels of gene expression (real-time PCR, western blot) as well as determining miRNA target-site specificity (luciferase reporter gene assays). Our study investigated two

miRNAs, miR-132 and miR-137, both of which have been shown to regulate neuronal function and development, and have been associated with schizophrenia from two distinct avenues of research, miR-132 from expression studies and miR-137 from genetic studies. We have successfully demonstrated miR-132 mediated down-regulation of neurotrophin 3 (*NTF3*), disrupted in schizophrenia 1 *(DISC1*), and ionotropic glutamate receptor kainate 5 (*GRIK5*) at the mRNA level, down-regulation of *GRIK5* at the protein level, and that miR-132 directly binds to its predicted target site in the 3'-UTR of *DISC1*. Additionally, we have successfully demonstrated miR-137 mediated down-regulation of transcription factor 4 (*TCF4*), voltage-dependent calcium channel alpha-1A subunit (*CACNA1C*), cyclin-dependent kinase 6 (*CDK6*), ankyrin 3 (*ANK3*), and zinc finger 804A (*ZNF804A*) at the mRNA level, and down-regulation of *TCF4*, *CACNA1C*, and *CDK6* at the protein level. Furthermore, we have demonstrated that miR-137 binds to specific target sites in the 3'-UTRs of *TCF4*, *CACNA1C*, and *CDK6*, suggesting miR-137 directly regulates these genes. Implications drawn from this study support the hypothesis that dysregulation of miRNA function may lead to human disease.

Materials and Methods

Post-mortem brain tissue

The Stanley Medical Research Institute (SMRI) provided 200mg of post-mortem brain tissue from the dorsolateral prefrontal cortex (Brodmann's area 46)[86]. The sample demographics are described in **Appendix 1**. Exclusion criteria included: (1) brain pathology, (2) history of pre-existing CNS disease, (3) poor RNA quality, (4) IQ < 70, (5) age < 30 and (6) substance abuse within one year of death.

RNA Isolation and quantification

All RNA was isolated as total RNA containing the small RNA fraction using the miRvana miRNA isolation Kit (Ambion, Austin, TX) following manufacturer's recommendations. RNA quality and concentration were subsequently measured on the 2100 Bioanalyzer (Agilent, Santa Clara, CA) as well as on the Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA). Average RNA integrity number (RIN) for RNA isolated from cell culture was 9.8.

For the Stanley post-mortem brain sample, RNA was isolated from 100mg of tissue using the miRvana miRNA isolation Kit (Ambion) following manufacturer's recommendations. The quality of RNA was assessed using the 2100 Bioanalyzer and average RIN for the entire sample was 7.2. Tissue for one BP sample was not received and 1 control sample gave poor RNA quality, both of which were excluded from further analyses.

Taqman Low-Density Array (TLDA)

miRNA reverse transcription

cDNA synthesis and expression detection were performed according to manufacturer's protocols (Applied Biosystems, Hercules, CA). Briefly, RNA was reverse transcribed using the miRNA reverse transcription kit in combination with the stem-loop Megaplex primer pool (ABI). 3 µl of total RNA (33.3 ng/µl) was combined with 0.8µl RT primer mix (10x), 0.8µl RT buffer (10x), 1.5 µl MultiScribe Reverse Transcriptase (10 U/µl), 0.2µl dNTPs with dTTP (0.5mM each), 0.9µl MgCl2 (3mM) and 0.1µl RNase inhibitor (0.25 U/µl) in a total reaction volume of 7.5µl. Reactions were run on an Eppendorf Mastercycler (Eppendorf, Westbury, NY)

in a 384-well plate for 40 cycles at 16°C for 2 min., 42°C for 1 min., 50°C for 1 sec., and then held at 85°C for 5 min.

miRNA Expression Detection

Prior to detecting miRNA expression, the RT product was pre-amplified following ABI's recommendations, allowing for increased detection sensitivity while preserving the miRNA[87-89]expression profile (**Appendix 2**). The reactions were diluted with 75µl of 0.1X TBE and stored at -20°C. 9µl of product and 450µl of TaqMan PCR Master Mix, were combined with 441µl nuclease free water, mixed and centrifuged for 30 sec. 100µl was loaded into each port of the appropriate 384 well TLDA array, centrifuged twice at 1200 rpm in a Sorvall Legend centrifuge (Thermo Scientific, USA), and sealed with a micro-fluidic card staker (ABI). The arrays were run on the 7900HT Real-Time PCR System according to manufacturer's protocol. Raw Cq values (RDML guidelines, http://www.rdml.org[90]) were calculated using the SDS software v.2.3 with automatic baseline settings at a threshold of 0.2.

PCR expression normalization

Under our experimental conditions, the three reference genes contained on the A and B TLDA arrays did not maintain a consistent ratio across samples (**Appendix 3**). Therefore, we normalized our data using the median-polish procedure[91], which has been used by others to evaluate global miRNA expression[92]. A description of the method is given in **Appendix 4.**

Quality Control (QC)

Potential technical artifacts, such as plate effects[93], were evaluated by plotting the sample profile correlations which were invariant under this normalization. Two bands of low correlations were observed, which we interpreted as plate effects independent of diagnosis

(**Appendix 5A**). Samples falling within these bands were excluded from further analyses as they also clustered separately from the main group (**Appendix 5B**). Thus, the final sample was reduced to 29 SZ, 27 BP and 31 CONT.

Statistical Methods—TLDA

The primary analyses were conducted using the rlm function in the R open-source statistical language (www.r-project.org) and the exploratory analyses were conducted using Robust Multiple Regression with Huber's method as implemented in the NCSS (http://www.ncss.com) software package. MiRNA variance was estimated using an F-test with corrected degrees of freedom and the Spearman (ρ) coefficient was used to estimate correlations of miRNA expressions. Correction for multiple testing was accomplished using the Benjamini-Hochberg[94] False Discovery Rate (FDR) at 10%. Gene targets for the differentially expressed miRNAs were predicted using the highly sensitive miRanda algorithm[53] (V.3) with default parameters.

Transfections

6-well

Transfections for gene expression assays were accomplished in 6-well plates. One day before transfection, HEK 293 (Human Embryonic Kidney) and Be2C (neuroblastoma) cells were seeded in media without antibiotics at 600,000 cells per well to be 90-95% confluent at the time of transfection. 4ug of either mir-137 precursor or scrambled hairpin control plasmid (Genecopoeia Inc, Rockville, MD) was diluted into 250uL of Opti-MEM without serum. In a separate tube, 10uL of Lipofetamine 2000 (Invitrogen, Carlsbad, CA) was diluted into 250uL Opti-MEM without serum and incubated for 5 minutes at room temperature. After incubation,

the DNA and Lipofectamine dilutions were combined and incubated for 20 minutes at room temperature to allow complex formation to occur. 500uL of the DNA/Lipofectamine complex was then added to each well containing cells and 3mL of media without antibiotic. The reactions were incubated at 37°C and harvested at 24 hours. To increase accuracy and to reduce assay variability, each sample was transfected in triplicate.

10cm Dish

Transfections for western blotting were accomplished in 10cm dishes. One day before transfection, HEK 293 (Human Embryonic Kidney) and Be2C (neuroblastoma) cells were seeded in media without antibiotics at ~$3.6x10^6$ cells per well to be 90-95% confluent at the time of transfection. 12ug of either mir-137 precursor or scrambled hairpin control plasmid (Genecopoeia) was diluted into 1.5mL of Opti-MEM without serum. In a separate tube, 35uL of Lipofetamine 2000 (Invitrogen, Carlsbad, CA) was diluted into 250uL Opti-MEM without serum and incubated for 5 minutes at room temperature. After incubation, the DNA and Lipofectamine dilutions were combined and incubated for 20 minutes at room temperature to allow complex formation to occur. 3mL of the DNA/Lipofectamine complex was then added to each well containing cells and 12mL of media without antibiotic. The reactions were incubated at 37°C and harvested at 24 hours.

96-well Transfection

The 96-well transfections were accomplished by following the alternate rapid protocol without pre-plating as outlined in Invitrogen's Lipofectamine 2000 manual. The following combination of miRNAs and targets were used to assess specificity of miRNA binding: 1) 180ng of mir-137 or miR-132 precursor (Genecopoeia) with 120ng of the target genes of interest, 2)

180ng of mir-137 or miR-132 precursor (Genecopoeia) with 120ng of *mutated* target genes of interest, 3) 180ng of mir-137 or miR-132 precursor (Genecopoeia) with 120ng of target genes of interest and 90nmol anti-miR-137 (Ambion), 4) 180ng of miR-377, an off-targeting miRNA precursor, with 120ng of target genes of interest, and 5) 180ng of miR-125a precursor (Genecopoeia) with 120ng of *lin-4*, a known miR-125a target gene (positive control). The respective reactions were then diluted into 20uL of Opti-MEM without serum. Next, for each well, 0.8uL of Lipofectamine 2000 (Invitrogen) was diluted into 24.2uL Opti-MEM without serum and incubated for 5 minutes at room temperature. After incubation, the DNA and Lipofectamine dilutions were combined and incubated for 20 minutes at room temperature to allow complex formation to occur. In the meantime, suspensions of HEK293 (Human Embryonic Kidney) or Be2C (neuroblastoma) cells were prepared to contain 120,000 cells in 100uL of media without antibiotics. The DNA-Lipofectamine complexes in each well were then mixed with 100uL of cells and incubated at 37°C and harvested after 24 hours. To increase accuracy and to reduce assay variability, each sample was transfected in quadruplicate.

Gene Expression Assays

cDNA was made from 1.5ug of RNA using the High Capacity cDNA Kit (ABI) according to manufacturer's recommendations. Gene expression assays were then performed by adding 0.25uL dH20, 0.25uL 20x Taqman Assay, 5.0uL Gene Expression Master Mix and 4.5uL cDNA diluted 1:10. The reactions for each gene were run in triplicate in a 384-well format on the ABI 7900HT according to manufacturer's recommendations. PCR-efficiency for each reaction was assessed using the LinRegPCR[95] program, which uses the raw real-time PCR data of each individual sample and performs a linear regression analysis to calculate starting concentrations of mRNAs and individual PCR efficiencies for each sample. A reference house-

keeping gene, importin 8 (*IPO8*), was also evaluated for each sample. The gene expression data was then normalized using the $2^{(-\Delta\Delta Ct)}$ method[96] with *IPO8* as the endogenous control. The Student's t-test was used to evaluate significant mean expression differences in GraphPad (GraphPad Software v.5.04, San Diego CA).

Western Blot

Protein was isolated from ~$2x10^7$ HEK293 or Be2C cells using 500uL of Mammalian Protein Extraction Reagent (MPER) following manufacturer's protocol (Thermo Fisher). Protein concentration was measure using the BCA Assay kit (Pierce) following the high range protocol for the Nanodrop 2000. 50ug of protein from each sample along with 10uL of the Precision Plus Dual Color Standard (Biorad) was loaded onto a 10% TGX gel (Biorad) in 1X Tris/Glycine/SDS buffer (Biorad) and run at 170V for 1 hour. The proteins were then transferred to a PVDF membrane in 1X Tris/Glycine/SDS buffer with 20% methanol and ice pack at room temperature with constant stirring for 1 hour at 100V. The membrane was then washed in 18mohm water 3 times for 5 minutes and then blocked with Superblock (Thermo Fisher) for 1 hour at room temperature. Once blocked, the primary antibody dilutions were made in Tris-Buffered Saline with Tween20 (TBST) and 10% blocking solution. The primary antibody was incubated overnight at 4°C. The membrane was then washed 5 times for 5 min with TBST. The secondary antibody was then added in TBST with 10% blocking solution and incubated at room temperature for 1 hour. The membrane was then washed 5 times for 5 min with TBST and developed using the SuperSignal West Pico (Pierce) kit according to manufacturer's recommendations. All antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Concentrations for the antibodies used were as follows: *GRIK5* 1:250 primary (sc-8915, goat polyclonal) and 1:20,000 donkey anti-goat secondary (sc-2020), *CDK6* 1:150 primary

(sc-271364, mouse monoclonal) and 1:10,000 goat anti-mouse secondary (sc-2005), *TCF4* 1:250 primary (sc-101095, mouse monoclonal) and 1:10,000 goat anti-mouse secondary (sc-2005), *CACNA1C* 1:1,000 primary (sc-25686, rabbit polyclonal) and 1:40:000 goat anti-rabbit secondary (sc-2030), and α-tubulin 1:40:000 primary (sc-23948, mouse monoclonal) and 1:20,000 goat anti-mouse secondary (sc-2005).

3'-UTR Target Site Cloning and Mutagenesis

Approximately 100 base pairs (50bp up- and downstream of the predicted target site) was cloned into the pEZX-MT01 vector (Genecopoeia) using the *AsiS*I and *XhoI* restriction sites in the multiple cloning region downstream of the luciferase reporter gene. All target-site cloning was performed by Genecopoeia, Inc. Cloned target-site sequences can be found in Tables 6 and 9 below. Mutagenesis was performed using the QuickChange II Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to manufacturer's protocol. All clones were then sequenced by the Nucleic Acids Research Facility (NARF) at Virginia Commonwealth University.

Luciferase Assay

The luciferase assays were accomplished using the Luc-Pair miR Luciferase Assay (Genecopoeia) microplate procedure. First, the media was aspirated and 100uL of Working Solution I (Solution I:Substrate I in a 1:200 ratio) was added to each well. After 10 minutes, firefly luciferase activity was measured in a Wallac Victor II luminometer (ThermoFisher). Next, 100uL of Working Solution II (Solution II:Substrate II in a 1:200 ratio) was added to each well. After 10 minutes, *Renilla* luciferase activity was measured. Next, the ratio of firefly to *Renilla* luciferase was calculated (F/R) for each well and the average ratio for each quadruplicate was taken. The average ratios were then normalized to the mock transfection, i.e. cells only.

Each set of miRNA and target experiment was performed at least three times and the Student's t-test (GraphPad) was used to calculate significant mean differences between each condition.

Results

TLDA

Overall Performance

Each cDNA sample (individual) was evaluated for the expression of 667 miRNAs. Of these, 226 miRNAs were excluded from further analyses due to lack of amplification, very low expression (Cq >35), or poor amplification efficiency across samples (missing expression values ≥ 80%). Thus, 441 miRNAs were included in all subsequent analyses and based on their expression, classified as highly (Cq 14-20), moderately (Cq 21-26), or low (Cq 27-34) expressed (**Appendix 6**). Being a global miRNA evaluation, it was reasonable to observe that not all miRNAs were brain expressed.

SMRI Demographic Covariates

Together with disease status, the SMRI provided additional information regarding potential confounders described in **Appendix 7**. Upon inspection, only postmortem interval (PMI), refrigerated interval (RI) and antipsychotics showed deviation from a normal distribution, which were log-transformed to approximate normality. In our analyses, none of the covariates showed consistent effects on miRNA expression. When fitting a regression model to a small number of samples, the number of estimated parameters cannot be large, therefore in all

analyses, only sex and pH were included as technical covariates as these were the only two showing a marginally significant effect on miRNA expression (Figure 15 in **Appendix 7**).

Detection of differentially expressed miRNAs in SZ and BP cases

Our main objective was to evaluate expression differences between SZ and BP cases and controls for all known human miRNAs using the TLDA high throughput real-time qPCR

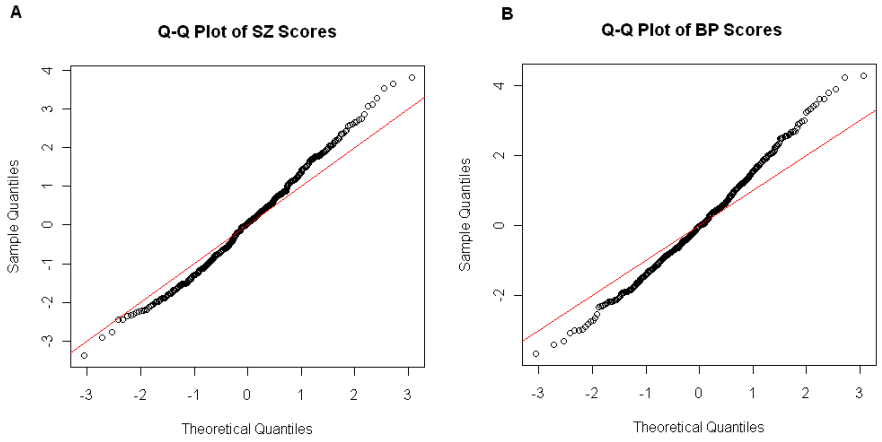


Figure 3: Q-Q plots of the distribution of T-scores within the (A) SZ and (B) BP groups. T-scores are obtained from the linear model.

approach. The T-score distribution from the linear model showed deviation from the expected normal distribution (Figure 3) and after controlling the FDR at 10% , 22 miRNAs were identified as significantly dysregulated in SZ and BP (Table 1). Notably, miR-7 and miR-212, which were previously reported to be dysregulated in the prefrontal cortex of individuals with SZ and schizoaffective disorder[79], were also dysregulated in our sample.

Table 1: MicroRNAs differentially expressed in schizophrenia (SZ) and bipolar disorder (BP) compared to controls at FDR of 10%. Bold=single-tube validated. B(i)=regression coefficient, SE=standard error, +/- sign for T-values reflect up/down regulation respectively, Q-value=FDR measure.

Status	miRNA	Aver. Cq	Express. Level	$\beta_{(i)}$	SE	T-Value	P-Value	Q-Value
SZ	**hsa-miR-34a**	21.82	Moderate	0.385	0.093	4.12	0.00004	0.018
SZ	**hsa-miR-132***	29.14	Low	0.364	0.092	3.98	0.00016	0.024
SZ	**hsa-miR-132**	17.47	High	0.212	0.053	3.97	0.00016	0.024
SZ	**hsa-miR-212**	23.04	Moderate	0.232	0.064	3.65	0.00047	0.048
SZ	hsa-miR-544	31.35	Low	0.839	0.243	3.46	0.00054	0.048
SZ	hsa-miR-7	23.1	Moderate	0.241	0.069	3.50	0.00078	0.054
SZ	**hsa-miR-154***	26.31	Moderate	0.511	0.153	3.33	0.00086	0.054
BP	hsa-miR-504	24	Moderate	0.710	0.169	4.19	0.00003	0.007
BP	hsa-miR-454*	30.53	Low	-0.925	0.226	-4.10	0.00004	0.007
BP	hsa-miR-29a	17.64	High	-0.297	0.073	-4.07	0.00005	0.007
BP	hsa-miR-520c-3p	30.11	Low	-0.617	0.165	-3.75	0.00018	0.020
BP	hsa-miR-140-3p	25.14	Moderate	-0.473	0.137	-3.47	0.00053	0.047
BP	**hsa-miR-145***	26.6	Moderate	0.572	0.171	3.35	0.00080	0.052
BP	hsa-miR-767-5p	26.87	Moderate	-0.329	0.100	-3.29	0.00102	0.052
BP	hsa-miR-22*	23.19	Moderate	0.465	0.142	3.27	0.00106	0.052
BP	hsa-miR-145	17.83	High	0.571	0.183	3.13	0.00177	0.066
BP	hsa-miR-874	21.9	Moderate	-0.391	0.125	-3.12	0.00181	0.066
BP	**hsa-miR-133b**	24.98	Moderate	0.359	0.116	3.11	0.00190	0.066
BP	hsa-miR-154*	26.31	Moderate	0.501	0.162	3.10	0.00195	0.066
BP	hsa-miR-32	29.18	Low	-0.724	0.235	-3.08	0.00209	0.066
BP	hsa-miR-573	29.03	Low	-0.939	0.308	-3.05	0.00227	0.067
BP	**hsa-miR-889**	24.83	Moderate	0.433	0.147	2.95	0.00321	0.089

SZ cases

Seven miRNAs were differentially expressed in the SZ group. Of these, miR-34a, located on chromosome 1 (1p36.22), was the most significantly differentially expressed (p=0.00004) and in contrast to a previous study showing high expression[97], this miRNA was moderately expressed in brain (average Cq = 21.8). MiR-132 and -132* are processed from the same precursor and together with miR-212, form a cluster located on the reverse strand of chromosome 17 (17p13.3). MiR-132 and -212 were previously reported to be expressed in brain and our data confirm this observation[98]. MiR-132 was highly expressed (average Cq=17.5) and hsa-mir-212 was moderately expressed (average Cq of 23). Both miRNAs were highly correlated (Spearman's $\rho = 0.87$), supporting previous observations for coordinated expression of clustered miRNAs[99-101]. MiR-132* however, was expressed at a lower level (average Cq=27.18) and was

less correlated with miR-132 (Spearman's ρ = 0.65) and miR-212 (ρ = 0.47). MiR-7 consists of three members located on three chromosomes; miR-7-1(9q21.32), miR-7-2 (15q21.6) and miR-7-3 (19p13.3). The miR-7 family showed moderate expression (average Cq=23.1), but since the mature sequence of these three miRNAs is identical, individual expression of each member could not be separated. Finally, miR-544 was low (Cq=31.35) and miR-154* was moderately (Cq=26.31) expressed. MiR-154* was the only miRNA showing expression differences in both SZ and BP.

BP cases

In the BP group, 15 miRNAs were observed to be differentially expressed. Seven were over-expressed and 8 were under-expressed (Table 1). The over-expressed miRNAs were all moderately to highly expressed and showed significant correlation with each other (Table 2). In contrast, the under-expressed miRNAs were moderately to low expressed and did not show any significant inter-correlations (Table 3). In the BP group, only two miRNAs were modestly correlated with each other (Spearman's ρ =0.35), miR-154* and -889, which cluster together on chr.14 (14q32.2).

Table 2: Correlation matrix of miRNAs over-expressed in the BP group. Bold=Statistically significant Spearman's ρ coefficients at p<0.05.

Variables	hsa-miR-504	hsa-miR-145	hsa-miR-145*	hsa-miR-22*	hsa-miR-133b	hsa-miR-154*	hsa-miR-889
hsa-miR-504	**1**	**0.420**	**0.503**	**0.608**	**0.512**	**0.611**	**0.633**
hsa-miR-145	**0.420**	**1**	**0.840**	**0.427**	**0.550**	**0.230**	**0.276**
hsa-miR-145*	**0.503**	**0.840**	**1**	**0.493**	**0.451**	**0.397**	**0.364**
hsa-miR-22*	**0.608**	**0.427**	**0.493**	**1**	**0.344**	**0.554**	**0.550**
hsa-miR-133b	**0.512**	**0.550**	**0.451**	**0.344**	**1**	0.141	**0.485**
hsa-miR-154*	**0.611**	**0.230**	**0.397**	**0.554**	0.141	**1**	**0.355**
hsa-miR-889	**0.633**	**0.276**	**0.364**	**0.550**	**0.485**	**0.355**	**1**

Table 3: Correlation matrix of under-expressed miRNAs in the BP group. Bold=Statistically significant Spearman's ρ coefficients at p<0.05.

Variables	hsa-miR-140-3p	hsa-miR-29a	hsa-miR-32	hsa-miR-874	hsa-miR-454*	hsa-miR-520c-3p	hsa-miR-573	hsa-miR-767-5p
hsa-miR-140-3p	**1**	0.127	**0.288**	0.062	**0.392**	0.051	−0.067	−0.057
hsa-miR-29a	0.127	**1**	0.163	0.017	**0.244**	0.165	−0.067	**0.245**
hsa-miR-32	**0.288**	0.163	**1**	0.012	**0.296**	**−0.226**	−0.079	−0.077
hsa-miR-874	0.062	0.017	0.012	**1**	−0.057	0.160	0.194	0.207
hsa-miR-454*	**0.392**	**0.244**	**0.296**	−0.057	**1**	**−0.335**	**−0.328**	0.076
hsa-miR-520c-3p	0.051	0.165	**−0.226**	0.160	**−0.335**	**1**	**0.403**	−0.149
hsa-miR-573	−0.067	−0.067	−0.079	0.194	**−0.328**	**0.403**	**1**	−0.171
hsa-miR-767-5p	−0.057	**0.245**	−0.077	0.207	0.076	−0.149	−0.171	**1**

TLDA Validation

All 22 miRNAs identified as differentially expressed from the TLDA approach were subsequently validated in single-tube real-time PCR reactions. One miRNA, miR-573, did not amplify and was therefore excluded from the validation analyses. The remaining 21 miRNA expressions were normalized by the $2^{-\Delta\Delta Ct}$ approach using RNU48 as an endogenous control. Eight miRNAs were successfully validated, 5 in SZ and 3 in BP (Table 1, bold). The discrepancy between the multiplex and singly validated miRNA data could be explained by biological (heterogeneity) or technical (PCR conditions or pre-amplification) differences. Additional expression studies will be needed to address such issues. However, many of the validated miRNAs reported here have also been previously reported in other studies using different platforms and/or samples.

Analysis of miRNA variances

Although miRNAs tend to be dysregulated among individuals with SZ or BP, only a limited number of miRNAs (22/441 or 5%) demonstrated a consistent direction of altered expression, i.e. the same miRNA following a similar direction of expression across the entire sample. This is not surprising considering the polygenic etiology of SZ and BP. However, when miRNA variances between groups were compared, many of the miRNAs showing significant

variance differences (**Appendix 8**) were present in both disease groups (Figure 4), thereby supporting the previously observed shared genetic risk in SZ and BP[19, 102].

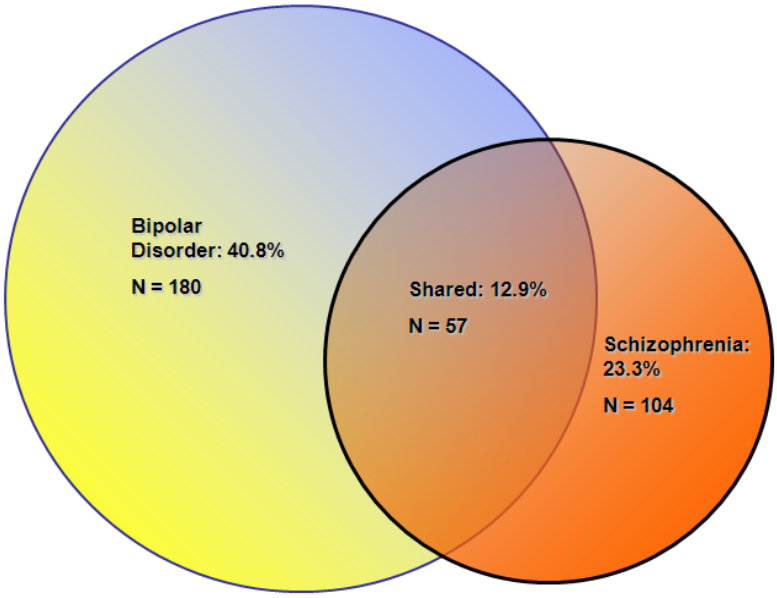


Figure 4: miRNAs with significant variance differences shared in schizophrenia and bipolar disorder.

Exploratory analyses

Exploratory analyses were performed to assess the effect of disease duration[103], smoking[104], suicide[105], and antipsychotic treatment on miRNA expression, as these traits were previously shown to affect expression of SZ candidate genes. The only significant effect was observed for miR-193b* ($p = 0.00002$) when suicide was analyzed in interaction with disease. Antipsychotic treatment did not show any systematic effect on miRNA expression with miR-218-2* ($p = 0.000012$) expression affected in the BP group only. A probable explanation is that in the SMRI sample, antipsychotic treatment is reported as a generalized measure (fluphenazine equivalents). Since different drugs may have different, if not opposing effects on miRNA expression, the individual drug effect is likely to be confounded.

In silico miRNA Target Prediction

As an initial evaluation, we used the most sensitive miRNA target prediction algorithm, miRanda[106], to capture the most targets for the 22 differentially expressed miRNAs in the SMRI sample. As a result, we identified 7,350 predicted gene targets for the seven miRNAs differentially expressed in the SZ group and 14,986 predicted gene targets for the 15 miRNAs differentially expressed in the BP group. We then assessed the nature of these gene targets using the Ingenuity Pathway Analysis (IPA) web tool. In IPA, we used our gene dataset as the reference set to minimize experimental or literature bias. The most enriched functional group of genes amongst the differentially expressed miRNA targets were genes forming networks related to nervous system function and disease, including SZ and BP. Therefore, knowing the 22 differentially expressed miRNAs target disease-related genes, we were confident to move forward with our investigation of validating target genes.

Of the 22 differentially expressed miRNAs, we chose to focus on the schizophrenia-related miRNAs as many (miR-7[107, 108] , miR-212[80],miR -132[31], and miR-34a[109, 110]) have been implicated in the development and function of the nervous system as well as in previous miRNA expression studies in nervous system diseases. Of the seven miRNAs differentially expressed in schizophrenia, we further investigated miR-132 due to it being high expressed and highly significant in our study as well as being implicated in other neurodevelopmental[31] and degenerative disorders[87] of the brain. MiR-132 has also been shown to regulate neuronal morphogenesis[111, 112], promote neurite outgrowth and spine formation[113, 114], and is transcriptionally controlled by the neurotrophin BDNF (brain-derived neurotrophic factor), which is important for neuronal development, survival, synapse formation and plasticity[115].

Bioinformatic Prediction of miR-132 Targets

Computational algorithms are prone to a high false-positive rate and the overall high sensitivity of miRanda, in particular, necessitates that additional steps are taken to reduce the type I error. Thus, it has been recommended and successfully demonstrated[116], that utilizing the intersection of at least two algorithms predicting the same binding site significantly reduces false-positives. Therefore, we utilized the miRecords database[64], which compiles the predictions from 11 established computational algorithms, to help derive the most plausible miR-132 specific gene targets. Using the intersection of at least two algorithms, 1,551 genes were predicted for miR-132. From this list the following genes were selected based on prior biological evidence and/or strong association with schizophrenia (Table 4). Glutamate receptor, ionotropic, kainate 5 (*GRIK5*), Glutamate receptor, ionotropic N-methyl-D-aspartate 3A (*GRIN3A*), and glutamate receptor, ionotropic, N-methyl-D-aspartate 2B (*GRIN2B*) all code for ionotropic glutamate receptors, the dysfunction of which has been shown to be related to schizophrenia in multiple brain regions[3, 117-121] . Disrupted in schizophrenia 1 (*DISC1)* was first discovered when a chromosomal rearrangement that disrupts the gene was linked to schizophrenia in an extended Scottish family[122] and has since been implicated in multiple association studies[123, 124] as well as being shown to affect multiple processes in neurodevelopment[125, 126]. Neurotrophin 3 (*NTF3*) belongs to a family of neurotrophins, including *NGF* (nerve growth factor) and *BDNF* (Brain Derived Neurotrophic Factor), and is an important regulator for survival, differentiation, and maintenance of nerve cells and acts to promote the growth and differentiation of new neurons[127]. Circadian locomotor output cycles kaput (*CLOCK*) encodes a basic helix-loop-helix transcription factor that is essential for

circadian rhythm[128] and has been shown to regulate dopaminergic transmission in the reward circuit[129]. Furthermore, disruption of *CLOCK* in mice yields a behavioral profile similar to human mania (hyperactivity, decreased sleep, lowered depression-like behavior, lower anxiety, and an increase in the reward value for cocaine) [130]. Finally, cardiomyopathy associated protein (*CMYA5*) has recently been implicated in two genome-wide association studies of schizophrenia[131,132].

Table 4: Schizophrenia-related mir-132 target genes predicted by 2 or more programs in miRecords.

Refseq	Symbol	Description	Predicted by
NM_001012958	*DISC1*	disrupted in schizophrenia 1	miRanda, PITA, RNAHybrid
NM_002527	*NTF3*	neurotrophin 3	miRanda, PITA, RNAHybrid
NM_133445	*GRIN3A*	glutamate receptor, ionotropic, N-methyl-D-aspartate 3A	miRanda, PITA, RNAHybrid
NM_600283	*GRIK5*	glutamate receptor, ionotropic, kainate 5	RNA22, RNAHybrid
NM_004898	*CLOCK*	circadian locomotor output cycles kaput	PITA, RNA22, RNAHybrid
NM_612193	*CMYA5*	cardiomyopathy associated protein	RNA22, RNAHybrid
NM_000834	*GRIN2B*	glutamate receptor, ionotropic, N-methyl-D-aspartate 2B	miRanda, PITA, RNA22, RNAHybrid

miR-132 Target Gene Validation

mRNA Expression

To detemine whether miR-132 down-regulates the bioinformatically predicted target genes at the mRNA level, we transfected either a plasmid expressing the miR-132 hairpin or a scrambled hairpin control into HEK293 (human embryonic kidney) and Be2C (neuroblastoma) cell lines. It is noteworthy that a plasmid expressing the miRNA hairpin was used as this allows for the miRNA to be processed by the normal cellular machinery. The HEK293 cell line was chosen for the following reasons: 1) miR-132 is not endogenously expressed in the cells, 2) high

transfection efficiency, and 3) the gene expression profile is similar to that of neurons[133]. The Be2C cell line was chosen for the following reasons: 1) miR-132 is not endogenously expressed in the cells, 2) highest transfection efficiency among neuronal cell lines, and 3) it is a neuronal cell line, which is related to our tissue of interest. After 24 hours, total RNA was isolated and gene expression was evaluated via real-time PCR. For each condition, miR-132 or scrambled hairpin transfected, expression of an endogenous control gene was also measured to which the target gene expression values were normalized against. Of the seven putative target genes tested, three were significantly down-regulated in the miR-132 transfected versus scrambled hairpin transfected conditions. *DISC1* was statistically significantly down-regulated in both HEK293($p=0.0001$, $n=27$) and Be2C($p=0.0019$, $n=27$) cell lines. *NTF3* was also down-regulated in both cell lines ($p=0.0002$, $n=27$ and $p=0.0001$, $n=27$) and *GRIK5* was down-regulated only in HEK293 ($p=0.0001$, $n=27$), as it was not endogenously expressed in Be2C cells (Figure 5). The remaining four genes *GRIN3A, GRIN2B, CLOCK,* and *CMYA5*, were expressed in both cell lines but did not demonstrate statistically significant expression differences between treatment conditions.

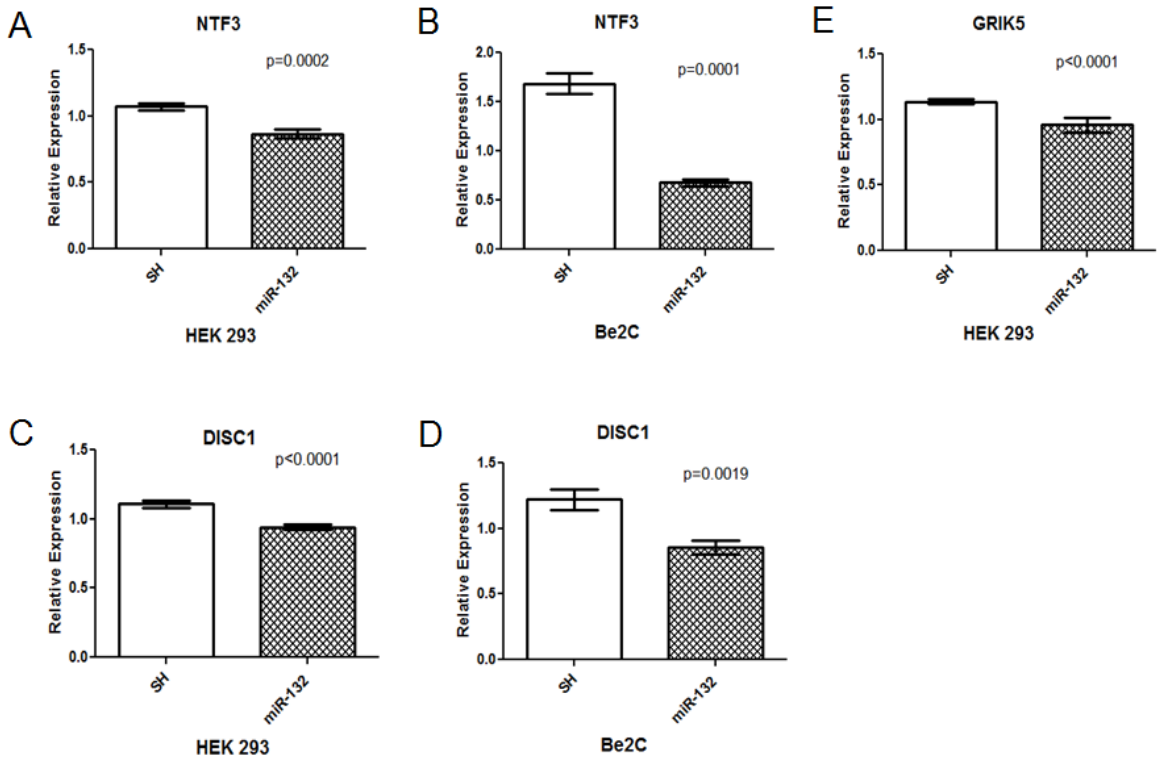


Figure 5: Gene expression for miR-132 target genes. *NTF3* gene expression in (A) HEK 293 and (B) Be2C cells. *DISC1* gene expression in (C) HEK 293 and (D) Be2C cells. *GRIK5* gene expression in (E) HEK 293 cells. N=27 for each bar, error bars represent SEM. Student's t-test was used to test for statistical significance between scrambled hairpin (SH) and miR-132 conditions.

Protein Expression

Having observed a miR-132 associated down-regulation of *DISC1*, *NTF3* and *GRIK5* at the mRNA level, we next evaluated whether the effect would be propagated at the protein level. To accomplish this, we transfected HEK293 cells with either miR-132 or a scrambled hairpin control and extracted whole cell protein lysates after 24 hours. Western blots were then performed using alpha-tubulin as a loading control. Densitometric quantification of the gel bands was then performed using the ImageJ program from the National Institutes of Health (http://rsbweb.nih.gov/ij/index.html). Of the three genes tested, only *GRIK5* was significantly down-regulated (p=0.0359) at the protein level in miR-132 transfected versus scrambled hairpin

transfected control (Figure 6). No statistically significant differences in protein level were observed for *NTF3* or *DISC1*.

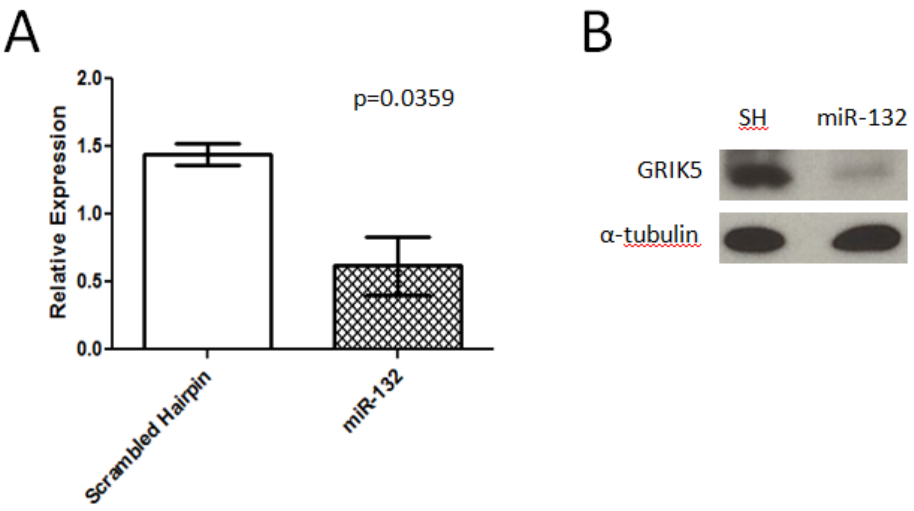


Figure 6: Western blot data for *GRIK5* protein expression. (A) *GRIK5* protein expression, (B) representative blot. N=4 for each bar, error bars represent SEM.

Validation of miRNA:mRNA Interactions

Demonstrating that miR-132 down-regulates *DISC1*and *NTF3* at the mRNA level, and *GRIK5* at both the mRNA and protein levels, we next sought to show miR-132 exerts its effects by binding to specific target-sites in the 3'-UTR of the respective target genes. To assess this, we used miRecords[64] to evaluate predicted miR-132 binding sites in the 3'-UTRs of *DISC1*, *NTF3* and *GRIK5*, the results of which are presented in Table 5. Three binding sites were predicted for *DISC1*, one of which, position 1160 in the 3'-UTR, was predicted by five programs and was therefore used as the major miR-132 target site. Four binding sites were predicted for *NTF3*, one of which, position 288 in the 3'-UTR, was predicted by five programs and was

Table 5: Predicted 3'-UTR binding sites for miR-132.

miRNA	RefSeq	Gene	Position in 3'-UTR	Prediction Programs
miR-132	NM_001012958	*DISC1*	1160	miRanda, PITA, DIANA-microT, RNAHybrid, RNA22
miR-132	NM_002527	NTF3	288	miRanda, PITA, TargetScan, RNAHybrid, RNA22
miR-132	NM_002088	*GRIK5*	2974	RNAHybrid, RNA22

Table 6: 3'-UTR sequences cloned into the luciferase reporter construct. WT=wild-type, Mut=Mutant, Bold=seed sequence, underline=site of mutation.

	3'-UTR Sequence
***DISC1* WT**	5' gttattctcaaattcagtcttccattcatctctccttcccttccctggcagacagacttctgca gccactg**ggactgtt**ggggtgaggaaaggcaagaaccgatgtttcattctctccct 3'
***DISC1* Mut**	5' gttattctcaaattcagtcttccattcatctctccttcccttccctggcagacagacttctgca gccactg**ggagacat**ggggtgaggaaaggcaagaaccgatgtttcattctctccct 3'
NTF3 WT	5' gttttgtgatccggctctcaggagtcactctgtaaaatctgtgtacaccagtattttgcattcag tattgtcaaggccat**gactgtt**gttttagtaaacttgttaaaatc 3'
NTF3 Mut	5' gttttgtgatccggctctcaggagtcactctgtaaaatctgtgtacaccag tattttgcattcagtattgtcaaggccat**gagacat**gttttagtaaacttgttaaaatc 3'
***GRIK5* WT**	5' gccccgccggcccccgggag ctggcggagccgagtgaccacgggcg**gggctgt**gcg ggcgcccggactgaccgaagggacggggcccgccccaggcc 3'
***GRIK5* Mut**	5' gccccgccggcccccgggagctggcggagccgagtgaccacgggcg**ggggaca**gcg ggcgcccggactgaccgaagggacggggcccgccccaggcc 3'

therefore used as the major target. Interestingly, only one site was predicted for *GRIK5*, position 2,974 in the 3'-UTR by two programs, and therefore by default it was used as the major target site. Once the putative target-sites were chosen for each gene, ~100bp fragment (~50bp up- and down-stream of the target site) was cloned into a reporter construct behind the luciferase gene. Mutant constructs were then made via site-directed mutagenesis, yielding a 4bp mutation in the target-site (Table 6). All constructs were subsequently sequenced to ensure the fidelity of the wild-type and mutant sequences. Next, to assess specificity of miRNA binding, the following combination of miRNAs and targets were transfected in quadruplicate in a 96-well plate: 1) mir-

132 with wild-type target 3'-UTR, 2) miR-132 with mutant target 3'-UTR, 3) miR-132 with wild-type target 3'-UTR and anti-miR-132, and 4) miR-377 (an off-targeting miRNA) with wild-type target 3'-UTR. All reactions were performed in HEK293 cells and assayed 24 hours later. The mean ratio of firefly (F) over renilla (R) luciferase was then calculated for each sample and normalized against the F/R ratio from mock transfected cells (Lipofectamine only).

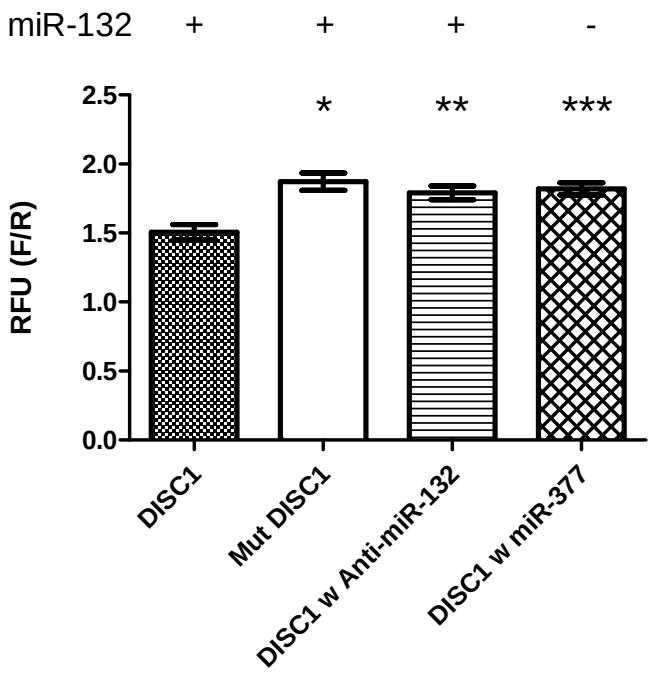


Figure 7: miR-132 target-site specificity. Relative flourescence units (RFU) for miR-132 transfected with the *DISC1* target and respective controls. Stars represent statistically significant differences compared to miR-132 with wild-type *DISC1*. *p=0.0227, **p=0.0323, ***p=0.0221. n=3 for each bar, error bars represent SEM.

Statistically significant differences were observed (Figure 7) between miR-132 transfected with the wild-type *DISC1* 3'-UTR versus the mutated *DISC1* 3'-UTR (p=0.0227, n=3), wild-type *DISC1* 3'-UTR versus wild-type *DISC1* 3'-UTR with anti-miR-132 (p=0.0323, n=3), and wild-type *DISC1* 3'-UTR versus wild-type *DISC1* 3'-UTR transfected with miR-377 (p=0.0221, n=3).

Thus, we conclude that miR-132 binds specifically to the *DISC1* 3'-UTR at position 1,160 and mediates down-regulation of luciferase gene expression, an effect which was alleviated by mutation in the seed sequence, addition of an anti-miR, and addition of an off-targeting miRNA. No significant differences were observed between miR-132 and *GRIK5* or *NTF3* 3'-UTR target constructs, indicating miR-132 does not bind to target sites at positions 2974 and 288 in the respective genes. However, mir-132 may bind to the other predicted binding sites in the 3'-UTR of *GRIK5* and *NTF3*, or binding of other miRNAs may be required to work synergistically with miR-132 for proper inhibition of gene expression to take place.

MiR-137 as a Schizophrenia Candidate Gene

MiR-137 has been directly implicated in the regulation of adult neurogenesis[85], dendritic development, and neuronal maturation[134] and was also found to be one of three microRNAs with targets significantly enriched for association with schizophrenia in a study integrating genetic data from a GWAS with brain imaging as a quantitative trait[135]. Moreover, in a recent genetic mega-analysis of unprecedented size (17,836 cases and 33,859 controls), the strongest novel finding for association with schizophrenia was to a variant within the precursor of miR-137, and four other schizophrenia loci achieving genome-wide significance in the study were predicted to be targeted by miR-137—transcription factor 4 (*TCF4)*, calcium channel, voltage-dependent, L type, alpha 1C subunit (*CACNA1C),* cub and sushi multiple domains 1(*CSMD1*) and chromosome 10 open reading frame 26 (*C10orf26*) (Gejman et al. in press). Furthermore, in the same study, *CACNA1C* and ankyrin 3 (*ANK3*) reached genome-wide significance in a joint analysis of 16,374 cases with schizophrenia or bipolar disorder and 14,044 controls. Interestingly, *ANK3* is also a predicted target for miR-137. Taken together, these data suggest miR-137 plays a role in schizophrenia pathogenesis.

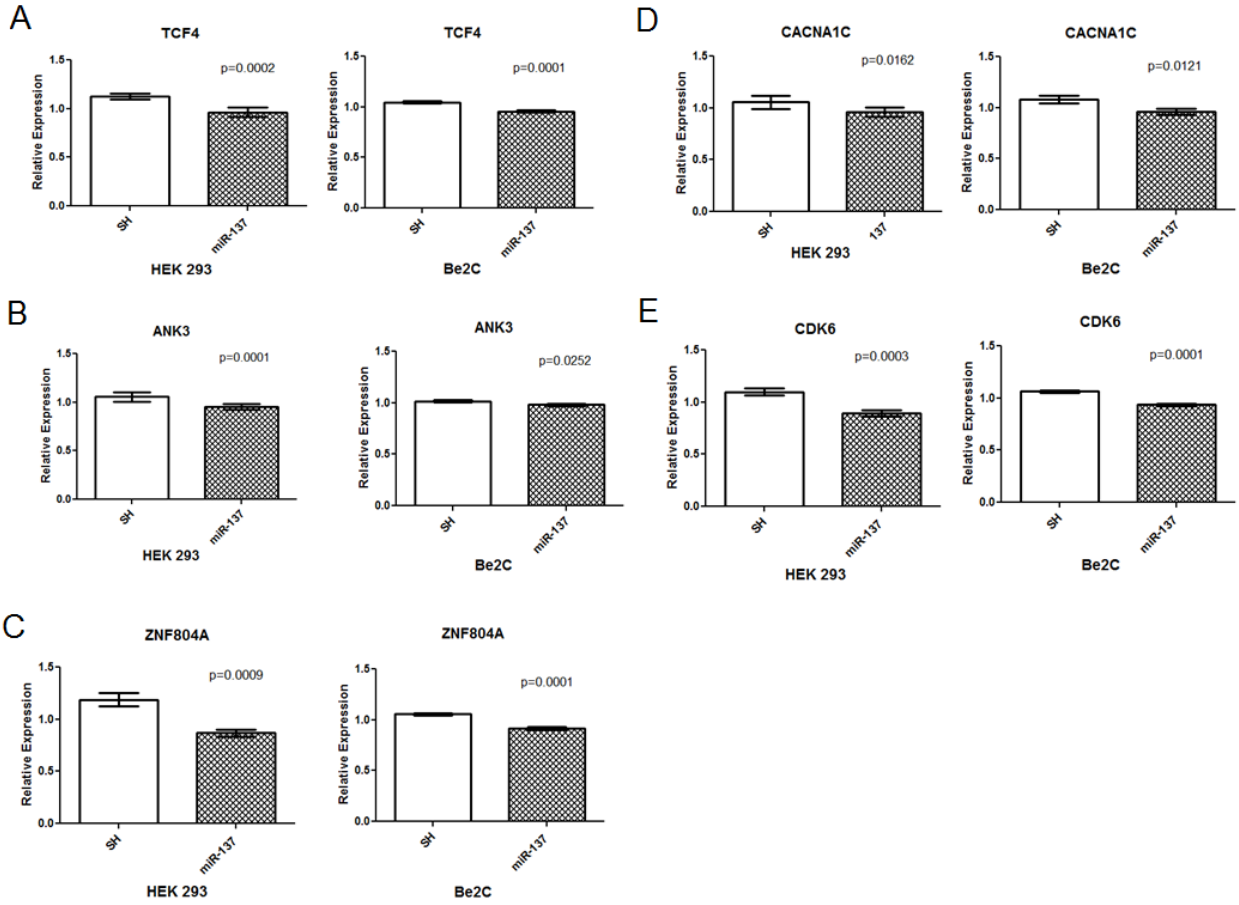
miR-137 Target Gene Validation

Gene Expression

Given that predicted gene targets of miR-137 were found to be associated with schizophrenia, we chose to evaluate three of the top candidates implicated in the mega-analysis, *TCF4*[136], *CACNA1C*[137], and *ANK3*[137] due to their prior association with SZ and BP. In addition to these genes, we used miRecords to compile bioinformatically predicted target genes for miR-137 and discovered two other schizophrenia candidate genes, *CDK6* and *ZNF804A* were among the top predicted targets of miR-137 as well. *CDK6* has been shown to bind directly to *B-Catenin* [138], thereby acting to negatively regulate the Wnt-signalling pathway, which has been shown to play a central role in normal brain development and has been previously implicated in schizophrenia[139-141] as well. *ZNF804A* encodes a protein of unknown function, however it has been associated with schizophrenia and bipolar disorder in multiple studies[142, 143], making it one of the most compelling schizophrenia candidate genes to date.

To assess whether miR-137 down-regulates the putative target genes identified by genetic studies, we transfected either a plasmid containing the miR-137 hairpin or a scrambled hairpin control into HEK293 and Be2C cell lines. After 24 hours, total RNA was isolated and gene expression was evaluated via real-time PCR. All five putative target genes tested were significantly down-regulated at the mRNA level in the miR-137 transfected versus scrambled hairpin transfected conditions in both cell lines (Figure 8). *TCF4* was statistically significantly down-regulated in HEK293($p=0.0359$, $n=27$) and Be2C($p=0.0019$, $n=27$), *CACNA1C* in HEK293($p=0.0162$, $n=27$) and Be2C($p=0.00121$, $n=27$), *ANK3* in HEK293($p=0.0001$, $n=36$) and

Be2C(p=0.0252, n=36), *CDK6* in HEK293(p=0.0003, n=27) and Be2C(p=0.0001, n=27), and finally *ZNF804A* in HEK293(p=0.0009, n=27) and Be2C(p=0.0001, n=27).



<u>Figure 8</u>: miR-137 target gene expression in HEK 293 and Be2C cell lines. SH=Scrambled Hairpin. Student's T-test was used to calculate p-values. N≥27 for each bar, error bars represent SEM.

Protein Expression

Having observed a miR-137 associated down-regulation of *TCF4*, *CACNA1C*, *ANK3, CDK6*, and *ZNF804A* at the mRNA level, we next tested whether the effect would be retained at the protein level. To accomplish this, HEK293 cells were transfected with a plasmid expressing either the miR-137 hairpin or a scrambled hairpin control and whole cell protein lysates were extracted after 24 hours. Western blots were then performed and of the five genes tested,

CDK6(p=0.0127), *TCF4*(p=0.0251) and *CACNA1C*(p=0.0164) were significantly down-regulated at the protein level in miR-137 transfected versus scrambled hairpin transfected control (Figure 9). However, no statistically significant difference in protein level was observed for *ANK3* and no antibody was available for detection of *ZNF804A*.

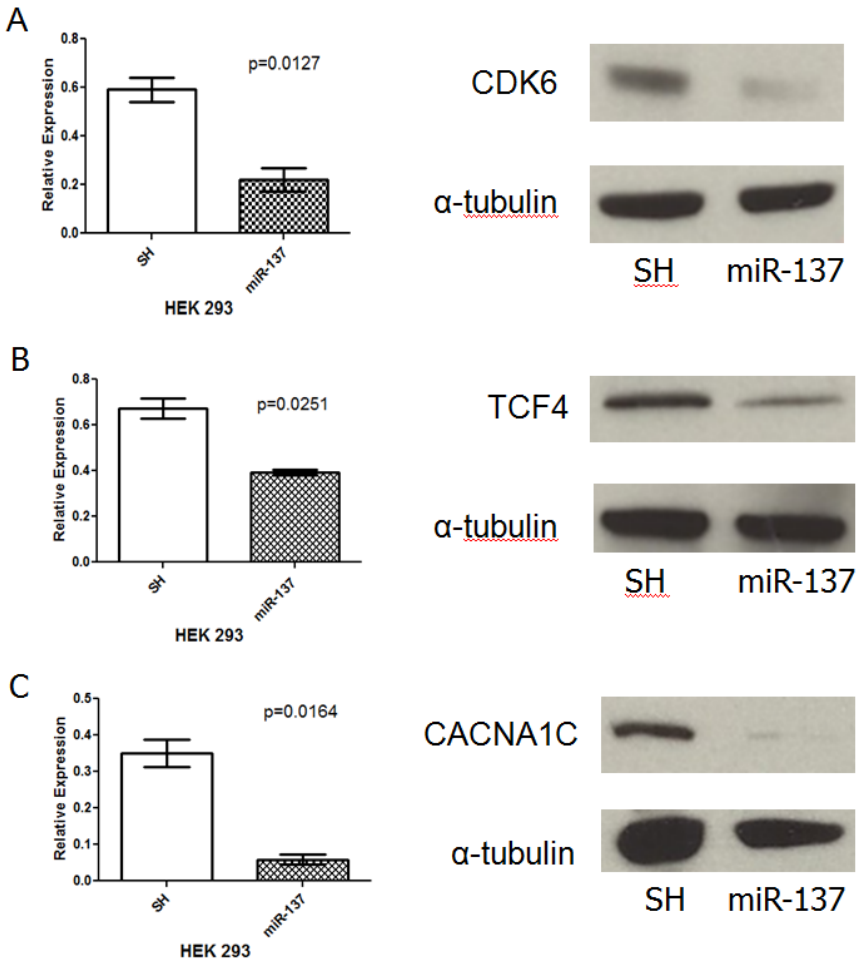


Figure 9: Western blot data for miR-137 gene targets. (A) *CDK6* protein expression (left), representative blot (right), (B) *TCF4* protein expression (left), representative blot (right), (C) *CACNA1C* protein expression, representative blot (right). N=3 for each blot, error bars represent SEM.

miR-137 Target-site Specificity

Given that miR-137 down-regulates *ZNF804A* and *ANK3* at the mRNA level, and *CDK6, TCF4* and *CACNA1C* at both the mRNA and protein levels, we next sought to demonstrate

specificity of miR-137 binding via reporter gene assays. First, miRecords was used to complile predicted miR-137 binding sites in the 3'-UTRs of *ZNF804A, ANK3, CDK6, TCF4,* and *CACNA1C,* the results of which are presented in Table 8. Seven binding sites were predicted for *CDK6*, one of which, position 7,140, was predicted by multiple programs and therefore was used as the major miR-137 target site. Three binding sites were predicted for *TCF4*, one of which, position 4565 was predicted by multiple programs and therefore was used as the major target. Two binding sites were predicted for *ZNF804A* by one program, thus we chose position 4,660 as the major target based on better alignment score. Only one binding site was predicted for *CACNA1C* and *ANK3,* position 1,410 and 230 respectively, and were therefore used as the major target sites for those genes. Once the putative target sites were chosen for each gene, ~100bp fragment (~50bp up- and down-stream of the target site) was cloned into a reporter construct behind the luciferase gene. Mutant constructs were then made via site-directed mutagenesis, yielding a 4bp mutation in the target site (Table 9).

Table 8: Predicted 3'-UTR binding sites for miR-137.

miRNA	RefSeq	Gene	Position in 3'-UTR	Prediction Programs
miR-137	NM_001259	*CDK6*	7140	miRanda, PITA, TargetScan, PicTar
miR-137	NM_003199	*TCF4*	4565	miRanda, PITA, TargetScan
miR-137	NM_194250	*ZNF804A*	4660	PITA
miR-137	NM_020987	*ANK3*	231	miRanda
miR-137	NM_000719	*CACNA1C*	1410	TargetScan

Table 9: 3'-UTR sequences of miR-137 targeted genes cloned into the luciferase reporter construct. WT=wild-type, Mut=Mutant, Bold=seed sequence, underline=site of mutation.

	3'-UTR Sequence
***CDK6* WT**	5'gtacccttacttgaaagtttctaatcttaagttttatgaaatgcaataatatgtatcagct**agcaata**tt tctgtgatcaccaacaactctcagtttgatcttaaagtctgaataataaa 3'
***CDK6* Mut**	5'gtacccttacttgaaagtttctaatcttaagttttatgaaatgcaataatatgtatcagct**aggttaa**tt ctgtgatcaccaacaactctcagtttgatcttaaagtctgaataataaa 3'
***TCF4* WT**	5' cattacctgtttcccatctgtagttattcgatgaagtcatgtacatgaccgttctgt**agcaata**aat gtgccatttttataaactgtttctgacacttgtttcatttcattttgcattgt 3'
***TCF4* Mut**	5' cattacctgtttcccatctgtagttattcgatgaagtcatgtacatgaccgttctgt**aggttaa**aat gtgccatttttataaactgtttctgacacttgtttcatttcattttgcattgt 3'
***ZNF804A* WT**	5' tttagacaaagctgatggcactatgttttgtatcatgttccttgaaactgtaaattcagtgaaaaatatc tctt**gcaata**aatttttgttaactatttaa 3'
***ZNF804A* Mut**	5' tttagacaaagctgatggcactatgttttgtatcatgttccttgaaactgtaaattcagtgaaaaatatc tctt**ggttaa**aatttttgttaactatttaa 3'
***ANK3* WT**	5' tgagccaatttacagagcatggaaatcactttcatttccggattggcgcgtgt**gcaatt** agcaatgcagtgtatatacaagaagccatgctgttaacagttta 3'
***ANK3* Mut**	5' tgagccaatttacagagcatggaaatcactttcatttccggattggcgcgtgt**ggttat** agcaatgcagtgtatatacaagaagccatgctgttaacagttta 3'
***CACNA1C* WT**	5' tctgcttctgaaacgggaatcagtaactctttgcattttctgtcccacaagatatgc aaaaacaat**gcaataa**tattcatttaaaaatacaattgtgagttgtgttggca 3'
***CACNA1C* Mut**	5' tctgcttctgaaacgggaatcagtaactctttgcattttctgtcccacaagatatgc aaaaacaat**ggttaaa**tattcatttaaaaatacaattgtgagttgtgttggca 3'

Next, we transfected the following combination of miRNAs and targets in quadruplicate in a 96-well plate to assess specificity of miRNA binding: 1) mir-137 with wild-type 3'-UTR, 2) miR-137 with mutant 3'-UTR 3) miR-137 with wild-type 3'-UTR and anti-miR-137, and 4) miR-377, an off-targeting miRNA with wild-type 3'-UTR. All reactions were performed in HEK293 cells and assayed 24 hours later. The results are shown in Figure 10.

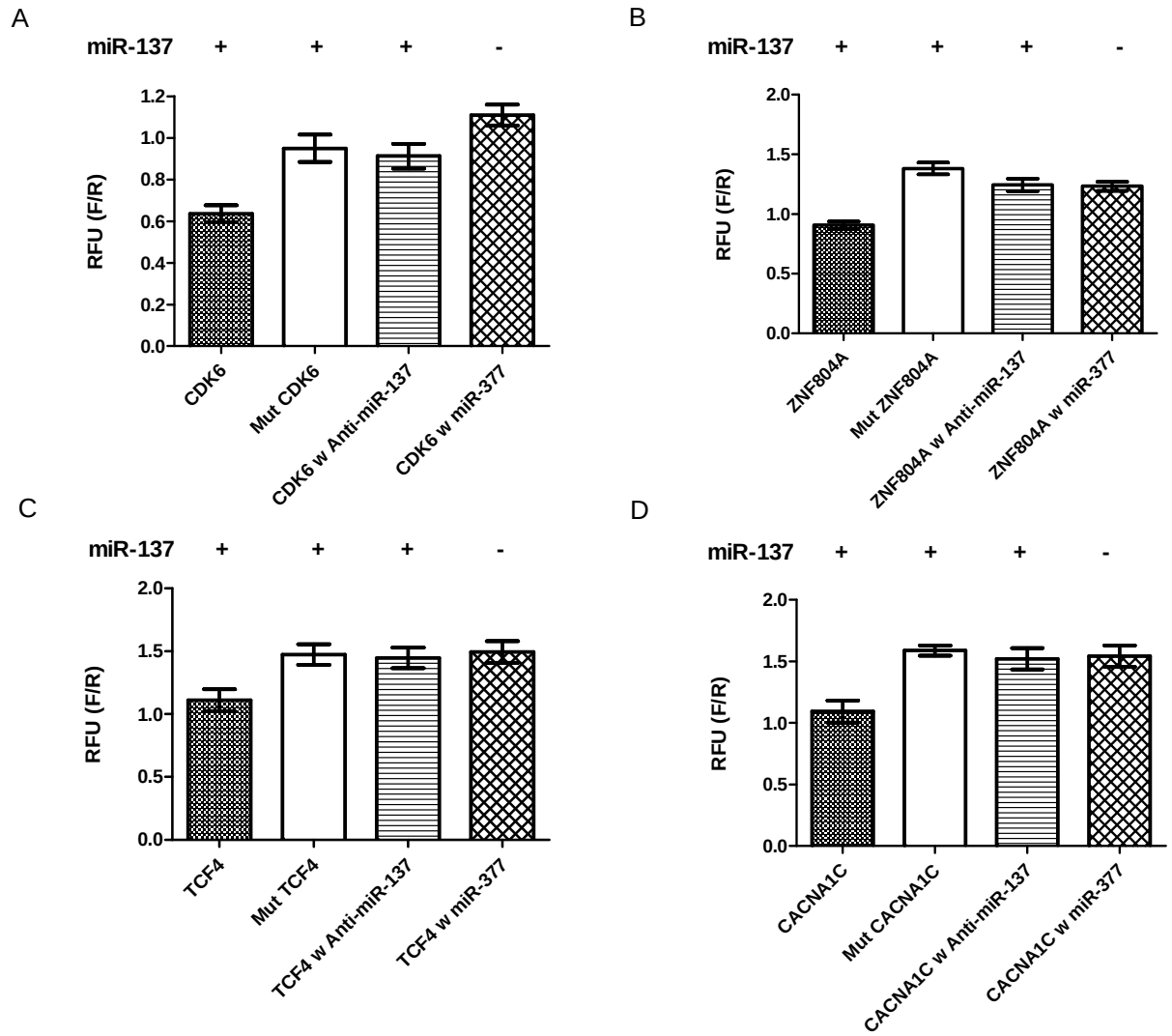


Figure 10: miR-137 target-site specificity. (A) *CDK6*—Statistically significant differences were observed between miR-137 transfected with the wild-type *CDK6* 3'-UTR versus the mutated *CDK6* 3'-UTR (p=0.0264,

n=3), wild-type *CDK6* 3'-UTR versus wild-type *CDK6* 3'-UTR with anti-miR-137 (p=0.0308, n=3), and wild-type *CDK6* 3'-UTR versus wild-type *CDK6* 3'-UTR transfected with miR-377 (p=0.0052, n=3). (B) *CACNA1C*— Statistically significant differences were observed between miR-137 transfected with the wild-type *ZNF804A* 3'-UTR versus the mutated *ZNF804A* 3'-UTR (p=0.004, n=3), wild-type *ZNF804A* 3'-UTR versus wild-type *ZNF804A* 3'-UTR with anti-miR-137 (p=0.0052, n=3), and wild-type *ZNF804A* 3'-UTR versus wild-type *ZNF804A* 3'-UTR transfected with miR-377 (p=0.0012, n=3). (C) *TCF4*— Statistically significant differences were observed between miR-137 transfected with the wild-type *TCF4* 3'-UTR versus the mutated *TCF4* 3'-UTR (p=0.0186, n=3), wild-type *TCF4* 3'-UTR versus wild-type *CDK6* 3'-UTR with anti-miR-137 (p=0.0228, n=3), and wild-type *TCF4* 3'-UTR versus wild-type *TCF4* 3'-UTR transfected with miR-377 (p=0.0311, n=3). (D) *CACNA1C*— Statistically significant differences were observed between miR-137 transfected with the wild-type *CACNA1C* 3'-UTR versus the mutated *CACNA1C* 3'-UTR (p=0.0463, n=3), wild-type *CACNA1C* 3'-UTR versus wild-type *CACNA1C* 3'-UTR with anti-miR-137 (p=0.0472, n=3), and wild-type *CACNA1C* 3'-UTR versus wild-type *CACNA1C* 3'-UTR transfected with miR-377 (p=0.0261, n=3).

Significant down-regulation of luciferase expression was observed for miR-137 binding to specific sites in the 3'-UTRs of *CDK6*, *ZNF804A*, *CACNA1C*, and *TCF4*, an effect which was alleviated by mutation in the seed sequence, addition of an anti-miR, and addition of an off-targeting miRNA. Thus, we have demonstrated miR-137 binds specifically to 3'-UTR target sites in *CDK6* (position 7,140) *, ZNF804A* (position 4,660), *TCF4* (position 4565)*, and CACNA1C* (position 1,410). Furthermore, having observed down-regulation at both the mRNA and protein levels for *CDK6*, *CACNA1C*, and *TCF4*, we conclude that miR-137 can regulate the expression of these genes by binding to specific sites in the 3'-UTR. No statistically significant difference was observed for miR-137 binding to the 3'-UTR target site in *ANK3,* indicating miR-137 does not exert its effect via the target site cloned.

Discussion

MiRNA Expression Profiling

miRNAs have key roles in regulating gene expression and brain development, and thus are likely genetic factors contributing to the etiology of psychiatric disorders. In the first part of this

study, we conducted an evaluation of miRNA expression in postmortem tissues from SZ and BP cases. Of the total number of analyzed miRNAs (N=441), we identified seven to be differentially expressed in SZ and 15 to be differentially expressed in BP, which constitutes ~5% of miRNAs studied. Similar observations have been made in other studies of SZ[79]and autistic[92] cases, respectively. Although relatively few miRNAs showed consistent unidirectional expression differences (22miRNAs, ~5%) between cases and controls, we did observe a large number of miRNAs with significant variance differences in SZ and BP, many of which were shared by both disease groups. Thus we, as others[92, 144], argue that the development of SZ and BP is likely to reflect two classes of miRNAs: the first class consisting of miRNAs showing consistent unidirectional expression across all cases in the disease groups, and the second class showing much greater individual variability of expression.

Several studies have attempted to address differential expression of miRNAs in the etiology of SZ. Perkins and colleagues[79] were the first to evaluate miRNA expression in a postmortem brain sample consisting of 13 individuals with SZ and 2 individuals with schizoaffective disorder. They identified 16 miRNAs dysregulated in cases, two of which, hsa-miR-7 and -212, were also differentially expressed in our study. In two separate studies, Beveridge and colleagues observed 26 differentially expressed miRNAs in the dorso-lateral prefrontal cortex (DLPFC) of SZ cases[78, 82], one of which, miR-7, was differentially expressed in our study. Finally, in a study of SZ and autistic cases[92], 28 miRNAs were shown to be dysregulated in cases, two of which, miR-7 and -132, were differentially expressed in our study.

In addition to being dysregulated in SZ and autistic cases, miR-7 has been shown to be involved in the development of Parkinson's disorder by repressing the expression of alpha

synuclein (α-Syn), a protein that accumulates in the nigral dopaminergic neurons[108]. MiR-7 was also shown to be involved in the development of glioblastoma by repressing the epidermal growth factor receptor (*EGFR*) gene and the *AKT1* signaling pathway[145]. The repressing effect of miR-7 on *AKT1* is noteworthy since *AKT1* has been shown to be associated with BP[146] disorder and with SZ across multiple populations[147-151].

MiR-132 and miR-212 form a cluster located on the reverse strand of chromosome 17 (17p13.3). Transcription of this cluster has been shown to be up-regulated by the neurotrophin *BDNF*, brain-derived neurotrophic factor[112, 115]. Neurotrophins are growth factors that are important for neuronal development and survival as well as synapse formation and plasticity[127]. MiR-132 has also been implicated in regulating neuronal morphogenesis[112] via the regulation of *p250GAP*[111, 114] and over-expression of miR-132 has been found to promote neurite outgrowth and dendritic spine formation, whereas inhibitors of miR-132 reduced these processes[113, 152]. Furthermore, *MECP2* (methyl CpG binding protein 2) and *SIRT1* (sirtuin 1), both of which can act as transcriptional regulators, have been suggested to be miR-132 targets[153, 154]. *MECP2* is required for maturation of neurons and mutations in *MECP2* underlie Rett syndrome, an X-linked neurodevelopmental disorder. Lastly, mir-132 and -212 expressions have been reported to be dysregulated in Alzheimer's disease[155], suggesting a broader impact of these miRNAs on diseases of brain development.

Functional Validation of miRNA Targets

miR-132

We focused on miR-132 because it was highly expressed and highly significant in our study, has been shown to be involved in several neuronal processes, and has been implicated in other miRNA expression studies of autism and Alzheimer's. We used bioinformatic algorithms to predict potential gene targets for miR-132, and chose to further investigate seven genes due to their biological relevance to the etiological hypotheses of schizophrenia and/or prior strong association with schizophrenia. Of the seven genes, three were significantly down-regulated at the mRNA level in miR-132 over-expressed versus scrambled hairpin control, *NTF3*, *DISC1* and *GRIK5*, suggesting miR-132 affects these transcripts. Taking these genes forward for investigation at the protein level, only *GRIK5* was observed to be significantly down-regulated. We also sought to demonstrate that miR-132 binds specifically to target-sites in the 3'-UTRs of *NTF3*, *DISC1* and *GRIK5*. After cloning the putative miR-132 target sites and respective mutants into a luciferase construct, we observed a significant down-regulation of luciferase activity compared to controls for *DISC1* only, indicating miR-132 binds specifically at position 1,160 in the *DISC1* 3'-UTR. Interestingly, we observed down-regulation of *DISC1* at the mRNA level and demonstrated that this effect can be mediated by miR-132 binding to a specific target-site in the 3'-UTR. However, we were unable to detect down-regulation of *DISC1* at the protein level, which can be reconciled by, 1) *DISC1* protein is not down-regulated at an appreciable level, i.e. a small change in protein that may not be detected by Western blot, or 2) an alternative mechanism exists that compensates for *DISC1* expression.

miR-132 and *NTF3*

Neurotrophins are important regulators for survival, differentiation, and maintenance of nerve cells and act to promote the growth and differentiation of new neurons[127]. *NTF3* belongs to a family of neurotrophins, which include *NGF* (nerve growth factor) and *BDNF* (Brain Derived Neurotrophic Factor), and has been shown to encourage axon elongation and branching[156] by inhibition of GSK-3β via the phophatidylinositol-3-kinase (PI3K)/Akt pathway (Figure 11). Similarly, in a reverse genetic study, Ma et al. found that ablation of NTF3 in the mouse neocortex resulted in reduction of a set of axonal bundles projecting from the thalamus through the cortical white matter. These bundles included thalamo-cortical axons that normally establish connections with the retrosplenial and visual cortex[157]. The retrosplenial cortex is part of the cingulate cortex and is implicated in the recall of episodic information and is one of several brain regions that produce anterograde amnesia when damaged. Taken together, miR-132 related down-regulation of NTF3 mRNA, particularly during development, may prevent axon elongation and branching and reduce thalamo-cortical connections leading to the manifestation of symptoms later in life. Thus, although miR-132 mediated down-regulation of *NTF3* protein was not observed in our study using cell lines, one might be able to observe a down-regulation in an animal model focusing on neurodevelopment.

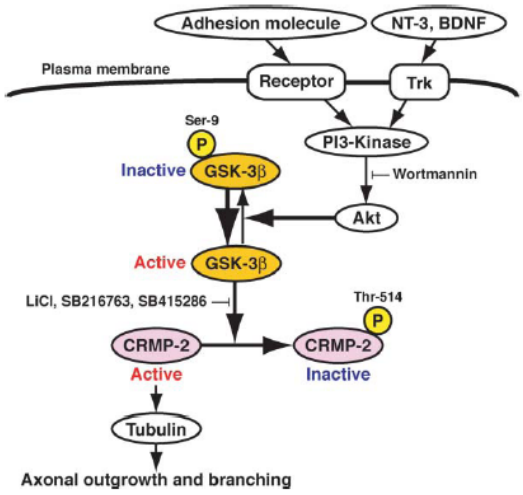


Figure 11: Schematic of the role of neurotrophins NTF3 and BDNF on the PI3K/Akt pathway. Adapted from Yoshimura et al., *Cell*, 2009.

miR-132 and *DISC1*

Linkage and association studies in multiple populations have identified *DISC1* as s susceptibility factor for schizophrenia[122-124]. Although the function of *DISC1* is unclear, multiple studies indicate a role for *DISC1* in the developing brain. In a yeast-2-hybrid analysis, Ozeki et al. demonstrated *DISC1* binds to NudE-like (NUDEL), a cytoskeletel protein essential for cortical development and axon growth[126]. Failure of this interaction has been shown to result in inhibition of neurite outgrowth *in vitro*[158] and abnormal cortical development *in vivo*[159]. Furthermore, observing *DISC1* expression during mouse development, Schurov et al. reported the amount of *DISC1* peaks in the mouse brain during the time of embryonic neurogenesis and again during puberty[160], two critical time points implicated in the pathophysiology of schizophrenia[161]. Thus, similar to *NTF3,* miR-132-mediated down-regulation of *DISC1* may play a key role in schizophrenia pathology during development.

miR-132 and *GRIK5*

Glutamate is the major excitatory neurotransmitter in the central nervous system. Nearly half of the neurons in the brain, including *all* neurons that project from the cerebral cortex, are believed to use glutamate as their neurotransmitter. Glutamate receptors are classified into two broad categories: ionotropic and metabotropic receptors. Ionotropic glutamate receptors, which include NMDA, kainate, and AMPA subtypes, initiate rapid depolarization by facilitating sodium or calcium entry into neurons through channels formed by the receptor itself. *GRIK5* codes for an ionotropic glutamate receptor in the kainate receptor family (Table 10).

Table 10: Ionotropic glutamate receptors. Adapted from Dingledine et al. *Pharmacol. Rev.*, 1999.

Receptor Family	Subunit	Gene	Chromosome (human)
AMPA	$GluR_1$	*GRIA1*	5q33
	$GluR_2$	*GRIA2*	4q32-33
	$GluR_3$	*GRIA3*	Xq25-26
	$GluR_4$	*GRIA4*	11q22-23
Kainate	$GluR_5$	*GRIK1*	21q21.1-22.1
	$GluR_6$	*GRIK2*	6q16.3-q21
	$GluR_7$	*GRIK3*	1p34-p33
	KA-1	*GRIK4*	11q22.3
	KA-2	*GRIK5*	19q13.2
NMDA	NR1	*GRIN1*	9q34.3
	NR2A	*GRIN2A*	16p13.2
	NR2B	*GRIN2B*	12p12
	NR2C	*GRIN2C*	17q24-q25
	NR2D	*GRIN2D*	19q13.1qter
	NR3A	*GRIN3A*	9q31.1
	NR3B	*GRIN3B*	19p13.3

The glutamate hypothesis of schizophrenia posits that the function of the N-methyl-D-aspartate (NMDA) receptor is compromised in disease. This hypothesis came about due to the observation that NMDA receptor antagonists such as phencyclidine (PCP) and ketamine induced schizophrenia-like psychosis in normal volunteers and exacerbated psychotic symptoms in schizophrenic patients[3, 162]. These data have been interpreted to suggest that NMDA receptor hypo-activity is associated with schizophrenia.

However, it is not apparent whether NMDA receptor hypo-activity results from a primary defect in NMDA receptors or from dysfunction in one of the other three glutamate receptor families that may secondarily lead to low levels of NMDA receptor activity. It has been shown that glutamate release from pre-synaptic kainate receptors can affect post-synaptic NMDA receptor-mediated neurotransmission[163, 164]. In this way, dysfunction of the *GRIK5* receptor could mimic abnormal NMDA receptor activity. Furthermore, expression studies in post-mortem brains have shown significantly lower levels of *GRIK5* mRNA in several brain regions including the prefrontal cortex[119], hippocampus[120] and thalamus[117]. Specifically, using *in situ*

hybridization, Meador-Woodruff et al. observed decreased *GRIK5* mRNA expression in Brodmann areas 9 and 46, both of which correspond to the prefrontal cortex, with the latter being the same brain region used in our miRNA profiling study[119]. In addition, Ibrahim et al. observed *GRIK5* mRNA was significantly reduced in the centromedial nucleus of the thalamus, which is a major thalamic relay between prefrontal, cingulate, and other limbic cortical areas, and the dorsomedial nucleus of the thalamus, which projects primarily to the prefrontal cortex[117]. Thus, given that we observed miR-132 was up-regulated in the prefrontal cortex (Brodmann area 46) of schizophrenic brains, and miR-132 down-regulated *GRIK5* at both the mRNA and protein levels, we provide strong evidence for a role of miR-132 disrupting glutamatergic transmission in schizophrenia.

miR-137

Although expression studies have not shown miR-137 to be differentially expressed in schizophrenia, in our study, miR-137 demonstrated significant variance differences in SZ compared to controls, with a trend toward over-expression (Figure 12).

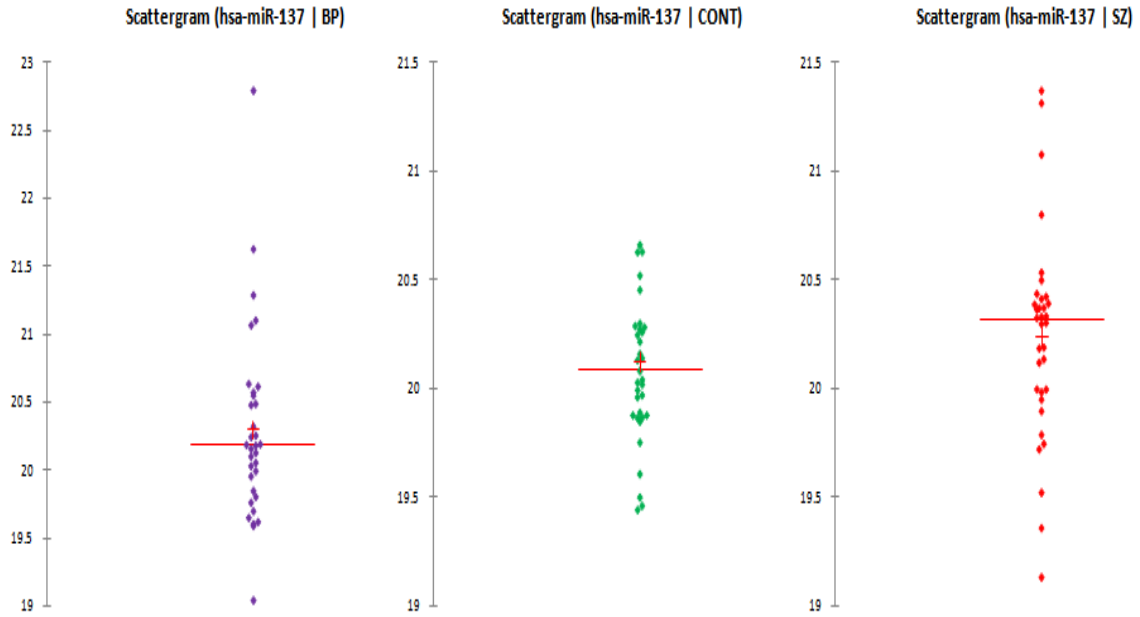


Figure 12: Scattergram of miR-137 expression in the SMRI postmortem brain sample. Horizontal line indicates mean expression value (Ct).

Several genetic studies of schizophrenia have implicated miRNAs to be associated with disease and recently, miR-137 was found to be the strongest novel finding in the largest genetic study of schizophrenia to date (Gejman et al. in press). Additionally, predicted gene targets of miR-137 were also significantly associated in the study. Thus, it was the goal of our study to validate the predicted interactions between a miRNA discovered by a genetic study, miR-137, and its predicted target genes. We investigated five genes predicted to be targeted by miR-137—*TCF4*, *CACNA1C*, *ANK3, CDK6*, and *ZNF804A*. All five were significantly down-regulated at the mRNA level in miR-137 over-expressed versus scrambled hairpin control and three, *CACNA1C, TCF4,* and *CDK6* were significantly down-regulated at the protein level as well. Going further, we also demonstrated that miR-137 binds specifically to target sites in the 3'-UTR

of *CACNA1C, TCF4,* and *CDK6*, suggesting that repression of these genes is directly mediated by miR-137.

miR-137 and *TCF4*

TCF4 belongs to to a subfamily of basic helix-loop-helix (bHLH) transcriptional regulators which have been shown to play important roles as neural progenitors in the developing nervous system[165] and mammalian cortex[166]. Two large genetic studies have implicated TCF4 as a promising candidate gene for schizophrenia. Stefansson et al. reported the C allele of the TCF4 rs9960767 polymorphism to be more frequent in patients with schizophrenia, leading to a 1.23-fold higher risk for the disorder[136]. This finding has been recently replicated in a mega-GWAS (Gejman et al. in press). Quednow et al. further investigated the influence of the disease-associated C allele on pre-pulse inhibition (PPI), an established endophenotype of schizophrenia, and found the schizophrenia-associated C allele to be strongly associated with reduced PPI[167]. PPI deficits have been implicated in the biological basis of schizophrenia and other neuropsychiatric disorders characterized by deficient suppression or 'gating' of irrelevant sensory, cognitive, or motor processes[168]. In addition to being associated with schizophrenia in two large genetic studies, haploinsufficiency of the *TCF4* gene has been shown to cause Pitt-Hopkins syndrome, a neurodevelopmental disease characterized by severe mental retardation, epilepsy, facial dysmorphisms, and intermittent hyperventilation[169-171]. In this way, mir-137 mediated down-regulation of TCF4 may in part, contribute to the pathogenesis of schizophrenia.

miR-137 and CDK6

The Wnt signalling pathway has been shown to play a central role in normal brain development, cell fate determination, and synaptic plasticity[172-174]. A schematic of the Wnt

signalling pathway is depicted in Figure 13. Expression of genes in the Wnt signalling pathway have also been shown to be altered in schizophrenia. Using immunohistochemical analysis of post-mortem brains, Miyaoka et al. observed signifcant increases in the number of Wnt-1 immunoreactive cells in CA3 and CA4 regions of the hippocampus[140]. In a similar study, Cotter et al. observed signifcant reductions of β-Catenin in the same hippocampal regions of postmortem brain tissue[139]. Taken together, these observations point to a malfunction of β-Catenin in schizophrenia, which in turn leads to a reactive increase in Wnt-1 expression. CDK6 has been shown to negatively regulate Wnt signalling by phosphorylating β-Catenin, thereby initiating its degradation[138]. It has been proposed that CDK6 participates in a negative feedback loop which could serve to restrict the duration or spread of β-Catenin mediated signals. Thus, miR-137- mediated down-regulation of CDK6 may disrupt the normal inhibition of β-Catenin-mediated gene expression , i.e. miR-137 inhibits an inhibitor, leading to uncontrolled expression of genes involved in schizophrenia pathogenesis.

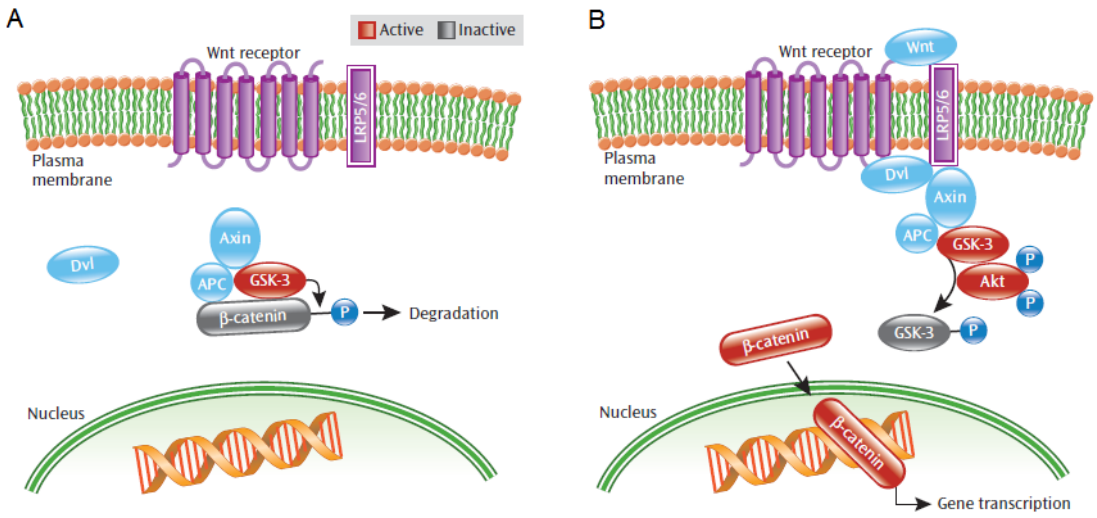


Figure 13: Schematic of the Wnt signalling pathway. (A) In the absence of Wnt receptor activation, B-catenin is constitutively phosphorylated by GSK-3, leading to the degradation of B-catenin and inhibition of B-catenin-mediated gene transcription. (B) Binding of Wnt to its receptor results in the formation of a

complex which phosphorylates GSK-3, preventing it from phosphorylating B-catenin. B-catenin consequently escapes degradation and accumulates in the nucleus where it regulates transcription through interactions with DNA transcription factors. Adapted from Freyberg et al., *Am. J. Psychiatry,* 2010.

miR-137 and *CACNA1C*

Voltage-gated Ca^{2+} channels have a central role in neuronal function and are essential for converting electrical activity into biochemical events. *CACNA1C* codes for the alpha 1C subunit of the L-type Ca^{2+} channel (LTC) and has been previously implicated as a risk factor for bipolar disorder[137], and more recently for schizophrenia[20] as well. Genetic variation in *CACNA1C* has been associated with decreased working memory processing in the prefrontal cortex[175] and a missense mutation in *CACNA1C* results in Timothy syndrome, which is characterized by multi-organ dysfunction, including cardiac arrhythmias and cognitive abnormalities[176]. Activation of LTCs have also been shown to specifically increase the expression of genes such as BDNF that are important for neuronal survival, learning, and adaptive responses in the nervous system[177]. Furthermore, a number of transcription factors essential for neuronal survival and plasticity such as CREB and MEF-2, have been shown to be dependent on Ca^{2+} fluxes through LTCs[178 179]. Given the important roles of LTCs in brain function, miR-137-mediated down-regulation of CACNA1C may lead to defects in calcium transmission in neurons, thereby contributing to schizophrenia pathology.

Conclusions

In this study, we evaluated 667 miRNAs, 441 of which showed reliable expression in a postmortem brain sample consisting of SZ, BP and control subjects. Twenty-two miRNAs were found to be differentially expressed between cases and controls, two of which, hsa-miR-7 and miR -212 were previously reported to be dysregulated in SZ. To our knowledge, we were also the first to report differentially expressed miRNAs in BP disorder, and in agreement with

previous observations of a shared genetic etiology of SZ and BP[20], we observed a large number of miRNAs with significant variance differences were shared by both disease groups.

This study also provides functional validation for two miRNAs implicated in schizophrenia by very different approaches, miR-132 via expression studies and miR-137 via a genetic study. MiR-132 was shown to target *NTF3*, *DISC1*, and *GRIK5* and mir-137 was shown to directly target *CACNA1C*, *TCF4*, and *CDK6*. Our study provides additional evidence for miRNA involvement in the etiology of schizophrenia. The nature of genes targeted by miR-132 and miR-137 strongly supports dysfunction of neurotransmission (*GRIK5, CACNA1C*) and disruption of signaling pathways during development (*NTF3*, *DISC1*, *TCF4*, and *CDK6*), both of which strengthen previously existing hypotheses of schizophrenia etiology.

Future Directions

Schizophrenia is a complex disease with many factors contributing to its etiology. While it is likely that multiple pathways are involved in the pathogenesis of schizophrenia, the Wnt/β-Catenin/GSK3β pathway appears to be a promising mechanism that may further elucidate the complexities of the disease. Thus, future work could focus on dissecting the pathway further by assessing in detail, how the miR-132 or miR-137 affects members of the pathway individually, or more interestingly, collectively. This can be accomplished by measuring gene expression for each member of the pathway after over-expression of the miRNAs *in vitro.* Given that miR-132 and miR-137 target genes involved in neurotransmission and development, it would also be interesting to assess the effects of miR-132 and miR-137 in animal models. This can be accomplished by over- or under-expression of the miRNA of interest in a mouse model and assessing the effects in specific brain regions of interest (DLPFC, STG) or more globally at different stages of development.

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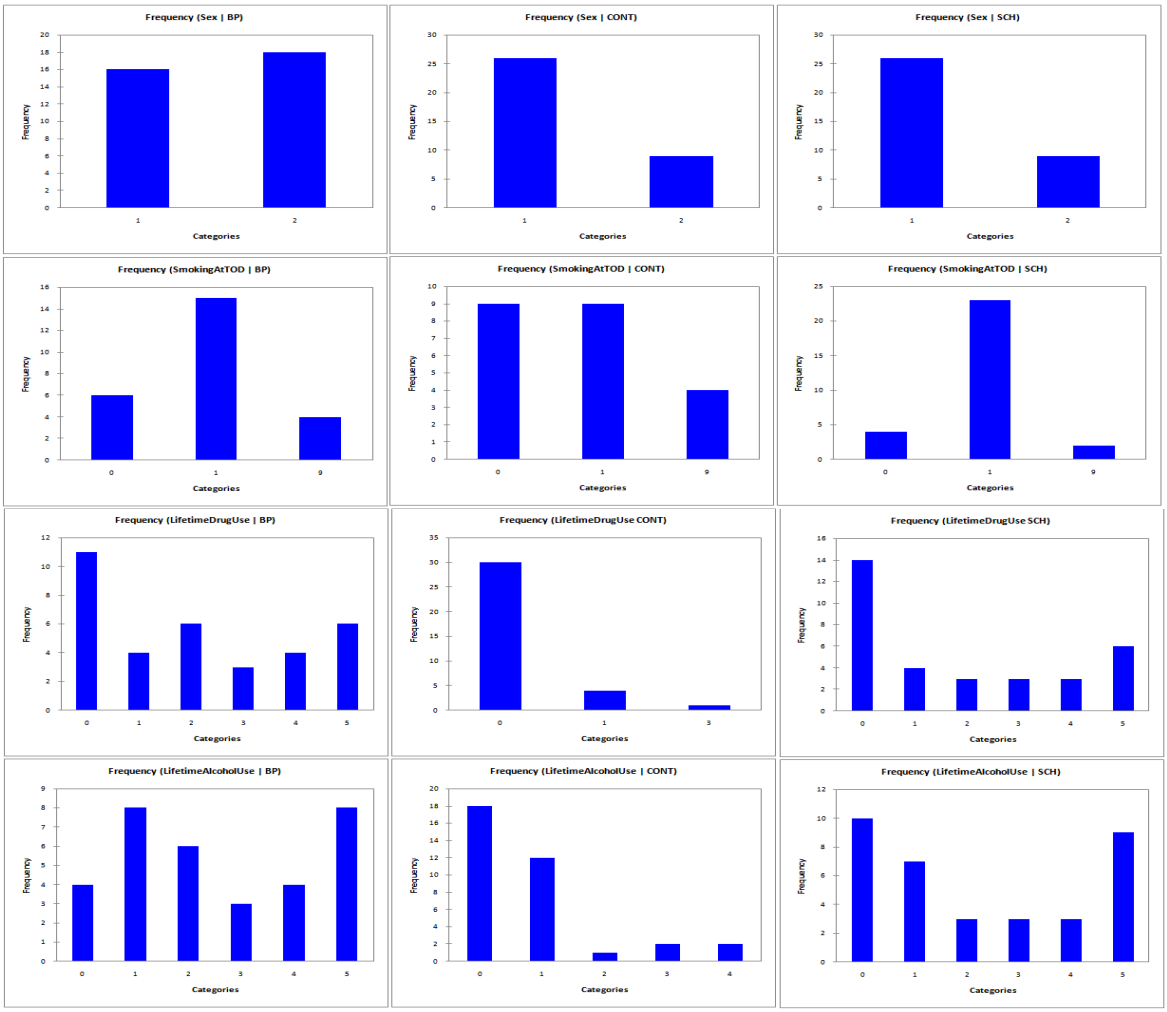
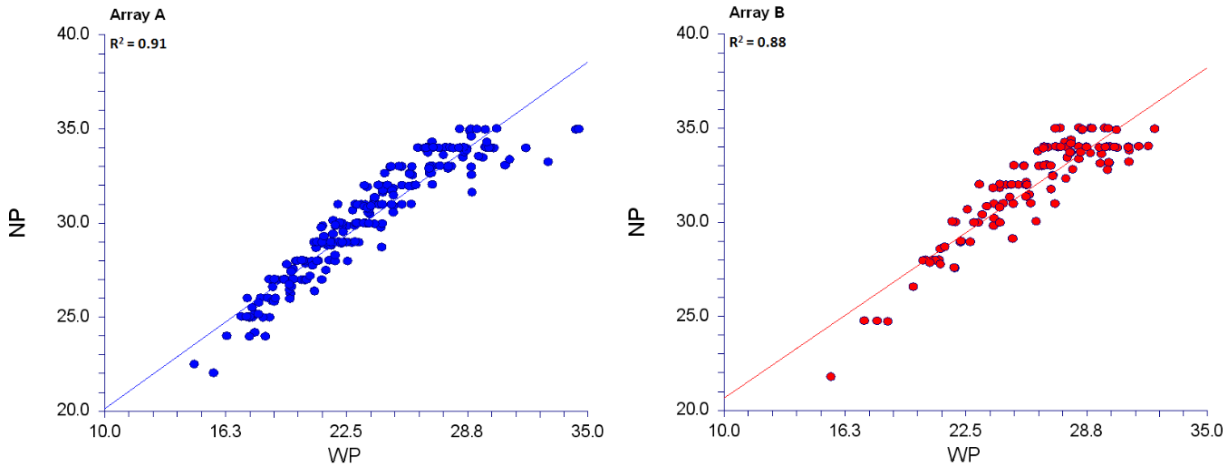
Appendix 1

Graphical representation of descriptive measures of the SMRI sample.



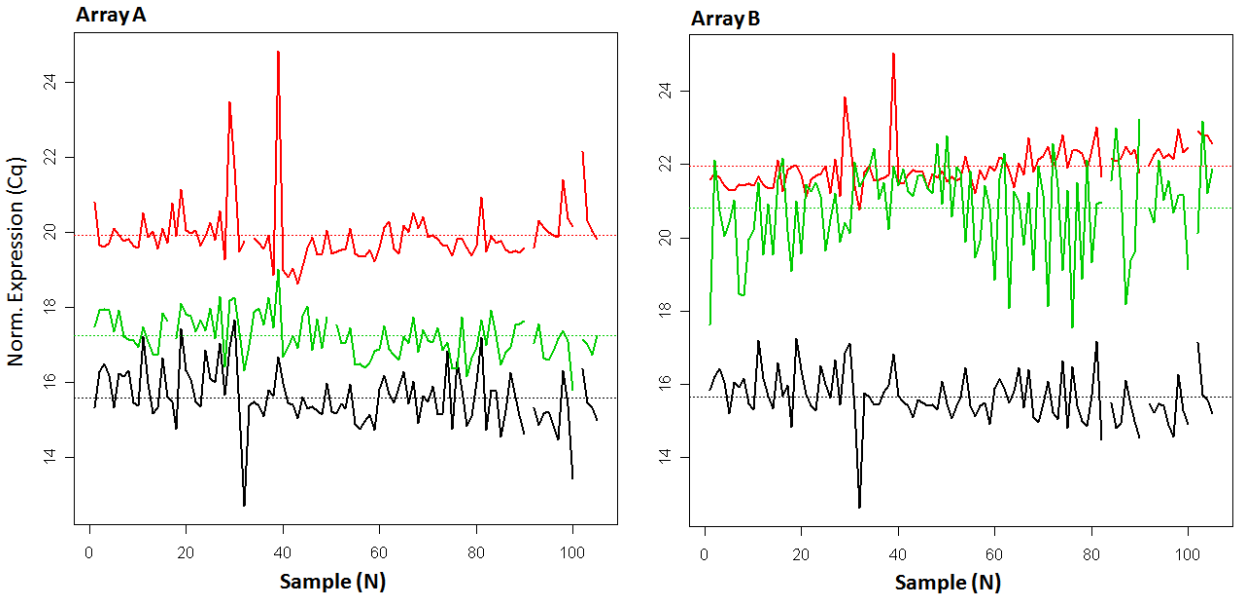
Appendix 2

Correlation plot of miRNA expression preamplified (WP) and not preamplified (NP).



Appendix 3

Expression of endogenous controls. Red (Top)=U6, Green (Middle)=RNU48 and Black (Bottom)=RNU44.



Appendix 4

Detailed description of the median polish normalization approach.

Artifacts of measurement may systematically raise or lower all the miRNA values measured from one sample to another. If we observe that all samples are raised simultaneously, it is more likely that this reflects a systematic bias in the sample rather than the miRNAs actually being more abundant. Therefore, we attempted to compensate for such artifacts using the U6, U44, and U48 RNAs included as controls within the TLDA arrays on the common assumption that these RNAs are expressed at the same levels in all samples. However, we found that they were as variable across samples as many of the experimental miRNAs, thereby demonstrating that in our sample, the provided controls cannot be used for normalization. Thus, we adopted an alternate method used in medium sized microarray gene expression data sets to estimate a sample-specific compensation value.

First, we used the median polish procedure to estimate a centering constant using the most stably expressed genes. Median polish is a robust centering procedure, which is insensitive to a large number of truly differentially expressed miRNA genes. The procedure assumes that the majority of genes considered are unaffected by the conditions under study, and tries to fit centering constants for each sample. The idea is that differences in the medians of the samples reflect biases having to do with the sample preparation. The median polish algorithm iteratively subtracts medians of rows and columns until all rows and columns have a median of zero, while keeping track of the amounts subtracted from each row and column. In this case we worked iteratively: each of the two arrays (A and B) were processed separately; for array for array A we pre-selected the 34 miRNA genes and for array B 26 miRNA genes with the smallest variance (< 0.1) under the assumption that they were most likely to be stable. We then fit the median polish

to those genes only, and normalized the data by subtracting the sample means. We then determined the genes of smallest variance again and repeated the procedure until no change occurred.

Next, we tested post-hoc whether the assumptions of the normalizing procedure were valid. We were concerned that perhaps some genes were stable in the control subjects, but not in the case subjects. In fact, the within-diagnosis standard deviations of miRNA genes were highly correlated across the different groups ($0.79 < r < 0.86$, Figure 14). Furthermore, the correlations between samples of the set of reference genes used for normalization across samples was very high (mean correlation: 0.99; minimum pair-wise correlation: 0.96), confirming that these genes were indeed stably expressed in our samples. The normalization constants for each array were computed separately.

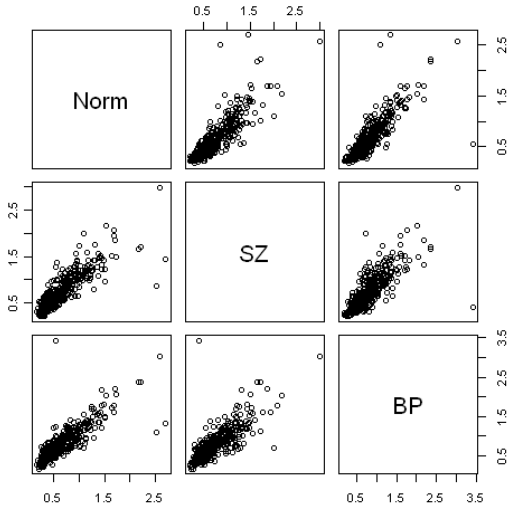
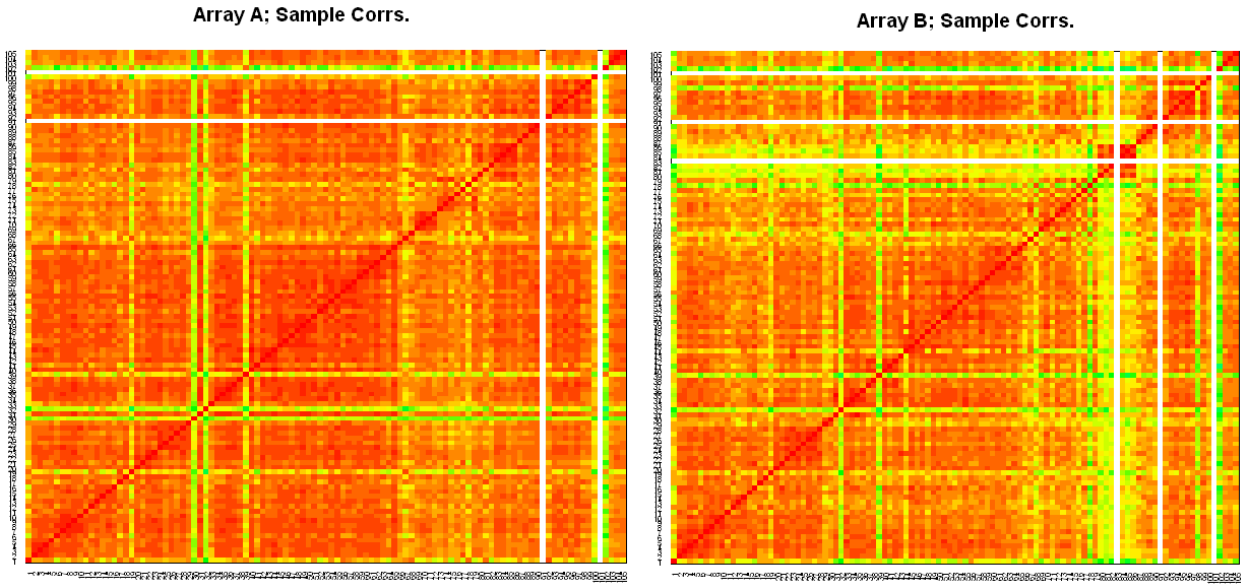


Figure14: Correlations of the reference set of genes used for normalization across samples.

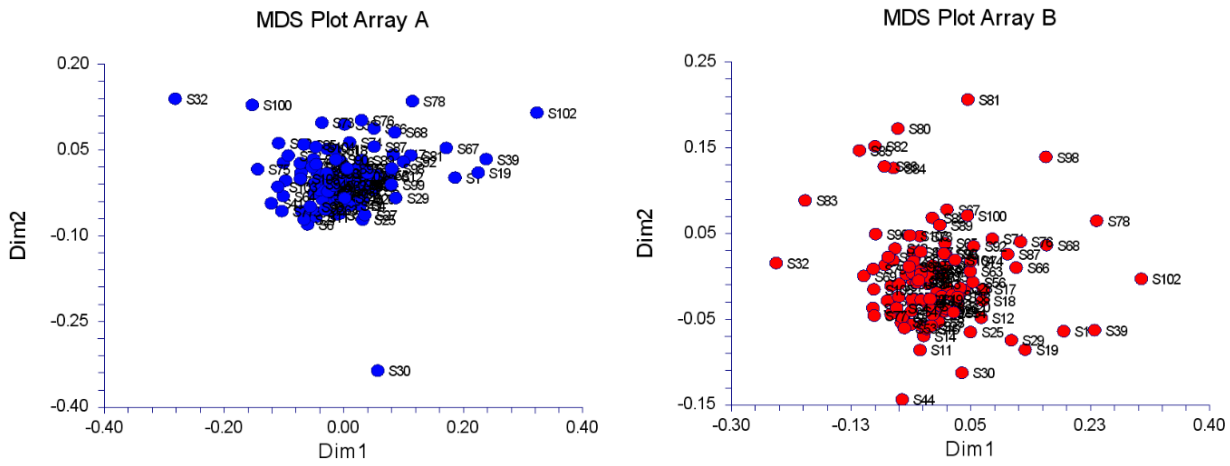
Appendix 5A

Within plate correlation plots.



Appendix 5B

Multi-dimensionality plot showing clustering of samples.



Appendix 6

Statistics of normalized expression values for the 441 miRNAs.

ID	Count	Aver. (Cq)	SD	CV %	Range	Skewness	Kurtosis
hsa-let-7a	87	18.92	0.43	2.30%	2.2701	0.3165	0.2851
hsa-let-7a*	87	28.14	0.77	2.73%	4.2356	0.8174	1.5093
hsa-let-7b	87	17.47	0.28	1.61%	1.3833	-0.9034	0.1916
hsa-let-7b*	87	27.03	0.78	2.87%	4.9655	4.2710	6.7163
hsa-let-7c	87	19.66	0.41	2.10%	2.4212	-5.2548	7.7019
hsa-let-7d	87	19.96	0.33	1.66%	2.044	5.8947	9.6632
hsa-let-7e	87	17.26	0.41	2.37%	2.2308	-1.1356	1.6802
hsa-let-7e*	87	29.06	0.56	1.93%	2.4563	1.2395	-1.0567
hsa-let-7f	87	21.49	0.50	2.33%	2.2821	-1.2064	-0.1848
hsa-let-7f-1*	87	28.40	0.80	2.82%	4.3268	-2.1882	2.6576
hsa-let-7f-2*	87	29.08	0.37	1.27%	2.0931	2.9943	2.4451
hsa-let-7g	87	20.28	0.33	1.63%	1.9367	3.6690	3.7113
hsa-let-7g*	87	28.27	1.61	5.71%	5.5744	1.6361	6.4364
hsa-let-7i*	87	29.48	0.63	2.13%	2.9014	0.0624	-0.4341
hsa-miR-1	87	26.68	0.68	2.57%	3.4263	3.3900	1.4303
hsa-miR-100	87	17.51	0.34	1.95%	1.4665	0.5593	-0.8982
hsa-miR-100*	87	30.57	0.82	2.67%	3.9843	2.6783	1.0260
hsa-miR-101	87	23.68	0.25	1.07%	1.5183	-0.3788	1.2102
hsa-miR-101*	87	29.72	0.88	2.97%	4.9734	-0.8521	0.6825
hsa-miR-103	87	20.18	0.21	1.04%	1.1272	-4.6704	5.0015
hsa-miR-105	87	27.17	0.53	1.96%	2.6583	-0.8954	-0.4686
hsa-miR-106a	87	20.26	0.42	2.05%	1.6684	-0.9735	-1.2973
hsa-miR-106b	87	21.75	0.32	1.48%	1.5013	-0.7111	-0.4000
hsa-miR-106b*	87	26.01	0.78	3.01%	5.3244	4.5852	6.5573
hsa-miR-107	87	23.86	0.44	1.86%	2.5924	-1.6183	2.0355
hsa-miR-10b*	87	29.68	1.02	3.44%	5.5488	-0.0950	1.6916
hsa-miR-122	87	26.85	2.06	7.69%	10.0607	-2.9378	0.4664
hsa-miR-124	87	20.81	0.38	1.81%	2.2141	1.5194	2.3255
hsa-miR-124*	87	25.59	0.53	2.06%	2.9863	2.1558	1.6603
hsa-miR-125a-3p	87	29.33	0.56	1.93%	2.9541	-1.8539	1.1719
hsa-miR-125a-5p	87	21.03	0.41	1.93%	1.9198	-1.3212	-0.8103
hsa-miR-125b	87	15.97	0.19	1.20%	1.0996	2.5733	2.7780
hsa-miR-125b-1*	87	27.45	0.63	2.28%	3.6651	-4.7028	5.0487
hsa-miR-125b-2*	87	24.49	0.35	1.43%	1.8304	-2.1258	2.6262
hsa-miR-126	87	17.60	0.39	2.20%	1.5321	0.3313	-1.8086
hsa-miR-126*	87	20.72	0.35	1.69%	1.4412	0.9034	-1.2456
hsa-miR-127-3p	87	19.17	0.34	1.80%	1.5058	0.3592	-0.8170
hsa-miR-127-5p	87	28.63	0.86	3.02%	3.8291	-1.7096	-0.8222
hsa-miR-128	87	18.73	0.38	2.05%	2.1199	2.0425	3.1106

hsa-miR-129-3p	87	20.48	0.42	2.05%	2.1721	1.8457	1.0158
hsa-miR-129-5p	87	23.92	0.38	1.60%	2.071	0.1565	1.6737
hsa-miR-130a	87	23.12	0.38	1.63%	1.9778	2.2928	0.9944
hsa-miR-130b	87	24.65	0.40	1.61%	1.9943	-3.0882	1.8232
hsa-miR-130b*	87	26.53	0.41	1.53%	2.0706	-2.3041	0.6714
miR-132	87	17.47	0.47	2.69%	2.8203	2.8944	3.4303
miR-132*	87	27.18	0.68	2.49%	3.4555	0.3625	1.0389
hsa-miR-133a	87	23.13	0.40	1.72%	2.2208	0.4538	0.7701
hsa-miR-133b	87	24.98	0.38	1.50%	1.9497	-0.0391	-0.0757
hsa-miR-134	87	23.60	0.43	1.84%	2.337	-0.1243	0.1106
hsa-miR-135a	87	22.09	0.46	2.07%	2.6091	0.3674	1.4821
hsa-miR-135a*	87	21.14	0.54	2.55%	2.5556	-2.7362	0.2393
hsa-miR-135b	87	25.08	0.28	1.11%	1.3005	1.9241	0.2659
hsa-miR-136	87	31.89	0.79	2.47%	3.3366	-1.5626	-0.5988
hsa-miR-136*	87	23.82	0.31	1.30%	1.3905	-0.1032	-0.4930
hsa-miR-137	87	20.13	0.40	1.98%	2.0606	-0.3194	0.9275
hsa-miR-138	87	17.94	0.33	1.85%	2.7019	7.4656	22.2351
hsa-miR-138-1*	87	25.02	0.33	1.31%	2.0654	-3.2734	4.3541
hsa-miR-138-2*	87	24.15	0.46	1.88%	2.1638	0.2955	-0.7896
hsa-miR-139-3p	87	21.57	0.43	1.97%	2.6348	0.4056	2.2260
hsa-miR-139-5p	87	18.22	0.44	2.42%	1.9286	-0.8852	-1.0970
hsa-miR-140-3p	87	25.14	0.43	1.72%	2.2255	-0.7223	-0.3805
hsa-miR-140-5p	87	20.53	0.27	1.33%	1.2184	0.4208	-0.8578
hsa-miR-141	87	30.20	0.86	2.84%	4.1337	-2.6053	1.3481
hsa-miR-142-3p	87	22.00	0.52	2.37%	2.6063	-2.1093	1.0701
hsa-miR-142-5p	87	28.54	0.76	2.67%	4.0169	-0.6223	0.9147
hsa-miR-143	87	21.51	0.65	3.01%	3.4916	-4.5894	3.8018
hsa-miR-144*	87	25.02	0.84	3.36%	4.0201	1.0794	-0.6909
hsa-miR-145	87	17.83	0.64	3.57%	3.5266	-1.9825	1.3806
hsa-miR-145*	87	26.60	0.68	2.56%	3.4221	-2.0168	0.7136
hsa-miR-146a	87	21.51	0.35	1.63%	1.4798	-0.6886	-1.2820
hsa-miR-146b-3p	87	28.03	0.41	1.46%	2.0241	2.5881	1.1239
hsa-miR-146b-5p	87	20.74	0.38	1.81%	1.6842	0.3731	-0.7504
hsa-miR-148a	87	24.78	0.48	1.95%	2.5307	0.5218	0.4675
hsa-miR-148b	87	24.60	0.38	1.53%	2.0791	-2.5783	2.5591
hsa-miR-148b*	87	29.27	0.44	1.49%	2.7151	1.7787	3.3533
hsa-miR-149	87	18.34	0.44	2.42%	2.1824	0.7603	-0.2744
hsa-miR-150	87	21.05	0.40	1.89%	1.8247	0.4700	-0.3314
hsa-miR-151-3p	87	22.04	0.57	2.59%	2.8735	-0.6982	-0.0761
hsa-miR-152	87	23.14	0.33	1.43%	1.8354	-1.8806	1.2614
hsa-miR-153	87	26.98	0.62	2.30%	3.1414	0.5577	0.8549
hsa-miR-154	87	27.77	0.62	2.22%	3.2457	2.2368	1.8775
hsa-miR-154*	87	26.31	0.68	2.59%	3.614	3.0711	2.4678
hsa-miR-15a	87	22.88	1.36	5.95%	13.6252	15.5518	63.1890
hsa-miR-15a*	87	27.20	0.45	1.66%	2.2119	-1.2509	1.1206

hsa-miR-15b	87	22.03	0.40	1.81%	2.3341	1.1943	1.4732
hsa-miR-16	87	19.47	0.50	2.57%	2.5271	-1.1617	0.2453
hsa-miR-16-1*	87	28.19	0.67	2.36%	3.1842	0.5733	0.4160
hsa-miR-17	87	20.07	0.41	2.04%	1.6213	0.2446	-1.4738
hsa-miR-17*	87	27.70	0.78	2.83%	4.121	1.0159	-0.6803
hsa-miR-181a	87	18.35	0.53	2.90%	2.9363	0.4191	0.5338
hsa-miR-181a*	87	23.91	0.44	1.86%	2.1818	-1.1201	0.0890
hsa-miR-181a-2*	87	24.91	0.38	1.54%	1.72	-1.5483	-0.4374
hsa-miR-181c	87	22.72	0.47	2.07%	2.5333	1.1857	0.6402
hsa-miR-181c*	87	23.25	0.32	1.39%	2.0113	0.9785	3.0822
hsa-miR-182	87	33.08	1.69	5.11%	7.6459	-2.8495	0.6042
hsa-miR-184	87	26.44	0.44	1.68%	2.1143	0.4286	-0.2667
hsa-miR-185	87	23.49	0.47	2.02%	2.0788	0.7608	-0.3664
hsa-miR-186	87	22.17	0.25	1.12%	1.2903	0.4069	0.1411
hsa-miR-187	87	25.92	0.80	3.10%	5.3938	-2.6110	5.7338
hsa-miR-188-5p	87	25.68	0.82	3.19%	4.2685	-2.3392	1.3768
hsa-miR-18a	87	24.77	0.48	1.96%	2.6443	-1.1691	1.4458
hsa-miR-18a*	87	29.50	0.76	2.59%	3.8157	-0.3771	0.0028
hsa-miR-18b	87	24.36	0.34	1.41%	2.3833	-4.7088	9.6091
hsa-miR-190	87	25.79	0.43	1.66%	1.8305	-0.5560	-0.9296
hsa-miR-190b	87	30.65	0.73	2.37%	3.5096	0.5310	-0.5975
hsa-miR-191	87	18.84	0.42	2.21%	1.6696	-0.9946	-1.1823
hsa-miR-192	87	25.68	0.48	1.86%	2.3049	-0.2541	0.1634
hsa-miR-192*	87	30.62	0.52	1.70%	3.2939	3.0681	5.3559
hsa-miR-193a-3p	87	30.12	1.21	4.01%	6.1	-1.1374	1.0431
hsa-miR-193a-5p	87	25.23	0.47	1.88%	2.3593	-1.6999	-0.0448
hsa-miR-193b	87	20.74	0.41	1.99%	1.9822	-0.4180	-0.4114
hsa-miR-193b*	87	25.96	0.55	2.13%	2.5074	-0.4129	-0.6098
hsa-miR-194	87	25.58	0.33	1.29%	1.4261	0.2985	-1.2154
hsa-miR-195	87	20.54	0.31	1.50%	1.6889	2.8017	1.4674
hsa-miR-197	87	20.91	0.47	2.25%	2.6035	-0.7538	1.3099
hsa-miR-199a-3p	87	23.99	0.63	2.62%	3.2026	-1.3596	0.0022
hsa-miR-199a-5p	87	28.55	0.95	3.34%	4.6276	0.1311	-0.5590
hsa-miR-199b-5p	87	28.82	0.71	2.46%	4.6123	0.1634	2.5528
hsa-miR-19a	87	22.22	0.50	2.26%	2.8502	-4.4479	6.1215
hsa-miR-19b	87	17.38	0.36	2.06%	1.8254	-1.9553	0.9810
hsa-miR-19b-1*	87	29.05	0.61	2.10%	3.3292	0.2485	0.2629
hsa-miR-200a	87	24.88	0.60	2.42%	2.7774	-0.4772	-0.6009
hsa-miR-200b	87	26.21	0.50	1.91%	2.5907	0.1336	0.1661
hsa-miR-200c	87	26.66	0.43	1.61%	2.5003	2.2497	2.4018
hsa-miR-202	87	29.03	1.38	4.76%	6.164	-0.7220	-1.0702
hsa-miR-203	87	23.31	0.39	1.66%	1.9311	0.8256	-0.3526
hsa-miR-204	87	20.10	0.31	1.54%	1.5067	0.0860	0.0748
hsa-miR-205	87	32.02	1.23	3.86%	6.7576	2.3491	1.2164
hsa-miR-206	87	31.20	0.73	2.33%	4.1648	2.4354	1.6443

hsa-miR-20a	87	20.03	0.42	2.10%	2.4962	-0.4270	1.2913
hsa-miR-20a*	87	26.92	0.59	2.18%	2.8892	1.4192	0.3082
hsa-miR-20b	87	22.73	0.33	1.43%	1.7347	-1.8776	0.0482
hsa-miR-20b*	87	32.09	1.60	4.98%	8.9037	-0.3853	2.1889
hsa-miR-21	87	19.99	0.50	2.48%	2.6267	0.3443	0.5302
hsa-miR-21*	87	29.21	0.99	3.39%	4.4701	-0.2039	-0.5986
hsa-miR-210	87	22.20	0.46	2.07%	2.2139	-1.1587	-0.1885
hsa-miR-212	87	23.04	0.54	2.36%	3.1163	2.0453	2.2044
hsa-miR-214	87	24.44	0.67	2.74%	3.368	-1.2799	0.0650
hsa-miR-214*	87	28.35	0.80	2.81%	3.8914	-1.4320	-0.1119
hsa-miR-215	87	25.70	0.39	1.52%	2.2111	-2.9435	4.9399
hsa-miR-216b	87	29.65	1.13	3.80%	5.6674	1.1889	0.2556
hsa-miR-217	87	31.34	1.23	3.94%	6.8314	1.6268	1.4686
hsa-miR-218	87	18.22	0.38	2.08%	2.4744	0.9099	3.2209
hsa-miR-218-2*	87	29.90	0.56	1.87%	2.422	-0.0017	-1.1275
hsa-miR-219-2-3p	87	21.06	0.87	4.13%	4.3646	1.5532	0.3272
hsa-miR-219-5p	87	20.11	0.93	4.61%	4.4281	1.3551	-0.0507
hsa-miR-22	87	21.44	0.55	2.58%	3.7524	-0.0796	4.2355
hsa-miR-22*	87	23.19	0.52	2.23%	2.453	2.2130	-0.3719
hsa-miR-221	87	19.39	0.35	1.80%	1.7726	1.4205	0.7313
hsa-miR-221*	87	28.93	0.49	1.68%	2.5785	3.8111	3.0580
hsa-miR-222	87	18.46	0.40	2.15%	2.219	2.4654	2.7550
hsa-miR-222*	87	25.87	0.88	3.40%	4.3797	0.4590	0.0180
hsa-miR-223	87	19.60	0.49	2.48%	2.6463	-2.6955	2.1490
hsa-miR-223*	87	28.92	0.78	2.70%	3.2223	-0.7672	-1.1056
hsa-miR-224	87	28.39	0.76	2.67%	3.6252	-0.9306	-0.2840
hsa-miR-23b	87	22.06	0.50	2.25%	2.7013	0.5501	0.6236
hsa-miR-24	87	17.11	0.25	1.47%	1.2732	0.0286	0.1246
hsa-miR-25	87	23.50	0.36	1.54%	1.8257	-0.7096	0.6084
hsa-miR-26a	87	17.94	0.25	1.42%	1.4163	2.8493	2.2414
hsa-miR-26a-1*	87	28.20	0.57	2.02%	2.481	-0.5544	-0.8955
hsa-miR-26a-2*	87	29.15	0.97	3.33%	5.6657	-3.3561	3.1734
hsa-miR-26b	87	21.00	0.37	1.75%	2.7542	-1.3667	6.4597
hsa-miR-26b*	87	27.79	0.34	1.23%	1.721	-1.7352	1.0943
hsa-miR-27a	87	23.05	0.35	1.53%	1.7225	-0.7769	0.4044
hsa-miR-27a*	87	29.43	0.83	2.83%	4.9145	-0.7297	1.2658
hsa-miR-27b	87	21.37	0.37	1.74%	2.1213	0.7003	1.5250
hsa-miR-27b*	87	25.84	0.45	1.76%	2.2952	0.7933	-0.3419
hsa-miR-28-3p	87	23.09	0.45	1.93%	3.4311	8.1041	23.3063
hsa-miR-28-5p	87	23.51	0.22	0.92%	1.0848	-0.6670	-0.0598
hsa-miR-296-5p	87	23.67	0.43	1.81%	1.9928	0.0409	-0.7087
hsa-miR-299-5p	87	27.46	0.61	2.23%	3.204	3.7068	2.1575
hsa-miR-29a	87	17.64	0.23	1.32%	1.0223	-0.3084	-0.6439
hsa-miR-29a*	87	24.91	0.39	1.55%	2.1759	4.1430	4.6904
hsa-miR-29b	87	21.30	0.39	1.82%	2.2267	1.1587	1.3419

hsa-miR-29b-2*	87	26.23	0.30	1.15%	1.8765	5.8686	9.4044
hsa-miR-29c	87	20.10	0.25	1.23%	1.5466	1.8456	2.3149
hsa-miR-29c*	87	23.71	0.27	1.13%	1.5767	5.0536	5.2358
hsa-miR-301a	87	21.49	0.32	1.51%	1.564	-0.3127	-0.3375
hsa-miR-301b	87	27.78	0.37	1.33%	2.1808	0.5409	2.7999
hsa-miR-302a	87	31.54	1.39	4.41%	8.904	-2.4991	4.6879
hsa-miR-302a*	87	32.52	1.47	4.52%	7.837	-0.7376	2.5463
hsa-miR-302b	87	31.14	0.59	1.89%	2.9762	0.8044	0.5420
hsa-miR-302c	87	31.76	1.91	6.02%	8.5246	-2.6295	-0.1135
hsa-miR-30a	87	20.45	0.28	1.36%	1.3192	0.2114	-1.1273
hsa-miR-30a*	87	20.24	0.21	1.04%	1.2331	-2.6300	3.4824
hsa-miR-30b	87	17.22	0.37	2.17%	2.0399	2.4390	1.2340
hsa-miR-30c	87	16.81	0.30	1.76%	1.4857	0.1236	-0.0915
hsa-miR-30c-1*	87	27.37	0.55	2.02%	3.3174	3.7290	4.0428
hsa-miR-30d	87	21.92	0.38	1.75%	2.3889	-3.3431	6.0141
hsa-miR-30d*	87	25.71	0.30	1.15%	1.5849	1.6164	0.7561
hsa-miR-30e	87	21.87	0.20	0.90%	0.8044	-0.8268	-1.7647
hsa-miR-30e*	87	20.02	0.20	0.98%	1.1254	-0.9048	1.2045
hsa-miR-31	87	21.50	0.30	1.41%	1.6755	-2.2301	1.5875
hsa-miR-32	87	29.18	0.84	2.89%	4.8023	2.4062	2.7324
hsa-miR-320	87	21.74	0.59	2.70%	2.8032	-0.9462	-0.2666
hsa-miR-323-3p	87	22.40	0.36	1.60%	1.6264	2.3320	-0.3355
hsa-miR-324-3p	87	23.74	0.22	0.94%	1.0742	-0.9625	-0.4302
hsa-miR-324-5p	87	21.48	0.37	1.72%	1.6916	1.8813	-0.3928
hsa-miR-326	87	28.04	0.48	1.72%	2.4658	-0.1472	-0.0142
hsa-miR-328	87	18.52	0.29	1.59%	1.7475	4.5034	5.6440
hsa-miR-329	87	26.31	0.38	1.46%	1.8993	1.3412	0.2967
hsa-miR-330-3p	87	21.63	0.40	1.84%	1.8271	-0.8022	-0.6214
hsa-miR-330-5p	87	26.77	0.53	1.96%	2.4719	-0.6744	-1.0527
hsa-miR-331-3p	87	18.67	0.30	1.62%	1.8819	2.6233	2.7904
hsa-miR-331-5p	87	27.88	0.46	1.64%	2.5806	-0.1008	1.2568
hsa-miR-335	87	21.80	0.32	1.46%	1.6007	0.9162	0.5616
hsa-miR-335*	87	25.00	0.42	1.68%	2.0397	-2.0724	0.0676
hsa-miR-337-3p	87	26.68	0.76	2.83%	5.0823	0.2471	4.2107
hsa-miR-337-5p	87	29.39	1.32	4.49%	6.9669	4.1160	2.8735
hsa-miR-338-3p	87	23.79	0.83	3.48%	4.0882	-0.7053	-0.1190
hsa-miR-339-3p	87	23.81	0.34	1.41%	2.1237	1.0283	3.1958
hsa-miR-339-5p	87	23.91	0.40	1.69%	1.9126	-1.7554	0.0145
hsa-miR-33a*	87	27.20	0.45	1.64%	2.2603	-2.5612	1.6085
hsa-miR-340	87	21.90	0.46	2.08%	2.0941	0.6383	-0.3370
hsa-miR-340*	87	22.98	0.49	2.12%	2.1664	0.9281	-0.2301
hsa-miR-342-3p	87	18.08	0.36	1.98%	1.5964	-0.8807	-1.4193
hsa-miR-342-5p	87	25.89	0.44	1.70%	2.0357	-0.9114	-0.9061
hsa-miR-345	87	23.69	0.35	1.49%	1.5454	0.1758	-0.8081
hsa-miR-34a	87	21.82	0.38	1.75%	1.6124	1.7313	-0.5488

hsa-miR-34a*	87	26.12	0.35	1.33%	1.6497	0.6446	-0.5474
hsa-miR-34b*	87	27.22	0.81	2.97%	3.5598	1.1079	-0.9286
hsa-miR-34c-5p	87	25.70	0.74	2.89%	3.8366	0.6646	-0.0173
hsa-miR-361-3p	87	23.82	0.55	2.32%	3.5974	3.9507	7.6584
hsa-miR-361-5p	87	23.83	0.26	1.08%	1.5285	1.0531	2.3510
hsa-miR-362-3p	87	28.09	0.49	1.76%	2.614	2.6006	2.1986
hsa-miR-362-5p	87	26.79	0.31	1.17%	1.673	0.3343	0.3478
hsa-miR-363	87	26.34	0.32	1.20%	1.7829	0.6989	0.5843
hsa-miR-365	87	21.03	0.35	1.68%	1.6758	1.8187	-0.0811
hsa-miR-367	87	31.36	1.88	5.98%	11.1117	-0.8832	4.6070
hsa-miR-369-3p	87	29.04	0.70	2.42%	3.538	1.4679	0.5272
hsa-miR-369-5p	87	29.15	0.53	1.83%	2.695	1.6734	0.6065
hsa-miR-370	87	21.73	0.37	1.71%	1.8575	-0.1173	0.8297
hsa-miR-372	87	32.02	2.67	8.34%	12.688	-0.7476	-0.4479
hsa-miR-374a	87	21.80	0.43	1.96%	2.6492	1.0093	2.6856
hsa-miR-374a*	87	29.79	0.82	2.75%	5.626	6.8915	13.1055
hsa-miR-374b	87	21.60	0.45	2.07%	2.5281	3.0915	3.2646
hsa-miR-374b*	87	30.05	0.86	2.85%	5.2577	-4.6488	8.0278
hsa-miR-375	87	25.52	0.47	1.84%	2.9372	0.0246	1.7234
hsa-miR-376a	87	23.31	0.32	1.38%	1.6576	-0.0150	0.2917
hsa-miR-376a*	87	27.95	0.49	1.75%	2.396	2.3781	0.2769
hsa-miR-376c	87	22.59	0.26	1.14%	1.1947	-0.8527	-0.8033
hsa-miR-377	87	28.59	0.76	2.65%	4.342	1.9429	2.5011
hsa-miR-377*	87	27.68	0.50	1.81%	2.3278	0.6377	-0.5355
hsa-miR-378	87	25.98	0.38	1.48%	2.445	-1.9289	4.7760
hsa-miR-378*	87	30.03	0.41	1.38%	2.1715	-0.4978	5.2227
hsa-miR-379	87	22.62	0.41	1.82%	2.2375	0.5529	2.1352
hsa-miR-379*	87	28.65	0.62	2.17%	2.7437	-1.7587	-0.5575
hsa-miR-380	87	28.14	0.94	3.35%	4.2823	-1.8834	-0.0722
hsa-miR-380*	87	27.15	0.41	1.50%	1.9358	-0.3484	-0.9582
hsa-miR-381	87	29.13	0.54	1.84%	2.9734	0.7438	0.8320
hsa-miR-382	87	20.52	0.35	1.71%	1.8909	0.6971	0.8369
hsa-miR-383	87	21.68	0.39	1.81%	2.7751	3.6631	8.3822
hsa-miR-409-3p	87	22.03	0.51	2.30%	3.0612	-0.1851	1.7361
hsa-miR-409-5p	87	26.09	0.52	2.01%	2.5125	0.8050	-0.4895
hsa-miR-410	87	23.49	0.38	1.62%	2.1602	2.0876	2.1263
hsa-miR-411	87	22.78	0.38	1.65%	1.6122	-0.5449	-1.0115
hsa-miR-411*	87	26.99	0.40	1.50%	1.9328	-0.1164	-0.6901
hsa-miR-422a	87	29.94	0.52	1.75%	2.3978	1.0532	-0.2209
hsa-miR-423-5p	87	24.12	0.55	2.30%	2.8542	-0.8682	1.2729
hsa-miR-424	87	28.64	0.59	2.05%	2.9741	0.5664	-0.5622
hsa-miR-424*	87	25.48	0.69	2.71%	4.9076	1.0410	6.9127
hsa-miR-425	87	22.23	0.28	1.25%	1.2846	0.4850	-0.3057
hsa-miR-425*	87	25.98	0.34	1.30%	2.3555	-2.9515	7.7714
hsa-miR-429	87	30.03	0.59	1.98%	2.7815	1.1113	-0.3374

hsa-miR-431	87	26.98	1.11	4.12%	5.4307	1.5201	0.2728
hsa-miR-431*	87	26.86	1.19	4.44%	6.7544	-0.8758	0.0231
hsa-miR-432	87	23.70	0.41	1.72%	3.0775	-7.5521	20.1181
hsa-miR-433	87	22.68	0.35	1.55%	2.3559	1.0733	4.3338
hsa-miR-448	87	29.32	1.16	3.96%	5.658	-2.3581	0.9789
hsa-miR-449a	87	31.37	1.60	5.10%	8.208	-1.8597	0.9920
hsa-miR-449b	87	30.61	1.22	4.00%	7.1756	-1.7078	7.3111
hsa-miR-450a	87	29.26	0.69	2.37%	3.0832	-0.0142	-0.8315
hsa-miR-451	87	21.04	0.74	3.52%	4.1163	-0.5332	0.7486
hsa-miR-452	87	28.69	0.68	2.36%	3.8547	-0.8035	0.7374
hsa-miR-454	87	23.61	0.52	2.20%	2.1746	-1.2750	-1.1289
hsa-miR-454*	87	30.53	1.04	3.39%	6.582	2.5848	4.4825
hsa-miR-455-3p	87	25.40	0.54	2.13%	2.5456	-0.3328	-0.1728
hsa-miR-455-5p	87	26.47	0.40	1.50%	2.3705	-1.2120	2.2594
hsa-miR-483-5p	87	26.66	0.81	3.04%	3.6908	-0.2070	-0.3372
hsa-miR-484	87	19.75	0.35	1.77%	1.5947	-1.8024	-0.1831
hsa-miR-485-3p	87	23.30	0.45	1.91%	2.1891	2.7476	1.1189
hsa-miR-485-5p	87	22.42	0.88	3.91%	3.9679	-3.0570	0.4264
hsa-miR-486-3p	87	26.96	0.63	2.35%	3.5735	0.4927	2.1370
hsa-miR-486-5p	87	26.57	0.63	2.36%	3.2597	-0.2521	0.2950
hsa-miR-487a	87	26.61	0.40	1.49%	1.9389	0.4778	-0.0337
hsa-miR-487b	87	22.80	0.48	2.10%	2.5878	3.0554	2.4604
hsa-miR-488	87	24.69	0.36	1.45%	1.7402	-1.0800	-0.0846
hsa-miR-488*	87	26.83	0.83	3.09%	3.7127	2.7080	-0.1266
hsa-miR-489	87	28.07	0.92	3.29%	4.1609	-1.1035	-1.2450
hsa-miR-490-3p	87	26.82	0.60	2.23%	3.2608	-2.2773	2.4126
hsa-miR-491-5p	87	22.26	0.35	1.56%	1.9118	0.5318	0.4121
hsa-miR-493	87	30.49	1.24	4.08%	5.7348	-2.9167	0.6796
hsa-miR-493*	87	30.48	0.93	3.07%	4.6776	1.5547	0.6912
hsa-miR-494	87	25.53	0.36	1.40%	1.7777	-0.3752	0.4274
hsa-miR-495	87	21.34	0.41	1.92%	2.2878	2.5685	2.2310
hsa-miR-496	87	29.91	1.07	3.59%	6.2374	-2.0529	2.5059
hsa-miR-497	87	23.67	0.60	2.52%	3.508	-3.0352	3.8653
hsa-miR-499-5p	87	27.67	0.87	3.16%	4.6997	0.7810	0.7380
hsa-miR-500	87	27.16	0.38	1.39%	2.0771	-0.2873	0.4962
hsa-miR-501-5p	87	24.79	0.31	1.27%	1.6653	-1.0309	1.2514
hsa-miR-502-3p	87	28.03	0.38	1.34%	1.7678	1.2950	-0.0579
hsa-miR-502-5p	87	28.90	0.59	2.04%	2.7383	-1.9555	-0.0074
hsa-miR-503	87	28.18	0.86	3.05%	4.0705	-1.3751	-0.3175
hsa-miR-504	87	23.96	0.58	2.42%	2.4887	2.6098	-0.5576
hsa-miR-505	87	26.38	0.50	1.90%	2.1368	0.5987	-1.0277
hsa-miR-505*	87	26.70	0.56	2.10%	3.8466	1.9410	6.1812
hsa-miR-508-3p	87	30.27	1.45	4.81%	7.8562	4.8488	4.9598
hsa-miR-509-3p	87	27.49	0.52	1.88%	2.605	-0.4186	0.9210
hsa-miR-509-5p	87	27.67	0.54	1.94%	3.708	1.7619	6.0914

hsa-miR-511	87	30.14	0.90	2.98%	4.7662	1.7273	1.0922
hsa-miR-512-3p	87	30.02	1.06	3.54%	6.3551	1.0541	1.5855
hsa-miR-513-3p	87	29.04	0.98	3.39%	4.6866	-1.0150	-0.4232
hsa-miR-516a-3p	87	30.06	1.14	3.80%	5.7425	-1.7989	1.6966
hsa-miR-517a	87	28.23	0.84	2.97%	3.6765	0.6927	-1.0419
hsa-miR-517b	87	28.71	1.04	3.62%	4.8816	-0.0299	0.9497
hsa-miR-517c	87	28.05	0.71	2.52%	3.3924	1.7502	-0.0193
hsa-miR-518a-3p	87	30.51	1.16	3.80%	5.362	1.3533	0.7106
hsa-miR-518e	87	28.64	1.03	3.61%	5.7106	2.1742	2.3470
hsa-miR-518f	87	29.80	0.70	2.36%	3.1773	-2.4861	-0.1581
hsa-miR-519a	87	26.71	0.55	2.07%	3.6663	1.7505	2.8420
hsa-miR-519b-3p	87	29.99	0.97	3.25%	4.4808	-1.5938	-0.1082
hsa-miR-519d	87	30.04	1.34	4.45%	5.8157	-0.7343	-0.3909
hsa-miR-520c-3p	87	30.11	0.68	2.27%	3.7471	1.5296	0.9317
hsa-miR-522	87	28.20	0.79	2.78%	4.6921	1.1684	1.4871
hsa-miR-524-3p	87	27.91	0.60	2.15%	5.1625	12.5474	40.6848
hsa-miR-526b*	87	30.60	0.70	2.30%	3.7135	-0.5236	0.1711
hsa-miR-532-3p	87	24.37	0.23	0.93%	1.0835	0.0234	-0.5910
hsa-miR-532-5p	87	23.28	0.26	1.10%	1.3187	0.6427	0.6372
hsa-miR-539	87	22.08	0.39	1.78%	1.9667	-0.0754	0.2753
hsa-miR-541	87	28.08	1.02	3.62%	4.9179	-1.1218	0.0268
hsa-miR-542-3p	87	30.62	0.52	1.71%	2.4926	0.0285	0.1091
hsa-miR-542-5p	87	30.81	1.14	3.69%	5.7075	-0.6490	0.2851
hsa-miR-543	87	23.86	0.50	2.08%	2.3508	1.9931	0.2583
hsa-miR-544	87	31.35	1.07	3.43%	6.3722	-3.3555	5.3541
hsa-miR-545	87	28.59	0.63	2.22%	3.3303	0.4377	1.3515
hsa-miR-545*	87	31.07	0.93	2.99%	5.5556	-7.1073	13.1239
hsa-miR-548a-3p	87	32.59	1.33	4.08%	6.2029	-1.0821	0.2675
hsa-miR-548b-5p	87	30.20	1.24	4.11%	7.2319	-1.1296	1.8294
hsa-miR-548c-5p	87	31.15	1.32	4.22%	6.8768	0.2268	-0.0129
hsa-miR-548d-5p	87	30.13	1.06	3.51%	5.4862	-0.7121	-0.2316
hsa-miR-550	87	31.66	1.12	3.55%	5.2627	-0.6288	-0.3162
hsa-miR-550*	87	29.83	0.74	2.49%	3.494	-0.9458	-0.6337
hsa-miR-551b	87	27.93	0.45	1.62%	2.3743	-0.0355	0.0301
hsa-miR-551b*	87	29.62	0.83	2.82%	3.8727	-0.2279	-0.2755
hsa-miR-564	87	26.45	0.72	2.70%	3.4256	-1.1561	0.2090
hsa-miR-565	87	22.88	1.09	4.74%	5.5068	0.3885	0.6631
hsa-miR-570	87	31.15	1.09	3.51%	5.7753	-0.1848	0.9763
hsa-miR-572	87	29.01	0.86	2.97%	3.567	-1.5916	-0.6171
hsa-miR-573	87	29.03	0.93	3.21%	7.4359	1.1158	12.3170
hsa-miR-574-3p	87	20.91	0.65	3.12%	3.3896	-0.1285	-0.2373
hsa-miR-576-3p	87	29.99	0.45	1.52%	2.0575	-1.0686	-0.3983
hsa-miR-579	87	28.48	0.63	2.21%	3.0134	0.3992	-0.3416
hsa-miR-580	87	29.49	0.94	3.19%	5.2454	3.6888	3.5561
hsa-miR-582-3p	87	28.59	0.37	1.30%	1.9653	0.7083	0.8826

hsa-miR-582-5p	87	28.41	0.63	2.23%	2.7351	-1.5011	-0.4987
hsa-miR-584	87	24.65	1.02	4.13%	4.4694	0.8097	-1.0850
hsa-miR-589	87	29.76	0.62	2.08%	3.1784	0.8055	-0.4932
hsa-miR-589*	87	27.98	0.67	2.39%	3.6559	1.5268	0.8245
hsa-miR-590-5p	87	24.42	0.40	1.62%	2.0464	-1.4565	0.6058
hsa-miR-592	87	24.31	0.37	1.52%	1.9225	0.8407	0.4653
hsa-miR-597	87	26.11	0.40	1.54%	1.8074	0.6500	-1.4843
hsa-miR-598	87	22.00	0.34	1.54%	1.7238	3.4567	2.1315
hsa-miR-601	87	30.36	0.72	2.36%	3.7416	-0.8191	0.4323
hsa-miR-604	87	28.40	0.76	2.66%	5.5177	2.3643	9.4831
hsa-miR-610	87	27.45	0.93	3.40%	5.1605	1.4030	0.9475
hsa-miR-616*	87	28.83	0.63	2.19%	3.1673	-0.2763	-0.1598
hsa-miR-618	87	30.45	0.94	3.08%	6.0126	3.8193	5.1702
hsa-miR-624-	87	31.00	1.48	4.77%	8.6436	0.1277	1.8373
hsa-miR-625	87	25.30	0.68	2.70%	3.3277	0.1514	-0.6302
hsa-miR-625*	87	23.24	0.75	3.25%	4.0064	-0.0426	0.2926
hsa-miR-627	87	33.06	1.42	4.30%	7.1381	-2.7823	2.4342
hsa-miR-628-3p	87	25.44	0.49	1.94%	3.1666	-3.6135	4.6869
hsa-miR-628-5p	87	24.03	0.49	2.02%	2.4341	1.1174	0.7197
hsa-miR-629	87	30.04	0.58	1.92%	3.6583	1.8484	3.1220
hsa-miR-629*	87	28.82	0.69	2.38%	4.723	1.2463	3.8706
hsa-miR-630	87	27.74	0.57	2.05%	4.2825	-3.6295	9.6709
hsa-miR-632	87	28.43	0.67	2.35%	3.118	-2.6293	0.7284
hsa-miR-635	87	31.21	1.70	5.44%	7.7309	0.2793	-0.3055
hsa-miR-636	87	29.19	0.73	2.50%	3.6721	-2.5270	0.5664
hsa-miR-638	87	26.85	0.69	2.59%	4.4294	2.7810	3.4085
hsa-miR-639	87	27.43	0.81	2.95%	4.0131	-0.2351	0.2687
hsa-miR-641	87	28.11	0.66	2.33%	3.7886	-0.0863	1.6593
hsa-miR-642	87	25.29	0.85	3.34%	4.5925	3.3106	2.6008
hsa-miR-645	87	32.09	0.84	2.62%	6.2224	5.2804	12.9137
hsa-miR-651	87	31.43	0.97	3.08%	4.1627	1.5053	-0.2930
hsa-miR-652	87	23.69	0.38	1.62%	2.0338	0.9632	0.6927
hsa-miR-654-3p	87	29.85	0.61	2.04%	3.2812	1.0596	1.2859
hsa-miR-654-5p	87	26.08	0.68	2.61%	3.2108	-0.1440	-0.5892
hsa-miR-655	87	24.54	0.50	2.05%	2.3343	2.2047	-0.2257
hsa-miR-656	87	24.48	0.55	2.24%	2.4772	1.5192	-0.7547
hsa-miR-659	87	25.21	0.64	2.53%	3.2628	-1.1106	-0.3333
hsa-miR-660	87	23.40	0.22	0.93%	1.3372	0.1890	1.8780
hsa-miR-661	87	23.59	1.25	5.30%	5.9389	-0.9593	0.1131
hsa-miR-668	87	24.50	0.83	3.38%	4.5818	0.9264	1.3165
hsa-miR-671-3p	87	26.47	0.40	1.52%	1.9815	-1.1972	-0.5689
hsa-miR-7	87	23.10	0.50	2.17%	2.4649	1.2294	1.0397
hsa-miR-708	87	22.06	0.31	1.40%	1.6134	2.4587	1.9682
hsa-miR-7-1*	87	21.61	0.55	2.52%	2.5382	-0.3521	-0.7111
hsa-miR-7-2*	87	25.45	0.48	1.87%	2.2976	0.0963	-0.0039

hsa-miR-744	87	21.45	0.29	1.33%	1.2281	0.6223	-1.2352
hsa-miR-744*	87	25.19	0.26	1.04%	1.3522	2.3531	0.6751
hsa-miR-758	87	25.86	0.48	1.85%	2.5211	-0.7824	0.5814
hsa-miR-760	87	24.43	0.40	1.65%	2.0608	-3.4882	1.4101
hsa-miR-766	87	22.01	0.40	1.80%	2.2126	-0.1552	0.5090
hsa-miR-767-5p	87	26.87	0.41	1.51%	2.103	-2.2150	0.9308
hsa-miR-768-3p	87	19.99	0.45	2.25%	2.4696	-0.7559	1.0098
hsa-miR-769-3p	87	26.96	0.60	2.21%	2.7319	-0.0659	-0.7050
hsa-miR-769-5p	87	20.94	0.26	1.24%	1.4223	-3.1445	2.4059
hsa-miR-770-5p	87	24.41	0.47	1.94%	3.869	-10.6307	32.7795
hsa-miR-801	87	20.33	1.13	5.56%	5.0423	-0.8084	-0.7472
hsa-miR-873	87	28.28	0.57	2.03%	2.8843	-1.1280	0.3823
hsa-miR-874	87	21.90	0.47	2.14%	2.211	-0.4616	-0.3955
hsa-miR-875-5p	87	28.89	0.85	2.95%	7.2964	12.3539	39.0033
hsa-miR-876-3p	87	28.74	0.48	1.67%	2.8366	0.2205	1.9367
hsa-miR-876-5p	87	27.11	0.53	1.94%	2.7332	0.9714	0.3346
hsa-miR-877	87	25.67	0.39	1.50%	2.1519	-2.1742	2.3473
hsa-miR-885-5p	87	19.37	0.42	2.17%	2.0051	3.0987	1.1379
hsa-miR-886-3p	87	22.34	0.97	4.35%	5.1487	-1.1772	0.3410
hsa-miR-886-5p	87	24.22	1.19	4.91%	7.9606	-1.5284	3.4195
hsa-miR-888	87	31.57	0.94	2.97%	6.2342	-0.7815	3.4130
hsa-miR-889	87	24.83	0.49	1.97%	2.3482	0.2971	-0.0462
hsa-miR-891a	87	29.08	0.93	3.22%	4.4933	-1.2085	-0.3989
hsa-miR-9	87	14.24	0.33	2.30%	1.7025	0.3642	0.4329
hsa-miR-9*	87	17.32	0.36	2.07%	1.754	2.9107	1.2067
hsa-miR-922	87	30.26	1.31	4.33%	5.8424	-0.6403	-0.7653
hsa-miR-923	87	18.00	1.68	9.31%	9.6587	0.3224	1.6096
hsa-miR-92a	87	21.25	0.54	2.55%	2.9362	2.6723	1.1264
hsa-miR-92a-1*	87	29.63	1.30	4.40%	6.0496	0.7230	-0.8081
hsa-miR-93	87	21.97	0.78	3.55%	4.3472	-11.3817	18.9680
hsa-miR-93*	87	27.48	0.48	1.74%	3.0131	-3.6741	5.1601
hsa-miR-935	87	21.31	0.56	2.62%	2.5439	0.5209	-0.5144
hsa-miR-941	87	26.44	0.75	2.85%	3.7278	-0.3129	-0.4427
hsa-miR-942	87	29.62	0.67	2.26%	2.8587	-0.5127	-0.8253
hsa-miR-944	87	30.33	0.65	2.14%	5.8944	10.4312	35.9124
hsa-miR-95	87	21.28	0.34	1.59%	1.7749	-0.3805	-0.2620
hsa-miR-96	87	30.98	1.30	4.21%	6.366	-0.7816	0.2530
hsa-miR-98	87	20.94	0.55	2.63%	3.1522	-6.5607	7.9800
hsa-miR-99a	87	17.27	0.34	1.98%	1.7092	-0.7251	-0.4236
hsa-miR-99a*	87	25.43	0.21	0.83%	1.0857	-0.4597	0.2091
hsa-miR-99b	87	18.83	0.25	1.32%	1.1188	0.0502	-0.9098
hsa-miR-99b*	87	27.45	0.64	2.32%	5.4086	-2.8980	19.7534

Appendix 7

Inclusion of demographic covariates in the SMRI sample.

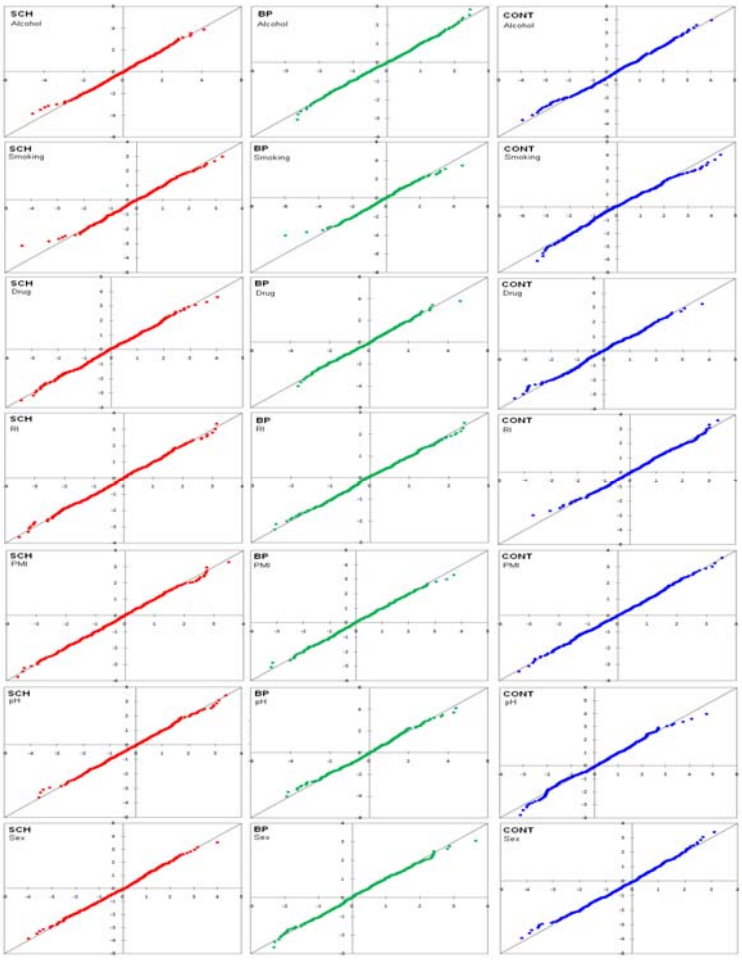


Figure15. Normal quantile plots of t-scores from seven major covariates tested in the SMRI postmortem brains. The diagonal line traces random distribution potential technical covariates.

In addition to disease status, SMRI provides information of potential confounding variables, which should be investigated carefully and, if necessary, included as covariates in subsequent analyses. The measures of miRNA abundance could be affected by a variety of factors, including: gender, age, post-mortem interval (PMI), refrigerator interval (RI), pH, RIN, drug use, alcohol use, anti-psychotic use, and duration of disease.

Ideally we would want to estimate each covariate's effect on each miRNA. However, with approximately 30 cases per condition, following the usual "statistician's rule-of-thumb" to estimate N parameters would require O(N) cases. Thus, in this specific sample we would be able to estimate only five of these parameters for each miRNA. Therefore, we tested for the effect of each covariate by itself within each diagnostic group separately in order to reduce the number of parameters in each fit. We then computed t-statistics for these effects. To our surprise, for almost all covariates the estimated effects were indistinguishable from random noise, as shown in Figure 15. We found no significant effects for age (not shown) or sex, and only few effects significant for pH, but since sex and pH are commonly used as covariates, these factors were explicitly fitted as such in the linear model together with diagnosis. Cumulative anti-psychotic use gave the most significant observation, which is discussed further below. We also estimated the coefficients of the effects within each group separately and then compared these estimates across groups. Generally, we found that estimates from different diagnostic groups were only very weakly correlated, supporting the idea that the corresponding factors had no systematic effect on miRNA abundance measures. The correlations between coefficients for the seven covariates estimated separately in BPD and SZ are shown in Figures 15 and 16. The highest correlations were 0.24 for pH estimated in BPD and control samples separately, and 0.20 for anti-psychotic use estimated within BPD and SZ separately.

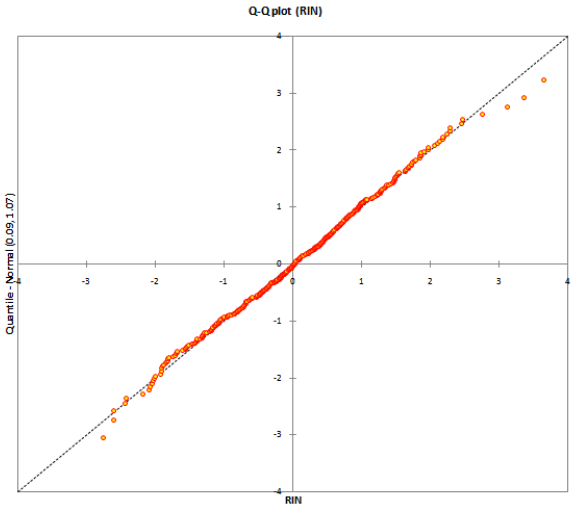


Figure 16: Normal quantile plots of t-scores from RIN measures tested in all three diagnostic groups of the SMRI postmortem brains. The diagonal line traces the normal distribution of expected values.

One drawback of the SMRI sample is the provided measurement of neuroleptics, which is given in fluphenazine equivalents. Because this measurement does not distinguish specific individual antipsychotic drug treatments, its effects on gene expression cannot be parsed out and hence cannot be measured separately. As different antipsychotic drugs are likely to have different, if not opposing effects on gene expression, we cannot provide an estimate of the individual drug effects on miRNA expression. Regardless, the effect of anti-psychotic use would be most crucial in the comparison of SZ and control groups. We first noted that the effect size estimated for SZ was little correlated with that estimated for BPD, and second the effect size was also much smaller within the SZ group (Figure 17, top left). Therefore, since the best estimate of the effect seemed to be so small, we decided not to use anti-psychotic use as a covariate because it would be completely confounded with the effect (disease) we were trying to estimate.

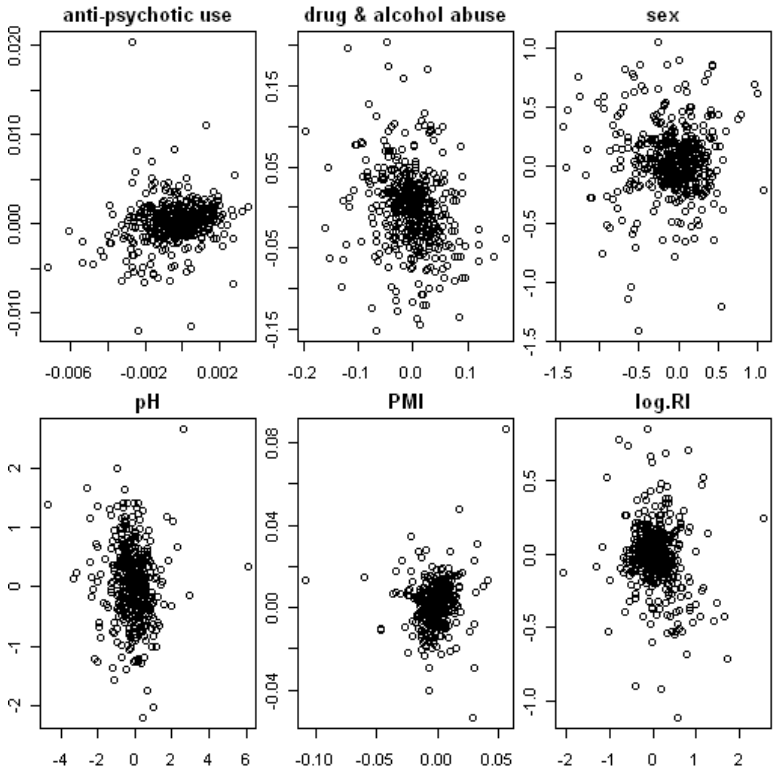


Figure 17: Correspondence between estimates of covariate effects in BPD (y-axis) and SZ (x-axis).

Appendix 8

miRNAs with significant variances in SZ and BP groups compared to controls. miRNAs in bold overlap in both disease groups.

BP miRNAs	P-value	Q-value	SCH miRNAs	P-value	Q-value
hsa-miR-98	1.05E-18	4.73E-16	hsa-miR-875-5p	1.67E-06	0.00075334
hsa-miR-218	1.04E-07	2.34E-05	hsa-miR-361-5p	1.78E-06	0.000401188
hsa-miR-299-5p	7.44E-07	0.000111644	hsa-miR-99b	1.05E-05	0.001184639
hsa-miR-129-3p	1.81E-06	0.000204075	**hsa-miR-29b-2**	1.53E-05	0.001381018
hsa-miR-138	2.47E-06	0.000222135	**hsa-miR-432**	5.10E-05	0.003827199
hsa-miR-374a	3.05E-06	0.000228435	**hsa-miR-375**	8.42E-05	0.005411178
hsa-miR-383	6.17E-06	0.000396473	**hsa-miR-381**	0.000137446	0.007731325
hsa-miR-433	2.21E-05	0.001244319	hsa-miR-29b	0.000151785	0.00758924
hsa-miR-876-3p	2.27E-05	0.001137289	**hsa-let-7d**	0.000188968	0.008503582
hsa-miR-491-5p	2.28E-05	0.001027239	**hsa-miR-363**	0.000231325	0.009463298
hsa-miR-758	3.09E-05	0.001262657	**hsa-miR-181a**	0.000238667	0.008949995
hsa-miR-369-5p	6.36E-05	0.002386368	hsa-miR-29a	0.000248598	0.008605322
hsa-miR-128	8.65E-05	0.00299527	hsa-miR-645	0.000397226	0.012767979
hsa-miR-382	0.000100962	0.003245214	**hsa-miR-27b**	0.000445602	0.013368052
hsa-miR-149	0.000134202	0.004026065	hsa-miR-15a	0.000486256	0.013675949
hsa-miR-139-3p	0.000144353	0.004059936	**hsa-miR-130b**	0.000629508	0.01666346
hsa-miR-221	0.000217727	0.005763365	**hsa-miR-199b-5p**	0.000807868	0.020196708
hsa-let-7d	0.000251463	0.006286567	hsa-miR-638	0.000986321	0.022192214
hsa-miR-17	0.000288892	0.006842176	**hsa-miR-154**	0.001076479	0.023067405
hsa-miR-625	0.000302578	0.006808014	**hsa-miR-125b**	0.001155641	0.023638117
hsa-miR-301b	0.000328164	0.007032081	**hsa-miR-128**	0.001283273	0.025107512
hsa-miR-379	0.000345211	0.007061142	**hsa-miR-551b**	0.001321223	0.024772937
hsa-miR-137	0.000347959	0.006807901	hsa-miR-101	0.00140852	0.025353365
miR-132	0.000366338	0.006868842	hsa-miR-190	0.001414796	0.024486853
hsa-miR-127-3p	0.00037765	0.006797695	hsa-miR-7	0.001432408	0.023873471
hsa-miR-30d	0.000399474	0.006913968	hsa-miR-652	0.001498502	0.024083072
hsa-miR-181a	0.000506896	0.008448264	**hsa-miR-383**	0.002098629	0.032564936
hsa-miR-222	0.000524302	0.008426275	hsa-miR-944	0.002110324	0.031654864
hsa-miR-31	0.000648658	0.010065386	hsa-miR-324-5p	0.002298239	0.033361538
hsa-miR-504	0.000698815	0.010482221	hsa-miR-376a	0.002430835	0.034183614
hsa-miR-323-3p	0.000733074	0.010641401	**hsa-miR-221**	0.002745997	0.037445407
hsa-miR-124	0.000775849	0.010910377	**hsa-miR-146b-5p**	0.003182978	0.040924004
hsa-miR-19b	0.000830577	0.011326052	**miR-132**	0.00347397	0.04342463
hsa-miR-107	0.000862912	0.011094586	**hsa-miR-23b**	0.00425432	0.051741735
hsa-miR-130b	0.000992179	0.012067047	**hsa-let-7g**	0.004530666	0.053652619
hsa-miR-19a	0.001025144	0.012139861	hsa-let-7b	0.005117286	0.059045611
hsa-miR-873	0.001030152	0.011886371	**hsa-miR-302b**	0.005685057	0.063956895
hsa-miR-668	0.001086738	0.012225805	hsa-let-7f-1	0.005703816	0.062602863
hsa-miR-26b-	0.001353912	0.014860005	**hsa-miR-629**	0.006871687	0.073625221

hsa-miR-20a	0.00142597	0.015278249	**hsa-miR-323-3p**	0.006934593	0.072571319
hsa-miR-410	0.001737357	0.018181641	hsa-miR-760	0.007681425	0.078560033
hsa-miR-26b	0.001815556	0.018568187	hsa-miR-488	0.009298286	0.09298286
hsa-miR-212	0.001822526	0.01822526	**hsa-miR-106b**	0.009383915	0.091799173
hsa-miR-487b	0.001844204	0.018041125	hsa-miR-125a-5p	0.010667947	0.102139922
hsa-miR-7-2	0.001936764	0.018157166	**hsa-miR-192**	0.011604207	0.108789441
hsa-miR-134	0.002043478	0.018766639	**hsa-miR-329**	0.011777834	0.108163785
hsa-miR-154	0.00212492	0.019124283	**hsa-miR-433**	0.012391434	0.111522907
hsa-miR-432	0.002165518	0.019107509	**hsa-miR-125b-2**	0.012662248	0.111725718
hsa-miR-187	0.002400987	0.020777775	**hsa-miR-767-5p**	0.013442403	0.116328484
hsa-miR-545	0.002409938	0.020461736	hsa-miR-190b	0.013555506	0.115093919
hsa-miR-140-5p	0.002457065	0.020475539	**hsa-miR-499-5p**	0.013734372	0.114453097
hsa-miR-589	0.002963526	0.024247028	hsa-miR-524-3p	0.014667599	0.12000763
hsa-miR-30b	0.003328908	0.026750157	**hsa-miR-511**	0.016407957	0.131849657
hsa-miR-331-3p	0.003512267	0.027728422	**hsa-miR-545**	0.01641655	0.129604343
hsa-miR-129-5p	0.003585407	0.027817809	hsa-miR-425	0.016625385	0.128990055
hsa-miR-488	0.003763286	0.028224643	**hsa-miR-410**	0.016702412	0.127391278
hsa-miR-27b	0.00379517	0.027997158	hsa-miR-576-3p	0.017023068	0.127673007
hsa-miR-768-3p	0.004159619	0.030190784	**hsa-miR-423-5p**	0.017471719	0.128889728
hsa-miR-329	0.004182062	0.029871869	hsa-miR-21	0.018068245	0.131140491
hsa-miR-125b	0.004221457	0.029682119	hsa-miR-130a	0.018438001	0.131700004
hsa-miR-138-1	0.004250934	0.029429547	**hsa-miR-16**	0.018553591	0.130454935
hsa-miR-135b	0.004344852	0.029623993	**hsa-miR-30d**	0.018783146	0.130037161
hsa-miR-642	0.004676412	0.031408734	**hsa-miR-32**	0.019813957	0.135095162
hsa-miR-146b-5p	0.004690498	0.031040061	hsa-miR-580	0.020141182	0.135276595
hsa-miR-380-	0.005258469	0.034294365	hsa-miR-601	0.020363445	0.134758094
hsa-miR-454	0.00532704	0.034245255	**hsa-miR-17**	0.021102795	0.137626925
hsa-miR-381	0.005360538	0.033975241	hsa-miR-150	0.021833272	0.140356748
hsa-miR-522	0.005443776	0.034023599	**hsa-miR-218**	0.022877329	0.144997153
hsa-miR-374b	0.00557577	0.034371184	hsa-miR-519b-3p	0.022904722	0.143154514
hsa-miR-181a	0.005825974	0.03542822	**hsa-miR-520c-3p**	0.022980235	0.141658982
hsa-miR-574-3p	0.006279842	0.037679055	hsa-miR-181c	0.023550566	0.143212898
hsa-miR-511	0.006411416	0.037962334	hsa-miR-126	0.023846916	0.143081498
hsa-miR-125b-2	0.006683615	0.039060086	**hsa-miR-222**	0.024033646	0.142304483
hsa-miR-192	0.006909738	0.039863874	**hsa-let-7e**	0.02423134	0.141611728
hsa-miR-539	0.007108796	0.040493139	**hsa-miR-628-5p**	0.024606605	0.14196118
hsa-miR-24	0.007121175	0.040056609	**hsa-miR-570**	0.025516492	0.145347104
hsa-miR-519a	0.007646843	0.042482463	hsa-miR-367	0.025769556	0.144953753
hsa-miR-499-5p	0.007764333	0.042609143	hsa-miR-199a-5p	0.028031925	0.155732919
hsa-miR-363	0.008049603	0.043642425	hsa-miR-513-3p	0.029155971	0.160002278
hsa-miR-106a	0.008060527	0.043181395	**hsa-miR-124**	0.03060438	0.165927362
hsa-miR-124	0.008224747	0.043542777	**hsa-miR-125b-1**	0.030717468	0.164557867
hsa-miR-520c-3p	0.008345135	0.043666404	**hsa-miR-409-5p**	0.031706125	0.167855955
hsa-miR-33a	0.009124257	0.04719443	**hsa-miR-181a**	0.032043621	0.167670112
hsa-miR-302b	0.009272412	0.047415743	**hsa-miR-539**	0.032047688	0.165763904

hsa-miR-495	0.009300501	0.047025003	hsa-miR-483-5p	0.03279019	0.167677109
hsa-miR-135a	0.009397144	0.046985718	**hsa-miR-149**	0.033489805	0.169330476
hsa-miR-214	0.009581388	0.047380491	**hsa-miR-340**	0.033559104	0.167795519
hsa-miR-100	0.009910584	0.048475682	**hsa-miR-454**	0.034372997	0.16997636
hsa-miR-449a	0.010159944	0.048638028	**hsa-miR-139-5p**	0.036967052	0.1808171
hsa-miR-203	0.010247505	0.048540811	hsa-miR-337-5p	0.037416661	0.181048359
hsa-miR-616	0.010819335	0.050715633	hsa-miR-26a	0.037992102	0.181877084
hsa-miR-34c-5p	0.010913185	0.050628177	hsa-miR-487a	0.038285127	0.181350603
hsa-miR-423-5p	0.010934061	0.050207423	hsa-miR-744	0.038443157	0.180202297
hsa-miR-570	0.01104799	0.050218139	**hsa-miR-382**	0.038935506	0.180628636
hsa-miR-125b-1	0.012166453	0.054749038	**hsa-miR-639**	0.039161178	0.179821736
hsa-miR-374a	0.012408073	0.055283492	**hsa-miR-487b**	0.040632039	0.184691088
hsa-miR-370	0.012685187	0.055964062	hsa-miR-106b	0.042704267	0.1921692
hsa-miR-23b	0.013017696	0.056873428	**hsa-let-7a**	0.043227806	0.192599134
hsa-miR-139-5p	0.013042298	0.056433018	hsa-miR-551b	0.043487648	0.19185727
hsa-miR-708	0.01310181	0.056150616	**hsa-miR-378**	0.044108067	0.192705149
hsa-miR-154	0.013147054	0.055812966	hsa-miR-146a	0.044507398	0.192580087
hsa-miR-221	0.013158439	0.055339228	hsa-miR-143	0.046226889	0.198115237
hsa-miR-625	0.013401781	0.055840755			
hsa-miR-579	0.013411907	0.055370259			
hsa-miR-25	0.013662936	0.055893829			
hsa-miR-184	0.013750648	0.055745869			
hsa-miR-769-5p	0.01423937	0.057211754			
hsa-miR-106b	0.014408797	0.057380166			
hsa-miR-153	0.014834114	0.058555711			
hsa-miR-409-5p	0.014855343	0.058129602			
hsa-miR-16	0.015220626	0.059045531			
hsa-miR-876-5p	0.015535925	0.059753558			
hsa-miR-455-5p	0.016015984	0.061077905			
hsa-miR-15b	0.016107474	0.060910615			
hsa-miR-340	0.016712411	0.062671541			
hsa-miR-133b	0.016893353	0.062826519			
hsa-miR-767-5p	0.0169668	0.06258246			
hsa-let-7i	0.017315253	0.063348486			
hsa-miR-505	0.017507328	0.06353466			
hsa-miR-152	0.017652969	0.063550689			
hsa-miR-340	0.017886019	0.06387864			
hsa-miR-517c	0.018061689	0.063998112			
hsa-miR-636	0.01830796	0.064363921			
hsa-miR-9	0.018573469	0.064791172			
hsa-miR-485-3p	0.018634558	0.064504238			
hsa-miR-376a	0.018706273	0.064258188			
hsa-miR-142-5p	0.019237274	0.065581617			
hsa-miR-628-5p	0.019404486	0.065654275			
hsa-miR-375	0.020726729	0.069604687			

hsa-miR-335	0.021411324	0.071371081			
hsa-miR-15a	0.0215993	0.071468271			
hsa-miR-518f	0.022494694	0.07388768			
hsa-miR-885-5p	0.022844241	0.074492089			
hsa-let-7g	0.022885915	0.074091092			
hsa-miR-564	0.022948359	0.073762583			
hsa-miR-590-5p	0.023176219	0.073966656			
hsa-miR-216b	0.023301759	0.073843602			
hsa-miR-29b-2	0.023414813	0.073682978			
hsa-miR-141	0.024028348	0.075088587			
hsa-miR-181a-2	0.024105331	0.074809646			
hsa-miR-500	0.024908348	0.076772307			
hsa-miR-886-3p	0.025183657	0.077092829			
hsa-miR-551b	0.02526875	0.07683066			
hsa-miR-199b-5p	0.026235974	0.079236164			
hsa-miR-545	0.026715191	0.080145573			
hsa-miR-197	0.027685816	0.082507398			
hsa-miR-338-3p	0.02830659	0.083802405			
hsa-miR-629	0.028664401	0.084307061			
hsa-miR-219-5p	0.02882042	0.084215513			
hsa-miR-20a	0.029092809	0.084462994			
hsa-miR-151-3p	0.029632088	0.085477177			
hsa-miR-93	0.029722787	0.085192702			
hsa-miR-105	0.029745672	0.084718685			
hsa-miR-671-3p	0.029926435	0.084697457			
hsa-miR-185	0.03060288	0.086070599			
hsa-miR-27b	0.030820071	0.086143055			
hsa-miR-582-5p	0.031281924	0.086894232			
hsa-miR-200b	0.031398359	0.086682585			
hsa-miR-328	0.032423273	0.088966298			
hsa-miR-32	0.032447305	0.08849265			
hsa-miR-508-3p	0.033810109	0.091653909			
hsa-miR-526b	0.034443733	0.092812455			
hsa-let-7e	0.035764282	0.095797183			
hsa-miR-639	0.036242873	0.096504692			
hsa-miR-378	0.036473565	0.096547671			
hsa-miR-766	0.037043755	0.097483567			
hsa-miR-411	0.03894087	0.101880183			
hsa-miR-490-3p	0.040434477	0.105176384			
hsa-miR-192	0.041519216	0.107377282			
hsa-miR-194	0.042170799	0.108439198			
hsa-let-7a	0.042609673	0.108945186			
hsa-miR-589	0.042926638	0.109135519			
hsa-miR-598	0.043765713	0.110643657			
hsa-miR-942	0.044128169	0.110936738			

hsa-miR-486-5p	0.044362633	0.110906582			
hsa-miR-219-2-3p	0.044711817	0.111161976			
hsa-miR-335	0.04519806	0.111753446			
hsa-miR-655	0.045936001	0.112957381			
hsa-miR-188-5p	0.048838855	0.119442851			
hsa-miR-877	0.049143901	0.11953922			