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Transcriptional consequences of schizophrenia candidate miR-137 manipulation in human neural progenitor cells

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ABSTRACT

MIR137, transcribed as the microRNA miR-137, is one of the leading candidate schizophrenia susceptibility genes to arise from large genome-wide association studies (GWAS) of the disorder. Recent data suggest that miR-137 modulates the expression of other schizophrenia susceptibility genes. Although bioinformatic resources are available with which to predict genes regulated by individual microRNA, there has been a lack of empirical data on genome-wide gene expression changes following miR-137 manipulation. We have therefore performed a genome-wide assessment of transcriptional changes in a human neural progenitor cell line after miR-137 over-expression and inhibition in order to elucidate molecular pathways by which genetic perturbation of miR-137 could promote susceptibility to schizophrenia. Bioinformatically-predicted miR-137 targets showed a small but highly significant down-regulation following miR-137 over-expression. Genes that were significantly down-regulated in association with miR-137 over-expression were enriched for involvement in neuronal differentiation. Differentially expressed genes that were confirmed by qPCR included others at genome-wide significant risk loci for schizophrenia (*MAD1L1* and *DPYD*) and *BDNF*. These data point to molecular pathways through which genetic variation at the *MIR137* locus could confer risk for schizophrenia.

1. Introduction

Large-scale genome-wide association studies (GWAS) have identified the chromosome 1 region containing *MIR137 host gene* (*MIR137HG*) as a susceptibility locus for schizophrenia (Schizophrenia Psychiatric GWAS Consortium, 2011; Ripke et al., 2013). This gene encodes the primary microRNA (pri-miRNA) from which microRNA-137 (miR-137; hsa-miR-137) is processed. MicroRNAs (miRNAs) are small non-protein coding RNAs that regulate the expression of other genes by binding to their mRNA and causing translational repression and/or mRNA destabilization (Bartel, 2009). miR-137 is expressed in both the developing and adult brain, where it has been shown to play an important role in the regulation of cell proliferation and differentiation (Silber et al., 2008; Smrt et al., 2010; Szulwach et al., 2010; Sun et al., 2011).

Intriguingly, genes that are bioinformatically-predicted to be regulated by miR-137 have been reported to be enriched for association with schizophrenia (Schizophrenia Psychiatric GWAS Consortium, 2011; Ripke et al., 2013). Genes with predicted miR-137 target sites include four (*TCF4*, *CACNA1C*, *CSMD1* and *C10orf26* [now *WBP1L*]) that are proximal to variants exhibiting genome-wide significant association with schizophrenia (Schizophrenia Psychiatric GWAS Consortium, 2011). The predicted miR-137 target and flanking sequences of these four genes have been shown to drive lower luciferase activity in the presence of miR-137 over-expression in HEK293 cells (Kwon et al., 2013). In addition, luciferase reporter and gene expression assays have provided evidence that the mRNA product of *ZNF804A*, containing another variant showing genome-wide significant association with schizophrenia (Williams et al., 2011), is also a direct target of miR-137 (Kim et al., 2012). These data suggest that genetic influences on miR-137 could confer risk for schizophrenia, in part, through a network of susceptibility genes (Wright et al., 2013).

Although destabilization of target mRNAs is a principal mechanism by which miRNAs regulate gene expression (Hendrickson et al., 2009; Guo et al., 2010), to date, only one published study has assessed genome-wide cellular RNA expression changes following miR-137 manipulation (Balaguer et al., 2010). In that study, the effects of miR-137 over-expression on global RNA expression were investigated in a human colon carcinoma cell line. As the extent to which these findings can be extrapolated to cells of the human brain is unknown, we have performed genome-wide RNA profiling of a human neural progenitor cell line following miR-137 manipulation, in order to identify molecular pathways through which genetic variation at the *MIR137HG* locus could confer susceptibility to schizophrenia.

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2. Materials and methods

2.1. Cell culture

Experiments were carried out using the CTXOE03 neural cell line obtained from ReNeuron Ltd (www.reneuron.com) under a material transfer agreement. This is a clinical-grade, karyotypically-normal clonal cell line, derived from human fetal cortical neuroepithelium (Pollock et al., 2006). The CTXOE03 line has been conditionally immortalized by genomic incorporation of the *c*-mycER^{TAM} transgene, to stimulate proliferation in the presence of the synthetic drug 4-hydroxy-tamoxifen (4-OHT). In their proliferative state, CTXOE03 cells stain positive for nestin, but are negative for both GFAP and β-III tubulin, consistent with neural progenitor cells (Pollock et al., 2006). Cells were cultured on laminin-coated T75 flasks using a modified DMEM:F12 media, as described previously (Hill et al., 2012).

2.2. Manipulation of miR-137 in cultured cells

Over-expression of miR-137 in CTXOE03 cells was achieved using a mirVana™ miRNA mimic of the miR-137 precursor (MC10513; Life Technologies). Inhibition of endogenous miR-137 activity was carried-out using a mirVana™ miRNA inhibitor (MH10513; Life Technologies). Transfection was performed using the N-TER™ reagent (Sigma) with mimic and inhibitors at a concentration of 10 nM. Four separate flasks of seeded cells were transfected with the miR-137 mimic, 4 with the miR-137 inhibitor and 4 with N-TER™ reagent alone as the negative control. In order to estimate transfection efficiency, an additional T75 flask of seeded cells was transfected with 10nM BLOCK-iT™ Alexa Fluor® Red Fluorescent oligonucleotide (Life Technologies), using the same N-TER™ reagent, and visualized after 24 h by fluorescence microscopy. 4-OHT was excluded from the media used for these experiments so that proliferation was not artificially stimulated through *c*-myc over-expression. Cells were harvested 72 h post-transfection.

2.3. RNA preparation

Pelleted cells were separately homogenized in 1 ml Tri-Reagent® (Life Technologies) and total RNA extracted according to manufacturer's instructions. Residual genomic DNA was removed by addition of 2 μl Turbo DNA-free™ (Life Technologies) and incubation at 37 °C for 30 min. Integrity of total RNA was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). All samples had an RNA integrity number (RIN) > 9.5.

2.4. Assessment of miR-137 over-expression

Mature miRNA and other small RNA were reverse transcribed from the four RNA samples in the mimic condition and the four RNA samples in the negative control condition using the TaqMan® MicroRNA reverse Transcription Kit and TaqMan® MicroRNA assay primers for hsa-miR-137 (Cat# 000593), hsa-miR-214 (Cat# 002306), hsa-miR-92b (Cat# 002343) and the endogenous small non-coding RNA controls U47 (Cat# 001223) and RNU44 (Cat# 001094)(Life Technologies). The microRNAs miR-214 and miR-92b have previously been shown to be expressed in human neural progenitor cells (Liu et al., 2012) and were assayed in order to test whether transfection of the miR-137 precursor mimic was affecting endogenous microRNA processing. Quantitative PCR (qPCR) was performed on all reverse transcription products in duplicate using the TaqMan® MicroRNA assays listed above and TaqMan® Fast Universal PCR Master mix (Life Technologies). The expression of mature miR-137, miR-214 and miR-92b was normalized against that of the U47 and RNU44 controls following the model of Pfaffl (2001) based on observed cycle thresholds and efficiencies for each reaction. The expression of miR-137 in the inhibitor condition was not assessed as it is designed to bind to (and thus inhibit the activity of) endogenous miR-137, rather than alter its expression.

2.5. Genome-wide RNA expression profiling

Biotin-labeled cRNA was generated from the 4 total RNA samples in each of the three experimental conditions (mimic, inhibitor and negative control) using the Illumina TotalPrep-96 RNA amplification kit (Life Technologies). Genome-wide expression profiling of each sample was performed using the Illumina HT-12 v4 BeadChip array (Illumina). Data were extracted from GenomeStudio software (Illumina) and variance stabilizing transformation and robust spline normalization applied using the lumi Bioconductor package (Du et al., 2008). All microarray data have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), with the accession number GSE49793. Microarray probes showing differences in expression between the mimic and negative control conditions and the inhibitor and negative control conditions were identified by individual t-tests (two-tailed) at two levels of statistical significance ($P < 0.05$ and $P < 0.01$). To limit spurious results arising from low expression genes, probes that were not detected in at least all four samples of one of the comparison conditions with a detection *P*-value > 0.95 were excluded.

2.6. Confirmation of downstream gene expression changes

We sought to confirm altered expression of selected genes using SYBR Green qPCR of cDNA reverse transcribed from the total RNA assayed by microarray. cDNA was synthesised from each DNase-treated RNA sample using random decamers and SuperScript® III (Life Technologies). qPCR primers were designed to target the same exons as the microarray probes showing altered expression (Supplementary Table 1). Reactions were carried out in a total volume of 20 μl, containing diluted cDNA, 1× HOT FIREPol® EvaGreen® qPCR Mix (Solis Biodyne, Tartu, Estonia) and primers at 200 nM, using an MJ Research Chromo 4 (Bio-Rad) and MJ Opticon Monitor analytic software (Bio-Rad). Given the small changes in gene expression indicated by the microarray analysis, we performed 8 technical replicate qPCR reactions for both the target gene and the internal control gene for each sample and quantified expression against a standard curve constructed by serial dilution of pooled cDNA. *GGA1* was identified as a suitable internal control gene on the basis of the genome-wide microarray data, where it showed the least variability (in terms of standard deviation/mean) across all samples. Target gene expression values (normalized by those of the internal control) were compared between samples of the mimic and negative control condition or inhibitor and negative control condition by individual t-tests (2-tailed).

2.7. Bioinformatic analyses

Microarray probes at which significant gene expression changes were detected were subject to Gene Ontology (GO) analysis through the DAVID Bioinformatics Resource 6.7 (Huang et al., 2009) using all biological process terms in the GOTERM_BP_FAT category. All probes with a detection *P*-value > 0.95 in at least all four samples of one of the comparison conditions were used as the background. To test for a general down-regulation of predicted miR-137 targets in the mimic relative to the negative control condition, and a general up-regulation of predicted miR-137 targets in the inhibitor relative to the negative control condition, fold-changes in the expression of all detected genes that are predicted to be miR-137 targets by the TargetScan 6.2 program with an aggregate probability of conserved targeting ≥ 0.9 (Friedman et al., 2009) were compared with fold-changes in the expression of all detected genes that are not predicted miR-137 targets using t-tests. All reported *P*-values are 2-tailed.

Table 1
Biological Process Gene Ontology terms that were significantly enriched among genes differentially expressed at $P < 0.01$ in association with the miR-137 mimic condition.

Gene Ontology term	*P*	Genes
GO:0045664 regulation of neuron differentiation	0.00066	*TGIF1*, *BDNF*, *TIMP2*, *TTC3*, *DBN1*, *CDK5RAP2*, *CCND2*
GO:0050767 regulation of neurogenesis	0.0023	*TGIF1*, *BDNF*, *TIMP2*, *TTC3*, *DBN1*, *CDK5RAP2*, *CCND2*
GO:0051960 regulation of nervous system development	0.0038	*TGIF1*, *BDNF*, *TIMP2*, *TTC3*, *DBN1*, *CDK5RAP2*, *CCND2*
GO:0060284 regulation of cell development	0.0069	*TGIF1*, *BDNF*, *TIMP2*, *TTC3*, *DBN1*, *CDK5RAP2*, *CCND2*
GO:0051789 response to protein stimulus	0.020	*TTC3*, *LOC387820*, *EIF2AK4*, *DNAJC4*, *HSPA4*
GO:0010033 response to organic substance	0.022	*GNG3*, *TIMP4*, *TTC3*, *LOC387820*, *EIF2AK4*, *DNAJC4*, *HSPA4*, *FKBP1A*, *ATP5G3*, *SREBF2*, *ASNS*, *CCND2*
GO:0042098 T cell proliferation	0.022	*CXCR4*, *FKBP1A*, *TNFSF14*
GO:0006470 protein amino acid dephosphorylation	0.033	*PTPN23*, *PPP2R3B*, *DUSP28*, *PTPN3*, *PPP1CB*
GO:0007411 axon guidance	0.042	*CXCR4*, *BDNF*, *NRP2*, *FEZ1*
GO:0006986 response to unfolded protein	0.042	*LOC387820*, *EIF2AK4*, *DNAJC4*, *HSPA4*

3. Results

3.1. Over-expression of mature miR-137 in human neural progenitor cells

Transfection efficiency, as indexed by uptake of Alexa Fluor® Red Fluorescent oligonucleotide, was estimated to be >90% (Supplementary Fig. 1). At harvest, the expression of mature miR-137 was >1000-fold higher in cells within the mimic condition compared with those in the negative control condition. Estimates were highly similar following qPCR normalization using either of the small non-coding RNA internal controls (RNU44 normalization: mean 1031-fold increase in miR-137 expression, $P < 0.0001$; U47 normalization: mean 1015-fold increase in miR-137 expression, $P < 0.0001$). In contrast, the expression of miR-214 and miR-92b did not significantly differ between the mimic and negative control conditions when normalized by either of the small non-coding RNA controls (all $P > 0.05$), indicating little effect of transfecting the mimic precursor on the microRNA processing machinery.

3.2. miR-137 manipulations result in small changes in RNA expression

Gene expression differed between the mimic and negative control conditions at 1080 microarray probes at $P < 0.05$, and at 202 microarray probes at $P < 0.01$. Of these, 524 were down-regulated at $P < 0.05$ and 96 down-regulated at $P < 0.01$. Mean fold-changes in gene expression associated with the miR-137 mimic condition ranged from 0.70 to 1.48. Gene expression differed between the inhibitor and negative control conditions at 1025 microarray probes at $P < 0.05$, and at 199 microarray probes at $P < 0.01$. Of these, 444 were up-regulated at $P < 0.05$ and 73 up-regulated at $P < 0.01$. Mean fold-changes in gene expression associated with the miR-137 inhibitor condition ranged from 0.71 to 1.47. Only one gene (*BBS7*) was differentially expressed in both the mimic versus the negative control condition and the inhibitor versus the negative control condition at $P < 0.05$ in the direction predicted if it were a direct target of miR-137 (i.e. down-regulated in the mimic and up-regulated in the inhibitor condition). All probes showing significant ($P < 0.05$) differences in gene expression between the mimic and negative control conditions and the inhibitor and negative control conditions are listed in Supplementary Tables 2 and 3, respectively.

3.3. Enrichment of gene expression changes involved in neuronal differentiation after miR-137 over-expression

Gene expression differences between the mimic and negative control condition at the more stringent threshold of $P < 0.01$ showed the most significant enrichment within particular Gene Ontology terms. This set was most significantly enriched for genes belonging to the biological process term 'regulation of neuron differentiation' (GO:0045664; $P = 0.00066$). The 7 differentially expressed genes belonging to this term were: *BDNF*, *TGIF1*, *TIMP2*, *TTC3*, *DBN1*, *CDK5RAP2* and *CCND2*. All significant ($P < 0.05$, uncorrected) GO terms for genes differentially expressed at $P < 0.01$ in association with the mimic condition are shown in Table 1. Given that the primary targets of a microRNA mimic would be predicted to be down-regulated, we then looked for enrichment of Gene Ontology terms in the 96 genes that showed reduced expression in the mimic relative to the negative control condition at $P < 0.01$. All 7 differentially expressed genes belonging to the 'regulation of neuron differentiation' term were down-regulated, and this term was again the most significant ($P = 0.000024$), surviving correction for all terms in the GOTERM_BP_FAT category (Bonferroni-corrected $P = 0.014$). For differentially expressed genes between the inhibitor and negative control conditions, the most significant biological process GO term was 'aging' (GO:0007568; $P = 0.005$) which was enriched among the gene set detected at $P < 0.05$. However, neither this nor any terms enriched in over-expressed genes associated with the inhibitor condition survived correction for multiple testing (Bonferroni-corrected $P > 0.99$).

3.4. Effect of miR-137 manipulation on predicted miR-137 targets

TargetScan predicts 305 miR-137 targets that each had a probability of conserved targeting > 0.9 (Friedman et al., 2009) and were detected on the array in all samples of the mimic or negative control conditions. These showed a very small but highly significant down-regulation in the mimic condition relative to the negative control condition (mean fold-change = 0.98; $P = 0.000019$). Of the predicted targets, 13 were down-regulated at $P < 0.05$ in the miR-137 mimic condition. These were *ATPAF1*, *CRKL*, *CSDA*, *EFR3A*, *GNAT1*, *IDH1*, *KIAA1671*, *LBH*, *MIA3*, *PPP1CB*, *STYX*, *TULP4* and *ZNF148*. Four of these genes (*CRKL*, *KIAA1671*, *PPP1CB* and *TULP4*) were down-regulated at $P < 0.01$ in association with the miR-137 mimic. TargetScan predicts 308 miR-137 targets that were detected on the array in all samples of the inhibitor or

negative control conditions. The expression of these genes did not differ between the inhibitor and the negative control condition (mean fold-change = 0.999; $P = 0.46$). Of the predicted miR-137 targets, only 3 were up-regulated at $P < 0.05$ (*ESRRG*, *FAM134C* and *XRN1*) and one up-regulated at $P < 0.01$ (*XRN1*) in association the miR-137 inhibitor condition.

3.5. Overlap with genes down-regulated after miR-137 over-expression in a human colon carcinoma cell line

Balaguer et al. (2010) reported 491 genes that were down-regulated by >2-fold in an individual sample of a human colon carcinoma cell line in which miR-137 was over-expressed, compared with an individual sample of the same cell line after negative control transfection. Of these, 441 were detected on the array in all samples of the mimic or negative control conditions in this study. Nineteen of these 441 genes were down-regulated at $P < 0.05$ in the miR-137 mimic condition of this study. These were *BDNF*, *C21ORF66*, *CSDA*, *DBN1*, *DPYSL3*, *EIF4B*, *EP400*, *FAM3C*, *FXR1*, *HSPD1*, *KIAA1671*, *LOC642817*, *PALLD*, *PCNA*, *PDHB*, *PGAM4*, *PPP1CB*, *SLC25A5* and *UBE3C*. Six of these genes (*BDNF*, *DBN1*, *DPYSL3*, *KIAA1671*, *PCNA* and *PPP1CB*) were down-regulated at the more conservative threshold of $P < 0.01$. Given that *BDNF* is one of the genes in the 'regulation of neuron differentiation' GO category and has been implicated in the pathophysiology of psychiatric disorders (Green et al., 2011; Monteleone et al., 2008; Molendijk et al., 2013) we further tested its down-regulation by qPCR. This confirmed a significant down-regulation of *BDNF* in the miR-137 mimic condition (fold-change = 0.78, $P = 0.03$).

3.6. Altered expression of other candidate schizophrenia susceptibility genes following miR-137 manipulation

In a recently published GWAS analysis (Ripke et al., 2013), 22 independent loci showed genome-wide significant association with schizophrenia. We explored potential effects of miR-137 manipulation on the expression of the gene that was closest to the most significant single nucleotide polymorphism (SNP) at each locus. Nine of these genes (*AS3MT*, *MAD1L1*, *CACNB2*, *SNX19*, *QPCT*, *C2orf82*, *AKT3*, *SDCCAG8* and *C12orf65*) were detected on the array in at least all 4 samples of one of the comparison conditions. Of these, *MAD1L1* was significantly down-regulated in the miR-137 mimic condition, and this was confirmed by qPCR (fold-change = 0.72, $P = 0.0003$). *AKT3* was significantly up-regulated in the inhibitor condition (fold-change = 1.07, $P = 0.004$), but this was not confirmed by qPCR (fold-change = 0.98, $P = 0.76$). In addition, *DPYD*, a gene that is adjacent to *MIR137HG* and another candidate schizophrenia susceptibility gene at that locus (Xu et al., 2012; Ripke et al., 2013), was significantly down-regulated in the miR-137 mimic condition and this confirmed by qPCR (fold-change = 0.86, $P = 0.024$). Of the 5 candidate schizophrenia susceptibility genes validated as miR-137 targets by Kwon et al. (2013) and Kim et al. (2012), only *TCF4* and *C10orf26* (now *WBP1L*) were detected on the array in at least all samples of one of the comparison conditions. Neither the miR-137 mimic nor the inhibitor was associated with any effect on *TCF4* RNA expression (mimic fold-change: 1.04, $P = 0.44$; inhibitor fold-change: 1.02, $P = 0.82$) or *C10orf26* RNA expression (mimic fold-change: 0.95, $P = 0.46$; inhibitor fold-change: 0.97, $P = 0.61$).

4. Discussion

MIR137 is one of the leading candidate schizophrenia susceptibility genes to arise from large-scale GWAS of the disorder (Schizophrenia Psychiatric GWAS Consortium, 2011; Ripke et al., 2013). Although bioinformatic resources are available with which to predict genes regulated by individual microRNA, there has been a dearth of empirical data on genome-wide gene expression changes following miR-137 manipulation. We have therefore performed a genome-wide assessment of transcriptional changes in a human neural cell line after miR-137 over-expression and inhibition in order to elucidate molecular pathways by which genetic perturbation of miR-137 could promote susceptibility to schizophrenia. We find that, within these cells, large changes in miR-137 expression resulted in only minor changes in the RNA expression of other genes. However, consistent with bioinformatic predictions and known functional roles of miR-137, predicted targets were, en masse, down-regulated following miR-137 over-expression, and differentially expressed genes were enriched for involvement in neuronal development. Genes showing significant changes in gene expression included others implicated in the etiology or pathophysiology of schizophrenia.

The extent to which the gene expression changes we observed will be relevant to schizophrenia will depend upon the timing and nature of the risk mechanism associated with genetic variation at the *MIR137HG* locus. The SNP at this locus most strongly associated with schizophrenia in the Psychiatric GWAS Consortium (2011) study is rs1625579, within an intron of *MIR137HG*. The most significant SNP at this locus in the more recent study of Ripke et al. (2013) is rs1198588, located 5′ of *MIR137HG*, and in strong linkage disequilibrium with rs1625579 (r^2 in CEU sample = 0.78). Homozygosity for the risk allele of rs1625579 has recently been reported to associate with reduced expression of the mature miR-137 transcript in dorsolateral prefrontal cortex from adult control individuals (Guella et al., 2013). Although we have generated data indicating an association between rs1625579 genotype and the cis-regulation of *MIR137HG* in human fetal brain (M.J. Hill & N.J. Bray, unpublished results), the effects of this SNP on miR-137 expression in neural progenitor cells are currently unknown. Given the findings of Guella et al. (2013), future studies might examine effects of miR-137 manipulation in differentiated cells akin to those of the adult human brain.

Although a large proportion of the significant gene expression changes we observed will be chance observations, the findings from our Gene Ontology analyses suggest that a reasonable number are genuinely the result of miR-137 manipulation. Consistent with known functional roles of miR-137 in the mouse brain, genes that were altered following miR-137 over-expression were significantly enriched for involvement in neuronal differentiation. Over-expression of miR-137 has been found to reduce the proliferation and induce premature differentiation of neural stem cells within the embryonic mouse brain (Sun et al., 2011) and adult subventricular zone (Silber et al., 2008), while the opposite effects have been reported in neural stem cells of the adult murine dentate gyrus (Smrt et al., 2010; Szulwach et al., 2010). Although the aim of the present study was to identify transcriptional consequences of miR-137 manipulation, future studies could explore the physiological effects of these manipulations in cells from the human brain. Effects of miR-137 perturbation on brain development could account for reported associations between the rs1625579 risk allele and regional brain volumes as well as earlier age of onset in schizophrenia (Lett et al., 2013).

One of the seven differentially expressed genes associated with the miR-137 mimic and annotated as belonging to the GO term 'regulation of neuron differentiation' is *BDNF*, encoding brain-derived neurotrophic factor, a molecule that has been reported to be reduced in the serum of patients with schizophrenia (Green et al., 2011) and other psychiatric disorders (Monteleone et al., 2008; Molendijk et al., 2013). Significant down-regulation of *BDNF* RNA after miR-137 over-expression was confirmed by qPCR. Only two of the microRNA target prediction programs included in the miRecords database (Xiao et al., 2009) – PITA (Kertesz et al., 2007) and RNAHybrid (Krüger and Rehmsmeier, 2006) – predict *BDNF* as a primary target of miR-137. However, that *BDNF* RNA has also been found to be reduced following miR-137 over-expression in another human cell line (Balaguer et al., 2010) suggests that, even if it is not a direct target, it does indeed serve as a downstream effector of miR-137 function.

Although TargetScan predicts several miR-137 targets among the genes that were differentially expressed, our methodology does not distinguish between gene expression changes resulting from the primary actions of miR-137 or their downstream consequences. It is likely that these will be differentially detected depending on the time-point at which the RNA is assayed, with later time-points detecting a greater proportion of secondary gene expression changes. Technology is now available with which to experimentally screen a large number of genic sequences to identify likely targets of individual miRNA (Gäken et al., 2012). However, downstream effects on gene expression are also important to identify since they might better reflect the cellular consequences of miR-137 manipulation as well as including additional molecular changes of potential relevance to schizophrenia pathogenesis.

Previous studies have reported that bioinformatically-predicted miR-137 targets are enriched for association with schizophrenia (Schizophrenia Psychiatric GWAS Consortium, 2011; Ripke et al., 2013) and several genes at loci exhibiting genome-wide significant association with the disorder have been validated as miR-137 targets by reporter gene assays (Kim et al., 2012; Kwon et al., 2013). Most recently, Ripke et al. (2013) cite unpublished findings from a similar study to our own in which miR-137 was over-expressed in human neural stem cells and where an enrichment of schizophrenia association signals in down-regulated genes was observed (A.L. Collins, Y. Kim, R. Bloom, D. Rubinow, W. Sun et al., unpublished results). Of genes at genome-wide significant risk loci for schizophrenia that were sufficiently expressed in our cells, we were able to confirm significant down-regulation of *MAD1L1* and *DPYD* in association with miR-137 over-expression. Although TargetScan predicts neither gene as a direct miR-137 target, the known role of *MAD1L1* in cell proliferation (e.g. Rottmann et al., 2005) makes it a good candidate for mediating miR-137 action. Our findings also suggest that effects of schizophrenia risk variation on miR-137 expression (Guella et al., 2013) could have a downstream effect on the expression of *DPYD*, another candidate schizophrenia susceptibility gene at the *MIR137HG* locus (Xu et al., 2012; Ripke et al., 2013).

We observed no significant effect of miR-137 manipulation on the RNA expression of *TCF4* or *C10orf26*, the two genes at genome-wide significant schizophrenia risk loci that have been previously validated as miR-137 targets (Kwon et al., 2013) and which were sufficiently expressed in our cells to be tested. Although mRNA destabilization is now considered to be a principle mechanism by which microRNA repress their targets (Guo et al., 2010), it is possible that the protein expression of these and other predicted miR-137 targets is regulated by translational repression in the absence of effects on mRNA levels. The application of proteomic technologies (e.g. Gold et al., 2012) following cellular miR-137 manipulation could address this question.

The changes in RNA expression we observed following miR-137 manipulation were very small. Although Balaguer et al. (2010) report a number of genes showing >2-fold expression differences following miR-137 over-expression in a human carcinoma cell line, it should be noted that this was in a comparison between a single control sample and single sample in which miR-137 was over-expressed, whereas we report the mean fold-change of expression over control from the 4 samples in each condition. The expression of any given gene will be determined by a multitude of regulatory factors, which might restore mRNA levels in spite of large changes in the expression of an interacting microRNA. These factors are likely to differ between cell types, such that some targets might be more vulnerable to microRNA manipulation in certain cells. In addition, genetic variation in the functioning of these targets and their other regulators might lead to variable effects of miR-137 perturbation between individuals.

In summary, we have produced a genome-wide assessment of transcriptional changes following miR-137 manipulation in a human neural progenitor line. We find altered RNA expression of genes involved in neuronal development, and a general down-regulation of bioinformatically-predicted miR-137 targets, following miR-137 over-expression. Differentially expressed genes included others implicated in the etiology or pathophysiology of schizophrenia. As well as illuminating molecular risk mechanisms associated with individual susceptibility genes, studies such as this will be important in establishing the extent to which these genes converge into shared biological pathways.

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Contributors

MJH, RS-F and NJB designed the study. MJH, JGD, RAN and NJB performed the laboratory work. MJH, CAM and NJB performed the analyses. NJB drafted the manuscript. All authors reviewed and approved the final manuscript.

Conflict of interest

All authors declare that they have no conflict of interest.

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References

Balaguer, F., Link, A., Lozano, J.J., Cuatrecasas, M., Nagasaka, T., Boland, C.R., Goel, A., 2010. Epigenetic silencing of miR-137 is an early event in colorectal carcinogenesis. Cancer Res. 70 (16), 6609–6618.

Bartel, D.P., 2009. MicroRNAs: target recognition and regulatory functions. Cell 136 (2), 215–233.

Du, P., Kibbe, W.A., Lin, S.M., 2008. lumi: a pipeline for processing Illumina microarray. Bioinformatics 24 (13), 1547–1548.

Friedman, R.C., Farh, K.K., Burge, C.B., Bartel, D.P., 2009. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 19 (1), 92–105.

Gäken, J., Mohamedali, A.M., Jiang, J., Malik, F., Stangl, D., Smith, A.E., Chronis, C., Kulasekararaj, A.G., Thomas, N.S., Farzaneh, F., Tavassoli, M., Mufti, G.J., 2012. A functional assay for microRNA target identification and validation. Nucleic Acids Res. 40 (10), e75.

Gold, L., Walker, J.J., Wilcox, S.K., Williams, S., 2012. Advances in human proteomics at high scale with the SOMAscan proteomics platform. Nat. Biotechnol. 29 (5), 543–549.

Green, M.J., Matheson, S.L., Shepherd, A., Weickert, C.S., Carr, V.J., 2011. Brain-derived neurotrophic factor levels in schizophrenia: a systematic review with meta-analysis. Mol. Psychiatry 16 (9), 960–972.

Guella, I., Sequeira, A., Rollins, B., Morgan, L., Torri, F., van Erp, T.G., Myers, R.M., Barchas, J.D., Schatzberg, A.F., Watson, S.J., Akil, H., Bunney, W.E., Potkin, S.G., Macciardi, F., Vawter, M.P., 2013. Analysis of miR-137 expression and rs1625579 in dorsolateral prefrontal cortex. J. Psychiatr. Res. 47 (9), 1215–1221.

Guo, H., Ingolia, N.T., Weissman, J.S., Bartel, D.P., 2010. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 466 (7308), 835–840.

Hendrickson, D.G., Hogan, D.J., McCullough, H.L., Myers, J.W., Herschlag, D., Ferrell, J.E., Brown, P.O., 2009. Concordant regulation of translation and mRNA abundance for hundreds of targets of a human microRNA. PLoS Biol. 7 (11), e1000238.

Hill, M.J., Jeffries, A.R., Dobson, R.J., Price, J., Bray, N.J., 2012. Knockdown of the psychosis susceptibility gene ZNF804A alters expression of genes involved in cell adhesion. Hum. Mol. Genet. 21 (5), 1018–1024.

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4 (1), 44–57.

Kertesz, M., Iovino, N., Unnerstall, U., Gaul, U., Segal, E., 2007. The role of site accessibility in microRNA target recognition. Nat. Genet. 39 (10), 1278–1284.

Kim, A.H., Parker, E.K., Williamson, V., McMichael, G.O., Fanous, A.H., Vladimirov, V.I., 2012. Experimental validation of candidate schizophrenia gene ZNF804A as target for hsa-miR-137. Schizophr. Res. 141 (1), 60–64.

Krüger, J., Rehmsmeier, M., 2006. RNAhybrid: microRNA target prediction easy, fast and flexible. Nucleic Acids Res. 34, W451–W454 (Web Server issue).

Kwon, E., Wang, W., Tsai, L.-H., 2013. Validation of schizophrenia-associated genes CSMD1, C10orf26, CACNA1C and TCF4 as miR-137 targets. Mol. Psychiatry 18 (1), 11–12.

Lett, T.A., Chakavarty, M.M., Felsky, D., Brandl, E.J., Tiwari, A.K., Gonçalves, V.F., Rajji, T.K., Daskalakis, Z.J., Meltzer, H.Y., Lieberman, J.A., Lerch, J.P., Mulsant, B.H., Kennedy, J.L., Voineskos, A.N., 2013. The genome-wide supported microRNA-137 variant predicts phenotypic heterogeneity within schizophrenia. Mol. Psychiatry 18 (4), 443–450.

Liu, J., Githinji, J., Mclaughlin, B., Wilczek, K., Nolta, J., 2012. Role of miRNAs in neuronal differentiation from human embryonic stem cell-derived neural stem cells. Stem Cell Rev. 8 (4), 1129–1137.

Molendijk, M.L., Spinhoven, P., Polak, M., Bus, B.A., Penninx, B.W., Elzinga, B.M., 2013. Serum BDNF concentrations as peripheral manifestations of depression: evidence

from a systematic review and meta-analyses on 179 associations (N = 9484). Mol. Psychiatry. http://dx.doi.org/10.1038/mp.2013.105 (Epub ahead of print).

Monteleone, P., Serritella, C., Martiadis, V., Maj, M., 2008. Decreased levels of serum brain-derived neurotrophic factor in both depressed and euthymic patients with unipolar depression and in euthymic patients with bipolar I and II disorders. Bipolar Disord. 10 (1), 95–100.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29 (9), e45.

Pollock, K., Stroemer, P., Patel, S., Stevanato, L., Hope, A., Miljan, E., Dong, Z., Hodges, H., Price, J., Sinden, J.D., 2006. A conditionally immortal clonal stem cell line from human cortical neuroepithelium for the treatment of ischemic stroke. Exp. Neurol. 199 (1), 143–155.

Ripke, S., O'Dushlaine, C., Chambert, K., Moran, J.L., Kähler, A.K., Akterin, S., Bergen, S.E., Collins, A.L., Crowley, J.J., Fromer, M., Kim, Y., Lee, S.H., Magnusson, P.K., Sanchez, N., Stahl, E.A., Williams, S., Wray, N.R., Xia, K., Bettella, F., Borglum, A.D., Bulik-Sullivan, B.K., Cormican, P., Craddock, N., de Leeuw, C., Durmishi, N., Gill, M., Golimbet, V., Hamshere, M.L., Holmans, P., Hougaard, D.M., Kendler, K.S., Lin, K., Morris, D.W., Mors, O., Mortensen, P.B., Neale, B.M., O'Neill, F.A., Owen, M.J., Milovancevic, M.P., Posthuma, D., Powell, J., Richards, A.L., Riley, B.P., Ruderfer, D., Rujescu, D., Sigurdsson, E., Silagadze, T., Smit, A.B., Stefansson, H., Steinberg, S., Suvisaari, J., Tosato, S., Verhage, M., Walters, J.T., Multicenter Genetic Studies of Schizophrenia Consortium, Levinson, D.F., Gejman, P.V., Kendler, K.S., Laurent, C., Mowry, B.J., O'Donovan, M.C., Owen, M.J., Pulver, A.E., Riley, B.P., Schwab, S.G., Wildenauer, D.B., Dudbridge, F., Holmans, P., Shi, J., Albus, M., Alexander, M., Campion, D., Cohen, D., Dikeos, D., Duan, J., Eichhammer, P., Godard, S., Hansen, M., Lerer, F.B., Liang, K.Y., Maier, W., Mallet, J., Nertney, D.A., Nestadt, G., Norton, N., O'Neill, F.A., Papadimitriou, G.N., Ribble, R., Sanders, A.R., Silverman, J.M., Walsh, D., Williams, N.M., Wormley, B., Psychosis Endophenotypes International Consortium, Arranz, M.J., Bakker, S., Bender, S., Bramon, E., Collier, D., Crespo-Facorro, B., Hall, J., Iyegbe, C., Jablensky, A., Kahn, R.S., Kalaydjieva, L., Lawrie, S., Lewis, C.M., Lin, K., Linszen, D.H., Mata, I., McIntosh, A., Murray, R.M., Ophoff, R.A., Powell, J., Rujescu, D., Van Os, J., Walshe, M., Weisbrod, M., Wiersma, D., Wellcome Trust Case Control Consortium 2, Management Committee, Donnelly, P., Barroso, I., Blackwell, J.M., Bramon, E., Brown, M.A., Casas, J.P., Corvin, A.P., Deloukas, P., Duncanson, A., Jankowski, J., Markus, H.S., Mathew, C.G., Palmer, C.N., Plomin, R., Rautanen, A., Sawcer, S.J., Trembath, R.C., Viswanathan, A.C., Wood, N.W., Data and Analysis Group, Spencer, C.C., Band, G., Bellenguez, C., Freeman, C., Hellenthal, G., Giannoulatou, E., Pirinen, M., Pearson, R.D., Strange, A., Su, Z., Vukcevic, D., Donnelly, P., DNA, Genotyping, Data QC and Informatics Group, Langford, C., Hunt, S.E., Edkins, S., Gwilliam, R., Blackburn, H., Bumpstead, S.J., Dronov, S., Gillman, M., Gray, E., Hammond, N., Jayakumar, A., McCann, O.T., Liddle, J., Potter, S.C., Ravindrarajah, R., Ricketts, M., Tashakkori-Ghanbaria, A., Waller, M.J., Weston, P., Widaa, S., Whittaker, P., Barroso, I., Deloukas, P., Publications Committee, Mathew, C.G., Blackwell, J.M., Brown, M.A., Corvin, A.P., McCarthy, M.I., Spencer, C.C., Bramon, E., O'Donovan, M.C., Stefansson, K., Scolnick, E., Purcell, S., McCarroll, S.A., Sklar, P., Hultman, C.M., Sullivan, P.F., 2013. Genome-wide association analysis identifies 13 new risk loci for schizophrenia. Nat. Genet. 45 (10), 1150–1159.

Rottmann, S., Menkel, A.R., Bouchard, C., Mertsching, J., Loidl, P., Kremmer, E., Eilers, M., Lüscher-Firzlaff, J., Lilischkis, R., Lüscher, B., 2005. Mad1 function in cell proliferation and transcriptional repression is antagonized by cyclin E/CDK2. J. Biol. Chem. 280 (16), 15489–15492.

Schizophrenia Psychiatric Genome-Wide Association Study Consortium, 2011. Genome-wide association study identifies five new schizophrenia loci. Nat. Genet. 43 (10), 969–976.

Silber, J., Lim, D., Petritsch, C., Persson, A., Maunakea, A., Yu, M., Vandenberg, S.R., Ginzinger, D.G., James, C.D., Costello, J.F., Bergers, G., Weiss, W.A., Alvarez-Buylla, A., Hodgson, J.G., 2008. miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. BMC Med. 6, 14.

Smrt, R.D., Szulwach, K.E., Pfeiffer, R.L., Li, X., Guo, W., Pathania, M., Teng, Z.Q., Luo, Y., Peng, J., Bordey, A., Jin, P., Zhao, X., 2010. MicroRNA miR-137 regulates neuronal maturation by targeting ubiquitin ligase mind bomb-1. Stem Cells 28 (6), 1060–1070.

Sun, G., Ye, P., Murai, K., Lang, M.-F., Li, S., Zhang, H., Li, W., Fu, C., Yin, J., Wang, A., Ma, X., Shi, Y., 2011. miR-137 forms a regulatory loop with nuclear receptor TLX and LSD1 in neural stem cells. Nat. Commun. 2, 529.

Szulwach, K.E., Li, X., Smrt, R.D., Li, Y., Luo, Y., Lin, L., Zhao, X., Jin, P., 2010. Cross talk between microRNA and epigenetic regulation in adult neurogenesis. J. Cell Biol. 189 (1), 127–141.

Williams, H.J., Norton, N., Dwyer, S., Moskvina, V., Nikolov, I., Carroll, L., Georgieva, L., Williams, N.M., Morris, D.W., Quinn, E.M., Giegling, I., Ikeda, M., Wood, J., Lencz, T., Hultman, C., Lichtenstein, P., Thiselton, D., Maher, B.S., Molecular Genetics of Schizophrenia Collaboration (MGS) International Schizophrenia Consortium (ISC), SGENE-plus, GROUP, Malhotra, A.K., Riley, B., Kendler, K.S., Gill, M., Sullivan, P., Sklar, P., Purcell, S., Nimgaonkar, V.L., Kirov, G., Holmans, P., Corvin, A., Rujescu, D., Craddock, N., Owen, M.J., O'Donovan, M.C., 2011. Fine mapping of ZNF804A and genome-wide significant evidence for its involvement in schizophrenia and bipolar disorder. Mol. Psychiatry 16 (4), 429–441.

Wright, C., Turner, J.A., Calhoun, V.D., Perrone-Bizzozero, N., 2013. Potential impact of miR-137 and its targets in schizophrenia. Front. Genet. 4, 58.

Xiao, F., Zuo, Z., Cai, G., Kang, S., Gao, X., Li, T., 2009. miRecords: an integrated resource for microRNA-target interactions. Nucleic Acids Res. 37, D105–D110 (Database issue).

Xu, B., Ionita-Laza, I., Roos, J.L., Boone, B., Woodrick, S., Sun, Y., Levy, S., Gogos, J.A., Karayiorgou, M., 2012. De novo gene mutations highlight patterns of genetic and neural complexity in schizophrenia. Nat. Genet. 44 (12), 1365–1369.