NRC Research Press

ARTICLE

Neuregulin 1-alpha regulates phosphorylation, acetylation, and alternative splicing in lymphoblastoid cells[1]

Mohammad M. Ghahramani Seno, Fuad G. Gwadry, Pingzhao Hu, and Stephen W. Scherer

Abstract: Neuregulins (NRGs) are signaling molecules involved in various cellular and developmental processes. Abnormal expression and (or) genomic variations of some of these molecules, such as NRG1, have been associated with disease conditions such as cancer and schizophrenia. To gain a comprehensive molecular insight into possible pathways/networks regulated by NRG1-alpha, we performed a global expression profiling analysis on lymphoblastoid cell lines exposed to NRG1-alpha. Our data show that this signaling molecule mainly regulates coordinated expression of genes involved in three processes: phosphorylation, acetylation, and alternative splicing. These processes have fundamental roles in proper development and function of various tissues including the central nervous system (CNS)—a fact that may explain conditions associated with NRG1 dysregulations such as schizophrenia. The data also suggest NRG1-alpha regulates genes (*FBXO41*) and miRNAs (miR-33) involved in cholesterol metabolism. Moreover, *RPN2*, a gene already shown to be dysregulated in breast cancer cells, is also differentially regulated by NRG1-alpha treatment.

Key words: NRG1-alpha, transcriptome, phosphorylation, acetylation, alternative splicing, schizophrenia.

Résumé : Les neurorégulines (NRG) sont des molécules signal impliquées dans divers processus cellulaires et développementaux. Une expression anormale ou des variations génomiques de certaines de ces molécules, comme la NRG1-alpha, ont été associées à des maladies comme le cancer et la schizophrénie. Afin d'acquérir une compréhension moléculaire approfondie des possibles sentiers/réseaux régulés par la NRG1-alpha, les auteurs ont réalisé une analyse transcriptomique chez des lignées cellulaires lymphoblastoïdes exposées à la NRG1-alpha. Ces données montrent que la molécule signal régule principalement l'expression coordonnée de gènes impliqués dans trois processus : la phosphorylation, l'acétylation et l'épissage alternatif. Ces processus jouent des rôles importants dans le bon développement et la bonne fonction de divers tissus incluant le système nerveux central—cela pourrait expliquer certaines des conditions associées à la dérégulation de la NRG1 comme la schizophrénie. Les données suggèrent aussi que la NRG1-alpha régulerait des gènes (*FBXO41*) et des miARN (miR-33) impliqués dans le métabolisme du cholestérol. De plus, *RPN2*, un gène déjà connu comme étant dérégulé dans le cancer du sein, est également exprimé de manière différentielle suite au traitement avec la NRG1-alpha.

Mots-clés : NRG1-alpha, transcriptome, phosphorylation, acétylation, épissage alternatif, schizophrénie.

Introduction

Neuregulins are a group of signalling proteins that are involved in development of various tissues including the central nervous system (CNS) (Falls 2003; Mei and Xiong 2008). The neuregulin family consists of four major groups of proteins named NRG1–NRG4; common to all is an epidermal growth factor (EGF)-like domain that mediates neuregulin-mediated activation of the ErbB receptors (Buonanno and Fischbach 2001). NRG1 has extensively been studied and shown to have essential roles in heart and breast development and function. Additionally, genomic variations at the *NRG1* locus have been associated with schizophrenia — a highly debilitating psychotic disorder that affects about 1% of the population — and breast cancer.

NRG1 is a large gene that encodes several NRG1 isoforms by using alternate promoters and (or) alternative splicing (Mei and Xiong 2008). NRG1s are expressed in various types of cells and can be categorized in two major groups: (1) those associated with cell membrane and exert their signalling on adjacent receptors or on cells far from them after being cleaved and released (type I, II, IV, V, and VI); and (2) nonsecreted NRG1 (type III) (Mei and Xiong 2008). NRG1s are further divided into alpha and beta classes based on slight difference in their EGF-like domains (Falls 2003).

NRG1s bind ErbB receptors (ErbB3 or ErbB4), where these receptors form homo- or heterodimers (which often include ErbB2). Upon NRG1–ErbBs interaction, the ErbB receptors, which are protein kinases, are activated by autophosphorylation on the tyrosine residues of their cytoplasmic domain. The activated ErbB receptors start a signaling cascade by activating major signalling complexes such as Ras/Map-kinase and PI3-kinase/Akt. This results in various cellular responses and processes such as cell proliferation, differentiation, migration, adhesion, apoptosis, myelination, myogenesis and, angiogenesis — depending on the target cell type (Birchmeier 2009).

NRG1-beta is important for heart development and function (Cote et al. 2005; Pentassuglia and Sawyer 2009; Zhu et al. 2010).

M.M. Ghahramani Seno.* The Centre for Applied Genomics, Program in Genetics and Genome Biology, The Hospital for Sick Children, Toronto, ON M5G 1L7, Canada; Department of Basic Sciences, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.
F.G. Gwadry* and P. Hu. The Centre for Applied Genomics, Program in Genetics and Genome Biology, The Hospital for Sick Children, Toronto, ON M5G 1L7, Canada.
S.W. Scherer. The Centre for Applied Genomics, Program in Genetics and Genome Biology, The Hospital for Sick Children, Toronto, ON M5G 1L7, Canada; McLaughlin Centre and Department of Molecular Genetics, University of Toronto, Toronto, ON M5S 1A8, Canada.

Corresponding author: Mohammad M. Ghahramani Seno (e-mail: mgseno@yahoo.com).
*These authors contributed equally to this work.

[1]This article is part of a Special Commemorative Issue marking the one-year anniversary of "Genomics: The Power and the Promise".

Several groups have studied this isoform functionally, describing global expression profiles induced by NRG1-beta (Amin et al. 2005; Nagashima et al. 2008). Compared to unaffected individuals, lymphoblastoid cell lines (LCLs) from individuals affected with schizophrenia respond differently to NRG1-alpha exposure. LCLs from schizophrenic individuals are slower in responding to chemotactic effect of NRG1-alpha compared to LCLs from unaffected individuals (Sei et al. 2007). This finding indicates that LCLs do express the NRG1 receptor(s), but LCLs from schizophrenic cases are defective or malfunctioning in signalling pathway(s) downstream of NRG1-alpha. Considering the importance of this finding and as there has not been a comprehensive study looking into the molecules involved in NRG1-alpha pathways, we studied global expression profile in LCLs treated with NRG1-alpha compared to their clonal untreated samples. This approach provided clear and unconfounded data indicating that several hundreds of genes are specifically regulated by NRG1-alpha. This list was very significantly enriched with genes that are active in acetylation, phosphorylation (kinase signalling), and alternative splicing processes. These three processes have already been shown to have important roles in CNS development and function, and to be associated with CNS disorders such as schizophrenia and autism (Gavin et al. 2008; Engmann et al. 2011; Kataoka et al. 2011).

Materials and methods

Cell culture, NRG1 treatment, and RNA extraction

LCLs were prepared from blood samples obtained from individuals who had consented to the study by signing related informed consent forms at the Hospital for Sick Children, Toronto, Canada. LCL cultures were grown and maintained in RPMI 1640 (Wisent, Canada) complemented with 15% FCS, 5 mmol/L L-glutamine (Wisent), 100 U/mL penicillin (Wisent), and 100 μg/mL streptomycin (Wisent). The cells were routinely split every 3 days. Two days before NRG1 treatment, the cells were subcultured at 3×10^5 cells/mL in 15 mL of complete media as detailed above. On the day of treatment, the cells were centrifuged and the pellets were resuspended in fresh complete media supplemented with 10% FCS at 6×10^5 cells/mL. For the treated group, 2 mL of complete RPMI media containing 10% FCS and 250 ng/mL NRG1 (R&D systems, cat. No. 296-HR) was added to 2 mL of the aforementioned cell preparations (giving a final concentration of 125 ng/mL of NRG1 on the cells). As the vehicle was BSA, a similar amount of BSA (Probumin, Millipore, cat. No. 81-063-3) was added to each culture in the control group. Cells were transferred to a 37 °C incubator. After the first treatment (27 h), NRG1 was added to the treated group at a final concentration of 40 ng/mL. Cultures in the control group were supplemented with the same amount of BSA. After the second NRG1 treatment (16 h), cells were transferred to room temperature and left for ~1 h to reach room temperature. The cells were then centrifuged at 200g for 10 min, and the pellets were used for total RNA extraction using a miRNA isolation kit (mirVana, Ambion) according to the manufacturer's recommended protocol. The extracted RNA was determined to be of high quality (RIN 9-10) using the Agilent Bioanalyzer (Santa Clara, USA). RNAs were stored at − 80 °C until used.

qPCR and primers

DNase-treated (DNase I, Invitrogen, USA) total RNA (1 μg) was reverse transcribed using the Superscript III reverse transcription kit (Invitrogen) according to the manufacturer's instructions. The product was diluted 5-fold in nuclease-free dH₂O, and 1–2 μL per reaction was used for qPCR. qPCR was performed using SYBR Green (Stratagene) and a relative standard curve method. The *TMEM32* gene was used as an internal normalizer, as it showed

minimal variations (based on our microarray data) among all samples. RT-qPCR primers (supplementary data, Table S1[2]) were designed using Primer 3 software (Rozen and Skaletsky 2000). For miRNA expression assays, the TaqMan microRNA assay kits were purchased from Applied Biosystems (USA) and the general protocol by the manufacturer was followed.

Microarray hybridization

Illumina HumanWG-6_V2 gene expression arrays and Illumina Universal-12 BeadChips (Illumina, Inc., San Diego, USA) were used for global gene and miRNA expression studies, respectively (for details on samples preparation, hybridization, and data acquisition, readers are referred to Ghahramani Seno et al. (2011)).

Statistical data analysis

Raw expression signals were preprocessed using the R (v12.12; http://cran.r-project.org/) Bioconductor package lumi (v2.8; http://bioconductor.org/). After removing the background signals, as defined by the signals from negative control probes, data were $\log_2$ transformed and normalized by quantile normalization. Normalized probe signals were considered acceptable and used for further statistical analyses if their associated detection *P* values were <0.01 for at least 75% of the samples in at least one of the two treated or control groups. Principal component analysis (PCA) was performed based on the selected probe sets to identify outlier arrays.

Differential expression was assessed by a moderated paired *t* test using the R Bioconductor package limma, which is a well-established and commonly used tool for differential expression analysis of data from microarray RNA experiments. The Benjamini and Hochberg method was used to correct for multiple hypothesis testing comparisons and to reduce the type I error rate. Cluster analysis was performed on the differentially expressed genes using average linkage hierarchical cluster analysis with a correlation metric and displayed in the form of a clustered image map (CIM) using CIMminer (http://discover.nci.nih.gov).

Gene ontology analysis

Possible functional enrichment among the differentially regulated genes based on their associated Gene Ontology (GO) terms was assessed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID v6.7; http://david.abcc.ncifcrf.gov). Of the 398 probes submitted, 337 had unique DAVID IDs, which were used for subsequent analyses. Enriched gene sets were graphically organized into networks using Cytoscape network software (v2.80; http://www.cytoscape.org) and the associated Enrichment Map plugin (v1.2; http://baderlab.org/Software/EnrichmentMap).

For a detailed description on how to use Enrichment Map tool for GO enrichment visualization, readers are referred to Merico et al. (2011). Briefly, the DAVID GO enrichment reports from DAVID's functional annotation chart report were loaded into the enrichment map analysis tool in Cytoscape. The "seed" gene sets were identified using a conservative false discovery rate (FDR) *q* value threshold of <0.001; then it was further relaxed to <0.01 and <0.05. The threshold of the weighted overlap coefficient was set at 0.375, which is considered to be moderately conservative.

Results

Experimental design and data quality control

Initially, we performed a sample size estimate to calculate the appropriate sample number required for this study (Liu and Hwang 2007). This analysis indicated eight samples would provide more than 80% power to detect the significant variations induced

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Fig. 1. Sample relation analysis based on normalized gene probe signals. The plot shows sample relations using hierarchical clustering based on probes with a coefficient of variance (standard deviation/mean) > 0.10. The analysis did not detect any major nonspecific source of variation, but as expected it showed relatedness (defined by paired samples) to be grouping the samples.
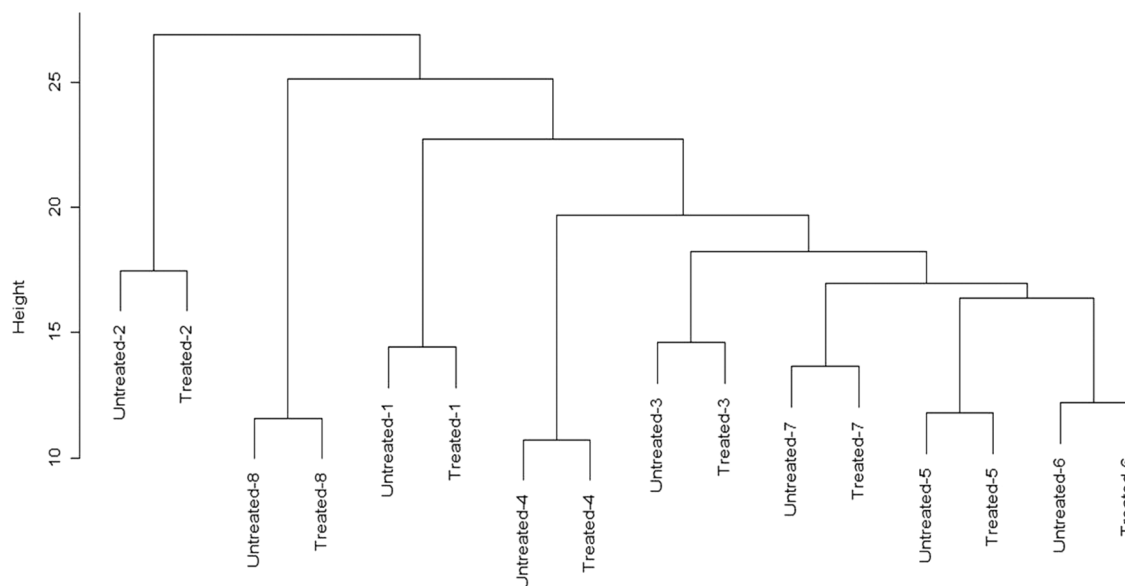
by a sole variant, i.e., NRG1-alpha treatment after multiple hypothesis testing correction (Fig. S1). LCLs from eight healthy males were grown under controlled conditions. Each culture was divided into two during the active growth stage, to act as NRG1-treated and control samples. High quality total RNAs from samples were assayed using Illumina gene and miRNA expression arrays. After applying quantile normalization, an initial detection call filtering was performed on the expression signals (see Materials and methods for details). Accordingly, 11 308 of the total 48 804 (~23%) and 341 of the total 733 (~47%) probe signals for gene and miRNA arrays, respectively, were selected as reliable for further analysis (Fig. S2 gives an overview of data analysis steps).

We took several approaches to qualitatively analyse the normalized expression data and evaluate their soundness. We used hierarchical clustering to estimate the sample relations based on probes with a coefficient of variance (standard deviation/mean) > 0.10. The plot did not detect any major nonspecific source of variation, but as expected it showed relatedness (defined by paired samples) to be grouping the samples (Fig. 1). Moreover, PCA did not detect any major nonspecific source of variation (Fig. S3). Whereas, PCA on the differentially expressed genes (fold change (FC) > 1.2; adjusted P value < 0.001) showed good separation of the NRG1-treated from the untreated group (Fig. 2a). Similarly, hierarchical cluster analysis performed on this gene list also clearly separated the two groups (Fig. 2b).

NRG1-associated transcriptome

When preparing the final list of the differentially expressed genes induced by NRG1-alpha treatment, a stringent criterion of adjusted P values < 0.001 (equivalent to raw P values < 0.000036) plus a fold change of 1.2 was applied. This rather conservative approach of keeping the probes with very small P values was to ensure the relevance of the data and minimize the false positive rate. Table S2 lists all 398 probes representing genes differentially expressed (204 upregulated and 194 downregulated) with FC > 1.2 in either direction and an adjusted P value of <0.001.

To have a comprehensive understanding of the genes differentially regulated by NRG1, we looked for possible module enrichments using GO terms. The differentially regulated genes annotated by GO terms were used to generate enrichment maps. Taking a very conservative setting of FDR q value < 0.001 resulted in a network

of four nodes and three edges (Fig. 3). The enrichment analysis clearly demonstrated significant effects of NRG1-alpha on genes associated with phosphorylation, acetylation, and alternative splicing. Table S3 summarizes the number of genes in each of these three nodes, indicating their common and unique genes. Briefly, the phosphoprotein gene set contained 181 genes, of which 109 were upregulated and 72 were downregulated. The set related to alternative splicing contained 167 genes, of which 106 were upregulated and 61 were downregulated. The acetylation node contained 90 genes, of which 54 were upregulated and 36 were downregulated. As also indicated in Fig. 3 and Table S3, there were many genes in common among these three nodes.

Figures S4 and S5 are enrichment maps generated using more relaxed FDR q values of <0.01 and <0.05, respectively. Using these criteria, the three nodes remain the main nodes associated with NRG1 treatment. The phosphoprotein node is the most significant ($P = 2.33 \times 10^{-11}$, FDR q value = 3.48×10^{-9}) followed by the acetylation ($P = 1.92 \times 10^{-11}$, FDR q value = 5.74×10^{-9}) and alternative splicing ($P = 4.24 \times 10^{-6}$, FDR q value = 4.23×10^{-4}) nodes.

NRG1 has been associated with breast cancer development (Huang et al. 2004; Britsch 2007; Chua et al. 2009). In locally advanced breast cancer, absent or low levels of NRG1-alpha are associated with poorer prognosis than for tumours with moderate to high levels of the protein (Raj et al. 2001). Our data showed that ribophorin II (*RPN2*) was downregulated (FC = –1.48, adjusted $P = 2.89 \times 10^{-5}$) upon NRG1-alpha stimulation of LCLs. In breast cancer cells, *RPN2* is upregulated, and downregulation of *RPN2* using RNAi reduces tumor growth by induction of apoptosis (Honma et al. 2008). p38 MAPK also negatively regulates the proteasome activity (which is often suppressed in breast cancer) by phosphorylating Thr-273 of *RPN2* (Lee et al. 2010). Furthermore, in hepatocelluar cancer, centromere protein A (*CENP-A*) is upregulated, and downregulation of this gene results in reduction of cell proliferation (Li et al. 2011). Chromosome 21 open reading frame 45 (C21orf45; also known as MIS18 kinetochore protein homolog A (MIS18A)) is a CENP-A interactor and its gene was downregulated (FC = –1.54, adjusted $P = 1.22 \times 10^{-5}$) by NRG1 in our experiment.

NRG1 has been shown to regulate cholesterol biogenesis in Schwann cells (Pertusa et al. 2007). F-box protein 41 (*FBXO41*), which is a gene downregulated (FC = –1.97, adjusted $P = 6.01 \times 10^{-7}$)

Fig. 2. Qualitative analysis of the differentially expressed genes by NRG1 treatment. (*a*) PCA based on the 398 differentially expressed genes demonstrated complete separation of NRG1-treated from the untreated group. The first three components accounted for 98.502% of the total variance: with PCs 1, 2, and 3 accounting for 95.84%, 2.09%, and 0.57% of the variance, respectively. Treated and Untreated stands for whether cells were treated with NRG1-alpha, and the numbers indicate sample number. (*b*) Hierarchical cluster analysis of the 398 differentially expressed genes demonstrates treatment as the major clustering index. Both expression patterns in individuals and genes were clustered. The color and intensity indicate direction and level of change: blue spectrum colors indicate downregulated expression; red spectrum colors, upregulated expression.
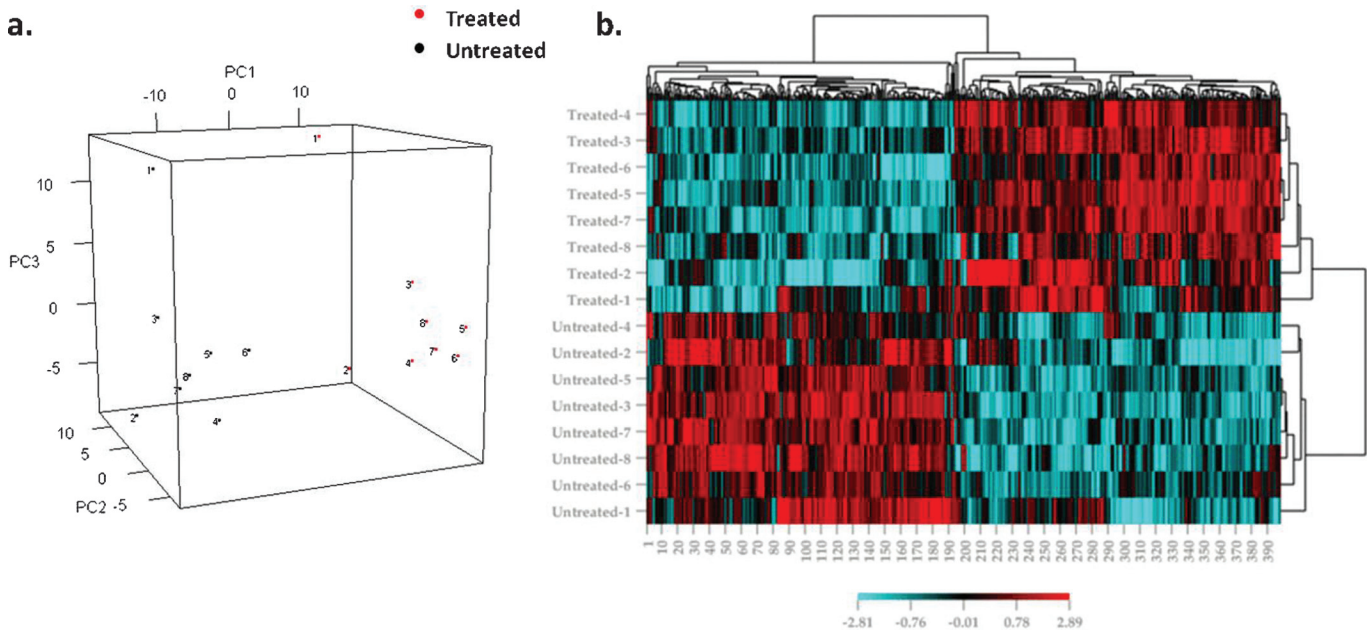

Fig. 3. Enrichment map representation of differentially regulated genes by NRG1 treatment. Enrichment results from DAVID were mapped as a network of gene sets (nodes) related by mutual overlap (edges). A conservative FDR *q* value threshold of < 0.001 was used. This resulted in a network of four nodes (circles) and three edges (links). Node size is proportional to the total number of genes in each set and edge thickness represents the number of overlapping genes between sets. The number in each node indicates the number of genes in that node. The number on each edge indicates the number of overlapping genes between the connecting nodes.
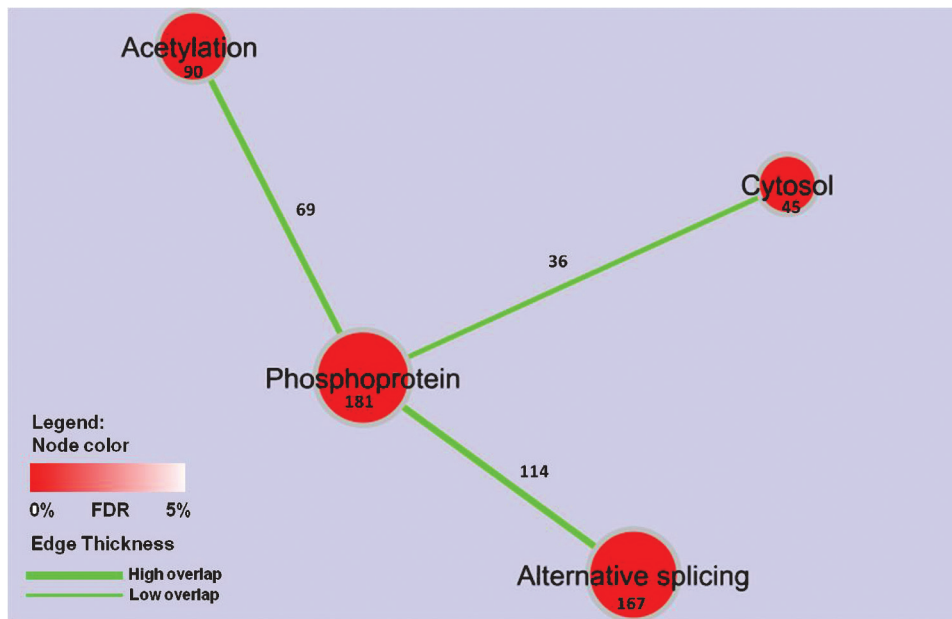

in our experiment, encodes apol-III-like domain, and apoL-III is involved in cholesterol transport (Hara et al. 1992; Kim et al. 2011).

We evaluated the expression levels of four genes, including those mentioned above, by qPCR in all 16 experimental samples (eight treated and eight untreated) (Fig. 4). In at least six of eight compari-sons (treated vs. untreated), the array outcomes were confirmed. Sample 1 was one of the two samples acting differently. Similarly, the PCA based on the differentially expressed genes (Fig. 2a) had shown that sample 1, with some level of variation, was not completely grouping with the rest of the samples.

Fig. 4. qPCR confirmation of microarray data. The expression levels of four genes were individually evaluated in all 16 samples (eight test and eight control samples) using relative standard curve method of qPCR. Each column shows the normalized differential expression level of the gene of interest obtained by comparing each test (NRG1-alpha treated) sample to its own control sample. As shown, the array data was confirmed in at least six out of eight comparisons. Sample 1 is one of the two samples acting differently. Similarly, PCA on array data (Fig. 2a) had already shown that sample 1 did not completely group with the rest of the samples.
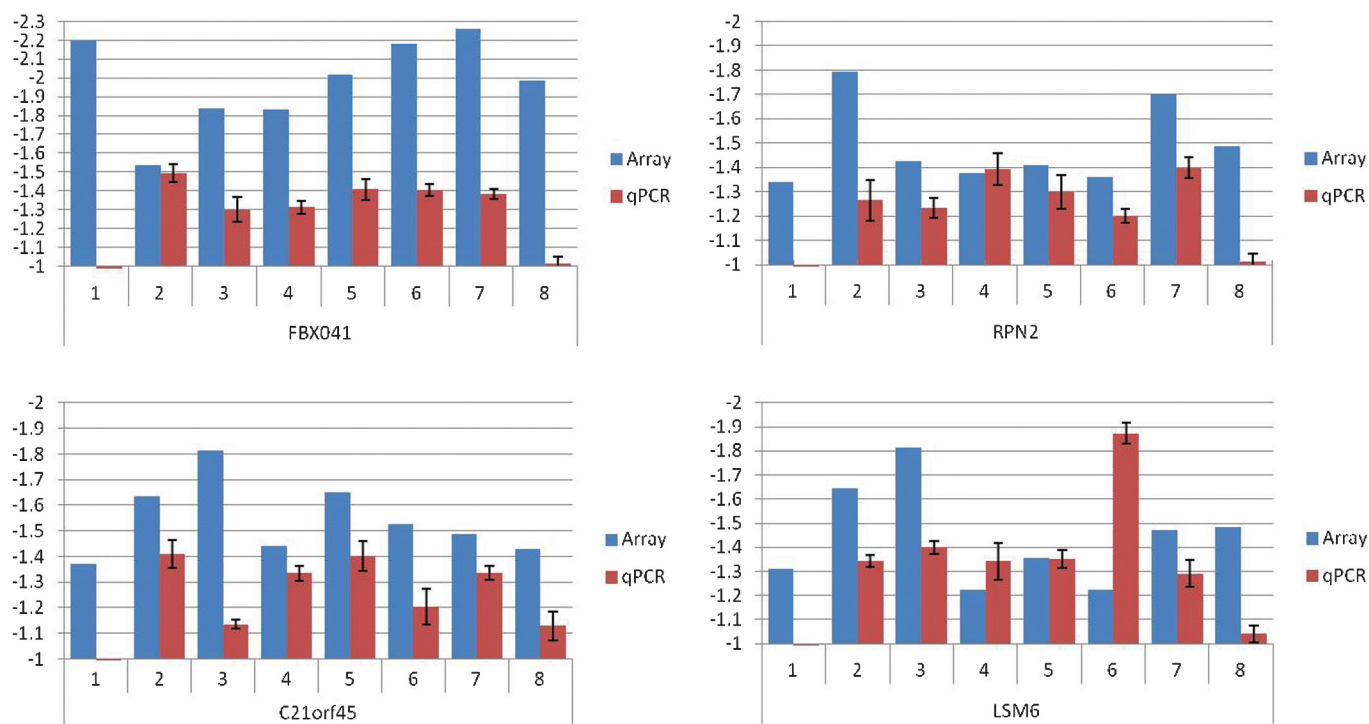
miRNAs differentially regulated by NRG1 treatment

We also evaluated which miRNAs were differentially regulated in LCLs by NRG1, using the same RNA used for expression arrays. Table 1 lists the 11 miRNAs (nine downregulated and two upregulated) differentially expressed with FC > 1.2 and adjusted P value < 0.01. Recently, miR-33a and miR-33b have been shown to be involved in cholesterol and fatty acid metabolism and in an insulin signaling pathway (Gerin et al. 2010; Horie et al. 2010; Marquart et al. 2010; Dávalos et al. 2011). These two miRNAs were amongst the differentially regulated miRNAs identified in our experiment (Table 1). We could not reliably confirm this change by qPCR, but the quality of RNA and microarray chips used and the fact that all the related probes signals, including replicates, had detection call P values < 0.01 offered reassurance of the validity. The notion that *NRG1* regulates cholesterol biogenesis (Pertusa et al. 2007) makes this observation intriguing, enticing us to further investigate the relationship among NRG1, miR-33, and cholesterol metabolism.

Discussion

The neuregulin gene family is involved in various cellular functions and processes including neuronal cells differentiation and migration, and synapse development and plasticity (Birchmeier 2009; Buonanno 2010). Moreover, in addition to being involved in heart and breast tissue development, *NRG1* variations have been associated with disorders such as cancer and schizophrenia (Falls 2003; Banerjee et al. 2010). Several studies have focused on NRG1-beta functions, but the function of NRG1-alpha has not been comprehensively studied. Because pathways downstream of NRG1-alpha had recently been shown to be altered in LCLs from schizophrenia cases (Sei et al. 2007), we studied gene expression profile in LCLs stimulated by NRG1-alpha to gain a comprehensive understanding of genes regulated or influenced by this factor.

For generating the list of differentially expressed genes between the NRG1-alpha treated versus the untreated group, a fold change of 1.2 and above associated with statistically stringent P values (adjusted P values of <0.001, equivalent to raw P values of <0.000036) was used as a cut-off value. Though a fold change of 1.2 may not seem significant in the biological contexts, but associating it with such stringent P values provides very close to real and consistent effects with minimal noise. Therefore, taking this notion we have achieved our goal of this study, i.e., pinpointing the transcripts, and hence gene networks, specifically affected by the NRG1-alpha treatment. Additionally, this study was conducted in LCLs and the effects are accordingly specific to these cell types. Obviously, the NRG1-alpha impact on other tissues such as nervous system can be expected to vary.

Expression profiles of LCLs triggered with NRG1-alpha indicated that NRG1 is, indeed, involved in major cellular signalling networks including phosphoprotein expression, alternative splicing, and acetylation (Fig. 3). The fact that neuregulin receptors, i.e., ErbBs, function as protein kinases, which are involved in protein phosphorylation, makes our finding relevant. However, significant enrichment of targets of protein kinases, i.e., phosphoproteins, amongst the genes whose expression was regulated by NRG1-alpha suggests that NRG1 acts through a mechanism by which the targets of protein kinases such as ErbBs, and their downstream pathways, are themselves regulated by NRG1.

The majority of cellular processes such as appropriate response to the environment by signal transduction, gene expression, cell division, differentiation, metabolic pathways, and apoptosis are regulated by reversible phosphorylation of phosphoproteins by kinases (Graves and Krebs 1999; Daub et al. 2008). Obviously, any deviations from normal expression of these signalling molecules can result in unfavourable phenotypes. Due to its sensitive function and structure, the brain is especially susceptible to subtle

Table 1. Differentially regulated miRNAs.

Probe ID	miRNA ID	Chromosome	FC	P	Adjusted P
ILMN_3167472	hsa-miR-32	9	−3.33	1.16×10^{-5}	0.0019
ILMN_3167691	hsa-miR-33a	22	−2.08	2.69×10^{-5}	0.0023
ILMN_3167212	HS_260	N/A	−1.78	1.72×10^{-5}	0.0019
ILMN_3168168	hsa-miR-519a	19	−1.47	4.44×10^{-4}	0.0079
ILMN_3166988	hsa-miR-33b	17	−1.40	4.24×10^{-4}	0.0079
ILMN_3167761	hsa-miR-212	17	−1.36	1.34×10^{-5}	0.0019
ILMN_3168433	HS_145.1	N/A	−1.29	1.75×10^{-4}	0.0049
ILMN_3168183	hsa-miR-129-5p	7,11	−1.28	2.70×10^{-4}	0.0057
ILMN_3168253	hsa-miR-512-5p	19	−1.25	4.78×10^{-4}	0.0081
ILMN_3167948	hsa-miR-18b	X	1.22	2.37×10^{-4}	0.0054
ILMN_3168213	hsa-miR-99a	21	1.30	2.36×10^{-4}	0.0054

Note: miRNAs with a fold change (FC) > 1.2 in either direction and an adjusted P value < 0.01 are displayed.

changes that would not normally result in any overt phenotype in other tissues; hence, the association of *NRG1* with schizophrenia may be explained. In fact, dysregulation in kinase activity has been suggested in the pathophysiology of some neurodevelopmental disorders such as schizophrenia and autism (Koros and Dorner-Ciossek 2007; Lovestone et al. 2007; Molteni et al. 2009; Freyberg et al. 2010; Kvajo et al. 2010; Ghahramani Seno et al. 2011). In line with this, the signaling pathway related to NRG1–ErbB interaction is mostly dependent on kinase action of intermediates (Banerjee et al. 2010).

Splice variants of genes result from alternative splicing of pre-mRNAs, and they enable the eukaryotic genome to increase proteomic diversity and functional capabilities (Nilsen and Graveley 2010). This phenomenon, which affects the majority of genes encoded by the human genome, is observed in various tissues and different developmental stages, and it has a fundamental biological role (Stamm et al. 2005; Möröy and Heyd 2007; Hartmann and Valcárcel 2009). The CNS is an organ system in which alternative splicing has a significant role for proper development and functions, such as those associated with learning and memory, neuronal cell recognition, neurotransmission, ion channel function, and receptor specificity (Grabowski and Black 2001; Grabowski 2011). Considering the sensitive function of this organ system, even the slightest changes in this process could result in overt phenotypes such as neuropsychiatric disorders. Abnormal splicing patterns of several genes, including *ERBB4* (Law et al. 2007; Goes et al. 2011), are associated with various neuropsychiatric disorders such as schizophrenia, bipolar disorder and major depressive disorder, suicide, substance abuse disorders, and autism (Glatt et al. 2011). Alternative splicing defects and aberrations are also involved in other pathologic conditions such as frontotemporal lobar dementia, tauopathies in the CNS, cancer, hypercholesterolemia, myotonic dystrophy, and spinal muscular atrophy (Tazi et al. 2009). Moreover, chromatin modifications such as phosphorylation and acetylation are processes with major roles in CNS-related functions, such as learning and memory development; aberrations in these processes are reported to be associated with some neuropsychiatric disorders including schizophrenia (Puckett and Lubin 2011).

In our experiment, *FBXO41* was downregulated upon NRG1-alpha treatment. FBXO41 has an apolipophorrin III-like domain (Jin et al. 2004). Apolipophorin-III (apo-Lp III) is involved in lipid transport in insects (Weers and Ryan 2006). Human apolipoprotein (apo) A-IV and apolipophorin III of the insect *Manduca sexta* cause cholesterol efflux from cholesterol-loaded mouse peritoneal macrophages, and reduce intracellularly accumulated cholesteryl ester, as a result of forming HDL-like particles with cellular lipids (Hara et al. 1992). This is interesting considering the finding that NRG1 is involved in cholesterol metabolism (Pertusa et al. 2007). Our miRNA profiling also indicated possible changes of miR-33 expression by NRG1 treatment; miR-33 has been in-

volved in cholesterol metabolism (Horie et al. 2010; Marquart et al. 2010).

All together, this study demonstrated several hundred molecules to be influenced by NRG1-alpha, with the majority involved in three cellular processes: phosphorylation, alternative splicing, and acetylation. These findings may have important implications for understanding disorders such as schizophrenia in which NRG1 plays an important role.

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