

# Integrated microRNA and protein expression analysis reveals novel microRNA regulation of targets in fetal down syndrome

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**Abstract.** Down syndrome (DS) is caused by trisomy of human chromosome 21 and is associated with a number of deleterious phenotypes. To investigate the role of microRNA (miRNA) in the regulation of DS, high-throughput Illumina sequencing technology and isobaric tagging for relative and absolute protein quantification analysis were utilized for simultaneous expression profiling of miRNA and protein in fetuses with DS and normal fetuses. A total of 344 miRNAs were associated with DS. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses were used to investigate the proteins found to be differentially expressed. Functionally important miRNAs were determined by identifying enriched or depleted targets in the transcript and the protein expression levels were consistent with miRNA regulation. The results indicated that *GRB2*, *TMSB10*, *RUVBL2*, the hsa-miR-329 and hsa-miR-27b, hsa-miR-27a targets, and *MAPK1*, *PTPN11*, *ACTA2* and *PTK2* or other differentially expressed proteins were connected with each other directly or indirectly. Integrative analysis of miRNAs and proteins provided an expansive view of the molecular signaling pathways in DS.

## Introduction

Genomic copy variations, including copy number variations and chromosome aneuploidies, offer biological diversity and lead to genetic disorders. Down syndrome (DS) is caused by trisomy of human chromosome 21 (chr21) and is associated with numerous deleterious phenotypes, including cognitive impairment, childhood leukemia and immune defects (1). It occurs in ~1/700 newborns worldwide (2). As the genetic basis for DS is known as an extra copy of chr21, several studies have focused on genes located on chr21. A number of genes located on chr21 are expressed at high levels in individuals with DS (3), however, several genes on other chromosomes are also disordered (4). Understanding the mechanism underlying how the extra chr21 causes various disease phenotypes can lead to improved management and, in the long term, treatment outcomes for individuals with DS. It is important to identify a safe and effective method to identify novel potential biomarkers.

MicroRNAs (miRNAs) are a class of small RNAs of ~22 nt, which are important in a number of key biological processes and several human diseases at the post-transcriptional level of gene expression (5). Previous studies have shown that miRNAs are important in the normal regulation of gene expression during cell proliferation and development (6). Identification of the differential expression of genome-wide known and novel miRNAs can facilitate in uncovering the molecular regulatory mechanisms underlying the progression of the complex and variable phenotype of DS. A previous finding suggested that miR-1246 may serve as a likely link between the p53 family and Down syndrome (7). Previous studies have focused predominantly on the Hsa21-derived miRNAs and have been performed on the tissues of humans with DS, whereas few studies have focused on the expression profile of miRNAs isolated from human blood samples (8-10), and investigation of novel methods in this area is warranted.

Elkan-Miller *et al* (11) used a novel method for the identification of functionally important miRNA-target interactions, integrating transcriptome, proteome and miRNA profiles, and advanced *in silico* analysis using the Functional Assignment of miRNA via Enrichment algorithm. These miRNAs were determined by identifying depleted or enriched targets in the

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**Abbreviations:** DS, Down syndrome; miRNA, microRNA; PAGE, polyacrylamide gel electrophoresis; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GO, gene ontology; ITRAQ, isobaric tagging for relative and absolute protein quantification

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**Key words:** Down syndrome, microRNAs, high-throughput nucleotide sequencing, proteomics, bioinformatics

protein and transcript datasets, with an expression consistent with the accepted model of miRNA regulation. To obtain an unbiased and complete view of the small RNA transcriptome and further investigate the role of miRNAs in early embryonic development of the DS fetus, the present study investigated the regulation of protein and miRNA expression as an initial step towards a better understanding of the regulation of gene expression in DS. The aim was to provide an expansive view of DS from the integrated bioinformatics analysis of proteomics and miRNA data sets.

## Materials and methods

**Patients and controls.** A total of six DS and six matched control fetal cord blood samples (18–22 weeks of gestation) were obtained from the Shenzhen People's Hospital (Shenzhen, China). The diagnosis of DS was confirmed through chromosome examination. The six DS and six control cord blood samples were combined to form pooled DS and control cord blood samples, respectively, for small RNA library construction and Illumina sequencing. The cord blood samples were obtained by puncture extraction with the assistance of a color Doppler ultrasound as the prenatal women were undergoing prenatal diagnosis. The characteristics of each case are provided in the Table I. The present study was approved by the Ethics Committee of Shenzhen People's Hospital. The CBMCs were separated using Ficoll-Paque (Sigma-Aldrich; Thermo Fisher Scientific, Inc., Waltham, MA, USA) density gradient centrifugation according to the manufacturer's protocol. Briefly, 2 ml of blood was layered on 3 ml of Ficoll-Paque and centrifuged for 25 min at 1,000  $\times$  *g* at room temperature. Mononuclear cells were aspirated with a pipette, washed twice in phosphate-buffered saline by centrifugation for 10 min at 700  $\times$  *g* at room temperature and dissolved in 1 ml TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.). These samples were stored at -80°C until further use (12).

The total plasma protein was extracted, and the concentration was measured using a BCA protein kit (Pierce Biotechnology, Rockford, IL, USA). In the present study, prior to proteomic analysis, 40  $\mu$ g of protein from each sample in each group was pooled.

Written informed consent was obtained from all guardians or subjects involved. The use of material for experiments was approved by the Ethics Committee of 181 Hospital (Guilin, China). The study was performed in accordance with the Helsinki Declaration on ethical principles for medical research involving human subjects.

**Deep sequencing.** Total RNA isolation from the CBMCs was performed using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Small RNA library preparation and sequencing were performed using Illumina sequencing technology (BGI, Shenzhen, China). In brief, the small RNA was isolated by separating 10  $\mu$ g of the total RNA using denaturing polyacrylamide gel electrophoresis (PAGE) and excising the region of the gel corresponding to 15–30 nt, based on standard oligonucleotide markers. Small RNAs were then reverse transcribed to cDNA using miRNA-specific stem-loop-like reverse transcription primers and amplified by the ABI PRISM 7500 Sequence Detection System (Applied

Biosystems; Thermo Fisher Scientific, Inc.). Finally, the amplified cDNAs were purified on a 6% Tris-Borate-EDTA PAGE and were sequenced on the Illumina Hi-seq 2000 system (Illumina, Inc., San Diego, CA, USA). Two small RNA sequencing data sets comprising the DS and control CBMCs were obtained from Illumina fast track sequencing services. The frequencies of each small RNA sequence reads were calculated as sequence tags, and only sequences of 18–30 nt were retained for further analysis. All unique sequence reads, which passed above the filters were mapped onto the reference human genome using the SOAP (version 2.0) program ([www.bioconductor.org/packages/2.4/bioc/html/KEGGSOAP.html](http://www.bioconductor.org/packages/2.4/bioc/html/KEGGSOAP.html)) with at most two mismatches (13). The differential expression of miRNAs was calculated by relative expression analysis between the DS and control.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** RT-qPCR was performed as described previously with a minor modification (14). In brief, total RNA was isolated from the CBMCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA was then reverse transcribed into cDNA using miRNA-specific stem-loop like RT primer (GenePharma, Shanghai, China). PCR was performed using an Applied Biosystems 7500 real-time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR reaction was conducted at 95°C for 5 min, followed incubation at 95°C for 15 sec, 65°C for 15 sec and 72°C for 32 sec for 40 cycles using SYBR Green PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan). Each PCR was repeated at least three times. The relative expression level of each miRNA was normalized against the level of RNU6B. Fold-changes were calculated according to the  $2^{-\Delta\Delta C_q}$  method (14).

**Isobaric tagging for relative and absolute protein quantification (iTRAQ) strong cation exchange (SCX)-tandem mass spectrometry (MS/MS) analysis.** The total protein of each corresponding group was blocked, digested and labeled using the iTRAQ protocol (Applied Biosystems; Thermo Fisher Scientific, Inc.). The labeled digests were combined into each sample mixture. Multidimensional liquid chromatography was used to separate the tryptic peptides prior to MS. The combined samples were separated into 10 SCX fractions using a 300Å, 35 $\times$ 0.3 mm, 3.5- $\mu$ m particle size column (Zorbax Bio-SCX; Agilent Technologies, Inc., Santa Clara, CA, USA) with a potassium formate gradient in 25% acetonitrile. The peptides in these fractions were separated on Tempo<sup>™</sup> LC nanoflow and MALDI spotting systems (Applied Biosystems; Thermo Fisher Scientific, Inc.) equipped with a reversed-phase Magic C18AQ column (Phenomenex, Inc., Torrance, CA, USA). Each chromatography run yielded ~380 MALDI spots on a stainless steel MALDI target plate. MS data acquisition was calculated using an Applied Biosystems 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Only a signal to noise ratio  $\geq$ 40 was selected for MS/MS. Mass spectra from 500 laser shots were obtained for each spot. The combined MS/MS data from 10 SCX fractions was used for a Paragon algorithm search engine and human V3.62 (European Bioinformatics Institute; [www.ebi.ac.uk](http://www.ebi.ac.uk)) (15).

Table I. Characteristics of each case.

Mother ID	Age (years)	Gestational age (weeks)	Karyotype result <sup>a</sup>
Patient 1	44	20	47, XX, +21
Patient 2	36	20	47, XX, +21
Patient 3	30	21	47, XY, +21
Patient 4	34	20	47, XX, +21
Patient 5	33	22	47, XY, +21
Patient 6	32	19	47, XX, +21
Control 1	37	21	46, XX
Control 2	36	20	46, XY
Control 3	30	20	46, XX
Control 4	34	18	46, XY
Control 5	33	22	46, XX
Control 6	32	21	46, XX

<sup>a</sup>The karyotype result indicates the total number of chromosomes, the sex chromosomes and the number of the extra chromosome.

#### Bioinformatics analysis

**miRNA expression profile and target gene prediction.** Three software programs were used to predict target genes: miRanda v5 ([www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/](http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/)), TargetScan 5.1 ([www.targetscan.org](http://www.targetscan.org)) and PicTar 2005 ([pictar.mdc-berlin.de/cgi-bin/PicTar\\_vertebrate.cgi](http://pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi)) (16).

The results were predicted by the three software programs at the same time and was considered reliable. The differentially expressed proteins were identified using iTRAQ analysis. Standard human gene symbols of these proteins were used to search the list of miRNA-targeted genes. Cytoscape software was then used to obtain the miRNA and target gene regulation network.

**Protein expression profile and gene interaction regulatory network.** The differentially expressed proteins were analyzed using the Mammalian Protein-Protein Interaction (MIPS) database ([mips.helmholtz-muenchen.de/proj/ppi/](http://mips.helmholtz-muenchen.de/proj/ppi/)), Kyoto Encyclopedia of Genes and Genomes (KEGG) SOAP and co-citation calculation in PubMed ([ncbi.nlm.nih.gov/pubmed](http://ncbi.nlm.nih.gov/pubmed)). A network was constructed by integrating the results of the differentially expressed proteins analyzed using the MIPS database. (17).

**Gene ontology (GO) and KEGG pathway analysis.** To further understand the functions of the identified proteins, the present study used the online GO tool, Web Gene Ontology Annotation Plot (WEGO; <http://wego.genomics.org.cn/>). GO and KEGG pathway mapping of the targeted genes were performed using the web-accessible Database for Annotation, Visualization and Integrated Discovery (DAVID) annotation system ([david.ncifcrf.gov](http://david.ncifcrf.gov)).

#### Results

**miRNA expression profile.** To investigate the expression profile of genome-wide miRNA in the umbilical cord blood (UCB),

Table II. Interaction count analysis of the target gene network.

Target gene	Interaction count
PSMA4	5
PTK2	10
PSMA6	6
COL1A2	7
C4B	6
YWHAG	6
EIF3I	6
MAPK1	11
SF3B1	5
CAV1	7
SERPINH1	5
SCARB2	5
TMSB10	14
RPL30	5
RUVBL2	8
PTPN11	8
GRB2	13
PSMA3	6
ACTA2	15
LAMB2	6
ALB	18
PLG	16
UBE2I	5
FN1	19
SUMO2	7
MARCKS	5
EIF3G	6
RPS14	6
RUVBL1	8
CRK	6
PSMD1	7
PSMD13	6
SNRPD2	6

Genes with an interaction count  $\geq 5$  are included. Interaction count indicates the number of interactions of a gene with other genes. If the number is higher, the target gene is considered of higher significance in the network.

the present study used Illumina sequencing technology to sequence the small RNA libraries of the DS group and normal group. A total of 344 miRNAs were detected as being differentially expressed, of which 46 miRNAs were upregulated and 298 miRNAs were downregulated in DS, compared with the normal control group. To validate the results of the Illumina sequencing, RT-qPCR assays were performed with specific stem-loop RT primers to examine the expression levels of the Hsa21-derived mature miRNAs and randomly selected significantly differentially expressed miRNAs, including four downregulated miRNAs (hsa-miR-16, miR-126, miR-21 and miR-223) and two upregulated miRNAs (hsa-miR-196b and



Table III. Predicted miRNA targets from three commonly used software programs in Down syndrome.

miRNA	Target gene
<b>Downregulated</b>	
hsa-miR-142-3p	RAB2A, LLGL2 TFG, COPG
hsa-miR-197	SSR3, OTUB1
hsa-miR-223	PARP1, POLDIP2
hsa-miR-139-5p	FGA
hsa-miR-150	BASP1
hsa-miR-192	RAB2A
hsa-miR-16	YWHAQ, TUBA1A SNCG, VAMP8 AP2A1, STXBP3
hsa-miR-874	PPP1CA
hsa-miR-590-3p	RAD23B, PPA1
hsa-miR-485-5p	MGST3
hsa-miR-132	PPP2CB, LRRFIP1 SLC2A1, CRK, FKBP2
hsa-miR-431	RBPMS
hsa-miR-411	SF3B3
hsa-miR-24	MPI, CRAT
hsa-miR-543	CASK
hsa-miR-30a	UBE2I, AP2A1
hsa-miR-30e	UBE2I, AP2A1
hsa-miR-329	GRB2
hsa-miR-25	COL1A2, PPP1R12C
hsa-miR-377	RBPMS, OGDHL
hsa-miR-374b	CALU
hsa-miR-107	VAMP8, SNCG SNX3, UMOD
hsa-miR-26a	MICAL3, COL1A2
hsa-miR-379	YARS
hsa-miR-29c	DNAJB11, ARF5 COL4A1, COL1A2 COL1A2, HMG3, TUBB2A
hsa-miR-376a	SUGT1, DLAT
<b>Upregulated</b>	
hsa-miR-206	DDX5, HMG3, SNX2, RRP1 DDX17, TRAPPC3, PGD
hsa-miR-196b	CASK, COL1A2
hsa-miR-183	PPP2CB
hsa-miR-424	TUBA1A, SNCG, VAMP8 AP2A1, CALU, STXBP3
hsa-miR-31	PPP2R2A
hsa-miR-324-5p	RAN
hsa-miR-224	DNAJC8, ZNF207
hsa-miR-28-5p	PSAP, OTUB1
hsa-miR-23b	CFDP1, ENTPD5, PRDX3
hsa-miR-27b	TMSB10, RUVBL2, FBLN2 XPO1, ACTA2
hsa-miR-494	PF4N4, F11R, ZNF207
hsa-miR-145	C6ORF115, ARPC5, AARS

Table III. Continued.

miRNA	Target gene
hsa-miR-363	VPS4B, COL1A2, PPP1R12C
hsa-miR-27a	TMSB10, RUVBL2 FBLN2, ACTA2
hsa-miR-101	RAB5A, ZNF207, FGA
hsa-miR-22	ENO1
hsa-miR-30d	UBE2I, AP2A1
hsa-miR-410	TRAPPC3, CASK
hsa-miR-495	RAN, SEPT7
hsa-miR-499-5p	ERO1L, MARCKS, EPB41L2
hsa-miR-186	EFEMP1, DNAJC8, TPR
hsa-miR-539	DDX5
hsa-miR-376b	SUGT1, DLAT
hsa-miR-23a	CFDP1, ENTPD5
hsa-miR-195	YWHAQ, TUBA1A, SNCG VAMP8, AP2A1, STXBP3
hsa-miR-30c	UBE2I, AP2A1
hsa-miR-221	ARF4
hsa-miR-29a	DNAJB11, ARF5, COL4A1 HMG3, TUBB2A XPNPEP1
hsa-miR-30b	UBE2I, AP2A1
hsa-miR-29b	HMG3, DNAJB11, TUBB2A ARF5, COL1A2
hsa-miR-92b	RRBP1, PPP1R12C
hsa-miR-182	ARF4

Results are the predictions of three software programs (PicTar, miRanda and TargetScan) at the same time, and was considered reliable. miR/miRNA, microRNA.

(84). The significant cellular component GO terms were genes involved in GO:0005829 cytosol (71), GO:0005739 mitochondrion (46), GO:0005768 endosome (15) and GO:0005794 Golgi apparatus (26). Molecular function ontology showed GO:0005515 protein binding (137), GO:0005198 structural molecule activity (23) and GO:0019825 oxygen binding (4). The GO terms indicated that *GRB2*, *TMSB10* and *RUVBL2*, the hsa-miR-329 and hsa-miR-27b, hsa-miR-27a targets, and the differentially expressed proteins were connected with each other either directly or indirectly. There was a direct association between *GRB2* and *MAPK1*, *PTK2* and *PTPN11*. There was also a direct association between *TMSB10*, *RUVBL2* and *ACTA2*. These results suggested that a set of abundant and significantly differentially expressed miRNAs may promote the progression of cognitive impairment in patients with DS by regulating genes in the pathway of nervous system development.

In addition, the present study obtained 28 KEGG pathways of the differentially expressed proteins in DS (Table V), including 'Focal adhesion' (Fig. 3), which was significantly enriched (P<0.05). The potential network of 'Focal adhesion' indicated that *GRB2*, hsa-miR-329 targets and the differentially expressed proteins were connected with each other,

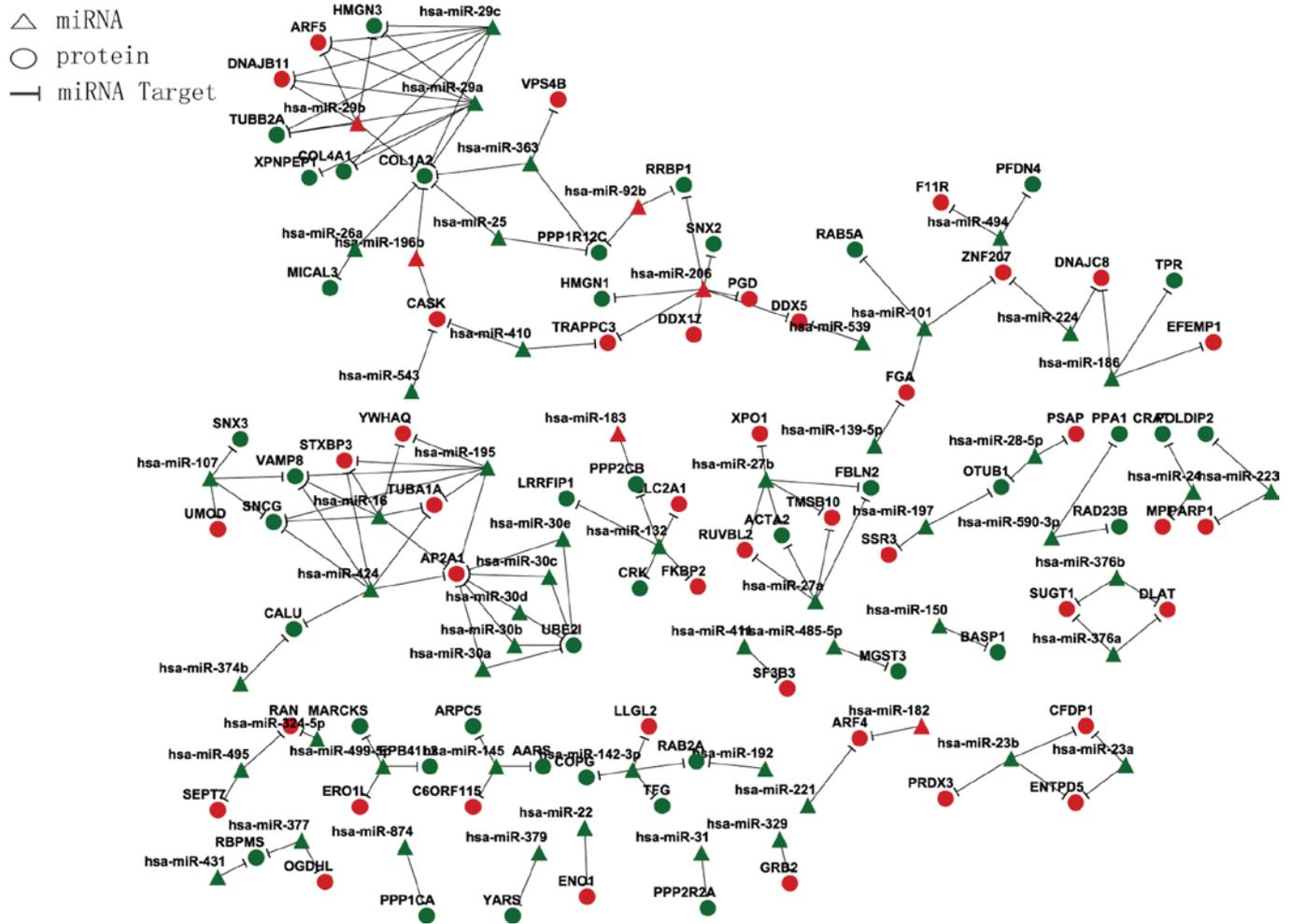


Figure 2. miRNA and target gene association regulatory network in Down syndrome. The miRNA target gene and proteins were considered reliable following prediction by three software programs at the same time. The network was limited to targets predicted by all three software programs. Triangles represent miRNAs, circles represent proteins. Green and red indicate downregulated and upregulated differentially expressed proteins or miRNAs, respectively. miRNA, microRNA.

either directly or indirectly; and there was a direct association of co-citation between *GRB2* and *MAPK1* involved in the MAPK signaling pathway. This suggested that the significantly differentially expressed proteins may promote the progression of cognitive impairment in patients with DS.

**Discussion**

The miRNA-guided regulation of gene expression may involve hundreds of miRNAs and their targets in animals. Genetic studies have successfully identified termed genetic switches of certain miRNA activities, which have intrinsic phenotypic consequences (18). miRNAs are crucial in post-transcription regulation, and the extra hsa21 in DS leads to the disordered expression of genes. The present study aimed to identify the protein and miRNA profiles, and reveal potential miRNA-targets in fetal DS using a combinatorial and novel approach involving iTRAQ quantitative proteomics, deep sequencing and bioinformatic analysis. A total of 58 known miRNAs were detected to have significantly different expression, which may be involved in the variable phenotypes of DS. Their predicted targets were found in the list of the differentially expressed proteins using iTRAQ analysis. This

may indicate a general connection between miRNA regulation of their coding genes and functional complexity of proteins. The present study also integrated miRNA and protein datasets and identified the three most differentially expressed miRNAs, seven differentially expressed proteins and one KEGG pathway in the DS fetus group. Functional analysis of miRNAs in DS is required.

miRNAs are involved in gene regulation, and have been recognized as predictive tools and important intervention targets for several diseases due to the convenience and stability of miRNA detection (19). In the present study, the challenge was to map miRNAs to specific gene targets and the molecular networks they regulate. Studies have provided insights into miRNA-mediated gene regulation in Ts65Dn mice, and the potential contribution to impaired hippocampal synaptic plasticity and neurogenesis, and the hemopoietic abnormalities observed in DS (20,21). The present study found evidence for the functional importance of several previously unknown miRNAs in DS. Specific miRNA expression profiles may point to the particular role of the miRNA in DS. miR-329 can inhibit cell proliferation in human glioma cells through regulating E2F1 (22). miRNA target genes can regulate cell development and differentiation, cell cycle, and apoptosis and miRNAs,

Table IV. Differentially expressed proteins annotation terms of the GO molecular function, cellular component and biological process categories in Down syndrome.

Term	P-value	Upregulated (downregulated) genes (n)	Significantly upregulated genes	Significantly downregulated genes
<b>Biological process</b>				
Cellular amino acid and derivative metabolic process	2.08E-06 <sup>a</sup>	11 (13)		
Transport	3.20E-06 <sup>a</sup>	49 (36)	MAPK1, GRB2	
Cellular component organization	0.001292 <sup>a</sup>	46 (38)	TMSB10, RUVBL2, MAPK1, GRB2	PTPN11
Translation	0.069213	6 (6)	MAPK1	
Protein modification process	0.216467	25 (12)	RUVBL2, MAPK1	PTPN11
Multicellular organismal development	0.288361	33 (32)	MAPK1, GRB2	PTPN11
Carbohydrate metabolic process	0.415425	9 (3)		
Cell communication	0.436129	20 (19)	MAPK1, GRB2	PTPN11
Cell cycle	0.537926	15 (6)	MAPK1	
Lipid metabolic process	0.931277	7 (5)		
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.946888	38 (30)	RUVBL2, MAPK1	
<b>Cellular component</b>				
Cytosol	7.25E-11 <sup>a</sup>	45 (26)	MAPK1, GRB2	PTPN11
Mitochondrion	5.85E-07 <sup>a</sup>	20 (26)	MAPK1	
Endosome	0.006439 <sup>a</sup>	6 (9)	GRB2	
Golgi apparatus	0.009079 <sup>a</sup>	10 (16)	GRB2	
Endoplasmic reticulum	0.012008	13 (14)		
Extracellular region	0.013983	28 (18)		
Cytoskeleton	0.024579	18 (18)	TMSB10, MAPK1	
Lysosome	0.026232	4 (5)		
Vacuole	0.031176	5 (5)		
Peroxisome	0.493033	1 (1)		
Ribosome	0.628763	2 (1)		
Plasma membrane	0.844492	27 (31)		
Nucleus	0.963673	41 (33)	RUVBL2, MAPK1, GRB2	
<b>Molecular function</b>				
Protein binding	2.61E-06 <sup>a</sup>	73 (64)	TMSB10, RUVBL2, PTPN11, MAPK1, GRB2	
Structural molecule activity	0.000156 <sup>a</sup>	11 (12)		
Oxygen binding	0.00194 <sup>a</sup>	3 (1)		
Catalytic activity	0.070336	47 (49)	RUVBL2, MAPK1	PTPN11
Nucleotide binding	0.09617	28 (18)	RUVBL2, MAPK1	
Carbohydrate binding	0.099071	7 (3)		
Lipid binding	0.126594	10 (1)		
Enzyme regulator activity	0.163896	13 (6)		
Transporter activity	0.54845	12 (6)		
Motor activity	0.587484	1 (1)		
Signal transducer activity	0.900132	14 (13)	MAPK1, GRB2	
Transcription regulator activity	0.939697	2 (2)		
Nucleic acid binding	0.990735	18 (20)	RUVBL2, MAPK1	

<sup>a</sup>P<0.05 was considered a statistically significant difference. Genes listed had high interaction counts with other genes and were considered of higher significance.

Table V. Kyoto Encyclopedia of Genes and Genomes pathways of the differentially expressed proteins in Down syndrome.

Pathway	P-value <sup>a</sup>	Upregulated (downregulated) gene number	Significantly upregulated genes
Complement and coagulation cascades	1.43E-09	14 (2)	PLG
Focal adhesion	0.001385	7 (10)	PTK2, MAPK1, FN1, GRB2
Chagas disease (American trypanosomiasis)	0.003458	6 (4)	MAPK1
Pertussis	0.010577	7 (0)	MAPK1
Pyruvate metabolism	0.025336	2 (2)	
Proteasome	1.06E-06	6 (4)	
Oxidative phosphorylation	0.044748	5 (4)	
Fc gamma R-mediated phagocytosis	0.005818	2 (7)	MAPK1
Amoebiasis	0.033113	4 (4)	PTK2, FN1
Glycolysis/gluconeogenesis	0.016705	4 (2)	
Thyroid cancer	0.029584	1 (2)	MAPK1
Pathogenic Escherichia coli infection	0.006884	3 (3)	
Glyoxylate and dicarboxylate metabolism	0.035541	1 (1)	
Staphylococcus aureus infection	2.46E-06	10 (1)	PLG
Systemic lupus erythematosus	0.026282	6 (4)	
Prion diseases	0.002715	5 (0)	MAPK1
Folate biosynthesis	0.014487	0 (2)	
Ribosome	0.032953	3 (4)	
Alanine, aspartate and glutamate metabolism	0.040724	2 (1)	
ECM-receptor interaction	0.000658	2 (8)	FN1
Cell adhesion molecules	0.04671	3 (6)	
Shigellosis	0.01202	1 (5)	MAPK1
Amino sugar and nucleotide sugar metabolism	0.000664	3 (4)	
Citrate cycle (TCA cycle)	0.00118	4 (1)	
RNA transport	0.000224	6 (10)	
Galactose metabolism	0.029584	2 (1)	
Bacterial invasion of epithelial cells	0.000115	3 (7)	PTK2, FN1
African trypanosomiasis	0.00363	5 (0)	

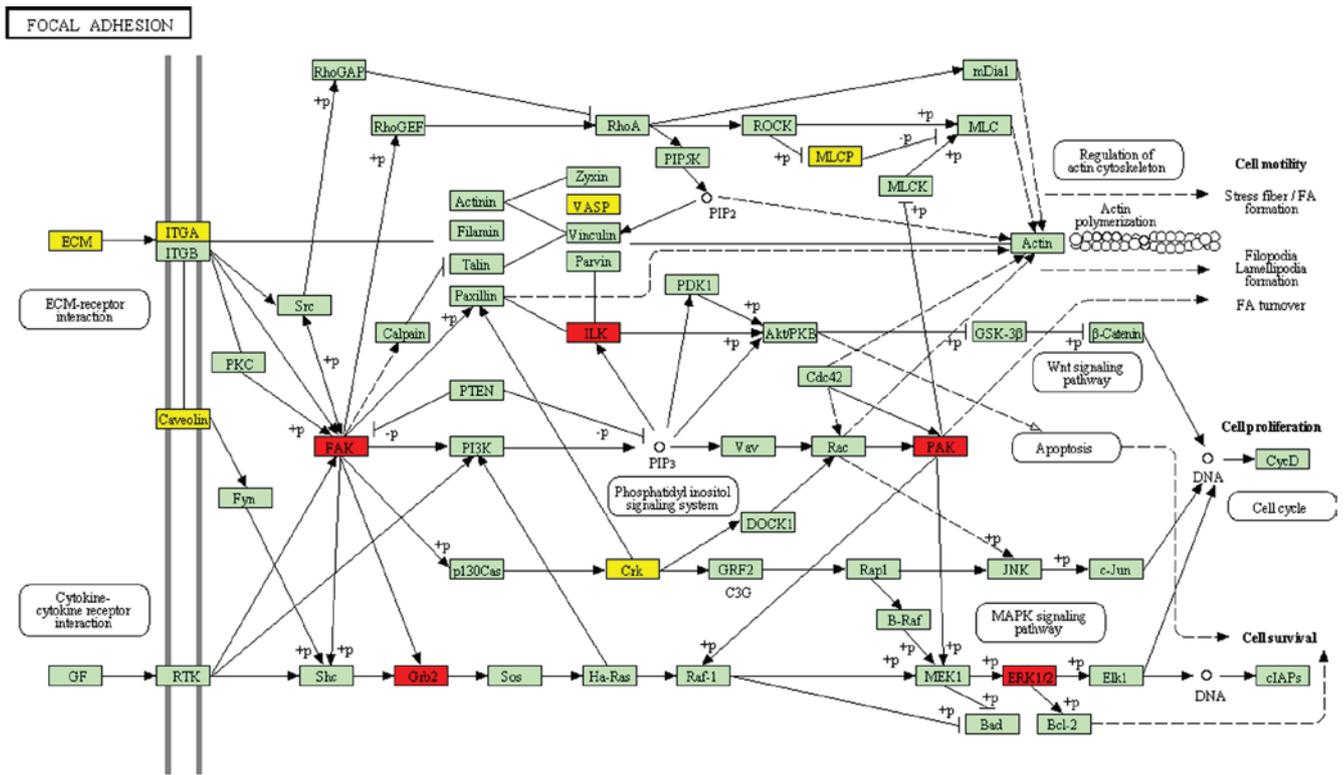
<sup>a</sup>P<0.05 was considered to indicate a statistically significant difference. Genes listed had high interaction counts with other genes and were considered of higher significance.

having an important regulatory role in cell biology (23). miR-27b and miR-27a have been found to negatively regulate adipocyte differentiation through the post-transcriptional regulation of the peroxisome proliferator-activated receptor  $\gamma$  (24). These findings suggest that miRNAs with significantly differential levels of expression are key in cell differentiation in DS. The focus of the present study was not centred on comparing miRNA between normal and DS samples. Therefore, more comprehensive clinical investigations are required to characterize the differential expression of the miRNA identified in DS.

As iTRAQ has previously been suggested to be suitable for identifying novel plasma biomarkers (25), this method has been used to detect for potential quantitative changes in the plasma proteome of fetuses with DS, compared with normal fetuses. These proteins are found in the sera of patients with Alzheimer's disease, which has a similar pathology to DS (26). In addition to the miRNA profile, the present study described changes of

the protein expression profile using iTRAQ. As a result, several proteomic changes in DS were revealed. A number of genes were identified in the analyses, and a comprehensive analysis of protein complexes, which may be coordinately regulated by miRNAs was performed. Gene network methods provide novel insights for elucidating the complexity of diseases, including DS. Hub nodes have been found to be key in several networks. Hub genes with high levels of connection are expected to be important in biology (27). In the present study, *GRB2*, *TMSB10*, *RUVBL2*, *MAPK1*, *PTPN11*, *PTK2* and *ACTA2* were identified as hub genes. These may be important in biological process, cellular component and molecular function in DS, and the proteins identified in DS each require in depth examination in order to understand their functional relevance.

Inhibition of the function of *GRB2* hinders the proliferation and transformation of various cell types and impairs developmental processes in various organisms. Thus, it is not unexpected that a targeted gene disruption of *GRB2* is lethal at



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Figure 3. Focal adhesion pathway of the differentially expressed 3 proteins in Down syndrome. Red indicates an upregulated gene; yellow indicates a down-regulated gene.

an early embryonic stage. Nonchimeric polytransgenic 152F7 mice, which have four human chromosome 21 genes within the DS critical region, present with learning and memory impairment. Decreased levels of *GRB2* in the 152F7 mice may contribute to impaired cytoskeletal functions in the hippocampus (28). *RUVBL2* is important in DNA damage repair, transcriptional regulation and chromatin remodeling (29). *PTK2* is a focal adhesion-associated protein kinase involved in spreading processes and cellular adhesion. Noonan syndrome is a fairly common autosomal, dominantly-inherited disorder. It is the most common syndromal cause of congenital heart disease following DS. In the case of Noonan syndrome, genetic diseases associated with *PTPN11*, mutations are broadly distributed in the coding region of the gene, however, all appear to lead to unregulated, or hyperactivated mutant forms of the protein (30). Impaired signaling in DS involving different signaling systems has been suggested. In addition, the availability of fetal brain and proteome technologies, identifying individual brain proteins, including *MAPK1*, led to the present study investigating individual signaling factors in the brain (31). The functional analysis of the miRNA-regulated protein complexes showed a clear bias towards signal transduction, transcriptional regulation, chromatin regulation and cell cycle. The method used in the present study provided improved candidate miRNA target lists, as demonstrated by a benchmark against large-scale, quantitative proteomics data.

The present study identified more than one potential miRNA-target pair from the predicted targets. Functional annotation indicated that they were involved in clusters of meaningful and significantly relevant biological processes.

Using the target analysis method enabled identification of the miRNA targets affected at the protein level. In order to elucidate the functions of the targets of miRNAs, KEGG pathway and GO term annotation were used to their target gene pool. KEGG annotation showed a significant change in the focal adhesions pathway in the DS group, compared with the normal group. Further investigation of the miRNA-gene network of the pathway showed that hsa-miR-329 may be the key regulators of the focal adhesion pathway. Focal adhesion showed a high level of enrichment and representation in the present study. This pathway includes several proteins, including *MAPK*. *MAPK* pathways can regulate cellular functions, including differentiation, proliferation, apoptosis and migration (32). Therefore, although the exact mechanism remains to be fully elucidated and requires further investigation, miRNAs may be involved in DS by regulating cell proliferation, differentiation and the cell signaling network. Their regulatory roles in the focal adhesions pathway may be involved in the pathogenesis of the DS.

In the present study, the miRNA target prediction and large-scale protein-protein interaction data used was found to be useful for improving current biological knowledge. Taken together, the three miRNAs (hsa-miR-329, hsa-miR-27b and hsa-miR-27a) and seven proteins (*GRB2*, *TMSB10*, *RUVBL2*, *MAPK1*, *PTPN11*, *ACTA2* and *PTK2*) with the highest level of differential expression in the DS fetuses were identified. The results also identified several directions for future investigations. Each possible miRNA-protein pair, which was identified in the present study, is a candidate for further extensive investigation to definitively confirm the presence of specific miRNA-protein interactions, thus providing a more

detailed understanding of the pathogenesis of DS. miRNAs and their target genes maintain a balance of gene expression regulatory networks; if this balance is disrupted, it leads to disease. Therefore, changes in specific miRNA and protein levels may affect gene expression in DS. An understanding of the gene regulatory networks controlled by miRNA in conjunction with protein in DS is required. The present study indicated that miRNAs are probable factors and potential biomarkers involved in the pathogenesis of DS. Further investigations are required to understand the roles of the identified miRNAs in the pathogenesis of DS. Integrating miRNA and protein data sets is a promising strategy for understanding the pathogenesis of DS. The findings of the present study provided insight into the potential contribution of anomalous regulated miRNAs to the abnormalities in DS. This may assist in structuring antenatal diagnostic biomarkers of DS, and identify novel therapeutic targets for the treatment of individuals with DS. The investigation of miRNAs may also lead to the identification of novel methods to prevent and treat other diseases.

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