A Proteomics Approach to Investigate miR-153-3p and miR-205-5p Targets in Neuroblastoma Cells

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Abstract

MicroRNAs are key regulators associated with numerous diseases. In HEK293 cells, miR-153-3p and miR-205-5p down-regulate alpha-synuclein (SNCA) and Leucine-rich repeat kinase 2 (LRRK2), two key proteins involved in Parkinson's disease (PD). We have used two-dimensional gel electrophoresis (2D-PAGE) coupled to mass spectrometry (MS) to identify a spectrum of miR-153-3p and miR-205-5p targets in neuronal SH-SY5Y cells. We overexpressed and inhibited both microRNAs in SH-SY5Y cells and through comparative proteomics profiling we quantified ~240 protein spots from each analysis. Combined, thirty-three protein spots were identified showing significant (p-value < 0.05) changes in abundance. Modulation of miR-153-3p resulted in seven up-regulated proteins and eight down-regulated proteins. miR-205 modulation resulted in twelve up-regulated proteins and six down-regulated proteins. Several of the proteins are associated with neuronal processes, including peroxiredoxin-2 and -4, cofilin-1, prefoldin 2, alpha-enolase, human nucleoside diphosphate kinase B (Nm23) and 14-3-3 protein epsilon. Many of the differentially expressed proteins are involved in diverse pathways including metabolism, neurotrophin signaling, actin cytoskeletal regulation, HIF-1 signaling and the proteasome indicating that miR-153-3p and miR-205-5p are involved in the regulation of a wide variety of biological processes in neuroblastoma cells.

Introduction

Parkinson’s disease (PD) is the most common neurodegenerative movement disorder characterized by degeneration of dopaminergic neurons in the substantia nigra pars compacta [1]. Most PD cases are sporadic but genetic lesions in alpha-synuclein (SNCA) [2], Parkin [3], PINK1 [4], DJ-1 [5] and Leucine-rich repeat kinase 2 (LRRK2) [6] have been associated with both early- and late-onset PD. Despite extensive studies the molecular pathways leading to the onset and progression of PD are poorly understood.
MicroRNAs have been used to decode different pathways associated with several diseases [7]. However, microRNA studies within neurodegeneration are limited. In terms of PD, miR-7/miR-153 and miR-205-5p have been shown to down-regulate SNCA and LRRK2, respectively whilst DJ-1 and Parkin are regulated by miR-34b/c [8, 9, 10]. Indirect regulatory effects on PD-associated proteins have also been reported for miR-133b, miR-433, miR-184” and let-7 [11, 12, 13]. Despite limited data on microRNA regulatory pathways associated with neurodegeneration [14], microRNAs are associated with neuronal stem cell differentiation and development, synapse formation and synaptic plasticity [11, 15].

Individual microRNAs can regulate several mRNAs [16]. Therefore, comparative proteomics profiling in cells with altered microRNA levels has the potential to reveal new microRNA target proteins. The aim of this study was to combine microRNA and proteomics technologies to identify new miR-153-3p and miR-205-5p targets in neuronal SH-SY5Y cells. We selected 2D-PAGE as opposed to LC-MS as although LC-MS analysis is more comprehensive 2D-PAGE offers the possibility of identifying more subtle changes in protein abundance. Several of the protein targets identified are associated with neuronal processes and key regulatory pathways, indicating that miR-153-3p and miR-205-5p are involved in a wide variety of biological processes.

Materials and Methods

Cell culture and transient cell transfection

SH-SY5Y cells (CRL-2266; ATCC) were cultured in a base medium mixture (Full medium: 1:1 DMEM/Ham’s-F12) (Invitrogen) supplemented with 10% v/v fetal bovine serum (Atlanta biologics) and 2 mM GlutaMAX (Invitrogen) in 5% CO₂ atmosphere at 37°C. Transfections were performed, in triplicate, with scrambled control mimic, miR-153-3p mimic, miR-205-5p mimic, scrambled control hairpin inhibitor, miR-153-3p hairpin inhibitor and miR-205 hairpin inhibitor, all mirVana™ (Life Technologies), at a final concentration of 20 nM. Cells were seeded in 6-well plates at 5x10⁵ cells/well. 2μl μRNA (20 μM), diluted with 100 μl of Opti-MEM, and 7 μl Lipofectamine RNAiMax (Invitrogen) diluted with 100 μl of Opti-MEM was incubated for 5 minutes (min) at room temperature (RT). The two solutions were mixed and incubated for 15 min at RT. The transfection mix was diluted to 2 ml with Opti-MEM, added to the wells and incubated at 37°C for 4–6 hours before replacing with full media. Cells were harvested after 24 hours for quantitative PCR (qRT-PCR) analysis and after 48 hours for Western blotting and 2D-PAGE analysis.

RNA isolation, RT-PCR and quantitative real-time PCR

RNA was isolated, in triplicate, 24 hours post-transfection using the miRCURY RNA isolation kit (Exiqon) treated with 1 unit/μg of RNA of DNasel (Thermo Scientific) for 30 minutes at 37°C followed by 10 min at 65°C with 50mM EDTA. cDNA was synthesized using the qScript™ microRNA cDNA Synthesis kit (Quanta Biosciences) and used for both semi-quantitative (25 cycles) and qRT-PCR. miR-153-3p forward primer (5’ GCCGGGCTTGCATAGTCACAA 3’), miR-205-5p forward primer (5’ GTTTCCTTCATTCCACCGG 3’), U6 forward primer (5’ CGCTTCGGCAGCACATATAC 3’), miR-153-3p forward primer (5’ GTTTCCTTCAT TCCACCGG 3’), U6 forward primer (5’ CGCTTCGGCAGCACATATAC 3’) and PerfeCTa Universal PCR primer along with PerfeCTa SYBR® GREEN SuperMix for IQ® were used for qRT-PCR in triplicates for each biological replicate.

Western blotting

Whole cell lysates were prepared using RIPA buffer (150mM NaCl, 1% w/v NP40, 50mM Tris pH 8.0, 0.5% w/v Sodium deoxycholate, 0.1% w/v SDS) 48 hours post-transfection and used for
western blot analysis following published protocol [17]. Primary antibodies used are shown in S1 Table. The secondary antibodies used were goat anti-rabbit or a goat anti-mouse secondary antibody (Jackson Immunoresearch). The reported western blot results are representative of \( n = 3 \).

Two-dimensional gel electrophoresis

**Sample preparation.** Total protein lysates were prepared using Urea solubilization buffer (7M Urea, 2M Thiourea, 4% (w/v) CHAPS and 30mM Tris, 1X protease/phosphatase inhibitor cocktail) and sonication. The supernatant of centrifuged lysates was concentrated using Amicon® Ultra centrifugal filters (10,000 MWCO). Protein concentrations were determined using the Bradford assay (Bio-Rad).

**2D-PAGE.** Protein lysates were diluted with rehydration buffer (7 M urea, 2M thiourea, 2% (w/v) CHAPS, 40 mM DTT, 0.5% IPG buffer, pH 3–10 NL, and 0.4% Bromophenol Blue) and applied to Immobiline™ DryStrip 7 cm, pH 3–10 NL (GE Healthcare) for overnight passive rehydration. Isoelectric focusing was conducted on a PROTEAN® IEF Cell, according to the manufacturer’s recommendation (Bio-Rad). Following rehydration, proteins were reduced with DTT and subsequently alkylated with iodoacetamide in equilibration buffer (6M Urea, 2.5% SDS, 50mM Tris, pH 8.8, 20% glycerol). Electrophoresis in the second dimension was performed on 12% SDS-PAGE at 100V. Gels were stained overnight with colloidal Coomassie Blue G-250 [18].

**Scanning and analysis of the images.** Gels were scanned using EPSON scan perfection V750 PRO software (Digital ICE technologies) at 600 dpi/16-bit grayscale. ImageMaster 2D platinum 7 (GE Healthcare) was used for spot detection, background subtraction, matching, and to identify statistically significant (ANOVA) differences between protein spots i.e. fold change over control. The experiments were performed in triplicate.

**In-gel digestion.** The differentially expressed protein spots were excised, cut into small pieces and placed in 0.6 ml Eppendorf tubes. The gel pieces were destained by incubating in 200 \( \mu l \) of 100 mM ammonium bicarbonate: acetonitrile (50:50 v/v) with shaking. When fully destained, the gel fragments were dehydrated with two washes of 100 \( \mu l \) of 100% acetonitrile (ACN) and were then dried in a vacuum centrifuge (Speed-Vac) for 5 min. The proteins were then cleaved enzymatically into peptides. For this, trypsin solution (2 \( \mu l \) of 0.02 \( \mu g/\mu l \)) was added to the wet the gel pieces and incubation was carried out for 4 hours at RT. Thirty \( \mu l \) of 50 mM ammonium bicarbonate was added to the gel pieces and left overnight at RT to allow for diffusion of the peptides from the gel. The digested proteins were stored at -80°C until further analysis.

**Peptide mass fingerprinting.** After digestion, POROS 20 R2 resin (Applied Biosystems) was added to the digested gel samples with 5% formic acid and 0.2% trifluoroacetic acid for extraction at 4°C for 4 hours on a shaker. Prior to MALDI-MS analysis, the peptide digests were further desalted using ZipTip C18 (Millipore). The ZipTips were conditioned with 10 \( \mu l \) of 0.1% TFA twice, 70% ACN/0.1% TFA twice, and 10 \( \mu l \) of 0.1% TFA twice. Samples containing the digest and bead mixture were transferred to the ZipTips and bound to the C18 resin. The loaded tips were then washed with 10 \( \mu l \) of 0.1% TFA. The peptide digests were eluted by placing 2 \( \mu l \) of 10 mg/mL CHCA matrix solution in 0.003% TFA, 13% ethanol, and 84% ACN onto the top of the ZipTips and slowly dispensing onto the MALDI plate. Mass spectrometric analysis was performed using a Thermo LTQ XL linear ion trap mass spectrometer (Thermo Scientific) equipped with a vacuum MALDI source, after the solvent evaporated at RT and the CHCA matrix was crystallized with peptides on the MALDI plate. A data-dependent acquisition was performed using Xcalibur software, in which the top 40 of the most abundant
precursor ions from the survey scan (mass range 700–3500 Da) were chosen and MS/MS acquisition was triggered to fragment them by CID (collision-induced dissociation). The normalized collision energy was 50%, and the isolation width was 3 Da. The raw-files from the LTQ mass spectrometer were analyzed by using Mascot Distiller 2.3.2 (Matrix Science, Boston, MA) for protein identification. Peptide masses were matched against the taxonomy Homo sapiens in the National Centre for Biotechnology Information non-redundant (NCBI) database. One missed trypsin cleavage per peptide was allowed and an initial mass tolerance of 0.3 Da was used in all searches. Complete carboxyamidation of cysteine sulfhydryls and partial oxidation of methionine were assumed [19].

Cell viability and reactive oxygen species measurements

Cell viability was measured using the neutral red uptake assay 48 hours post-transfection. Cells were washed with PBS, 100µl of neutral red working solution (40µg/ml) added to each well and plates were incubated for 2 hours. Cells were then washed with PBS, neutral red extracted using 150µl of destain solution (50% ethanol, 1% glacial acetic acid, 49% deionized water) per well and the plates were subjected to shaking for 10 min. Absorbance was measured at 540nm using an Epoch microplate spectrophotometer (BioTeck).

Cellular reactive oxygen species (ROS) were measured using 2’, 7’- dichlorofluorescein diacetate (DCF-DA) (Sigma-Aldrich). Cells were plated on solid black opaque plates at 5x10^4 cells per well and after 48 hours incubated with 100µl of DCF-DA (25µM) for 45 min. Fluorescence was measured using a GLoMas® Multi Detection System fluorescence plate reader (Promega) at 485nm (excitation) and 528nm (emission). The assays were performed in triplicate.

Image analysis, statistical analysis and contextual analysis

Western blot images were analyzed using IQTL software (GE Healthcare). Microsoft excel tools was used for two-tailed Student’s t-test. The standard error was used to display variation. The targets of miR-153-3p and miR-205-5p were used as input queries for the Partek Genomics Suite software, version 6.6 (Partek) to perform Gene ontology (GO) analysis and generate interactive maps and pathways.

Results and Discussion

Overexpression and inhibition of miR-153-3p and miR-205-5p in SH-SY5Y cells

miR-153 overexpression in HEK293 cells downregulate SNCA whilst miR-205 overexpression in HEK293 cells has been shown to downregulate LRRK2 [8, 9]. In this study, we selected the neuroblastoma cell line SH-SY5Y as its neuronal characteristics represents a better platform to dissect microRNA-regulated pathways and mechanisms associated with PD. miR-153-3p was successfully overexpressed using miR-153-3p mimic and inhibited using miR-153-3p antagomir in SH-SY5Y cells (Fig 1A and 1B). Similarly, miR-205-5p was successfully overexpressed using miR-205-5p mimic and inhibited using miR-205-5p antagomir (Fig 1A and 1B). Further, we used qPCR to verify the down regulation of both the microRNAs due to the antagomirs (Fig 1C).

Altered levels of miR-153-3p and miR-205-5p results in protein changes associated with a spectrum of biological processes

We next sought to identify additional targets of miR-153-3p and miR-205-5p in SH-SY5Y cells using 2D-PAGE analysis. However, before performing 2D-PAGE analysis we showed that
miR-153-3p and miR-205-5p transfections had no significant effect on SH-SY5Y cell viability ensuring that any observed protein changes were due to changes in miR-153-3p and miR-205-5p levels (Fig 1D).

We performed comparative 2D-PAGE analysis comparing control mimic and control antagomir transfected cells with cells transfected with the miR-153-3p mimic and the miR-153-3p antagomir, respectively (Fig 2). The same comparative analyses were performed for SH-SY5Y cells transfected with the miR-205-5p mimic and the miR-205-5p antagomir (Fig 3).

We identified thirty-three protein spots that showed significant abundance changes (fold change > 1.4, n = 3, p-value < 0.05) between control transfected and miR-153-3p/miR-205-5p-transfected SH-SY5Y cells. In response to altered levels of miR-153-3p seven protein spots were up-regulated whilst eight protein spots were down-regulated (Fig 2, Table 1). In response to miR-205-5p perturbations twelve protein spots were up-regulated whilst six protein spots were down-regulated (Fig 3, Table 1). The protein spots were subjected to MS and the fragment spectra were searched against the NCBInr database (taxonomy Homo sapiens) using Mascot Distiller revealing the identity of the differentially expressed proteins (S1 Fig; Table 1, S2 Table).

Regulation of key neuronal processes by miR-153-3p and miR-205-5p

Overexpression of miR-153-3p resulted in up-regulation of proteasome subunit alpha type-1 isoform 2 (PSMA1) (Table 1, spot 4; S2 Fig). miR-205-5p overexpression also increased the abundance of proteasome subunit p42 (PSMC6) (Table 1, spot 24; S2 Fig) and proteasome subunit alpha type-1 isoform 2 (PSMA1) (Table 1, spot 21). Efficient proteasome activity is vital in neurons as inappropriate degradation of misfolded proteins, such as amyloids and SNCA, results in aggregate formation, a hallmark of AD and PD [12, 20].

miR-153-3p overexpression also resulted in increase abundance of Prefolding subunit 2 (PFDN2) (Table 1, spot 7), which transfers misfolded proteins to chaperonin ensuring proper folding [21]. This indicates that miR-153-3p may up-regulate PFDN2 in response to increased levels of misfolded proteins as a neuroprotective mechanism.

We also found that cathepsin Z (CTSZ) (Table 1, spot 13) is down-regulated in response to miR-153-3p inhibition. In aging mouse brains cathepsin is upregulated, impairing neuronal
survival and neuritogenesis, indicating that miR-153-3p may regulate cathepsin levels to maintain a healthy neuronal population [22].

Inhibition of miR-153-3p also results in the up-regulation of the calcium activated chloride channel family member 4 (CLCA4) (Table 1, spot 9). Calcium activated chloride channels are highly expressed in microglia and activated microglia and a reduction in toxicity is seen in response to CLCA inhibitors [23]. CLCA4 inhibition by miR-153-3p may contribute to neuroprotection.

The stress-induced phosphoprotein 1 (STIP1) (Table 1, spot 4) is also up-regulated in response to the miR-153-3p mimic whilst the expression of mortalin (Heat shock 70kDa protein 9-HSPA9) (Table 1, spot 6) is down-regulated. The STIP1 protein forms a complex with HSC70 and HSP90 [24] and STIP1 is elevated in serum from patients with neuro-Behçet's disease [25]. We also found that 14-3-3 protein epsilon (14-3-3E) (Table 1, spot 15), involved in cell cycle regulation, PI3-Akt signaling, Hippo signaling, Neurotrophin signaling, and viral carcinogenesis (Fig 4), is down-regulated in response to miR-153-3p inhibition [26]. Several 14-3-3 isoforms are present in Lewy bodies suggesting the involvement of 14-3-3 proteins in neurodegeneration [27]. The regulation of 14-3-3 proteins by microRNAs has been documented where 14-3-3zeta is a direct target of miR-451 [28].
In response to miR-153-3p inhibition cofilin-1 was down-regulated (Table 1, spot 32), verified by western blot analysis (Fig 5B), whilst miR-205-5p overexpression resulted in cofilin-1 up-regulation (Table 1, spot 18). Cofilin-1 is involved in protein translocation, rod-shaped actin bundle formation and is activated by amyloid-beta (Abeta 1–42) [29, 30]. Rod-shaped actin bundles are sites for amyloid-precursor protein accumulation in AD [31]. Western blot analysis confirmed cofilin-1 regulation by the miR-205-5p mimic (Fig 5C) and the antagomir (Fig 5D).

Our data indicates that both miR-205-5p and miR-153-3p influence direct and peripheral processes associated with neurodegenerative disorders, providing clues towards the possible regulation of key pathways (S4 Table, Fig 4).

Peroxiredoxins are regulated by both miR-153-3p and miR205-5p. We found that miR-153-3p overexpression leads to an up-regulation of peroxiredoxin 2 (PRDX2) (Table 1, spot 1) whilst miR-153-3p inhibition results in peroxiredoxin-4 (PRDX4) precursor up-regulation (Table 1, spot 9). Similar effects were also observed for miR-205-5p (Table 1, spot 19 & 29). The PRDX family protects cells from oxidative stress-induced apoptosis and have been associated with neurodegeneration [32]. PRDX2 overexpression in MN9D neuronal cells results in a ROS decrease and prevention of 6-OHDA-induced ASK1 activation by regulating the redox

Fig 3. Comparative proteomic profiling in SH-SY5Y. Two-dimensional gels of control mimic, miR-205-5p mimic, control antagomir and miR-205-5p antagomir transfected cells. n = 3 for all experiments. Numbers (16–33) represent differentially expressed protein spots identified by MS, reported in Table 1. doi:10.1371/journal.pone.0143969.g003
Table 1. List of differentially regulated proteins in SH-SY5Y cells in response to mimics and antagomirs of miR-153-3p and miR-205-5p.

| Spot No. | UniProt Accession | Description | Sequence coverage | Fold change | Significance p-value |
|----------|-------------------|-------------|-------------------|-------------|----------------------|
| **miR-153-3p mimic—upregulated proteins** | | | | | |
| 1 | P32119 | Peroxiredoxin-2 | 27 | 1.66 ± 0.13 | 0.03 |
| 2 | P11177 | Pyruvate dehydrogenase beta subunit | 12 | 2.26 ± 0.19 | 0.04 |
| 3 | P09429 | High mobility group protein B1 (HMGB-1) | 26 | 1.64 ± 0.11 | 0.02 |
| 4 | P25786 | Proteasome subunit alpha type-1 isoform 2 | 20 | 1.49 ± 0.09 | 0.03 |
| | P31948 | Stress-induced-phosphoprotein 1 | | | |
| **miR-153-3p mimic—downregulated proteins** | | | | | |
| 5 | Q8IW75 | Serpin A12 precursor | 1 | 1.44 ± 0.04 | 0.04 |
| 6 | P38646 | Heat shock 70kDa protein 9 (mortalin) | 24 | 1.84 ± 0.22 | 0.003 |
| 7 | Q9UHV9 | Prefoldin subunit 2 | 16 | 2.34 ± 0.25 | 0.04 |
| **miR-153-3p antagomir—Upregulated proteins** | | | | | |
| 8 | P05388 | 60S acidic ribosomal protein P0 | 35 | 2.24 ± 0.13 | 0.05 |
| 9 | Q13162 | Peroxiredoxin-4 precursor | 30 | 2.03 ± 0.43 | 0.05 |
| 10 | Q14CN2 | Ca2+-activated chloride channel protein 2 | 3 | | |
| **miR-153-3p antagomir—downregulated proteins** | | | | | |
| 11 | P06733 | Alpha-enolase | 13 | 3.18 ± 0.09 | 0.01 |
| 12 | P13929 | Beta-enolase | 22 | 1.84 ± 0.13 | 0.03 |
| 13 | Q9UBR2 | Cathepsin Z precursor | 6 | 2.65 ± 0.16 | 0.01 |
| 14 | P23528 | Cofilin-1 | 53 | 2.11 ± 0.20 | 0.004 |
| 15 | P68104 | Elongation factor 1-alpha 1 | 10 | 2.55 ± 0.64 | 0.01 |
| 16 | P62258 | 14-3-3 protein epsilon | 24 | | |
| **miR-205-5p mimic—upregulated proteins** | | | | | |
| 16 | Q13765 | Nascent-polypeptide-associated complex alpha (HSD48) | 25 | 1.80 ± 0.28 | 0.05 |
| 17 | P63241 | Eukaryotic translation initiation factor 5A-1 isoform B | 22 | 2.28 ± 0.27 | 0.05 |
| 18 | P23528 | Cofilin-1 | 43 | 2.11 ± 0.77 | 0.009 |
| 19 | P32119 | Peroxiredoxin-2 | 21 | 1.99 ± 0.03 | 0.05 |
| 20 | P04083 | Annexin A1 | 7 | 2.08 ± 0.16 | 0.04 |
| 21 | P25786 | Proteasome subunit alpha type-1 isoform 2 | 9 | 1.61 ± 0.16 | 0.001 |
| 22 | Q13126 | Methylthioadenosine phosphorylase | 16 | 1.80 ± 0.24 | 0.04 |
| 23 | Q13347 | Eukaryotic translation initiation factor 3 subunit I | 13 | 1.55 ± 0.03 | 0.04 |
| 24 | P62333 | Proteasome subunit p42 | 3 | 1.90 ± 0.09 | 0.04 |
| **miR-205-5p mimic—downregulated proteins** | | | | | |
| 25 | P50213 | Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial precursor | 23 | 2.02 ± 0.24 | 0.007 |
| | P04406 | Glyceraldehyde-3-phosphate dehydrogenase | 4 | | |
| 26 | Q8IW75 | Serpin A12 precursor | 1 | 2.38 ± 0.50 | 0.03 |
| **miR-205-5p antagomir—upregulated proteins** | | | | | |
| 27 | Q13148 | TAR DNA-binding protein 43 | 7 | 2.03 ± 0.09 | 0.01 |
| 28 | Q07955 | Serine/arginine-rich splicing factor 1 isoform 1 | 53 | 2.24 ± 0.58 | 0.04 |
| 29 | Q13162 | Peroxiredoxin-4 precursor | 33 | 1.66 ± 0.09 | 0.04 |
| **miR-205-5p antagomir—downregulated proteins** | | | | | |
| 30 | P09382 | Human Galectin-1 | 52 | 1.80 ± 0.22 | 0.03 |
| 31 | P22392 | Human Nucleoside Diphosphate Kinase B (Nm23) | 24 | 1.55 ± 0.00 | 0.03 |
| 32 | P23528 | Cofilin-1 | 34 | 2.11 ± 0.30 | 0.02 |

(Continued)
status of thioredoxin (Trx), whilst PRDX2 knockdown causes a ROS increase [33]. Cytosolic PRDX2 (S3 Table) can also act as a chaperone protecting citrate synthase, insulin and SNCA from stress-induced aggregation [34, 35]. We verified the miR-153-3p- and miR-205-5p-mediated increase in PRDX2 by western blot analysis (Fig 5A and 5C). PRDX4 is putative tumor driver where down-regulation of PRDX4 in glioblastoma multiformes (GBMs) results in decreased cell growth and increased levels of ROS, DNA damage, and apoptosis [36].

The regulation of PRDXs by miR-153-3p and miR-205-5p suggest that miR-153-3p and miR-205-5p may affect cellular ROS levels. Indeed, overexpression of miR-153-3p and miR-205-5p causes significant ROS reduction (Fig 5E). Combined this indicate that miR-153-3p and miR205-5p influence PRDX levels, which may affect ROS levels (Fig 5F).

**miR-153-3p and miR-205-5p alter known cell cycle regulators**

Numerous microRNAs are involved in the cell cycle, cancer proliferation and metastasis [37]. In response to miR-153-3p inhibition we identified Nucleoside diphosphate Kinase B (Nm23) (Table 1, spot 10) and tumor suppressor alpha-enolase (Table 1, spot 11), two cell cycle

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**Table 1. (Continued)**

| Spot No. | UniProt Accession | Description | Sequence coverage | Fold change | Significance p-value |
|----------|-------------------|-------------|-------------------|-------------|---------------------|
| 33       | Q13765            | Nascent-polypeptide-associated complex alpha (HSD48) | 25               | 1.69 ± 0.00 | 0.05               |
| Q01105   | Protein SET       |             | 5                 |             |                     |

**X:** Single peptides identified from individual protein spots in two (*), three (**) or seven (***) independent MALDI-MS detections. All the protein spots were picked and analyzed from at least two independent experiments. Fold change ± error are calculated with respect to control mimic and control inhibitor by ImageMaster 2D platinum 7 (GE) software. The significance was calculated using two-tailed t-test.

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*Fig 4. Protein association network showing interconnecting relationships between miR-153-3p and miR-205-5p target proteins through key regulatory pathways. YWHAE: 14-3-3 epsilon protein; ENO1: Alpha-enolase; ENO3: beta-enolase; IDH3A: Isocitrate dehydrogenase [NAD] subunit alpha; PDHB: Pyruvate dehydrogenase complex beta subunit; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.*

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miR-153-3p and miR-205-5p regulatory proteins [38, 39]. miR-153-3p inhibition results in increased abundance of Nm23 (Fig 2, spot 10), known as a transcriptional activator of c-myc [40]. In contrast, alpha-enolase is down-regulated in response to miR-153-3p inhibition (Table 1). Interestingly, alpha-enolase can bind to the c-myc promoter, but in contrast to Nm23, represses c-myc expression [41].

We also found that altered levels of miR-205-5p affect proteins associated with tumor proliferation and invasion (Table 1). miR-205-5p inhibition down-regulates Nm23 (Table 1, spot 31) and Protein SET (Fig 2, spot 33). Protein SET, part of the inhibitor of acetyltransferases (INHAT) complex, is up-regulated in numerous tumors [42]. Interestingly, a jun co-activator, Nascent-polypeptide-associated complex alpha (HSD48), was up-regulated (Table 1, spot 16) by miR-205-5p overexpression whilst its expression decreased (Table 1, spot 33) by miR-205-5p inhibition [43, 44]. HSD48 (NACA) regulation by miR-205-5p was confirmed by western blot analysis (Figs 5C and 4D).

Galectin-1, a beta-galactoside binding protein associated with cell proliferation and differentiation is also down-regulated in response to miR-205-5p inhibition (Table 1, spot 30) [45].

Combined these results indicate that miR-153-3p and miR-205-5p may play a role in cell proliferation and migration involving various target proteins.
miR-153-3p and miR-205-5p have roles in regulating proteins involved in metabolic pathways

Glucose stimulation increases miR-153 expression and miR-153 expression is reduced in PTPRN2 (Protein tyrosine phosphatase receptor type N polypeptide 2) mouse knockout models [46]. We found that the expression of adipokine Serpin A12 (SERPINA12) (Table 1, spot 5) is down-regulated in response to miR-153-3p overexpression whilst the pyruvate dehydrogenase complex beta subunit (PDHB) (Table 1, spot 2), a key enzyme linking the glycolytic pathway to the TCA cycle, is up-regulated (Table 1) [47].

miR-153-3p overexpression also resulted in the up-regulation of High mobility group protein B1 (HMGB1) (Table 1, spot 3), involved in remodeling chromatin affecting gene expression (S3 Table) [48]. HMGB1-deficient mice have lethal hypoglycemia causing death within 24 hours [49]. To verify the up-regulation of HMGB1 in response to miR-153-3p overexpression we performed western blot analysis (Fig 5A). Interestingly, cofilin-1 (CFL1) (Table 1, spot 14), which decreases in abundance as a result of miR-153-3p inhibition, is shown to act as glucocorticoid receptor inhibitor [50].

Similar to miR-153-3p, miR-205-5p also down-regulates Serpin A12 (Table 1, spot 26). Furthermore, miR-205-5p down-regulates isocitrate dehydrogenase [NAD] subunit alpha (IDH3A) (Table 1, spot 25), a key enzyme in the TCA cycle and GAPDH (Table 1, spot 25)[51, 52]. miR-205-5p also up-regulates Annexin A1 (Table 1, spot 20), a protein that regulates phospholipase A2 activity [53].

Collectively, miR-153-3p and miR-205-5p appear to regulate proteins involved in metabolic pathways and in particular carbohydrate metabolism (S4 Table).

miR-205-5p is associated with transcriptional regulation

miR-205-5p appears to be affecting the abundance of proteins that influence mRNA expression and processing (Table 1 and Fig 2). The serine/arginine-rich splicing factor 1 (SRSF-1) (Table 1, spot 28), which ensures splicing accuracy and regulates alternative splicing, is up-regulated in response to miR-205-5p inhibition [54]. Indeed, HSD48 (Table 1, spot 16 & 33), which is regulated by miR-205-5p, is a transcription regulator [55]. miR-205-5p inhibition also causes increased abundance of the TAR DNA-binding protein 43 (TDP-43) (Table 1, S3 Table, spot 27) that promotes CFTR exon skipping and regulates transcription [56]. Nm23, a gene expression modulator, is also regulated by miR-205-5p showing decreased levels in response to miR-205-5p inhibition (Table 1, spot 31) [57].

As microRNAs are most commonly involved in translational regulation, the up-regulation of eukaryotic translation initiation factor 5A-1 isoform B (EIF5A) (Table 1, spot 17) and (EIF3I) (Table 1, spot 23), in response to miR-205-5p was not surprising.

Concluding remarks

MicroRNA biology is complex and we have shown that miR-153-3p and miR-205-5p influences the abundance of numerous proteins integral to many biological processes in neuroblastoma cells (Fig 4, S3 Table). Interestingly, we observed that some proteins (cofilin-1 and HSFD48) show reciprocal regulatory effects in response to miRNA mimic and antagonir whilst other proteins did not show this reciprocal regulation. This suggests that the proteins identified in this study represent a combination of direct and indirect targets of miR-153-3p and miR-205-5p.

Some of these processes associated with the identified proteins are fundamental in nature whilst others are specifically associated with cell survival, cell proliferation and...
neuroprotection. Although we acknowledge that the altered abundance of a small number of proteins in a pathway may not necessarily indicate that the entire pathway is affected, our study highlights that to fully understand microRNA-mediated processes a holistic approach is needed, which will pave the way for further insight into neuronal processes associated with normal development and disease.

Supporting Information

S1 Fig. MS/MS annotated spectra of the proteins identified with single peptide for (A) spot 5 (Q81W75), Serpin A12 precursor (Mascot score: 32, score > 16 indicates homology, expect 0.0072); (B) spot 33 (Q01105), Protein SET (Mascot score: 58, score > 37 indicates identity, expect 6.2e-05); (C) spot 24 (P62333), Proteasome subunit p42 (Mascot score: 19, score > 18 indicates homology, expect 0.05); (D) spot 25 (P04406), Glyceraldehyde-3-phosphate dehydrogenase (Mascot score: 67, score > 38 indicates identity, expect 2.6e-05). Shown are representative spectra for the peptide sequence shown at the top of each spectrum. The spectra are derived from Mascot search results.

(TIF)

S2 Fig. Molecular map of the 26S proteosome showing proteins involved in ubiquitin mediated proteolysis. Proteasome subunit alpha type-1 isoform 2 (PSMA1) (regulated by miR-153-3p and miR-205-5p) and proteasome subunit p42 (PSMC6) (regulated by miR-205-5p) are integral parts of the 26S proteosome.

(TIF)

S1 Table. List of primary antibodies used in this study.

(DOCX)

S2 Table. Details of identified proteins by mass spectrometry.

(XLSX)

S3 Table. GO annotation of all proteins identified showing molecular function and cellular location.

(DOCX)

S4 Table. Cellular processes and pathway analysis of the differentially regulated proteins in response to mimics and antagomirs of miR-153-3p and miR-205-5p.

(DOCX)

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Author Contributions

Conceived and designed the experiments: KP IB RP HPH GA EJC JPL SGM. Performed the experiments: KP IB RP HPH EJC. Analyzed the data: KP IB RP HPH GA EJC JPL SGM. Contributed reagents/materials/analysis tools: EJC SGM. Wrote the paper: KP IB RP GA EJC JPL SGM.
References

1. Hirsch E, Graybiel AM, Agid YA. Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson’s disease. Nature. 1988; 334: 345–8. PMID: 3339295

2. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson’s disease. Science 1997; 276: 245–7. PMID: 9137268

3. Kitada T, Asakawa S, Hatton N, Matsumine H, Yamamura Y, Minihime S, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature. 1998; 392: 605–8. PMID: 9560156

4. Valente EM, Salvi S, Ialongo T, Morongiello A, Elia AE, Caputo V, et al. PINK1 mutations are associated with sporadic early-onset parkinsonism. Ann Neurol. 2004; 56: 336–41. PMID: 15349860

5. Bonifati V, Rizzu P, Quittner F, Krieger E, Vanacore N, van Swieten JC, et al. DJ-1 (PARK7), a novel gene for autosomal recessive, early onset Parkinson’s disease. Neurol Sci. 2003; 24: 159–60. PMID: 14598065

6. Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M, Lincln S, et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron. 2004; 44: 601–7. PMID: 15541309

7. Ardekani AM, Naeini MM. The Role of MicroRNAs in Human Diseases. Avicenna J Med Biotechnol. 2010; 2: 161–79. PMID: 23407304

8. Doxakis E. Post-transcriptional Regulation of alpha-Synuclein Expression by mir-7 and mir-153. J Biol Chem. 2010; 285: 12726–12734. doi: 10.1074/jbc.M110.096827 PMID: 20106983

9. Cho HJ, Liu G, Jin SM, Parisaiedou L, Xie C, Yu J, et al. MicroRNA-205 regulates the expression of Parkinson’s disease-related leucine-rich repeat kinase 2 protein, Hum Mol Genet. 2012; 24: 608–20. doi: 10.1093/hmg/ddt470 PMID: 21325283

10. Miñones-Moyano E, Porta S, Escaramís G, Rabionet R, Iaioila S, Kagerbauer B, et al. MicroRNA profiling of Parkinson’s disease brains identifies early downregulation of miR-34b/c which modulate mitochondrial function. Hum Mol Genet. 2011; 20: 3067–78. doi: 10.1093/hmg/ddr210 PMID: 21558425

11. Kim J, Inoue K, Ishii J, Vanti WB, Voronov SV, Murchison E, et al. A MicroRNA feedback circuit in midbrain dopamine neurons. Science. 2007; 317: 1220–4. PMID: 17761882

12. Wang WX, Rajeev BW, Stromberg AJ, Ren N, Tang G, Huang Q. The expression of microRNA miR-107 decreases early in Alzheimer’s disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1. J Neurosci. 2008; 28: 1213–23. doi: 10.1523/JNEUROSCI.0506-07.2008 PMID: 18234899

13. Gehret S, Imai Y, Sokol N, Lu B. Pathogenic LRRK2 negatively regulates microRNA-mediated translational repression. Nature. 2010; 466: 637–41. doi: 10.1038/nature09191 PMID: 20671708

14. Rege SD, Geetha T, Pondugula SR, Zizza CA, Wernette CM, Babu JR. Noncoding RNAs in Neurodegenerative Diseases. ISRN Neurol. 2013; 2013: 375852. doi: 10.1155/2013/375852 PMID: 23738143

15. Bushati N, Cohen SM. MicroRNAs in neurodegeneration. Curr Opin Neurobiol. 2008; 18: 292–296. doi: 10.1016/j.conb.2008.07.001 PMID: 18662781

16. Hashimoto Y, Akiyama T, Yasuda T, Kawauchi T, Yamaguchi E, Tanaka S, et al. Multiple-to-multiple relationships between microRNAs and target genes in gastric cancer. PLoS One. 2013; 8: e62589. doi: 10.1371/journal.pone.0062589 PMID: 23667495

17. Patil CS, Basak I, Lee S, Abdullah R, Larsen JP, Meller SG. PARK13 regulates PINK1 and subcellular relocation patterns under oxidative stress in neurons. J Neurosci Res. 2014; 92: 1167–77. doi: 10.1002/jnr.23396 PMID: 24738695

18. Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Camenotta B et al. Blue silver: a very sensitive colloidial Coomassie G-250 staining for proteome analysis, Electrophoresis. 2004; 25: 1327–33. PMID: 15174055

19. Basak I, Pal R, Patil KS, Dunne A, Ho HP, Lee S, et al. Arabidopsis ATPARK13, which confers thermotolerance, targets misfolded proteins. J Biol Chem. 2014; 289: 14458–69. doi: 10.1074/jbc.M114. 548156 PMID: 24719325

20. Abdullah R, Basak I, Patil KS, Alves G, Larsen JP, Meller SG. Parkinson’s disease and age: the obvious but largely unexplored link. Exp Gerontol. 2014; 69: 33–8. doi: 10.1016/j.exger.2014.09.014 PMID: 25261764

21. Vainberg IE, Lewis SA, Rommelaere H, Ampe C, van der Kerkhove J, Klein HL et al. Prefoldin, a chaperone that delivers unfolded proteins to cytosolic chaperonin, Cell. 1998; 93: 863–73. PMID: 9630229

22. Obermajer N, Doljak B, Jamičnik P, Fonović UP, Kos J. Cathepsin X cleaves the C-terminal dipeptide of alpha- and gamma-enolase and impairs survival and neuritogenesis of neuronal cells. Int J Biochem Cell Biol. 2009; 41: 1685–96. doi: 10.1016/j.biocel.2009.02.019 PMID: 19433310

23. Skaper SD. Ion channels on microglia: therapeutic targets for neuroprotection. CNS Neurol Drug Targets. 2011; 10: 44–56. PMID: 21143139
24. Silverstein AM, Galigniana MD, Chen MS, Owens-Grillo JK, Chinkers M, Pratt WB. Protein phospha-
tase 5 is a major component of glucocorticoid receptor-hsp90 complexes with properties of an FK506-
binding immunophilin. J Biol Chem. 1997; 272: 16224–30. PMID: 9195923
25. Vural B, Uğurel E, Tüzün E, Kürtüncü M, Zuliani L, Cavus F, et al. Anti-neuronal and stress-induced-
phosphoprotein 1 antibodies in neuro-Behçet's disease. J Neuroimmunol. 2011; 239: 91–7. doi: 10.
1016/j.jneuroim.2011.08.008 PMID: 21875754
26. Yang X, Lee WH, Sobott F, Papagrigoriou E, Robinson CV, Grossmann JG, et al. Structural basis for
protein-protein interactions in the 14-3-3 protein family. Proc Natl Acad Sci. 2006; 103: 17237–42.
PMID: 17085597
27. Berg D, Holzmann C, Riess O. 14-3-3 proteins in the nervous system, Nat Rev Neurosci. 2003; 4: 752–
62. PMID: 12951567
28. Bergamaschi A, Katzenellenbogen BS. Tamoxifen downregulation of miR-451 increases 14-3-3ζ
and promotes breast cancer cell survival and endocrine resistance. Oncogene. 2012; 31: 39–47. doi: 10.
1038/onc.2011.223 PMID: 21580268
29. von Blume J, Duran JM, Forlanelli E, Alleaume AM, Egorov M, Polishchuk R. Actin remodeling by ADF/
cofilin is required for cargo sorting at the trans-Golgi network. J Cell Biol. 2009; 187: 1055–69. doi:10.
1083/jcb.200908040 PMID: 20026655
30. Gohla A, Birkenfeld J, Bokoch GM. Chronophin, a novel HAD-type serine protein phosphatase, regu-
lates cofilin-dependent actin dynamics. Nat Cell Biol. 2005; 7: 21–9. PMID: 15580268
31. Maloney M, Bamburg J. Cofilin-mediated neurodegeneration in Alzheimer's disease and other amyloi-
dopathies. Mol Neurobiol. 2007; 35: 21–44. PMID: 17519504
32. Basso M, Giraudo S, Corpillo D, Bergamasco B, Lopiano L, Fasano M. Proteome analysis of human
substantia nigra in Parkinson's disease, Proteomics. 2004; 4: 3943–52. PMID: 15526345
33. Hu X, Weng Z, Chu CT, Zhang L, Cao G, Gao Y, et al. Peroxiredoxin-2 protects against 6-hydroxydopa-
nine-induced dopaminergic neurodegeneration via attenuation of the apoptosis signal-regulating
kinase (ASK1) signaling cascade. J Neurosci. 2011; 31: 247–61. doi: 10.1523/JNEUROSCI.4589-10.
2011 PMID: 21209210
34. Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, et al. Two enzymes in one: two yeast peroxire-
doxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone func-
tion. Cell. 2004; 117: 625–35. PMID: 15163410
35. Moon JC, Hah YS, Kim WY, Jung BG, Jang HH, Lee JR, et al. Oxidative stress-dependent structural
and functional switching of a human 2-Cys peroxiredoxin isotype II that enhances HeLa cell resistance
to H2O2-induced cell death. J Biol Chem 2005; 280: 28775–84. PMID: 15941719
36. Garzon R, Calin GA, Croce CM. MicroRNAs in Cancer. Annu Rev Med. 2009; 60: 167–79. doi: 10.
1146/annurev.med.59.053006.104707 PMID: 19630570
37. Choudhuri T, Murakami M, Kai R, Sahu SK, Mohanty S, Verma SC, et al. Nm23-H1 can induce cell
cycle arrest and apoptosis in B cells. Cancer Biol Ther, 2010; 9: 1065–78. PMID: 20448457
38. Song Y, Luo Q, Long H, Hu Z, Que T, Zhang H, et al. Alpha-enolase as a potential cancer prognostic
marker promotes cell growth, migration, and invasion in glioma. Mol Cancer. 2014; 13: 65. doi: 10.
1186/1476-4598-13-65 PMID: 24650096
39. Subramanian A, Miller DM. Structural analysis of alpha-enolase Mapping the functional domains
involved in down-regulation of the c-myc protooncogene. J Biol Chem. 2000; 275: 5958–65. PMID:
10681589
40. Cervoni N, Delich N, Seo SB, Chakravarti D, Szyf M. The oncoprotein Set/TAF-1beta, an inhibitor of
histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and tran-
scriptional silencing, J Biol Chem. 2002; 277: 25026–31. PMID: 11978794
41. Möller I, Beatrix B, Kreibich G, Sakai H, Lauring B, Wiedmann M, et al. Unregulated exposure of the
ribosomal M-site caused by NAC depletion results in delivery of non-secretory polypeptides to the
Sec61 complex. FEBS Lett. 1998; 441: 1–5.
42. Quélô I, Gauthier C, Hannigan GE, Dedhar S. Integrin-linked kinase regulates the nuclear entry of the
c-Jun coactivator alpha-NAC and its coactivation potency. J Biol Chem. 2004; 279: 43893–9. PMID:
15299025
45. He J, Baum LG. Presentation of galectin-1 by extracellular matrix triggers T cell death, J Biol Chem. 2004; 279: 4705–12. PMID: 14617626

46. Mandemakers W, Abuhatzira L, Xu H, Caromile LA, Hebert SS, Snellinx A, et al. Co-regulation of intragenic microRNA miR-153 and its host gene Ia-2 beta: identification of miR-153 target genes with functions related to IA-2beta in pancreas and brain. Diabetologia. 2013; 56: 1547–56. doi: 10.1007/s00125-013-2901-5 PMID: 23595248

47. Kato M, Wynn RM, Chuang JL, Tso SC, Machius M, Li J, et al. Structural basis for inactivation of the human pyruvate dehydrogenase complex by phosphorylation: role of disordered phosphorylation loops, Structure. 2008; 16: 1849–59. doi: 10.1016/j.str.2008.10.010 PMID: 19081061

48. Klune JR, Dhupar R, Cardinal J, Billiar TR, Tsung A. HMGB1: endogenous danger signalling. Mol Med. 2008; 14: 476–84. doi: 10.2119/2008-00034.Klune PMID: 18431461

49. Calogero S, Grassi F, Aguzzi A, Voigtlander T, Ferrier P, Ferrier S, et al. The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. Nat Genet. 1999; 22: 276–80. PMID: 10391216

50. Rüegg J, Holsboer F, Turck C, Rein T. Cofilin 1 is revealed as an inhibitor of glucocorticoid receptor by analysis of hormone-resistant cells. Mol Cell Biol. 2004; 24: 9371–82. PMID: 15485906

51. Hara MR, Agrawal N, Kim SF, Cascio MB, Fujimuro M, Ozeki Y, et al. S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. Nat Cell Biol. 2005; 7: 665–74. PMID: 15951807

52. Campanella ME, Chu H. Low P.S. Assembly and regulation of a glycolytic enzyme complex on the human erythrocyte membrane. Proc Natl Acad Sci. 2005; 102: 2402–7. PMID: 15701694

53. Kwon JH, Lee JH, Kim KS, Chung YW, Kim IY. Regulation of cytosolic phospholipase A2 phosphorylation by proteolytic cleavage of annexin A1 in activated mast cells. J Immunol. 2012; 188: 5665–73. doi: 10.4049/jimmunol.1102306 PMID: 22539796

54. Kohzt JD, Jamison SF, Will CL, Zuo P, Luhmann R, Garcia-Blanco MA, et al. Protein-protein interactions and 5′-splice-site recognition in mammalian mRNA precursors. Nature. 1994; 368: 119–24. PMID: 8139654

55. Yotov WV, Moreau A, St-Arnaud R. The alpha chain of the nascent polypeptide-associated complex functions as a transcriptional coactivator. Mol Cell Biol. 1998; 18: 1303–11. PMID: 9488445

56. Buratti E, Baralle FE. Characterization and functional implications of the RNA binding properties of nuclear factor TDP-43, a novel splicing regulator of CFTR exon 9. J Biol Chem. 2001; 276: 36337–43 PMID: 11470789

57. Postel EH, Berberich SJ, Rooney JW, Kaetzel DM. Human NM23/nucleoside diphosphate kinase regulates gene expression through DNA binding to nuclease-hypersensitive transcriptional elements. J Bioenerg Biomembr. 2000; 32: 277–84. PMID: 11768311