

RESEARCH ARTICLE

# Comprehensive Evaluation of microRNA Expression Profiling Reveals the Neural Signaling Specific Cytotoxicity of Superparamagnetic Iron Oxide Nanoparticles (SPIONs) through N-Methyl-D-Aspartate Receptor

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## Abstract

Though nanomaterials are considered as drug carriers or imaging reagents targeting the central nervous system their cytotoxicity effect on neuronal cells has not been well studied. In this study, we treated PC12 cells, a model neuronal cell line, with a nanomaterial that is widely accepted for medical use, superparamagnetic iron oxide nanoparticles (SPIONs). Our results suggest that, after treated with SPIONs, the expression pattern of the cellular miRNAs changed widely in PC12 cells. As potential miRNA targets, NMDAR, one of the candidate mRNAs that were selected using GO and KEGG pathway enrichment, was significantly down regulated by SPIONs treatment. We further illustrated that SPIONs may induce cell death through NMDAR suppression. This study revealed a NMDAR neurotoxic effect of SPIONs and provides a reliable approach for assessing the neurocytotoxic effects of nanomaterials based on the comprehensive annotation of miRNA profiling.

## Introduction

The rapid development of nanotechnology provides a flexible platform for generating nanomaterials with specific functions for medical applications due to their unique properties. For example, nanoparticles, which usually are 10–100 nm in diameter, can easily flow through blood capillaries to reach their target sites. Nanoparticles can be technologically engineered and modified so that they carry components for medical imaging, cancer therapy or drug release [1–9]. Because of the versatile nature of the conjugate, nanomaterial has been widely used in diagnosis and treatment. Different bulk forms can be generated by different nanotechnology procedures

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and may endow a given nanomaterial with new properties, in some cases involving completely unexpected physical and chemical properties [10–13]. For this reason, the *de novo* capabilities of nanomaterials are still under investigation. Besides the emerging applications of nanomaterials in biological systems, the cellular effects of nanomaterials are still unclear. Several studies based on the toxicity of nanomaterial in biological systems indicate the need for a new scientific discipline focused on nanotoxicity [14–18]. Due to the complexity of nanomaterials and their effects on living organisms, few studies have been able to make a robust conclusion about the cytotoxicity of certain nanomaterials. Indeed, for some nanomaterials that are suggested to have low cytotoxicity, their cellular effect and long-term safety need further inspection. Unfortunately, except for the conventional toxicity assay, there is a lack of reliable methodology for systematically assessing the overall cellular effects of specific materials.

The overwhelming majority of assays to test nanotoxicity in biological systems are performed *in vitro* with the advantages over *in vivo* studies of providing less ethical ambiguity, being easier to reproduce and carrying less expense. Conventional tests for cellular nanotoxicity include assays for cytotoxicity, genotoxicity or altered gene expression, and these assessments are carried out using standard *in vitro* assays, such as Northern blotting, real-time PCR, or microarray analyses [19–21]. Based on high-throughput microarray and bioinformatics analyses, gene expression profiling may provide a systematic method for examining the biocompatibility of nanomaterials. However, mRNA assays may not accurately reflect the response state of a cell due to the inescapable degradation of a portion of the mRNAs during sample preparation and the regulation of proteins through post-transcriptional mechanisms such as effects on translation.

MicroRNAs (miRNAs) are short RNA molecules working as post-transcriptional regulators by binding to complementary sequences on target mRNA transcripts. By enacting gene silencing through translational repression or target degradation, miRNAs may regulate comprehensive biological processes, including cell viability, proliferation, development and differentiation [22–26]. Methods have been developed for profiling miRNA expression, for example, the deep sequencing technique [27]. Based on the increased stability of miRNA during sample processing, miRNA expression profiling may provide a more reliable method for evaluating the biocompatibility of nanomaterial. Several studies have revealed that miRNAs is regulated in response to the cytotoxicity of nanomaterial [28–30]. Recently, miRNAs involved in the cytotoxicity of CdTe quantum dots in NIH/3T3 cells has been illustrated, demonstrating that SOLiD sequencing-based miRNA expression profiling provides a viable method for analyzing the nanomaterial cytotoxicity [31].

Engineered superparamagnetic iron oxide nanoparticles (SPIONs) can be used as advanced carriers for delivering therapeutic reagents and noninvasive magnetic resonance imaging (MRI) [32–34]. Because capable of crossing through the blood-brain barrier, SPIONs are engineered for targeted imaging and therapy for brain diseases [35, 36]. With regards to the central nervous system (CNS), the interaction between nanoparticles and neurons needs to be inspected. Several studies have investigated the effects of nanomaterials on PC12 cells, a neuroendocrine cell line with the capability of producing the neurotransmitter dopamine and activating functional metabolic pathways in response to it [37–39]; however, the comprehensive effects of nanomaterials on neurons need to be further explored. In this study, the effect of SPIONs on the PC12 cell line were systematically evaluated based on differential miRNA expression profiling analyzed with a newly modified mathematical model and web-based bioinformatics. The fact that that SPIONs may induce cell death through NMDAR suppression was also revealed for the first time by bioinformatics analysis. These results are consistent with protein electrophoresis analysis. Thus, this paper provides a systematic biological approach for evaluating nanomaterial biocompatibility with neurons.

## Methods

### 1. Preparation of nanoparticles

SPIONs were donated by Jiangsu Laboratory for Biomaterials and Devices. Transmission electron microscopy (TEM) images were taken with a Tecnai 20 microscope (Oregon, USA). The TEM samples were prepared by dropping the freshly prepared solution onto a 300-mesh carbon-coated copper grid (SPI supplies, USA). For the details of preparing SPIONs and more information of characterize of SPION, such as zeta potential and DLS data, please refer to studies reported previously[40–42].

### 2. Cell culture

Rat pheochromocytoma (PC12) cells were obtained from the cell bank of Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China) and as described previously[43], were cultured in Ham's F12K (Gibco, Canada), containing 2 mM L-glutamine (Gibco), with 15% horse serum (Gibco), 2.5% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in a 5% CO<sub>2</sub> incubator (Hera Cell 150, Thermo, US).

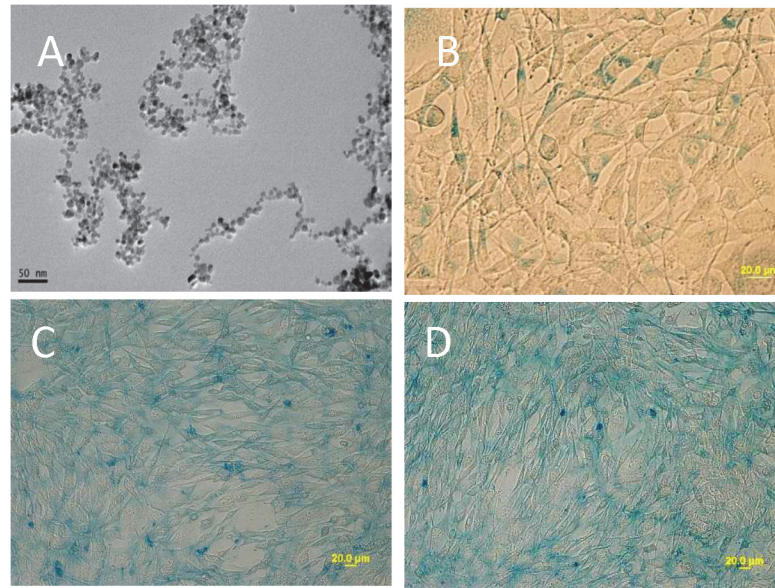
### 3. Small RNA extraction, SOLiD sequencing and Bioinformatics analysis

The cells were plated at an initial density of  $1 \times 10^5$  cells/ml in 35 mm culture dishes. After 24 h, they were exposed to SPIONs (214 µg/ml) for an additional 24 h. The cells were harvested with trypsin to extract the small RNAs using the mirVana miRNA isolation kit (Ambion, TX, USA) according to the manufacturer's protocol.

The small RNAs were converted into a double-stranded cDNA library using a SOLiD Small RNA Expression Kit (Invitrogen, CA, USA), which is compatible with the Applied Biosystems SOLiD System (Life Technologies, CA, USA). The results of SOLiD sequencing were obtained in the form of nucleotide sequences and their coverage. The registered miRNAs were identified by comparing them to sequences in Genbank ([www.ncbi.nlm.nih.gov/GenBank](http://www.ncbi.nlm.nih.gov/GenBank)) and miRbase (<http://www.mirbase.org>). The dysregulated expression of miRNAs in the nanomaterial treated PC12 cells was analyzed and the comprehensive repression effect on each mRNA of the whole miRNA profile was calculated following the method reported previously [31, 44]. Finally, the significantly affected genes were assigned to either the KEGG pathways or the GO biological processes through DAVID.

### 4. Western blotting

After culturing in the indicated conditions, protein was extracted from the cells. The protein concentrations of homogenized lysates was measured by Nanodrop (Thermo, DE, USA), and 10 µg of protein extracted from cancer cells was separated on a 10% SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane (BioRad, CA, USA). After blocking in a powdered nonfat milk solution (5% in PBS with 0.05% Tween-20 (Thermo)), the blot was incubated with polyclonal rabbit anti-rat antibodies against NMDAR2A (Abcam, Cambridge, MA), NMDAR2C (Abcam), NMDAR2D (Millipore, Billerica, MA, USA), caspase-12 (Abcam), cytochrome (Cyt)-c (Abcam), or actin (Abcam) at a 1:1000 dilution in 5% blocking solution (Abcam) overnight at 4°C. An anti-rabbit IgG antibody (Abcam) at a dilution of 1:5000 was used as a secondary antibody. Protein expression patterns were detected with an enhanced chemiluminescence kit (ECL; Amersham Bioscience/GE Healthcare, Little Chalfont, UK).



**Fig 1. Morphology and uptake of SPIONs in PC12 cells.** A confocal image of the SPIONs is shown (A). Prussian blue staining was performed to measure the uptake of SPIONs (214 µg/ml) in PC12 cells at 0 (B), 12 (C) and 24 h (D). The majority of cells can incorporate SPIONs at 24 h (D).

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## 5. Statistical analysis

The results were presented as the mean  $\pm$  standard deviation (SD). The statistical significance of the differences was tested with the Student's t-test. In all comparisons, values of  $p < 0.05$  (\*) were considered statistically significant.

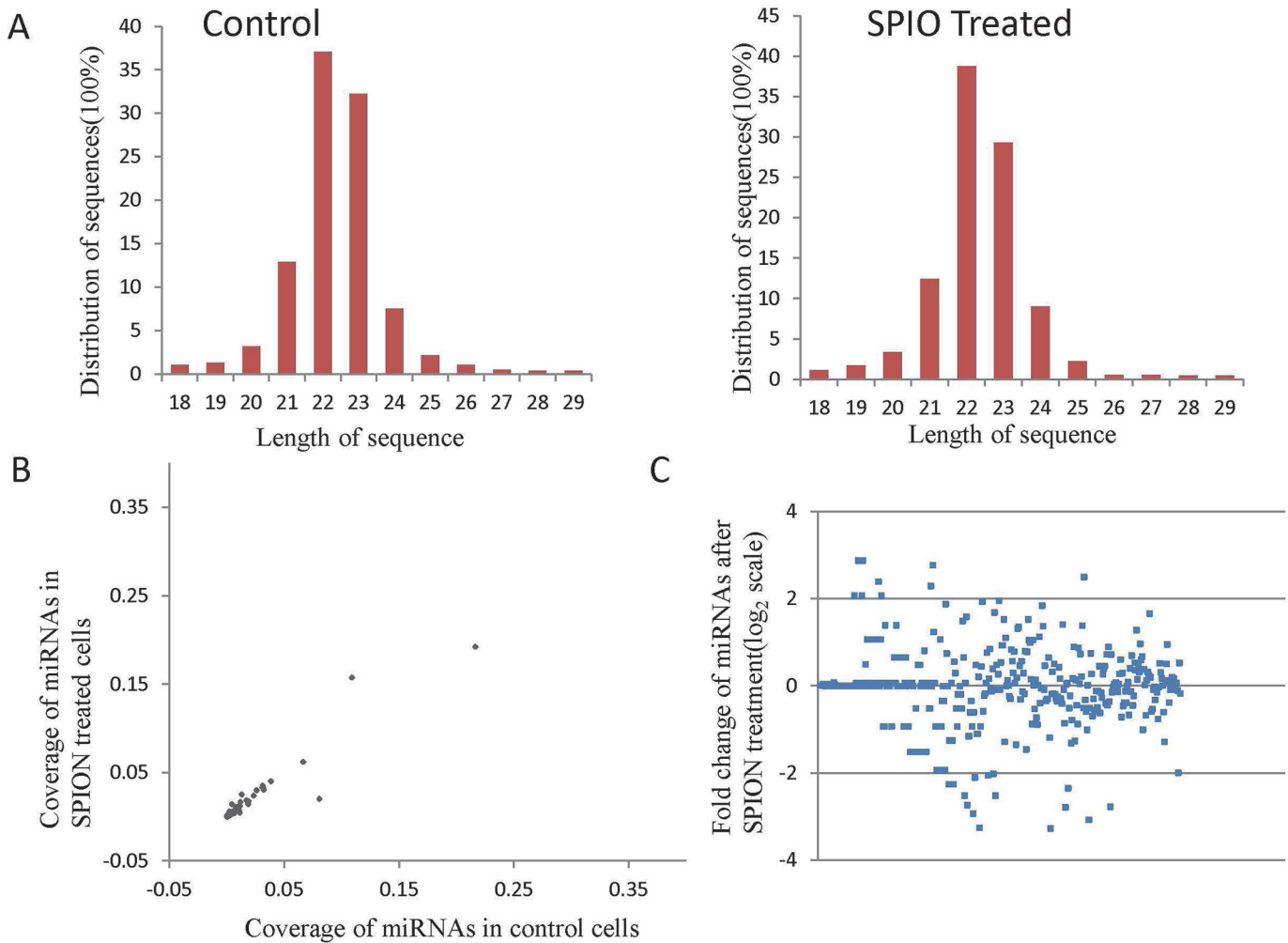
## Results and Discussion

### 1. SPION treatment induces a change in the microRNA expression profile in neurons without impairing their viability

A microscopic image of the SPIONs is shown in Fig. 1A. In order to address their cellular effect, PC12 cells were treated with 214 µg/ml of SPIONs. Prussian blue staining indicated the translocation of SPIONs into PC12 cells following 0 h (Fig. 1B), 12 h (Fig. 1C) and 24 h exposure (Fig. 1D). Results indicated that the majority of cells can incorporate SPIONs at 24 h. a significant increasing of nanoparticle uptake has not been observed after 24 h. Endocytosis have been regarded as the possible mechanism of mammalian cells for nanoparticle internalization [45]. Generally, it was thought that particles with sizes  $< 0.5$  µm is through pinocytosis, while particles with sizes  $> 0.5$  µm is through phagocytosis. Since the diameter of SPIONs used in our study is about 4–7 nm, it is possible that SPIONs were internalized through pinocytosis. Pinocytosis process has been further subcategorized into macropinocytosis, clathrin-dependent endocytosis, caveolin-dependent endocytosis, and clathrin- and caveolin-independent endocytosis. Intensive studies have been done for identifying the pathway of internalization of nanoparticle, however, there still no consensus about the transport mechanism due to the different cell type and different properties of nanoparticle used in the experiments [46–49]. Once nanoparticles were internalized in to the cells, biological process of the cells can be wildly changed. In this study, we concerned about the neurotoxicity effect of SPIONs [50–53].

To investigate the potential neurotoxicity of SPIONs, miRNAs were extracted from PC12 cells treated with 214 µg/ml SPIONs and compared to untreated control cells. The expression





**Fig 2. miRNA high-throughput deep sequencing for PC12 cells treated with or without SPIONs.** Deep sequencing results show that the majority of the reads were 18–25nt, with the most abundant size of 22nt in both the control and SPIONs treated group (A). The abundance of many miRNAs is less than 0.1% (B). Correlation analysis between the convergence of miRNAs in SPIONs treated or non-treated cells shows that the relative amounts of some miRNAs deviated strikingly, although most miRNAs were regulated somewhat (C).

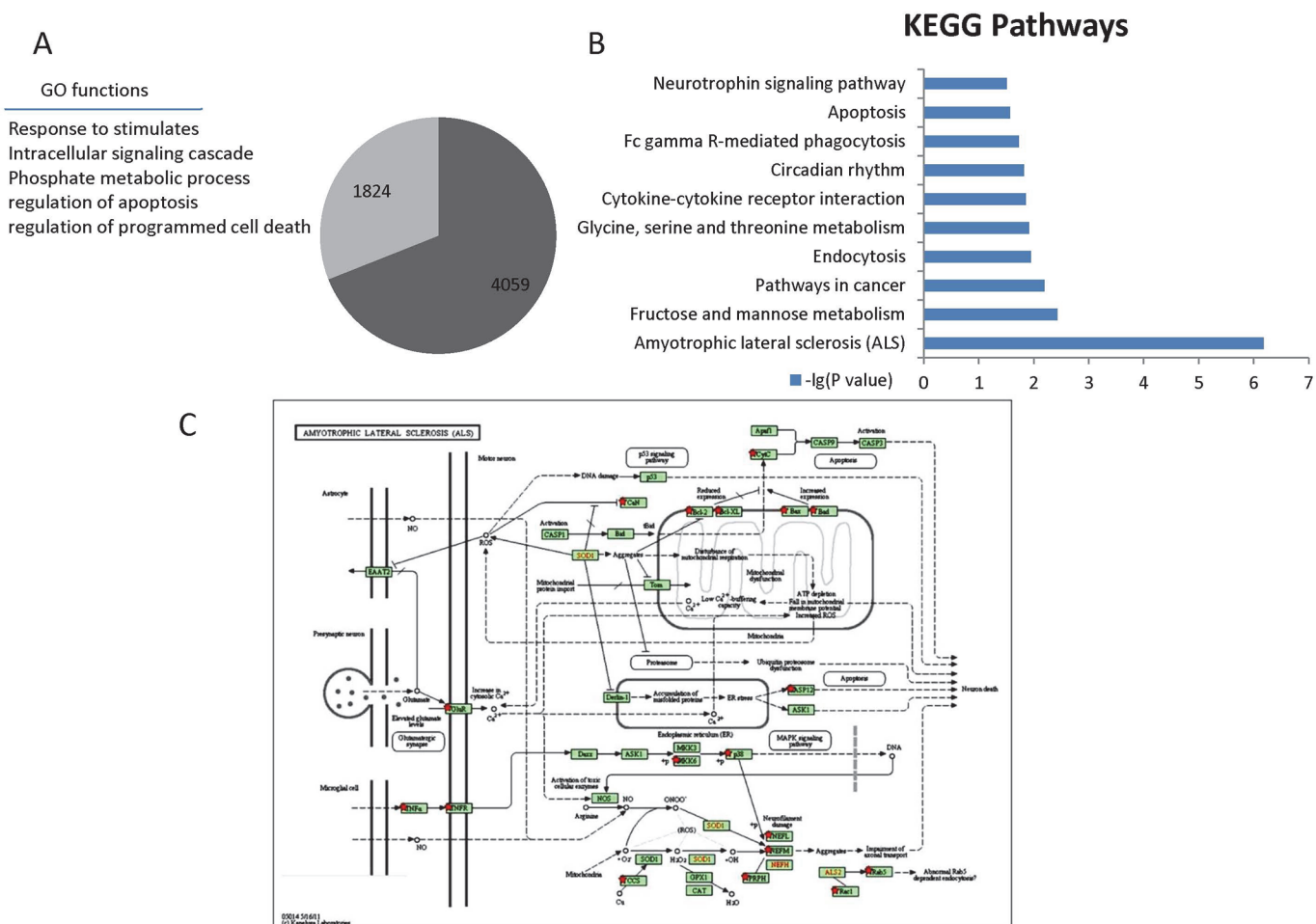
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patterns of miRNAs were analyzed using a high-throughput deep sequencing method. A total of tens millions SOLiD sequencing events were obtained for the miRNA samples. According to the size distribution of the sequencing reads, the majority of the miRNAs were 18–25 nt, with the most abundant size of 22 nt in both the control and SPION treated groups (Fig. 2A). After filtering the contaminated reads (those contaminated by other RNA pieces), a total of 388 miRNAs were identified with hundreds of thousands of copies in each sample. The low coverage (0.1%) of many miRNAs guaranteed the feasibility of the SOLiD sequencing data (Fig. 2B). The comparison of miRNAs in SPIONs treated and non-treated cells was analyzed. As shown, the expression of some miRNAs was obviously different in the two groups, although most miRNAs were regulated somewhat (Fig. 2C). The accuracy of SOLiD sequencing results was verified by qRT-PC, which is generally accepted as a reliable method for measuring relative expression levels of miRNAs. Correlation between the relative expression levels of miRNAs obtained with SOLiD sequencing and qRT-PC was clear and statistically consistent (data not shown).

## 2. Profiling of the comprehensive translational suppression by miRNAs after SPION treatment

With the method reported previously [44], the comprehensive suppression effects of a given miRNA profile on the translation of mRNAs can be predicted. There were 5883 mRNAs predicted to be regulated in the cells treated with SPIONs. For selecting the mRNAs that may be significantly repressed, a Z-test was performed comparing the differences between the repression of miRNAs in control cells and SPION treated cells. As a result, approximately 31% of the targeted mRNAs were predicted to be significantly regulated after nanomaterial treatment. This gene set was enriched for gene ontology (GO) functions associated with the cellular response to stimulation, stress or phosphate metabolic processes and contained multiple markers associated with cell death, apoptosis or programmed cell death (Fig. 3A).

Based on the mRNAs that were predicted to be significantly regulated by the integrated miRNA expression profile, we sought to investigate the intracellular changes in PC12 cells induced by SPION exposure. Because the KEGG pathway database records networks of molecular interactions within cells, we enriched for the mRNAs that were predicted to be significantly dysregulated by analysis of KEGG pathways through DAVID. Fig. 3B shows the KEGG



**Fig 3. Assessment of neuronal cytotoxicity of SPIONs.** Based on their miRNA target profile, 5883 mRNAs were predicted to be regulated in cells following treatment with SPIONs. Approximately 31% of the mRNA candidates were significantly regulated by nanomaterial treatment. This gene set was enriched for gene ontology (GO) functions (A). We also sorted the mRNAs into KEGG pathways through DAVID. The “Amyotrophic Lateral Sclerosis (ALS) pathway” (C) was of the most highly affected KEGG pathway with  $p < 10^{-7}$  (B).

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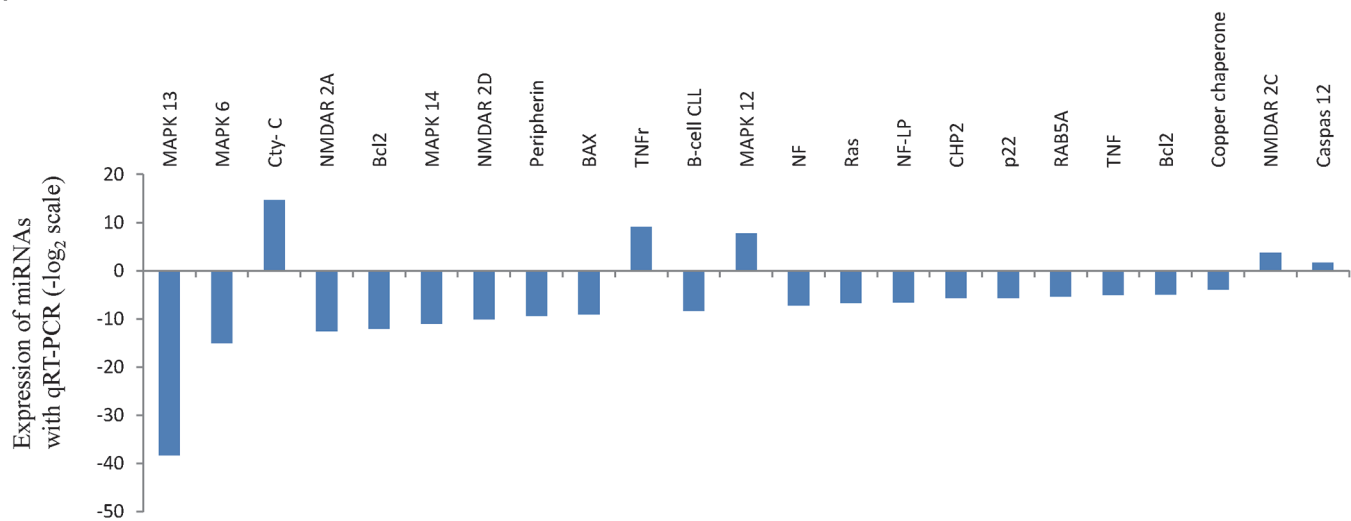
pathways that were significantly affected by SPION treatment. Among these, the “Amyotrophic Lateral Sclerosis (ALS) pathway” (illustrated diagrammatically in Fig. 3C; This also can be found online: <http://www.genome.jp/kegg/pathway/hsa/hsa05014.html>) was predicted to be most highly affected with a  $P < 10^{-7}$ , which indicates that SPION exposure may trigger neuron degeneration pathways.

The mRNAs within the ALS pathway are elaborated in Fig. 4A according to their calculated Z value. Within this list, p38 mitogen-activated protein family kinases were dramatically dysregulated. Mitogen-activated protein kinase 13 (MAPK13) and mitogen-activated protein kinase 14 (MAPK14) are predicted to be significantly suppressed by miRNAs compared with the control group. Given that the p38-isoform is mainly distributed in the adrenal gland and pituitary, it is not surprise that it comprises the most dramatically regulated isoform [54–56]. Interestingly, our data showed that miRNAs targeted to mitogen-activated protein kinase 12 (MAPK12), which is mainly expressed in skeleton muscle, had an increased expression pattern.

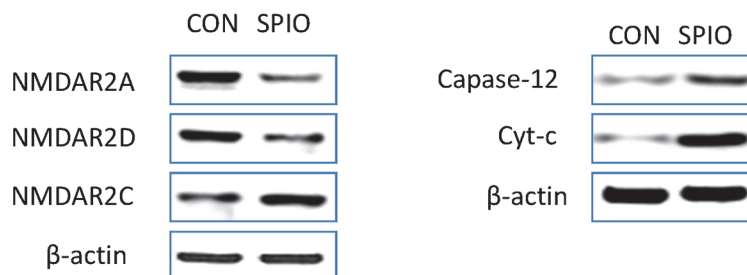
### 3. SPIONs induce neuron death through the NMDAR pathway

PC12 cells are derived from chromaffin cells of the adrenal medulla and are widely used as a model system for sympathetic ganglion-like neurons [57,58]. It has been shown that N-

A



B



**Fig 4. SPIONs induce neuronal death through the NMDAR pathway.** A selected group of the genes predicted to be regulated by SPION treatment are shown (A). The expression of NMDAR2A and NMDAR2D were suppressed, while NMDAR2C showed a slight elevation expression pattern, which was verified by western blotting (B). The expression of apoptosis-related proteins, capase-12 and Cyt-c were also predicted to be unregulated and were verified to be increased following treatment with SPION (C). NF: Neurofilament. NF-LP: Neurofilament-light peptide.

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methyl-D-aspartate receptor (NMDAR), a glutamate receptor, is expressed on PC12 cells [59–61]. NMDAR is important for processes underlying neuronal plasticity, outgrowth and survival. The functional NMDAR receptor is assembled from an essential subunit, NMDAR subunit 1 (NMDAR1), together with one or more of a second family of subunits termed NMDAR subunits 2A–D (NMDAR2A–D) [62, 63]. As a ligand-gated ion channel, NMDAR is highly permeable to  $\text{Ca}^{2+}$ . Excitatory amino acids, such as glutamate, may cause membrane depolarization and accumulation of cytosolic  $\text{Ca}^{2+}$  by enhancing cellular  $\text{Ca}^{2+}$  influx and releasing  $\text{Ca}^{2+}$  from intracellular compartments. However, several studies have illustrated that suppression of NMDAR induces the activation of a series of neurotoxic events, including cell death through the NMDAR-Caspase pathway [64–67]. Fig. 4A shows that after SPION treatment the expression of NMDAR2A and NMDAR2D was predicted to be suppressed and NMDAR2C was elevated. The apoptosis-related proteins Cyt-C and Caspase 12 are also thought to function within the NMDAR pathway of cell death (Fig. 3A). Each of these proteins was shown to follow the expected pattern of expression in response to SPION treatment (Fig. 4B). This is the first report of a role for these apoptosis-related proteins in NMDAR signaling. Taken together, these results derived from comprehensive assessment of miRNA expression profiling suggest that SPIONs may induce cell apoptosis through the NMDAR pathway in neuron cells.

## Conclusions

Although SPIONs are widely accepted as magnetic resonance imaging reagents for disease diagnosis, including brain disease, as well as drug carriers targeting the CNS, the effect of SPIONs on neuronal cell has not been well studied. To elaborate their effects on neuronal cells, the expression profiles of miRNAs in neurons were comprehensively analyzed with a method we developed. Our results suggest after PC12 was treated by SPIONs, the miRNA expression pattern was widely changed. To identify potential targets, candidate mRNAs were selected using GO and KEGG pathway enrichment. Genes related to cell death or apoptosis pathways were identified. This indicated that SPIONs may trigger neuron degeneration pathways, including the ALS pathway. Based on this comprehensive analysis, we illustrated that SPIONs may induce neuron death through the glutamate receptor, NMDAR in sympathetic ganglion-like neurons. Thus, we systematically analyzed the interaction between neurons and SPIONs, a widely used nanoparticle, by a new method based on miRNA sequencing technology and demonstrated that NDMAR may play an important role in the cell death induction by SPIONs.

## Author Contributions

Conceived and designed the experiments: BS ZDX. Performed the experiments: BS NY RL. Analyzed the data: BS RL NY. Contributed reagents/materials/analysis tools: BS ZDX. Wrote the paper: BS ZDX.

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