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RESEARCH ARTICLE

Effects of superparamagnetic iron oxide nanoparticles and static magnetic fields on neural stem cell differentiation by transcriptomic techniques

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Abstract

Neural stem cells (NSCs)-based cell therapy provides promising treatment for the neurodegenerative diseases. The success of stem cell therapy relies on the efficient differentiation of transplanted stem cells into functional cells. Therefore, directed differentiation of NSCs into neurons is essential for its application in the neurodegenerative diseases. In recent years, magnetic fields and superparamagnetic iron oxide nanoparticles (SPIOs) have shown potential in the regulation of stem cell behaviors. Here, we investigated the regulatory effects of static magnetic fields (SMFs) and the combination with SPIO on NSC differentiation by transcriptome sequencing analysis techniques. Our results found that SPIOs caused more differentially expressed genes than SMF alone. Interestingly, the number of differentially expressed genes induced by the combination was less than that of SPIO alone, which may imply that the regulation is not a simple superposition effect. Our findings provide experimental evidence for the regulation of SMF and SPIO on NSC differentiation at the transcriptomic level.

Keywords: *SPIO*; *SMF*; *NSC*; *transcriptomic*

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Nowadays, the probability of suffering from degenerative diseases of the central nervous system such as Alzheimer's disease (AD) gradually rises with the increasing of the average human lifespan (1, 2). The degeneration, damage, or irreversible loss of neurons are responsible for those degenerative diseases of the central nervous system. Stem cell transplantation to regenerate to neurons is an effective approach to treat neurodegenerative diseases, especially the neural stem cells (NSCs).

NSCs are pluripotent stem cells with the potential to differentiate into neurons. Therefore, NSC transplantation would be a promising choice for the treatment of a variety of neurodegenerative diseases. For example, human NSCs (hNSCs) transplanted into a mouse model of AD can rescue impaired memory function by regulating multiple mechanisms (3). Another report demonstrated that the transplantation of NSCs originated from embryonic mouse into the hippocampus of the mice enhanced the basal forebrain synaptic function and protected the cholinergic neurons (4). However, the low differentiation efficiency to neurons and uncontrollability of NSCs *in vivo* have become an obstacle for its application in disease treatment. Therefore, directed induction of NSCs differentiation into neurons has attracted more and more attention.

The fate of NSCs is decided by the interaction between its surroundings with cells, which is called niche, including biochemical factors, physical factors, extracellular matrix components, and the interactions among cells (5).

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Physical factors as a crucial clue in cell fate regulation are setting off a research boom. As early as 1970, single physical factors that have been confirmed can regulate the growth of neuronal neurites (6). Magnetic field, as a physical factor, is believed to play an important role in modulating various cellular processes. Specifically, the magnetic field not only affects the membrane properties of cells, cell morphology, skeletal organization, cell cycle, etc. but also has specific effects on cell proliferation, differentiation, and migration (7–17). Superparamagnetic iron oxide nanoparticles (SPIOs) taken as an excellent biocompatibility magnetic nanomaterial are widely used in various medical research fields. SPIOs with small size, surface area, and excellent biocompatibility have been widely used in drug-targeted transport. A recent study also has found that SPIOs modulated the fate of stem cells (18). Recently, one research reported that SPIOs-labeled mesenchymal stem cells (MSCs) could migrate into the damaged olfactory tissue under the magnetic traction of a permanent magnet, which resulted in a more significant homing effect of MSCs in a mouse model of olfactory damage (19), which provides a new strategy for treatment with an effective stem cell transplantation method.

In this research, we aimed to study the regulation of the differentiation-related genes and related signaling pathways during NSC differentiation under the presence of SPIO and magnetic field.

Materials and methods

NSCs culture

The NSCs were isolated from the hippocampus of FVB mice (embryonic day 18.5). NSCs were then purified and cultured with medium containing DMEM-F12 medium (Gibco, Grand Island, NY), 2% B-27 (Gibco), 20 ng/mL EGF (R&D Systems, Minneapolis, MN), 20 ng/mL FGF-2 (R&D Systems), and 1% penicillin-streptomycin (P/S, Sigma, St. Louis, MO) in 5% CO_2 at 37°C. The cells were passaged every 3–5 days during the culture period. NSC differentiation was achieved by replacing EGF and FGF-2 of above medium with differentiation kit (Stem Cell, Canada). Animal studies were approved by the Care and Use of Animals Committee of Southeast University. All efforts were made to reduce the number of mice and their suffering.

Static magnetic field (SMF)

The NSCs were cultured in the center area of 35 mm dishes, which were placed in the middle of the box. Two permanent magnets were placed on both sides of the box to generate an SMF in the same direction. The intensity of SMF at the center of the dish was measured and recorded with a Gauss meter according to the published paper (20).

RNA extraction for RNA-seq analysis

Total RNA from NSCs cultured in 200 μg/mL SPIOs, 60 ± 10 mT SMF, SPIOs (200 μg/mL) + SMF (60 ± 10 mT), and control groups differentiated for 3 days were extracted and then were split into three fractions for separate replicates. RNA-Seq libraries of NSCs were generated using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing and the Illumina mRNA-Seq Sample Prep Kit, and library quality was analyzed by an Agilent2100 Bioanalyzer. Illumina Novaseq 6,000 platform was applied for transcriptome sequencing preparation, and 150 bp paired-end reads were generated finally. Then, the original data were recorded in FASTQ format, which included sequence information and corresponding sequencing quality information.

RNA-seq data were analyzed by the STAR (version 2.5.2) and feature Counts (version 1.6.4) pipeline and mapped the clean reads of the mouse reference genome (mm9). Differential gene expression (DGE) analysis was conducted using the R package edgeR (version 2.6.0), and significantly differentially expressed genes recognized as the t with P-values < 0.05 and log|FC| >1. Functional enrichment analysis of Gene Ontology (GO) and pathway enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed using the R package clusterProfiler (version 3.12.0), and the statistical significance threshold level for GO and pathway enrichment analyses was P-value < 0.05.

Results and discussion

Global gene expression profile of SPIOs and SMF-treated NSCs

Wang et al. reported that SPIOs may induce MSC differentiation into osteoblasts via MAPK signaling pathway (18). SMF also has been shown to regulate the osteogenic differentiation of MSCs through the fibronectin leucine-rich transmembrane protein (FLRT)/bone morphogenetic protein (BMP) signaling pathway (21). SMF combine with SPIOs for MSCs transplanting has also been reported (19). However, studies on the combined use of SMF with SPIOs in the regulation of NSC differentiation are rare, and the regulatory mechanisms remain unclear.

In this research, RNA-Seq analysis was used to explore the potential molecular mechanism and DGE of NSCs differentiation under different conditions such as SMF and SPIOs. Briefly, we obtained NSC samples from the control group, the SPIOs group, the SMF group, and the SMF + SPIOs group, and after sequencing, we analyzed datasets according to the description in the "Methods" section. NSCs were cultured for 3 days before collection for gene expression profile assay. By the way, the method of synthesis of SPIOs and related property parameters refer to the published articles (22). Differential expression

levels of transcripts in mRNAs obtained from SPIOs (200 μg/mL), SMF (60 ± 10 mT), and SMF (60±10 mT) + SPIOs (200 μg/mL)-treated NSCs were compared with negative control.

RNA-Seq analysis was performed to identify differentiated genes under different culture conditions and to explore the regulatory mechanisms. Duplicate samples for each population showed high reproducibility (Pearson's r was greater than 0.98 in all groups) (Fig. 1A). Overall, NSCs treated with SMF caused the least number of differentially expressed genes (87) when compared to the other two groups, which had 26 upregulated genes and 61 downregulated genes (Fig. 1B and 1D, respectively). While the SPIOs group alone caused the highest number of differentially expressed genes (309) compared to the other two groups, slightly more genes were upregulated (183) than downregulated genes (126) (Fig. 1B and 1C). However, the SMF + SPIOs group caused the second highest number of differentially expressed genes (212). Among those genes, there were

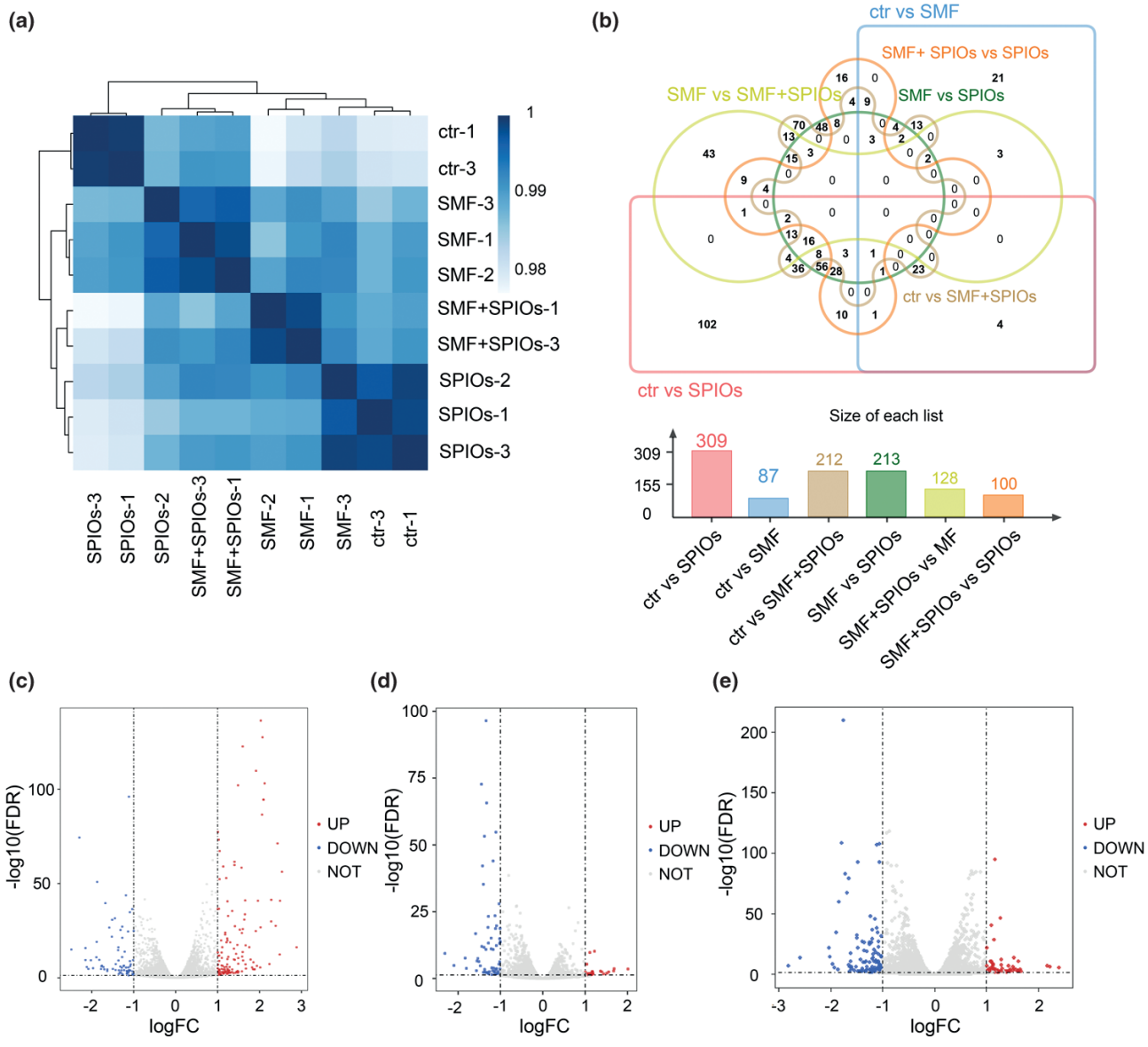

Fig. 1. Transcriptomic analysis of NSCs sourced from different treatment groups and control group. (a) Sample clustering analysis for all replicates in different groups of NSCs. (b) Venn diagram performing the number and distribution relationship of differentially expressed genes in different groups. (c), (d), and (e) present volcano plots of differentially expressed gene between SPIOs vs control group, SMF vs control group, the SMF + SPIOs vs control group, respectively. For the convenience of mapping, the – log10(FRD) is often taken as the ordinate and so is the transverse coordinate. Red dots: upregulated expression; blue dots: downregulated expression; gray dots: no obvious differential expression.

128 downregulated and 84 upregulated genes (Fig. 1B and 1E). Then, we chose Venn diagrams to perform the association between differentially expressed genes in each experimental group (Fig. 1B).

Those results demonstrate that the SPIOs incubation only has the largest effects on the gene expression level of NSCs, and 60±10 mT SMF stimulation provided a weaker effect on the gene expression level of NSCs that compare with it. However, the addition of SMF could reduce the number of SPIOs-induced differentially expressed genes in differentiation of NSCs, and its means the participation of 60±10 mT SMF could weaken the regulatory effect of SPIOs.

Differentially expressed genes of SPIOs and SMF-treated NSCs

To further refine the concentrated distribution of differentially expressed genes in the NSCs treated under the three different conditions (SPIOs, SMF, SMF + SPIOs) compared to the control group, we screened the top 20 differentially expressed levels genes up- and downregulated in each group and shown them in Fig. 2.

These figures indicate that there are only trace duplications of differentially abundantly expressed genes in SPIOs, SMF, and SMF + SPIOs groups with very large differences compared with the control group. For example, the *Il6*, *Gm16685*, *Hspb1*, *Ctgf*, *Serpine1*, *Papss2*, etc. were significantly highly expressed in the SPIOs group (Fig. 2A), *Pla2g5*, *Gm10180*, *Slc35f2*, *D030055H07Rik*, etc. were significantly highly expressed in the SMF group (Fig. 2B), and *Il6*, *Slc35f2*, *Hspb1*, *Mc2r*, *Ovgp1*, etc. were significantly highly expressed in the SMF + SPIOs group (Fig. 2C). *Mmp2*, *Adh7*, *Gucy2e*, *Pla2g5*, *Gm10180*, *Slc35f2*, *Rxrg*, *Plxdc1*, *Irf7*, etc. were highly expressed in the control group.

GO and KEGG signaling pathway analysis

In order to more clearly elucidate the mechanism of the regulatory effects of the three conditions on NSCs differentiation, we performed GO analysis and signaling pathway enrichment analysis on those differentially expressed genes. GO analysis refers to an international standard classification system for gene function, which consists of cellular component (CC), biological process (BP), and molecular function (MF) (23). GO enrichment analysis results for each group compared to the control group differentially expressed genes involved in MF, BP, and CC.

The number of differentially expressed genes was the highest for SPIOs alone group compared to the control group, and therefore, the number of GO-enriched entries was relatively high. Analysis revealed that more of the enriched entries were related to stem cell differentiation regulate, such as cell differentiation, cell adhesion, MAPK-related signaling pathway, and neuron development (Fig. 3A). It is also known that the fate of stem cells is regulated by multiple signaling pathways. According the results of our KEGG analysis, we particularly noted multiple signaling pathways that have been reported to be associated with the regulation of stem cell differentiation fate, such as MAPK signaling pathway and PI3K−Akt signaling pathway. MAPK signaling pathways are an important signaling pathway regulating the behavior of NSCs, which can regulate proliferation (24), differentiation, and migration (25). The PI3K kinase/Akt signaling pathway not only is associated with the survival of NSCs [30] but also has an inducing effect on NSC proliferation and differentiation [31]. The differentially expressed genes from SMF compared to control were analyzed by GO method and found fewer entries associated with the regulation of stem cell differentiation except for some

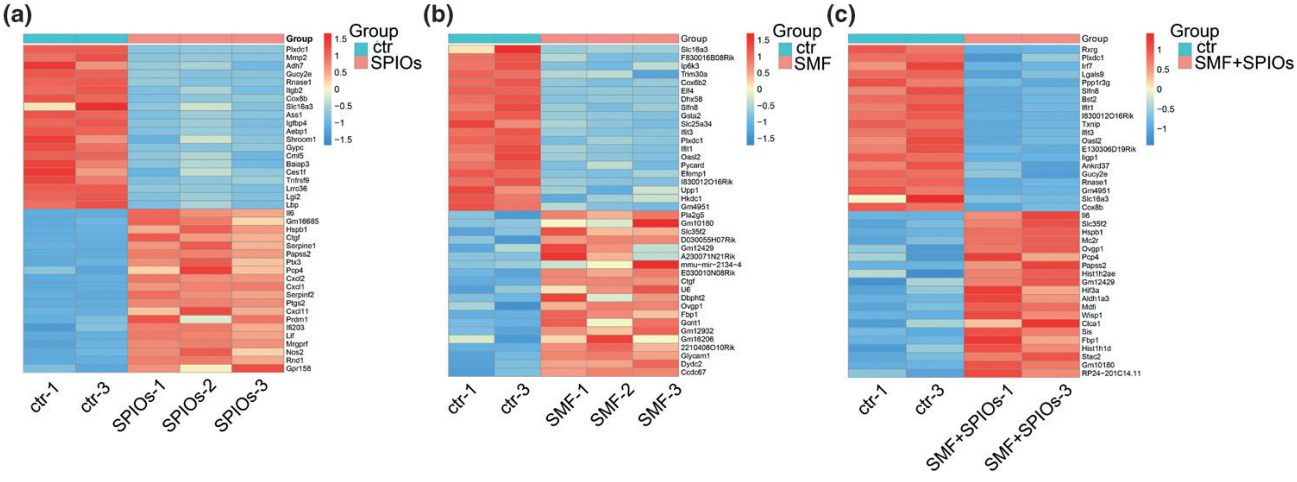

Fig. 2. The heatmap of top 20 significantly high and low expressing genes in different groups. Top 20 significantly upregulated and downregulated expressing genes were shown in SPIOs group (a) (red bar), SMF group (b) (red bar), and SMF + SPIOs (c) (red bar) compared with the control group (green bar).

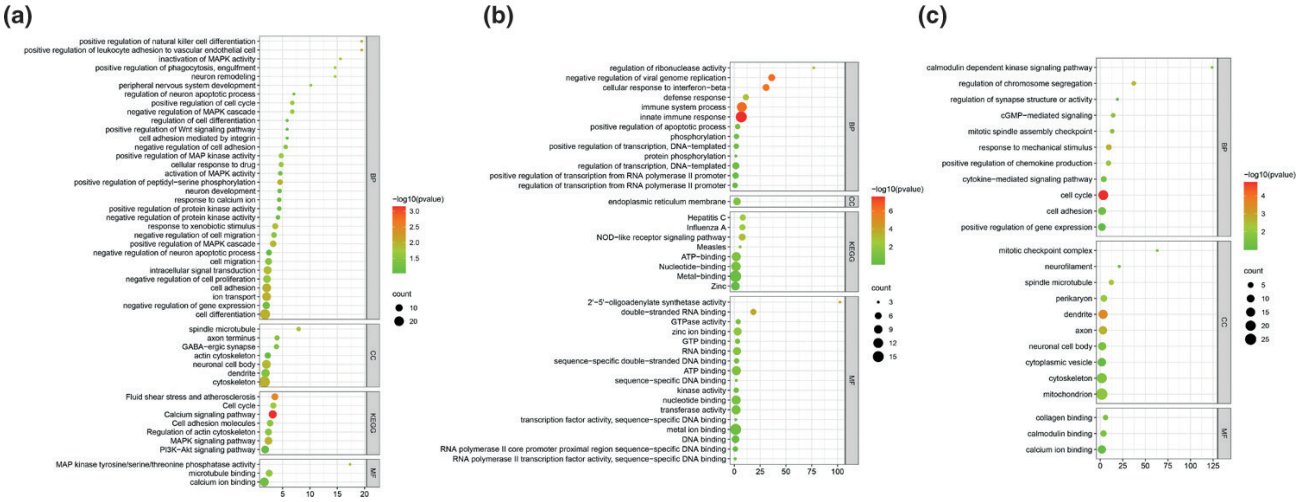

Fig. 3. GO and KEGG analyses of differentially expressed genes in different treatment groups compared with the control group. (a) Analysis of differentially expressed genes GO and KEGG enrichment in the SPIOs group compared with the control group. (b) Analysis of differentially expressed genes GO and KEGG enrichment in the SMF group compared with the control group. (c) Analysis of differentially expressed genes GO enrichment in the SMF + SPIOs group compared with the control group. The size of the dots represents the number of differentially expressed genes enriched, and the larger dots represent more genes enriched; the color of the dots represents the *P* value of the significance level of differentially expressed genes, and the redder the color represents the larger the *P* value and greener colors represent smaller *P* values.

regulating neuronal development (Fig. 3B). Although the number of differentially expressed genes in the SMF + SPIOs group was more than twice compared to the control group, their GO analysis results were also largely unrelated to cell differentiation with the entries of SMF versus control group, and the results of KEGG signaling pathway enrichment were also similar (Fig. 3C).

Therefore, we suggest that the 200 μg/mL SPIOs used in the experiment have a modulating effect on the differentiation of NSCs. However, the MF under experimental conditions could neither have an effect on the differentiation of NSCs nor have the SMF + SPIOs.

In the present study, we found the interesting phenomenon that only SPIO already causes large changes in the transcripts of NSCs, while the addition of SMF attenuated this effect. In this regard, we suspect that this may be related to the intensity and time of the SMF, as numerous articles have reported that SMF regulates a variety of stem cell behaviors (18, 21, 26). In future work, we intend to refine more on the effect of processing time and intensity of the SMF, to answer the regulatory effect of magnetic field as a physical factor on stem cells.

Conclusion

We used gene transcriptomic analysis to explore the crucial genes or signaling pathways that may be involved in regulating the process of NSCs differentiation under different conditions. Our study demonstrates that SPIOs have more significant effects on NSC differentiation than SMF and induce more differentially expressed genes. These differentially expressed genes were found to be enriched in multiple signaling pathways related to the regulation of NSC differentiation by KEGG signaling pathway enrichment analysis.

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