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Low frequency pulsed electromagnetic field promotes differentiation of oligodendrocyte precursor cells through upregulation of miR-219-5p in vitro

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ABSTRACT

Aim: Spinal cord injury (SCI) is a common demyelinating disorder of the central nervous system. The differentiation of oligodendrocyte precursor cells (OPCs) into mature oligodendrocytes (OLs), which induce myelination, plays a critical role in the functional recovery following SCI. In this study, the effect of low frequency pulsed electromagnetic field (PEMF) on the differentiation of OPCs and the potential underlying mechanisms were investigated.

Main methods: OPCs were randomly divided into the PEMF and non-PEMF (NPEMF) groups. Immunofluorescence and western blot assays were performed to assess the expression levels of OLs stage-specific markers after 3, 7, 14, and 21 days of PEMF or NPEMF exposure. qRT-PCR was used to further assess the expression levels of miR-219-5p, miR-338, miR-138, and miR-9, which are associated with OPCs differentiation, and the expression levels of genes associated with miR-219-5p. Finally, following PEMF or NPEMF exposure, qRT-PCR and western blot assays were performed to explore the relationship between miR-219-5p and Lingo1 and between miR-219-5p and PEMF in promoting OPCs differentiation.

Key findings: PEMF promoted the differentiation of OPCs. PEMF upregulated the expression level of miR-219-5p and downregulated the expression level of Lingo1 during the differentiation of OPCs. Under PEMF exposure, miR-219-5p targeted Lingo1 and reversed the inhibitory effect of miR-219-5p inhibitor on OPCs differentiation. In addition, PEMF synergized with miR-219-5p to promote OPCs differentiation.

Significance: Our results, for the first time, indicated that PEMF promoted OPCs differentiation by regulating miR-219-5p activity in vitro.

1. Introduction

Spinal cord injury (SCI) is a severe traumatic demyelinating disorder usually caused by accidents, and is characterized by high morbidity, disability rate, and cost of treatment [1–3]. SCI induces primary neural injury and secondary tissue damage [1,4,5]. Secondary tissue damage includes axonal demyelination, neuronal cell death, and inflammation [6–9]. Axonal demyelination is considered to be a key mechanism underlying secondary injury, and the degree of functional loss is proportional to the degree of axonal demyelination [9,10]. Oligodendrocytes (OLs) are myelinating cells that are widely distributed in the central nervous system; they block any inhibition on action potential conduction and provide nutritional support to axons [11,12]. Therefore, promoting oligodendrocyte precursor cells (OPCs) differentiation could be useful in treating secondary injury following SCI.

Oscillatory field stimulation (OFS) has been shown to promote the function recovery after SCI in rodents. Our previous studies have shown that OFS can promote the differentiation of OPCs into OLs and subsequent remyelination [13,14]. However, implantation of the OFS device at the SCI site can cause significant trauma to rats. Low frequency pulsed electromagnetic field (PEMF) is widely used as a non-invasive, safe, and effective treatment method for various tissues and biological systems [15–18]. Several studies have shown that PEMF promotes the alleviation of symptoms and delays disease progression in neurodegenerative diseases, indicating that PEMF plays an important role in neurotransmission and autoimmune mechanisms responsible for demyelination [17,19,20]. However, PEMF-mediated OPCs differentiation and the underlying mechanisms remain poorly understood.

miRNAs are non-coding RNA molecules that bind to target mRNA sequences to induce mRNA degradation to regulate biological processes [21]. Recent studies have shown that miRNAs play a key role in the differentiation of OLs. miR-219, miR-138, and miR-338 are abundantly

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expressed in mature OLs and play an essential role in the differentiation of OPCs into mature OLs [22,23]. In central nervous system, recent studies have confirmed that miR-219 inhibits the expression of OPCs differentiation inhibitors, such as Sox6, FoxJ3, ZFP238, and Lingo1 to promote OPCs differentiation and myelination [22,23]. In an in vitro study, miR-9 was shown to inhibit PMP22 expression and negatively regulate the differentiation of $A2B5^+$ OPCs to premyelinating $GalC^+$ OLs [24]. To identify miR-219-5p targeted genes, we acquired a list of the target genes predicted using TargetScan 7.2. Lingo1 is a transmembrane protein selectively expressed in central nervous system oligodendrocytes. As a component of NgR1/p75 and NgR1/Taj signaling complexes, it regulates myelin regeneration and axonal growth [25]. A recent study confirmed that Lingo1 can negatively regulate OPCs differentiation and myelination via Rho A-GTP signaling [26]. The study has shown that antagonizing the function of Lingo1 can downregulate the expression of Rho A-GTP and promote the differentiation and myelination of OPCs [26].

Here, we demonstrated that PEMF promotes the differentiation of OPCs in vitro. We also discovered that the effects of PEMF regulated miR-219-5p expression, which, in turn, targeted Lingo1 to modulate OPCs differentiation. We propose a novel, non-invasive, safe, and effective approach to promote OPCs differentiation.

2. Materials and methods

2.1. Animals

Ten P1–2 Sprague–Dawley rats were purchased from the Experimental Animal Center of Anhui Medical University (SPF, Certificate No. 2011-002). The animals were housed individually in special feeding cages. The room was kept on 12 h of light/dark cycle under a controlled ambient temperature (22 ± 2 °C) and humidity (50 ± 5%). All procedures were performed by following the guidelines of the Animal Care and Use Committee of Anhui Medical University and the National Institutes of Health guide for the care and use of Laboratory animals. All procedures were approved by the Ethics Committee of Anhui Medical University.

2.2. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), PDGF-AA, bFGF, and 3,3′,5-triiodo-L-thyronine (T3) were purchased from Gibco (Carlsbad, CA, USA). The miR-219-5p mimic, miR-219-5p inhibitor, negative control (NC), and U6 were synthesized by RiboBio Company (Guangzhou, China). Lipofectamine 2000 reagent was purchased from Invitrogen (CA, USA). Rabbit-anti-chondroitin sulfate proteoglycan (NG2, ab129051, Abcam), mouse-anti-myelin basic protein (MBP, ab62631, Abcam), and rabbit-anti-Lingo1 (ab63231, Abcam) were purchased from Abcam (Cambridge, UK). Rabbit-anti-Galactosylceramidase (Galc, 11991-1-AP, Proteintech) and rabbit anti-β-Actin (PR-0255, ZSGB-BIO) antibodies were purchased from Proteintech (Wuhan, China). CY3-conjugated goat anti-mouse (GB21302, Servicebio) and anti-rabbit secondary antibodies (GB21303, Servicebio) were purchased from Servicebio (Wuhan, China). HRP-linked goat anti-mouse (ZDR-5307, ZSGB-BIO) and anti-rabbit secondary antibodies (ZDR-5306, ZSGB-BIO) were purchased from ZSGB-BIO (Beijing, China). RIPA lysis buffer, PMSF, and BCA protein assay kit were obtained from Beyotime Institute of Biotechnology (Shanghai, China). ECL plus western blotting substrate were purchased from Thermo Fisher Scientific (USA).

2.3. Oligodendroglial cell culture

Primary OPCs were obtained from the cerebral cortex of P1–2 Sprague-Dawley rat pups as previously described [27]. Rabbit anti-NG2 (1:200 dilution), a marker of OPCs, showed that the cultures consisted of 98% OPCs (data not shown). OPCs were cultured in proliferation medium containing DMEM high glucose supplemented with 10% FBS, 2% B27, PDGF-AA, and bFGF. After achieving 75–85% confluency, the medium was changed to differentiation medium containing 40 ng/mL T3. OPCs were divided into two groups: the PEMF group and NPEMF group. Then, the cells were placed in PEMF exposure system and cultured at 37 °C in an incubator containing 5% CO_2.

2.4. PEMF exposure system

The device used for mediating PEMF exposure was designed by the Institute of Electrical Engineering of the Chinese Academy of Sciences. PEMF exposure system consisted of a DC power supply, an STM32 control system, a pulsed signal generator, and two air-cored circular Helmholtz coils. One of the circular Helmholtz coils could excite and provide electromagnetic field. The system could produce a continuously adjustable output of 0 to 100 Hz and a maximum magnetic induction of 100 mT. Each Helmholtz coil consisted of 1500 coils of enameled copper wire with a diameter of 0.67 mm. In this study, the output pulsed square signal of 50 Hz and 1.8 mT were produced as previously described [28]. The Helmholtz coils were housed inside a cell culture incubator ruling out thermal effect with consistent temperature

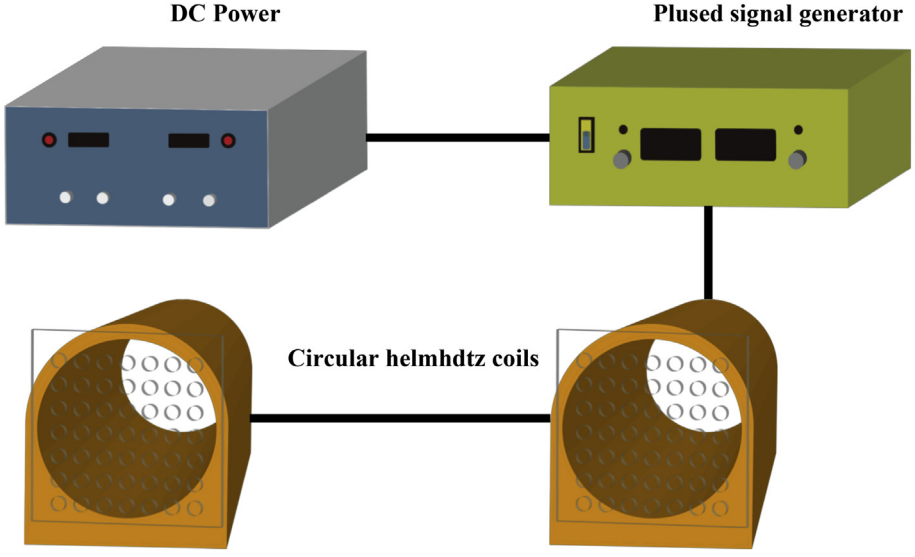

Fig. 1. Schematic illustration of the PEMF exposure system.
The PEMF exposure system consisted of a DC power supply, an STM32 control system, a pulsed signal generator, and two air-cored circular Helmholtz coils. Each Helmholtz coil consisted of 1500 coils of enameled copper wire with a diameter of 0.67 mm. One of the circular Helmholtz coils could excite and provide electromagnetic field and the thermal effects of the two circular Helmholtz coils are the same.

Table 1
Primers for real-time RT-PCR.

Gene	Forward primer sequence (5′- > 3′)	Reverse primer sequence (5′- > 3′)
GAPDH	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT
Elmod2	CGTAGAGCGTCGTTTCCGTT	TTGAAAACTGCTGTCCTTCTCG
Tpcn1	TCGCTCAGGCTTCTAGTCCT	CTCCCCTACCCACCTAGCTT
Cxxc4	TTTACAGGGGGTGATGTGCAA	ATCCACAAGGCTGTTCAGGG
Olig1	AAGGCATGTCGTAGCGTAGG	ATCACAAGCCAAGACGCAGA
Zic5	TCCACACACCTTCCAGCAAT	TCCCTGCAATGTACAAGTCCAA
Erbb2	TGATCATCATGGAGCTGGCG	TGAACTTCCTGGATGTCCTGC
Olig2	GAACCCCGAAAGGTGTGGAT	TTCCGAATGTGAATTCGATTTGAGG
Sox6	AGATTTCCATGGCTTTGTCACT	GCTGTCCCAGTCAGCATCTT
Foxj3	TTCTCTGGCATTGGGGCAAA	TCTGGCCACAAAGAGAATCTTAAC
Rims1	TCTGATGCAGACAGAACGCA	ACTTGGAGTATTGCTCGTGGT
Lingo1	CTTTCCCCTTCGACATCAAGAC	AAGACGGACCACGACGAC

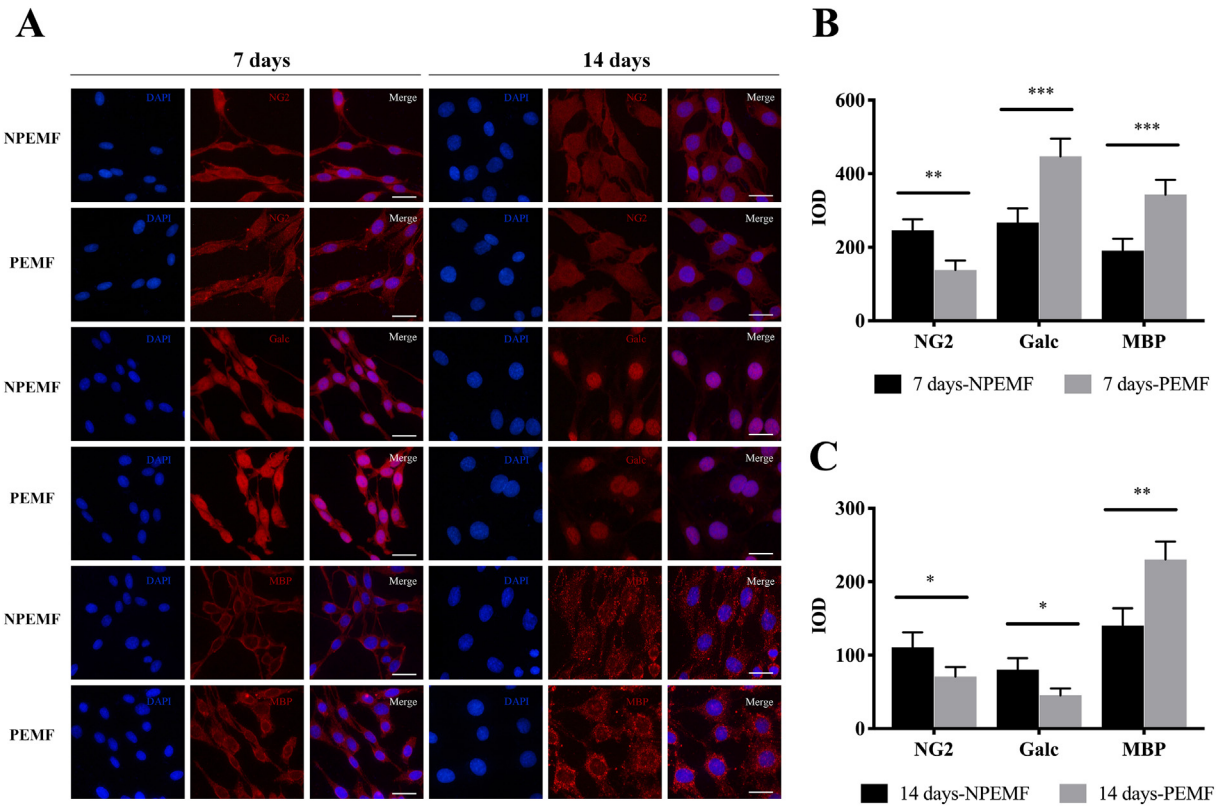

Fig. 2. The effect of PEMF on the differentiation of OPCs was assessed by immunofluorescence.
Immunofluorescence was performed to assess the OPCs differentiation after PEMF or NPEMF stimulation. (A) Representative images of OLs stained with stage-specific markers (NG2, Galc, and MBP) at early (7 days) and late (14 days) stage (400× magnification). Scale bar = 25 μm. (B) Optical density quantification of single cell belonging to PEMF and NPEMF stimulated OLs at early (7 days) stage. (C) Optical density quantification of single cell belonging to PEMF and NPEMF stimulated OLs at late (14 days) stage. Each bar shows mean ± SD of three independent experiments performed in quadruplicates. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus NPEMF group, Student's *t*-test.

monitoring. Schematic illustration of the PEMF exposure system is shown in Fig. 1. Both groups of OPCs were exposed in the morning and afternoon for 2 h for 3, 7, 14, and 21 days.

2.5. Cell transfection

OPCs were divided into the following groups: (1) PEMF + NC group; (2) PEMF + miR-219-5p mimic group; (3) PEMF + miR-219-5p inhibitor group; and (4) NPEMF + miR-219-5p inhibitor group; (5) NPEMF + miR-219-5p mimic group. Before 18 h of transfection, OPCs were passaged thrice and seeded in 6-well plates containing the differentiation medium without penicillin and streptomycin. When the cells reached 80%–90% confluency, cell transfection was performed using Lipofectamine 2000 reagent, following the manufacturer's instructions. Within 5 h after transfection, OPCs were exposed to PEMF or NPEMF, according to the predetermined grouping. Then, the medium was replaced with fresh differentiation medium without penicillin and streptomycin and cultured at 37 °C under 5% CO_2 for 48 h.

2.6. Immunofluorescence

To determine whether PEMF affected the differentiation of OLs, OLs were stained during differentiation at four time points. Immunostaining on paraformaldehyde-fixed cultured OLs was performed as described previously [29]. The antibodies used in this assay were: rabbit anti-NG2 (1:200 dilution), a marker of OPCs; rabbit anti-Galc (1:200 dilution), a marker of immature OLs; mouse anti-MBP (1:200 dilution), a marker of mature OLs; CY3-conjugated goat anti-mouse (1:200 dilution); and anti-

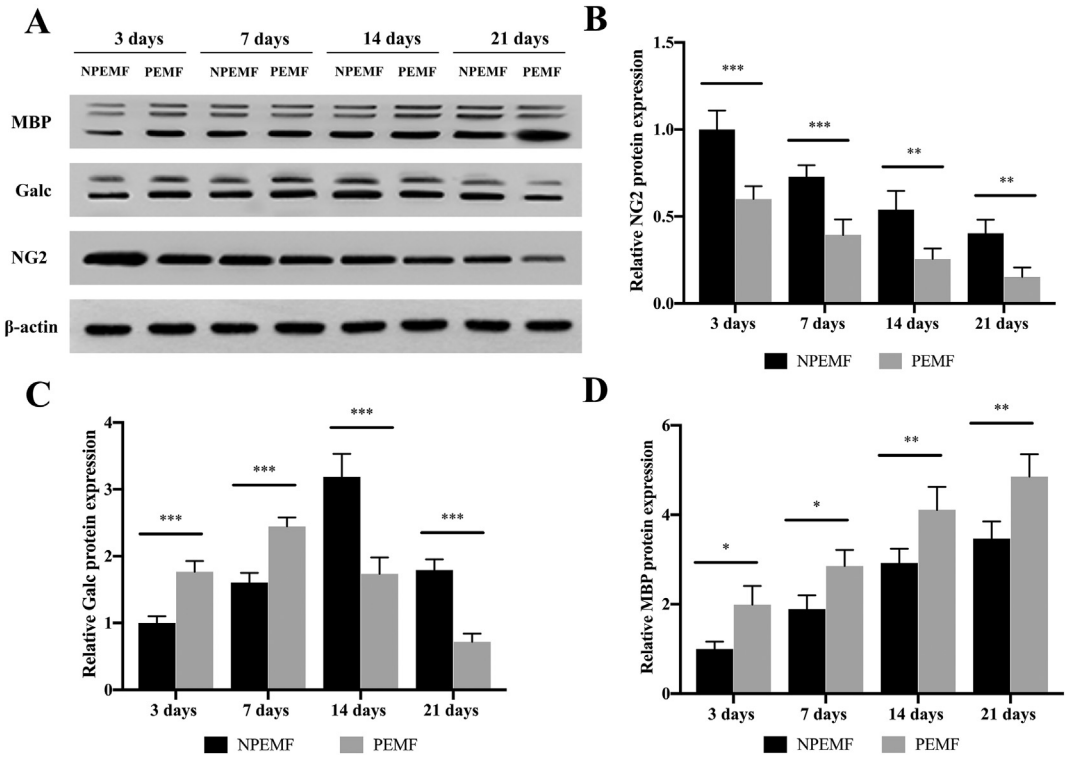

Fig. 3. The effect of PEMF on the differentiation of OPCs was assessed by western blot assay.
The western blot assay was performed to assess OPCs differentiation under PEMF or NPEMF stimulation at early and late stages. (A) Results of western blot analysis at early and late stages in the PEMF and NPEMF groups. (B–D) Quantitative analysis of western blot results. Each bar shows mean ± SD of three independent experiments performed in quadruplicates. (B) Treatments: F = 24.35 and DF = 1, times: F = 388.3 and DF = 3, subjects: F = 31.32 and DF = 4. (C) Treatments: F = 6.656 and DF = 1, times: F = 57.31 and DF = 3, subjects: F = 1.428 and DF = 4. (D) Treatments: F = 63.22 and DF = 1, times: F = 53.48 and DF = 3, subjects: F = 0.7739 and DF = 4. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to the NPEMF group, two-way ANOVA test.

rabbit secondary antibody (1:200 dilution). The fluorescence signals were obtained using confocal laser scanning microscope system (LSM 880, Zeiss, Oberkochen, Germany) by comparing and processing all images of the same light intensity and filtering with the Zen blue software. Image J was used for quantitative analysis. Two coverslips were used in each group. The number of immunopositive cells in each of the six randomly selected regions of each coverslip was counted, and then, the optical densities of the individual cells were calculated and compared.

2.7. Western blot analysis

To further identify whether PEMF alone or in conjugation with miR-219-5p affected the differentiation of OLs, the relative expression levels of NG2, Galc, MBP, and Lingo1 were quantified by western blot analysis, as described previously [27]. Primary and secondary antibodies used were: rabbit anti-NG2 (1:1000 dilution), rabbit anti Galc (1:1000 dilution), mouse anti-MBP (1:1000 dilution), rabbit anti-Lingo1 (1:1000 dilution), rabbit anti-β-Actin antibody (1:1000 dilution), HRP-conjugated goat anti-mouse (1:10,000 dilution), and anti-rabbit secondary antibody (1:10,000 dilution). The protein band signals were obtained using Tanon5200 system (Tanon, Shanghai, China). Image J was used for quantification analysis. The intensity of actin bands was used for normalization.

2.8. RNA isolation, cDNA synthesis, and quantitative real time-polymerase chain reaction

According to the manufacturer's instructions, total RNA was extracted from OLs at four time points and transfected OPCs exposure to PEMF or NPEMF using the RNeasy Mini Kit (Qiagen, Hilden, Germany). In order to quantify miRNAs, 10 ng of total RNA was reverse transcribed using a TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, NY, USA). Each reverse transcription (RT) reaction mixture contained 10 ng total RNA, 0.19 μL RNase inhibitor (20 U/μL), 6 μL RT Primer, 1.5 μL 10× RT Buffer, 0.3 μL dNTPs with dTTP (100 mM), 3 μL MultiScribe Reverse Transcriptase (50 U/μL), and 1.01 μL ddH_2O (RNase/DNase free). The 15 μL reaction mixture was incubated at 16 °C for 30 min, at 42 °C for 30 min, at 85 °C for 5 min, and then, held at 4 °C using Flex Cycler Multi-Functional PCR System (Jena, Thuringia, Germany). Following the RT step, the total volume of each qPCR reaction mixture was 20 μL, containing 1 μL 20× TaqMan® MicroRNA Assays, 10 μL TaqMan® Universal Master Mix II (2×), 0.16 μL cDNA, and 8.84 μL ddH_2O (RNase/DNase free).The qPCR reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s, and finally held at 4 °C using StepOne RT-PCR System (Applied Biosystems). The relative expression of miRNAs was standardized against the relative expression of U6 snRNA as an endogenous standardized control. TargetScan 7.2 was used to acquire a list of putative miR-219-5p targets. In order to quantify mRNA, 1200 ng of total RNAs were reverse transcribed into cDNA using PrimeScript™ RT Master Mix (Takara Biotechnology, Dalian, China). Then, quantitative real-time PCR was performed using Maxima SYBR Green/qPCR Master Mix (Takara Biotechnology, Dalian, China). Briefly, the total volume of each qPCR reaction mixture was 10 μL, containing 1 μL sense and antisense primers (5 μmol/μL), 1 μL cDNA (10 ng), 2 μL 2× SYBR GreenMaster Mix, and 8 μL ddH_2O (RNase/DNase free), which was subjected to 45 cycles of amplification, involving denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s using StepOne RT-PCR System (Applied Biosystems). The relative expression levels of mRNA were normalized against the relative mRNA expression of GAPDH used as an endogenous standardized control. The miRNA and

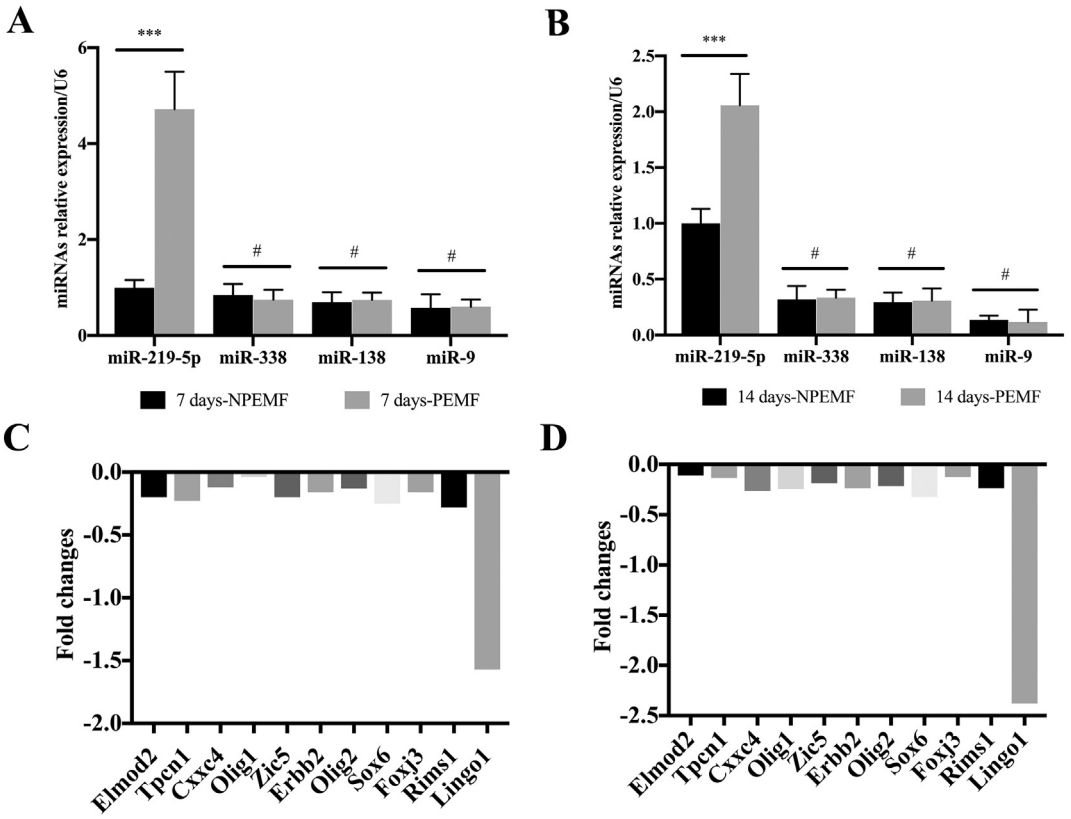

Fig. 4. The effect of PEMF on the relative expression of miRNAs and miR-219-5p target gene were assessed using qRT-PCR assay at early (7 days) and late (14 days) stages.

qRT-PCR was performed to assess the relative expression levels of miRNAs associated with OPCs differentiation under PEMF or NPEMF exposure at the early (7 days) and late (14 days) stages. The miR-219-5p targeted PCR assay was used to assess the effect of PEMF on the relative expression of possible target genes. (A, B) The relative expression levels of miRNAs at the early (7 days, A) and late (14 days, B) stages of the PEMF and NPEMF groups. (C, D) The expression levels of putative target genes of miR-219-5p represented as fold changes in the PEMF group compared to those in the NPEMF group in early (7 days, C) and late (14 days, D) stages. Each bar shows the mean ± SD of three independent experiments performed in quadruplicates. $^{\#}p > 0.05$, $^{***}p < 0.001$ compared to the NPEMF group. Student's *t*-test.

mRNA relative expression levels were measured using the $2^{-\Delta\Delta CT}$ method (Life Technologies) in quadruplicate (Table 1).

2.9. Statistical analysis

All experiments were performed independently at least three replicates in quadruplicates and quantified blindly. The data were presented as mean ± standard deviation (SD). Statistical analysis was carried out with SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) software using independent sample Student's *t*-test to analyze the differences between the two groups. Two-way analysis of variance (ANOVA) was performed to analyze the effect of variables “exposure time” and “treatment”. One-way analysis of variance was performed using Tukey's multiple comparisons test when comparing multiple groups. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. PEMF promotes the differentiation of OPCs

To determine whether PEMF affected the differentiation of OPCs, immunofluorescence and western blot assays were performed to observe the relative expression levels of OLs stage-specific markers at early (3 days and 7 days) and late stages (14 days and 21 days). As shown in Fig. 2A, all of cells were NG2-positive and exhibited typical OPC morphology after 7 days. Although MBP is a specific molecular marker of mature OLs, differentiated OLs still exhibited a low relative MBP expression at early stage. To assess the effect of PEMF, we compared the relative expression of stage-specific markers. At early and late stages, there was a significant difference in the expression levels of NG2, Galc, and MBP between the PEMF group and the NPEMF group (Fig. 2B, C). The effect of PEMF was further validated by western blot assays. As shown in Fig. 3A–D, the relative expression of OLs stage-specific markers was significantly different between the two groups. The PEMF group exhibited accelerated OPCs differentiation unlike the NPEMF group. These results indicate that PEMF promoted the differentiation of OPCs.

3.2. PEMF upregulates the expression level of miR-219-5p and downregulates the expression level of Lingo1 during OPCs differentiation

To investigate the possible mechanism of PEMF-mediated promotion of OPCs differentiation, we analyzed differential expression profiles of miRNAs associated with OPCs differentiation between the two groups using qRT-PCR assays at early and late stages. As shown in Fig. 4A–B, the differentiation of OPCs under PEMF exposure was positively correlated with miR-219-5p expression at early (7 days) and late (14 days) stages. The PEMF group showed higher relative expression of miR-219-5p than the NPEMF group (Fig. 5A). These results suggested that the differentiation of OPCs under PEMF exposure was associated with miR-219-5p expression. We further determined the expression profile of genes which negatively regulated OPCs differentiation in the two groups using miR-219-5p qPCR assay at early (7 days) and late (14 days) stages (Fig. 4C–D). In the early (7 days,

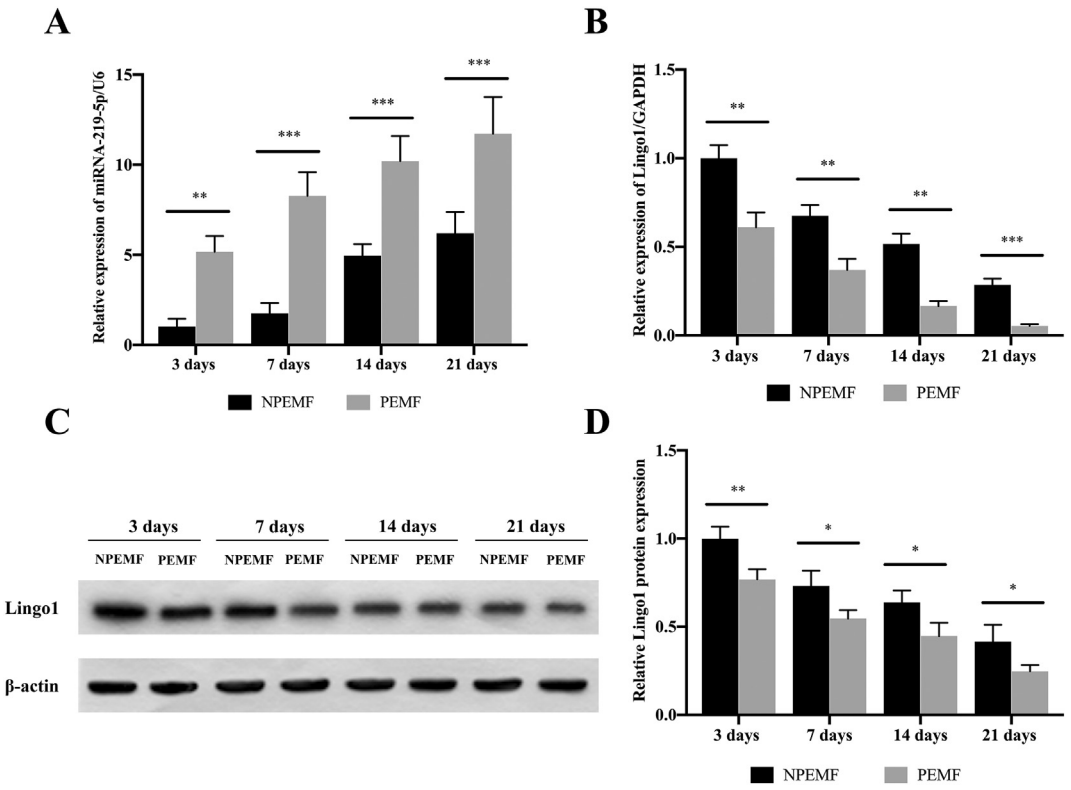

Fig. 5. PEMF upregulated the relative expression levels of miR-219-5p and downregulated the relative expression levels of Lingo1 during OPCs differentiation. The qRT-PCR and western blot assays were performed to evaluate the effect of PEMF on the relative expression of miR-219-5p and Lingo1. (A) The relative expression levels of miR-219-5p in the PEMF and NPEMF groups during OPCs differentiation. (B) The relative expression levels of Lingo1 mRNA in the PEMF and NPEMF groups during OPCs differentiation. (C) Western blot results of Lingo1 protein in the PEMF and NPEMF groups during OPCs differentiation. (D) Quantitative analysis of western blot results. Each bar shows mean ± SD of three independent experiments performed in quadruplicates. (A) Treatments: $F = 80.34$ and $DF = 1$, times: $F = 37.67$ and $DF = 3$, subjects: $F = 1.962$ and $DF = 4$. (B) Treatments: $F = 98.95$ and $DF = 1$, times: $F = 205.1$ and $DF = 3$, subjects: $F = 2.856$ and $DF = 4$. (D) Treatments: $F = 128.2$ and $DF = 1$, times: $F = 53.91$ and $DF = 3$, subjects: $F = 0.2998$ and $DF = 4$. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to the NPEMF group, two-way ANOVA test.

Fig. 4C) and late stages (14 days, Fig. 4D) of OPCs differentiation, PEMF significantly downregulated the expression level of Lingo1 mRNA. As depicted in Fig. 5B, Lingo1 mRNA expression levels were significantly different between the PEMF group and the NPEMF group. Changes in the expression level of Lingo1 protein during the OPCs differentiation were further validated through western blot assays (Fig. 5C–D). These results suggested that PEMF played a critical role in downregulation of Lingo1 protein expression at early and late stages. Thus, we speculated that miR-219-5p and Lingo1 were associated with the differentiation of OPCs under PEMF exposure. The mechanism of PEMF-mediated promotion of OPCs differentiation may involve PEMF-mediated upregulation of the expression level of miR-219-5p and downregulation of the expression level of Lingo1.

3.3. miR-219-5p targets Lingo1 under PEMF exposure

To investigate the relationship between downregulation of Lingo1 and upregulation of miR-219-5p under exposure to PEMF, OPCs were transfected, and then, were exposed to either PEMF or NPEMF.

The qRT-PCR assay revealed that the expression level of miR-219-5p was significantly upregulated after transfection with miR-219-5p mimic and downregulated after transfection with miR-219-5p inhibitor. Meanwhile, PEMF reversed the inhibitory effect of miR-219-5p inhibitor by promoting the expression level of Lingo1 (Fig. 6A). As shown in Fig. 6B, the relative expression levels of Lingo1 mRNA were significantly downregulated by miR-219-5p overexpression and upregulated by miR-219-5p inhibition compared to those in the corresponding controls under PEMF exposure. PEMF reversed the effects of miR-219-5p inhibitor on Lingo1 expression level. These results were further confirmed by western blot assays (Fig. 6C–D). These results indicated that the miR-219-5p targeted Lingo1 under PEMF exposure.

3.4. PEMF and miR-219-5p synergistically promote OPCs differentiation

To further explore the effect of PEMF and miR-219-5p in promotion of OPCs differentiation, western blot assay was performed to determine the relative expression levels of stage-specific markers following transfection under PEMF or NPEMF exposure. As shown in Fig. 7A, C, D, and E, there were significant differences in OPCs differentiation among the five groups. Unlike in the NPEMF + miR-219-5p mimic group, PEMF synergized with miR-219-5p to significantly promote OPCs differentiation in the PEMF + miR-219-5p mimic group. Compared with that in the NPEMF + miR-219-5p inhibitor group, PEMF significantly antagonized the inhibitory effect of miR-219-5p inhibitor in the PEMF + miR-219-5p inhibitor group. Taken together, these data indicate that PEMF and miR-219-5p synergistically promoted OPCs differentiation. In conclusion, miR-219-5p and Lingo1 promoted the differentiation of OPCs into myelinating OLs under PEMF exposure (Fig. 7B).

4. Discussion

The present study clearly indicated that direct exposure to 50 Hz PEMF promoted the differentiation of OPCs into mature OLs. Exposure to 50 Hz PEMFs upregulated the relative expression of miR-219-5p in OLs. Further analysis of miR-219-5p and its target genes revealed that

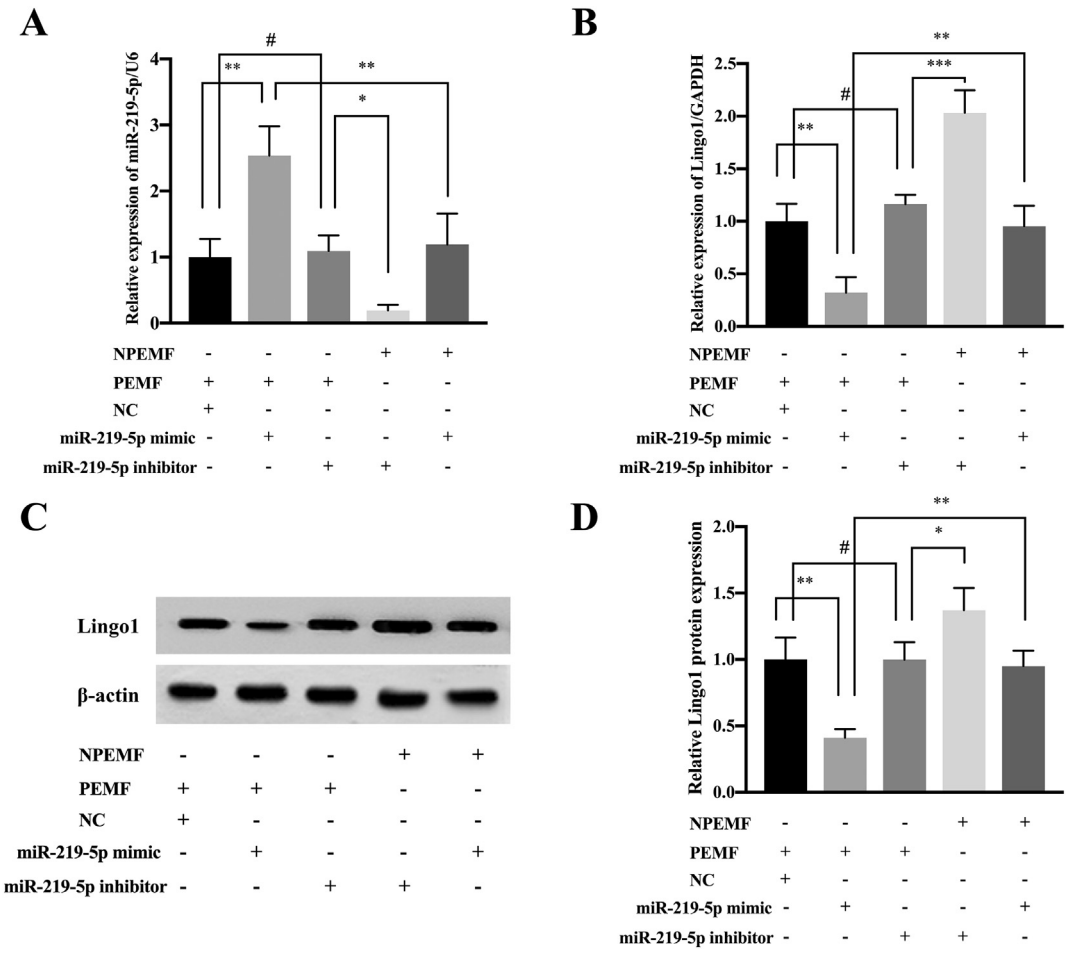

Fig. 6. miR-219-5p targets Lingo1 under the exposure of PEMF during OPCs differentiation.
Cell transfection was conducted under the exposure of PEMF or NPEMF to evaluate whether miR-219-5p targeted Lingo1 during OPCs differentiation. (A) The relative expression levels of miR-219-5p in the PEMF and NPEMF groups after transfection. (B) The relative expression levels of Lingo1 mRNA under PEMF or NPEMF exposure after transfection. (C) The western blot results of Lingo1 protein under PEMF or NPEMF exposure after transfection. (D) Quantitative analysis of western blot results. N = 3 transfections. Each bar shows mean ± SD of three independent experiments performed in quadruplicates. (A) F = 19.42, DF = 4. (B) F = 39.87, DF = 4. (D) F = 19.60, DF = 4. *p < 0.05, **p < 0.01, ***p < 0.001, #p > 0.05, compared to the NC, NPEMF, or inhibitor groups, one-way ANOVA with Tukey's multiple-comparison test.

miR-219-5p and Lingo1 plays an important role in the differentiation of OPCs after exposure to 50 Hz PEMF. Therefore, we showed, for the first time, that miR-219-5p regulation plays a key role in mediating the biological effects of 50 Hz PEMF on the differentiation of OPCs.

Previous studies have shown that PEMF could exert different physiological effects on different species and cell lines [28,30–32]. Based on studies related to osteogenic effects of PEMF, PEMF administration is approved as a safe and effective method for the treatment of osteoporosis and as a non-binding therapy for bone by the US Food and Drug Administration [33]. In contrast, the effects of PEMF on OPCs are less investigated. It has previously been reported that static magnetic field stimulation enhances OLs differentiation via upregulation of intracellular Ca^{2+} influx and, in turn, Ca^{2+} expression, which is consistent with previous studies on the utility mechanism of PEMF [31,34]. However, the potential role of the increase in levels of Ca^{2+} in the PEMF-induced enhancement of OPCs differentiation is unclear. Previous studies established that the effect of PEMF depended on exposure time, exposure frequency, exposure intensity, type of cells, and reaction of cells [28,30,31]. Several studies have demonstrated that exposure to high-frequency PEMF for a long duration could increase the risk of neurodegenerative diseases, but low-frequency PEMF exposure was considered to be safe and effective under controlled conditions [35,36]. Currently, 1–160 Hz PEMF are commonly used to induce cellular physiological responses. Among traded commodities, 50 Hz PEMF had been considered to activate the signaling cascades, thereby some researchers use 50 Hz PEMF as a classic frequency for their studies [28,31]. We successfully constructed the adjustable PEMF and explored the potential effect of PEMF on the differentiation of OPCs. Three days after exposure of primary OPCs to PEMF, we found that OPCs exhibited good growth. Interesting, we found that 14 days was a critical time in the differentiation of OPCs into mature OLs under exposure to PEMF. During the differentiation of OPCs, the changes observed in expression levels of NG2, Galc, and MBP were consistent with previous studies [37].

Substantial evidences have demonstrated that the miRNAs mediate differentiation of OLs and facilitate functional recovery after SCI [22,23]. To elucidate the mechanism of PEMF-mediated promotion of OPCs differentiation, we evaluated whether PEMF affected the expression of miRNAs that were associated with the differentiation of OPCs. miRNA-219-5p expression level was found to be significantly increased with the increase in PEMF exposure time compared to that in the NPEMF group. In contrast, other miRNAs, including miR-138, miR-9, and miR-338, did not exhibit similar trend between two groups. In addition, miR-219 exhibits secondary expression in OPCs with main expression in differentiating OLs [22,23]. Inhibition of miR-219 severely impaired the differentiation of OLs; miR-219 alone promotes OLs

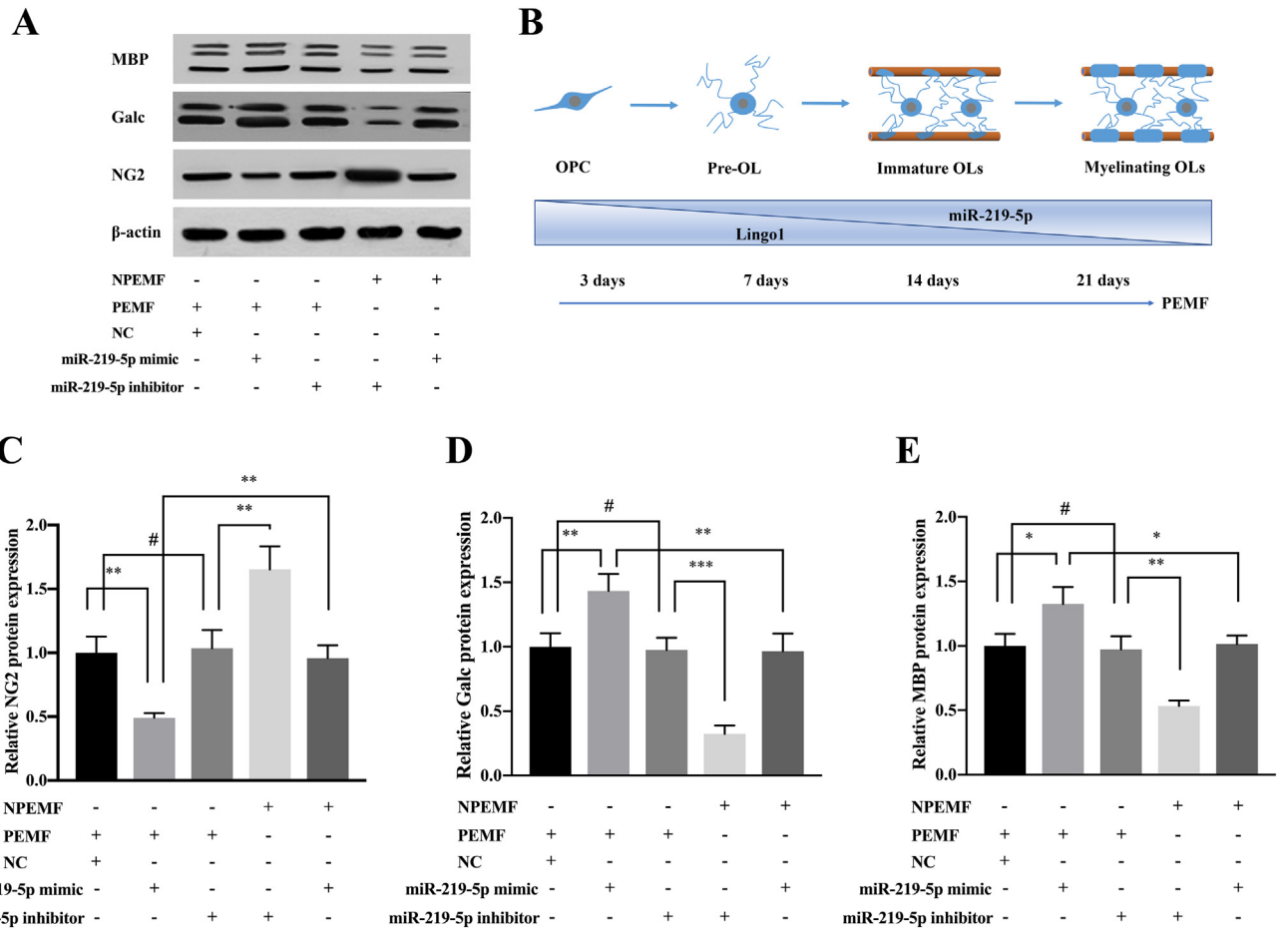

Fig. 7. PEMF and miR-219-5p synergistically promote the differentiation of OPCs.
The western blot assay was performed to assess the relationship of PEMF and miR-219-5p with OPCs differentiation. (A) The western blot results of OPCs stage-specific markers (NG2, Galc, and MBP) in the PEMF and NPEMF groups after transfection. (B) Stages of OPCs differentiation toward oligodendroglial lineage: OPC, pre-OL, immature OLs, myelinating OLs. PEMF promoted OPCs differentiation into myelinating OLs via interaction of miR-219-5p with Lingo1. (C–E) Quantitative analysis of western blot results. Each bar shows mean ± SD of three independent experiments performed in quadruplicates. (C) $F = 31.51$, $DF = 4$. (D) $F = 39.76$, $DF = 4$. (E) $F = 23.86$, $DF = 4$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#}p > 0.05$, compared to the NC, NPEMF, or inhibitor groups, one-way ANOVA with Tukey's multiple-comparison test.

differentiation after demyelination to form neonatal myelin, and targets critical genes or signaling pathways that inhibit the differentiation and myelination of OLs [22]. The proliferation and differentiation of OPCs are inseparable, and cessation of proliferation triggers their differentiation. miR-219-5p targets specific genes that promote the proliferation and inhibit the differentiation of OPCs. These genes include Sox6, FoxJ3, ZFP238, Lingo1, etc. [22,23]. PEMF may inhibit the proliferation of OPCs during OPCs differentiation, which may be one of the mechanisms of PEMF-mediated promotion of OPCs differentiation [22,23].

From the relationship between the expression level of miR-219-5p and PEMF, we speculated that promoting differentiation was due to increase the expression level of miR-219-5p by the direct exposure to PEMF. In fact, this was consistent with the results of previous studies that miR-219-5p promoted the differentiation of OPCs [22,23]. Moreover, we evaluated the expression levels of several negatively regulated genes during the differentiation of OPCs. Several studies have suggested that Lingo1 is a key negative regulator of OLs differentiation and myelination [25,26]. Overexpression of Lingo1 inhibits OL differentiation and myelination, whereas Lingo1 antagonists promote OLs differentiation.

We observed that changes in the expression of miR-219-5p and Lingo1 in OLs were significantly reversed under direct exposure to the magnetic field. A recent study has shown that miR-219 can target Lingo1 to promote myelin repair in the central nervous system [23]. In addition, inhibition of Lingo1 expression by salvaging miR-219-5p inhibitor with PEMF further promoted the pivotal role of PEMF similar to the effect of miR-219-5p in OPCs. We investigated the similar effect of PEMF on miR-219-5p levels during the differentiation of OPCs. During this process, PEMF and miR-219 exhibited a synergistic effect, while antagonizing the inhibitory effect of miR-219 inhibitor or inhibitor genes on the differentiation of OPCs.

Our study confirmed the effect of PEMF on the differentiation of OPCs, aiming to provide novel potential therapeutic strategies for SCI. Unfortunately, our study was limited to the effect of PEMF on the differentiation of OPCs. The effects of PEMF on the proliferation, apoptosis, remyelination, and axon elongation in neuron culture of OPCs need further elucidation. Our current work is mainly in vitro, and related in vivo experiments are underway. Moreover, the effects of PEMF on OPCs under different frequencies, different intensities, and different exposure times need further exploration. In addition to OPCs, the participating cells in spinal cord injury include nerve cells, vascular endothelial cells, astrocytes, and macrophages. The effects of PEMF on these cells are still unclear and needs to be explored.

5. Conclusion

In conclusion, our study showed that exposure to 50 Hz PEMF

promoted the differentiation of OPCs via upregulation of the interaction between miR-219-5p and Lingo1. PEMF promoted OPCs differentiation via miR-219-5p regulation and suppression of the inhibitory effects of the miR-219-5p inhibitor on OPCs differentiation. Thus, non-invasive PEMF stimulation may have a potentially positive impact on the functional recovery process following SCI.

Author contributions

Research design and protocol: Fei Yao, Li Cheng, and Juehua Jing. Research development: Fei Yao, Ziyu Li, and Li Cheng. Data collection: Liqian Zhang and Xiaowei Zha. Data analysis and interpretation of results: Fei Yao, Li Cheng, and Juehua Jing. Drafting manuscript: Fei Yao and Ziyu Li. All authors have read and agreed with the manuscript and conclusions.

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Competing interests

The authors declare that they have no competing interests.

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