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# Effects of electroacupuncture and the retinoid X receptor (RXR) signalling pathway on oligodendrocyte differentiation in the demyelinated spinal cord of rats

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## ABSTRACT

**Objectives** In spinal cord demyelination, some oligodendrocyte precursor cells (OPCs) remain in the demyelinated region but have a reduced capacity to differentiate into oligodendrocytes. This study investigated whether ‘Governor Vessel’ (GV) electroacupuncture (EA) would promote the differentiation of endogenous OPCs into oligodendrocytes by activating the retinoid X receptor  $\gamma$  (RXR- $\gamma$ )-mediated signalling pathway.

**Methods** Adult rats were microinjected with ethidium bromide (EB) into the T10 spinal cord to establish a model of spinal cord demyelination. EB-injected rats remained untreated (EB group, n=26) or received EA treatment (EB+EA group, n=26). A control group (n=26) was also included that underwent dural exposure without EB injection. After euthanasia at 7 days (n=5 per group), 15 days (n=8 per group) or 30 days (n=13 per group), protein expression of RXR- $\gamma$  in the demyelinated spinal cord was evaluated by immunohistochemistry and Western blotting. In addition, OPCs derived from rat embryonic spinal cord were cultured in vitro, and exogenous 9-cis-RA (retinoic acid) and RXR- $\gamma$  antagonist HX531 were administered to determine whether RA could activate RXR- $\gamma$  and promote OPC differentiation.

**Results** EA was found to increase the numbers of both OPCs and oligodendrocytes expressing RXR- $\gamma$  and RALDH2, and promote remyelination in the remyelinated spinal cord. Exogenous 9-cis-RA enhanced the differentiation of OPCs into mature oligodendrocytes by activating RXR- $\gamma$ .

**Conclusions** The results suggest that EA may activate RXR signalling to promote the

differentiation of OPCs into oligodendrocytes in spinal cord demyelination.

## INTRODUCTION

Multiple sclerosis (MS) is a common autoimmune disorder of the central nervous system (CNS). Although the underlying cause has not been fully clarified, it is generally attributed to failure of myelin-producing cells—oligodendrocytes and oligodendrocyte precursor cells (OPCs). To date, there is no curative treatment for MS. However, based on the aforementioned presumption, protection or stimulation of endogenous OPCs represents a potential therapeutic option for MS. After demyelination, surviving endogenous OPCs are able to activate, proliferate, migrate to the demyelinated site and differentiate into mature oligodendrocytes to support axonal remyelination.<sup>1 2</sup> However, this process is limited in MS.<sup>3 4</sup> Therefore many treatment strategies aim to stimulate endogenous OPCs by promoting their ability to proliferate and differentiate.

Electroacupuncture (EA) has historically been used as an adjuvant therapy to treat MS.<sup>5 6</sup> We previously reported that ‘Governor Vessel’ (dorsal midline) EA (GV-EA) increased numbers of endogenous OPCs and oligodendrocytes, promoted remyelination and enhanced functional improvement in spinal cord demyelination induced by ethidium bromide (EB),<sup>2</sup> indicating that GV-EA



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stimulates endogenous OPCs. However, the molecular mechanism underlying this functional recovery has not been fully identified. A recent study reported up-regulation of retinoid acid receptor (retinoid X receptor  $\gamma$ , RXR- $\gamma$ ) expression in cells of the oligodendrocyte lineage involved in remyelination and in remyelinated MS lesions.<sup>7</sup> Blockade or activation of the RXR signalling pathway can inhibit or promote OPC differentiation and remyelination, respectively, suggesting that this signalling pathway plays a critical role in oligodendrocyte development and myelination. In light of the above, we sought to determine whether or not activation of the RXR signalling pathway is involved in GV-EA-induced stimulation of endogenous OPCs in demyelination secondary to EB.

## METHODS

### Experimental animals and surgery

A total of 78 female Sprague-Dawley rats (220–250 g) were provided by the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). The animals were reared in separate cages in a room under controlled light, relative humidity (50%) and temperature ( $20 \pm 2^\circ\text{C}$ ) conditions. All animals had free access to basic feed and tap water. The production licence (ID: SCXK2009-0011) and utilisation licence (ID: SYXK2007-0081) for the use of experimental animals were provided by the Guangdong Provincial Department of Science and Technology. Animal experiments were approved by the Experimental Animal Ethics Committee of Sun Yat-sen University (Ethic Committee of ZSSOM on Laboratory Animal Care (#2015-045) and experimental protocols complied with the ethical requirements of the Ministry of Science and Technology (People's Republic of China). All efforts were made to minimise the number of animals used and their suffering. The rats were randomly divided into three groups: (1) the Control group, which was subjected to dorsal laminectomy and exposure of the dura only; (2) the EB group, which received EB injection after dorsal laminectomy; and (3) the EB+EA group, which received EA treatment after EB injection and dorsal laminectomy. Each group was further divided into three subgroups that were euthanased at 7-day ( $n=5$  per group), 15-day ( $n=8$  per group) and 30-day ( $n=13$  per group) time points for observation.

Rats were anaesthetised using an intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg). Dorsal laminectomies were performed at the T9/T10 vertebral levels and a  $2 \times 2 \text{ mm}^2$  area of the dura was exposed.  $1 \mu\text{L}$  of EB (0.1 mg/mL) was stereotaxically injected into the dorsal funiculus (DF) of the spinal cord at T10 to a depth of 0.6–0.8 mm using a  $1 \mu\text{L}$  fixed-needle Hamilton syringe.<sup>2 8</sup> In the Control group, laminectomies and exposure of the dura were carried out without EB injection. After surgery, all

animals received intramuscular injections of penicillin (160 000 U/mL/day) and were caged separately on thick soft bedding.

### EA therapy

EA was administered every other day to rats in the EB+EA group only, starting from the third day post-surgery until the day of euthanasia (longest treatment period 26 days). EA treatment was performed at two classical GV acupuncture points, namely GV6 (*Jizhong*) and GV9 (*Zhiyang*).<sup>9 10</sup> GV6 and GV9 were located on the posterior midline in the depression below the spinous process of the 11th and seventh thoracic vertebra, respectively, in the prone position. A pair of stainless steel needles (0.35 mm diameter, 50 mm length; Jiangsu Medical Instruments Inc, China) were connected to the output terminals of an EA apparatus (model no. G 6805-2, Shanghai Medical Electronic Apparatus Company, China) and EA was applied using alternating strings of dense-sparse waves at alternating frequencies (60 Hz for 1.05 s and 2 Hz for 2.85 s, pulse width 0.5 ms). The intensity was adjusted to induce slight twitching of the hindlimbs ( $\leq 1 \text{ mA}$ ) and each treatment lasted 20 min.

### Tissue processing

Forty-five rats ( $n=5$  per time point per group) were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) under deep terminal anaesthesia using sodium pentobarbital (60 mg/kg intraperitoneally). Segments of spinal cord containing the lesioned sites in the EB group ( $n=15$ ) and the EB+EA group ( $n=15$ ) and corresponding segments in the Control group ( $n=15$ ) were collected. The tissue from the epicentre of the demyelination site was prepared and cryosectioned for subsequent histochemical and immunohistochemical analyses (see below). In an additional five animals per group at the 30-day time point only, semi-thin sections were prepared for ultrastructural analysis following toluidine staining (see below). In the remaining nine rats ( $n=3$  per group at the 15- and 30-day time points only) the tissues were harvested, dissolved and centrifuged, and the supernatant was collected and stored at  $-80^\circ\text{C}$  for subsequent Western blotting.

### Immunohistochemical staining

Sections of spinal cord from  $n=5$  animals per group per stage were post-fixed overnight in 4% paraformaldehyde, and incubated in 0.1 M phosphate buffered saline (PBS) containing 30% sucrose at  $4^\circ\text{C}$  until they sank. Transverse cryosections ( $25 \mu\text{m}$  thickness) of the spinal cord were cut and mounted on gelatin-coated slides for immunohistochemical staining, as previously described.<sup>3</sup> The primary antibodies used were mouse monoclonal anti-neural/glial antigen (NG)2 (MAB5384, 1:200, Millipore), mouse monoclonal anti-adenomatous polyposis coli (APC, OP80, 1:200, Millipore) and rabbit

polyclonal anti-RXR- $\gamma$  (sc-555, 1:100, Santa Cruz). Secondary antibodies included cyanine (Cy)3-conjugated goat anti-mouse (115-165-146, 1:300, Jackson ImmunoResearch, Inc) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit (111-095-144, 1:50, Jackson ImmunoResearch, Inc) antibodies.

RXR- $\gamma$  expression in OPCs and oligodendrocytes was determined by counting individual cells double-labelled for NG2/RXR- $\gamma$  and APC/RXR- $\gamma$ , respectively, within a fixed unit area ( $300 \times 300 \mu\text{m}^2$ ) in the epicentre of the demyelination site. Five randomly selected sections were counted in each rat per group. Only positive cells with a well-defined cell body were counted. We also investigated retinaldehyde (RA) synthesis in OPCs of the demyelinated spinal cord by double-staining for NG2 and RA dehydrogenase (RALDH)-2 using goat polyclonal anti-RALDH2 primary (1:100, Santa Cruz) and FITC-conjugated rabbit anti-goat secondary antibodies (1:50, Jackson Immunoresearch, Inc).

#### Toluidine blue staining

In five animals per group at the 30-day time point only, 3 mm thick sections of spinal cord tissues including the epicentre of the demyelinated site were cut and quickly post-fixed in a fixative solution containing 2.5% glutaraldehyde overnight at 4°C. After washing several times in PBS, the sections were post-fixed with 2% osmium tetroxide (Oxkem Limited, Reading, UK) for 1 hour at room temperature in the dark, dehydrated in a graded series of ethanol (70% ethanol for 15 min, 95% ethanol for 15 min, 100% ethanol for 15 min, repeated twice), absolute acetone (twice for 15 min), and immersed in 50% Epon812 in acetone (1:1 Epon812:acetone) for 2 hours followed by 100% Epon812 for 12 hours. Thereafter, sections were embedded individually in beam capsules and solidified at 60°C for 24 hours. Afterwards, semi-thin sections (1  $\mu\text{m}$ ) were cut and stained with toluidine blue. For ultrastructural analysis, ultra-thin sections were made, stained with lead citrate and uranyl acetate, and then observed under transmission electron microscopy (TEM; Philips CM10). For quantitative analysis, four semi-thin sections from each animal (n=5 per group) were selected and a total of 20 sections per group (Control, EB and EB+EA groups) were used. The numbers of three types of myelin sheath (normal, degenerated and newly formed (remyelinated)) were counted within a fixed unit area ( $50 \times 50 \mu\text{m}^2$ ) at  $\times 400$  magnification. Four randomly selected fields from three layers (superficial, middle and deep) along the depth of the DF from each section were counted. The investigator was blind to treatment group allocation in order to avoid bias.

#### Western blot analysis

Western blot analysis was performed in a subset of animals (n=3 per group) at the 15- and 30-day time

points only. First, total protein concentration of the stored supernatant was determined. After routine electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and non-specific binding sites were blocked. Next, membranes were incubated with rabbit anti-RXR- $\gamma$  (1:500, Santa Cruz) for nine rats in the 15-day group, anti-myelin basic protein (MBP; AB5864, 1:1000, Merck Millipore) for nine rats in the 30-day group, and mouse anti- $\beta$ -actin (A2228, 1:1000, Sigma) for rats in both groups, respectively, overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit (111-035-003) or goat anti-mouse antibody (115-035-003), 1:5000, Jackson ImmunoResearch, Inc) for 2 hours. Protein bands were visualised using an enhanced chemiluminescence (ECL) Western blot substrate kit (Pierce, Illinois, USA) and exposed on X-ray films. Intensity of the protein bands was measured and quantified with NIH Image J V1.37 (National Institutes of Health, Bethesda, Maryland, USA). The intensity values of RXR- $\gamma$  were obtained by normalising firstly against controls and secondly against  $\beta$ -actin loading ratios. All Western blot experiments were performed in triplicate.

#### Spinal cord-derived neural progenitor cell culture

E16 embryos of Sprague-Dawley rats were used to isolate and culture spinal cord-derived neural progenitor cells (NPCs) according to the method of Reynolds and Weiss with minor modifications, approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong.<sup>11 12</sup> The NPC culture medium was composed of the following reagents from Invitrogen (1 $\times$ Dulbecco's modified Eagle's medium/F12, 1% N2, 2% B27, 0.06% glucose, 2 mM/L glutamine and 20 ng/mL basic fibroblast growth factor (bFGF)) and Sigma (1.34 mM/L sodium bicarbonate, 0.5 mM/L N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid (HEPES), 2  $\mu\text{g}/\text{mL}$  heparin, freshly supplemented with 20 ng/mL epidermal growth factor (EGF)).

#### Induction and passage of OPC culture

To induce OPCs, freshly dissociated NPCs were seeded at  $1 \times 10^5$  cells/mL in NPC medium for 1 or 2 days to stimulate the development of small neurospheres, before induction and passage in OPC medium according to the method of Fu *et al.*<sup>13</sup> The OPC medium contained NPC medium (without bFGF/EGF) that was freshly supplemented with 0.1% bovine serum albumin (Amresco, Solon, Ohio, USA), 10 ng/mL biotin (Sigma), 10 ng/mL recombinant rat platelet-derived growth factor (PDGF-AA, R&D, Minneapolis, MN, USA) and 15 ng/mL bFGF (Invitrogen). To examine the purity of the OPCs, the cells were fixed for immunohistochemical staining.

### Effect of RXR- $\gamma$ signalling pathway on differentiation of spinal cord OPCs in vitro

The OPCs derived from rat E16 spinal cord were seeded onto 200  $\mu\text{g}/\text{mL}$  PLO (poly-L-ornithine)-coated coverslips at a density of  $1 \times 10^5$  cells/slip. For the differentiation of OPCs, PDGF/bFGF was withdrawn from the OPC medium. After this, 1% fetal bovine serum (FBS) and variable concentrations (4  $\mu\text{M}$ , 2  $\mu\text{M}$ , 1  $\mu\text{M}$  or 0.1  $\mu\text{M}$ ) of HX531 (3912, Tocris Bioscience) were added, respectively, to the medium for the first 2 days to inhibit differentiation. Then, 50 nM 9-cis-RA (R4643, Sigma) was added to the OPC culture for another 3 days to promote the differentiation of OPCs into oligodendrocytes. At the end of differentiation, cells were fixed for immunocytochemical staining. Antibodies against NG2 (MAB5384, Millipore), receptor interaction protein (RIP; 1:50, gifted by Dr X M Xu, Indiana University School of Medicine, Indianapolis, IN, USA) and RXR- $\gamma$  were used to identify respective cell types in the OPC culture. The OPC culture was divided into seven groups (namely Control, DMSO (dimethyl sulfoxide), 50 nM 9cRA, 0.1  $\mu\text{M}$  HX531 +50 nM 9cRA, 1  $\mu\text{M}$  HX531+50 nM 9cRA, 2  $\mu\text{M}$  HX531+50 nM 9cRA, and 4  $\mu\text{M}$  HX531+50 nM 9cRA groups). Three wells were used for each group, and for each well five randomly selected fields at  $\times 400$  magnification were used for enumeration of double positive cells.

### Statistical analysis

All data are represented as mean  $\pm$  SD. One-way analysis of variance (ANOVA) or repeated-measure ANOVA was used to test differences among groups. If equal variances were verified, post-hoc tests of least-significant difference (LSD) were applied; otherwise, the Tamhane's T2 test was used. Significance levels were set at  $p < 0.05$ .

## RESULTS

### EA increased the number of endogenous OPCs expressing RXR- $\gamma$ and RALDH2 in demyelinated spinal cord

By NG2/RXR- $\gamma$  double immunofluorescence labelling, some OPCs in the DF of the spinal cord in the Control group exhibited RXR- $\gamma$  expression (figure 1A1–A3). The number of RXR- $\gamma$  positive OPCs appeared to be increased in the EB group (figure 1B1–B3), although the difference was not statistically significant. However, EA treatment promoted the apparent increase, and at 7-day post-surgery significantly more NG2<sup>+</sup>/RXR- $\gamma$ <sup>+</sup> OPCs were observed in the lesioned area in the EB+EA group than in the Control or EB groups (figure 1C1–C3 and D). With the passage of time, the number of RXR- $\gamma$ <sup>+</sup> OPCs was further increased in both the EB and EB+EA groups at 15 days postoperatively ( $p = 0.001$  and  $p < 0.001$ , respectively, vs Control), and the incidence of RXR- $\gamma$ <sup>+</sup> OPCs in the EB+EA group was comparatively higher than the EB group ( $p = 0.029$ ). By 30 days post-surgery, although

the overall number of RXR- $\gamma$ <sup>+</sup> OPCs in the EB+EA and EB groups was lower, it remained at a higher level than the Control group ( $p = 0.045$  and  $p < 0.001$ , respectively). The EB+EA group showed the highest incidence of RXR- $\gamma$ <sup>+</sup> OPCs. Similar results were observed for quantitative analysis of RXR- $\gamma$  expression by Western blotting in the demyelinated spinal cord at the 15-days postoperative time point; the EB+EA group displayed a significantly higher level of RXR- $\gamma$  expression than the Control or EB groups (figure 1E). Overall the results indicate that EA may promote an early increase in the number of endogenous OPCs expressing RXR- $\gamma$ .

Figure 2A–C shows the results of double staining for NG2/RALDH2. In the DF of the spinal cord from the Control group, RALDH2 expression in NG2<sup>+</sup> OPCs was weak (figure 2A1–A3). However, in both the EB group (figure 2B1–B3) and the EB+EA group (figure 2C1–C3), expression of RALDH2 was notably increased in NG2<sup>+</sup> OPCs. Quantitative analysis showed that there were more NG2<sup>+</sup>/RALDH2<sup>+</sup> double-labelled OPCs per unit area of demyelinated DF in the EB and EB+EA groups than the equivalent region in the Control group at 7, 15 and 30 days after surgery, respectively (figure 2D). Moreover, at 15 days postoperatively, the number of NG2<sup>+</sup>/RALDH2<sup>+</sup> double-labelled OPCs in the EB+EA group was significantly higher than that in the EB group ( $p = 0.007$ ), consistent with our observation that RXR- $\gamma$  expression in the EB+EA group was maximal at 15 days post-surgery and significantly increased compared with the EB group (figure 1). These results suggest that EA may enhance the RXR- $\gamma$  signalling pathway in OPCs by both increasing the expression of RA receptor RXR- $\gamma$  and promoting RA synthesis by elevating RALDH2 levels.

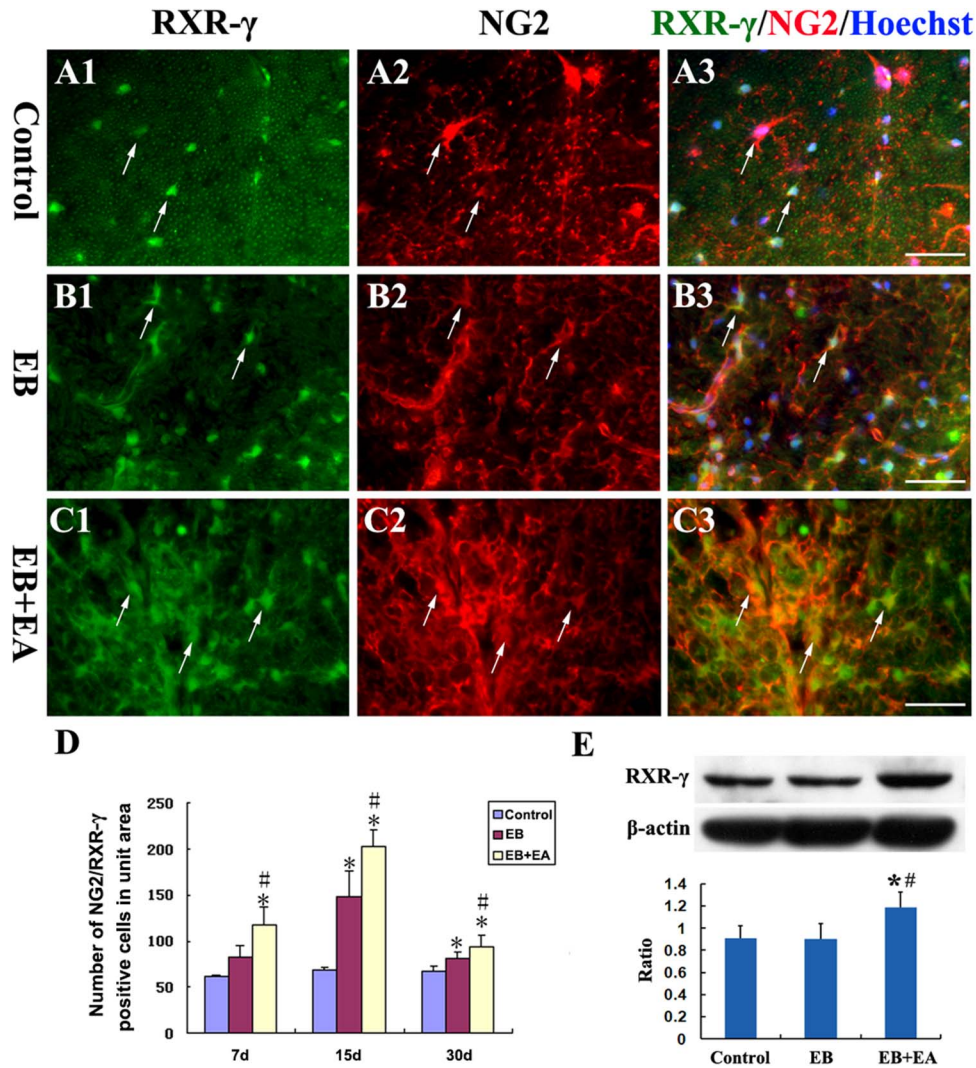
### EA promoted the number of oligodendrocytes expressing RXR- $\gamma$ in the demyelinated spinal cord

APC<sup>+</sup> cells in the DF of the spinal cord expressed RXR- $\gamma$  in the Control group (figure 3A1–A3). However, the number of APC<sup>+</sup>/RXR- $\gamma$ <sup>+</sup> cells in the demyelinated DF of the spinal cord from the EB and EB+EA groups was significantly increased in comparison with the Control group at all time points (figure 3B1–B3, C1–C3 and D). There were no significant differences between the EB+EA and EB groups at 7 or 30 days post-surgery, but the number of APC<sup>+</sup>/RXR- $\gamma$ <sup>+</sup> cells in the EB+EA group was higher than that in the EB group at 15 days post-surgery (figure 3D;  $p < 0.001$ ), suggesting that EA may increase the intermediate number of endogenous oligodendrocytes expressing RXR- $\gamma$ .

### EA promotes remyelination of the demyelinated spinal cord

Figure 4 shows the results from the toluidine blue staining and TEM. Thirty days post-surgery, the number of myelinated axons decreased significantly in





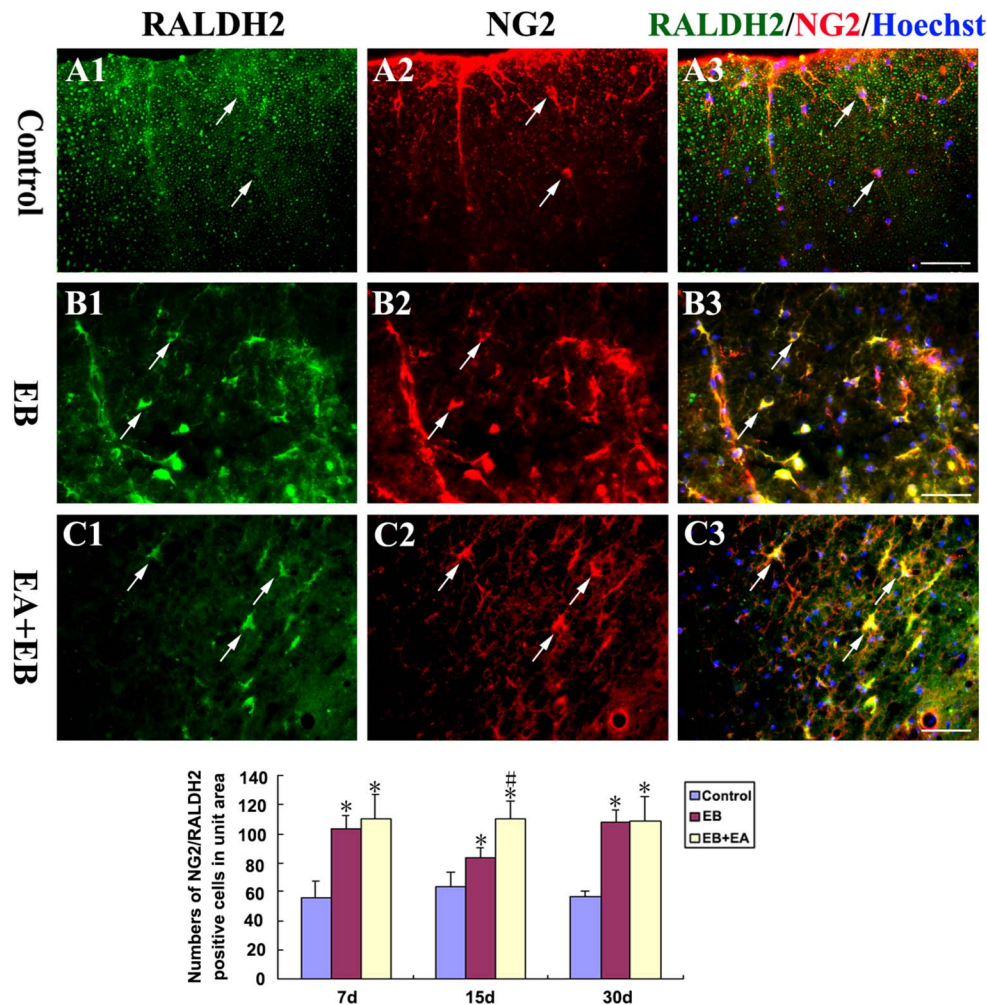
**Figure 1** Representative immunofluorescence images (A–C), showing (left to right) staining for retinoid X receptor  $\gamma$  (RXR)- $\gamma$  (green), neural/glia antigen (NG2) (red) and nuclei using Hoechst solution (blue), and numbers of NG2<sup>+</sup>/RXR- $\gamma$ <sup>+</sup> double-positive oligodendrocyte precursor cells (OPCs) per unit area ( $300 \times 300 \mu\text{m}^2$ ) (D), in the dorsal funiculus of the spinal cord of rats subjected to dorsal laminectomy and dural exposure only (Control group,  $n=15$ ) or dorsal laminectomy followed by ethidium bromide (EB) injection that remained untreated (EB group,  $n=15$ ) or received electroacupuncture (EB+EA group,  $n=15$ ) before euthanasia at 7, 15 or 30-day time points ( $n=5$  per group per stage). (E) Representative gels and quantification of protein expression of RXR- $\gamma$ , measured by Western blotting and expressed relative to  $\beta$ -actin, in an additional three animals per group at the 15-day stage only. Scale bar=50  $\mu\text{m}$ . \* $p < 0.05$  vs Control group; # $p < 0.05$  vs EB group.

both the EB and EB+EA groups compared with the Control group (figure 4A1–A2, B1–B2, C1–C3 and E;  $p < 0.001$ ). However, there were a greater number of myelinated and remyelinated axons in the EB+EA group (figure 4C1–C3 and E;  $p = 0.009$ ). Ultrastructural analysis using TEM also showed similar results. Normal myelinated axons with thick myelin sheaths were widely distributed in the Control group (figure 4A3), but notably lower in number in the EB+EA group and rare in the EB group. Tissues of the EB group featured large areas of naked axons in regions of demyelination, displaying a typical onion-like appearance with disorganised, loose myelin lamellae (figure 4B3). A greater number of remyelinated axons with thinner myelin sheaths relative to normal sheaths were observed in the EB+EA group

(figure 4C3). Regenerating myelin was noted to be thin and formed by oligodendrocytes that touched and wrapped around the axons (figure 4D1–D2). The inner and outer ends of the spiralling cytoplasmic processes of oligodendrocytes were evident (figure 4D2). MBP protein expression at 30 days, measured using Western blotting, was increased by EA treatment after injury to a level comparable to that in the EB group (figure 4F;  $p = 0.007$ ). Overall, these results suggest that EA treatment promotes endogenous remyelination in the demyelinated spinal cord.

#### RA enhanced OPCs to differentiate into oligodendrocytes in vitro

Figure 5A1–A2 shows NPCs expressing nestin derived from E16 embryonic rat spinal cord. Successful

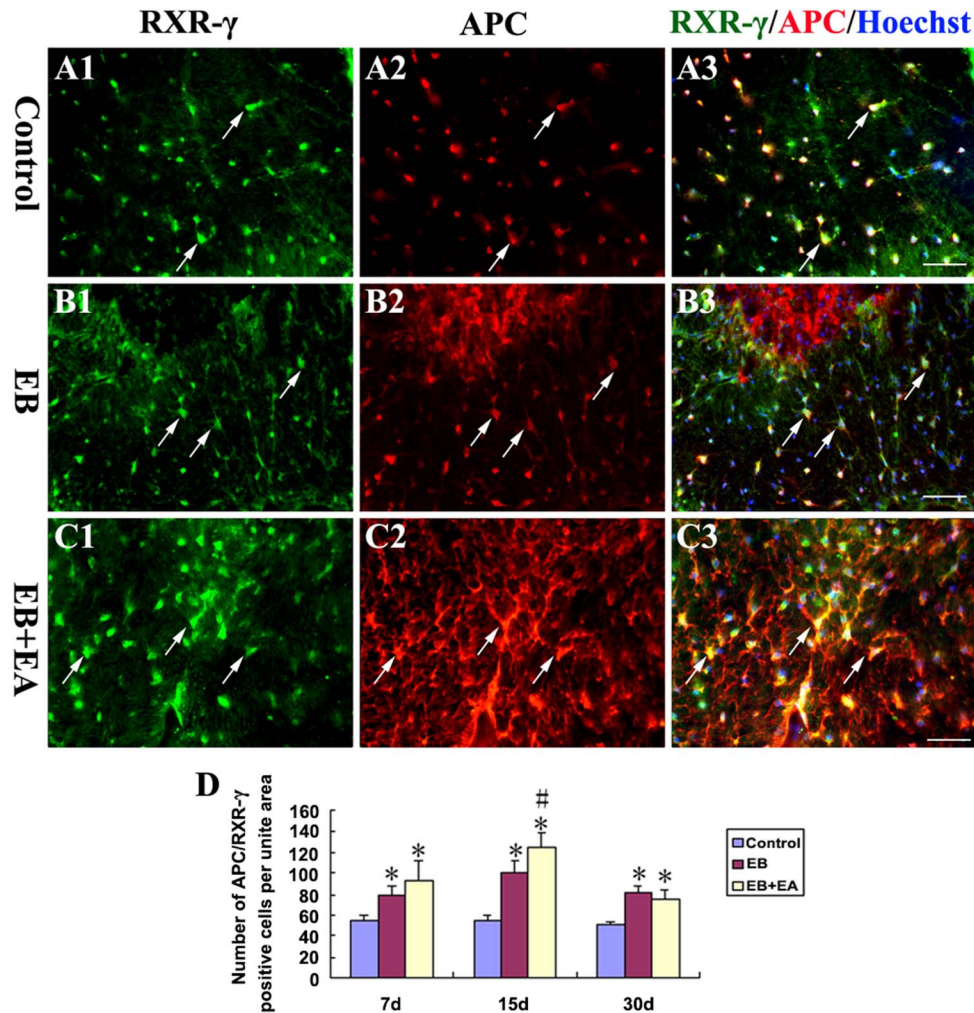


**Figure 2** Representative immunofluorescence images (A–C), showing (left to right) staining for retinaldehyde dehydrogenase (RALDH)2 (green), neural/glial antigen (NG)2 (red) and nuclei using Hoechst solution (blue), and numbers of NG2/RALDH2<sup>+</sup> double-positive oligodendrocyte precursor cells (OPCs) per unit area (300×300 μm<sup>2</sup>) (D), in the dorsal funiculus of the spinal cord of rats subjected to dorsal laminectomy and dural exposure only (Control group, n=15) or dorsal laminectomy followed by ethidium bromide (EB) injection that remained untreated (EB group, n=15) or received electroacupuncture (EB+EA group, n=15) before euthanasia at 7, 15 or 30-day time points (n=5 per group per stage). Scale bar=50 μm. \*p<0.05 vs Control group; #p<0.05 vs EB group.

establishment of a pure OPC culture was verified by the multipolar phenotype as well as co-labelling of Olig2 and NG2 (figure 5B and C1–C3). The purity of the OPCs was approximately 83.94±1.71% (cell count based on NG2 expression). Before differentiation, nearly all NG2 positive OPCs expressed RXR-γ (figure 5D1–D3). After switching the differentiation media, OPCs started to differentiate. Figure 5E1–E4 illustrates the inhibitory effects on OPC differentiation of adding 1% FBS containing basal OPC medium plus different concentrations of HX531 (4, 2, 1 and 0.1 μM, respectively) to the OPC cultures over 2 days, as well as the effects of adding 50 nM 9-cis-RA (a RXR-γ ligand) for another 3 days to promote the differentiation of OPCs into oligodendrocytes. A greater proportion of differentiated oligodendrocytes were observed when 50 nM 9-cis-RA was added, when compared with the Control and DMSO groups (26.28

±5.73% vs 18.64±4.21% and 19.87±3.91%, respectively; p=0.022 and p=0.049, figure 5E1–E2 and F). The effect of 9-cis-RA in promoting differentiation of OPCs into mature oligodendrocytes was significantly inhibited by HX531 (a RXR-γ antagonist) in a concentration-dependent manner (figure 5E3–E4 and F). A low dose of HX531 at 0.1 μM concentration was sufficient to abrogate the stimulatory effect of 9-cis-RA on OPC differentiation to a level comparable to the Control group (figure 5F). HX531 at a higher concentration (4 μM) was found to reduce the percentage of differentiated oligodendrocytes when compared with the Control group (figure 5F). Overall, the results indicate that 9-cis-RA promotes the differentiation of OPCs into mature oligodendrocytes mainly by activating the RXR signal pathway in vitro. In addition, RXR-γ was expressed in RIP<sup>+</sup> oligodendrocytes (figure 5G).





**Figure 3** Representative immunofluorescence images (A–C), showing (left to right) staining for retinoid X receptor  $\gamma$  (RXR)- $\gamma$  (green), adenomatous polyposis coli (APC; red) and nuclei using Hoechst solution (blue), and numbers of APC<sup>+</sup>/RXR- $\gamma$ <sup>+</sup> double-positive oligodendrocytes per unit area ( $300 \times 300 \mu\text{m}^2$ ) (D), in the dorsal funiculus of the spinal cord of rats subjected to dorsal laminectomy and dural exposure only (Control group,  $n=15$ ) or dorsal laminectomy followed by ethidium bromide (EB) injection that remained untreated (EB group,  $n=15$ ) or received electroacupuncture (EB+EA group,  $n=15$ ) before euthanasia at 7, 15 or 30-day time points ( $n=5$  per group per stage). Scale bar= $50 \mu\text{m}$ . \* $p < 0.05$  vs Control group; # $p < 0.05$  vs EB group.

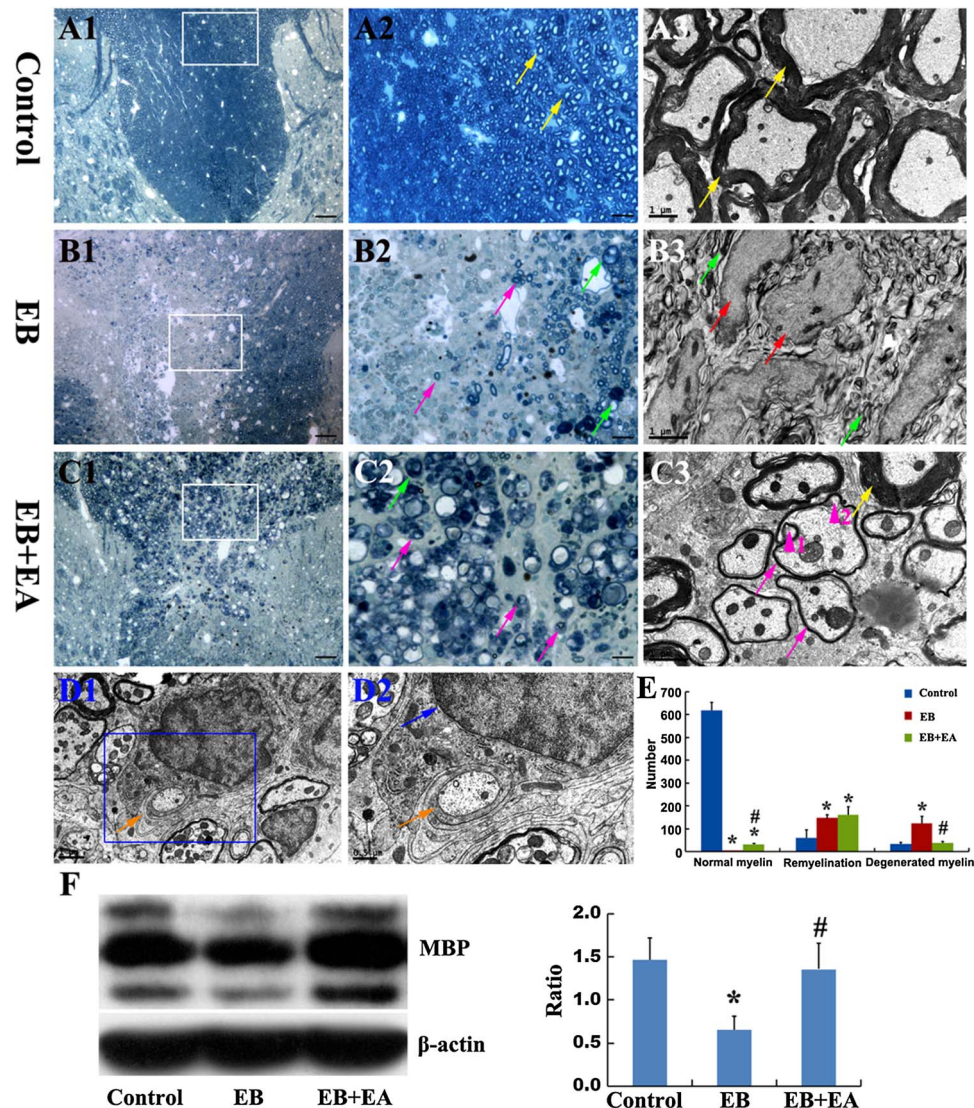
## DISCUSSION

The results of the present study have shown that EA induces RXR- $\gamma$  expression and increases the number of OPCs and oligodendrocytes expressing RXR- $\gamma$  in the demyelinated DF of the spinal cord induced by EB administration in rats. Furthermore, in vitro experiments using HX531 and 9-cis-RA indicate that exogenous 9-cis-RA promotes cultured OPCs to differentiate into oligodendrocytes through the RXR- $\gamma$  pathway.

As far as can be ascertained, this is the first report of the promotion of RXR- $\gamma$  expression by EA treatment in the demyelinated spinal cord. However, the underlying mechanism of action remains uncertain. It has been reported that EA increases the expression of RXR in rat liver,<sup>14</sup> indicating that common mechanisms of stimulation may exist across different cell types. RXRs are nuclear receptors that regulate cell proliferation and differentiation.<sup>15 16</sup> Huang *et al*<sup>7</sup>

recently found that RXR- $\gamma$  was highly expressed in cells of oligodendrocyte lineage during the regenerative phase of CNS remyelination; also, activation of RXR stimulated oligodendrocyte differentiation to enhance remyelination. Likewise, herein we showed that the number of oligodendrocyte lineage cells expressing RXR- $\gamma$  in the demyelinated DF of the spinal cord increased after EA treatment, suggesting that promotion of remyelination by EA may involve the RXR signalling pathway.

The activation of the RXR signalling pathway involves RXR and its ligand RA. RA activity is mainly regulated by the local activity of RA-synthesising enzymes including RALDH2. RALDH2 has been identified as a crucial enzyme for RA synthesis.<sup>17 18</sup> In the present study, EA increased the number of RALDH2<sup>+</sup>/NG2<sup>+</sup> double-labelled OPCs in the demyelinated spinal cord, indicating that EA may also promote



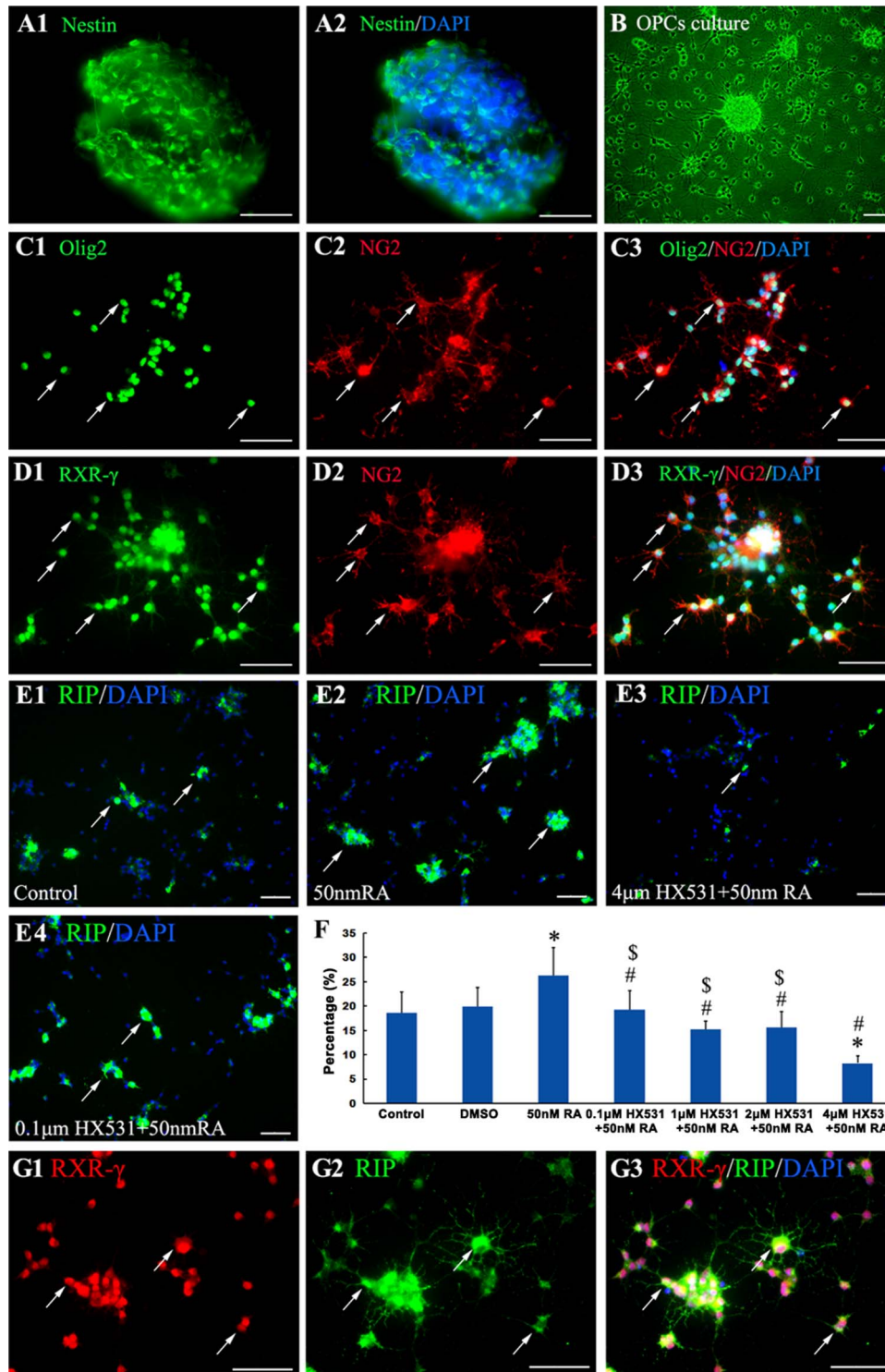
**Figure 4** Representative toluidine blue stained images of semi-thin sections of spinal cord (A1–C1 and A2–C2), transmission quantitative analysis of three different types of myelination (normal, degenerated and newly formed (remyelinated)) (E, n=5 per group), plus representative Western blots and quantitative protein expression of myelin basic protein (MBP), measured by Western blotting and expressed relative to  $\beta$ -actin (F, n=3 per group), in rats subjected to dorsal laminectomy and dural exposure only (Control group, n=8) or dorsal laminectomy followed by ethidium bromide (EB) injection that remained untreated (EB group, n=8) or received electroacupuncture (EB+EA group, n=8) before euthanasia at 30 days. A3–C3 and D1–D2 are representative images of transmission electron microscopy. The insert box in D1 is zoomed in showing a higher magnification in D2. The yellow, green, red and pink arrows indicate normal myelin sheaths, degenerated myelin, naked axons and newly formed myelin (remyelination), respectively, in the demyelinated white matter regions. Pink arrowheads 1 and 2 indicate the inner and outer ends, respectively, of the spiralling cytoplasmic processes of oligodendrocytes (characteristic features of central nervous system (CNS)-type remyelination). The blue arrow shows an oligodendrocyte spiralling and wrapping around an axon to form a myelin sheath (orange arrow) in the EB+EA group. (A2–C2) (scale bar 20  $\mu$ m) are magnifications of the insets in A1–C1 (scale bar 80  $\mu$ m). \* $p$ <0.05 vs Control group. # $p$ <0.05 vs EB group.

synthesis of RA by OPCs. Previous reports have characterised RALDH2<sup>+</sup>/NG2<sup>+</sup> cells as a unique RA-synthesising subpopulation of activated oligodendrocyte precursors.<sup>17 18</sup> Moreover, Hong *et al*<sup>19</sup> showed that EA increases mRNA and protein expression of RA in rats following ischaemia-reperfusion injury. To demonstrate directly that RA can promote the differentiation of OPCs into oligodendrocytes by activating the RA/RXR- $\gamma$  signalling pathway, we

investigated the role of 9-cis-RA and HX531 on the differentiation of OPCs derived from rat embryonic spinal cord. The results demonstrated that 9-cis-RA promoted the differentiation of OPCs into oligodendrocytes by activating RXR- $\gamma$ . Our results are consistent with those of Huang *et al*,<sup>7</sup> who used OPCs derived from rat neonatal cerebral tissue.

It is unclear, however, exactly how the RXR signalling pathway exerts an influence on the differentiation and





**Figure 5** Culture and induction of oligodendrocyte precursor cells (OPC) differentiation through the retinoid X receptor  $\gamma$  (RXR- $\gamma$ ) signalling pathway in vitro. Neural progenitor cells (NPCs) were detected by nestin immunofluorescence staining (A1–A2) and induced into OPCs with a typical bipolar or tripolar morphology (B). OPCs were identified by Olig2 (green) and neural/glial antigen (NG)2 (red) immunofluorescence staining (C1–C3). RXR- $\gamma$  protein (green) was expressed in NG2<sup>+</sup>OPCs (red) (D1–D3). The nucleus was stained blue by DAPI (4',6-diamidino-2-phenylindole). Receptor interaction protein (RIP)<sup>+</sup> oligodendrocytes (green) in the Control (E1), 50 nM-RA (E2), 4  $\mu$ M HX531+50 nM-RA (E3) and 0.1  $\mu$ M HX531+50 nM-RA (E4) groups, were counterstained by DAPI (blue). Scale bar=50  $\mu$ m. (F) The percentage of RIP<sup>+</sup> oligodendrocytes in DAPI<sup>+</sup> cells counted in each of the seven different groups (see Methods). (G) RXR- $\gamma$  (green) was expressed in RIP<sup>+</sup> oligodendrocytes (red) induced by 9cRA. \* $p$ <0.05 vs Control or DMSO group. # $p$ <0.05 vs 50 nM-RA group. \$ $p$ <0.05 vs 4  $\mu$ M HX531+50 nM-RA group.

myelination of the cells of the oligodendrocyte lineage. Pombo *et al*<sup>20</sup> reported that 9-cis-RA-dependent activation of the MBP promoter required the presence of DNA binding and the ligand-dependent transactivation domain AF-2 in RXR *in vitro*. Furthermore, 9-cis-RA stimulated MBP expression in the CG-4 cell line after differentiation to oligodendrocytes and increased the number of cells expressing MBP protein in optic nerve glial cell cultures. Recently, Huang *et al*<sup>7</sup> generated a comprehensive transcriptional profile in separate stages of spontaneous remyelination following focal demyelination in the rat CNS and found that transcripts encoding RXR- $\gamma$  were differentially expressed during remyelination.

Endogenous OPC differentiation and remyelination are activated through the RA/RXR signalling pathway in the demyelinated spinal cord, but spontaneous restoration is limited in MS.<sup>3 4</sup> In our study, we also found that EA increased the quantity of normal myelin and expression of MBP protein, and decreased the amount of degenerated myelin, which is consistent with our previous report.<sup>2</sup>

## CONCLUSIONS

The results of the *in vivo* and *in vitro* experiments of the present study suggest that EA promotes OPCs to differentiate into oligodendrocytes through a mechanism involving activation of the RXR signalling pathway of OPCs. Thus, EA would be expected to prevent inhibition of spontaneous remyelination and to accelerate the differentiation of mature OPCs. These findings suggest that EA may represent a promising therapeutic strategy and that the RXR signalling pathway may be a potential drug target in regenerative therapy for demyelinating disorders.

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