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ORIGINAL ARTICLE

Ethyl Pyruvate–Derived Transdifferentiation of Astrocytes to Oligodendrogenesis in Cuprizone-Induced Demyelinating Model

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Abstract

Astrocytes redifferentiate into oligodendrogenesis, raising the possibility that astrocytes may be a potential target in the treatment of adult demyelinated lesion. Upon the basis of the improvement of behavior abnormality and demyelination by ethyl pyruvate (EP) treatment, we further explored whether EP affects the function of astrocytes, especially the transdifferentiation of astrocytes into oligodendrogenesis. The results showed that EP treatment increased the accumulation of astrocytes in myelin sheath and promoted the phagocytosis of myelin debris by astrocytes *in vivo* and *in vitro*. At the same time, EP treatment induced astrocytes to upregulate the expression of CNTF and BDNF in the corpus callosum and striatum as well as cultured astrocytes, accompanied by increased expression of nestin, Sox2, and β-catenin and decreased expression of Notch1 by astrocytes. As a result, EP treatment effectively promoted the generation of $NG2^+$ and PDGF-Ra^+ oligodendrocyte precursor cells (OPCs) that, in part, express astrocyte marker GFAP. Further confirmation was performed by intracerebral injection of primary astrocytes labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). As expected, $NG2^+$ OPCs expressing CFSE and Sox2 were elevated in the corpus callosum of mice treated with EP following transplantation, revealing that EP can convert astrocytes into myelinating cells. Our results indicate the possibility that EP lead to effective myelin repair in patients suffering from myelination deficit.

Key Words Ethyl pyruvate · cuprizone-induced demyelination · astrocytes · transdifferentiation · oligodendrogenesis

Highlights

1. EP increased the accumulation of astrocytes in myelin sheath and promoted the phagocytosis of myelin debris by astrocytes.
2. EP induced astrocytes to upregulate the expression of CNTF and BDNF in the corpus callosum and striatum.
3. EP increased the expression of nestin, Sox2, and β-catenin and decreased expression of Notch1 by astrocytes.
4. As a result, EP effectively promoted the generation of $NG2^+$ and PDGF-Ra^+ oligodendrocyte precursor cells (OPCs) that were, in part, derived from transdifferentiation of astrocytes to oligodendrogenesis.

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Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS), characterized by oligodendrocyte loss, demyelinated plaques, and axonal degeneration. In the brain, chronic inflammation leads to axonal myelin sheath destruction and the progressive loss of neurological functions with neuronal death. In white matter lesions of MS, oligodendrocyte precursor cells (OPCs) present a stop state and a nondifferentiation into myelinating oligodendrocytes [1–3]. However, detailed cellular and molecular mechanism leading to oligodendrocyte loss and consequently demyelination is not fully elucidated. Cuprizone (CPZ)-induced demyelination, unlike experimental autoimmune encephalomyelitis (EAE), is independent of autoimmune attack and is also often used to mimic the pathology of human MS [4, 5]. Several similarities of histopathological changes between CPZ-induced model and postmortem MS brain have been observed. For instance, the density of mitochondria within demyelinated axons was increased in active MS lesions [6] and cuprizone-induced white matter lesions [7]. In addition, preapoptotic oligodendrocytes expressed active caspase-3 during the process of pathological lesions in MS [8] and CPZ-induced demyelination [9].

Currently, disease-modifying therapy (DMT) is widely accepted as an option in the treatment of relapsing-remitting MS, but its efficacy is still limited for the progressive MS [10, 11]. Besides, DMT does not target demyelination and neurodegeneration, which are two very important pathological manifestations. Therefore, the direct challenge for MS treatment will focus on how to target neuroprotection and remyelination [12]. Future MS treatment paradigms, therefore, are likely to comprise a combinatorial approach that involves both immunomodulatory and regenerative treatments. Current approaches to remyelination include 1) blocking inhibitors of remyelination, 2) improving the clearance of myelin debris, 3) increasing the number of OPCs, and 4) stimulating OPC differentiation. To date, no therapies have led to robust remyelination [13]. In the future, it is, however, imaginable that those treatments might be effective, for example, in an early stage of progression and, therefore, be applicable to a certain subgroup of progressive MS patients [14]. CPZ-induced demyelination provides a good experimental model to study demyelination and remyelination and is a suitable pharmacological model for developing novel promising drugs for the remyelination and/or neuroprotection.

Astrocytes are the most abundant glial cells in white matter and gray matter of the CNS and have a crucial role in participating in synaptogenesis, mediating the immune response, and promoting cell migration, differentiation, and maturation of the CNS [15, 16]. Astrocytes directly affect the proliferation and survival of the oligodendrocyte line [17], suggesting that astrocytes are strongly associated with remyelination. Activated astrocytes could dedifferentiate into OPCs and redifferentiate into mature oligodendrocytes, raising the possibility that astrocytes may be a potential source of OPCs in the adult demyelinated spinal cord [18]. A recent study showed that testosterone promoted oligodendrocyte remyelination via astrocyte recruitment [7]. In addition, astrocytes also promote oligodendrogenesis after white matter damage by producing neurotrophic factors [19, 20]. Therefore, targeting astrocytes has become an attractive direction in the treatment of demyelinating diseases [21].

Ethyl pyruvate (EP), a simple derivative of the endogenous energy substrate pyruvate, provides strong anti-inflammatory and antioxidative properties [22]. In contrast to pyruvate, EP yielded a higher potency, probably due to its lipophilic properties and significant stability *in vivo* [22, 23]. Despite the existence of chemical structural similarity, differential pharmacological effects have been observed when EP and pyruvate have been compared head-to-head in models of inflammation and/or redox stress. Besides the anti-inflammatory effect, treatment with EP improved survival and/or ameliorated organ dysfunction in a wide variety of experimental models, including stroke [24], spinal cord injury [25], and Parkinson's disease [26]. In addition, EP was able to provide potent protection after white matter injury through blocking the inflammatory responses and modulating the apoptosis of oligodendrocytes [27]. Our previous study clearly indicate that EP treatment improved behavioral dysfunction and demyelinating lesion and promoted M2 polarization of microglia and phagocytosis of myelin debris in CPZ-induced model [28]. So far, we still know very little about whether and how EP influences astrocytes for the myelin protection and/or regeneration in CPZ-induced demyelinating model. In this study, we try to explore the potential effect of EP targeting astrocytes for the myelin protection and regeneration.

Methods and Materials

Animals and Drug

Male C57BL/6 mice (10–12 weeks old) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The study was approved by the Council for Laboratory and Ethics Committee of Shanxi University of Chinese Medicine, Taiyuan, China. The experimental protocol was performed according to the International Council for Laboratory Animal Science guidelines. Mice were maintained and housed under pathogen-free conditions in a reversed 12:12-h light/dark cycle for 1 week prior to experimental manipulation. EP was purchased from Sigma-Aldrich (California, USA).

Cuprizone-Induced Demyelination Model

Mice were fed with 0.2% (w/w) CPZ (Sigma-Aldrich, USA) in chow diet *ad libitum* for a total of 6 weeks. Mice were

randomly divided into three groups ($n = 8$ per group) as follows: 1) normal group, healthy mice fed a normal diet; 2) normal + EP group, healthy mice injected with EP for 14 days; 3) CPZ group, fed CPZ for 6 weeks, and intraperitoneally injected with normal saline during the 4th to 6th weeks of CPZ feeding; and 4) EP-treated group, fed CPZ for 6 weeks, and intraperitoneally injected with EP (20 mg/kg) during the 4th to 6th weeks of CPZ feeding. For the control of CPZ-containing diet intake, both body weights and food consumption were closely monitored twice daily. Since there was not any difference about behavior and pathology between control group and control + EP group, the following studies were limited to control group, CPZ group, and EP-treated group.

Tissue Preparation

Half of the mice were deeply anesthetized with 10% chloral hydrate and perfused intracardially with saline, followed by 4% paraformaldehyde in PBS (0.01 M, pH = 7.4). Brains were removed and cryoprotected by immersion in 15%, 25%, and 30% sucrose solutions 1 day for each concentration. Brain coronal sections (10 μm) were cut using a cryostat microtome (Leica CM1850, USA) for immunofluorescence staining. Another half of the mice were deeply anesthetized with 10% chloral hydrate and perfused intracardially with saline, without 4% paraformaldehyde. Brains were removed and quickly stored at − 80 °C for ELISA and Western tests.

Immunohistochemistry Staining

Briefly, the slides of brain (10 μm) and cells (astrocytes) were blocked with 1% BSA (Sigma, USA) at room temperature (RT) for 30 min for blocking nonspecific binding. The slides of brain were added with the following antibodies: anti-GFAP (Abcam, USA), anti-MBP (Abcam, USA), anti-BDNF (Abcam, USA), anti-CNTF (Abcam, USA), anti-nestin (Abcam, USA), anti-sox2 (Abcam, USA), anti-β-catenin (Sigma, USA), anti-notch1 (Abcam, USA), anti-NG2 (Millipore, USA), anti-PDGF-Ra (R & D, USA) at 4 °C overnight, and followed by corresponding secondary antibodies at RT for 2 h. Meanwhile, additional sections were treated similarly, but the primary antibodies were omitted as a negative control. Results were visualized under fluorescent microscopy (BX60, Olympus Imaging America Inc., USA) in a blinded fashion. Quantification was performed by Image-Pro Plus 6.0 software for three sections per mouse.

Primary Astrocytes

Primary astrocytes were prepared from postnatal (1 day old) C57BL6 mice. Under aseptic conditions, cerebral cortices were dissected and placed in sterile phenol red free Dulbecco's modified Eagle's medium (DMEM, 4500 mg/l glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, Thermo Scientific) containing 1% of streptomycin (10,000 μg/ml)-penicillin (10,000 units/ml). The cells were dissociated into a single cell suspension with a Pasteur pipette and then seeded in 75-cm flask culture plates (Corning, USA) at a density of 2.5×10^5 cells/well in a humidified incubator at 37 °C and 5% CO_2. When mixed glial cultures reached 90% confluence (typically 8–10 days), the microglia in the cultures was removed by shaking constantly for 6 h. The above process was repeated at least twice until GFAP immunocytochemical staining showed that the purity of astrocytes was close to 100%.

CFSE Labeling of Astrocytes

Primary astrocytes were resuspended with a sterile PBS and labeled in 50 μM carboxyfluorescein succinimidyl ester (CFSE) solution for 30 min at RT protected from light. After centrifugation at 14,800*g* for 10 min at 4 °C, the supernatant was discarded and pellet was washed with PBS for three times. After the final wash, CFSE-labeled astrocytes were resuspend with sterile PBS.

In Vitro Phagocytosis of Myelin Debris by Astrocytes

Primary astrocytes (1×10^5/well) were plated in 12-well plates and incubated with CFSE-conjugated myelin debris (5 mg/ml) in the presence and absence of EP (30 μg/ml) for 24 h at 37 °C with 5% CO_2. Unphagocytosed myelin debris was washed out, and the capacity of phagocytosis was measured under fluorescence microscopy and by multifunctional Synergy H1 Hybrid Reader (BioTek, USA) using fluorescence 485 excited light.

In Vitro Expression of Neurotrophic Factors by Astrocytes

Primary astrocytes (2×10^5/well) were plated in 6-well plates and treated with EP (30 μg/ml) for 24 h at 37 °C with 5% CO_2. The cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS for 3 times, then anti-CNTF, anti-BDNF, and anti-bFGF were added overnight at 4 °C. Negative control used PBS to replace the first antibody. The next day, after washing with PBS, the corresponding second antibody was added at RT for 2 h. Results were visualized under fluorescent microscopy (BX60, Olympus Imaging America Inc., USA) in a blinded fashion. Quantification was performed by Image-Pro Plus 6.0 software.

In Vivo Cell Transdifferentiation Assay

CFSE-labeled astrocytes (200,000 cells in 2 μl PBS) were injected into the corpus callosum of CPZ-induced demyelinated mouse brain. In brief, a burr hole was made

and a 30-gauge needle was inserted through the burr hole into the corpus callosum (coordinates: − 1.06 mm posterior, 1.8 mm ventral, and 0.6 mm lateral to the bregma) (Fig. 9b). Injection was performed during 5 min and the needle was kept in place for additional 5 min before removal. Each mouse received daily CPZ feeding and EP injection (20 mg/kg, intraperitoneally) for consecutive 14 days. Animals were sacrificed for immunohistofluorescence at 2 weeks after cell transplantation. All analyses were performed by a blinded observer.

Statistical Analysis

Data were expressed as the mean ± SEM (standard error of mean). For parametric data, comparison of different groups was performed by one-way analysis of variance, followed by Tukey's post hoc test for multiple comparisons. The statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

Results

EP Improves Behavioral Dysfunction and Demyelinating Lesion

Our previous results demonstrated that anxiety- and depression-like behavior and cognitive impairment were observed in CPZ-induced demyelinating mode, while EP treatment improved these behavioral abnormalities as compared with CPZ group [28].

Histologically, CPZ-fed mice had a marked demyelinating lesion in the corpus callosum, while EP treatment increased the intensity of myelin staining by Black Gold II, as compared to CPZ-fed mice, indicating that CPZ-fed mice showed severe demyelination, which was significantly improved by EP treatment in CPZ-induced demyelinating model [28].

EP Enhances the Enrichment and Phagocytosis of Myelin Debris by Astrocytes

Immunohistochemical observation in Fig. 1 showed that $GFAP^+$ astrocytes in CPZ-fed mice were obviously enriched in the corpus callosum (CC1), lateral corpus callosum (CC2), and striatum (ST) of myelin sheath tissues compared with normal mice (Fig. 1, $p < 0.0001$ for ST and $p < 0.0001$ for CC1). EP treatment further increased the enrichment of astrocytes (Fig. 1, $p < 0.01$ and $p < 0.0001$ respectively).

Recently, it was reported that besides microglia, astrocytes also engulf myelin debris after myelin damage [29]. Further immunohistochemical staining showed that some MBP expression was colocalized with $GFAP^+$ astrocytes and $GFAP^+$ astrocytes expressing MBP were obviously enhanced after EP treatment, as compared with normal and CPZ groups (Fig. 2a, $p < 0.01$ and $p < 0.001$, respectively), suggesting that astrocytes might engulf myelin debris in damaged myelin sheath tissue.

To further confirm that astrocytes engulf myelin debris and that EP promotes the phagocytosis of myelin debris by astrocytes, we established the culture of primary astrocytes *in vitro*. The results showed that CFSE-coupled myelin debris was localized in the cytoplasm of astrocytes after washing (Fig. 2b), suggesting that astrocytes should phagocytize myelin debris. Comparing with control well without CFSE-myelin debris, the intensity of intracellular CFSE-myelin debris was elevated after the addition of myelin debris by quantitative counting under microscope (Fig. 2c, $p < 0.05$) and fluorescence intensity of multifunctional reader (Fig. 2d, $p < 0.001$). EP treatment further enhanced the ability of astrocytes to phagocytize myelin debris (Fig. 2c, d, $p < 0.05$ and $p < 0.001$, respectively), revealing that EP promotes the phagocytosis of myelin debris by astrocytes.

EP Promotes the Expression of Neurotrophic Factors by Astrocytes

Neurotrophic factors are well known to be important for the oligodendrogenesis. For example, astrocytes promoted oligodendrogenesis after white matter damage via astrocyte-derived neurotrophic factors [19]. In this study, the expression of CNTF was slightly enhanced in the corpus callosum of brain after CPZ feeding compared to normal mice, though it did not reach statistical significance (Fig. 3a, $p = 0.077$). EP treatment further increased the expression of CNTF that was colocalized with $GFAP^+$ astrocytes (Fig. 3a, $p < 0.001$). Simultaneously, compared with normal mice, astrocytes in the striatum increased after CPZ feeding, but did not express more BDNF (Fig. 3b). $GFAP^+$ astrocytes expressing BDNF increased significantly after EP treatment in CPZ-induced demyelination (Fig. 3b, $p < 0.001$). The enlarged image clearly showed an increased astrocyte expressing CNTF and BDNF (Fig. 3a, b).

To further confirm that EP can promote the expression of CNTF and BDNF on astrocytes, we carried out cell experiments *in vitro*. The results showed that EP treatment effectively promoted the expression of CNTF (Fig. 4a, $p < 0.001$) and BDNF (Fig. 4b, $p = 0.056$) on astrocytes, including bFGF (Fig. 4c, $p < 0.001$), although the difference of BDNF was not statistically significant. Based on previous reports and these results, it can be assumed that astrocyte-derived CNTF and BDNF should be beneficial for the oligodendrogenesis after myelin sheath damage by CPZ feeding.

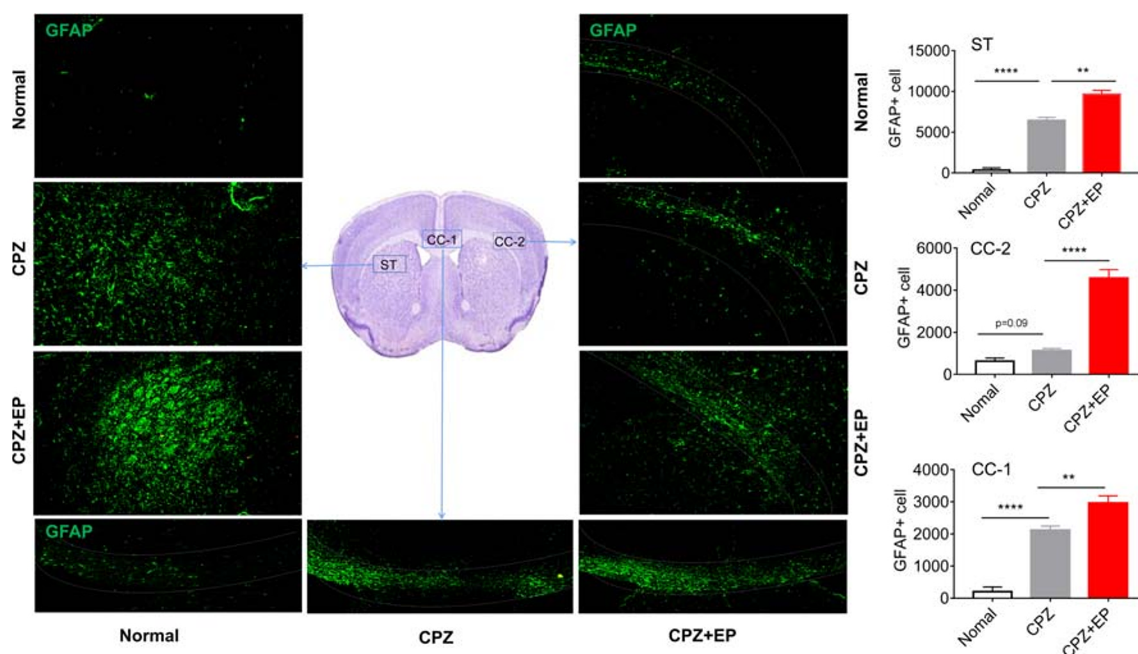

Fig. 1 EP enhanced the accumulation of astrocytes in myelin tissues of CPZ-induced demyelinating mice. Mice were fed with chow containing 0.2% CPZ for 6 weeks and were intraperitoneally injected with EP, starting from 4th weeks to 6th weeks for consecutive 14 days. GFAP^{+} astrocytes in the corpus callosum (CC1), lateral corpus callosum (CC2), and striatum (ST) were observed by immunohistochemistry. The results were obtained from 3 to 4 mice in each group. Micrographs are representative of brain slices in each group with similar results. The results represent the mean ± SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$

EP Induces the Expression of Embryonic Protein by Astrocytes

In adult brain, nestin is also expressed in some astroglial cells [30] and may be re-expressed in reactive astrocytes under pathophysiological conditions [31], considering as embryonic protein. To determine the role of EP in the differentiation of astrocytes in CPZ-induced demyelinating mice, we detected the expression of nestin on GFAP^{+} astrocytes. The results showed that the expression of nestin in the corpus callosum, external capsule, and striatum was significantly elevated compared with normal mice (Fig. 5a–c, $p < 0.001$, $p < 0.01$, and $p < 0.001$, respectively). EP treatment further enhanced the expression of nestin, mainly in astrocytes (Fig. 5a–c, $p < 0.001$, respectively).

Because of these findings that astrocytes can be converted into oligodendrocyte lineage cells in adult mice demyelinated brains by Sox2 [32], we further investigated whether EP induces the expression of Sox2 by astrocytes in CPZ-induced demyelination. As shown in Fig. 6a, b, compared with normal mice, GFAP^{+} astrocytes expressing Sox2 were declined in the corpus callosum ($p < 0.05$) and elevated in the striatum ($p <$ 0.05) after CPZ feeding. However, EP treatment obviously upregulated GFAP^{+} astrocytes expressing Sox2 in both the corpus callosum and striatum (Fig. 6a, b, $p < 0.001$, respectively). We carried out triple staining of GFAP, nestin, and Sox2 in the brain of CPZ-fed mice treated with EP. The results showed that in the corpus callosum near subventricular zone (SVZ), some GFAP^{+} astrocytes indeed expressed both nestin and Sox2 (Fig. 6c).

Previous studies have demonstrated that Sox2 stimulates β-catenin, which in turn induces Sox2. At the same time, β-catenin can stimulate nestin [33], while Notch signal can inhibit the formation and maturation of OPCs [34]. Based on these findings (Fig. 7a), we then observed the expression of β-catenin and Notch1 by immunohistochemistry staining. The expression of β-catenin on GFAP^{+} astrocytes was upregulated in the striatum after CPZ feeding, as compared with normal mice (Fig. 7b, $p < 0.01$), while EP treatment further increased the expression of β-catenin on GFAP^{+} astrocytes (Fig. 7b, $p < 0.001$). Consistent with previous studies, the upregulation of β-catenin may be related to increased nestin expression on astrocytes (Fig. 5).

As expected, the expression of Notch1 on astrocytes in the striatum was obviously elevated in CPZ mice compared with normal group (Fig. 7c, $p < 0.001$), which was declined by EP treatment (Fig. 7c, $p < 0.001$). We carried out triple staining of GFAP, Notch, and β-catenin in the brain of CPZ-fed mice treated with EP. The results showed that in the striatum, not in the corpus callosum near SVZ, some GFAP^{+} astrocytes also expressed both Notch1 and β-catenin, but the intensity of β-catenin expression was stronger than Notch1 expression (Fig. 7d). Taken together, these results indicate that Sox2 positively controls β-catenin and nestin signaling, while β-catenin also positively regulates Sox2 and nestin signaling, which may contribute to the proliferation and differentiation of astrocytes.

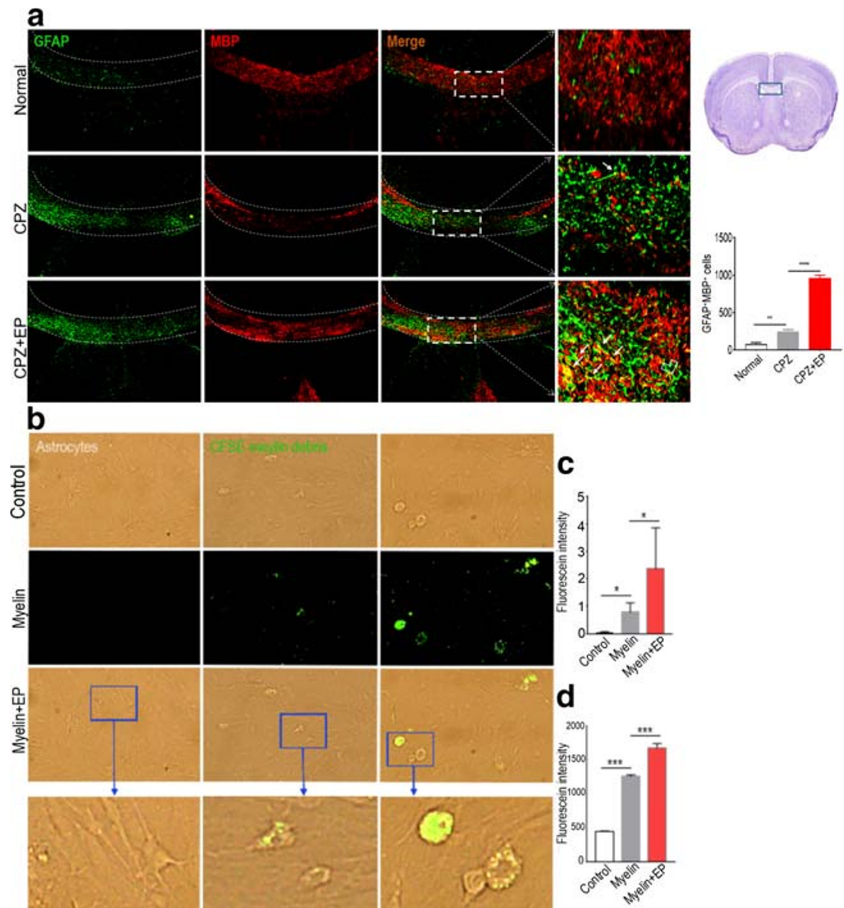

Fig. 2 EP promoted astrocytes to engulf myelin debris around the myelin sheaths. Mice were fed with chow containing 0.2% CPZ for 6 weeks and were intraperitoneally injected with EP, starting from 4th weeks to 6th weeks for consecutive 14 days. (a) Double immunohistochemical staining with anti-GFAP and anti-MBP in the corpus callosum of brain. The 4th column is an enlarged image from the dotted grid in the 3rd column. The results were obtained from 3 to 4 mice in each group. Micrographs are representative of brain slices in each group with similar results and quantified by Image-Pro Plus 6.0 software. (b) *In vitro* phagocytosis of myelin debris by primary astrocytes. The data were obtained from two independent experiments with similar results and quantified by Image-Pro Plus 6.0 software and by multifunctional Synergy H1 Hybrid Reader. The 4th row is an enlarged image from the dotted grid in the 3rd row. The results represent the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$

In addition, Notch1 may negatively regulate the formation and maturation of OPCs.

EP Promotes the Generation of OPCs Derived from Astrocytes

Increasing evidence indicate that astrocyte-derived neurotrophic factors promoted the oligodendrogenesis after white matter damage [19] and that Sox2/β-catenin/nestin regulated the proliferation and differentiation of oligodendrogenesis [35, 36]. On the other hand, a series of studies have reported the transdifferentiation of astrocytes to oligodendrocyte lineage cells [37–39]. First, we detected the number of $NG2^+$ and PDGF-Ra^+ OPCs. The results showed that $NG2^+$ OPCs were enhanced (Fig. 8a, $p < 0.001$) and EP treatment further increased $NG2^+$ OPCs in the striatum compared with CPZ model (Fig. 8a, $p < 0.001$). Similarly, PDGF-Ra^+ OPCs in the striatum and corpus callosum was also elevated (Fig. 8a, $p < 0.0001$ and $p < 0.05$ respectively) and EP treatment further increased PDGF-Ra^+ OPCs compared with CPZ model (Fig. 8a, $p < 0.001$ and $p < 0.01$). Next, we observed the possibility of astrocyte transdifferentiation to OPCs in CPZ-induced demyelination model by EP treatment. Most interestingly, the results showed that only a few spontaneous astrocytes might be colocated GFAP with $NG2^+$ or PDGF-Ra^+ under laser confocal in the striatum of mice treated with EP (Fig. 8b, c, yellow arrow), revealing that EP treatment could promote the transdifferentiation of astrocytes into OPCs in CPZ-induced demyelinating model.

To confirm the transdifferentiation of astrocytes into OPCs *in vivo* after EP treatment, CFSE-labeled astrocytes were transplanted into the corpora callosa of brains from adult mice fed with CPZ for 4 weeks, followed by CPZ feeding and EP treatment for additional 2 weeks (Fig. 9a). Animal brain sections were prepared and the expression of GFAP, Sox2, and NG2 was checked for the transdifferentiation of astrocytes into OPCs. However, there were very low numbers of transplanted CFSE-labeled astrocytes expressing Sox2 and NG2 in CPZ-fed mice (Fig. 9b). There was no significant difference observed between normal and CPZ groups (Fig. 9b). Indeed, transplanted CFSE-labeled astrocytes expressing Sox2 and NG2 significantly increased in CPZ-fed mice that received EP treatment (Fig. 9b, $p < 0.001$), but these differentiated cells could still be a very small proportion, because most of the endogenous $GFAP^+$ astrocytes in the brain of mice did not express NG2 and Sox2 in the brain of mice without the implantation of exogenous CFSE-astrocytes (Fig. 9c).

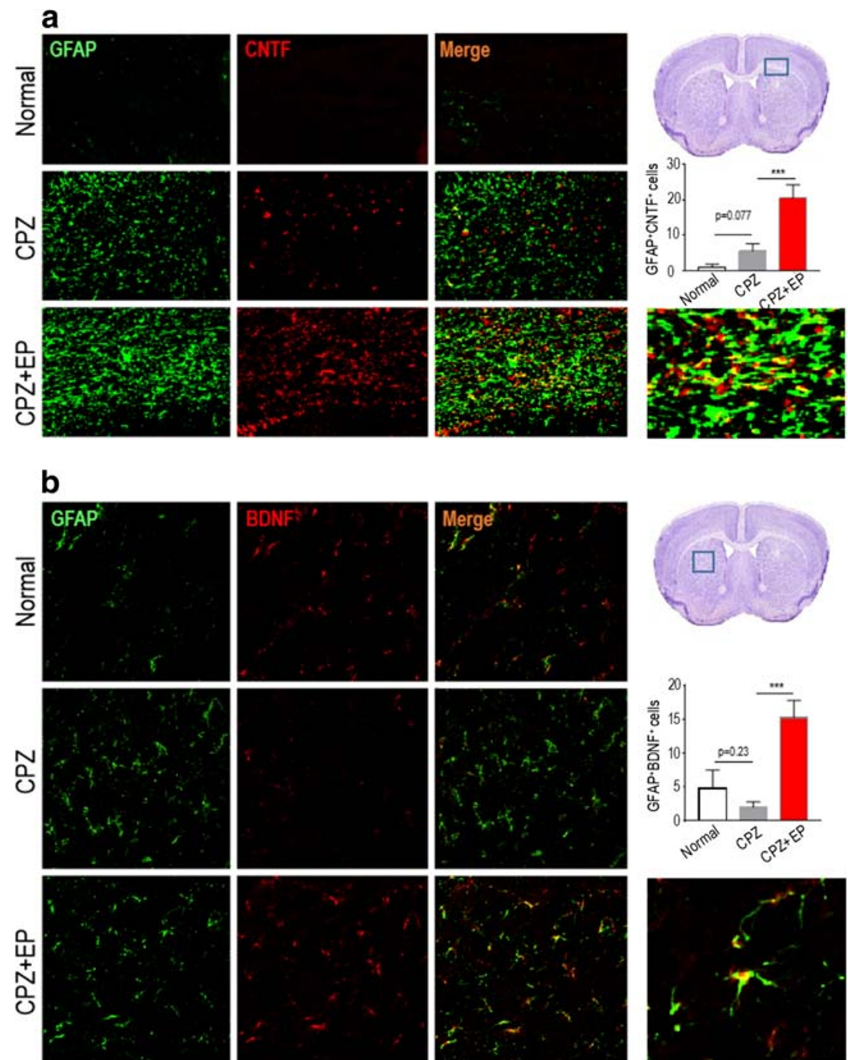

Fig. 3 EP promoted the expression of CNTF and BDNF by astrocytes *in vivo*. Mice were fed with chow containing 0.2% CPZ for 6 weeks and were intraperitoneally injected with EP, starting from 4th weeks to 6th weeks for consecutive 14 days. (a) Double immunohistochemical staining with anti-GFAP and anti-CNTF in the corpus callosum of brain. (b) Double immunohistochemical staining with anti-GFAP and anti-BDNF in the striatum of brain. The 4th column is an enlarged image from the dotted grid in the 3rd column. The results were obtained from 3 to 4 mice in each group. Micrographs are representative of brain slices in each group with similar results and quantified by Image-Pro Plus 6.0 software. The results represent the mean ± SEM. $^{***}p < 0.001$

Discussion

In this study, CPZ feeding caused demyelination, accompanied by astrocyte enrichment in the corpus callosum. However, EP treatment further promoted astrocyte enrichment. Previous work showed that hypertrophic astrocytes in active MS lesions contain myelin fragments, suggesting that myelin can be phagocytosed by astrocytes [29]. These myelin-positive astrocytes were concentrated at the advancing edge of myelin damage [29]. Consistent with these results, we also found that astrocytes enriched in myelin sheath were colocalized with MBP in CPZ-induced demyelination lesions, revealing that there may be the phagocytosis of myelin debris by astrocytes at the site of myelin damage. *In vitro* experiments further confirmed that EP treatment can promote the phagocytosis of myelin debris by astrocytes. Engulfment has traditionally been attributed to professional phagocytes, such as macrophages, microglia, and dendritic cells. However, it was reported that astrocytes also engulf smaller fragments, such as axonal or myelin debris [29, 40]. Functionally, myelin debris was taken up by astrocytes through receptor-mediated endocytosis and resulted in astrocyte activation and chemokine production that leads to recruitment of astrocytes to the lesion site [29].

The next question is whether astrocytes engulfing myelin debris are beneficial or harmful for myelin regeneration. It has long been thought that astrocytes provide a supporting microenvironment for neuronal function in the healthy and inflamed CNS. In recent years, growing evidence indicates that astrocytes play an active and dual role in the CNS diseases such as MS [41]. Some experimental data implicate astrocytes as actual mediators of inflammation, as observed in sites of injury, ultimately limiting neuronal repair and remyelination [42]. By contrast, other evidence suggests that astrocytes provide support and protection for oligodendrocytes and neurons [43, 44]. The latter provides the possibilities for targeting astrocytes in the treatment of the CNS diseases. In this study, it was clearly showed that EP treatment promoted the expression of CNTF

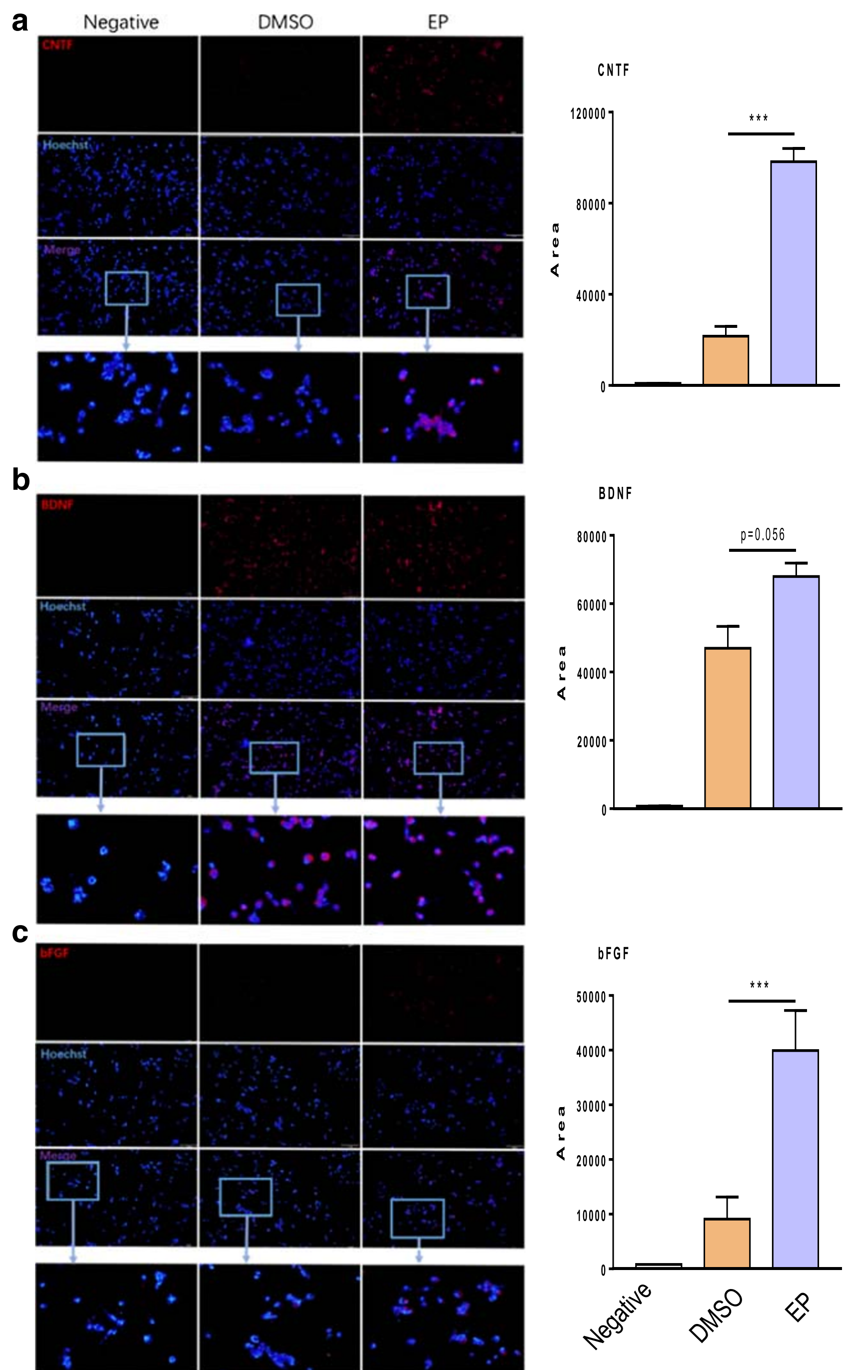

Fig. 4 EP promoted the expression of CNTF and BDNF by astrocytes *in vitro*. Cultured astrocytes were treated with EP and stained with anti-CNTF, anti-BDNF, and anti-bFGF. Negative control used PBS to replace the first antibody. (a) CNTF, (b) BDNF, and (c) bFGF. The 4th row is an enlarged image from the dotted grid in the 3rd row respectively. The data were obtained from two independent experiments with similar results and quantified by Image-Pro Plus 6.0 software. The results represent the mean ± SEM. ***$p < 0.001$

and BDNF by astrocytes, suggesting that EP treatment should contribute to the regeneration of myelin sheath. It has been reported that CNTF directly affected the differentiation of oligodendrocytic cells after CPZ-induced demyelination [45], controlled the directed migration of SVZ-derived progenitors and oligodendrocyte precursors [46], and promoted the survival and differentiation of oligodendrocyte precursor cells *in vitro*, but failed to promote remyelination *in vivo* [47]. In addition, astrocytes promoted oligodendrogenesis by secreting BDNF during endogenous recovery from white matter damage [19]. Our results, together with previous findings, provide new therapeutic insight into demyelination and remyelination, in which EP induced astrocyte-derived CNTF and BDNF that supports oligodendrogenesis and regeneration after demyelination.

Previous studies have demonstrated that neural precursor cells (NPCs) from the adult SVZ can generate new oligodendrocytes after demyelination [48]. Adult neural stem cells (NSC) were recruited from the subventricular zone to populate demyelinated lesions by the differentiation of

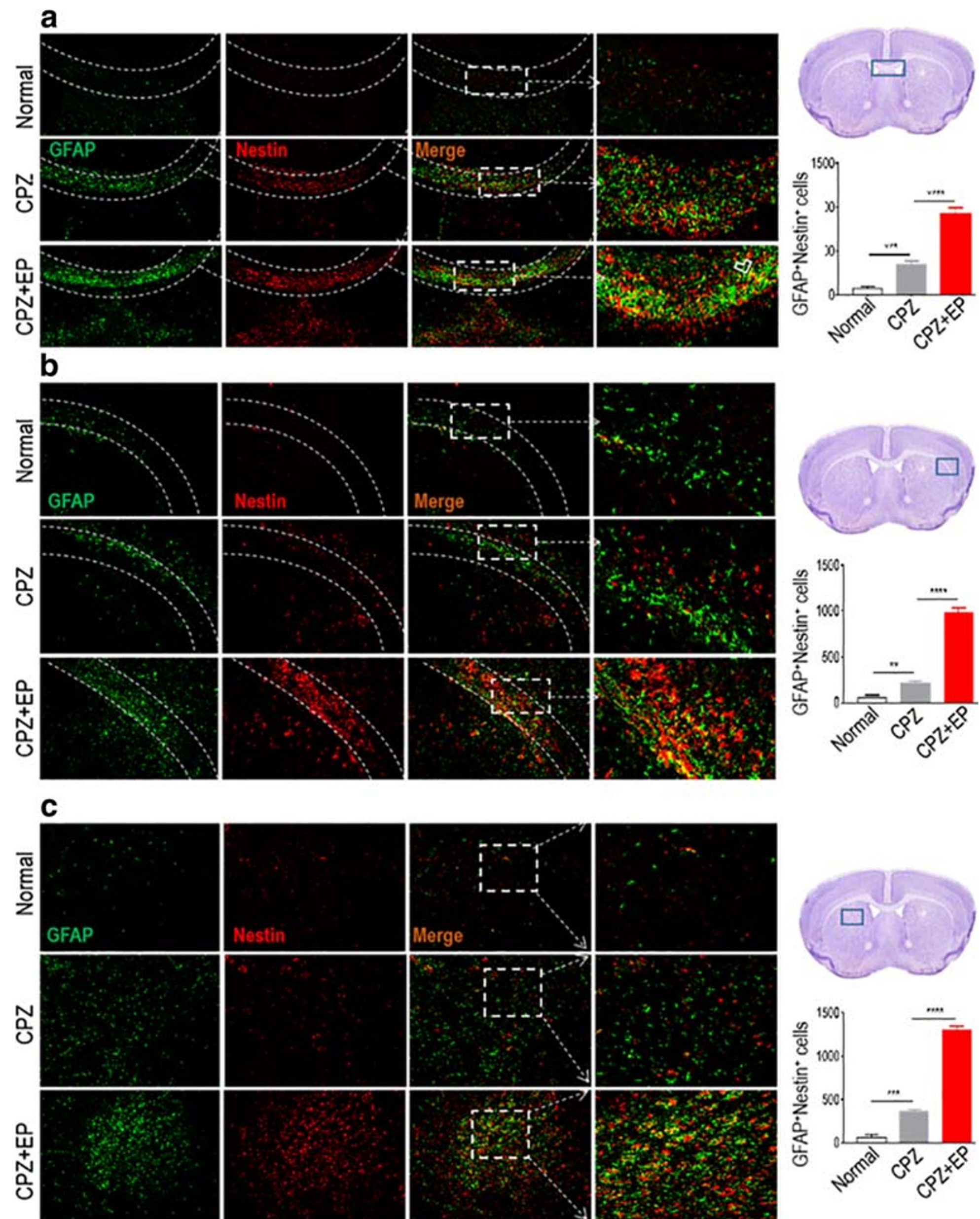

Fig. 5 EP promoted the expression of nestin by astrocytes. Mice were fed with chow containing 0.2% CPZ for 6 weeks and were intraperitoneally injected with EP, starting from 4th weeks to 6th weeks for consecutive 14 days. (a) Double immunohistochemical staining with anti-GFAP and anti-nestin in the corpus callosum of brain, (b) in the external capsule, and (c) in the striatum. The 4th column is an enlarged image from the dotted grid in the 3rd column. The results were obtained from 3 to 4 mice in each group. Micrographs are representative of brain slices in each group with similar results and quantified by Image-Pro Plus 6.0 software. The results represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

oligodendrocytes and were enhanced by genetic or pharmacological inhibition of Gli1 [49]. The most interesting finding in this study is that EP can effectively induce the transdifferentiation of astrocytes into oligodendrogenesis, accompanied by increased expression of nestin, Sox2, and β-catenin by astrocytes. The expression of nestin is a marker of pre-O2A cells that is maintained in proliferating glial progenitors, and was induced in reactive astrocytes by brain injuries [50]. Here, the expression of nestin was upregulated in CPZ-induced demyelinating model, while EP treatment further increased the expression of nestin in astrocytes during CPZ-induced demyelination. Reactive astrocytes in active MS lesions re-expressed nestin, which is important for the astroglial adaptation to pathological situations [51]. These results suggest that nestin+ cells that also express GFAP may be dedifferentiated from mature astrocytes and, thus, re-enter the cell cycle as a source of cells with stem cell potential within the injury site, contributing to the spontaneous remyelination process.

The transcription factor Sox2 has been widely concerned because of its functional connection to stem cell state [52, 53]. Sox2 plays a crucial role in coordinating OPC proliferation and oligodendrocyte differentiation and is required for developmental myelination [54]. It was reported that Sox2 could reprogram astrocytes to neural progenitors/neural stem cells [55, 56], converting astrocytes to myelinating cells [32]. Therefore, Sox2-mediated reprogramming of astrocytes may contribute to a wide range of neuron repair and myelin

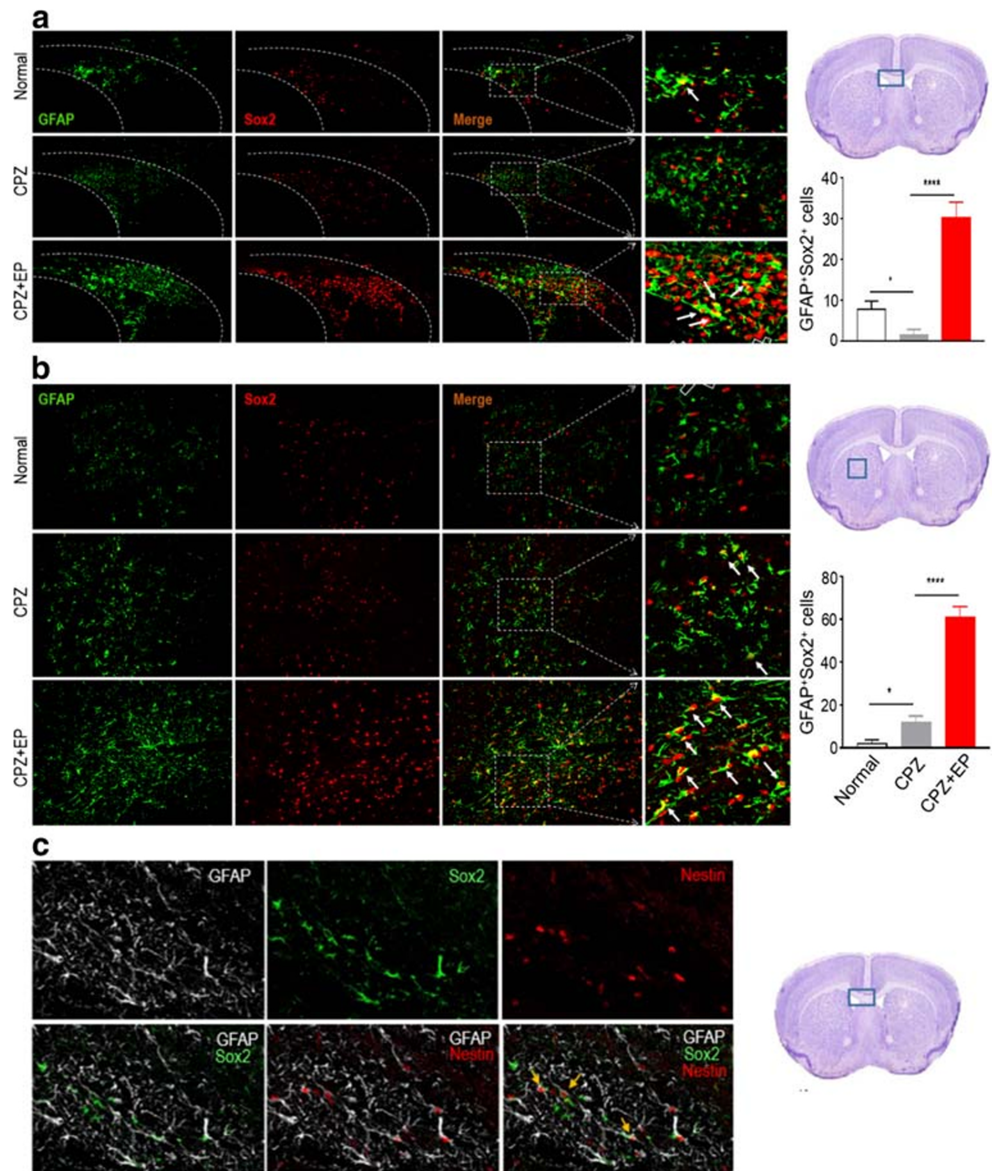

Fig. 6 EP promoted the expression of Sox2 by astrocytes. Mice were fed with chow containing 0.2% CPZ for 6 weeks and were intraperitoneally injected with EP, starting from 4th weeks to 6th weeks for consecutive 14 days. (a) Double immunohistochemical staining with anti-GFAP and anti-Sox2 in the corpus callosum of brain and (b) in the striatum. The 4th column is an enlarged image from the dotted grid in the 3rd column. (c) Triple staining of GFAP, nestin, and Sox2. The results were obtained from 3 to 4 mice in each group. Micrographs are representative of brain slices in each group with similar results and quantified by Image-Pro Plus 6.0 software. The results represent the mean ± SEM. $^{*}p < 0.05$, $^{****}p < 0.001$

regeneration by endogenous-induced progenitors. Similar to Sox2, Notch is also a marker for undifferentiated human and mouse progenitors [57]. However, some contrary and unexpected results often appear, in which Notch signaling prevented oligodendrocyte maturation and myelin formation by blocking OPC differentiation in the developing CNS [58]. It was therefore speculated that Notch signaling might be activated in MS lesions to prevent OPCs from differentiating into myelin-forming oligodendrocytes [59]. Another evidence also demonstrated that activation of Notch1 receptor mediated by reactive astrocytes inhibited OPC differentiation, leading to delayed remyelination and fewer mature, myelinating oligodendrocytes [60]. The inhibition of Notch increased hair cell differentiation from stem cells that express stem cell markers such as Sox2 and have the capacity for proliferation and transdifferentiation for a short period [61], and enhanced tissue repair in MS model by promoting remyelination and reduced axonal damage [62].

β-Catenin signaling has been implicated as an important signal controlling OPC maturation and CNS myelination [63]. Although it is widely accepted that Wnt/β-catenin signaling has an inhibitory effect on OPC differentiation and oligodendrocyte maturation [64, 65], opposite observations showed a promyelinating effect of Wnt/β-catenin signaling. The selective inhibition of Wnt/β-catenin blocks the expression of myelin protein in mouse oligodendrocytes, while activation of Wnt/β-catenin promotes myelin gene expression and myelinogenesis [66]. The activation of Akt promotes β-catenin, thereby inducing OPC differentiation [67]. In the peripheral nervous system, the treatment of GSK3β inhibitor promotes remyelination by enhancing β-catenin expression [68]. In addition, naringin treatment has the potential to promote

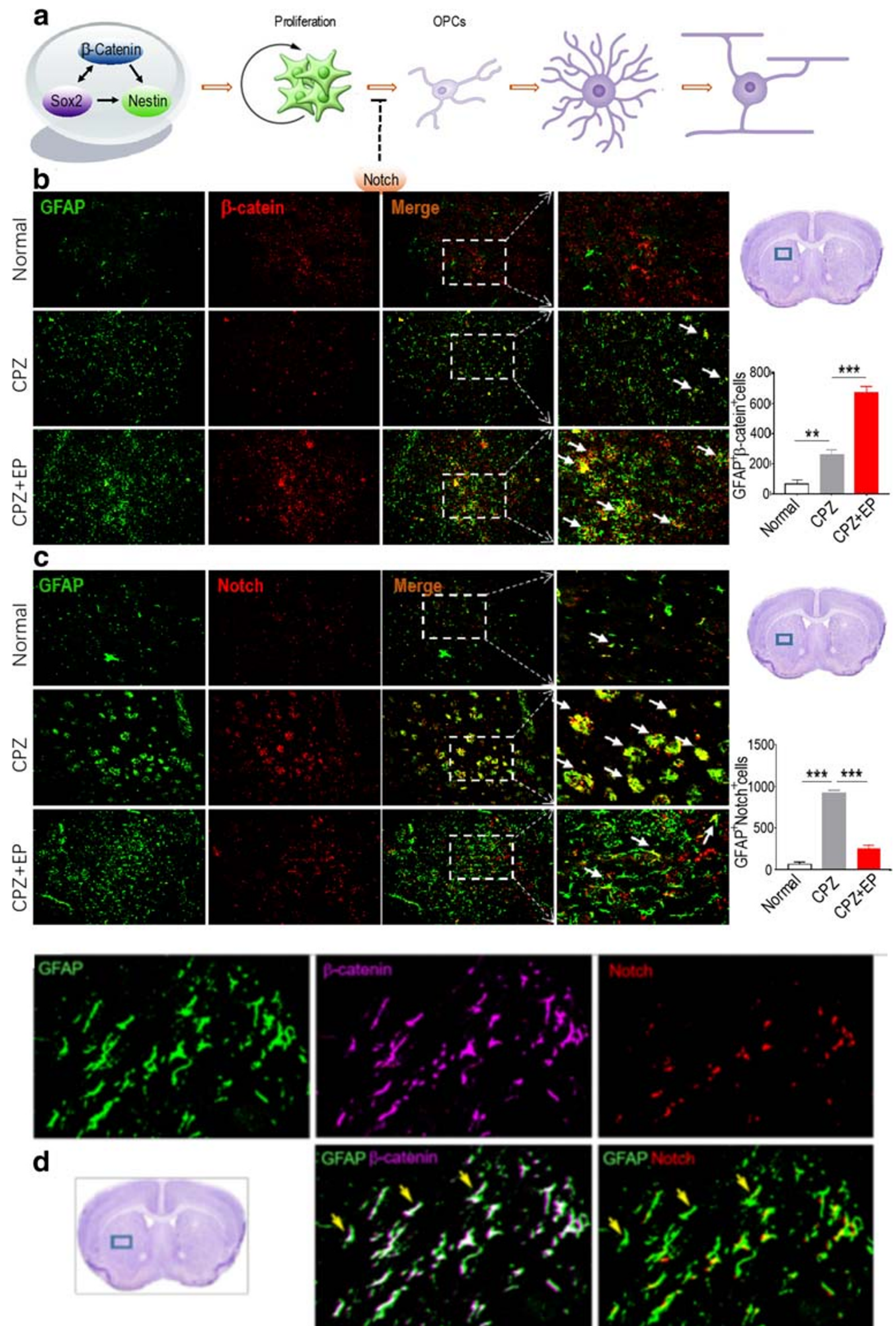

Fig. 7 EP increased the expression of β-catenin and decreased the expression of Notch1 by astrocytes. Mice were fed with chow containing 0.2% CPZ for 6 weeks and were intraperitoneally injected with EP, starting from 4th weeks to 6th weeks for consecutive 14 days. (a) Ideograph of possible roles of β-catenin and Notch1 during oligodendrogenesis, (b) double immunohistochemical staining with anti-GFAP and anti-β-catenin in the striatum of brain, (c) double immunohistochemical staining with anti-GFAP and anti-Notch1 in the striatum of brain, and (d) triple staining of GFAP, Notch, and β-catenin. The 4th column is an enlarged image from the dotted grid in the 3rd column. The results were obtained from 3 to 4 mice in each group. Micrographs are representative of brain slices in each group with similar results and quantified by Image-Pro Plus 6.0 software. The results represent the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$

OPC differentiation and remyelination, accompanied by increased β-catenin expression. These results also support our finding that EP treatment promotes myelin regeneration, which may be related to upregulation of β-catenin expression. Altogether, there is no doubt that the role of β-catenin in the pathogenesis and remyelination in MS patients and EAE animals appears complicated: either as a foe (a pathogenic factor) or a friend (a remyelinating factor). The reason for the contradictory event is not clear, depending on timing, extent of activation, downstream molecules involved, and fine-tuning regulatory mechanisms. Based on these findings from our lab and other groups, we speculated that EP treatment

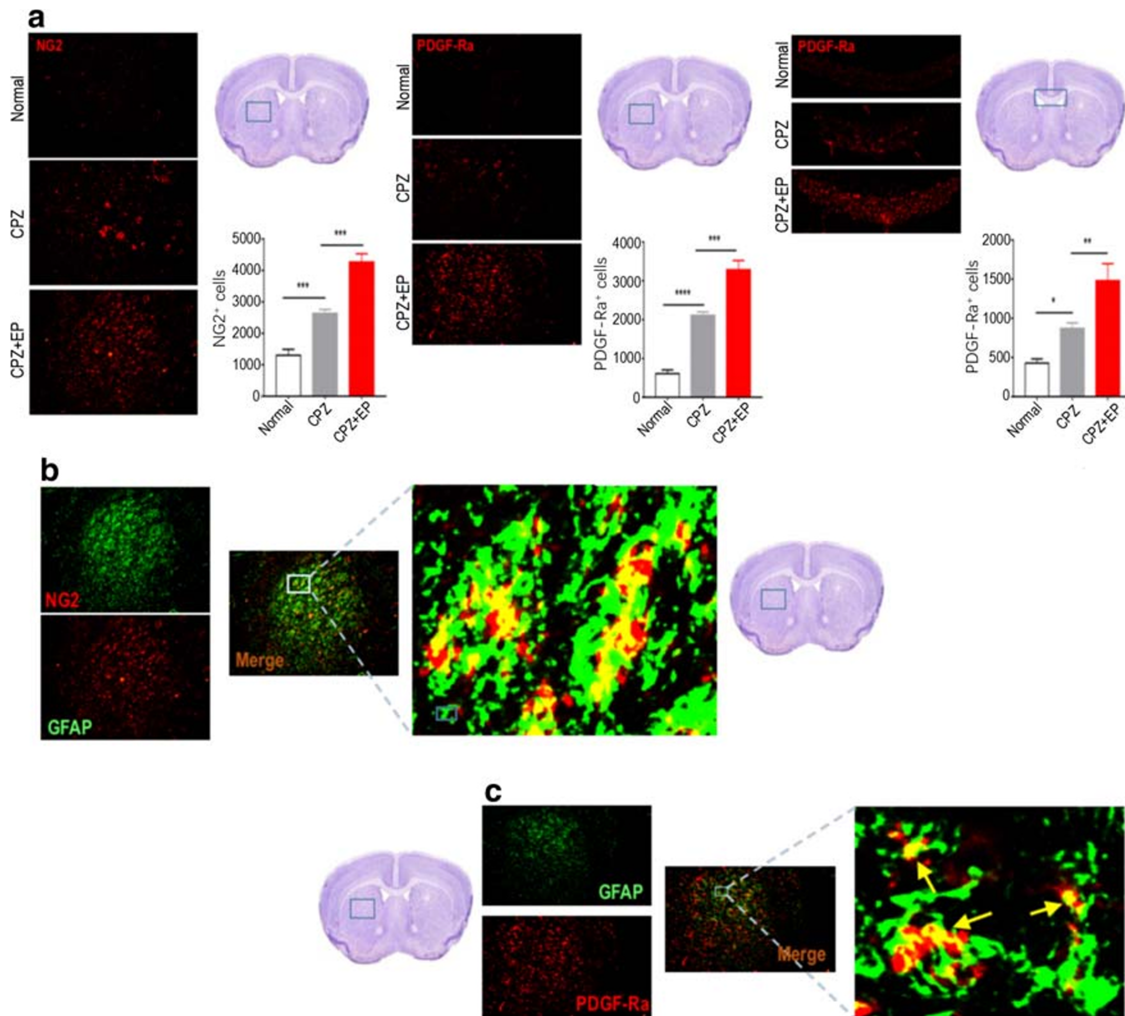

Fig. 8 EP induced the generation of NG2$^+$ and PDGF-Ra$^+$ OPCs. Mice were fed with chow containing 0.2% CPZ for 6 weeks and were intraperitoneally injected with EP, starting from 4th weeks to 6th weeks for consecutive 14 days. (a) NG2$^+$ and PDGF-Ra$^+$ OPCs in the striatum and corpus callosum of brain. The results were obtained from 3 to 4 mice in each group and quantified by Image-Pro Plus 6.0 software. (b) Double immunohistochemical staining with anti-GFAP and anti-NG2 in the striatum of mice treated with EP and (c) double immunohistochemical staining with anti-GFAP and anti-PDGF-Ra in the striatum of mice treated with EP. The right is an enlarged image from the dotted grid in the left. Micrographs are representative of brain slices in each group. The results represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$

could induce astrocytes to upregulate the expression of nestin, Sox2, and β-catenin, and promote astrocytes to enter into the transdifferentiation of OPCs and maturation of oligodendrocytes. Of course, the precise mechanism how EP induces astrocytes to upregulate nestin, Sox2, and β-catenin and how nestin, Sox2, and β-catenin interact to promote myelin regeneration remains to be further explored.

Besides the result that EP effectively increased the number of NG2$^+$ or PDGF-Ra$^+$ OPCs expressing GFAP, further confirmation was performed by intracerebral injection of primary astrocytes labeled with CFSE. As expected, NG2$^+$ OPCs expressing CFSE and Sox2 were elevated in the corpus callosum of mice treated with EP following transplantation, revealing that EP can convert astrocytes into myelinating cells. Our results indicate the possibility that EP lead to effective myelin repair in patients suffering from myelination deficit. Transdifferentiation converts mature cells from one specialized cell type into another for neuroregeneration and remyelination. Astrocytes, the most abundant cells in the CNS, undergo a process termed "reactive gliosis" wherein they exhibit dramatic changes in morphology and protein expression. Direct conversion of human astrocytes into functional dopamine neurons via forced expression of transcription factors has been reported in previous experiment [69, 70]. Similarly, *in vivo* conversion of astrocytes to oligodendrocyte lineage cells was also observed [37, 38]. As a limitation of this study, we have not elucidate:1) how EP induces astrocytes to up regulate the expression of CNTF and BDNF, 2) how it

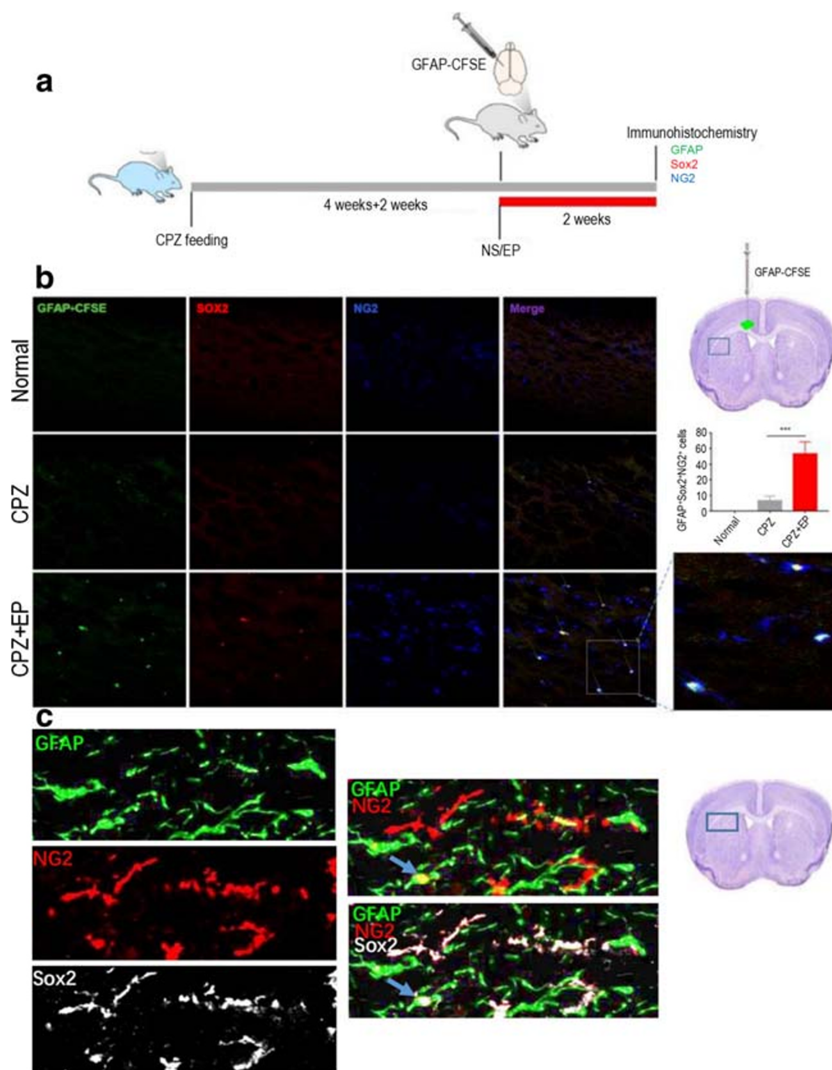

Fig. 9 EP induced transdifferentiation of astrocytes into OPCs. Mice were fed with chow containing 0.2% CPZ for 6 weeks. In the 4th week, CFSE-labeled astrocytes were injected into the brain, and mice were intraperitoneally injected with EP for consecutive 14 days. (a) Experimental protocol for intracerebral astrocyte injection and (b) immunohistochemical staining with anti-GFAP, anti-Sox2, and NG2 in the brain of mice injected with CFSE-labeled astrocytes. The 4th column is an enlarged image from the dotted grid in the 3rd column. The green dot in the pattern brain is the injection site of astrocytes. The results were obtained from 3 to 4 mice in each group and quantified by Image-Pro Plus 6.0 software. (c) Triple staining of GFAP, NG2, and Sox2 in the brain of mice noninjected with CFSE-labeled astrocytes. Micrographs are representative of brain slices in each group with similar results. The results represent the mean ± SEM. *** $p < 0.001$

affects the changes of nestin, Sox2, β - catenin and Notch1 in astrocytes, 3) whether neurotrophic factors and progenitor cell markers are involved in EP-mediated myelin regeneration, and 4) whether EP induces the differentiation of oligodendrocyte-like cells driven by neural sten cells in CPZ-demyelinating model. These questions are being carefully designed and performed in the future.

In conclusion, EP promoted migration and enrichment of astrocytes to demyelinated tissue and induced astrocytes to express neurotrophic CNTF and BDNF as well as translation factor nestin, Sox2, and β-catenin, which should contribute to astrocytes to differentiate of oligodendrogenesis. At the same time, EP promoted astrocytes to phagocytized myelin debris for removing the harmful substances of myelin regeneration (Graphical Abstract).

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Author Contributions Xiao BG, Ma CG, and Huang JJ contributed to study design, data analysis, and manuscript modification. He Y, An J, and Yin JJ contributed to animal model and laboratory measurement (immunohistochemistry). Miao Q and Sui RX contributed to *in vitro* cell experiments. Han QX and Ding ZB contributed to astrocyte culture, labeling, and intracranial injection. All authors were involved in analysis of results and manuscript preparation and approval.

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Compliance with Ethical Standards

The study was approved by the Council for Laboratory and Ethics Committee of Shanxi University of Chinese Medicine, Taiyuan, China. The experimental protocol was performed according to the International Council for Laboratory Animal Science guidelines.

Disclosures The authors declare that they have no conflict of interest.

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