

# Therapeutic time window for the effects of erythropoietin on astrogliosis and neurite outgrowth in an in vitro model of spinal cord injury

Hea Nam Hong, PhD<sup>a</sup>, Ju Hee Shim, MS<sup>a</sup>, You Jin Won, PhD<sup>a</sup>, Jong Yoon Yoo, MD, PhD<sup>b,\*</sup>, Chang Ho Hwang, MD, PhD<sup>c,\*</sup>

## Abstract

**Background:** The objective of this study was to investigate the underlying molecular mechanisms and the therapeutic time window for preventing astrogliosis with erythropoietin (EPO) treatment after in vitro modeled spinal cord injury (SCI).

**Methods:** Cultured rat spinal cord astrocytes were treated with kainate and scratching to generate an in vitro model of SCI. EPO (100U/mL or 300U/mL) was added immediately or 2, 4, or 8 hours after injury. Some cultures were also treated with AG490, an inhibitor of the EPO-EPO receptor (EpoR) pathway mediator Janus kinase 2 (JAK2). To evaluate neurite extension, rat embryonic spinal cord neurons were seeded onto astrocyte cultures and treated with EPO immediately after injury in the presence or absence of anti-EpoR antibody.

**Results:** EPO treatment at up to 8 hours after injury reduced the expression of axonal growth inhibiting molecules (glial fibrillary acidic protein, vimentin, and chondroitin sulfate proteoglycan), cytoskeletal regulatory proteins (Rho-associated protein kinase and ephrin A4), and proinflammatory cytokines (tumor necrosis factor- $\alpha$ , transforming growth factor- $\beta$ , and phosphorylated-Smad3) in a dose-dependent manner ( $P < .001$ ). Most effects peaked with EPO treatment 2–4 hours after injury. Additionally, EPO treatment up to 4 hours after injury promoted expression of the EpoR ( $>2$ -fold) and JAK2 ( $>3$ -fold) in a dose-dependent manner ( $P < .001$ ), whereas co-treatment with AG490 precluded these effects ( $P < .001$ ). EPO treatment up to 4 hours after injury also enhanced axonal b-III tubulin-immunoreactivity ( $>12$ -fold), and this effect was precluded by co-treatment with an anti-EpoR antibody ( $P < .001$ ).

**Conclusions:** EPO treatment within 8 hours after injury reduced astrogliosis, and EPO treatment within 4 hours promoted neurite outgrowth. EPO therapy immediately after spinal cord injury may regulate glia to generate an environment permissive of axonal regeneration.

**Abbreviations:** AKT = protein kinase B, BSA = bovine serum albumin, CNS = central nervous system, CSPGs = chondroitin sulfate proteoglycans, EphA4 = ephrinA4, EPO = erythropoietin, EpoR = EPO receptor, GFAP = glial fibrillary acidic protein, IL = interleukins, JAK2 = Janus kinase 2, KA = kainic acid, NG2 = neuron-glia antigen 2, pAKT = phosphorylated protein kinase B, PI3K = phosphoinositide 3-kinase, p-Smad3 = phosphorylated-Smad3, rhEPO = recombinant human EPO, ROCK = Rho-associated protein kinase, SCI = spinal cord injury, SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis, STAT = signal transducer and activator of transcription, TGF- $\beta$  = transforming growth factor- $\beta$ , TNF- $\alpha$  = tumor necrosis factor- $\alpha$ .

**Keywords:** astrocyte, erythropoietin, neurite growth, spinal cord injury

Editor: Anser Azim.

This work was supported by a grant (2011-412) from the ASAN Institute for Life Science, Seoul, Korea and by the National Research Foundation of Korea (NRF) grant funded by the Korea government (Ministry of Science, ICT & Future Planning) (NRF-2017R1A2B4011478).

The authors have no conflicts of interest to disclose.

<sup>a</sup> Department of Anatomy, <sup>b</sup> Department of Rehabilitation Medicine, Asan Medical Center, <sup>c</sup> Department of Physical Medicine and Rehabilitation, Ulsan University Hospital, University of Ulsan College of Medicine, Republic of Korea.

\* Correspondence: Chang Ho Hwang, Department of Physical Medicine and Rehabilitation, Ulsan University Hospital, University of Ulsan College of Medicine, 290-3 Jeonha-dong, Dong-gu, Ulsan 682-714, Republic of Korea (e-mail: chhwang1220ciba@gmail.com), Jong Yoon Yoo, MD, PhD, Department of Rehabilitation Medicine, Asan Medical Center, University of Ulsan College of Medicine, 388-1 Pungnap-dong, Songpa-gu, Seoul 138-736, Republic of Korea (e-mail: jyoo@amc.seoul.kr).

Copyright © 2018 the Author(s). Published by Wolters Kluwer Health, Inc. This is an open access article distributed under the Creative Commons Attribution-NoDerivatives License 4.0, which allows for redistribution, commercial and non-commercial, as long as it is passed along unchanged and in whole, with credit to the author.

Medicine (2018) 97:9(e9913)

Received: 2 August 2017 / Received in final form: 8 November 2017 / Accepted: 25 January 2018

<http://dx.doi.org/10.1097/MD.0000000000009913>

## 1. Introduction

The limited functional recovery after spinal cord injury (SCI) is partly related to a growth-inhibiting environment created by activated glia. In response to SCI, astrocytes undergo reactive astrogliosis, which is characterized by the upregulation of 2 intermediate filaments, glial fibrillary acidic protein (GFAP) and vimentin. Reactive astrocytes also secrete various neuro-inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs)<sup>[1]</sup> and produce pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), transforming growth factor- $\beta$  (TGF- $\beta$ ), and interleukins (IL-1, IL-6).<sup>[2]</sup> Although these changes lead to transient beneficial effects in the early phase of injury,<sup>[2]</sup> activated glia ultimately create an environment precluding axonal regeneration and may facilitate secondary neuronal damage after primary insult.<sup>[3]</sup> In particular, activation of the Rho-associated protein kinase (ROCK) pathway after SCI is associated with neuronal apoptosis<sup>[4]</sup> and ephrinA4 (EphA4) signaling prevents axonal regeneration after SCI.<sup>[5]</sup> Accordingly, regulation of astrogliosis is an important potential therapeutic approach to improving functional recovery after SCI.

Erythropoietin (EPO) promotes neural regeneration after the central nervous system (CNS) injury by decreasing glutamate

toxicity, increasing the generation of anti-apoptotic factors, reducing inflammation, and decreasing nitric oxide-mediated injury through direct and indirect antioxidant effects.<sup>[6]</sup> EPO-mediated neuro-protection is partly attributed to Epo receptor (EpoR) activation and subsequent Janus kinase 2 (JAK2) signaling as well as the activation of other downstream signaling pathways such as the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway.<sup>[7–9]</sup> Although the neuro-protective effects of EPO are well known, few studies to date have evaluated the therapeutic time window for administering EPO to prevent astrogliosis after SCI.

The goal of this study was to investigate the molecular mechanisms and therapeutic time window for the effects of recombinant human EPO (rhEPO) on glial activation after SCI. Specifically, authors used an *in vitro* model of SCI to examine the effects of rhEPO on the generation of inhibitory molecules, pro-inflammatory cytokine expression, EPO-EpoR signaling, and neurite outgrowth.

## 2. Materials and methods

### 2.1. Cell culture

Authors developed an *in vitro* model of SCI that combines chemical [(kainic acid (KA))] and mechanical (scratch) injury to simulate real-time SCI.<sup>[10]</sup>

### 2.2. rhEPO treatments

Ethical approval was not necessary because it was an *in-vitro* experiment.

After scratching and incubation with 50  $\mu$ M KA, cells were rinsed twice in Hank's balanced salt solution and the medium was replaced with fresh Dulbecco's modified Eagle's medium. rhEPO (Epoquine, 10,000 units/mL; CJ Pharmaceutical Co., Republic of Korea) was added at different concentrations (100 U/mL or 300 U/mL) was added at  $t=0$  (immediately), 2, 4, or 8 hours after injury. Some cultures were also treated with the EPO-EpoR pathway inhibitor (AG490) at the indicated time points.

### 2.3. Western blotting

Cells were lysed by the addition of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 7.8% glycerol, 4.5% mercaptoethanol and 0.1% bromophenol blue) and subsequent boiling for 5 minutes at 100°C. Lysates were cleared by centrifugation and then supernatant protein concentrations were determined relative to a bovine serum albumin (BSA) standard. Cell lysates were subjected to SDS-PAGE on a 4% stacking gel and 10% polyacrylamide separating gel for 70 minutes at 130 V. Then, proteins were transferred onto nitrocellulose membranes with a Bio-Rad transfer unit for 120 minutes at 200 mA. Protein blots were incubated in blocking buffer (2% BSA in Tween20/Tris-buffered saline) for 1 hour at room temperature on a rotating platform. Blots were incubated overnight with primary antibodies against GFAP, CSPG, vimentin, ROCK, EphA4, TNF- $\alpha$ , TGF- $\beta$ , phosphorylated-Smad3 (p-Smad3: a mediator of TGF- $\beta$  actions), EpoR, or JAK2. Then, blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour, followed by 3 washes. Immuno-reactive bands were visualized by chemiluminescence reagents (PIERCE, Rockford, IL). Band optical densities were analyzed with an Imaging

Densitometer (Bio-rad, GS-670). More than 3 independent experiments were performed for each experiment.

### 2.4. Neurite outgrowth analysis

Spinal cord neurons were prepared from embryonic day 16 (E16) Sprague-Dawley rats as previously described.<sup>[11]</sup> After 7 days *in vitro*, spinal neurons were trypsinized and seeded onto astrocytes immediately after model SCI injury. A concentration of 100 U/mL rhEPO was added at  $t=0$  (immediately), 2, 4, or 8 hours after injury. Some cultures were also treated with anti-rhEPO receptor antibody (AbEpoR, 4  $\mu$ g/mL, R&D Systems, Minneapolis) at the indicated times. After treatment, cultures were incubated in neuro-basal medium for 48 hours and subsequently fixed with 4% paraformaldehyde. Three independent experiments were performed ( $n=2$  wells per group per experiment), and 10 fields per well were randomly selected at a magnification of  $\times 10$ . Neurite outgrowth was evaluated in each field by measuring the area fraction occupied by neurites stained with anti-Tuj1 antibody (anti-Tubulin, beta III isoform, Millipore, Darmstadt, Germany) using ImageJ software (version 1.43u, National Institutes of Health, Bethesda, MA).

### 2.5. Statistical analysis

Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) was used for image quality optimization and figures preparation. Data were analyzed using GraphPad Instat 3.05 (GraphPad, San Diego). Statistical comparisons were performed using a one-way analysis of variance with Tukey-Kramer *post hoc* test. A  $P$ -value  $> .05$  was the threshold for statistical significance.

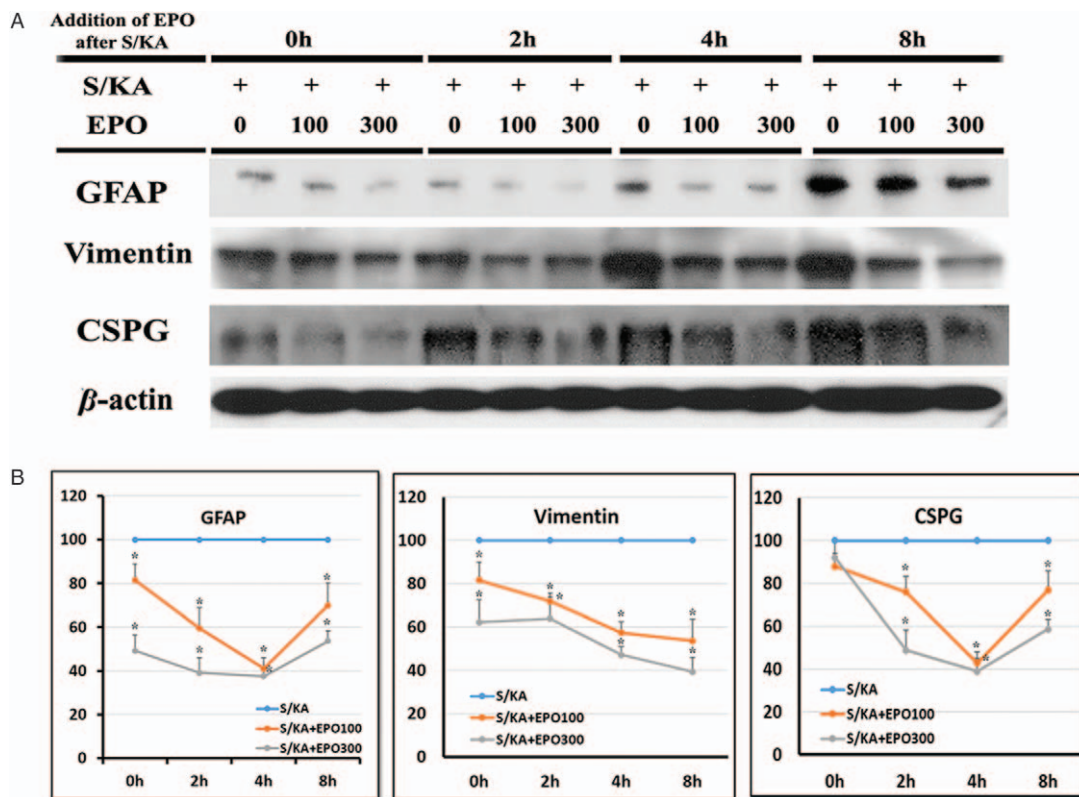
## 3. Results

### 3.1. EPO treatment prevents astroglial expression of neuroinhibitory molecules after model SCI

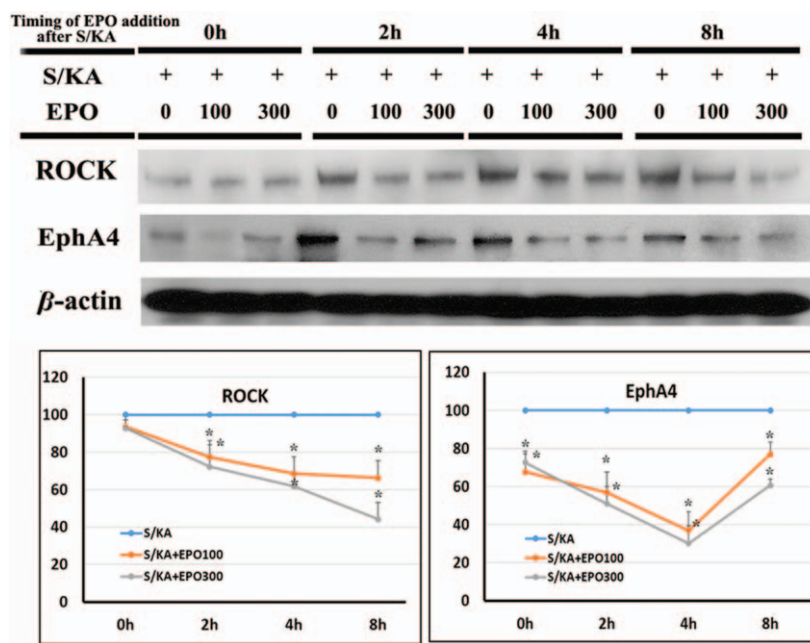
Western blotting revealed that EPO treatment reduced the expression of GFAP, vimentin, and CSPG compared to control at 48 hours after treatment. GFAP expression was decreased when EPO was applied immediately after injury (100U:  $81.1 \pm 8.3\%$ ; 300U:  $49.5 \pm 2.7\%$ ) and 4 hour after injury (100U:  $41.6 \pm 6.4\%$ ; 300U:  $38.6 \pm 9.7\%$ ) compared to the control level. Vimentin expression was most remarkably decreased when EPO was applied 8 hours after injury (300U:  $39.6 \pm 2.2\%$ ) while CSPG expression was most notably decreased by EPO treatment 4 hours after injury (300U:  $39.1 \pm 8.3\%$ ) compared to control. Thus, EPO treatment reduced GFAP and CSPG expression compared to control when applied up to 8 hours after injury (Fig. 1A and B).

### 3.2. EPO treatment decreases the expression of cytoskeletal protein regulators after model SCI

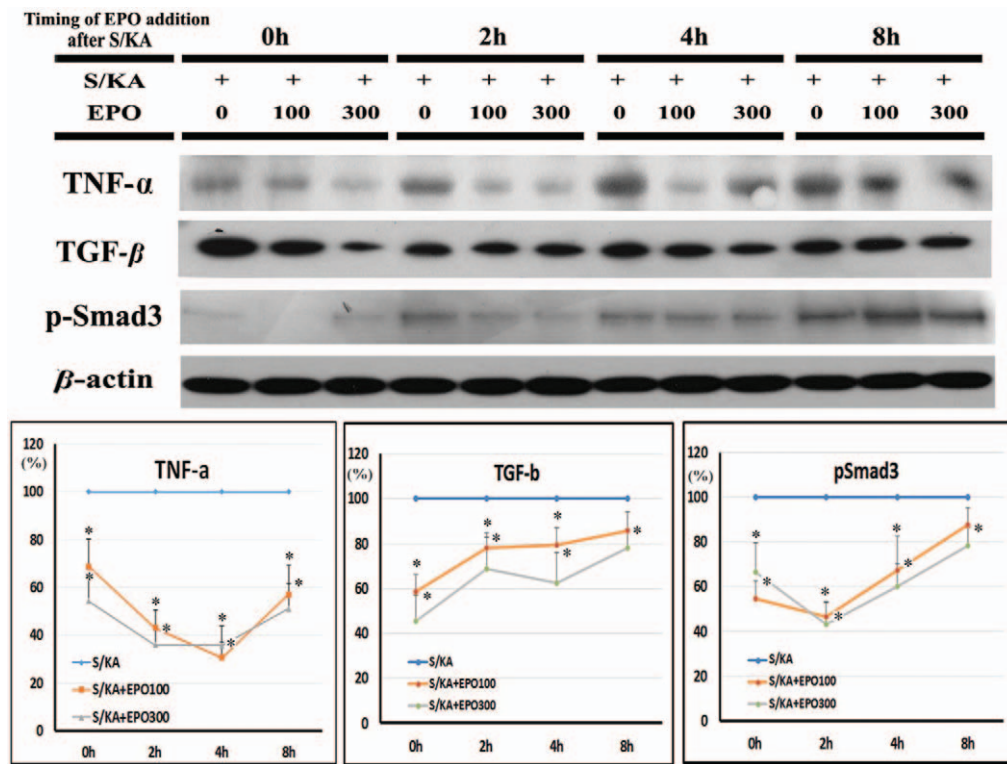
EPO treatment reduced the expression of ROCK compared to control when applied up to 8 hours after injury (100U:  $63.7 \pm 6.9\%$ ; 300U:  $43.0 \pm 7.6\%$ ). EPO treatment decreased EphA4 expression below that in control cells all time points with a maximum decrease observed at 4 hours after injury (100U:  $38.7 \pm 4.1\%$ ; 300U:  $29.0 \pm 6.6\%$ ); this decrease was still evident when EPO was applied 8 hours after injury (100U:  $78.4 \pm 3.9\%$ ; 300U:  $60.9 \pm 2.6\%$ ) (Fig. 2). Meanwhile, the effect of 300 U EPO on the expression of EphA4 at both 0 hour and 2 hours were opposed with the time point 4 and 8 hours.



**Figure 1.** Effects of EPO treatment on astroglial expression of axonal growth inhibiting molecules. (A) Western blots showing the relative expression of target proteins with  $\beta$ -actin as a loading control. (B) Quantification of bands shown in panel A relative to the control condition. Values represent the mean  $\pm$  standard deviation ( $n=3$  per time point in each group). CSPG=chondroitin sulfate proteoglycan, EPO=erythropoietin, GFAP=glial fibrillary acidic protein, S/KA=scratch and kainate injury model. \* indicates  $P < .001$  vs the S/KA group.



**Figure 2.** Effects of EPO on cytoskeletal protein regulatory molecule expression. Top: Western blots showing the relative expression of target proteins with  $\beta$ -actin as a loading control. Bottom: Quantification of bands shown in the top panel relative to the control condition. Values represent the mean  $\pm$  standard deviation ( $n=6$  per time point in each group). EphA4=ephrinA4, EPO=erythropoietin, ROCK=rho-associated protein kinase, S/KA=scratch and kainate injury model. \* indicates  $P < .001$  vs the S/KA group.



**Figure 3.** Effects of EPO on TNF- $\alpha$ , TGF- $\beta$ , and p-Smad3 expression. Top: Western blots showing the relative expression of target proteins with  $\beta$ -actin as a loading control. Bottom: Quantification of bands shown in the top panel relative to the control condition. Values represent the mean  $\pm$  standard deviation ( $n=6$  per time point in each group). EPO = erythropoietin, S/Ka = scratch and kainate injury model, TGF- $\beta$  = transforming growth factor-beta, TNF- $\alpha$  = tumor necrosis factor-alpha. \* indicates  $P < .001$  vs the S/Ka group.

### 3.3. EPO treatment decreases pro-inflammatory cytokine expression after model SCI

EPO treatment reduced the expression of TNF- $\alpha$ , TGF- $\beta$ , and p-Smad3 compared to control when applied up to 4 to 8 hours after injury, with a peak effect when applied 4 hours after injury. Effects on TGF- $\beta$  and p-Smad3 expression were not statistically significant when EPO was applied at 8 hours after injury (Fig. 3). Meanwhile, the effect of 300 U EPO on the expression of p-Smad3 at both 0 hour and 2 hours were opposed with the time point 4 and 8 hours and the effect of 300 U EPO on the expression of TNF- $\alpha$  at both 4 and 8 hours were opposed with the time point 0 hour and 2 hours.

### 3.4. Effects of EPO treatment on EpoR signaling after model SCI

EPO treatment increased EpoR expression nearly 2-fold compared to control when applied 2 hours after injury, but showed little-to-no effect when applied 4 hours after injury. In contrast, EPO treatment increased JAK2 expression when applied 4 hours after injury. EPO treatment also reduced the expression of phosphorylated protein kinase B (pAKT) when applied 4 hours after injury. Co-treatment with AG490 prevented EPO-mediated decreases in TGF- $\beta$  expression after application at 2 hours after injury and reductions in pAKT after application at 4 hours after injury (Fig. 4).

### 3.5. EPO treatment increases neurite outgrowth after model SCI

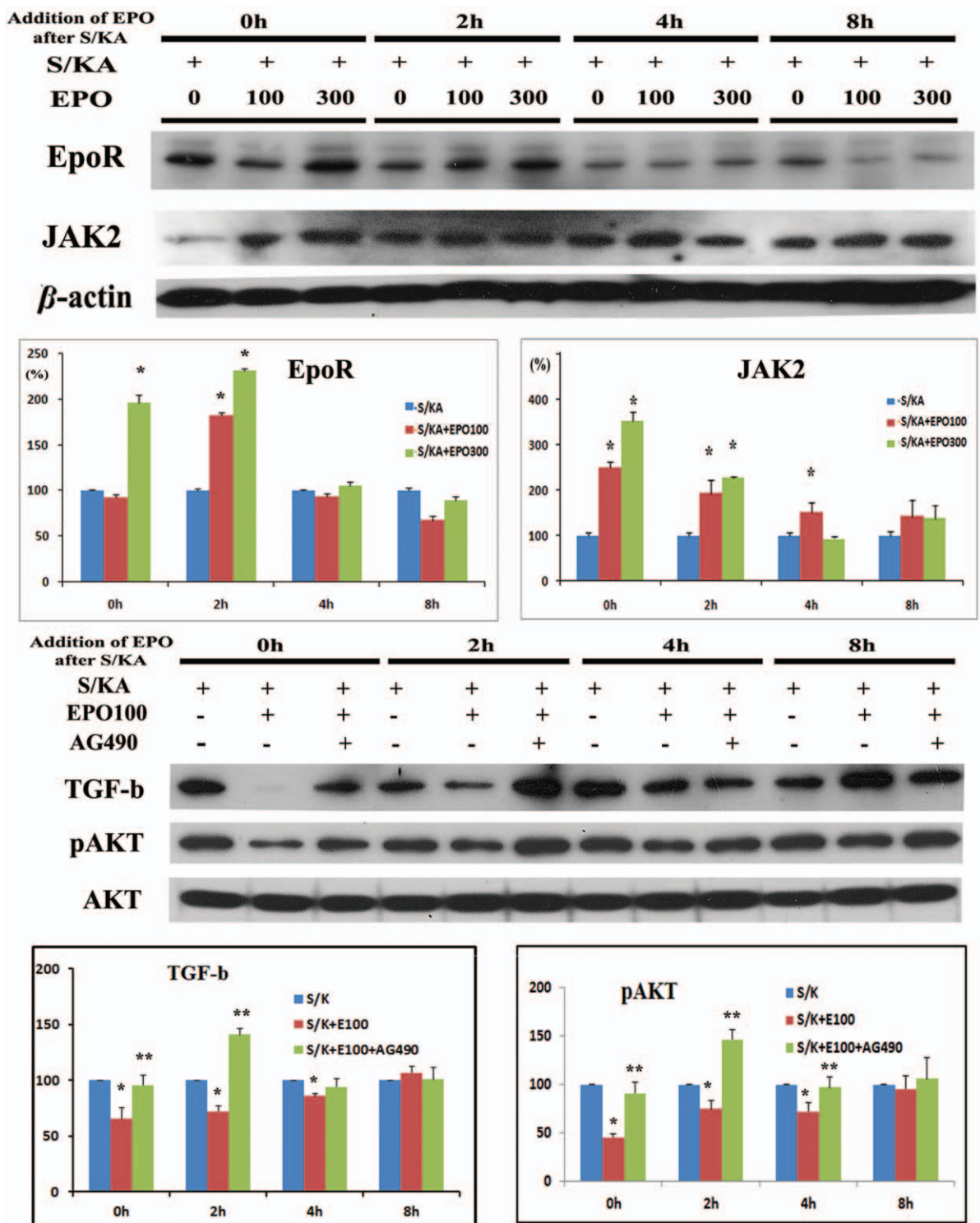
Spinal cord neurons grown in the absence of EPO were shorter and fewer in number compared to those grown in the presence of

EPO. EPO treatment dramatically increased the number of  $\beta$ -III tubulin-immuno-reactive axons when applied up to 4 hours after injury. EPO treatment immediately after injury increased neurite outgrowth by almost 13-fold ( $12.71 \pm 1.76$ ) compared to control, whereas treatment at 8 hours after injury had no significant effect on neurite outgrowth. Co-treatment anti-EpoR antibody prevented EPO-mediated increases in neurite outgrowth at all time points (Fig. 5A–C).

## 4. Discussion

In the present study, authors examined the ability of EPO to limit the extent of astrogliosis after modeled SCI in vitro and found that EPO applied up to 8 hours after injury regulated the expression of axonal growth inhibiting molecules, cytoskeletal regulatory proteins, pro-inflammatory cytokines, and EPO-EpoR signaling molecules. Additionally, EPO applied up to 4 hours after injury promoted neurite extension in seeded spinal neurons. Effects were dose dependent and partially prevented by co-application of AG490 or anti-EpoR antibody.

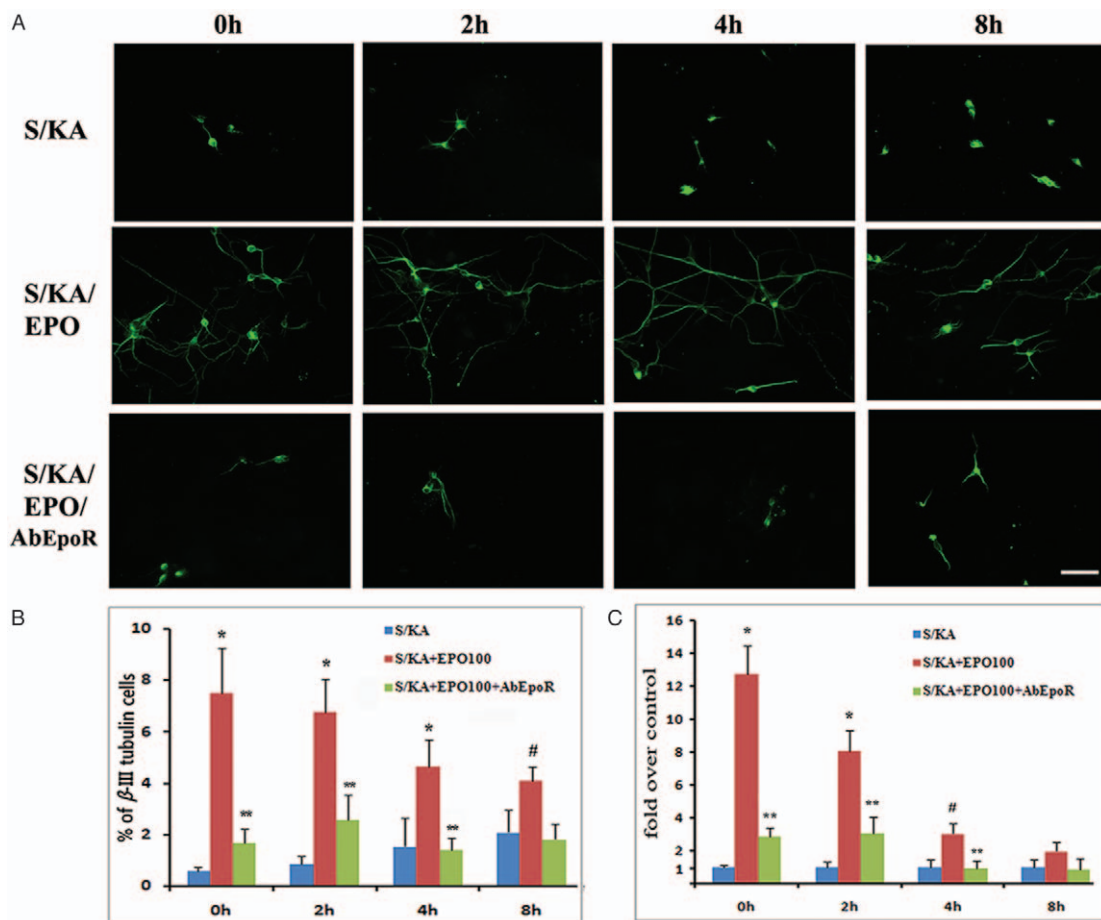
Authors' finding that EPO treatment mitigated increases of astrocytic GFAP, vimentin, and CSPG expression after injury is compatible with the findings reported in previous literature describing the anti-apoptotic and neuroprotective effects of EPO in SCI<sup>[12,13]</sup> as well as the reported ability of EPO to prevent gliosis.<sup>[14,15]</sup> Previous research has focused on the expression of neuron-glial antigen 2 (NG2) and phosphacan in glial scarring and demonstrated important roles for these molecules in the inhibition of neuronal regeneration after human SCI.<sup>[16]</sup> It was



**Figure 4.** Effects of EPO on EpoR and JAK2 expression. Top panels: Western blots showing the relative expression of target proteins with  $\beta$ -actin as a loading control (upper) and AKT as a total protein control (lower). Bottom panels: Quantification of bands shown in the top panel relative to the control condition. Values represent the mean  $\pm$  standard deviation ( $n=3$  per time point in each group). EPO=erythropoietin, EpoR=EPO receptor, JAK2=Janus kinase 2, pAKT=phosphorylated protein kinase B, S/KA=scratch and kainate injury model, TGF- $\beta$ =transforming growth factor-beta. \* indicates  $P < .001$  vs the S/KA group and \*\* indicates  $P < .001$  vs the S/KA + EPO100 U group.

reported that, among other CSPGs, phosphacan was selectively decreased by EPO treatment after SCI.<sup>[14,15]</sup> Our results challenge this notion and suggest that other glial scar components may be regulated by EPO treatment after SCI.

Authors also found that EPO treatment attenuated the expression of cytoskeletal regulatory proteins, ROCK, and EphA4, after modeled SCI. Astroglial activation is associated with increases in ROCK and ephrin expression, which regulate



**Figure 5.** Effects of EPO on neurite outgrowth. (A) Immunocytochemical staining with anti-Tuj1 antibody. (B, C) Quantification of immunofluorescence shown in panel A relative to the control condition. Values represent the mean  $\pm$  standard deviation ( $n=6$ ). Scale bar = 100  $\mu$ m. AbEpoR = anti-rhEPO receptor antibody, EPO = erythropoietin, S/KA = scratch and kainate injury model. \* indicates  $P < .001$  vs the S/KA group, \*\* indicates  $P < .001$  vs the S/KA + EPO100 U group, and # indicates  $P < .005$  v. the S/KA group.

cytoskeletal proteins via RhoA and Eph/ephrin signaling, respectively.<sup>[17,18]</sup> The EphA4 receptor is highly expressed on astrocytes following SCI and is a major inhibitor of neurite regrowth.<sup>[19]</sup> In contrast, ROCK is a member of a family of serine-threonine kinases that inhibits cell migration by promoting actin stabilization and the secondary loss of actin monomers. Accordingly, ROCK inhibitors such as Fasudil and Y-27632 increase neurite regeneration and outgrowth in various CNS disorders.<sup>[20]</sup> From this perspective, the inhibitory effects of EPO treatment on ROCK and EphA4 expression in astrocytes may be an important component of its neuroregenerative mechanism.

It is generally recognized that EPO exerts antiapoptotic and anti-inflammatory effects in the CNS disorders.<sup>[21,22]</sup> Here, authors found that EPO treatment reduced the expression of pro-inflammatory cytokines after modeled SCI. TNF- $\alpha$  is a cytokine involved in systemic inflammation and apoptosis. EPO has been previously reported to reduce the expression of IL-6 and TNF- $\alpha$  as well as astroglial reactivity.<sup>[12]</sup> TGF- $\beta$  stimulates astrocytes and fibroblasts to form glial scars.<sup>[23,24]</sup> Buss et al<sup>[25]</sup> reported that TGF- $\beta$ 1 expression was dramatically increased at 2 days after SCI and followed by the induction of TGF- $\beta$ 2 expression. This finding suggests a role for TGF- $\beta$ 1 in induction of acute inflammatory response and glial scar formation, and a role for TGF- $\beta$ 2 in glial scar maintenance. A recent study by Fang et al<sup>[26]</sup>

revealed that EPO treatment decreased TGF- $\beta$  expression after SCI and effectively prevented a number of injury-related pathological alterations. However, TGF- $\beta$  regulates many biological processes through the Smad and DAXX pathways.<sup>[27,28]</sup> On the basis of these findings and authors' results, authors hypothesize that the ability of EPO treatment to affect TGF- $\beta$  expression after model SCI is critical for preventing glial scar formation and facilitating neuroregeneration.

Protective effects of EPO in nonhematopoietic tissues are thought to be mediated by EPO-EpoR signaling and subsequent activation of JAK2 and other downstream mediators including signal transducer and activator of transcription (STAT) and PI3K/AKT.<sup>[7-9]</sup> In the current study, EpoR expression was increased when EPO was applied 2 hours after injury, whereas JAK2 expression was increased when by EPO was applied 4 hours after injury. Apparent dose-dependency and significant effects of EPO treatment on EpoR and JAK2 expression are consistent with previous studies investigating the neuroprotective effects of EPO. Authors' findings are also consistent with the observation of Ostroseki et al., who reported that AG490 co-treatment abolished the neuroprotective effects of EPO in cultured brain neuron.<sup>[7]</sup> Contrary to authors' expectation and previous reports, AKT expression was reduced when EPO was applied 4 hours after injury, and this effect was sensitive to

AG490 co-treatment. Miljus et al.<sup>[29]</sup> reported that AG490, but not PI3K inhibitor co-treatment, abolished the protective effects of EPO in insect brain neurons, and proposed that the PI3K/AKT signaling pathway was not involved in the mechanism of EPO mediated neuroprotection. Considering the heterogeneity of the nonhematopoietic EpoRs<sup>[30,31]</sup> and the phylogenetic age of these receptors,<sup>[7]</sup> undiscovered interactions in the EPO-EpoR pathway may account for the effects of EPO on astrocytes.

Finally, authors observed a facilitative effect of EPO on neurite outgrowth. Several previous reports have indicated a positive effect of EPO on axonal and/or dendritic outgrowth,<sup>[32-34]</sup> in agreement with authors' finding. However, previous studies utilized cultured brain cell and especially hippocampal cell, such that this is the first study to demonstrate an effect of EPO on the growth of spinal neurons. It is notable that the peak effect of EPO on neurite outgrowth was observed when EPO was applied immediately after injury, in contrast with other effects of EPO that peaked when EPO was applied at 2 or 4 hours after injury. This finding revives controversy about whether EPO inhibits further neural damage or promotes neuronal regeneration after SCI study.<sup>[35]</sup>

In case of being treated before injuries, EPO showed the dose-dependent, protective effects on hippocampal, cortical neurons and spinal neurons on normal conditions in wide range of concentration (0–30 pM or 0–100 U) in a study by Morishita et al and by Yoo et al.<sup>[36,37]</sup> However, at higher concentration (300 pM or 200–300 U), it showed no dose-dependency and even revealed decreased effects. It might happen by super-saturation or ceiling effects of EpoRs. As thermal injuries can induce denaturation of EpoRs,<sup>[38]</sup> chemical or mechanical injuries can also make any changes in structure of EpoRs.<sup>[39]</sup> In the present study, the threshold of supersaturation or ceiling effects might be shifted so that subsequent dose-dependency could be noticed again at higher concentrations (300 U). However, the effect of 300 U EPO on the expression of p-Smad3, EphA4, and TNF- $\alpha$  at some time points was opposed with the different time points. It might take different time, depending on the different downstream cascades (ROCK vs EphA4 and TNF- $\alpha$  vs p-Smad3), how quickly denatured EpoR readapt to changes in molecular or genetic levels. In addition, heterogeneity of the non-hematopoietic EpoRs with the different phylogenetic age could booster these diversities.<sup>[7,30,31]</sup>

The present work had some limitations. First, despite authors' efforts to simulate SCI in vitro, it is notable that various neuronal populations react differently to injury, and even similarly sized neurons within a population can exhibit different responses to astrocytic scar-like co-cultures.<sup>[40,41]</sup> Thus, authors' model was not a definitively reliable representation of SCI. Second, it is difficult to compare authors' findings with those of previous studies, as previous studies have used: different rat strains, sexes, and weights; various methods and devices for SCI induction; and various schedules of therapeutic treatment.<sup>[42]</sup> No positive control for the EPO and no control group for the EPO on normal conditions are another issue. It is clear that delay between EPO and SCI in part mediates the neuro-protective efficacy of treatment. Therefore, future studies should expand our findings to an in vivo model of SCI.

## 5. Conclusions

The application of EPO to astrocytic cultures after modeled SCI enhanced EPO-EpoR signaling and specifically JAK signaling, but not PI3K/AKT signaling. These changes were associated with

decreased TNF- $\alpha$ , TNF- $\beta$ , and Smad expression and the reduced expression of cytoskeletal regulatory proteins ROCK and EphA4. Finally, EPO treatment promoted neurite outgrowth when applied within 4 hours after injury. Our findings suggest that it may be effective if provided around 2 to 4 hours after injury in the inhibition of further neuronal deterioration point of view, and it may be optimal if administered immediately after injury in the neuro-regenerative point of view.

## References

- Silver J, Miller JH. Regeneration beyond the glial scar. *Nat Rev Neurosci* 2004;5:146–56.
- Voskuhl RR, Peterson RS, Song B, et al. Reactive astrocytes form scar-like perivascular barriers to leukocytes during adaptive immune inflammation of the CNS. *J Neurosci* 2009;29:11511–22.
- Fitch MT, Silver J. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Exp Neurol* 2008;209:294–301.
- Dergham P, Ellezam B, Essagian C, et al. Rho signaling pathway targeted to promote spinal cord repair. *J Neurosci* 2002;22:6570–7.
- Arocho LC, Figueroa JD, Torrado AI, et al. Expression profile and role of EphrinA1 ligand after spinal cord injury. *Cell Mol Neurobiol* 2011;31:1057–69.
- Juul S. Erythropoietin in the central nervous system, and its use to prevent hypoxic-ischemic brain damage. *Acta Paediatr Suppl* 2002;91:36–42.
- Ostrowski D, Ehrenreich H, Heinrich R. Erythropoietin promotes survival and regeneration of insect neurons in vivo and in vitro. *Neuroscience* 2011;188:95–108.
- Kamishimoto J, Tago K, Kasahara T, et al. Akt activation through the phosphorylation of erythropoietin receptor at tyrosine 479 is required for myeloproliferative disorder-associated JAK2 V617F mutant-induced cellular transformation. *Cell Signal* 2011;23:849–56.
- Maiese K. Regeneration in the nervous system with erythropoietin. *Front Biosci (Landmark Ed)* 2016;21:561–96.
- Yoo JY, Hwang CH, Hong HN. A model of glial scarring analogous to the environment of a traumatically injured spinal cord using kainate. *Ann Rehabil Med* 2016;40:757–68.
- Yang H, Cheng XP, Li JW, et al. De-differentiation response of cultured astrocytes to injury induced by scratch or conditioned culture medium of scratch-insulted astrocytes. *Cell Mol Neurobiol* 2009;29:455–73.
- Villa P, Bigini P, Mennini T, et al. Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis. *J Exp Med* 2003;198:971–5.
- Matis GK, Birbilis TA. Erythropoietin in spinal cord injury. *Eur Spine J* 2009;18:314–23.
- Vitellaro-Zuccarello L, Mazzetti S, Madaschi L, et al. Erythropoietin-mediated preservation of the white matter in rat spinal cord injury. *Neuroscience* 2007;144:865–77.
- Vitellaro-Zuccarello L, Mazzetti S, Madaschi L, et al. Chronic erythropoietin-mediated effects on the expression of astrocyte markers in a rat model of contusive spinal cord injury. *Neuroscience* 2008;151:452–66.
- Buss A, Pech K, Kakulas BA, et al. NG2 and phosphacan are present in the astroglial scar after human traumatic spinal cord injury. *BMC Neurol* 2009;9:32.
- Chan CC, Wong AK, Liu J, et al. ROCK inhibition with Y27632 activates astrocytes and increases their expression of neurite growth-inhibitory chondroitin sulfate proteoglycans. *Glia* 2007;55:369–84.
- Puschmann TB, Turnley AM. Eph receptor tyrosine kinases regulate astrocyte cytoskeletal rearrangement and focal adhesion formation. *J Neurochem* 2010;113:881–94.
- Goldshmit Y, Galea MP, Wise G, et al. Axonal regeneration and lack of astrocytic gliosis in EphA4-deficient mice. *J Neurosci* 2004;24:10064–73.
- Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* 2003;4:446–56.
- Agnello D, Bigini P, Villa P, et al. Erythropoietin exerts an anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis. *Brain Res* 2002;952:128–34.
- Gorio A, Gokmen N, Erbayraktar S, et al. Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma. *Proc Natl Acad Sci U S A* 2002;99:9450–5.

- [23] Hirano S, Yonezawa T, Hasegawa H, et al. Astrocytes express type VIII collagen during the repair process of brain cold injury. *Biochem Biophys Res Commun* 2004;317:437–43.
- [24] Ihn H. Autocrine TGF-beta signaling in the pathogenesis of systemic sclerosis. *J Dermatol Sci* 2008;49:103–13.
- [25] Buss A, Pech K, Kakulas BA, et al. TGF-beta1 and TGF-beta2 expression after traumatic human spinal cord injury. *Spinal Cord* 2008;46:364–71.
- [26] Fang XQ, Fang M, Fan SW, et al. Protection of erythropoietin on experimental spinal cord injury by reducing the expression of thrombospondin-1 and transforming growth factor-beta. *Chin Med J (Engl)* 2009;122:1631–5.
- [27] Zhang Y, Zhang J, Navrazhina K, et al. TGFbeta1 induces Jagged1 expression in astrocytes via ALK5 and Smad3 and regulates the balance between oligodendrocyte progenitor proliferation and differentiation. *Glia* 2010;58:964–74.
- [28] Zode GS, Sethi A, Brun-Zinkernagel AM, et al. Transforming growth factor-beta2 increases extracellular matrix proteins in optic nerve head cells via activation of the Smad signaling pathway. *Mol Vis* 2011;17:1745–58.
- [29] Miljus N, Heibeck S, Jarrar M, et al. Erythropoietin-mediated protection of insect brain neurons involves JAK and STAT but not PI3K transduction pathways. *Neuroscience* 2014;258:218–27.
- [30] Sanchez PE, Fares RP, Risso JJ, et al. Optimal neuroprotection by erythropoietin requires elevated expression of its receptor in neurons. *Proc Natl Acad Sci U S A* 2009;106:9848–53.
- [31] Dumont F, Bischoff P. Non-erythropoietic tissue-protective peptides derived from erythropoietin: WO2009094172. *Expert Opin Ther Pat* 2010;20:715–23.
- [32] Choi M, Ko SY, Lee IY, et al. Carbamylated erythropoietin promotes neurite outgrowth and neuronal spine formation in association with CBP/p300. *Biochem Biophys Res Commun* 2014;446:79–84.
- [33] Oh DH, Lee IY, Choi M, et al. Comparison of neurite outgrowth induced by erythropoietin (EPO) and carbamylated erythropoietin (CEPO) in hippocampal neural progenitor cells. *Korean J Physiol Pharmacol* 2012;16:281–5.
- [34] Ransome MI, Turnley AM. Erythropoietin promotes axonal growth in a model of neuronal polarization. *Mol Cell Neurosci* 2008;38:537–47.
- [35] Kwon BK, Okon EB, Plunet W, et al. A systematic review of directly applied biologic therapies for acute spinal cord injury. *J Neurotrauma* 2011;28:1589–610.
- [36] Morishita E, Masuda S, Nagao M, et al. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. *Neuroscience* 1997;76:105–16.
- [37] Yoo JY, Won YJ, Lee JH, et al. Neuroprotective effects of erythropoietin posttreatment against kainate-induced excitotoxicity in mixed spinal cultures. *J Neurosci Res* 2009;87:150–63.
- [38] Narhi LO, Aoki KH, Philo JS, et al. Changes in conformation and stability upon formation of complexes of erythropoietin (EPO) and soluble EPO receptor. *J Protein Chem* 1997;16:213–25.
- [39] Espada J, Brandan NC, Dorado M. Effect of chemical and enzymatic agents on the biological activity of erythropoietin. *Acta Physiol Lat Am* 1973;23:193–201.
- [40] Wanner IB, Deik A, Torres M, et al. A new in vitro model of the glial scar inhibits axon growth. *Glia* 2008;56:1691–709.
- [41] Ondarza AB, Ye Z, Hulsebosch CE. Direct evidence of primary afferent sprouting in distant segments following spinal cord injury in the rat: colocalization of GAP-43 and CGRP. *Exp Neurol* 2003;184:373–80.
- [42] Mann C, Lee JH, Liu J, et al. Delayed treatment of spinal cord injury with erythropoietin or darbepoetin—a lack of neuroprotective efficacy in a contusion model of cord injury. *Exp Neurol* 2008;211:34–40.