The Sonic Hedgehog Pathway Mediates Carbamylated Erythropoietin-enhanced Proliferation and Differentiation of Adult Neural Progenitor Cells*

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Carbamylated erythropoietin (CEPO), a well characterized erythropoietin (EPO) derivative, does not bind to the classical EPO receptor and does not stimulate erythropoiesis. Using neural progenitor cells derived from the subventricular zone of the adult mouse, we investigated the effect of CEPO on neurogenesis and the associated signaling pathways in vitro. We found that CEPO significantly increased neural progenitor cell proliferation and promoted neural progenitor cell differentiation into neurons, which was associated with up-regulation of Sonic hedgehog (Shh), its receptor ptc, and mammalian achaete-scute homolog 1 (Mash1), a pro-neuron basic helix-loop-helix protein transcription factor. Blockage of the Shh signaling pathway with a pharmacological inhibitor, cyclopamine, abolished the CEPO-induced neurogenesis. Attenuation of endogenous Mash1 expression by short-interfering RNA blocked CEPO-promoted neuronal differentiation. In addition, recombinant mouse Shh up-regulated Mash1 expression in neural progenitor cells. These results demonstrate that the Shh signaling pathway mediates CEPO-enhanced neurogenesis and Mash1 is a downstream target of the Shh signaling pathway that regulates CEPO-enhanced neuronal differentiation.

Ischemic stroke induces neurogenesis (1–4). Erythropoietin (EPO)2 has neuroprotective effects for treatment of acute stroke via interaction with its receptor (EPOR) (5). Recent studies show that EPO increases neurogenesis in the subventricular zone (SVZ) of adult rodent brain under normal and stroke conditions via interaction with EPOR (6–8). Systemic administration of EPO enhances stroke-induced neurogenesis (6). However, EPO also elevates hematocrit levels, which could lead to adverse effects on stroke recovery (6, 9).

Carbamylated erythropoietin (CEPO), a non-erythropoietic derivative of EPO that does not bind to the classical EPOR (10), is neuroprotective for acute stroke but does not elevate hematocrit levels. The effect of CEPO on neurogenesis has not been investigated.

Sonic hedgehog (Shh) is a member of the family of the hedgehog proteins known to exert important regulatory functions in patterning and growth in a large number of tissues during embryogenesis (11–13). In the mammalian brain, Shh plays an important role for the regulation of progenitor cell proliferation and differentiation (14–17). Shh binds to the transmembrane receptor protein patched (ptc), which, in the absence of Shh, exerts an inhibitory effect on the seven-transmembrane receptor smoothened (Smo) (18, 19). Binding of Shh to ptc blocks the inhibitory effect of ptc on Smo. Once activated, Smo induces a complex series of intracellular reactions that target the Gli family of transcription factors (11). Gli1 is the principal effector of Shh signaling in neural progenitor cells (20). In the present study, using neural progenitor cells derived from the SVZ of adult mice, we tested the hypothesis that CEPO via Shh signaling promotes proliferation and differentiation of neural progenitor cells.

EXPERIMENTAL PROCEDURES

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. Male mice (C57BL6/J, 6–8 weeks) were purchased from The Jackson Laboratory (Bar Harbor, ME). EPOR null mice (ΔEPOR mice, C57BL6 background) were provided by Dr. Constance Tom Noguchi at NIDDK, National Institutes of Health (44).

Neurosphere Culture—SVZ neural progenitor cells were dissociated from normal (n = 10) and ΔEPOR mice (n = 10) as previously reported (21, 22). The cells were plated at a density of 2 × 10^4 cells/ml in growth medium. Growth medium contains Dulbecco’s modified Eagle’s-F-12 medium (Invitrogen), 20 ng/ml of epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN), and 20 ng/ml of basic fibroblast growth factor...
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(bFGF) (R&D Systems). Dulbecco’s modified Eagle’s-F-12 medium contains l-glutamine (2 mM), glucose (0.6%), putrescine (9.6 μg/ml), insulin (0.025 mg/ml), progesterone (6.3 ng/ml), apo-transferrin (0.1 mg/ml), and sodium selenite (5.2 ng/ml). The generated neurospheres (primary sphere) were passaged by mechanical dissociation and reseeded as single cells at a density of 20 cells/μl in bFGF- and EGF-containing media (passage 1 cells). Passage 1 cells were processed for various experiments in the present study. These cells were cultured in reduced growth medium containing 10 ng/ml of EGF and 10 ng/ml of bFGF.

To analyze the formation of secondary neurospheres, passage 1 neurospheres were collected and digested with 0.05% trypsin-EDTA (Invitrogen) for 5 min at 37 °C. They were then gently triturated with a fire-narrowed Pasteur pipette, spun down at 400 rpm for 3 min, resuspended in the reduced growth medium containing 2% fetal bovine serum but without the growth factors for 7 days. This medium was referred to as the reduced growth medium 18 h prior to termination of incubation. The generated neurospheres were counted as single cells. These cells at a density of 20 cells/ml in each well of a 24-well plate (Corning). The number of neurospheres was counted at 7 days in vitro.

To analyze cell proliferation, bromodeoxyuridine (BrdUrd) (20 μg/ml), the thymidine analog that is incorporated into the DNA of dividing cells during S-phase was added into the reduced growth medium 18 h prior to termination of incubation. BrdUrd-positive cells were measured.

To dynamically measure dividing neural progenitor cells, time-lapse microscopy was employed (23). Cultured neurospheres were incubated in a stage top chamber with 5% CO2 and 37 °C (LiveCell Control Unit), which was placed on the stage of a Nikon TE2000-U inverted microscope equipped with a motorized Z stage. A ×10 objective with ×1.5 electronic zoom was used for acquiring images. A stack of images (30 images with a 5-μm step in z-axes) were acquired at 15-min intervals for a total of 20 h using a CCD camera (CoolSnap, 5000) and MetaView software (Universal Imaging, West Chester, PA) (23).

To analyze phenotypes of neural progenitor cells, neurospheres were mechanically dissociated as single cells. These cells (2.5 × 104 cells/cm2) were plated directly onto laminin-coated glass coverslips in Dulbecco’s modified Eagle’s-F-12 medium containing 2% fetal bovine serum but without the growth factors for 7 days. This medium was referred to as the differentiation medium. Immunocytochemistry was performed with various antibodies (see below) to determine phenotypes of neural progenitor cells.

Experimental Protocol—1) To examine whether Shh/Gli signaling pathway is involved in the effects of CEPO on neurogenesis, neural progenitor cells were incubated in the presence of CEPO (0, 1, 10, 100 ng/ml) (H. Lundbeck A/S, Denmark) with or without cyclopamine (a specific inhibitor of Smo, 5 μM; Calbiochem, San Diego, CA). Neural progenitor cell proliferation and differentiation and activation of the Shh/Gli pathway were measured. In addition, neural progenitor cells were transfected with mouse Gli1 siRNA and incubated with CEPO (10 ng/ml). Neuronal differentiation and Mash1 expression, neural progenitor cells were incubated in the presence of recombinant mouse Shh (Shh- N terminus, 0, 10, 100, 1000 ng/ml; RR&D Systems) with or without cyclopamine (5 μM).

Mouse siRNA Synthesis and Transfection—Mouse Mash1 siRNA cassettes were designed according to the Mouse Mash1 sequence in GenBank™ (NM_008553) using siRNA target finder (GenScript Corp., Piscataway, NJ). The selected sequences were chemically synthesized, and the cassettes were constructed by PCR, which consists of a 505-bp human H1 promoter and terminator sequence flanking a DNA insert encoding a small hairpin RNA (GenScript Corp.). A BLAST search against the mouse genome was performed for the specificity of all target sequences and the scrambled sequences. All cassettes were labeled at the 5’-end with Cy3 for control of transfection efficiency (Fig. 7). Mouse Gli1 siRNA was purchased from Dharmaco, Inc. Neural progenitor cells were transfected using the Mouse NSC Nucleofector™ kit (Amax Inc.) following the manufacturer’s instructions. The total amount of siRNA per transfection was kept constant to 0.5 μg/ml. mRNA and protein levels were measured 48 and 72 h after transfection.

Real-time RT-PCR—Quantitative PCR was performed using the SYBR Green real-time PCR method (24). Total RNA was isolated from neural progenitor cell cultures using the Stratagene Absolutely RNA MicroRNA isolation kit (La Jolla, CA). Quantitative RT-PCR was performed on an ABI 7000 PCR instrument (Applied Biosystems, Foster City, CA) using three-stage program parameters provided by the manufacturer, as follows; 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during PCR. PCR products were run on 2% agarose gels to confirm that correct molecular sizes were present. Each sample was tested in triplicate, and samples obtained from three independent experiments were used for analysis of relative gene expression using the 2−ΔΔCT method (25). The following primers for real-time PCR were designed using Primer Express software (ABI): Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (FWD, AGA ACA TCA TCC CTG CAT CC; REV, CAC ATT GGG GGT AGG AAC AC); Shh (FWD, CCT TTA CCC TAC AAG CAG TTT ATT GC; REV, GTA ATT GGG GGT GAG TCT AAA TC); ptcI (FWD, TAG CGC CTT CTT TTG GA; REV, GTG GAA GTT GGT GGA CGA GT); Gli1 (FWD, TCC ACA CGC CCC CTA GTG; REV, TGG CAA CAT TTT CGG TGA TG); Mash1 (FWD, TCT CCT GGG AAT CGG CTA TTG GAT TGC); Immunocytochemistry and Quantification—Single and double immunofluorescent stainings of cultured cells were performed as previously described (2, 26). The following primary antibodies were used in the present study: mouse anti-BrdUrd (1:100; Roche Applied Science), mouse anti-β-tubulin III (TuJ-1, 1:500; Covance), mouse anti-microtubule-associated...
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protein 2 (MAP2) (1:200; Chemicon), mouse anti-NeuN (1:100; Chemicon), rabbit anti-glia fibrillary acidic protein (GFAP) (1:500; Dako Cytomation, Carpinteria, CA), rabbit anti-Gli1 (1:300; Abcam Inc. Cambridge, MA), rabbit anti-nestin (1:100; BD Biosciences), rabbit anti-SOX2 (1:50; Santa Cruz Biotechnology), and mouse anti-Mash1 monoclonal (1:250; BD Biosciences). Cultured cells were fixed in 4% paraformaldehyde for 15–20 min at room temperature. Nonspecific binding sites were blocked with 5% normal goat serum for 60 min at room temperature. The cells were then incubated with the primary antibodies listed above and with CY3-conjugated or fluorescein isothiocyanate-conjugated secondary antibodies. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). The number of BrdUrd-, TuJ1-, MAP2-, NeuN-, and GFAP-positive cells and total DAPI cell number were counted, and the percentage of each cell type was determined.

**Western Blot Analysis**—Western blots were performed according to published methods (27). Briefly, lysates from neural progenitor cells were sonicated for 10 s and centrifuged at 14,000 × g for 20 min. Protein concentration in the supernatants of cell extract was determined using a BCA protein assay kit (Pierce Biotechnology, Inc.). Equal amounts of proteins were loaded on 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes, and the blots were subsequently probed with the following antibodies: Shh (N-19) (1:200; Santa Cruz Biotechnology), patched (G-19) (1:200; Santa Cruz Biotechnology), Gli1 (1:5000; Abcam Inc.), and Mash1 (1:250; BD Biosciences). For detection, horseradish peroxidase-conjugated secondary antibodies were used (1:2000) followed by enhanced chemiluminescence development (Pierce). Normalization of results was ensured by running parallel Western blots with β-actin antibody. The optical density was quantified using an image processing and analysis program (Scion Image, Ederick, MA).

**Measurements of Neurite Outgrowth**—To analyze neurite outgrowth, TuJ1-positive cells were digitized using a ×20 objective (Zeiss) via the MCID computer imaging analysis system (Imaging Research, St. Catharines, Ontario, Canada). Neurite outgrowth was quantified using a software program developed in our laboratory that includes measurements of the number, length, and diameter of branches (27). Fifty TuJ1-positive cells per group were measured, and all measurements were performed by experimenters blinded to each culture condition.

**Statistical Analysis**—One-way analysis of variance followed by Student-Newman-Keuls test was performed. The data were presented as means ± S.E. A value of *p* < 0.05 was taken as significant.

**RESULTS**

**CEPO Induces Proliferation and Differentiation of Neural Progenitor Cells**—To examine the effect of CEPO on adult neural progenitor cells, we employed a neurosphere assay that has been widely used for investigating the biology of neural progenitor cells (24, 28–31). When neural progenitor cells harvested from the SVZ of the adult mouse were plated at a density of 20 cells/μl on the non-adhesive culture surface in the reduced growth medium (10 ng/ml of bFGF and 10 ng/ml of EGF), these cells formed spheres 7 days *in vitro*. The vast majority of cells in neurospheres were nestin-immunoreactive (98.8 ± 0.3%, Fig. 1A) and Sox2-immunoreactive (74.6 ± 0.34%, Fig. 1B), markers of neural progenitor cells. Double immunostaining revealed that 69 ± 0.02% of cells in neurospheres were BrdUrd-positive, an index of proliferating cells, and nestin-positive, suggesting that most of the cells are proliferating. When single cells dissociated from neurospheres were reseeded on laminin-coated glass coverslips (2.5 × 10^4^ cells/ml) in medium without the growth factors for 7 days, these cells differentiated into TuJ1-positive (Fig. 1C), a marker of immature neurons, GFAP-positive, a marker of astrocytes (Fig. 1D), and O4-positive cells, a marker of oligodendrocytes (Fig. 1E). However, NeuN-positive cells, a marker of mature neurons, were not detected until 14 days in culture, which is consistent with published studies (32). These data indicate that SVZ cells have the capacity of self-renewal and undergo multilineage differentiation, characteristics of neural progenitor cells (33). We then determined whether CEPO promotes neural progenitor cell proliferation. Single SVZ cells were cultured in the reduced growth medium containing CEPO (0, 1, 10, 100 ng/ml). Exposure of single SVZ cells to CEPO (10 and 100 ng/ml) resulted in a significant (*p* < 0.05) increase in the number and size of primary neurospheres (Fig. 2, B, G, and H) compared with that in the control group (Fig. 2, A, G, and H). We further measured the number of the secondary neurospheres in which the CEPO-treated neurospheres were dissociated and reseeded at 1 × 10^4^ cells/ml in the reduced growth medium. Compared with the control group, the number of the secondary neurospheres derived from the CEPO-treated (10 ng/ml) group was significantly (*p* < 0.05) increased (459 ± 14%, *n* = 8 versus 349 ± 18%, *n* = 8, in control). Furthermore, treatment with...
CEPO significantly \((p < 0.05)\) increased the number of BrdUrd-positive cells (Fig. 2, E and I) and the number of BrdUrd- and nestin-positive cells (Fig. 2, J and K), suggesting that increases of number and size of neurospheres result from an increase of progenitor cells but are not induced by an increase of cell aggregation. In parallel, time-lapse microscopic analysis revealed that 15 cells that migrated out of the neurosphere were dividing in the CEPO \((10 \text{ ng/ml})\) group during a 20-h experimental period, whereas only 4 dividing cells were detected in the control group at the same time period. Collectively, these results demonstrate that CEPO augments neural progenitor cell proliferation.

We next determined the effect of CEPO on neural progenitor cell differentiation. Single cells dissociated from neurospheres were reseeded on laminin-coated glass coverslips \((2.5 \times 10^4\) cells/ml) and treated with CEPO \((0, 1, 10, 100 \text{ ng/ml})\) for 7 days in differentiation medium without the growth factors. Treatment with CEPO \((10 \text{ and } 100 \text{ ng/ml})\) significantly increased the number of TuJ1-positive (Fig. 3, B and C) and MAP2-positive \((3.8 \pm 0.01\%\), \(n = 8\) in CEPO \(10 \text{ ng/ml}\) versus \(1.7 \pm 0.04\%\), \(n = 8\), in control) cells and decreased the number of GFAP-positive cells, although it did not reach statistical significance compared with the number in the untreated group (Fig. 3, A and C). The increase of TuJ1-positive cells could reflect a decrease of cell death. To address the possibility that CEPO may act as a survival factor for neural progenitor cells, we measured the number of terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL)-positive cells and found that the number of TUNEL-positive cells in the CEPO group \((12 \pm 1.2\%\), \(n = 4\)) was not significantly different from the number in the control group \((11 \pm 1.1\%\), \(n = 4\)). These data suggest that CEPO selectively enhances neural progenitor cell differentiation into neurons.

To examine whether CEPO enhances neurite outgrowth, neurite outgrowth was quantified by measuring the length and number of branches extending from TuJ1-positive cell soma. In the control group, TuJ1-positive cells exhibited a few short branches (Fig. 4E). However, TuJ1-positive cells exhibited complex branching patterns when neural progenitor cells were incubated with CEPO (Fig. 4F). Quantitative analysis revealed that treatment with CEPO significantly \((p < 0.05)\) increased the number and length of branches (Fig. 4, J and K), suggesting that CEPO promotes neurite outgrowth.

We then measured the effect of CEPO on SVZ cells harvested from EPOR mice. Treatment with EPO \((10 \text{ units/ml})\) significantly \((p < 0.05)\) increased the number of TuJ1-positive cells in neural progenitor cells derived from wild-type mice \((4 \pm 0.32\%\), \(n = 8\) versus \(2 \pm 0.2\%\), \(n = 8\), in control) but did not increase the number of TuJ1-positive cells in EPOR neural progenitor cells \((3 \pm 0.5\%\), \(n = 8\) versus \(3 \pm 0.3\%\), \(n = 8\), in control), which is consistent with our previous studies (6, 27). However, treatment of EPOR neural progenitor cells with CEPO \((10 \text{ ng/ml})\) significantly \((p < 0.05)\) increased the number of TuJ1-positive cells \((5 \pm 0.5\%\), \(n = 8\)) compared with the number in the control group \((3 \pm 0.3\%\), \(n = 8\)). These results suggest that EPOR is not required for CEPO-enhanced neurogenesis.

The Shh/Gli1 Signaling Pathway Regulates the Effect of CEPO on Neural Progenitor Cell Proliferation and Differentiation—The Shh/Gli1 signaling pathway is required for regulating neurogenesis in the adult rodent brain (20, 34). To determine whether CEPO induces Shh/Gli1 signaling activation, we
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FIGURE 4. CEPO enhances neuronal differentiation and neurite outgrowth. Panels A–H are representative images of TuJ1-immunoreactive cells (red) and DAPI nuclei (blue). Treatment with CEPO significantly ($p < 0.05$) increased the numbers of TuJ1-positive cells (B, F and I) and neurite outgrowth (F, J, and K) compared with the control group (A, E, J, and K), whereas siRNA-Mash1 (C, G, I, J, and K) or cyclopamine (D, H, I, and K) blocked CEPO-increased TuJ1 cells (C, D, I, and J) and neurite outgrowth (G, H, I, and K). Panels I–K are quantitative data showing the percentage of TuJ1-positive cells (I), total branch length of neurites (J), and total branch number of neurites (K). *, $p < 0.05$ and #, $p < 0.05$ versus the control and CEPO groups, respectively. Bar, 100 μm. $n = 8$ for each group. +Si, siRNA-Mash1 cassettes; +SS, scrambled cassettes. CY, cyclopamine.

examined expression of Shh, its receptors, and Gli1. Real-time RT-PCR and Western blot analysis revealed that neural progenitor cells expressed Shh, ptc, and Gli1 and treatment with CEPO significantly up-regulated these genes (Fig. 5, A and B). However, in the absence of EGF or bFGF, CEPO failed to augment mRNA levels of Shh (1.09 ± 0.07 versus 1.0 ± 0.09 in control), ptc (0.9 ± 0.03 versus 1.0 ± 0.08 in control), and Gli1 (0.9 ± 0.12 versus 1.0 ± 0.16 in control), suggesting that CEPO activates the Shh pathway in cooperation with EGF and bFGF. Moreover, CEPO in the presence of 20 ng/ml EGF and 20 ng/ml bFGF did not significantly increase the number of neurospheres (442 ± 18, $n = 8$) compared with the number in control group (379 ± 16.9, $n = 8$). These data are consistent with findings that EGF at lower concentration has a synergistic effect with Shh in the control of proliferation of SVZ neural progenitor cells (16). To test whether Shh signaling is necessary for CEPO to promote proliferation and differentiation of neural progenitor cells, we treated neural progenitor cells with CEPO in the presence of the Shh antagonist cyclopamine. Application of cyclopamine (5 μM) abolished the ability of CEPO to induce ptc and Gli1 expression but did not suppress CEPO-induced Shh expression (Fig. 5, A and B). In addition, cyclopamine significantly reduced the numbers of CEPO-increased BrdUrd-positive (Fig. 2, F and J) and TuJ1-positive (Fig. 4, D and I) cells compared with the numbers in the control group. Furthermore, cyclopamine blocked CEPO-induced neurite outgrowth (Fig. 4, H, J, and K). These data indicate that the Shh/Gli1 pathway regulates CEPO-enhanced neuronal progenitor cell proliferation and differentiation.

To directly examine the effect of Shh on neural progenitor cell proliferation and differentiation, we measured the number of BrdUrd- and TuJ1-positive cells after treatment with Shh-N. At a concentration of 5 ng/ml EGF, Shh has a synergistic effect on neural progenitor cells (16). Therefore, SVZ neural progenitor cells were cultured in the growth medium containing EGF (5 ng/ml) with or without Shh-N (100 ng/ml) for 7 days. Shh-N treatment led to a significant ($p < 0.05$) increase of BrdUrd-positive cells from 61 ± 2% in the control group to 73 ± 2%. In addition, Shh-N treatment substantially increased the number of TuJ1-positive cells in a dose-dependent manner, with maximal increases at a dose of 1,000 ng/ml (11 ± 0.3%, $n = 8$) for 100 ng/ml and 16 ± 0.7%, $n = 8$ for 1,000 ng/ml versus 2 ± 0.2%, $n = 8$ in control) when neural progenitor cells were cultured in the differentiation medium. These data indicate that Shh induces neural progenitor cell proliferation and differentiation, which is consistent with published results (35).

CEPO Up-regulates Mash1 Expression Resulting in Neuronal Differentiation in a Shh-dependent Manner—Mash1, a proneural bHLH transcription factor, promotes neuronal differentiation (36). Immunocytochemistry analysis revealed that all Mash1-positive cells were nestin-immunoreactive, suggesting that these cells are intermediate neural progenitor cells (Fig.
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To examine whether Mash1 is a potential target for CEPO-induced neuronal differentiation, we first examined Mash1 expression. Treatment of neural progenitor cells with CEPO (10 ng/ml) significantly (p < 0.05) increased Mash1 mRNA (Fig. 6, B and C) and protein (Fig. 6, D and E) levels at 1 and 7 days in vitro, respectively, compared with levels in the control group. We then tested whether an increase in Mash1 regulates CEPO-promoted neuronal differentiation. We constructed siRNA cassettes against mouse Mash1 (Fig. 7A, siRNA-Mash1). Real-time RT-PCR and Western blot analysis showed that siRNA-Mash1, but not scrambled, cassettes significantly (p < 0.05) attenuated Mash1 mRNA and protein levels in neural progenitor cells 48 and 72 h after transfection, respectively (Fig. 7, B–E), suggesting that the siRNA-Mash1 cassettes are effective in reducing endogenous Mash1 expression. In addition, siRNA-Mash1 cassettes substantially suppressed CEPO-up-regulated Mash1 (Fig. 7, F and G). Furthermore, treatment with CEPO for 48 h did not significantly (p < 0.05) increase the number of TuJ1-positive cells in neural progenitor cells transfected by siRNA-Mash1 cassettes compared with the number in neural progenitor cells transfected by scrambled cassettes or non-transfected neural progenitor cells (Fig. 4, C and J). Blockage of endogenous Mash1 by siRNA-Mash1 cassettes also significantly inhibited CEPO-increased neurite outgrowth compared with CEPO alone and CEPO with scramble cassette groups (Fig. 4, G, J, and K). These data suggest that Mash1 mediates CEPO-promoted neuronal differentiation. In addition to Mash1, CEPO increased Ngn1 mRNA levels (2.2 ± 0.5) in neural progenitor cells compared with levels in the control group. We then tested whether an increase in Mash1 regulates CEPO-promoted neuronal differentiation. In vitro experiments also significantly (p < 0.05) increased Mash1 mRNA (Fig. 7, B and C) and protein (Fig. 6, D and E) levels at 1 and 7 days in vitro, respectively, compared with levels in the control group. We then tested whether an increase in Mash1 regulates CEPO-promoted neuronal differentiation. We constructed siRNA cassettes against mouse Mash1 (Fig. 7A, siRNA-Mash1). Real-time RT-PCR and Western blot analysis showed that siRNA-Mash1, but not scrambled, cassettes significantly (p < 0.05) attenuated Mash1 mRNA and protein levels in neural progenitor cells 48 and 72 h after transfection, respectively (Fig. 7, B–E), suggesting that the siRNA-Mash1 cassettes are effective in reducing endogenous Mash1 expression. In addition, siRNA-Mash1 cassettes substantially suppressed CEPO-up-regulated Mash1 (Fig. 7, F and G). Furthermore, treatment with CEPO for 48 h did not significantly (p < 0.05) increase the number of TuJ1-positive cells in neural progenitor cells transfected by siRNA-Mash1 cassettes compared with the number in neural progenitor cells transfected by scrambled cassettes or non-transfected neural progenitor cells (Fig. 4, C and J). Blockage of endogenous Mash1 by siRNA-Mash1 cassettes also significantly inhibited CEPO-increased neurite outgrowth compared with CEPO alone and CEPO with scramble cassette groups (Fig. 4, G, J, and K). These data suggest that Mash1 mediates CEPO-promoted neuronal differentiation. In addition to Mash1, CEPO increased Ngn1 mRNA levels (2.2 ± 0.02 versus 1.0 ± 0.08 in control group), another pro-neuronal bHLH transcription factor (27).

Expression of Mash1 can be regulated by Shh, bone morphogenetic proteins, and the Wnt pathway (37–40). Our real-time RT-PCR analysis showed that CEPO did not alter mRNA levels of BMP2 (1.12 ± 0.6) and Wnt3a (1.5 ± 0.5) in neural progenitor cells compared with levels in the control group (1.0 ± 0.1). Thus, the Shh/Gli1 signaling pathway could be a potential intermediate signal in regulating CEPO-promoted neuronal differentiation. If Shh is the intermediate signal between CEPO and Mash1 signaling, then we would expect that blockage of Shh signaling would abolish CEPO-up-regulated Mash1 expression and consequently inhibit CEPO-promoted neuronal differentiation. Real-time RT-PCR and Western blot analysis revealed that incubation of neural progenitor cells with CEPO (10 ng/ml) in the pres-
ence of cyclopamine (5 μM) significantly attenuated CEPO-increased Mash1 mRNA and protein levels (Fig. 6, B–E) and suppressed CEPO-increased TuJ1-positive cells (Fig. 4, D and I). In addition, blockage of Gli1, the effector of Shh signaling, with siRNA-Gli1 significantly attenuated CEPO-up-regulated Mash1. Inhibition of endogenous Mash1 with siRNA-Mash1 cassettes suppressed CEPO-promoted neuronal differentiation. These data indicate that CEPO enhances in vitro neurogenesis which is mediated by the Shh signaling pathway and that Mash1 is a downstream target of the Shh signaling pathway and regulates CEPO-enhanced neuronal differentiation.

Using a commonly employed in vitro assay, we show that the treatment of neural progenitor cells derived from the SVZ of adult mouse with CEPO substantially increased the numbers of proliferative cells and neurons but did not increase astrocytes. These data demonstrate for the first time that in addition to its neuroprotective effect, CEPO augments neural progenitor cell proliferation and selectively promotes neural progenitor cell differentiation into neurons.

The Shh signaling pathway orchestrates neurogenesis in the adult brain (16, 34). Shh acts as a mitogen in cooperation with EGF to regulate proliferation of neural stem cells in adult SVZ (41). Exogenous Shh increases neurogenesis in the SVZ, whereas blockage of Shh signaling in adult mice diminishes expression of Gli1 and reduces SVZ cell proliferation (16). Using Gli1-CreERT2 mice, in which an inducible Cre recombinase (CreERT2) is expressed from Gli1, Ahn and Joyner (20) have recently demonstrated in vivo that Shh regulates neural stem and progenitor cells in the SVZ and the subgranular zone of adult mouse. Here, we show that CEPO activated the Shh signaling pathway with low doses of EGF and bFGF but CEPO on its own is not sufficient to up-regulate the Shh pathway genes. The effects of CEPO on the Shh pathway are specific, as coinubcation of neural progenitor cells with CEPO and cyclopamine, a pharmacological inhibitor of the Shh pathway, abolished CEPO-induced Gli1 expression and conse-

**DISCUSSION**

The present study demonstrates that CEPO promotes adult neural progenitor cell proliferation and differentiation. Blockage of Shh with the Shh antagonist cyclopamine abolished the effect of CEPO on neural progenitor cells, while blockage of Gli1 with siRNA-Gli1 attenuated CEPO-up-regulated Mash1. Inhibition of endogenous Mash1 with siRNA-Mash1 cassettes suppressed CEPO-promoted neuronal differentiation. These data indicate that CEPO enhances in vitro neurogenesis which is mediated by the Shh signaling pathway and that Mash1 is a downstream target of the Shh signaling pathway and regulates CEPO-enhanced neuronal differentiation.
CEPO mediates neuroprotection in the central nervous system by binding to a heteroreceptor consisting of the classical EPOR and the common β receptor (CD131) (10, 43). Our data obtained from ΔEPOR mice suggest that EPOR is not required for the effect of CEPO on adult neural progenitor cells. Future studies of whether other receptors regulate the biological function of CEPO in neural progenitor cells are warranted.

In summary, the present study demonstrates that the Shh signaling pathway mediates CEPO-enhanced neural progenitor cell proliferation and differentiation.

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