Effects of Erythropoietin in Murine-Induced Pluripotent Cell-Derived Panneural Progenitor Cells

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Induced cell fate changes by reprogramming of somatic cells offers an efficient strategy to generate autologous pluripotent stem (iPS) cells from any adult cell type. The potential of iPS cells to differentiate into various cell types is well established, however the efficiency to produce functional neurons from iPS cells remains modest. Here, we generated panneural progenitor cells (pNPCs) from mouse iPS cells and investigated the effect of the neurotrophic growth factor erythropoietin (EPO) on their survival, proliferation and neurodifferentiation. Under neural differentiation conditions, iPS-derived pNPCs gave rise to microtubule-associated protein-2 positive neuronlike cells (34% to 43%) and platelet-derived growth factor receptor positive oligodendrocytelike cells (21% to 25%) while less than 1% of the cells expressed the astrocytic marker glial fibrillary acidic protein. Neuronlike cells generated action potentials and developed active presynaptic terminals. The pNPCs expressed EPO receptor (EPOR) mRNA and displayed functional EPOR signaling. In proliferating cultures, EPO (0.1–3 U/mL) slightly improved pNPC survival but reduced cell proliferation and neurosphere formation in a concentration-dependent manner. In differentiating cultures EPO facilitated neurodifferentiation as assessed by the increased number of β-III-tubulin positive neurons. Our results show that EPO inhibits pNPC self-renewal and promotes neurogenesis.

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INTRODUCTION

Induced pluripotent (iPS) cells are generated by reprogramming of somatic cells by induced expression of the four transcription factors Oct4, Sox2, Klf4 and c-Myc (1–3). Similar to embryonic stem cells (ESC), iPS cells are able to differentiate into all of the different cell types that comprise the organs of an adult body including neuronal subpopulations (1–6). However, individual iPS cells are heterogeneous in respect to gene expression and epigenetic patterns (7–10).

Some iPS cell lines have a differentiation bias toward their cell type of origin, which seems to be linked to somatic memory mechanism, a cell-type specific epigenetic memory they retain following reprogramming (7,8,11,12). A requirement for the use of iPS cell-derived neurons for pharmacological or therapeutic applications is an efficient and stable neurogenesis (13). Recent studies have indicated a role for the activin/nodal, BMP, Notch, Shh or Fgf pathways (6,14) as well as several miRNAs (15) to exit the pluripotent state and to initiate differentiation.

Hypoxia promotes self-renewal and proliferation of tissue-specific stem cells and ESCs (16). In human iPS cells, hypoxia promotes reprogramming and enhances iPS cell production (17) and neuronal differentiation (18). Hypoxia also is a potent inducer of gene expression of the hematopoietic growth factor erythropoietin (EPO) (19). EPO and its receptor (EPOR) are best known for their role in regulating erythroid proliferation and differentiation (19) but EPO and EPOR also are expressed in the nervous system (20) where they exert potent cytoprotective and trophic activities (20–23). A substantial amount of effort has been devoted to characterize the mechanism of the neuroregenerative actions of EPO (24,25). For example, inhibition of apoptosis and inflammation seem to mediate EPO-induced neuroprotection after acute
brain injuries (24,25) while the trophic and myelination promoting effects of EPO may be important for its ability to counteract chronic neurodegeneration and neuroinflammation (26–29). Direct effects on neural stem cells also may play a role in the neurorestorative actions of EPO in rodent models of ischemic and traumatic brain injury (30–33). In these in vivo models, EPO improves injury-induced neurogenesis (31–33) and stimulates oligodendrogliosis (33). Accordingly, deletion of EPOR in the nervous system reduces the size of the neural stem cell pool and impairs injury-induced adult neurogenesis (30,34). Hypoxic preconditioning of neural stem cells induces EPO and improves neuronal differentiation (35) but it is not known whether EPO can stimulate neurogenesis from pluripotent stem cells. In this study, we examined whether EPO and EPOR play a role in the generation of neuronal cells from iPS cells. For this purpose we first characterized neurogenesis of iPS cells (1), and studied the effects of EPO on proliferation and neurodifferentiation of the iPS cell-derived neural progenitor cells.

**MATERIAL AND METHODS**

**iPS/ES Cell Maintenance**

For all experiments, iPS cells reprogrammed from murine neural stem cells by ectopic Oct4, Klf4, c-Myc and Sox2 expression, expressing lacZ from the rosa26 locus and carrying a Oct4-GFP transgene (36) was used as a positive control for pluripotent gene expression (Figure 1C). Cells were cultured on inactivated murine embryonic fibroblasts (MEF) in 60-mm plates (BD Biosciences, Heidelberg, Germany) in DMEM medium supplemented with 15% FCS, 1% nonessential amino acids, 1% penicillin/streptomycin, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamate, 10 mmol/L L-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) (all from PAA Laboratories GmbH, Hamburg, Germany), 0.1 mmol/L β-mercaptoethanol (Sigma-Aldrich, Taufkirchen, Germany) and leukemia inhibitory factor at 37°C/5% CO2 and passed every 2–3 d. Medium was changed every second day. For passaging, cells were washed once with PBS and detached from the culture plates by trypsin/EDTA (PA Laboratories GmbH) treatment for 5 min at 37°C. The reaction was stopped after 5 min with DMEM medium supplemented with 10% FCS and cells were collected, washed and replated onto fresh feeders.

**Neural Differentiation of iPSC Cultures**

iPS cells and ESCs were differentiated into panneural progenitor cells (pNPCs) using a monolayer differentiation protocol (37) with the following modifications: iPS cells and ESCs were passaged and the cells were plated onto 100 mm plates (Greiner Bio-One, Frickenhausen, Germany) coated with 0.1% gelatin from bovine skin in PBS (Sigma-Aldrich) and incubated at 37°C to separate iPS/ES cells and MEFs. After 45 min, iPS or ES cells were collected from the gelatin-coated plates, washed, counted and replated on gelatin-coated 60 mm plates (BD Biosciences) in GMEM medium (Life Technologies GmbH, Münster, Germany). In addition, pNPC-spheres were passaged every 2–3 d. Medium was replaced every second day. For passaging protocol: pNPC-spheres were first incubated in AccuMax (PA Laboratories GmbH) for 5 min at 37°C and mechanically triturated into single cells with a fire-polished Pasteur pipette. pNPCs were replated in 7 mL pNPC medium density of 1,000,000–1,500,000 cells per flask.

**Analysis of Oct4-GFP Fluorescence**

GFP fluorescence was analyzed with an Olympus IX70 fluorescence microscope (Olympus, Münster, Germany) and images were taken using the Analysis-SIS software (Soft Imaging System GmbH, Münster, Germany). In addition, the loss of GFP fluorescence during differentiation was analyzed by flow cytometry. Cells at d 0 and 5 of differentiation were detached from the culture plates as described, collected, washed, resuspended in PBS (Sigma-Aldrich) and analyzed using a BD FACS Canto and FACSDiva software (BD Biosciences). FACS data was analyzed using the software Weasel.

**Differentiation of pNPCs**

Secondary pNPC-spheres were dissociated into single cells and plated on poly-d-lysine (PDL; Sigma-Aldrich) coated glass coverslips (Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany) in 24-well cell culture plates (Greiner Bio-One) at a density of 25,000 cells/well in B27 medium consisting of neurobasal medium supplemented with 2% B27 (both from Life Technologies GmbH) and leukemia inhibitory factor at 37°C/5% CO2 and passed every 5 to 6 d. Medium was replaced every third day. pNPC-generated spheres were passaged on d 5 or 6 according to the following protocol: pNPC-spheres were first incubated in AccuMax (PA Laboratories GmbH) for 5 min at 37°C and mechanically triturated into single cells with a fire-polished Pasteur pipette. pNPCs were replated in 7 mL pNPC medium density of 1,000,000–1,500,000 cells per flask.
Figure 1. Characterization of in vitro neurogenesis of IPS cells and neural differentiation of IPS pNPCs. (A) Time scale diagram for neural differentiation of IPS cells and pNPC expansion as neurospheres. Left panel shows typical alkaline phosphatase positive IPS cell colonies (red). The rest of the panels depict overlays of phase contrast images and the GFP signal of Oct-4-driven GFP. IPS cells were positive for GFP; pNPCs, neurospheres and expanded pNPCs were negative for GFP. (B) FACS quantification of the Oct-4-GFP positive cells (P1) at day 0 and day 5 of differentiation for clonal line 1 (C1) and 2 (C2). Data are represented as mean ± SD, n = 3. (C) Expression analysis of different pluripotency and neural genes by RT-PCR in IPS cells and pNPCs. Mouse embryonic stem cell line (ESC wtB1), wtB1-derived pNPCs, neural stem cells (mNSC) and mouse embryonic fibroblasts (MEF) were used as controls. (D) Percentage of cells from C1 and C2 expressing markers for neurons (MAP-2), oligodendrocytes (PDGFR) and astrocytes (GFAP) at day 10 of differentiation. Data is represented as mean ± SD, n = 3. (E) Representative images of marker expression during pNPC differentiation. At day 0 of differentiation cells expressed the neural stem cell marker nestin and the neuronal progenitor marker Pax6. After 10 days in culture pNPCs expressed the neuronal markers MAP-2 and TAU, the astrocytic marker GFAP and the oligodendrocytic marker PDGFR. Staining for the oligodendrocyte marker O4 became visible after 14 days of differentiation. After 22 days, cells expressed the presynaptic marker synapsin, the postsynaptic marker Homer1 and myelin basic protein, a marker for mature oligodendrocytes. (F) Immunohistochemical labeling of active synapses. Fluorescent picture showing staining for synapsin (green) and synaptotagmin I (red) and the mask for analyzing the frequency of colocalized dots (yellow, arrowhead). Graph shows the quantification of the mean percentage of double stained dots. Data represent mean ± SD, n = 3. (G) Electrophysiological characterization of pNPCs at day 13/14. I/V curves of VC-stimulation in pNPC C1 neuron-like cells before and after treatment with ttx (tetrodotoxin, sodium channel blocker) or TEA (tetraethylammonium, potassium channel blocker). Stimulation potential (mV) is plotted against the maximum measured inward or outward current (current was normalized to cell size [pA/pF], data represent mean ± SD; n = 6, *p < 0.05, **p < 0.01 (paired t test for normal and Wilcoxon test for not normal distributed samples). A representative trace of membrane potential responding to current injection by VC depolarization (depolarizations: black lines; hyperpolarizations: gray lines) and a representative trace of whole cell currents in voltage clamp mode responding to step depolarization by current injection. Current injections (-50 pA, +10 pA) into pNPC-derived neurons in current clamp (CC)-mode. Stimulation via stepwise increase of membrane potential (-80 mV to +55 mV, step size 15 mV) in VC-mode. Scale bars (A): first and fourth subpanels from left, 100 μm; second, third and fifth subpanels from left, 50 μm; (E): top subpanels and second through fourth bottom subpanels, 25 μm; first and fifth bottom subpanels, 10 μm. Continued on the next page.
GmbH), 1% penicillin/streptomycin and 2 mmol/L L-glutamine (both from PAA Laboratories GmbH). pNPCs were cultured at 37°C under 5% CO₂/95% air and 90% humidity and medium was replaced every fourth day.

**RT PCR**

Total RNA was isolated using TRizol (Life Technologies GmbH) according to the manufacturer’s instructions. 1 μg of total RNA was treated with 1 U DNAseI (Fermentas, St. Leon-Rot, Germany) for 40 min at 37°C to remove remaining DNA. mRNA was transcribed into cDNA with the Maxima Reverse transcriptase (Fermentas) at 50°C for 60 min. cDNA fragments from the genes of interest were amplified by standard PCR using gene-specific primers (Supplementary Table S1). Mouse β-actin primers were used as internal standard.

**Immunocytochemistry**

pNPCs were fixed for 20 min in 4% PFA (Sigma-Aldrich) after 0, 10, 14 or 22 d in differentiation, permeabilized and blocked in 0.2% Triton X-100 (Roche, Penzberg, Germany) with 10% normal horse serum (NHS) in phosphate buffered saline (PBS) (Dianova, Hamburg, Germany) for 30 min. Cells were then incubated with primary antibodies (Supplementary Table S2) against neural cell markers in 1% NHS at 4°C overnight, followed by incubation with a secondary antibody (Supplementary Table S2) in 1% NHS for 1 h. Cells were mounted in Mowiol with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear counter staining. Cells were photographed with a Leica LSM TCS SP5 confocal microscope (Leica, Wetzlar, Germany). Images were processed using the ImageJ software (MBF ImageJ for Microscopy) (38) and CorelDRAW Graphics Suite X5.

**Cell Counting**

pNPCs differentiated for 10 d were fixed and stained for microtubule-associated protein 2 (MAP-2), glial fibrillary acidic protein (GFAP) or platelet-derived growth factor receptor α.
(PDGF receptors) as described above. For each marker, photos from three coverslips (five randomly chosen fields/coverslip) were taken using an Axiophot2 fluorescence microscope (Zeiss, Jena, Germany) with a SPOT Insight camera (Visitron Systems, Puchheim, Germany). From each image the number of living and dead (DAPI) as well as MAP-2, GFAP or PDGFR positive cells were counted. The experiment was replicated twice.

**Synaptic Vesicle Recycling**

pNPCs differentiated for 22 d, were incubated with a rabbit anti-synaptotagmin I antibody (1:100, Synaptic Systems, Göttingen, Germany) against the luminal domain of the protein in high potassium Krebs-Ringer-HEPES buffer (KRH) (55 mmol/L KCl, 85 mmol/L NaCl, 10 mmol/L HEPES, pH 7.4, 10 mmol/L glucose, 2.6 mmol/L CaCl2 and 1.3 mmol/L MgCl2) for 1 min at 37°C. The antibody solution was removed and cells were washed thrice in KRH buffer (5 mmol/L KCl, 140 mmol/L NaCl, 10 mmol/L HEPES, pH 7.4, 10 mmol/L glucose, 2.6 mmol/L CaCl2 and 1.3 mmol/L MgCl2) for 5 min. Cells were fixed and incubated with a mouse anti-synapsin I antibody (1:1,000; Synaptic Systems) followed by an incubation with a goat anti-mouse-DL488 and goat anti-rabbit-Cy3 antibody (1:1,000, both from Dianova, Hamburg, Germany) as described above. Photographic images from three coverslips (three randomly chosen fields/coverslip) were taken using an Axiophot2 fluorescence microscope with a SPOT Insight camera. Pictures were processed in ImageJ (38) using the following protocol: images were smoothed using a gaussian filter with σ = 2, Laplace filtered and thresholded to isolate small maxima in fluorescence intensity corresponding to synaptic buttons. To increase the signal-to-noise ratio a particle filter with a threshold of 16 pixels was applied. The total number of synapsin I positive buttons and the number of buttons colocalizing with synaptotagmin I staining were counted. The experiment was replicated twice.

**Whole Cell Patch-Clamp Analysis**

Cells were transferred into a recording chamber and continuously superfused with extracellular solution containing 125 mmol/L NaCl, 25 mmol/L NaHCO3, 2.5 mmol/L KCl, 1.25 mmol/L Na2PO4, 2 mmol/L CaCl2, 2 mmol/L MgCl2 purged by 95% CO2/5% O2. The ion channel antagonists tetraethylammonium chloride (TEA) or tetrodotoxin (ttx) (both from Sigma-Aldrich) were added to the extracellular solution with a concentration of 30 mmol/L and 1 μmol/L, respectively. All experiments were performed at room temperature using an EPC 10 double patch clamp amplifier and pulse software (HEKA, Lambrecht, Germany). Electrodes were pulled from thick-walled borosilicate glass (Warner Instruments, Hamden, CT, USA) and filled with intracellular solution (120 mmol/L K-gluconate, 32 mmol/L KCl, 10 mmol/L HEPES, 4 mmol/L NaCl, 0.5 mmol/L EGTA, 4 mmol/L MgATP, 0.4 mmol/L Na2-GTP, pH 7.2, 290-300 mOsml−1) and had a resistance between 4 and 6 MΩ. Cells were held in whole-cell configuration at ~70 mV and were discarded if the series resistance was higher than 25 MΩ at the beginning of the measurements.

**EPOR Activity Assay**

Activation of the EPOR-associated signaling was investigated by Western blot analysis of the phosphorylation of ERK 1/2. Secondary pNPC-spheres were dissociated into single cells as described above and seeded into 25-cm2 suspension culture flasks with a density of 1,500,000 cells. After 24 h, 1 U/mL human recombinant EPO (epoetin beta, NeoRecormon, Roche, Welwyn Garden City, UK) or PBS was added to the suspension culture and total cell protein was extracted 5 min after treatment using PhosphoSafe (Merck, Darmstadt, Germany) according to the manufacturer’s instructions. Protein concentration was measured with the BCA Protein Assay (Thermo Fisher Scientific, Schwerte, Germany) and stored afterward at ~ −80°C. Five volumes of protein solution were mixed with 1 volume of Laemmli buffer (250 mmol/L Tris HCl, pH 8.3, 8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 0.04% pyronin Y) and boiled for 5 min at 95°C. Ten to fifteen μg protein was run on 15% polyacrylamide gels (Roth, Karlsruhe, Germany) for 2 h at 150 V and transferred to a nitrocellulose membrane for 1 h at 400 mA using a miniVE electrophoresis and electro transfer unit (Amersham Bioscience/GE Healthcare, Munich, Germany). After blocking with 5% milk powder in Tween20-Tris-buffered saline (TTBS), at room temperature for 1 h, the membranes were incubated with primary antibodies for mouse anti-pMAPK (1:2,000, Sigma-Aldrich) or rabbit anti-MAPK (1:5,000, Sigma-Aldrich). Immunoreactive bands were visualized using a secondary anti-mouse (1:5,000) or anti-rabbit (1:15,000) antibody coupled to horseradish peroxidase (both from Sigma-Aldrich) by enhanced chemiluminescence (ECL Prime Western blotting detection kit, Amersham Bioscience/GE Healthcare). Densitometric analysis of protein bands was performed using the ImageJ software.

**Cell Survival Assay**

For the pNPC survival assay, secondary spheres were dissociated into single cells and plated at a density of 50,000 cells on PDL coated glass coverslips in 24-well cell culture plates. pNPCs were cultured in B27 medium with 0.1, 1 or 10 U/mL EPO or PBS. After 24 h or 48 h MT-cell survival assay was performed according to the following protocol: pNPCs were incubated in B27 medium supplemented with 30 mmol/L thiozolyl-blue-tetrazolium-bromide (MTT; Sigma-Aldrich) for 2 h at 37°C/5% CO2. Cells were washed with PBS and treated for 5 min with cell lysis buffer (50% N,N-dimethylformamide, 20% sodium dodecylsulfate, pH 4.7). The absorption of the
formazan product that is produced by metabolization of the MTT reagent was measured at 570 nm. Cell vitality of the EPO treated cells was calculated from the absorption as follows:

\[
\text{Cell vitality [%]} = \frac{\text{Mean Abs}_{570\text{nm}} \text{ EPO treatment}}{\text{Mean Abs}_{570\text{nm}} \text{ control treatment}} \times 100.
\]

Control was set as 100%. Four independent experiments in six replicates of every treatment group were performed for each clone.

**pNPC Dilution Assay**

Secondary pNPC-spheres were dissociated into single cells and seeded with a density of 1, 10, 25, 50, 100, 250, 500, 750, 1000, 1500 or 2500 cells per well into 96-well suspension culture plates (Greiner Bio-One) containing 200 μL of pNPC medium. Cells were treated immediately either with 0.3 or 1 U/mL EPO or placebo and cultured for 7 d without changing the medium. At d 7, all spheres/well were photographed using an Olympus IX70 inverse microscope and the AnalySIS software and counted. Sphere diameter was measured using Imagej (38).

Analysis was performed blinded. Four independent experiments were performed for each clone. For each experiment, three replicates of every treatment group were analyzed.

**pNPC Proliferation Assay**

Single pNPCs derived from secondary spheres were labeled with 0.2 μmol/L carboxyfluorescein-succinimidyl-ester (CFSE) (Sigma-Aldrich) in PBS (1,000,000 cells/mL) for 10 min at 37°C, mixture of ice-cold PBS and pNPC medium without hEGF and bFGF2 was added and cells were incubated for 5 min on ice followed by three washing steps in PBS. Immediately after washing an aliquot of CFSE-labeled and unlabeled cells were analyzed using a BD FACSCanto and FACSDiva software.

Labeled cells were seeded into 25-cm² suspension culture flasks with a density of 1,000,000 cells/flask and grown in pNPC medium supplemented with 1 U/mL EPO or placebo. After 48 h, newly formed pNPC-spheres were dissociated into single cells and analyzed by flow cytometry. FACS data was evaluated using Weasel software. For each clone, four independent experiment were performed.

In a separate set of experiments, CFSE-labeled cells were incubated in 5 mL pNPC medium without growth factors containing either DMSO, 10 μmol/L LY294002 or 10 μmol/L U0126. After 20 min, placebo or 1 U/mL EPO was added and cells were incubated for an additional hour. Afterward, cells were washed twice in PBS and seeded into 25-cm² suspension culture flasks in 5 mL pNPC medium and incubated at 37°C under 5% CO₂/ 95% air and 90% humidity. After 48 h, CFSE fluorescence was analyzed by flow cytometry as described above. For each clone, three to four independent experiments were performed.

**Cell Cycle Analysis**

pNPCs were derived from secondary spheres as described above. One million cells in 5 mL pNPC medium were incubated in the presence of 5 μg/mL aphidicolin (SERVA Electrophoresis GmbH, Heidelberg, Germany) for 14 h. After 14 h, cells were washed twice in PBS and incubated in 5 mL pNPC medium supplemented with 1 U/mL EPO or placebo for 10 and 24 h. Afterward, cells were washed twice in PBS and fixed in 3% PFA for 15 min. After fixation cells were washed once in PBS and stained in 500 μL PBS containing 50 μg/mL propidium iodide (PI, Sigma-Aldrich), 0.1 mg/mL RNaseA (Fermentas) and 0.05% Triton X-100 for 40 min at 37°C. PI labeled cells were analyzed using a FACSCanto and FACSDiva software. FACS data was evaluated using Weasel software. For each clone, three to four independent experiments were performed.

**Neuronal Differentiation Assay**

Secondary spheres were dissociated into single cells and plated on PDL coated glass coverslips in 24-well cell culture plates with a density of 12,500 cells/coverslip and grown in B27 medium with 3 U/mL EPO or placebo. After 2, 4 or 6 d, cells were fixed and stained with a mouse-anti-β-III-tubulin primary (1:500, Sigma-Aldrich) and an anti-mouse-Cy3 (1:1000, Dianova) secondary antibody, and counterstained with DAPI. Pictures from two coverslips (eight randomly chosen fields/coverslip) were taken using an Axiosphot2 fluorescence microscope with a SPOT Insight camera. The number of living (DAPI) and β-III-tubulin (TuJ1)-positive cells was counted. In addition, the length of each process of TuJ1-positive cells was measured using the NeuronJ plugin for the ImageJ software (21). Cells with processes shorter than the cell soma’s diameter were excluded from further analysis. Measurements and data analysis was performed blinded.

**Statistical Analysis**

Data are expressed as mean ± SD for indicated number of repetitions (n). Data were tested for normal distribution using a graphical approach (quantile-quantile Plot) and the Shapiro–Wilk test. Group differences with normal distribution were analyzed by Student t test and the Wilcoxon test was used for not normally distributed datasets. Data plotting and statistical analysis were done using Microsoft Excel and R (39). Differences were considered significant at \( p < 0.05 \).

All supplementary materials are available online at www.molmed.org.

**RESULTS**

**Neurogenesis of iPS Cells**

To assess the potential of iPS cells for in vitro neurogenesis, we first induced their differentiation into pNPCs by culturing the cells for 5 d followed by pNPC expansion as neurospheres for additional 10 to 12 d (Figure 1A). During neural differentiation the iPS cells lost the expression of an Oct-4-driven GFP-transgene within 5 d (Figures 1A,1B). At this stage, the pNPCs were negative for the pluripotency genes
Nanog, Rex1 and Fgf4 (Figure 1C) and started to express neural stem cell markers nestin and musashi (see Figure 1C). As expected, pNPCs but not iPS cells expressed genes involved in forebrain (Emx2, Dlx5) or midbrain/hindbrain development (Nkx6.1, Dlx2 and Hexa2) (40–42).

Neural Differentiation and Maturation of iPS pNPCs

When the cells were cultured under neuronal differentiation conditions for 10 d pNPCs became positive for neuronal and glial markers (Figure 1D,1E). Counting of frequencies of neural cell types after 10 d of differentiation (see Figure 1D) revealed that 33% (clonal line C1) and 43% (clonal line C2) of all cells expressed the neuron-specific protein, microtubule-associated-protein 2 (MAP2), whereas 25% (C1) and 21% (C2) expressed the oligodendrocyte marker platelet-derived growth factor receptor (PDGFR). Less than 1% of all cells were positive for the astrocyte specific protein, glial fibrillary acidic protein (GFAP) (see Figure 1D). Figure 1E shows representative images of cells at various stages of differentiation. Immediately after plating iPS pNPCs were positive for the neural precursor marker nestin. Some of them also expressed the neuronal progenitor marker Pax6 (see Figure 1E). In 10 d old cultures, the expression of the neuronal markers MAP2 and TAU as well as the oligodendrocyte marker PDGFR were prominent and the astrocyte marker GFAP was detectable in few cells. Staining for the oligodendrocyte marker O4 (see Figure 1E) and the presynaptic marker synapsin became visible after 14 d of differentiation. After 22 d in culture, synapsin expression was pronounced and cells expressed the postsynaptic marker homer1 (see Figure 1E). Expression of myelin basic protein (MBP), a marker for mature oligodendrocytes became also detectable after 22 d in culture (see Figure 1E). At this stage of in vitro maturation, roughly one fourth (23% to 24%) of the neuronlike cells showed the presence of active presynaptic terminals after induction of presynaptic vesicle fusion by a brief exposure to 55 mmol/L KCl (Figure 1F).

To further confirm that the iPS-derived neuronlike cells develop functional neuronal properties, we performed whole cell patch clamp recordings at d 13 and 14 of differentiation. The cells were stimulated in current clamp (CC) mode with depolarizing current injections (–50 pA to +130 pA, step size 20 pA) over a time period of 500 ms. In 11 out of 16 (C1) and 10 out of 20 (C2) recorded cells current injection elicited action potentials, multiple action potentials could be elicited (Figure 1G). The average resting membrane potential for the recorded cells was –44.81 ± 9.01 mV (C1) and –48.38 ± 13.38 mV (C2), respectively. In voltage clamp (VC) mode we stepwise depolarized the cells (~80 mV to +55 mV, step size 15 mV) to induce action potentials and measured the corresponding inward and outward currents. Neuronlike cells from both clonal lines showed a similar pattern of maximum inward and outward currents plotted against the voltage stimulus, however C2 showed a tendency for lower outward currents compared with clone 1 (Figure 1G and not shown). As expected, treatment of cells with the Na⁺-channel inhibitor tetrodotoxin (ttx) or the K⁺-channel inhibitor tetraethylammonium (TEA) completely abolished inward (Na⁺) and reduced outward (K⁺) currents (see Figure 1G).

Effect of EPO on iPS pNPCs

We first confirmed that pNPCs from both iPS clones express EPOR mRNA and functional EPOR by monitoring the EPO-induced activation of MAPK signaling (Figure 2A). When the cells were cultured for 24 h under different concentra-
tions of EPO, cell survival in C2 was improved by EPO concentrations of 1 U/mL and 10 U/mL (Figure 2B). A tendency for a better cell survival after EPO also was observed in C1 (see Figure 2B). A similar pattern of cell survival also was seen in cells cultured for 48 h (see Figure 2B). When the effect of EPO on cell proliferation was tested by CFSE cell proliferation assay, EPO (1 U/mL) reduced cell proliferation in both cell clones (Figure 2C). Since binding of EPO to EPOR activates ERK1/2 and Akt-1 signaling in neurons (21–23), we tested whether pretreatment with inhibitors of these pathways can modify the effect of EPO on pNPC proliferation. Treatment with the PI3K inhibitor LY294002 (10 μmol/L) or the MAPK inhibitor U0126 (10 μmol/L) failed to influence the antiproliferative effect of EPO consistently (Figures 3A–B). Cell cycle analysis of pNPCs showed no changes in cell cycle progression after EPO treatment (Figure 3C).

To analyze the effect of EPO on neurosphere formation, pNPCs were plated with different densities from 1 up to 2,500 cells/well and were allowed to form spheres for 7 d in the presence of 0.3 or 1 U/mL EPO or placebo. After 7 d, EPO suppressed sphere formation in a dose dependent manner (Figure 2D). For assessment of the effect of EPO on neuronal differentiation, pNPCs were cultured under culture conditions favoring differentiation in the presence or absence of 3 U/mL EPO. EPO treatment more than doubled the number of TuJ1-positive neurons at d 4 and 6 (Figure 2F). Together this indicates that EPO facilitated neurodifferentiation of iPS-derived pNPCs.

**DISCUSSION**

Efficient generation of neural cell types from pluripotent embryonic or iPS cells have been the subject of intensive research (6,14,43–46), but factors that lead to a robust formation of pNPCs from pluripotent stem cells and/or their differentiation into neural subtypes still need to be identified. In the present study we first show that the hematopoietic growth factor EPO directs iPS pNPC neurogenesis into neuronal fate by inhibiting proliferation and promoting neuronal differentiation.

We first confirmed the potential of iPS cells to generate proliferating pNPCs, which further differentiated into mature neurons and oligodendrocytes. iPS cell-derived neuronlike cells showed the ability to generate action potentials, possessed membrane characteristics similar to newly formed neurons (47,48) and were capable of forming active presynaptic terminals. In this regard our findings are in accordance with several other
studies that showed the ability of murine iPS cell lines to generate different neural subtypes, including functional neurons (6,45,49,50). The iPS cell-derived progenitors in our study produced mainly neurons and oligodendrocytes. In contrast to earlier studies showing the generation of 10% astrocytes from murine iPS cells (6,51), we detected less than 1% astrocytes. These differences might be explained by differences in the reprogramming process and the origin of the somatic cell used for iPS cell generation, as our cells might have a differentiation bias toward neurons due to their type of origin (1,5,43,52,53). Culture conditions such as the concentration of EGF and bFGF2 in culture media also may have influenced the neural cell fates (54,55).

In the second part of the study, we analyzed the potential of EPO to modulate neurogenesis in two clones of iPS pNPCs which expressed functional EPOR. We observed that EPO increased cell viability of pNPCs. This is not surprising given the fact that the antiapoptotic and cytoprotective effects of EPO have been reported in several in vivo and in vitro experimental settings (25). An alternative explanation for the increase in viable cells after EPO treatment would be a stimulation of cell proliferation. However, we found that EPO had an inhibitory effect on cell proliferation. Treatment with EPO thus seems to improve the maintenance of a homogenous and stable neural cell type. Furthermore, the competence of pNPCs to form spheres was drastically reduced by EPO. In agreement with our findings, Shingo and colleagues described an inhibitory effect of EPO on neurosphere formation and growth from neural stem cells isolated from fetal rat brain (35). Others have reported stimulation of proliferation of adult neural stem cells in response to EPO treatment (34,56–58). These opposing results may be related to different cell culture types and to the use of higher EPO concentrations.

MAPK and PI3/AKT signaling have been shown to support neurosphere formation and/or growth in neural stem cells (59). These signaling pathways also have been shown to be activated by EPO in neurons (21–23,28,57,60). Even if iPS cell-derived pNPCs expressed EPOR mRNA and functional EPOR-associated intracellular signaling pathways, neither inhibition of MAPK nor PI3K/AKT counteracted the effect of EPO on iPS pNPC proliferation. Therefore, relevant pathways mediating the EPO-induced inhibition of cell proliferation remain to be determined.

Finally, we show that in differentiating cultures of iPS pNPCs treatment with EPO led to increased numbers of TuJ1-positive neurons. These findings agree with previous reports on EPO-induced improvements in neuronal differentiation of fetal and adult neural stem cells (35,61).

CONCLUSION

We generated panneural progenitor cells (pNPCs) from mouse iPS cells and investigated the effect of the neurotrophic growth factor erythropoietin on their survival, proliferation and neuro differentiation. We show that EPO inhibits self-renewal and promotes neurogenesis of these cells. This novel effect of EPO may bear clinical relevance, as EPO might be used to improve the production and maintenance of a homogeneous and stable neural stem cell population from human iPS cells.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

EPO AND NEUROGENESIS OF iPS CELLS


