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STAT3 and SOCS3 regulate NG2 cell proliferation and differentiation after contusive spinal cord injury

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ABSTRACT

NG2 cells, also known as oligodendrocyte progenitors or polydendrocytes, are a major component of the glial scar that forms after spinal cord injury. NG2 cells react to injury by proliferating around the lesion site and differentiating into oligodendrocytes and astrocytes, but the molecular mechanism is poorly understood. In this study, we tested the role of the transcription factor STAT3, and its suppressor SOCS3, in NG2 cell proliferation and differentiation after spinal cord injury. Using knockout mice in which STAT3 or SOCS3 are genetically deleted specifically in NG2 cells, we found that deletion of STAT3 led to a reduction in oligodendrogenesis, while deletion of SOCS3 led to enhanced proliferation of NG2 cells within the glial scar after spinal cord injury. Additionally, STAT3 and SOCS3 were not required for astrogliogenesis from NG2 cells after spinal cord injury. Interestingly, genetic deletion of STAT3 and SOCS3 did not have opposing effects, suggesting that SOCS3 may have targets other than the STAT3 pathway in NG2 cells after spinal cord injury. Altogether, our data show that both STAT3 and SOCS3 play important, yet unexpected, roles in NG2 cell proliferation and differentiation after spinal cord injury.

1. Introduction

Oligodendrocyte progenitor cells (OPCs), also known as NG2 cells, are capable of differentiating into oligodendrocytes in the developing and the adult CNS (Nishiyama et al., 2009) and comprise approximately 70% of all dividing cells in the adult spinal cord (Horner et al., 2000). Although most studies focus on the role of NG2 cells in remyelination after spinal cord injury (SCI), less is known about their contribution to the glial scar (Levine, 2015; Son, 2015). Several studies implicate their production of chondroitin sulfate proteoglycans (CSPGs) (Dou and Levine, 1994; Levine, 1994; Petrosyan et al., 2013; Tan et al., 2006) and capacity to form synapses with dystrophic axons (Filous et al., 2014) to be largely inhibitory for axon regeneration. In addition, NG2 cells react to injury in a similar manner to reactive astrocytes in that they become hypertrophic and proliferate in the penumbra surrounding the injury site (McTigue et al., 2001; Zai and Wrathall, 2005). In fact, NG2 cells have been shown to differentiate into GFAP$^+$ astrocytes after several models of CNS injury (Komitova et al., 2011; Sellers et al., 2009; Tripathi et al., 2010; Zawadzka et al., 2010). However, the signaling pathways involved in the proliferation and differentiation of NG2 cells after SCI are not well understood.

Cytokines such as CNTF and LIF have been suggested to be important for NG2 cell proliferation and differentiation into oligodendrocytes and astrocytes *in vitro* as well as development of oligodendrocytes *in vivo* (Barres et al., 1996; Ishibashi et al., 2009; Mayer et al., 1994). The best characterized signaling pathway for many of these cytokines is activation of the JAK–STAT3 pathway through the gp130 receptor. This pathway is negatively regulated by SOCS3, which binds to the gp130–JAK complex. Cytokine expression is increased in the glial scar region after contusive SCI (Tripathi and McTigue, 2008; Zai et al., 2005) and high levels of phospho-STAT3, which is nearly undetectable in the uninjured spinal cord, are found in NG2 cells in this region (Hesp et al., 2015; Tripathi and McTigue, 2008). The JAK-STAT3 signaling pathway has also been implicated in astrocyte differentiation from Nestin$^+$ cortical precursor cells due to the binding of STAT3 to the GFAP promoter (Bonni et al., 1997; Nakashima et al., 1999b), and astrogliogenesis and oligodendrogenesis is impaired in LIF KO mice and gp130 KO mice (Bugga et al., 1998; Nakashima et al., 1999a). Furthermore, both STAT3 and SOCS3 have been implicated in astroglial scar formation after SCI (Herrmann et al., 2008; Okada et al., 2006; Wanner et al., 2013), but their role in NG2 cells after SCI is not known.

Abbreviations: CNTF, Ciliary neurotrophic factor; CSPG, Chondroitin sulfate proteoglycan; DAPI, 4′,6-Diamidino-2-phenylindole; EdU, 5-Ethynyl-2′-deoxyuridine; GFAP, Glial fibrillary acidic protein; JAK, Janus kinase; LIF, Leukemia inhibitory factor; NG2, Neural glial antigen 2; OPC, Oligodendrocyte progenitor cell; SCI, Spinal cord injury; SOCS3, Suppressor of cytokine signaling 3; STAT3, Signal transducer and activator of transcription 3; TUNEL, Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling.

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We hypothesized that STAT3 is necessary for NG2 cell proliferation and differentiation after contusive SCI. We tested this hypothesis *in vivo* after SCI using genetic deletion of STAT3 or its suppressor SOCS3 specifically in NG2 cells. Our data indicate that after SCI, SOCS3 is an important regulator of NG2 cell proliferation, while STAT3 is important for oligodendrogenesis. Additionally, we determined that STAT3 and SOCS3 were dispensable for astrogliogenesis from NG2 cells after contusive SCI. Interestingly, genetic deletion of STAT3 and SOCS3 did not have opposing effects, revealing an unexpected molecular mechanism of NG2 cell proliferation and differentiation after SCI.

2. Materials and methods

2.1. Animals

NG2-CreER mice, obtained from The Jackson Laboratory (stock 008538; Zhu et al., 2011), were bred to Rosa26-tdTomato reporter mice (kindly donated by Dr. Fan Wang, Duke University, Durham, NC; Arenkiel et al., 2011) to produce NG2-CreER^{+}/Rosa26-tdTomato$^{F/+}$ offspring, referred to as NG2-tdTomato or WT mice. To generate NG2 cell-specific deletion of STAT3, NG2-tdTomato mice were bred to STAT3 floxed mice, obtained from The Jackson Laboratory (stock 016923; Moh et al., 2007), to produce NG2-CreER^{+}/Rosa26-tdTomato-$^{F/+}$/STAT3$^{F/F}$ mice, referred to as NG2-STAT3 KO mice. To generate NG2 cell-specific deletion of SOCS3, NG2-tdTomato mice were bred to SOCS3 floxed mice, obtained from The Jackson Laboratory (stock 010944; Mori et al., 2004), to produce NG2-CreER^{+}/Rosa26-tdTomato-$^{F/+}$/SOCS3$^{F/F}$ mice, referred to as NG2-SOCS3 KO mice. Since the only commercially available antibodies against RFP were produced in a rabbit, and tdTomato fluorescence was destroyed by our antigen retrieval technique, we generated NG2-EYFP mice (NG2-CreER bred to Rosa26-EYFP from The Jackson Laboratory (stock 006148;Srinivas et al., 2001) in which a chicken GFP antibody can be used with rabbit antibodies that we could not use for co-labeling studies in NG2-tdTomato tissue. All mice were of pure C57BL/6 genetic background. All procedures involving animals were approved by the University of Miami Institutional Animal Care and Use Committee and followed NIH guidelines.

2.2. Surgery

Six to 8 week old female mice were injected i.p. (intraperitoneal) with 0.124 mg/g body weight of tamoxifen (MP Biomedicals) as previously described (Lee et al., 2009) for 5 consecutive days. One week after the last injection, mice were anesthetized (ketamine/xylazine, 100 mg/15 mg/kg i.p.) and received contusive SCI as previously described (Lee and Lee, 2013; Zhu et al., 2015). Mice received a T8 laminectomy, and the spinal column stabilized using spinal clamps, and then received a moderate (75 kDyne) SCI using an Infinite Horizons impactor device (Precision Systems and Instrumentation, LLC). Mice received post-operative treatment of antibiotics (Baytril, 10 mg/kg), and analgesics (buprenorphine, 0.05 mg/kg) diluted into 1 mL of Lactated Ringer's solution injected subcutaneously twice per day for the first week following surgery. Mice received manual bladder expression twice daily until the end of the experiment.

2.3. Histology

Mice were anesthetized and then transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). Spinal cords were dissected and postfixed in 4% paraformaldehyde in PBS for 2 h, and cryoprotected in 30% sucrose in PBS overnight. 8 mm segments of the spinal cord centered at the injury site were embedded in OCT compound (Tissue-Tek) and sagittal serial sections (16 μm) were cut using a cryostat. Sections were immunostained by incubating in primary antibodies in 5% Normal Goat Serum in PBS with 0.3% Triton X-100 overnight at 4 °C. Primary antibodies used were: RFP (Rockland 600-401-379S, 1:1000), GFAP (Invitrogen 130300, 1:1000 or Dako Z033429, 1:1000), APC/CC1 (Millipore OP80 Ab-7, 1:500), NG2 (Millipore AB5320, 1:200), GFP (Abcam ab13970, 1:2000), PDGFRβ (Abcam ab32570, 1:200), Iba1 (Wako 019-19741, 1:500), Olig2 (Millipore AB9610, 1:200), and phospho-STAT3 Y705 (Cell Signaling 9145S, 1:100). Following primary antibody incubation, sections were washed and incubated in species-appropriate Alexa Fluor IgG (H + L) secondary antibodies (Invitrogen, 1:500) at room temperature for 1 h. For CC1, goat-anti-mouse IgGγ2b secondary antibody was used (Invitrogen A-21141, 1:500). Slides were mounted using Vectashield with DAPI (Vector Laboratories H-1200). Images were obtained using a Nikon Eclipse Ti fluorescent microscope or an Olympus FluoView 1000 confocal microscope. For phospho-STAT3 staining, we performed antigen retrieval by incubating slides in L.A.B. solution (Polysciences, Inc.) for 15 min at 60 °C prior to immunostaining.

2.4. EdU proliferation assay

Mice were injected i.p. with 50 mg/kg of EdU (Invitrogen A10044) in 2% DMSO in PBS at 2, 3, and 4 days after SCI and sacrificed at 7 days after SCI. Tissues were prepared for histology as described above, and EdU was detected by using Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen C10337) according to the manufacturer's instructions. Sections were subsequently immunostained as described above. All sections were counterstained with DAPI, and only EdU^{+} cells that were also DAPI^{+} were included in the quantification.

2.5. TUNEL apoptosis detection

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed as described previously (Gavrieli et al., 1992) with some modifications. Frozen sections were permeabilized in 2 parts ethanol, 1 part acetic acid for 5 min at −20 °C, then washed thoroughly with PBS. Sections were incubated in PBS at 37 °C for 30 min then incubated with 40 μg/mL proteinase K in PBS for 15 min at 37 °C. Digestion was stopped by washing with PBS and then slides were pretreated with TdT Buffer (25 mM Tris–HCl, 200 mM sodium cacodylate, 0.25 mg/ml BSA, 1 mM cobalt chloride, Roche) at 37 °C for 10 min. To perform end-labeling, TdT Buffer was combined with terminal transferase (Roche, 400 U/slide) and Biotin-16-dUTP (Roche, 4 μM) and added to slides for 1 h at 37 °C. Slides were thoroughly washed with PBS then blocked for 30 min with 5% normal goat serum in PBS with 0.3% Triton-X, then incubated with Alexa-Fluor conjugated streptavidin (Invitrogen, 1:500) for 30 min at 37 °C. Slides were subsequently immunostained as described above. All sections were counterstained with DAPI, and only TUNEL^{+} cells that were also DAPI^{+} were included in the quantification.

2.6. Histology quantification

For histological quantifications, three consecutive sagittal spinal cord sections (per animal) centered on the injury epicenter and spaced 160 μm apart were imaged on a Nikon Eclipse Ti fluorescent microscope. Using NIS Elements AR Software, a contour was drawn around the GFAP-negative area (lesion site) and used as a reference point to delineate 250 μm intervals rostral and caudal from the lesion site (thus all sections were co-stained with GFAP). The area for each interval was determined using NIS Elements AR Software. Regions 0–250 μm and 250–500 μm were considered as within the glial scar whereas the 1000–1250 μm area was considered outside the glial scar based on the hypertrophic and interweaving GFAP processes that reactive astrocytes typically display. For these quantifications, all tdTomato^{+} cells on each section were counted, excluding those with obvious tubular pericyte morphology (as shown in Fig. 2F–I, T–Y), and the percentage that colocalized with each antigen was calculated. Only tdTomato^{+} cells