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Research Paper

Regenerative Potential of Ependymal Cells for Spinal Cord Injuries Over Time

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ABSTRACT

Stem cells have a high therapeutic potential for the treatment of spinal cord injury (SCI). We have shown previously that endogenous stem cell potential is confined to ependymal cells in the adult spinal cord which could be targeted for non-invasive SCI therapy. However, ependymal cells are an understudied cell population. Taking advantage of transgenic lines, we characterize the appearance and potential of ependymal cells during development. We show that spinal cord stem cell potential *in vitro* is contained within these cells by birth. Moreover, juvenile cultures generate more neurospheres and more oligodendrocytes than adult ones. Interestingly, juvenile ependymal cells *in vivo* contribute to glial scar formation after severe but not mild SCI, due to a more effective sealing of the lesion by other glial cells. This study highlights the importance of the age-dependent potential of stem cells and post-SCI environment in order to utilize ependymal cell's regenerative potential.

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1. Introduction

Spinal cord injury (SCI) is currently a chronic incurable disease with reported incidences ranging from 9.2 to 246 cases per million of the population a year depending on the area surveyed (Siddiqui et al., 2015). The majority of affected people are 10–40 years old at the time of injury (Siddiqui et al., 2015) and it deeply affects the quality and expectancy of life in young people (The National Spinal Cord Injury Statistical Center, 2015). Indeed, SCI typically results in permanent functional impairment in locomotion and sensation below the injury level, and can also cause neuropathic pain, spasticity and incontinence (Westgren and Levi, 1998).

Upon traumatic injury, the cellular and molecular response of the spinal cord is complex and characterized by acute and chronic phases (Silver and Miller, 2004). The spinal cord attempts repair but it is never complete (Silver and Miller, 2004). The key factors leading to the lack of complete regeneration and recovery of function are the formation of an inhibitory glial environment, neural cell death, demyelination, axonal degeneration and lack of regrowth and inflammatory response (Barnabe-Heider and Frisen, 2008; Gregoire et al., 2015).

Even though it has been suggested that the glial scar has inhibitory effects on self-repair and neuroregeneration after SCI, recent studies showed that astrocytes, ependymal and inflammatory cells have also pro-regenerative properties (Anderson et al., 2016; Barnabe-Heider et al., 2010; Rolls et al., 2009; Sabelstrom et al., 2013). Indeed, the central nervous system shows an innate ability to partially regenerate after traumas (Gregoire et al., 2015). At the anatomical level, the glial scar can be divided into two regions: the border, rimmed primarily by resident reactive astrocytes, and the lesion core, formed mainly by migrating ependymal cells and infiltrating stromal cells (Barnabe-Heider et al., 2010; Goritz et al., 2011; Sabelstrom et al., 2014). Therapeutically, several regenerative approaches have been tested to cure SCI, such as stem cell transplantation (Charsar et al., 2016; Granger et al., 2014). However, these studies have shown beneficial effects in animal models but have important practical limitations in a human context (Granger et al., 2014; Charsar et al., 2016). An alternative way will be to recruit and activate endogenous stem cells after SCI (Qin et al., 2015).

During adulthood, the spinal cord stem cell potential is restricted to ependymal cells, the cell population surrounding the central canal (Barnabe-Heider et al., 2010; Meletis et al., 2008). Ependymal cells are activated by traumatic SCI, self-renew and differentiate into astrocytes and oligodendrocytes (Barnabe-Heider et al., 2010; Meletis et al., 2008). Moreover, when the proliferation of ependymal cells is impaired,

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the formation of the glial scar after SCI is heavily compromised, detrimentally affecting neuronal survival (Sabelstrom et al., 2013).

Altogether, these reports showed that ependymal cells are the endogenous stem cells in the adult spinal cord and therefore constitute an attractive cell population to further investigate and target in order to treat SCI. However, ependymal cells are an understudied cell population and how ependymal cells and other endogenous cell populations influence each other during glial scar formation is unknown, especially during pre-adult (juvenile) stages.

Taking advantage of inducible and non-inducible FoxJ1 transgenic mouse lines, where transgene expression is restricted to cells with motile cilia and thereby specifically targets ependymal cells in the spinal cord (Meletis et al., 2008; Barnabe-Heider et al., 2010), we have investigated the developmental origin and stem cell potential of ependymal cells during juvenile stages. We demonstrated that the first appearance of ependymal cells around the central canal is at embryonic day (E) 15.5 and that, at early postnatal age, ependymal cells fully surround the central canal. We also showed that the *in vitro* spinal cord stem potential is confined to ependymal cells from postnatal day (P) 10, and that this potential decreases over time. More interestingly, and in contrast to the situation in adults (Barnabe-Heider et al., 2010; Sabelstrom et al., 2013), we show that juvenile (P21) ependymal cells respond to severe but not mild traumatic SCI *in vivo*, and that this reduced response is accompanied by an increased contribution of astrocytes, pericytes and microglia. By using FoxJ1-CreER^{T2}-Rasless::YFP mice to specifically block the proliferation of ependymal cells (Sabelstrom et al., 2013), we further confirm that the juvenile mice has greater self-recovery potential due to higher reactivity of other glial cells.

Our experiments reveal that juvenile mice have a higher intrinsic regenerative potential and that they respond to SCI in an age- and lesion depth-dependent manner, greatly influenced by the environment.

2. Materials and Methods

2.1. Mice

All experiments were conducted in accordance with the guidelines of the Swedish Board of Agriculture (ethical permit N329/11, N217/14) and were approved by the Karolinska Institutet Animal Care Committee. We used the tamoxifen-inducible FoxJ1-CreER^{T2}::YFP transgenic mice (Ostrowski et al., 2003; Jacquet et al., 2011; Jacquet et al., 2009). The FoxJ1 promoter is highly expressed by ependymal cells in the central nervous system from development to adulthood. FoxJ1-CreER^{T2}-Rasless::YFP mice were used to specifically delete the N-, K-, H-*ras* genes to block the proliferation of ependymal cells (Sabelstrom et al., 2013). To induce recombination, we injected 60 mg/kg of body weight once daily for 5 and 3 days in adult and juvenile mice, respectively. Clearance of tamoxifen was allowed for 5 days before the start of the spinal cord injury experiments. Embryonically and early postnatally, tamoxifen at the same concentration was injected intraperitoneally to pregnant mice or pups' dam from embryonic day E13–14, E15–17, E17–18, postnatal day P0–P4 and P5–P9. These animals were sacrificed one day after the last injection. Non-inducible FoxJ1-EGFP (Ostrowski et al., 2003) with FoxJ1 promoter driving GFP expression were used for embryo collection at E13.5, E15.5, E17.5, P0, P5 and P10.

2.2. Surgical Procedure and Postoperative Care

Mice were kept under anesthesia with a mixture of 2% isoflurane (Baxer) and 1 l/min O_2. Body temperature was maintained around 37 °C–38 °C keeping the animals on a thermo-pad for the entire surgery. The back of the animal was shaved and disinfected with 70% EtOH. The skin was incised, the superficial fat gently shifted and the muscle tissue dissected to expose laminae T9–T11. A T10 laminectomy was performed and the dura mater was removed. A dorsal funiculi transection or a dorsal hemisection were performed with a microknife (FST).

After surgery, mice were placed back in their home cages. Mice underwent daily checks for general health, mobility within the cage, wounds, swelling, infections or autophagy of the toes. The animals showed neither skin lesions, infection nor autophagy throughout the study. Bladders were manually expressed after operation until needed.

2.3. Tissue Preparation and Sectioning

At the end of the survival period, the animals were deeply anesthetized with sodium pentobarbital (APL) (150 mg/kg body weight) and perfused transcardially with 0.1 M PBS, pH 7.4, and 4% PFA in PBS, pH 7.4 (Life Technologies). Dissected spinal cords were further postfixed in 4% PFA in PBS at 4 °C overnight and cryoprotected in 30% sucrose (Life Technologies) for at least 48 h. After embedding in Tissue-Tek OCT compound (Sakura), the spinal cords were cut sagittally or coronally to 16 μm thickness. Sections were collected 1:12 accordingly to stereological principles and stored at −20 °C until further use.

2.4. Immunohistochemistry

Full details of the primary antibodies used are reported in Table 1.

Spinal cord sections were blocked with 10% normal donkey serum (Jackson Immunoresearch), 0.3% Triton X-100 (Sigma) in PBS for 1 h at room temperature. Primary and secondary antibodies were diluted in 2% BSA (bovine serum albumin; Sigma), 0.2% Triton X-100 PBS. Primary antibodies were incubated at room temperature overnight and secondary antibodies were incubated for 1 h. Secondary antibodies were conjugated with Alexa Fluor fluorophores. Counterstaining was performed with DAPI (1:10,000) in PBS and sections were coverslipped with Vectashield mounting media (BioNordika).

2.5. Neural Stem Cell Cultures

2.5.1. Culture

Animals were sacrificed for control culture or one week after SCI. Spinal cord cells were dissociated and neurosphere cultures were established as described (Meletis et al., 2008). All cells isolated from one spinal cord were plated in 10 cm culture dishes. First, neurospheres were harvested after 2 weeks in culture and then were dissociated into single cells for passage or differentiation. Approximately 100,000 cells per animal were plated in a 10 cm culture dish for the next generation of neurospheres, and all the new neurospheres (second, third and fourth generations) were harvested after one week in culture. Dissociated primary neurospheres, approximately 50,000 cells/well, were plated in poly-D-lysine-coated chamber slides (Sigma) for differentiation with growth factors-free medium supplemented with 1% fetal bovine serum. Two to four independent experiments per group were performed.

Table 1
Antibody table. Details of sources and concentrations of antibodies used for histo/immunochemistry in this study.

Antibody table			
Antibody	Species	Dilution	Company (Catalog#)
GFP	Chicken	1:500	Aves (GFP-1020)
Ki-67	Rabbit	1:500	ThermoScientific (RM-9106)
GFAP	Rabbit	1:500	Millipore (AB5804)
Iba1	Rabbit	1:200	Wako (019–19,741)
PDGFR-β	Rabbit	1:200	abcam (ab32570)
CNPase	Mouse	1:200	Millipore (MAB326R)
Tuj1	Mouse	1:500	Covance (MMS435P)
Anti-rabbit cy3 secondary antibody	donkey	1:500	Jackson Immuno Research (711–166-152)
Anti-mouse cy3 secondary antibody	donkey	1:500	Jackson Immuno Research (715–165-140)
Anti-chicken Alexa 488 secondary antibody	donkey	1:500	Jackson Immuno Research (703–545-155)

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