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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.

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

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and oligodendrocytes (Barnabe-Heider et al., 2010; Meletis et al., 2008). Moreover, when the proliferation of ependymal cells is impaired, 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

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)

growth factors-free medium supplemented with 1% fetal bovine serum. Two to four independent experiments per group were performed.

2.5.2. Immunocytochemistry

After two-week's primary neurosphere culture, approximately 300–500 recombined neurospheres were plated in poly-D-lysine-coated chamber slides for one day, followed by immunocytochemistry for quantification of recombined neurospheres with anti-GFP antibody. For differentiation assay, after 10 days in growth factor-free differentiation condition, immunocytochemistry was performed as described with antibodies as listed in Table 1.

2.5.3. Cell Culture Analysis

For neurosphere assay, neurosphere media was taken 4 times randomly from one culture dish (250 ml per time) and the number of neurospheres were counted; therefore, the total numbers of neurospheres was calculated with total volume of media (11–14 ml per dish). For differentiation assays, after immunocytochemistry, quantification of the percentage of markers was randomly recorded with at least 6 views (20× objective magnification) per well under fluorescence. At least two wells for one marker per animal were used for quantification. The total number of cells was obtained counting all nuclei stained with DAPI.

2.6. Image Acquisition and Tissue Analysis

Confocal representative images of the lesion site and spinal cords were acquired using the Zeiss LMS700 microscope setup. Quantification of the lesion and cell infiltration areas was performed using the Zeiss AxioSkop2 microscope setup and AxioVision software. The areas were measured at the epicenter of the lesion. Quantification of the number of cells were performed using the Zeiss Apotome2 microscope setup. The quantification of cells was performed in 2–4 sections per animal. For each experimental group and staining, 3–9 animals were analyzed.

2.7. Statistical Analysis

Statistics were run with Student's *t*-test for comparing two groups and Student's *t*-test with Bonferroni's correction for more than two groups' comparisons. For all the data analysis, Student's *t*-test with or without Bonferroni's correction or one-way ANOVA with Bonferroni's multiple comparison was used as indicated ($^{*}P < 0.05$, $^{**}P < 0.01$,

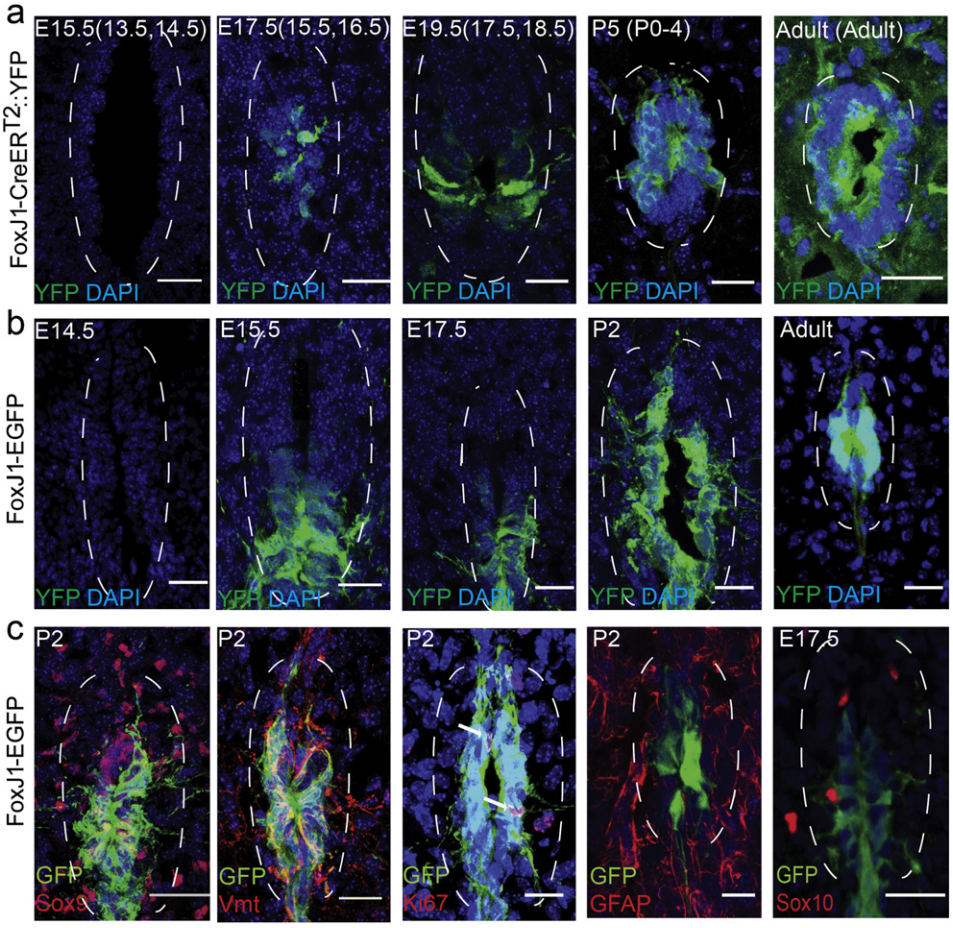
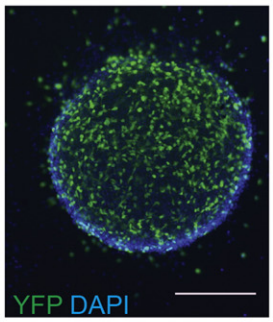


Fig. 1. Ependymal cells appear at mid-embryonic stage in the developing mouse spinal cord. (**A**) Representative coronal section images of YFP labeled ependymal cells around the central canal (dashed lines) of tamoxifen recombined FoxJ1-CreER^{T2}::YFP. YFP expression is induced by tamoxifen at E13.5–14.5, E15.5–17.5, E17.5–18.5, P0–4 and adult (control). Embryonic or postnatal spinal cords were collected at E15.5, E18.5, E19.5, P5 and adult (control), respectively. (**B**) Representative coronal section images of GFP labeled ependymal cells around the central of non-inducible FoxJ1-EGFP spinal cords at different developmental stages (E14.5, E15.5, E17.5 and P2) and adult (control). (**C**) Representative coronal section images of FoxJ1+ ependymal cells stained for precursor marker sox9 and vimentin, astrocyte marker GFAP, proliferation marker Ki67 and oligodendrocyte marker sox10 of either embryonic stage E17.5 or postnatal spinal cord at P2. Scale bar = 25 μm. E = embryonic day, P = postnatal day. Dashed lines indicate ventricular zone/ central canal. Arrows indicate co-staining of GFP and Ki67.

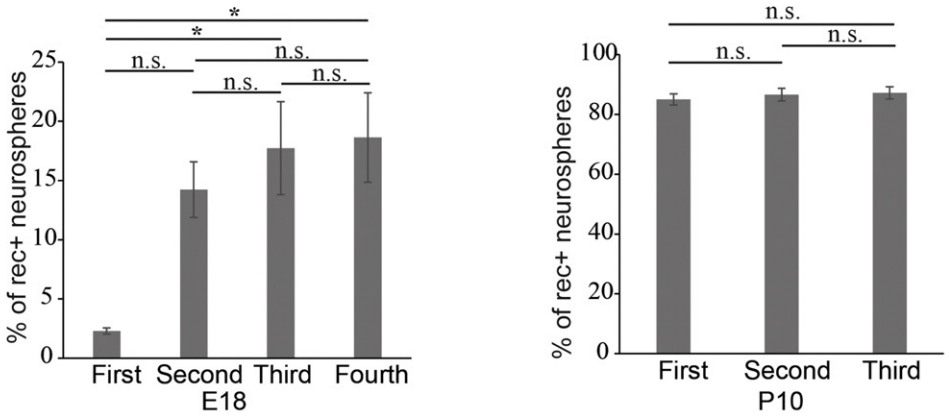
a



b

Timepoint	rec+ neurospheres /total neurospheres	In vivo recombination CC
E15 (TAM at E13-14)	0	0
E18 (TAM at E15-17)	2.3 ± 0.26%	2.4 ± 0.32%
P5 (TAM at P0-5)	74.09 ± 2.02%	74.5 ± 2.88%
P10 (TAM at P5-10)	85.0 ± 1.85%	85 ± 2.4%
Adult	85 ± 2.2%*	80-90%*

c



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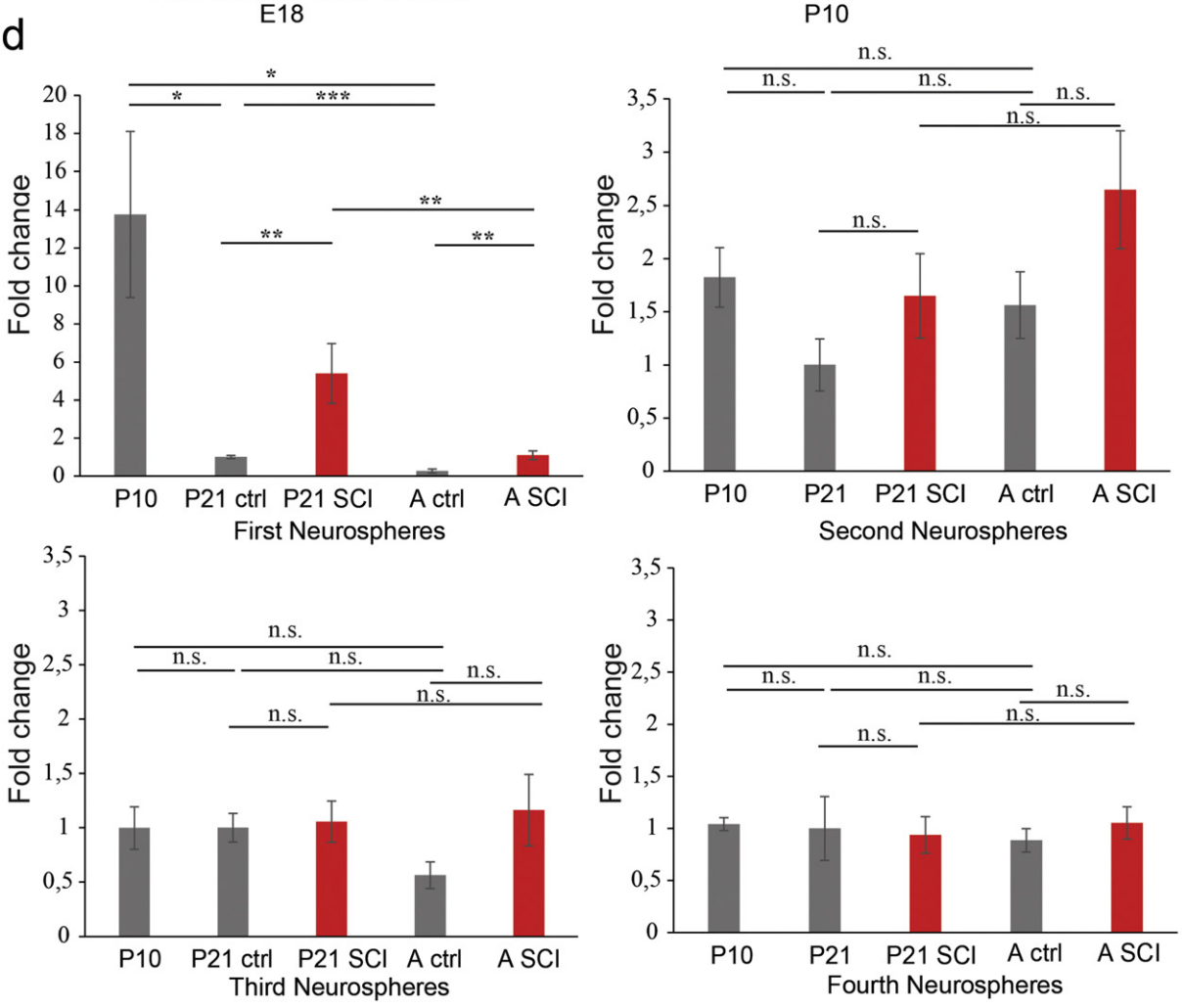


***$P < 0.001$). For each experimental group and staining, 3–9 animals were analyzed.

3. Results

3.1. Ependymal Cells Appear at Mid-Embryonic Stage in the Developing Mouse Spinal Cord

It has been shown that ependymal cells are the only cells retaining stem cell potential in the adult spinal cord (Meletis et al., 2008; Barnabe-Heider et al., 2010) but the generation of ependymal cells during development in the mouse spinal cord has not been characterized. To study the appearance of ependymal cells in the developing mouse spinal cord, we used both inducible and non-inducible FoxJ1 promoter-driven transgenic mouse lines to specifically label ependymal cells (Meletis et al., 2008; Ostrowski et al., 2003; Jacquet et al., 2011; Jacquet et al., 2009). In order to identify the appearance of ependymal cells during development, we induced YFP expression in ependymal cells via tamoxifen-dependent recombination in FoxJ1-CreER^{T2}::YFP mice at different embryonic and postnatal developmental windows: embryonic day (E) E13.5–14.5, E15.5–17.5, E17.5–18.5 and postnatal day (P) P0–4 (Fig. 1A). Embryonic or postnatal spinal cords were collected one day after the last tamoxifen administration.

Analysis of YFP expression revealed that FoxJ1 + cells first appear around the central canal in the E15.5-E17.5 window (Fig. 1A), which was confirmed using the non-inducible FoxJ1-EGFP transgenic mouse line, where the reporter protein EGFP labels exclusively ependymal cells but not their progeny (Fig. 1B). FoxJ1-EGFP mice were sacrificed at different time points during embryonic development and early postnatal age, and similarly, we found that the first GFP + cells appeared at E15.5 (Fig. 1B). In both animal models, the labeled cells showed the typical morphology of adult ependymal cells (Fig. 1A,B) (Bruni and Reddy, 1987). As development proceeded, the number of reporter-expressing cells increased until postnatal stages (P0-P5), when GFP + cells fully surround the central canal, similarly to adulthood (Fig. 1A).

We further confirmed that GFP + cells are ependymal cells by the expression of the classical markers Sox9 and Vimentin. Some of these cells also expressed Ki67 +, showing that proliferation of ependymal cells continues after their differentiation (Fig. 1C). Moreover, during embryonic and early postnatal stages, none of the GFP + cells express astrocytic (GFAP) or oligodendrocytic (Sox10) markers (Fig. 1C), further confirming their ependymal cell identity.

3.2. Higher Self-Renewal Potential of Ependymal Cells during Development and after SCI In vitro

It has been described that neural stem cells, including adult ependymal cells in the spinal cord, are able to generate neurospheres *in vitro*, and the dissociated cells from neurospheres can further generate new neurospheres (Weiss et al., 1996). Using this neurosphere assay can reveal the degree of self-renewal capacity of stem cells. To study whether all or only part of the newly born ependymal cells have self-renewal potential, we used FoxJ1-CreER^{T2}::YFP mice to perform neurosphere assays. Embryos at different time points and early postnatal pups were sacrificed after two or five days of tamoxifen administration to pregnant or lactating mothers, respectively. Almost the full embryonic spinal cords were dissociated for neurosphere assay, and a small cervical spinal cord biopsy from the same animal was kept for immunohistochemistry to compare the *in vitro*/*in vivo* recombined ratios. Recombined neurospheres or cells indicated neurospheres derived from ependymal cells *in vitro* or ependymal cells *in vivo*, respectively. Analysis showed that the ratio of recombined neurospheres was similar to the ratio of recombined-ependymal cells at the same time points, meaning that all the ependymal cells have self-renewal potential. More importantly, the number of cells expressing the YFP reporter drastically increased from E15–17 to P5–10 (Fig. 2A and B) (Meletis et al., 2008).

To confirm the self-renewal capacity of newly born ependymal cells, we serially passaged the first recombined neurospheres. We found that the earliest-born recombined neurospheres (E18.5, tamoxifen induced at E15.5–17.5), grew with passages and postnatally, by P10, all recombined neurospheres maintained their ratio over passages (Fig. 2C). These data suggest that all the newly-born ependymal cells have self-renewal potential and are able to maintain it, and that the self-renewal potential is confined to ependymal cells in the spinal cord from an early postnatal stage.

To further investigate the self-renewal potential of ependymal cells and the effect of SCI during postnatal development, we cultured neurospheres from P10, P21 (juvenile) and adult mouse spinal cord. In our experiments, we defined a "unit of self-renewal potential" as the ratio between the numbers of neurospheres generated per 10,000 primary cells plated. We set the ratio obtained from P21 control cultures as one-fold for further comparison with the other conditions. In primary neurosphere culture, we observed that during the postnatal development, the self-renewal potential of ependymal cells significantly decreased by approximately 14-fold (P10 vs. P21) and 4-fold (P21 vs. adult), respectively (Fig. 2D).

Despite the dramatic decrease of self-renewing ependymal cells from mature adults, this potential can be highly recruited after SCI by showing more neurospheres (Barnabe-Heider et al., 2010). Given the higher self-renewal ability of juvenile (P21) spinal cord ependymal cells and the beneficial effect of the recruitment of spinal cord ependymal cells after adult SCI, we investigated whether juvenile ependymal cells show an even higher self-renewal ability after SCI. We performed dorsal funiculus transection on both P21 and adult mice at thoracic (T) 10 level. Spinal cords were collected for primary cell cultures one week after injury. The self-renewal potential unit of P21 non-injured group was defined as one-fold, as described above. When we compared P21 and adult cultures prepared from injured spinal cords, we observed a five-fold increase in self-renewal potential between different conditions: specifically including non-injured P21 vs. non-injured adult, P21 injured vs. adult injured, P21 injured vs. P21 non-injured conditions (Fig. 2D). Furthermore, when we passaged the neurosphere cultures for four generations, we observed that the effect of injury on the self-renewal potential was gradually lost (Fig. 2D). These data indicate that SCI can highly increase the self-renewal capacity of ependymal cells at both time points but only temporally, and that juvenile ependymal cells have a higher recruitment potential after SCI than adult ones.

3.3. Juvenile Ependymal Cells Show Higher Oligodendrogenesis Potential after SCI In vitro

To characterize the potential of differentiation *in vitro* during development and after injury, spinal cord primary neurospheres were harvested, dissociated and cultured in differentiation media at different developmental stages (E18.5, P10, P21), adulthood and after SCI (P21 and adulthood). We observed that E18.5 primary neurospheres can

Fig. 2. Juvenile ependymal cells show a greater self-renewal potential than adult in control conditions and after SCI *in vitro*. (**A**) Representative image of a recombined neurosphere *in vitro* from FoxJ1-CreER^{T2}::YFP mice. (**B**) Quantification of recombined neurospheres *in vitro* and recombined ependymal cells *in vivo*. Rec + = recombined. (**C**) Quantification of the ratio of E18.5 (tamoxifen administration at E15–17) and P10 (tamoxifen administration at P5-P9) recombined neurospheres over the total number of neurospheres in culture. (**D**) Quantification in fold change of neurospheres grown in culture from spinal cords at the indicated ages and experimental conditions during four generations of neurosphere. One-fold change is defined based on the number of neurospheres per 10,000 cells plated of cell extracted from P21 control spinal cords. Folds are shown ± SEM. First, second, third, fourth = primary, secondary, tertiary and fourth neurosphere passages, respectively. A = adult. Ctrl = control. P = postnatal day. SCI = spinal cord injury. N = 4–11 animals/condition. Scale bar = 100 μm. P-value: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001. Student's t-test with or without Bonferroni's correction or one-way ANOVA with Bonferoni's multiple comparison was used. Two to four independent cultures were performed for each condition.

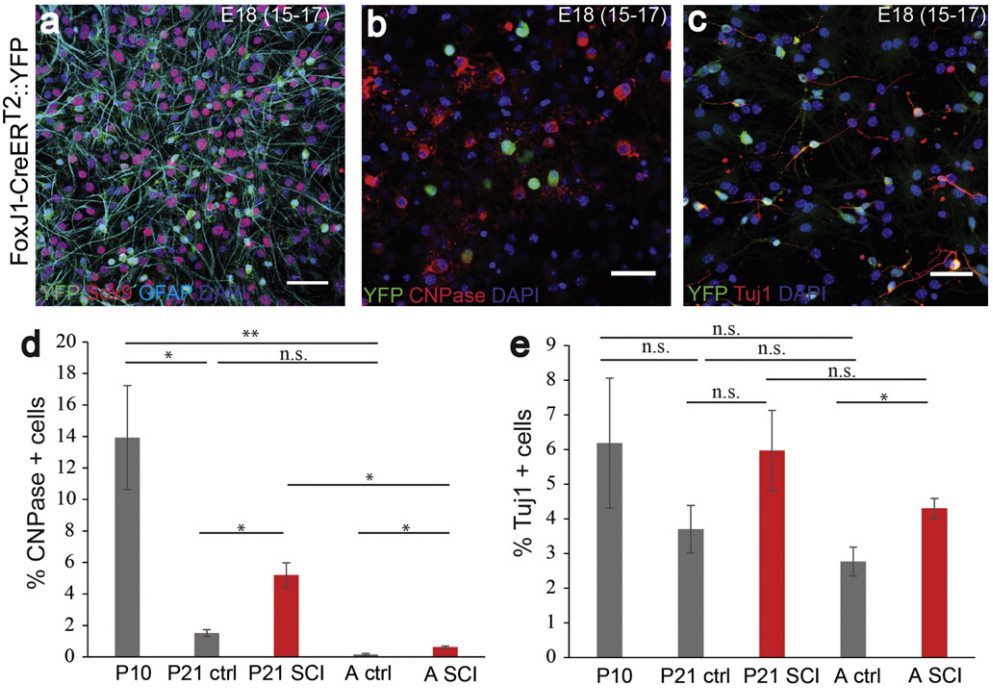


Fig. 3. Juvenile ependymal cells show higher differentiation potential towards the oligodendrocytic lineage. (**A-C**) Representative images of E18.5 spinal cord primary stem cell culture after 10 days of differentiation (FoxJ1-CreER^{T2}::YFP mice). This culture can generate differentiated (**A**) astrocytes (GFAP and Sox9), (**B**)oligodendrocytes (CNPase) and (**C**) neurons (Tuj1). (**D**) Quantification (by percentage) of the expression of CNPase + oligodendrocytes from differentiated primary neurospheres during postnatal development, adulthood and after SCI. (**E**) Quantification (by percentage) of the expression of Tuj1 + cells from primary differentiated neurospheres during postnatal development, adulthood and after SCI. Percentages are shown ±SEM. *P*-value * ≤ 0.05, ** ≤ 0.01. Scale bar = 100 μm. *N* = 3–4 animals per condition. A minimum of 500 cells were counted per each condition. A = adult. Ctrl = control. SCI = spinal cord injury, CNPase = cyclic nuecleotide phosphodiesterase, Tuj1 = neuron-specific beta III tubulin. Student's *t*-test with or without Bonferroni's correction or one-way ANOVA with Bonferoni's multiple comparison was used. Two to four independent cultures were performed for each condition.

differentiate into astrocytes (YFP +/Sox9 +/GFAP + cells), oligodendrocytes (YFP +/CNPase + cells) and neurons (YFP +/Tuj1 + cells) (Fig. 3A–C). However, ependymal cells gradually lose the capability to differentiate into oligodendrocytes. Indeed, we observed almost 10-fold decrease between P10 to P21 and to adulthood (Fig. 3D).

Interestingly, from a therapeutic perspective, we observed that SCI enhances differentiation into oligodendrocytes of both P21 and adult ependymal cells by almost 5-fold compared to non-injured conditions when neurospheres are grown in serum (Fig. 3D). Moreover, P21-derived generated more oligodendrocytes than adult-derived neurospheres after SCI. These observations may have positive implications for the remyelination potential of juvenile compared to adult spinal cords after traumatic injury. However, the capacity to generate neurons (Tuj1 + cells) only displayed a decreasing tendency without statistical significance during development from P10 to P21 and to adult (Fig. 3E). SCI increased neurogenesis from ependymal cells *in vitro* mildly, while the more profound effect recorded was on oligodendrocyte generation. The differentiation data suggest that the intrinsic differentiation capability of ependymal cells to other cell types varies during development and after SCI and preferentially stirs towards gliogenesis.

3.4. The Recruitment of Ependymal Cells at the Injury Site Is Age- and Lesion Depth-Dependent

Previous studies showed that adult ependymal cells are activated and recruited at the lesion site in a similar manner after both mild and severe traumatic SCI (Barnabe-Heider et al., 2010; Sabelstrom et al., 2013). We investigated whether a similar or different pattern of migration and activation was observed in juvenile (P21) spinal cord.

We used the FoxJ1-CreER^{T2}::YFP mouse model to fate map the ependymal cells and follow their progeny activation and migration after SCI in juvenile and adult mice. Five days after tamoxifen-induced specific expression of the YFP reporter in ependymal cells, we performed mild or severe SCI at mid-thoracic level (T9–10) in juvenile (P21) and adult (2–5 months old) mice. We used dorsal funiculi transection (DFT) and dorsal hemisection (DH) as mild and severe SCI models, respectively (Cheriyan et al., 2014; Kwon et al., 2002). Animals were then sacrificed one week or four months after lesion to investigate contribution of ependymal-derived progeny and anatomical changes during both post-injury acute and chronic phases.

Previous reports showed that adult ependymal cells are massively recruited towards the lesion site after SCI (Barnabe-Heider et al., 2010; Okada et al., 2006). Here, we also observed that adult ependymal cells migrate towards the middle of the lesion site after SCI (Fig. 4A,B,E). Surprisingly, juvenile ependymal cells were recruited only after severe (DH) and not mild (DFT) SCI (Fig. 4A,B,E). The total number of recombined ependymal cells was comparable in the animals at the different ages tested with a different distribution between the cells remaining around the central canal and those having migrated to the lesion site (Fig. 4C), assuring that the effect on migration we observed was not due to recombination artifacts.

3.5. The Scar Formation and the Lesion Environment Affect the Recruitment of Ependymal Cells

SCI induces activation and recruitment of several cell types, including astrocytes, microglia, blood-derived macrophages and stromal cells (fibroblasts and pericytes). Therefore, we analyzed other aspects of glial scar formation in order to further understand the different responses of P21 and adult ependymal cells.

Firstly, the lesion area after SCI can be subdivided in a lesion core where reactive astrocytes do not infiltrate and an astrogliotic area where reactive astrocytes accumulate. We observed that the lesion core area was smaller in juvenile than adult mice following DFT (Fig. 4A,D), indicating that juvenile spinal cord can seal the lesion site more efficiently than adult after mild injury ($P < 0.01$ at 1 wpi and $P < 0.05$ at 4 mpi). In

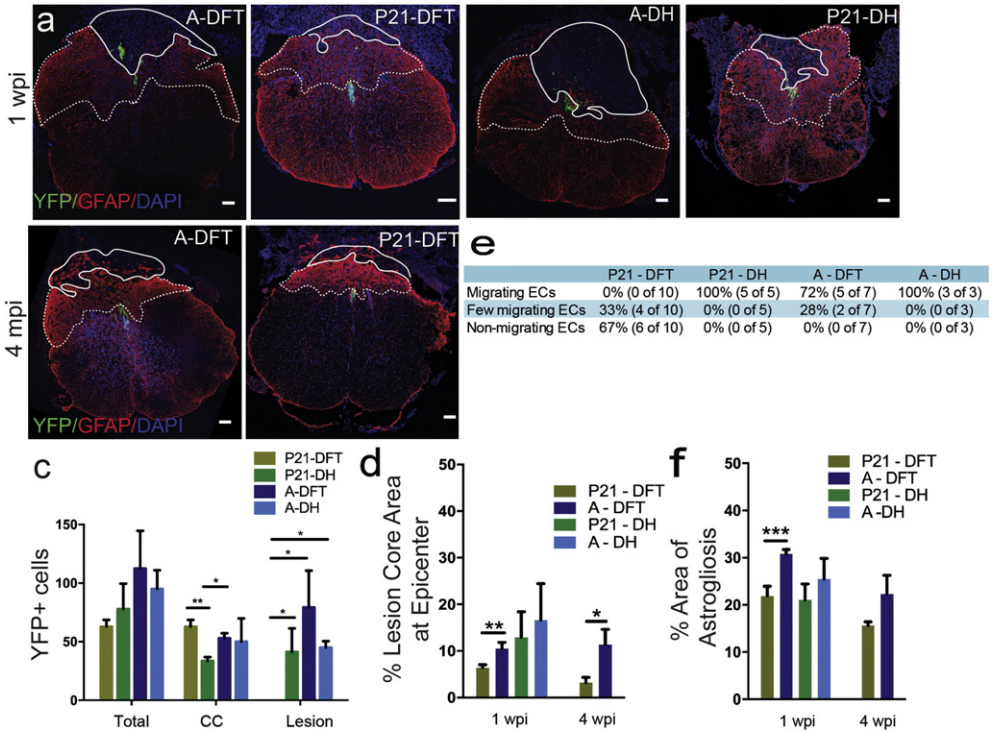


Fig. 4. Ependymal cells recruitment depends on age and spinal cord lesion depth. (**A**,**B**) Representative pictures of coronal spinal cord sections of adult DFT, P21 DFT, adult DH and P21 DH spinal cord 1 week after injury and adult DFT and P21 DFT 4 months after SCI (FoxJ1-CreER^{T2}::YFP mice). Sections were stained with GFP, GFAP and DAPI. (**C**) Quantification of the number of ependymal cells around the central canal and at the lesion site in juvenile and adult spinal cords one week after DFT or DH. (**D**) Quantification ((by percentage) of the extension of the lesion core and (**F**) astrogliosis areas in juvenile and adult spinal cords one week and four months after DFT or DH. (**E**) Summary table showing the correlation between ependymal cells migration towards the lesion site and age or lesion depth. Scale bars are 100 μm. Full and dashed lines outline lesion core and astrogliotic area, respectively and were used for quantification. Percentages are shown ± SEM. *P*-value * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001. Only statistical significance were marked with *, otherwise no significance. P21 = postnatal day 21, A = adult, DFT = dorsal funiculi transection, DH = dorsal hemisection, CC = central canal, ECs = ependymal cells, wpi = week post-injury, mpi = months post-injury. Few migrating ECs = <5 ECs in the lesion site/section. *N* = 3 to 10 animals per condition. Student's *t*-test with or without Bonferroni's correction was used.

contrast, we observed a lesion core area comparable to adult tissue when a severe injury (DH) was performed in juvenile animals (Fig. 4A,D).

Secondly, since astrocytes also contribute to the sealing of the lesion site (Okada et al., 2006), we measured the area of astrogliosis around the core of the lesion. We observed an astrogliosis area smaller or similar to adult when a mild (DFT) ($P < 0.001$) or severe (DH) injury was performed in juvenile animals (Fig. 4F), respectively. Importantly, we did not observe a statistically significant difference in lesion core or astrogliosis areas in adult mice following mild (DFT) or severe (DH) injuries (Fig. 4A,D,F). Altogether, our data suggest that the size of the lesion impacts the migration of ependymal cells in juvenile mice.

Activated microglia and blood-derived macrophages are heterogeneous cell populations. They are subdivided in M1 and M2 macrophages that show proinflammatory and anti-inflammatory properties, respectively (Kigerl et al., 2009). This importantly affects the self-repair properties of the spinal cord after injury. Moreover, pericytes have recently emerged as a pivotal cell type in sealing the lesion following SCI (Goritz et al., 2011).

To assess whether microglia, blood-derived macrophages and stromal cells can also influence the age- and lesion-dependent differential recruitment of ependymal cells after SCI, we stained the lesion site of juvenile and adult mice after mild and severe SCI for Iba1 and PDGFRβ, markers of microglia-macrophages and stromal cells, respectively.

Similarly to our analysis of the lesion area, we measured a smaller area infiltrated by activated microglia and blood-derived macrophages ($P < 0.05$) or stromal cells ($P < 0.05$) at the lesion site in juvenile compared to adult mice after DFT (Fig. 5A–D). We also observed Iba1$^+$ and PDGFRβ$^+$ areas comparable to adult only after performing a severe lesion in juvenile mice (Fig. 5A–D). Iba1 analyses were performed also at 4 mpi (month post-injury). However, during the chronic phase after SCI, the inflammatory response retrogresses and we did not observe any significant activation of microglia/blood-derived macrophages (data not shown). Altogether, this suggests that the glial scar formation is significantly different in juvenile and adult mice due to the differential response of the other glial cell populations and pericytes.

3.6. Lesion Sealing Is More Efficient and Ependymal-Cell Independent in Juvenile than Adult Spinal Cord

Adult ependymal cells contribute to sealing and restrict second enlargement of the lesion (Sabelstrom et al., 2013). Our data in juvenile mice showed that ependymal cells are recruited to the lesion site depending on the severity of the injury. Therefore, we assessed the contribution of juvenile ependymal cells to the sealing and restriction of secondary enlargement in juvenile animals taking advantage of the FoxJ1-CreER^{T2}-Rasless::YFP mouse model. In this mouse model, removal of the Ras genes in ependymal cells blocks their cell cycle and proliferation after SCI (Sabelstrom et al., 2013).

We performed DH in juvenile and adult mice and sacrificed the animals one month after injury when the injury depth reaches its maximum and no further enlargement is observed (Sabelstrom et al., 2013). We stained for GFAP and PDGFRβ in order to visualize the border of the lesion and the stromal cap, respectively. We observed that when ependymal cells could not be activated in juvenile or adult mice, the lesion was properly sealed in all the analyzed juvenile animals while 50% of the adult mice showed a cavity (Fig. 6A-C). We also quantified the astrogliotic area and the infiltrating stromal cell area. For normalization, we set the areas measured on animals injured at P21 as 100%.

We found that both astrogliosis and stromal infiltration areas at the injury site of animals injured at P21 were significantly smaller than

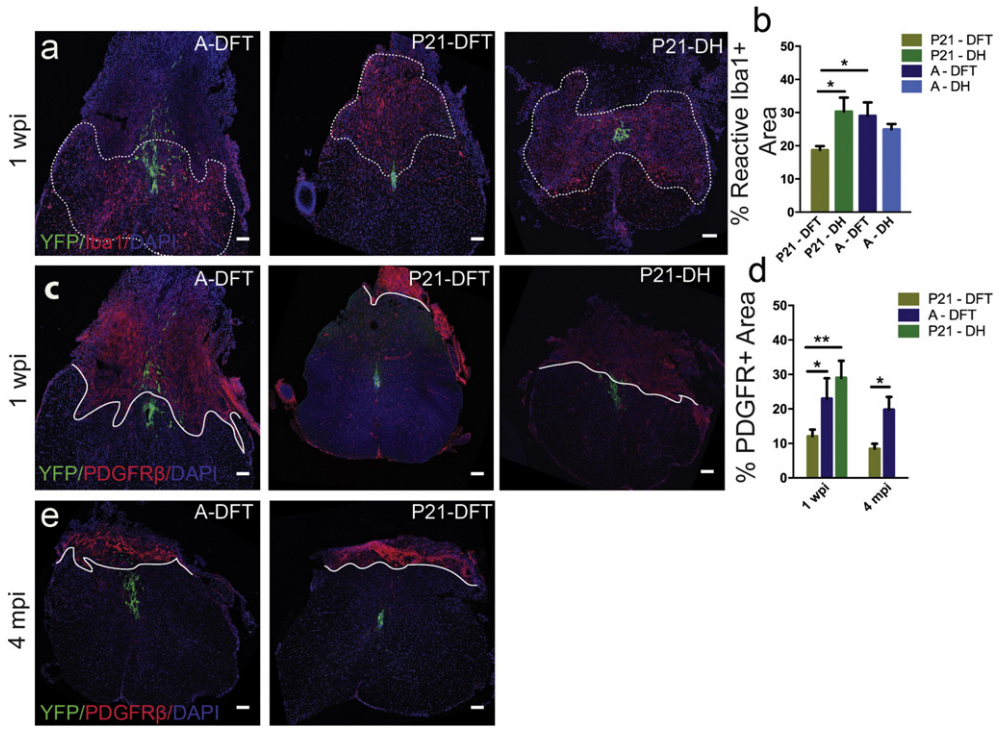


Fig. 5. Activation of microglia/macrophages and scar-forming stroma cells infiltration depends on age and spinal cord lesion depth. Representative pictures of coronal spinal cord sections of adult DFT, P21 DFT and P21 DH spinal cord (FoxJ1-CreERT2::YFP mice): **(A,B)** activated microglia/macrophages area (Iba1$^+$ area) and quantification of Iba1$^+$ area 1 week after injury, (**C-E**) pericyte-derived stromal cells area (PDGFRβ$^+$) and quantification of PDGFRβ$^+$ area 1 week or 4 months after injury. Quantification was shown in percentage. Scale bars are 100 μm. Dashed and full lines outline the reactive Iba1$^+$ cells and pericyte areas of infiltration, respectively and were used for quantification. Percentages are shown ± SEM. *P*-value * ≤ 0.05, ** ≤ 0.01. Only statistical significance were marked with *, otherwise no significance. P21 = postnatal day 21, A = adult, DFT = dorsal funiculi transection, DH = dorsal hemisection, wpi = week post-injury, mpi = months post-injury. *N* = 3 to 9 animals per condition. Student's *t*-test with or without Bonferroni's correction was used.

when injury was performed during adulthood (Fig. 6D). These data confirm that in juvenile mice, ependymal cells are not recruited because other glial cells have sufficient lesion sealing properties. This suggests that ependymal cells are the spinal cord endogenous reservoir to promote self-repair when severe injury occurs and in adulthood.

In summary, our data showed that the formation of the glial scar after SCI is influenced by both the mouse age and severity of injury. Furthermore, we showed that juvenile ependymal cells, the stem cells of the spinal cord, have higher intrinsic stem cell potential than adult ones. However the potential for self-repair and regeneration is highly influenced by age and the lesion environment.

4. Discussion

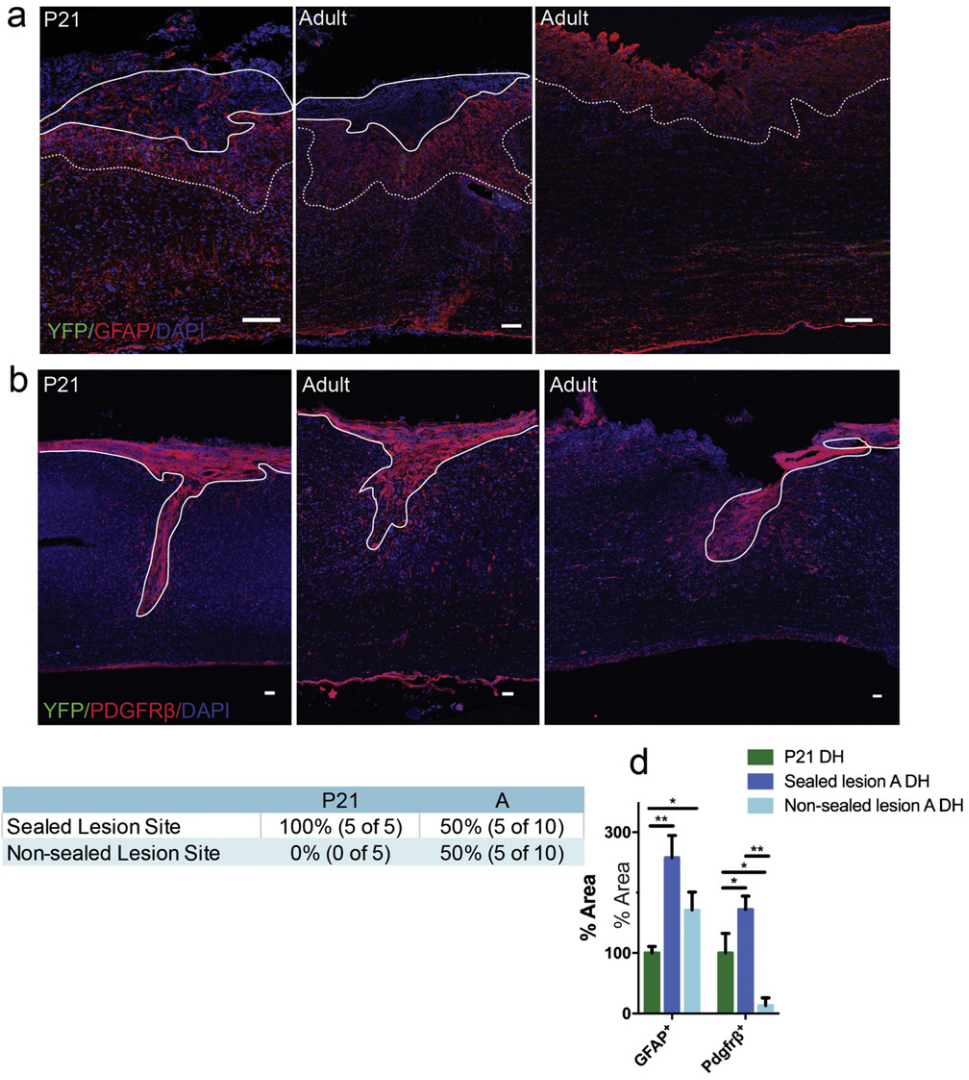
We have studied and characterized the potential of ependymal cells during development and after SCI. By comparing the *in vivo* and *in vitro* recombined ratios of FoxJ1-CreER^{T2}::YFP mouse line during embryonic and early postnatal development, we found that *in vitro* spinal cord stem cell potential is gradually confined to ependymal cells starting from their embryonic appearance at E15.5. The self-renewal potential is all confined to ependymal cells from P10, but the capability of self-renewal dramatically decreases after postnatal development and enhanced by SCI.

Juvenile ependymal cells have higher intrinsic stem cell potential than adults both before and after SCI *in vitro*. However, *in vivo* ependymal cells react as a backup mechanism for self-repair and get recruited when the other glial cell types fail to seal the lesion. This suggests that there might be higher self-repair potential in juvenile than adult spinal cords.

In the spinal cord, ependymal cells originate at mid embryonic stages (E15.5) and completely surround the central canal by birth (P0). Two waves of ependymogenesis have been described in rats: the first seems to derive from radial glial progenitors during embryonic development (E17.5) and the second wave is formed during postnatal life (Qin et al., 2015). However, most studies on ependymogenesis have been based on immunohistochemistry analysis with pan markers of ependymal and astrocytic progenitor cells, such as Sox9 and Vimentin. These markers lack specificity and do not allow precise birth-dating of mature ependymal cells. More importantly, this has limited further study of the stem cell potential of endogenous ependymal cells during spinal cord development and upon injury.

To determine the developmental time point at which mouse ependymal cells arise in the spinal cord, we took advantage of inducible FoxJ1-CreER^{T2}::YFP and non-inducible FoxJ1-EGFP transgenic mouse lines. FoxJ1 promoter-driven reporter expression can specifically label ependymal cells in the adult spinal cord (Meletis et al., 2008; Barnabe-Heider et al., 2010). We showed that recombined ependymal cells line around the central canal the earliest at E15.5 in the mouse embryonic spinal cord. Combining with the markers previously used to characterize ependymal cells (Sox9 and Vimentin), we confirmed that ependymal cells first appear in mouse spinal cord at E15.5, and virtually fully surround the central canal by P10, while showing proliferative activity.

Neurosphere assay reveals the self-renewal potential of neural stem cells. Our data showed that virtually all neurospheres are derived from ependymal cells from P10 to adulthood, indicating that the spinal cord stem cell potential is fully confined to ependymal cells from a very young age. We suggest that therapies targeting endogenous stem cells after SCI should focus on ependymal cells. Our neurosphere assay showed that juvenile have a greater self-renewal potential than adult ependymal cells and are more highly recruited after SCI. Previous studies have suggested that neural stem cells lose their stem cell potential during ageing and after traumatic brain injury (Sun et al., 2005; Conover and Shook, 2011). Our data are in line with these studies.



	P21	A
Sealed Lesion Site	100% (5 of 5)	50% (5 of 10)
Non-sealed Lesion Site	0% (0 of 5)	50% (5 of 10)

Fig. 6. Activation of ependymal cells is not required to seal the lesion site in juvenile spinal cords. (**A,B**) Representative picture of longitudinal spinal cord sections of the lesion site epicenter in juvenile and adult spinal cord one month after dorsal hemisection (FoxJ1-CreER^{T2}-Rasless::YFP mice). Tissues were stained by GFAP and PDGFRβ. (**C**) Summary table showing the correlation between age, lesion sealing and ependymal cells activation. Scale bars are 100 μm. (**D**) Quantification (by percentage) of reactive astrocyte (GFAP^{+}) and pericyte-derived stromal cells (PDGFRβ^{+}) area. P21 GFAP^{+} or PDGFRβ^{+} area were set as 100% for normalization. Percentages are shown ± SEM. *P*-value * ≤ 0.05, ** ≤ 0.01. Only statistical significance were marked with *, otherwise no significance. Dashed lines outline the astrogliotic lesion border. Full lines outline the non-astrogliotic lesion cord or the infiltration area by pericyte-derived stromal cells. P21 = postnatal day 21, A = adult, DH = dorsal hemisection, mpi = months post-injury. *N* = 3 to 6 animals per condition. Student's *t*-test with or without Bonferroni's correction was used.

Additionally, we provide previously unreported evidence that there is a higher recruitment of juvenile than adult ependymal cells after SCI.

Juvenile ependymal cells also display a higher potential of differentiation into oligodendrocytes compared to adult both prior or after SCI. However, the potential of generating neurons is not dramatically affected by different ages or by SCI *in vitro*. Previous work has shown that embryonic spinal cord stem cells and adult ependymal cells give rise to different proportions of neural cell types (Kalyani et al., 1997; Barnabe-Heider et al., 2010), our data support that further therapies should focus on the recruitment of ependymal cells and their differentiation to oligodendrocyte rather than into neurons to increase functional recovery. Previous studies showed that exogenous growth factors can modulate oligodendrocyte proliferation and suggested that this would be a beneficial target for further drug discovery and therapies for SCI (Corns et al., 2015). Our data agrees with this argument and further indicates that modulating stem cell differentiation towards the oligodendrocyte lineage will be influenced by age-dependent differences in intrinsic stem cell potential.

Ependymal cells respond to SCI by migrating towards the core of the lesion site, where they give rise to astrocytes and, to a smaller extent, oligodendrocytes (Barnabe-Heider et al., 2010). It has been suggested that ependymal cell-derived astrocytes positively contribute to the self-repair of the injured spinal cord (Sabelstrom et al., 2013). However, the self-recovery potential of the spinal cord after injury seems to decline during ageing (DeVivo et al., 1990; Furlan et al., 2010; Wyndaele and Wyndaele, 2006). In this regard, we investigated whether and how ageing affects the capacity of ependymal cells to respond to injury.

Ependymal cell-derived astrocytes are located at the core of the lesion site, while resident astrocytes proliferate and are recruited at the border of the lesion site after spinal cord injury (Barnabe-Heider and Frisen, 2008; Burda and Sofroniew, 2014). It was demonstrated that ageing changes the astrocyte physiology and the sensitivity to oxidative stress, and age-related astrocytic changes in the central nervous system results in reduced VEGF and FGF-2 signaling, which in turn limits neural stem cell and progenitor cell maintenance and contributes to decreased neural stem cell potential (Bernal and Peterson, 2011; Lin et al., 2007).

We observed that juvenile ependymal cells are not recruited to the lesion site *in vivo* after mild SCI in juvenile mice. In parallel, we also observed that the sealing of the lesion is more efficient in juvenile

compared to adult mice when we performed SCI of the same severity. This suggests that juvenile resident astrocytes may contribute to sealing the lesion more efficiently than during adulthood. This may lead to a more pro-regenerative environment and the activation, recruitment and differentiation of ependymal cells into astrocytes is not required in juvenile mice as much as during adulthood following SCI. However, in case of severe SCI, juvenile ependymal cells migrate towards the core of the lesion site, showing the same phenotype that was previously described in response to both mild and severe SCI in adult mice (Barnabe-Heider et al., 2010; Sabelstrom et al., 2013).

Importantly, one previous study showed that blocking the recruitment and proliferation of adult ependymal cells results in development of large cysts at the lesion sites, implying that ependymal cell progeny functions as a scaffold within the scar to restrict secondary enlargement of the lesion after the initial insult (Sabelstrom et al., 2013). We showed here that when the cell cycle is blocked in juvenile ependymal cells, no cysts develop in the mouse spinal cord after severe SCI. This supports the idea that juvenile ependymal cells play a secondary role in the sealing and enlargement of the lesion site as other glial cell populations are already responding to the lesion. Moreover, this sheds light on the importance of the environment to efficiently recruit endogenous stem cells upon SCI.

Altogether, our data show that despite the presence of ependymal cell stem cell potential in both juvenile and adult spinal cords, they react *in vivo* as backup participants in the response to SCI in juvenile mice, i.e., only when the injury is too severe to be sufficiently sealed by the other resident glial cells, such as astrocytes. This highlights that efficient modulation of ependymal cells and the glial scar response both have to be considered in the therapeutic context.

Besides resident astrocytes and ependymal cells, other glial cell types, including microglia, blood-derived macrophages and stromal cells, also have a major role in glial scar formation after SCI and influence the reactivity of resident astrocytes (Goritz et al., 2011; Jin and Yamashita, 2016; Schwartz, 2010). We investigated whether these mechanisms would also influence the migration and activation of ependymal cells in mild and severe SCI in juvenile and adulthood.

During glial scar formation, glial cells influence each other response and recruitment to the lesion site (Stenudd et al., 2015). In juvenile mice, we report that mild SCI results in reduced activation and recruitment of microglia and blood-derived macrophages to the lesion site compared to their response following severe SCI. Also, the microglia/blood-derived macrophages activation and recruitment reaches the extent observed in adulthood only when a severe injury is performed in juvenile mice. Therefore, we suggest that juvenile reactive astrocytes are more pro-regenerative than in the adult.

Pericytes are the major cell type forming the fibrotic cap filling the lesion site after SCI (Goritz et al., 2011). We observed that in juvenile mice the stromal cap is reduced in size compared to adult spinal cords when the same kind of injury is performed. The differences among several cells types involved in self-repair reveal that the regenerative and recovery potential of the spinal cord decreases not only during development but also during ageing. That could be due to the age-dependent transcriptome changes in terms of inflammation or metabolism (Saunders et al., 2014; Noor et al., 2011). Notably, there is clinical evidence that people who have sustained SCI during pediatric rather than adult ages enjoy better overall health and functional recovery as well as reduced pain (Ma et al., 2016). Consist with this, our data on the formation of the glial and stromal scars suggest that juvenile spinal cords have a more pronounced potential for self-repair than adult cords, which could be highly relevant to the development of optimal individual SCI therapies.

Our findings of developmental changes in the injury responses of endogenous stem cells have particular therapeutic relevance for SCI. Recruitment of endogenous stem cells is a conceptually different approach to achieving the functional benefits previously observed with cell transplantation, which can carry significant ethical, tumorigenic and immunological/infectious risks (Barnabe-Heider and Frisen, 2008; Coutts and Keirstead, 2008). Considering the high prevalence of SCI in the teenager/young adult population, better understanding of changing stem cell properties during development and ageing is essential for developing age-targeted endogenous stem cell approaches. Indeed, virtually all published studies to date on endogenous spinal cord stem cells have been conducted using adult and not juvenile animals. This study opens the door for tackling the challenge of manipulating proliferation, migration and differentiation of endogenous stem cells across different age/developmental periods.

In summary, we show age-related differences in ependymal stem cell potential and contribution to scar formation and sealing of the lesion following SCI. Our results highlight that the juvenile spinal cord is more prone to self-repair, and suggest that recruitment of ependymal cell occurs mainly when other spinal cord self-repair mechanisms are insufficient to restrict lesion site damage. Properly tuning the response of both ependymal and other glial cell populations should be explored as a way to promote spinal cord repair following SCI.

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Conflicts of Interest

We declare that we have no conflict of interest.

Author Contributions

F.B.H. conceptualized the project.
X.L., E.M.F. and K.T. designed and performed experiments.
X.L., E.M.F., N.G. and F.B.H. performed analysis.
K.J.F. provided tissues and scientific suggestions.
X.L., E.M.F., N.G. and F.B.H. wrote the article.

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