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# p57kip2 regulates glial fate decision in adult neural stem cells

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## SUMMARY

Our recent studies revealed p57kip2 as an intrinsic regulator of late gliogenesis and demonstrated that in oligodendroglial precursor cells p57kip2 inhibition leads to accelerated maturation. Adult neural stem cells have been described as a source of glial progenitors; however, the underlying mechanisms of cell fate specification are still poorly understood. Here, we have investigated whether p57kip2 can influence early events of glial determination and differentiation. We found that Sox2/GFAP double-positive cells express p57kip2 in stem cell niches of the adult brain. Short-hairpin RNA-mediated suppression of p57kip2 in cultured adult neural stem cells was found to strongly reduce astroglial characteristics, while oligodendroglial precursor features were increased. Importantly, this anti-astrogenic effect of p57kip2 suppression dominated the bone morphogenetic protein-mediated promotion of astroglial differentiation. Moreover, we observed that in p57kip2 knockdown cells, the BMP antagonist chordin was induced. Finally, when p57kip2-suppressed stem cells were transplanted into the adult spinal cord, fewer GFAP-positive cells were generated and oligodendroglial markers were induced when compared with control cells, demonstrating an effect of *in vivo* relevance.

**KEY WORDS:** Astrocyte fate, Oligodendroglial differentiation, Multiple sclerosis, Regeneration, Spinal cord injury, Rat, Cdkn1c

## INTRODUCTION

Adult neurogenesis mainly occurs in two regions of the central nervous system (CNS), the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Alvarez-Buylla et al., 2001). Both regions contain adult neural stem cells (NSCs), which have the capacity to self-renew and differentiate into neurons and glia (Gritti et al., 1999; Kriegstein and Alvarez-Buylla, 2009). NSCs and progenitor cells are embedded in a cellular and extracellular micro-environment, the so-called stem cell niche (Palmer et al., 2000; Doetsch, 2003). SVZ-derived NSCs are able to generate neuronal-determined progenitors and tangentially migrate along the rostral migratory stream (RMS) to the olfactory bulb to be incorporated as interneurons (Doetsch and Alvarez-Buylla, 1996). Furthermore, it has been demonstrated that glial fibrillary acidic protein (GFAP)-expressing radial glia astrocytes of the SVZ also possess stem cell properties (Doetsch et al., 1999; Laywell et al., 2000). NSCs from the SGZ divide along the hilus and generate a continuous pool of differentiating neuronal precursors, which functionally integrate into the granular hippocampal cell layer (Kuhn et al., 1996; van Praag et al., 2002). However, these hippocampal-derived NSCs are not restricted to generate only neurons but they can also give rise to oligodendroglial cells in culture (Rivera et al., 2006) as well as *in vivo* (Jessberger et al., 2008). Of note, under inflammatory conditions, SVZ NSCs are also able to generate oligodendroglial cells (Picard-Riera et al., 2002; Menn et al., 2006; Nait-Oumesmar

et al., 2007). In addition, it has been shown that NSCs from the subcallosal zone, a caudal extension of the SVZ, migrate into the corpus callosum to become oligodendrocytes (Seri et al., 2006).

The CNS has a limited capacity for regeneration, which is why traumatic injuries, demyelinating or degenerative diseases generally result in irreversible deficits. In the pathology of multiple sclerosis (MS), an immune-driven inflammatory disease featuring myelin loss, axonal degeneration as well as astrogliosis (Trapp et al., 1999; Bitsch et al., 2000), transient phases of recovery occur owing to remyelination (Franklin and Ffrench-Constant, 2008). Functional cell replacement is a consequence of oligodendrocyte precursor cell (OPC) or NSC activation and differentiation (Chang et al., 2000; Picard-Riera et al., 2002; Menn et al., 2006; Nait-Oumesmar et al., 2007; Rivera et al., 2010). Despite its spontaneous character, the overall remyelination efficiency remains low and is further decreased with disease progression, which is thought to be due to the presence of inhibitory factors (Scolding et al., 1998; Wolswijk, 1998; Chang et al., 2000; Chang et al., 2002; Kuhlmann et al., 2008; Kremer et al., 2009; Kremer et al., 2011). Identification of such inhibitory components and knowledge about signal transduction pathways to interfere with thus allow development of strategies to promote cell replacement and endogenous remyelination.

We have investigated inhibitory processes of myelinating glial cell differentiation and found that the p57kip2 protein (Cdkn1) efficiently blocks both peripheral Schwann cell maturation as well as OPC differentiation (Küry et al., 2002; Heinen et al., 2008; Kremer et al., 2009). Upon long-term suppression, a number of differentiation associated processes (such as cell-cycle exit, morphological maturation, gene expression and myelin production) were affected and maturation was promoted.

In view of its strong inhibitory effect on progenitor cells we wondered whether glial fate determination as an early differentiation step might also be under the control of p57kip2. We therefore investigated its expression among stem cells in adult CNS niches and studied the consequences of decreased p57kip2 expression levels in cultured and transplanted NSCs. This revealed

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a strong anti-astrogenic effect, generation of oligodendroglial features as well as induction of BMP antagonists upon p57kip2 suppression in cultured NSCs. Modulation of p57kip2 expression or activity could therefore be used to promote myelinating glial cell replacement and to limit the degree of astrogliosis at the same time.

## MATERIALS AND METHODS

### Animal subjects

Adult female Wistar rats with a weight of 160–180 g (3–4 months old) were used as donors for the NSC isolation and for immunohistochemical analysis of stem cell niches. Adult female Fischer 344 rats were used for transplantation experiments. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and institutional guidelines.

### NSC preparation

Adult rats were anesthetized using ISOFLURAN (DeltaSelect, Langenfeld, Germany) and killed by decapitation. Brains and spinal cords were removed and put in 4°C phosphate-buffered saline (PBS; PAA Laboratories, Pasching, Austria). Overlying meninges and blood vessels were removed. Hippocampus (HC) and ependymal zones, including subependymal and subventricular zones from the lateral wall of the lateral ventricle (SVZ), were aseptically removed and dissociated mechanically. The cell suspension was washed in PBS and further digested in PPD solution containing papain (0.01%, Worthington Biochemicals, Lakewood, USA), 0.1% dispase II (Boehringer, Ingelheim, Germany), DNase I (0.01%, Worthington Biochemicals) and 12.4 mM MgSO<sub>4</sub>, dissolved in HBSS (PAA Laboratories) for 30 minutes at 37°C. The cell suspension was triturated every 10 minutes until the tissue was completely digested, spun down and washed in Neurobasal (NB) medium (Gibco BRL, Karlsruhe, Germany) supplemented with B27 (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin/0.1 mg/l streptomycin (PAN Biotech, Aidenbach, Germany). Cells were resuspended in NB medium supplemented with 2 µg/ml heparin (Sigma-Aldrich, Taufkirchen, Germany), 20 ng/ml FGF-2 (R&D Systems, Wiesbaden-Nordenstadt, Germany) and 20 ng/ml EGF (R&D Systems). Cultures were maintained as neurospheres in uncoated culture flasks at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Single cells began to form spheres within 5–7 days and continued to grow during the following weeks. Half of the medium was changed every 3 days and cell cultures were passaged weekly by means of limited accutase (PAA Laboratories) digestion at 37°C for 10 minutes and subsequent trituration. A total of  $5 \times 10^4$  cells/ml were seeded in T75 culture flasks in fresh growth medium. In order to allow differentiation to take place, dispersed cells were plated on poly-L-ornithine/laminin (100 µg/ml and 5 µg/ml, Sigma-Aldrich)-coated cell culture dishes or glass cover slips and incubated in control culture medium [ $\alpha$ -MEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (PAA)] in absence or presence of 10 ng/ml BMP2 and 10 ng/ml BMP4 (R&D Systems).

### Stem cell transfection and immunocytological procedures

Neurosphere cultures from passage numbers three to six were used to generate dispersed NSCs. Transfection with shRNA-mediated suppression constructs [H1: pSUPER empty control vector and H1-kip2: p57kip2\_1 suppression vector (Heinen et al., 2008)] were carried out in combination with a citrine expression vector (Heinen et al., 2008) using a Lonza nucleofection device (program A-033 high efficiency) and the adult rat NSC nucleofactor kit (Lonza, Basel, Switzerland). Following this procedure, ~30–50% of surviving cells were transfected. For marker expression analysis, NSCs were fixed with 4% paraformaldehyde/PBS solution, PBS washed, blocked for 2 hours using 10% normal goat serum in PBS and subjected to antibody incubation at 4°C overnight: rabbit anti-p57kip2 (1:200, Sigma-Aldrich), rabbit anti-caspase3 (1:500, Cell Signaling Technology, Leiden, The Netherlands), rabbit anti-Ki67 (1:500, Millipore, Schwalbach, Germany), rabbit anti-GFAP (1:1000, Dako, Hamburg, Germany), mouse anti-*nestin*, (1:300, Millipore), mouse anti-GFAP (1:1000, Millipore), rabbit anti-AQP4 (1:500, Sigma-Aldrich), mouse anti-GalC (1:350, Millipore), mouse anti-O4 (1:1000, Millipore) and rabbit anti-GST- $\pi$  (1:1000, Enzo). Following PBS washes secondary

anti-mouse and anti-rabbit antibodies conjugated with either Alexa Fluor594, Alexa Fluor350 fluorescent dyes (1:1000, Invitrogen), Cy3 or FITC (1:500, Millipore) were added for 2 hours at room temperature. Cells were mounted under Citifluor (Citifluor, Leicester, UK) and analyzed using an Axio Cam HRc microscope (Zeiss, Jena, Germany) or for confocal images with a Zeiss LSM 510 Axiovert 200 M microscope (Zeiss, Jena, Germany). Citrine-positive cells on nine different fields per glass coverslip were counted and analyzed for marker expression. For live cell imaging, adult neural stem cells were photographed every 8 hours starting at time point 24 hours post-transfection lasting up to 192 hours.

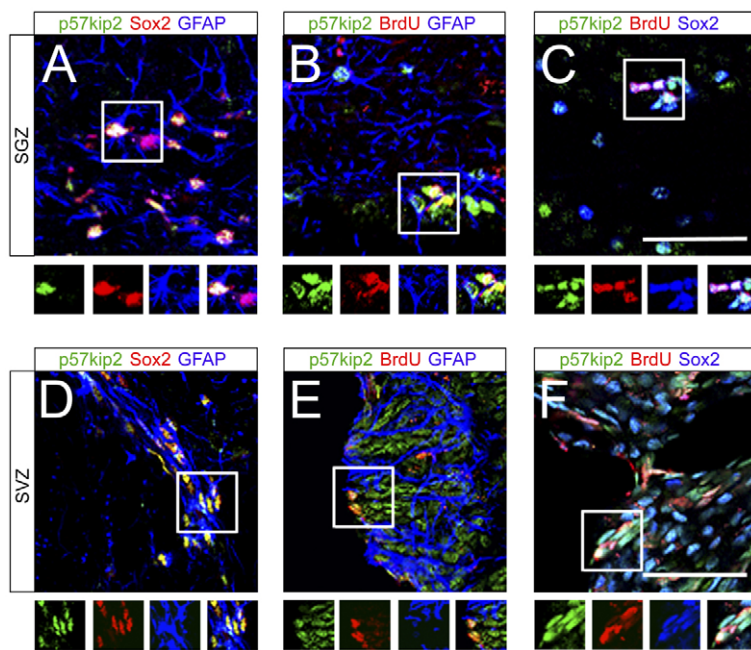
For preparation of cryosections, neurospheres were allowed to settle for 20 minutes at room temperature and then fixed with 4% PFA for 45 minutes at room temperature. Cryoprotection was carried out in 30% sucrose for 5 hours at 4°C. Embedded in Tissue Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands), neurospheres were cut into 14 µm sections and processed for immunohistochemistry. Sections were blocked using 1% normal goat serum with 0.1% Triton in PBS for 1 hour at room temperature and subjected to antibody incubation (mouse anti-*nestin*, 1:300, Millipore; mouse anti-GFAP, 1:1000, Millipore; rabbit anti-p57kip2, 1:200, Sigma-Aldrich) at 4°C overnight in antibody incubation solution (1% normal goat serum with 0.03% Triton in PBS). Staining with secondary antibodies was performed as described above. Data are shown as mean values  $\pm$  s.e.m. and *t*-test was applied in order to determine statistical significance and was performed with Prism 5.0 (GraphPad Software).

### RNA preparation, cDNA synthesis and quantitative reverse transcription (RT)-PCR

Transfected NSCs were isolated after 24 hours using fluorescence-activated cells sorting (FACS) by means of citrine co-expression (FACSaria; BD, Franklin Lakes, NJ, USA) and then cultured for up to 7 days. Total RNA purification from cells was carried out using the RNeasy procedure (Qiagen, Hilden, Germany). Isolated RNA was reverse transcribed using the high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Quantitative determination of gene expression levels was performed on a 7900HT sequence detection system (Applied Biosystems) using Power SybrGreen universal master mix (Applied Biosystems). Primer sequences were determined using PrimerExpress 2.0 software (Applied Biosystems) and tested for the generation of specific amplicons: GFAP\_fwd, CTGGTGTGGAGTGCCTTCGT; GFAP\_rev, CACCAACCAGCTTCCGAGAG; CGT\_fwd, CCGGCCACCCTGTCAAT; CGT\_rev, CAGGGAGACGAGTCAACAGT; EZH2\_fwd, GGGCTGCACACTGCAGAAA; EZH2\_rev, CATGGTTAGAGGAGCCGTCC; p57kip2\_fwd, CAGGACGAGAATCAGGAGCTGA; p57kip2\_rev, TTGGCGAAGAAGTCGTTTCG; chordin\_fwd, ACTAGCTCACGTCCCCTTGAAG; chordin\_rev, GGCCTGGAGCTCTCGAAGTA; noggin\_fwd, CTGGTGGACCTCATCGAACA; noggin\_rev, GCGTCTCGTTTCAGATCCTTCTC; GAPDH\_fwd, GAACGGGAAGCTCACTGGC; GAPDH\_rev, GCATGTCAGATCCACAACGG; ODC\_fwd, GGTTCAGAGGCCAAACATC; ODC\_rev, GTTGCCACATTGACCGTGAC. GAPDH and ODC were used as reference genes, and relative gene expression levels were determined according to the  $\Delta\Delta$ Ct method (Applied Biosystems). Each sample was measured in quadruplicate; data are shown as mean values  $\pm$  s.e.m. and *t*-test was applied in order to determine statistical significance (Prism 5.0; GraphPad Software).

### Cell transplantation into the intact spinal cord

Animals were anesthetized with a cocktail of ketamine (62.5 mg/kg; WDT, Garbsen, Germany), xylazine (3.175 mg/kg; WDT, Garbsen, Germany) and acepromazine (0.625 mg/kg, Sanofi-Ceva, Düsseldorf, Germany) in 0.9% sterile saline solution. Prior to NSC implantation, animals received a partial laminectomy at cervical level C3. Then a total volume of 2 µl cell suspension containing either  $1.2 \times 10^5$  control NSC/µl (H1 and citrine vector co-transfected; *n*=6) or  $1.2 \times 10^5$  p57kip2 suppressed NSC/µl (H1-kip2 and citrine vector co-transfected; *n*=6) was injected under stereotactical guidance (mediolateral midline, dorsoventral 0.8 mm) into the dorsal spinal cord through a pulled glass micropipette (100 µm internal



**Fig. 1. p57kip2 is expressed in adult brain stem cell niches.** (A-F) Immunohistochemical stainings revealed overlapping p57kip2, Sox2, GFAP and BrdU signals in SGZ (A-C) and SVZ (D-F) niches, demonstrating p57kip2 expression in stem cells in vivo. All cells are shown as single and merged pictures. Scale bars: 50  $\mu$ m.

diameter) using a Picospritzer II device (General Valve, Fairfield, USA). Air-driven pulses of 15 nl per pulse, 20 pulses per site, were delivered. The micropipette tip remained in place for 25 seconds before withdrawal. The implantation site was covered with gelfoam (Gelita Tampon; Braun, Germany) before readapting muscular layers and stapling the skin above the lesion. Four days post-operation, animals were transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Spinal cords were removed, post-fixed overnight and cryoprotected in 30% sucrose, cut into sagittal 35  $\mu$ m cryostat sections and processed for immunohistochemistry. Immunofluorescence stainings were performed on free-floating sections to assess NSC differentiation parameters using primary antibodies at 4°C overnight: goat anti-GFP/citrine (1:500; Biotrend, Cologne, Germany), rabbit anti-GFAP (1:1000, Dako), rabbit anti-GST- $\pi$  (1:2000, Enzo) and mouse anti-adenomatous polyposis coli for oligodendrocytes (APC; CC1; 1:500, Calbiochem, Darmstadt, Germany). Sections were incubated with rhodamine-X- (1:500, Dianova, Hamburg, Germany), Alexa Fluor488- (1:1000, Molecular Probes) or Cy5- (1:500, Dianova) conjugated donkey secondary antibodies for 2 hours at room temperature. Sections were mounted onto glass slides and covered with Prolong Antifade (Invitrogen GmbH, Karlsruhe, Germany). Analysis was performed by confocal scanning laser microscopy (Leica TCS-NT, Wetzlar, Germany). Colocalization of citrine-labeled NSCs with differentiation markers was determined by analyzing between 30-35 optical sections through the z-axis of the coronal section at 400 $\times$  magnification. Colocalization was confirmed once the differentiation marker was spatially associated with the citrine signal through subsequent optical sections in the z-axis. Expression sites were analyzed with two sections per marker and by analyzing one field of view in three different regions: (1) directly at the injection site in the white matter (ISWM); (2) caudal to the injection site in the white matter (WM); and (3) above the injection site in the grey matter (GM). Data are shown as mean values  $\pm$  s.e.m. and *t*-test was applied in order to determine statistical significance (Prism 5.0; GraphPad Software).

#### BrdU labeling and immunohistochemical staining procedures

In vivo labeling of dividing cells was performed by intraperitoneal injection of 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich) at 50 mg/kg of body weight using a sterile solution of 10 mg/ml BrdU dissolved in 0.9% (w/v) NaCl solution. Injections were performed daily on 4 consecutive days (day 1 to 4). At day 30, rats were anesthetized and sacrificed by transcardial perfusion. Brains were removed, post-fixed in paraformaldehyde overnight at 4°C, cryoprotected in 30% (w/v) sucrose, 0.1 M sodium phosphate

solution (pH 7.4) and cut into 40  $\mu$ m sagittal sections using a sliding microtome on dry ice. Free-floating sections were treated with 0.6% H<sub>2</sub>O<sub>2</sub> in Tris-buffered saline [TBS; 0.15 M NaCl, 0.1 M Tris-HCl (pH 7.5)] for 30 minutes. For BrdU detection, tissue pre-treatment was performed as described previously (Couillard-Despres et al., 2005). Sections were blocked [TBS, 0.1% Triton X-100, 1% bovine serum albumin, 0.2% teleostean gelatine (Sigma, Taufkirchen, Germany)] and primary antibodies were applied overnight at 4°C: rat anti-BrdU (1:500, Oxford Biotechnology, Oxford, UK), rabbit anti-GFAP (1:1000, Dako), guinea pig anti-GFAP (1:500, Progen, Germany), goat anti-Sox2 (1:500, Santa Cruz Laboratories, Santa Cruz, USA), rabbit anti-p57kip2 (1:400, Sigma-Aldrich). Sections were washed and incubated with fluorochrome-conjugated secondary antibodies overnight at 4°C: donkey anti-goat, -mouse, -rabbit or -rat conjugated with Alexa Fluor488 (1:1000, Molecular Probes, Eugene, USA), rhodamine X (1:500, Dianova, Hamburg, Germany), Cy5 or biotin (1:500, Jackson ImmunoResearch, West Grove, USA). Sections were put onto slides and mounted under Prolong Antifade kit (Molecular Probes). Photodocumentation and analysis were performed by confocal scanning laser microscopy.

## RESULTS

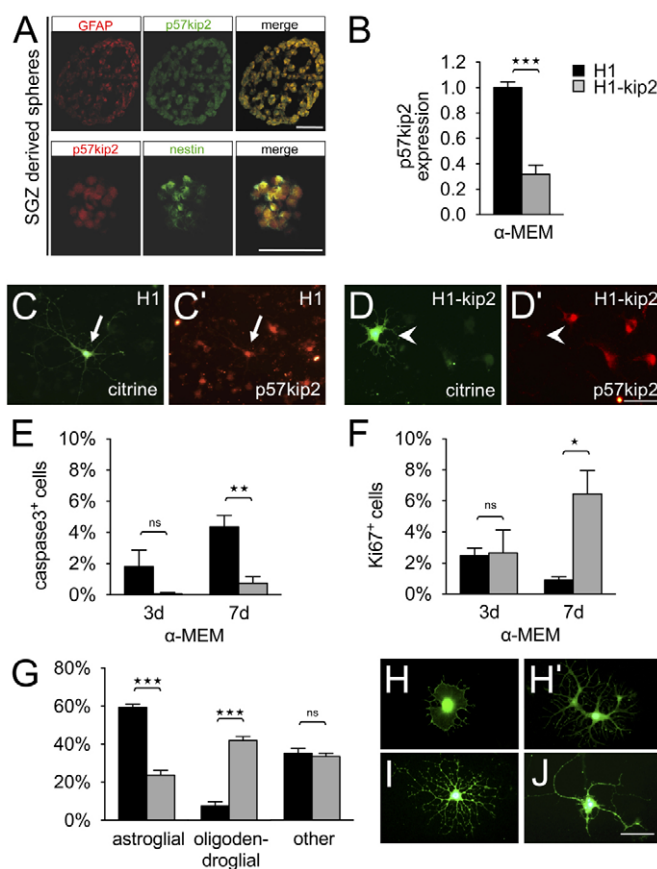
### p57kip2 is expressed by adult NSCs of the two neurogenic niches

Immunohistochemical analysis revealed that the p57kip2 protein is expressed in the neural stem cell niches SGZ and SVZ (Fig. 1). Sox2/GFAP-expressing cells have been proposed to be resident NSCs within these brain regions (Graham et al., 2003) and we found that Sox2/GFAP double-positive cells also expressed p57kip2 in SGZ and SVZ (Fig. 1A,D). NSCs are slow proliferating cells with a self-renewal capacity, which is typically identified by their label-retaining potential. Thus, we examined the expression of p57kip2 in GFAP- and Sox2-expressing cells that had retained 5-bromo-2-deoxyuridine (BrdU) 26 days after a daily injection of BrdU for 4 consecutive days. This revealed in both niches a large number of BrdU label-retaining GFAP-expressing cells that were also p57kip2 positive (Fig. 1B,E), as well as BrdU-positive Sox2/p57kip2 co-expressing cells (Fig. 1C,F). Of note, no colocalization of p57kip2 with NeuN was detected, suggesting that p57kip2 is absent in mature DG neurons (data not shown).

## p57kip2 suppression in cultured adult neural stem cells

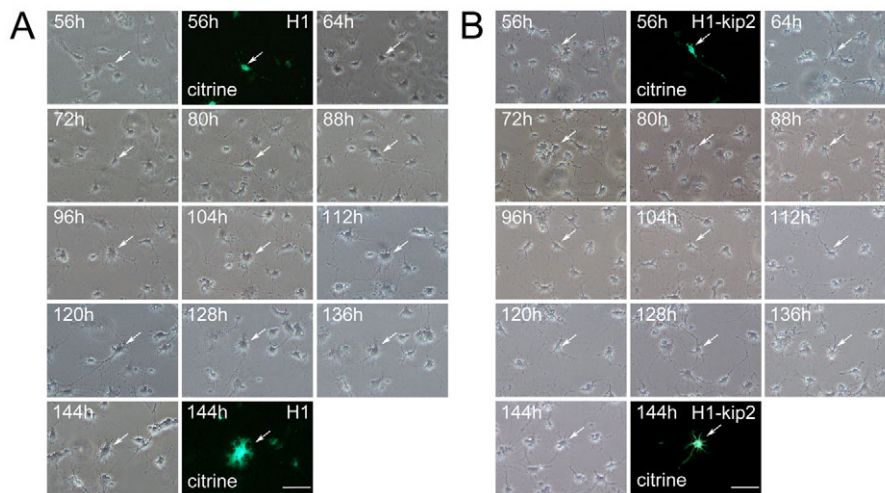
Following the demonstration of p57kip2-positive cells in stem cell niches of the adult brain, we tested whether neurosphere cells express p57kip2 and whether colocalization with stem/progenitor cell markers such as GFAP and nestin could be observed in vitro. This revealed that all neurosphere cells were p57kip2 positive (Fig. 2A) and that expression was maintained in dispersed and adherent cells (data not shown). We next addressed the role of p57kip2 in stem cell differentiation and suppressed its expression using short-hairpin RNAs (shRNAs). This procedure has already successfully been applied to glial progenitor cells and was found to be specific for p57kip2 without inducing a non-specific defense reaction, the so-called interferon response (Heinen et al., 2008; Kremer et al., 2009). Dispersed adult SGZ and SVZ-derived neurosphere cells were transfected with suppression and control constructs in presence of a citrine-expressing vector, FACS isolated after 24 hours for gene expression measurements and plated on laminin-coated surfaces in differentiation permissive medium ( $\alpha$ -MEM) (Rivera et al., 2006). Real-time quantitative RT-PCR confirmed the knockdown of p57kip2 transcription (Fig. 2B) and anti-p57kip2 staining showed that p57kip2 protein levels were also strongly reduced in suppressed but not in control transfected cells (identified by means of citrine expression; Fig. 2C-D'). To elucidate whether the gene suppression affects apoptotic cell death or proliferation, we performed anti-caspase3 and anti-Ki67 stainings, respectively (Fig. 2E,F). This revealed a significantly reduced cell death rate upon p57kip2 suppression while the proliferation rate of differentiating stem cells was slightly increased. Note that, under the conditions applied, the majority of cultured cells were not proliferating. In general, when cell specific markers were evaluated using immunocytofluorescent stainings, only transfected cells (visualized by means of citrine co-expression) were scored.

Interestingly, we observed different cellular morphologies between p57kip2 suppressed and control-transfected cells (for both populations SGZ and SVZ) and therefore quantitatively analyzed the distribution of cells adopting astroglial, oligodendroglial or other (neuronal) morphologies (7 days post transfection). Our analyses revealed that the majority of control-transfected NSCs (termed H1 in Fig. 2G,H,H') adopted a flat astrocyte type I-like or multipolar astrocyte type II-like morphology without secondary processes, whereas most of the p57kip2-suppressed NSCs acquired a multipolar shape with secondary processes resembling an oligodendroglial-like morphology (H1-kip2 in Fig. 2G,I). We detected eight times more oligodendroglial-like phenotypes in p57kip2-suppressed NSCs than in control-transfected cells, whereas the number of cells with a non-glial shape was not affected (Fig. 2G,J). In order to study single cells upon modulation of p57kip2 expression, we performed live cell imaging and followed control-transfected (H1) and p57kip2-suppressed (H1-kip2) cells during a period from day 1 to 8 (hours 24 to 192) in control medium ( $\alpha$ -MEM), as well as following stimulation with bone morphogenetic proteins 2 and 4 (BMP2/4) (Gross et al., 1996). We conducted two independent experiments and observed in total 284 H1 transfected and 224 H1-kip2 transfected single cells during the whole period (24 to 192 hours). We discriminated between cells with oligodendroglial morphologies and flat cells with astrocytic appearance. This analysis revealed that in both media p57kip2 knockdown promotes oligodendroglial maturation. In  $\alpha$ -MEM we found a shift from  $24.47 \pm 3.94\%$  (H1) to  $67.44 \pm 1.61\%$  (H1-kip2) of cells with oligodendroglial appearance (*t*-test,  $P=0.0097$ ). In the presence of BMPs, we detected a shift from  $25.86 \pm 1.86\%$  (H1) to



**Fig. 2. Characterization of neurospheres prepared from the SGZ of the adult hippocampal dentate gyrus.** (A) Confocal images of cryosectioned neurospheres stained for p57kip2 and stem/progenitor cell markers revealed co-expression with GFAP and nestin. (B) Adult neural SGZ neurospheres were dissociated and dispersed cells were transfected with gene suppression constructs in order to mediate downregulation of p57kip2. Quantitative RT-PCR analysis of sorted control transfected (H1) and p57kip2 suppressed (H1-kip2) cultured NSCs demonstrated that p57kip2 transcript levels were significantly lowered. (C-D') Anti-p57kip2 immunostaining 3 days after NSC transfection (visualized by means of citrine expression; arrow marks an expressing cell, arrowhead marks absent expression), showed that H1-kip2 transfected NSCs were devoid of p57kip2 protein. (E,F) Anti-caspase3 and anti-Ki67 immunostainings showed that 3 and 7 days after p57kip2 suppression, apoptotic cell death was reduced and that proliferation rate was slightly increased after 7 days. Suppression of p57kip2 induces morphological alterations. (G) Determination of relative cell numbers with astroglial, oligodendroglial and other morphologies in control transfected (H1) and p57kip2-suppressed (H1-kip2) NSCs derived from the SVZ (similar results observed in SGZ cells, data not shown). (H-J) Representative H1 transfected cells with astroglial-like (H,H'), non glial- (I) and an H1-kip2 transfected cell with oligodendroglial-like (I) features. Data are mean  $\pm$  s.e.m. derived from  $n=3$  (caspase3, Ki67) and  $n=6$  (morphology) independent experiments. *t*-test (\*\*\* $P<0.001$ ; \*\* $P<0.01$ ; \* $P<0.05$ ; ns, not significant). Scale bars: 50  $\mu$ m.

$63.76 \pm 1.95\%$  (H1-kip2) of cells with oligodendroglial appearance (*t*-test,  $P=0.0050$ ). This is shown for a representative p57kip2-suppressed (H1-kip2) stem cell developing an oligodendroglial morphology (Fig. 3B) and a representative control-transfected (H1) stem cell adopting a flat astrocytic morphology (Fig. 3A). Morphological changes were first observed after  $\sim 72$  hours and that



**Fig. 3. Live cell imaging.** (A,B) A representative SGZ-derived stem cell transfected with the empty control vector (H1) (A) and a representative SGZ-derived stem cell transfected with the p57kip2 suppression vector (H1-kip2) (B) observed during the time window 56 to 144 hours post-transfection (cultured in  $\alpha$ -MEM). After 72 hours, morphological changes become apparent and the p57kip2-suppressed cell develops an oligodendroglial morphology, whereas the control transfected cell adopts a flat astrocytic morphology. No signs of selective proliferation or cell death could be observed. Arrows mark transfected and citrine-labeled cells. Scale bars: 50  $\mu$ m.

in this analysis no dividing (neither H1- nor H1-kip2 transfected) cells were observed. This is in contrast to the slight proliferation differences shown in Fig. 2F and might be due to the much lower cell number that has been studied when compared with the Ki67 expression analysis. In addition, only transfected cells that we were able to follow during the whole observation period were finally scored for morphological changes, as we wanted to reveal when and to what extent differentiation occurs.

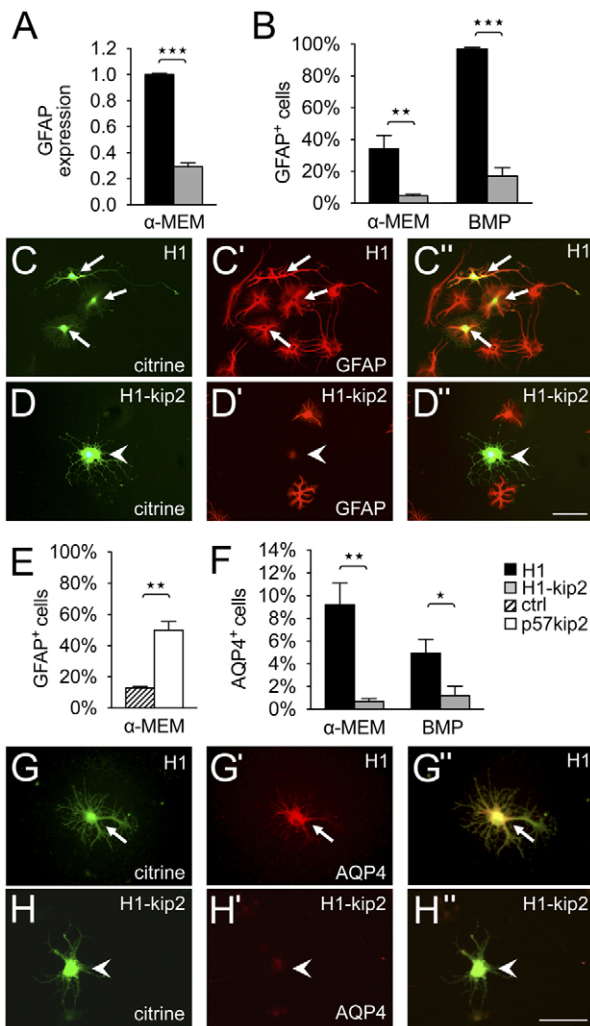
### Astrocyte differentiation is downregulated upon p57kip2 suppression

We next examined whether the expression of glial lineage markers was dependent on p57kip2 expression levels. We observed a strong reduction of GFAP transcript levels in p57kip2-suppressed cells compared with control transfected cells derived from the SGZ (Fig. 4A). This downregulation also resulted in a significant decrease of the percentage of GFAP-positive cells (Fig. 4B), both under standard conditions as well as under dominant astrocyte-promoting conditions such as the stimulation with BMP2/4 (Gross et al., 1996). GFAP-negative p57kip2-suppressed NSCs displayed oligodendroglial-like morphologies, whereas GFAP-expressing control cells strongly resembled astrocytes (Fig. 4C-C',D-D'). Three different p57kip2 suppression constructs were used, all of which resulted in decreased GFAP expression levels. For reasons of simplicity, only data of experiments carried out with the first construct (termed H1-kip2) (Heinen et al., 2008) are shown. However, p57kip2 overexpression (Heinen et al., 2008) substantially increased the percentage of GFAP-positive cells, thus enhancing astrogenesis (Fig. 4E). H1-kip2 transfected SVZ-derived stem cells downregulated GFAP to the same degree (data not shown) and were furthermore shown to turn down aquaporin 4 (AQP4) expression upon p57kip2 knockdown in control medium, as well as in presence of BMPs (Fig. 4F,G,H'). AQP4 encodes an astroglial end-foot marker and we found that under the conditions applied some SVZ, but no SGZ-derived stem cells expressed it during the differentiation process.

### p57kip2 suppression leads to an increase of OPC markers

Since astrogenic features were revealed to be inhibited upon p57kip2 suppression, we determined whether this gene knockdown also affected oligodendroglial parameters. p57kip2-

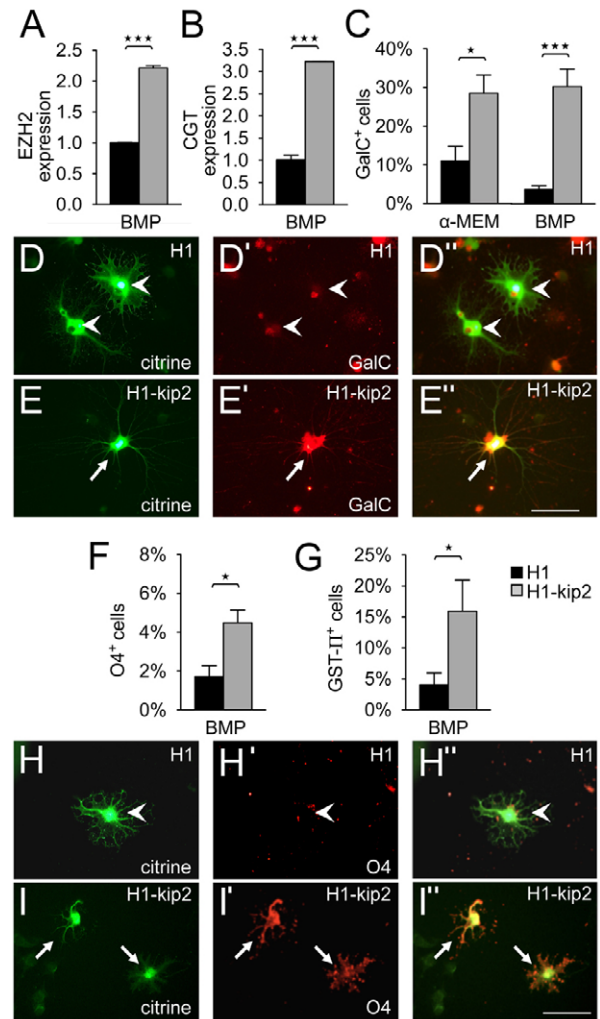
suppressed SGZ cells displayed raised transcript levels of the polycomb group protein EZH2 (Fig. 5A), which was shown to be expressed at high levels in NSCs that differentiate into OPCs (Sher et al., 2008). In addition, the OPC marker ceramide galactosyl transferase (CGT) was upregulated in p57kip2-suppressed cells (Fig. 5B) and we observed a significant increase in the percentage of galactocerebroside-positive (GalC) cells after p57kip2 knockdown (Fig. 5C,D-E'). Interestingly, in presence of BMP2/4, this induction process was even more emphasized. Despite the fact that almost 30% of the p57kip2-suppressed cells differentiated into GalC-expressing cells in presence of BMP2/4 (Fig. 5C), we could not detect differences in the expression of mature oligodendrocyte markers such as myelin basic protein (MBP) or 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase; data not shown). In order to reinforce these results, we evaluated the effect of p57kip2 suppression in SVZ-derived NSCs. We found that, in these cells, the expression of O4, another early oligodendroglial marker, was elevated upon reduction of p57kip2 expression (Fig. 5F,H-I') as was the expression of GST- $\pi$  (Fig. 5G). Note that GST- $\pi$  positivity was also significantly elevated in p57kip2-suppressed SGZ-derived NSCs (data not shown). This suggests that the p57kip2 suppression induces an OPC stage and that additional signals are necessary to further promote the generation of mature oligodendrocytes. To find out whether selection (i.e. increased proliferation or promoted survival) accounts for the observed effects, we performed anti-Ki67 and anti-caspase3 staining in combination with GalC, O4, GFAP and AQP4 antibodies at two different time points (3 days and 7 days) after transfection of SGZ and SVZ cells. This analysis revealed that, at both time points, none of the O4- or GalC-positive cells were Ki67 or caspase3 labeled (data not shown), indicating that the generation of these cells is most likely not due to an increased proliferative activity or enhanced survival. Given that suppression of p57kip2 exerts an overall cell protective effect (Fig. 2E), we can also exclude that the strong difference among GFAP-positive cells results from an increased death in response to p57kip2 suppression. This was also confirmed using GFAP/caspase3 double stainings (data not shown). In addition, we observed that the total cell numbers between the two conditions (control and p57kip2-suppressed cells) were quite stable. We therefore conclude that p57kip2 can regulate cellular fate switching.



**Fig. 4. Downregulation of astrocyte markers following p57kip2 suppression.** (A–D'') GFAP downregulation, as revealed by transcript levels in sorted (FACS) cells (A) as well as by immunostaining of control (H1) and p57kip2-suppressed (H1-kip2) SGZ cells 7 days post-transfection (B–D''). (E) Induced GFAP expression by p57kip2 overexpression. (F–H'') AQP4 protein downregulation in SVZ cells upon p57kip2 knockdown. Arrows mark expressing cells; arrowheads mark absent expression. Data are mean $\pm$ s.e.m. derived from  $n=6$  (GFAP) and  $n=3$  (AQP4) independent experiments.  $t$ -test (\*\* $P<0.001$ ; \* $P<0.01$ ; \* $P<0.05$ ; ns, not significant). Scale bars: 50  $\mu$ m.  $\alpha$ -MEM, control medium; BMP, BMP2/4-containing astrocyte-inducing medium.

### Evaluation of neuronal and stem cell marker expression

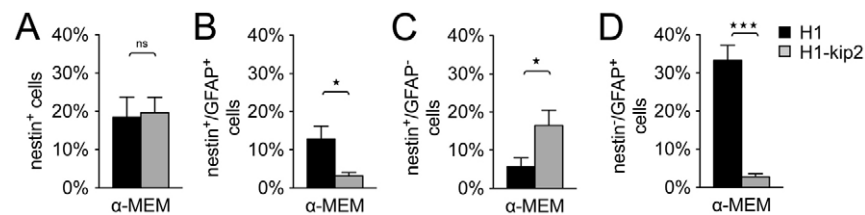
In order to find out whether p57kip2 levels also affect neurogenesis, we performed anti- $\beta$ -tubulin, anti-Map2ab and anti-DCX staining on transfected SVZ and SGZ cells. In SVZ cells, the number of  $\beta$ -tubulin-positive cells was downregulated upon p57kip2 suppression, whereas SGZ cells showed no  $\beta$ -tubulin reaction. When compared with the GFAP downregulation, the reduction of  $\beta$ -tubulin-positive cells was small ( $12.08\pm1.4\%$  to  $6.28\pm3.3\%$  from control to p57kip2 suppressed cells; mean $\pm$ s.e.m.;  $n=4$ ). Both cell types non-significantly downregulated levels of



**Fig. 5. Upregulation of oligodendroglial precursor markers following p57kip2 suppression.** Adult SGZ neural stem cells were sorted (FACS via citrine co-expression) after p57kip2 knockdown and plated on laminin-coated coverslips for 7 days in control ( $\alpha$ -MEM) or astrocyte-promoting (BMP) conditions. (A,B) Increased EZH2 (A) and CGT (B) transcript levels were observed in sorted and p57kip2-suppressed NSCs. (C–E'') In addition, an increased percentage of GalC-positive SGZ cells upon p57kip2 knockdown was observed. (F,H–I'') Induction of O4 expression in p57kip2-suppressed SVZ cells. Arrows mark expressing cells; arrowheads mark absent expression. (G) Induction of GST- $\pi$  positivity in p57kip2-suppressed SVZ cells. Data are mean $\pm$ s.e.m. derived from  $n=6$  (EZH2, CGT, GalC, O4, GST- $\pi$ ) independent experiments.  $t$ -test (\*\* $P<0.001$ ; \* $P<0.05$ ). Scale bars: 50  $\mu$ m.  $\alpha$ -MEM, control medium; BMP, BMP2/4 containing astrocyte-inducing medium.

Map2ab-positive cells ( $6.46\pm1.78\%$  to  $4.67\pm0.77\%$ ; mean $\pm$ s.e.m.;  $n=3$ ). In SGZ cells, the amount of DCX positivity was also slightly lowered ( $29.03\pm1.19\%$  to  $25\pm1.75\%$  from control to p57kip2-suppressed cells; mean $\pm$ s.e.m.;  $n=3$ ). We conclude that a mild reduction of neuronal marker expression accompanies the strong reduction of astrocyte markers.

Of note, interesting observations resulted when we performed anti-nestin stainings. As a consequence of the p57kip2 knockdown, the number of nestin/GFAP double-positive cells was reduced, but the number of nestin<sup>+</sup>/GFAP<sup>+</sup> cells was increased. However, the overall number of nestin-positive cells remained unchanged (this



**Fig. 6. p57kip2-suppressed SVZ-derived NSCs show no difference in the expression of the stemness marker nestin.**

(A–D) Immunofluorescent determination shows no significant change in the number of nestin-positive cells between control transfected and p57kip2-suppressed cells 7 days post-transfection (A), whereas the number of nestin/GFAP double-positive cells was decreased (B), the number of nestin-positive/GFAP-negative cells was increased (C) and the number of nestin-negative/GFAP-positive was strongly decreased (D). Data are mean±s.e.m. derived from  $n=9$  independent experiments.  $t$ -test (\* $P<0.05$ ; \*\*\* $P<0.001$ ; ns, not significant).  $\alpha$ -MEM, control medium.

applies for SGZ and SVZ cells and is shown for SVZ cells in Fig. 6A–D). This indicates that the expression of nestin as a stem cell marker is not dependent on p57kip2.

### Transplantation of p57kip2 suppressed stem cells

In order to reveal whether these observations are of in vivo relevance and whether the previously observed tendency to generate astrocytes from transplanted stem cells (Setoguchi et al., 2004) can be counteracted by means of modulated p57kip2 expression, we injected transfected SGZ-derived NSCs into the dorsal spinal cord of healthy rats. Owing to the transient nature of our genetic modulation of stem cells, spinal cords were removed at an early time point (4 days following transplantation) and GFAP, GST- $\pi$  and CC1 expression on citrine-labeled stem cells was investigated. Similar to the studies on cultured stem cells, empty vector transfected non-suppressed cells were used as controls. Consistent with our previous in vitro observations, significantly fewer transplanted NSCs expressed GFAP protein when p57kip2 was suppressed prior to transplantation. These modulated cells showed a significant induction of the oligodendroglial precursor marker GST- $\pi$  (Rivera et al., 2009) and tended to induce expression of the oligodendroglial marker CC1 [Fig. 7A; representative pictures of injection site in the white matter (ISWM) shown in Fig. 7B–E’]. This indicated that lowered p57kip2 levels influence the capacity of transplanted cells to react to astrogenic signals as they occur in the dorsal spinal cord (Fuller et al., 2007) and to induce OPC features instead.

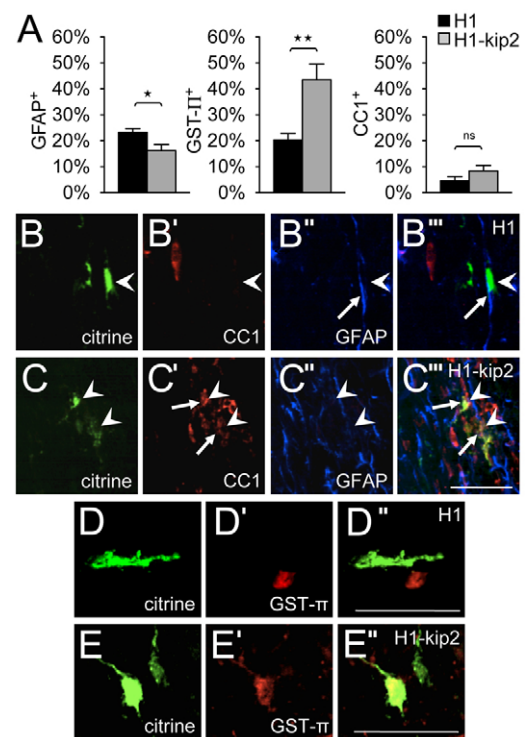
### BMP antagonists are upregulated upon p57kip2 suppression

To elucidate the underlying mode of action, we investigated levels of gene expression of major BMP antagonists such as chordin, noggin and follistatin (Jablonska et al., 2010; Walsh et al., 2010). Consistent with our findings that upon p57kip2 suppression astrocytic markers were downregulated, and that these cells lost astrocytic morphological features, we detected a strong induction of chordin expression in FACS isolated cells (Fig. 8A). Interestingly, noggin also showed a slight induction; however, only when cells were cultured in control medium but not in the presence of BMPs (Fig. 8B). Expression levels of follistatin were not affected by the gene modulation (data not shown).

### DISCUSSION

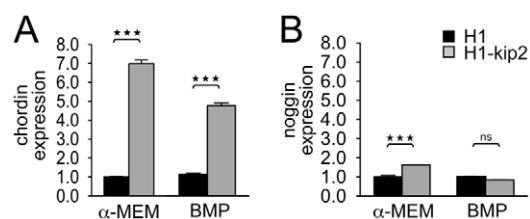
Adult neural stem cell differentiation is regulated by the interplay of multiple extrinsic and intrinsic factors. Here, we demonstrate that NSCs in both neurogenic niches as well as in neurospheres and isolated NSCs in culture express p57kip2, a protein that we have

previously identified as potent negative regulator of myelinating glial cell differentiation (Heinen et al., 2008; Kremer et al., 2009). Our functional data on cultured and transplanted NSCs (derived from both niches) provide strong evidence that p57kip2 additionally acts as an astrogenic determinant and that suppression of this gene strongly interferes with astrocyte generation and promotes the accumulation of OPC characteristics. The observation that glial cell derivatives were not preferentially surviving or



**Fig. 7. Transplantation of p57kip2-suppressed NSCs into the intact dorsal rat spinal cord.**

(A) The percentage of GFAP-positive transfected SGZ cells 4 days post-transplantation (only citrine-positive cells were evaluated) was significantly decreased upon p57kip2 suppression. This was accompanied by significantly increased numbers of GST- $\pi$ -positive cells, as well as increased numbers of CC1-expressing cells. (B–E’) Representative GFAP, GST- $\pi$  and CC1 immunostaining of control transfected (H1) and p57kip2-suppressed (H1-kip2) SGZ cells upon injection into the white matter. Arrows indicate expressing cells; arrowheads mark absent expression. Data are mean±s.e.m. derived from six operated rats each.  $t$ -test (\* $P<0.01$ ; \*\* $P<0.01$ ; ns, not significant). Scale bars: 100  $\mu$ m in C’’, 50  $\mu$ m in D’’, E’.



**Fig. 8. Gene expression of SGZ-derived adult neural stem cells.**

NSCs transfected with control (H1) or p57kip2 suppression vectors (H1-kip2) were sorted (FACS). After 7 days in control (α-MEM) or astrocyte-promoting conditions (BMP), cells were lysed and gene expression levels were determined using quantitative RT-PCR. (A,B) Chordin transcript levels were strongly increased under both experimental conditions, whereas noggin expression was found to be only slightly upregulated in absence of BMPs. Data are shown as mean±s.e.m. derived from  $n=3$  independent experiments.  $t$ -test (\*\*\*) $P<0.001$ ; ns, not significant).

proliferating at higher rates in response to p57kip2 knockdown indicates that p57kip2 is an intrinsic adult neural stem cell regulator and that it plays a role in glial fate decision. During development, p57kip2 was shown to inhibit Mash1 (Ascl1) expression and to prevent neuronal differentiation of embryonal neural stem and progenitor cells (Joseph et al., 2009). No overlap of p57kip2 and neuronal markers such as NeuN was detected in our SGZ sections. Interestingly, in the adult SGZ, Mash1 overexpression led to increased generation of oligodendroglial at the expense of neuronal cells (Jessberger et al., 2008). However, we observed that both SGZ and SVZ cells do not show significantly increased *Mash1* gene expression levels upon p57kip2 knockdown. Furthermore, we found that the number of Mash1-positive cells under our experimental conditions is generally low and not significantly changed in response to altered p57kip2 levels ( $2.25\pm1.05\%$  in control cells to  $1.9\pm0.8\%$  in p57kip2-suppressed cells). We therefore conclude that Mash1 regulation is not part of the p57kip2-dependent mechanism and suggest that high levels of Mash1 might artificially impose an oligodendrocyte identity onto precursor cells following overexpression.

As adult CNS NSCs (this study) and OPCs (Kremer et al., 2009) were found to express p57kip2, it will be important to investigate and compare the underlying differentiation mechanisms. This will reveal to what extent similar interactions and binding partners are involved in these separate processes or whether they involve different signaling pathways.

### Nature of generated cells

The observation that p57kip2 suppression leads to GFAP reduction might also reflect a loss of stem cell character given its expression among adult neural stem cells in vivo (Doetsch et al., 1999; Laywell et al., 2000). However, dispersed NSCs in culture develop GFAP expression only over time and we demonstrated that both markers GFAP as well as AQP4 are reduced in p57kip2 knockdown cells. AQP4 is a water channel protein located in astroglial end-feet and thus constitutes a specific functional component (Wolburg, 1995). In addition, expression of nestin as a stem cell marker was unaffected upon p57kip2 suppression in NSCs. Therefore, our observations currently support a downregulation of astrocyte rather than of stem cell characteristics.

It remains to be shown whether the modulation of p57kip2 expression exerts a direct effect on oligodendrogenesis, or whether the prevention of astrocyte generation is indirectly promoting it.

However, as we did not observe induction of neuronal markers, this would argue for a direct regulation. The appearance of OPC features, but not of late oligodendrocyte markers, as we have observed it, might be a consequence of media composition (serum content or presence of BMPs) and needs to be addressed in future investigations. Nevertheless, such a limited differentiation switch could also be an advantage in the naturally occurring remyelination process in the context of (exogenous) cell replacement. Indeed, transplanted p57kip2 suppressed stem cells were able to induce expression of the OPC marker GST- $\pi$  and to initiate the expression of the mature marker CC1, indicating that environmental factors can further promote their differentiation. In this regard, long-term observations of transplanted NSCs will show whether these cells are able to distribute in the tissue, successfully interact with axons and restore myelin. Future in vivo studies conducted in a myelin-deficient background such as in the shiverer mouse are likely to provide an answer to this question. As OPC supporting signals might also derive from injured, demyelinated or inflamed tissues, modulated NSCs should then also be transplanted into injured or diseased animals. The observation of a p57kip2 suppression-dependent positive differentiation effect in diseased animals could be of particular importance as the plasticity of the stem cell compartment, especially upon CNS inflammation, was shown to be negatively affected (Pluchino et al., 2008; Wang et al., 2008). In the inflamed demyelinated CNS, as found in rodent experimental autoimmune encephalomyelitis (EAE) or in MS, increased numbers of SVZ-derived progenitors were shown to be mobilized towards lesions and to give rise to new oligodendrocyte precursors (Picard-Riera et al., 2002; Menn et al., 2006; Nait-Oumesmar et al., 2007). This behavior differs from their default neuronal differentiation and indicates that stem cell niches sense altered signals. It will therefore be of importance to analyze whether this shift is reflected by lowered p57kip2 levels in NSCs and whether this could represent an endogenous mechanism to restore myelination.

### Underlying molecular mechanisms

Although the underlying mode of action remains to be elucidated in more detail, p57kip2 is likely to interfere with BMP-related signaling cascades. It has been proposed that BMP2 and BMP4 promote astroglial lineage commitment (Gross et al., 1996), and we could demonstrate that suppression of p57kip2 blocks the BMP-dependent astrogenic effect on adult NSCs. It is therefore conceivable that either BMP-receptor expression or activation is affected, or that signaling components further downstream are modulated. Chordin and noggin are potent antagonists of BMP signaling (Bachiller et al., 2000) and we investigated whether p57kip2 is involved in the regulation of their expression. The observed strong induction of chordin and the moderate induction of noggin (Fig. 8) indicate that, upon p57kip2 suppression, these antagonists are produced and that they mediate the anti-astrocyte effects. It is possible that, in the p57kip2-dependent glial fate regulation process, chordin and noggin act as cell-intrinsic safeguards in a BMP-enriched environment, as it was shown to occur upon transplantation into the spinal cord (Setoguchi et al., 2004; Hampton et al., 2007; Matsuura et al., 2008). In addition, the recent demonstration that chordin promotes oligodendrogenesis from adult NSCs in vitro and in vivo (Jablonska et al., 2010) suggests that a cell-autonomous induction of chordin actively contributes to the establishment of oligodendroglial features. However, because in this study chordin infusion induced Mash1 positivity, which we did not observe upon p57kip2 suppression, it remains to be shown whether similar molecular processes are

involved or whether the p57kip2-dependent mechanism exerts a unique mode of action. Whether these molecules account for all observed changes or whether further molecular regulators, such as Id4, Smad4, Smad1/5/8 or Stat, also depend on p57kip2 expression and control the fate of transfected NSCs remains to be shown in future experiments.

### Biomedical relevance

Gliosis, i.e. enhanced astrocyte generation, as well as oligodendroglial turnover and remyelination are important features in pathological CNS conditions such as spinal cord injury (SCI) or MS. In both situations, appropriate cell replacement is required in order to restore axonal functions but endogenous regeneration is usually insufficient or skewed towards production of astrocytes. After demyelinating injury, BMPs were shown to promote gliosis (Setoguchi et al., 2001) and subsequent glial scar formation, which interferes with axonal restoration as well as remyelination. Similarly, glial scar formation following spinal cord injury was also shown to be responsible for failure of axonal regeneration (Silver and Miller, 2004). However, supply of appropriate myelinating glial cells such as olfactory ensheathing cells or Schwann cells was reported to enhance the repair process (Li and Raisman, 1995; Barnett et al., 2000). This demonstrates the necessity of reducing the number of astrocytes and simultaneously providing myelinating glial cells in order to either restore axonal function (MS) or to allow axons to grow through the lesion zone (SCI). The observation that p57kip2-suppressed NSCs are directed towards the oligodendroglial lineage and at the same time display significantly reduced cell death rates, indicates that such a modulated cell population could represent a valuable tool for functional cell replacement.

In summary, we provide evidence that the inhibitory regulator of myelinating glial precursor cell differentiation, p57kip2, exerts a strong influence over adult neural stem cell fate decision and might represent an important element that is responsible for glial cell specificity. These findings are not only of interest in terms of endogenous cell turnover and stem cell biology, but they could also provide the basis for future CNS repair strategies that aim at the generation of appropriate cell types, prevention of gliosis and restoration of brain function.

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### Author contributions

J.J.J. and F.J.R. performed experiments, data collection and analysis, data interpretation and manuscript writing; A.T. and B.S. performed experiments, data collection and analysis; Mahesh Kandasamy collected data; N.W. and O.A. carried out data collection, analysis and interpretation; H.-P.H. was responsible for conception and design, data interpretation and manuscript writing; L.A. was responsible for conception and design, data analysis and interpretation; P.K. was responsible for conception and design, data analysis and interpretation, manuscript writing, and final approval of the manuscript.

### Competing interests statement

The authors declare no competing financial interests.

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