

## Novel stem/progenitor cells with neuronal differentiation potential reside in the leptomeningeal niche

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### Abstract

Stem cells capable of generating neural differentiated cells are recognized by the expression of nestin and reside in specific regions of the brain, namely, hippocampus, subventricular zone and olfactory bulb. For other brain structures, such as leptomeninges, which contribute to the correct cortex development and functions, there is no evidence so far that they may contain stem/precursor cells. In this work, we show for the first time that nestin-positive cells are present in rat leptomeninges during development up to adulthood. The newly identified nestin-positive cells can be extracted and expanded *in vitro* both as neurospheres, displaying high similarity with subventricular zone-derived neural stem cells, and as homogeneous cell population with stem cell features. *In vitro* expanded stem cell population can differentiate with high efficiency into excitable cells with neuronal phenotype and morphology. Once injected into the adult brain, these cells survive and differentiate into neurons, thus showing that their neuronal differentiation potential is operational also *in vivo*. In conclusion, our data provide evidence that a specific population of immature cells endowed of neuronal differentiation potential is resident in the leptomeninges throughout the life. As leptomeninges cover the entire central nervous system, these findings could have relevant implications for studies on cortical development and for regenerative medicine applied to neurological disorders.

**Keywords:** neural stem cells • leptomeninges • stem cell niche • neuronal differentiation

### Introduction

During recent years, embryonic and adult neural stem cells (NSCs) gained attention as major candidates for regenerative and cell replacement therapies in various neurodegenerative diseases. In this setting, stem cell-based therapies raised important ethical, technical and immunological concerns [1].

The clinical application of adult NSCs, despite their properties of self-renewal, neuro-glial differentiation potential and their possible use in autologous setting, is still of debate. Among technical

concerns, of relevance is that NSCs have hardly accessible site of sampling, are difficult to expand *in vitro* as homogeneous stem cell population and show low rate of *in vivo* neuronal differentiation efficiency [2].

Both during development and in adulthood, neurogenesis from endogenous neural stem/precursor cells has been shown to occur in discrete areas of the brain where complex microenvironments, or niches, ensure a balance between proliferation and self-renewal [3, 4]. NSCs have been found in the main neurogenic regions of the brain, that is, hippocampus, subventricular zone (SVZ), olfactory bulb [5, 6] and in some non-neurogenic regions, that is, spinal cord [7]. In SVZ, NSCs are present up to adulthood and are in tight contact with astrocytes, neuroblasts, ependymal cells, endothelial cells and growth factor-rich basal lamina [8–11].

In this work, we asked whether other brain sites could host stem cell niches. To investigate the stem cell distribution in rat central nervous system (CNS), we analyzed the expression pattern

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in rat cortex of the stem/progenitor cells marker nestin. Nestin is an intermediate filament of neuroepithelial derivation [12] that has been detected in stem/progenitor cells of neural and non-neural tissues [13]. We found that a nestin-expressing cell population is present in rat leptomeninges during embryonic stages up to adulthood. Leptomeninges, which include arachnoid and pia mater, cover the entire CNS and are filled with cerebrospinal fluid produced by choroid plexi. All the major arteries supplying the brain pass through leptomeninges and form branches while penetrating the cortex [14]. Interestingly, every parenchymal vessels inside the CNS are surrounded by a perivascular space (Virchow–Robin space) formed by the extroflexions of leptomeninges (arachnoid and pia mater) filled with cerebrospinal fluid [15–17]. Thus, leptomeninges are widely spread inside the CNS parenchyma, including the choroid plexus.

Leptomeninges form a complex microenvironment that has important functions for the normal cortex development [18]. They are present since the very early embryonic stages of cortical development, when columnar neuroepithelium is located between ventricle surface and pial basal membrane. Leptomeninges are involved in multiple interactions among a large number of molecular and chemotactic factors (e.g. SDF-1/CXCR4, reelin, oxidative state) [19–21], cell types (e.g. pia mater cells, radial glia, neural precursor cells, Cajal Retzius cells, glia limitans cells) [22, 23] and extracellular matrices (e.g. laminin, collagen IV, fibronectin) [24–26] that ensure correct cortical development. Abnormal function/structure of leptomeninges causes altered cortical histogenesis, as in the case of cobblestone lissencephaly (type II), where the fragmentation of pia mater basal membrane leads to the formation of cortical neurons protruding into the sub-arachnoid space [27].

The peculiar spatial relationships of leptomeninges in CNS, their role in cortex development and our serendipitous discovery of nestin-positive cells prompted us in determining whether leptomeninges could be a possible stem cell niche hosting stem/progenitor cells with neuronal differentiation potential.

In this work, we show that nestin-positive cells can be extracted from leptomeninges and expanded *in vitro* both as neurospheres, displaying high similarity with SVZ-derived NSCs, and as homogeneous cell population with stem cell features. *In vitro* expanded stem/progenitor cells can be induced to differentiate with very high efficiency in excitable cells with neuronal morphology and phenotype. When injected into adult brain, these cells survive and differentiate into neurons, thus showing that their neural differentiation potential is operational also *in vivo*.

## Material and methods

### Brain perfusion and immunofluorescence

The animals were perfused with 4% paraformaldehyde in PBS. Brains were dissected, fixed in 4% paraformaldehyde solution and then left in 10% and subsequently 30% sucrose solution. By freezing microtome 30- $\mu$ m-thick

coronal brain sections were cut and processed by immunofluorescence as previously described [28]. Briefly, brain slices were incubated for 2 hrs in blocking solution (PBS/5%FCS/3%BSA/0.3% Triton X-100). Slices were then incubated for 12 hrs at 4°C in floating, with antibodies. Primary antibodies were detected with appropriated secondary antibodies for 4 hrs at 4°C in blocking solution.

### Antisera

The following primary antibodies were used: mouse monoclonal antibodies anti-nestin, anti-CD106 (VCAM-1), anti-CD90, anti-CD31, anti-CD45, anti-CD105 (endoglin) and anti-BrdU (all from Pharmingen/Becton Dickinson); anti-EGFP (rabbit, 1:2000, Invitrogen, San Giuliano Milanese, Milan, Italy), MAP2 (mouse, 1:1000, Sigma, Milan Italy), GFAP (mouse, 1:1000, BD Pharmingen, Buccinasco, Italy), Laminin (rabbit, 1:1000 Sigma), Nestin (mouse, 1:1000, BD Pharmingen), NG2 (rabbit, 1:500, Chemicon, Milan, Italy), Neurofilament 160 (mouse, 1:100, Sigma).

The following secondary antibodies were used: goat anti-mouse Ig/Alexa Fluor 488, IgM/FITC, IgG/PE and chicken anti-rabbit/Alexa Fluor 488 (all from Molecular Probes, San Giuliano Milanese, Milan, Italy), goat anti-mouse/Cy3, goat anti-rabbit/Cy3 (all from Amersham, Milan, Italy).

### Cell cultures

Cells were harvested from the first cortical layers and overhead leptomeninges of Sprague–Dawley rats or from enhanced green fluorescent protein (EGFP) transgenic rats [29] at post-natal days 15 ( $n = 6$  experiments, 10 animals each). Tissues were sampled with stereo-microscope from brain coronal sections. Mechanically and enzymatically dissociated tissue extracts from first cortical layers of P15 rats were cultured in neurosphere-inducing and adherent conditions.

- 1 Neurosphere culture: tissue extracts were seeded into 6-well plates (Falcon, Buccinasco, Italy) in 3 ml of neurosphere culture medium. Medium supplemented with fresh growth factors was added every 2–3 days. Neurospheres could be detected under phase optics after 7–10 days.
- 2 Adherent culture: tissue extracts were seeded into culture flasks (Falcon) with growing medium; after 72 hrs, non-adherent cells were removed. In 10 days, clear colony-forming units could be detected. Differentiation was performed after three to six passages by plating  $1 \times 10^5$  cells/cm<sup>2</sup> in poly-L-lysine (40  $\mu$ g/ml, Sigma)-coated cover slip in the differentiation medium. For media composition, see below.

### Media

Neurosphere culture medium: Neurobasal Medium, (Gibco, San Giuliano Milanese, Milan, Italy) containing 2% B27 supplements (Gibco), 1% N2 supplement (Gibco), 200 mM glutamine, 1% penicillin-streptomycin plus 20 ng/ml epidermal growth factor (EGF) and 10 ng/ml basic fibroblast growth factor (bFGF).

Growing medium: Dulbecco modified Eagle medium (DMEM), with high glucose concentration, GLUTAMAX I, 18% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (all from GibcoBRL/Life Technologies, Milan, Italy). Differentiation medium: Neuron Chow (Neurobasal Medium [Gibco] containing 2% B27 supplements (Gibco), 200 mM glutamine, 10 mM glutamate and 1% penicillin-streptomycin-fungizole) plus 50 ng/ml brain-derived nerve growth factor (BDNF).

## Flow cytometric analysis

Samples of fresh tissue extracts and cultured cells were analyzed by flow cytometry using standard methods [30].

## Intracellular calcium imaging

Intracellular calcium levels (ratio 340/380) were evaluated by ratiometric imaging techniques. Cells were loaded with Fura2-AM for 30–45 min. at 37°C. The loading solution contained 5  $\mu$ M Fura 2-AM in HEPES buffered solution. Loading solution was removed and the culture was incubated for another 10 min. at 37°C in HEPES buffered solution to allow de-esterification of Fura2-AM. The cover slips were then placed onto a recording chamber, connected by a Tygon tube to a 1-ml syringe used for substance fast application.

All image processing and analysis was performed using OpenLab software (Improvision, Tübingen, Deutschland, Europe) with an inverted Zeiss microscope (Zeiss, Oberkochen, Germany). Wavelength of 340 and 380 nm were used to excite Fura2 and the emitted light was collected at 510 nm.

## Colony forming unit (CFU) assay

Dissociated tissue extracts were plated at five different cell concentrations. After 14 days of culture, every single cell colony with more than 50 cells was considered as CFU. CFU assay was performed every following passage by plating cells at four different concentrations. CFUs were stained with May–Grunwald Giemsa and then counted (mean  $\pm$  S.D. of two different cultures).

## Cells immunofluorescence

Cells on cover slips were immunostained as previously described [31]. Briefly, cells were first fixed, blocked and permeabilized. Slides were incubated for 10 min. with the nuclear dye DAPI (Sigma); then, immunostaining was performed with standard procedure using primary antibodies and fluorochrome-conjugated secondary antibodies.

## 5-bromo-2-deoxyuridine (BrdU) labelling

Cells were incubated for 12 hrs with BrdU (3  $\mu$ M) before starting the differentiation protocol. After differentiation, cells were fixed on cover slips with 4% paraformaldehyde and rinsed with PBS. Cells were then treated for 15 min. in 2N HCl/0.5% Triton X-100 at room temperature, and the reaction were neutralized with 0.1 N  $B_4Na_2$  (pH 8.5).

## Stereotaxical surgery in brain

All *in vivo* experiments were in accordance with the Italian Legislative Decree N.116/92. Male Sprague–Dawley adult rats (Harlan, Milan, Italy) ( $n = 8$ ) were anaesthetized and a guide cannula (26G, Plastics One, Roanoke, VA, USA) was stereotaxically (stereotaxic frame; Kopf Instruments, Tujunga, CA, USA) lowered to Hippocampus (AP -2.3mm, L 2.2 mm, V 3.4mm, incisor

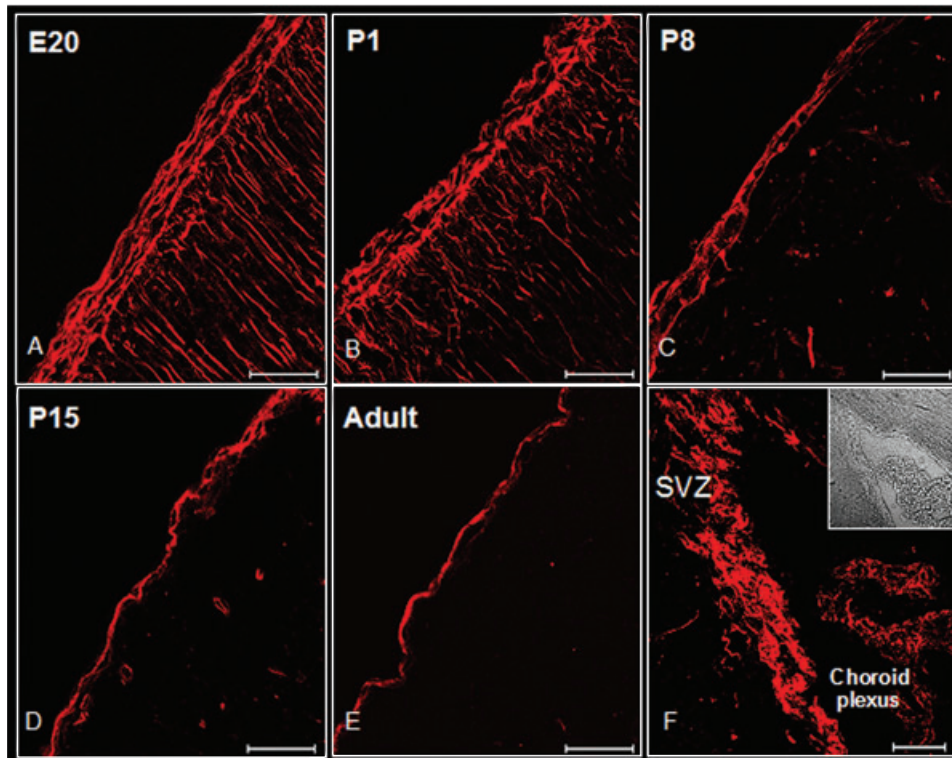
bar 3.0 below horizontal line; Paxinos and Watson). Either cell suspension or vehicle (PBS) were loaded in a Hamilton syringe connected with a Tygon tube (ID 0.020 mm, OD 0.060 mm, Saint-Gobain PPL Corp., Charny, France) and with a needle (33G, Plastics One). The needle was inserted in the guide cannula. A volume of 1  $\mu$ l ( $3 \times 10^3$  EGFP + cells) was then infused with a syringe pump over 5 min. and left in place for additional 5 min.

## Transplanted cell counts

Frequency estimation of transplanted EGFP+/MAP2+ leptomeningeal cells were performed in hippocampal slices of four rats injected with EGFP+ leptomeningeal cells and one not-injected rat as negative control. Five of serial coronal sections (30  $\mu$ m) of hippocampus were randomly selected and stained with antibodies against EGFP and MAP2. Five random fields for each slice were acquired with a 40 $\times$  objective in confocal microscopy. EGFP-positive cells and MAP2/EGFP double-positive cells were then counted. The number of MAP2-positive injected cells was expressed as percentage of EGFP-positive cells examined.

## Quantitative real-time RT (reverse transcription)–PCR analysis (qPCR)

Total RNA was purified with Trizol reagent (Invitrogen) and retrotranscribed to cDNA by reverse transcriptase AMV contained in the First Strand cDNA Synthesis Kit (Roche, Milan, Italy). qPCR reactions were carried out in 20  $\mu$ l total volume containing 10 ng of cDNA (RNA equivalent), 1 $\times$  Power SYBR Green I Master Mix or Taqman Universal PCR Master Mix (Applied Biosystems, Monza, Milan, Italy), 0.4  $\mu$ M primers forward and reverse or 1/20 Taqman probe. After a starting denaturation for 10 min. at 95°C, 40 PCR cycles (15 sec. 95°C and 1 min. 60°C) were carried out on ABI PRISM 7900HT SDS instrument (Applied Biosystems). The Taqman assays (Applied Biosystems) were as follows: Rn00566603\_m1 for *Gfap*; Rn00565046\_m1 for *Mtap2*; Rn00564394\_m1 for *Nes*; Rn00578849\_m1 for *Cspg4*; Rn00667869\_m1 for *Actb*. Forward and reverse 5'-3' primer sequences and PCR product lengths were as follows: *TdGF1*, GCTGGTGAA-GACCTCGACGT, CGGAAGGCACAAGCTGGA, 106 bp; *Smad4*, GCACTAC-CACCTGGACTGGAA, TGTGAACCGCCAGTAATGTC, 126 bp; *Pou5f1*, GCCAAGCTGCTGAAACAGAAG, CTGGCTGAACACCTTTCCAAA, 96 bp; *Nanog*, GGCCTGACTCAGAAGGGCTC, TGCCCCATACTGGAAGGTTTC, 106 bp; *Sox2*, CGCCGAGTGGAACTTTTGT, CGCGGCCGGTATTATAATC, 111 bp; *Klhl1*, GCTCATAGGCTTGTCTGAGCT, GCTTGGCTTCACAGACATCG-3', 75 bp; *Eng*, GGACAGCCTCTCCTCCAGC, TGCTCACCTGTACGAAGCCC, 101 bp; *Col1a1*, GCAGATTGAGAACATCCGAG, CCAGTACTCTCCGCTCTTCCA, 106 bp; *Cd44*, CAACGCTATCTGTGCAGCCA, CAAGAGGAGCTGAG-GCATTGA, 101 bp; *Fgfr1*, AAATTCAAATGCCGTCGAG, GCGTAACGAAC-CTTGAGCC, 111 bp; *Fgfr2*, TTGGCAGCCAGAAATGTGC, CTTGACTG-GAAGTCGCCCAT, 126 bp; *Fgfr3*, CAAGGTGTACAGCGACGCAC, GTGGTGT-TAGCTCTGCAGTCTT, 126 bp; *Kit*, GGCATCACCATCAAAAACGTC, GGGATAGCTTTGATGGCTGC, 131 bp; *Cd34*, CACCAGCCATCTCAGAGACCA, CAGTGTGACGGTTG-GGTAAGTC, 113 bp. The probe signal was normalized to an internal reference, and a cycle threshold (Ct) was taken significantly above the background fluorescence. The Ct value used for subsequent calculation was the average of three replicates. The relative expression level was calculated using transcript level of *Actb* as endogenous reference. Data analysis was done according to the comparative method following the User Bulletin No. 2 (Applied Biosystems).



**Fig. 1** Distribution of nestin-positive cells in rat cortex at different ages. Confocal images of coronal sections of parietal cerebral cortex: rats at embryonic day 20 (E20) (A), postnatal day +1 (P1) (B), +8 (P8) (C) and +15 (P15) (D) and adults (E). Immunolabelling with the anti-nestin (red) antibody. (F) Subventricular zone (SVZ) of P15 rats as positive control and choroid plexus. Scale bar 50  $\mu\text{m}$ .

## Statistical analysis

Data were analyzed using GraphPad Prism4 software. Results were expressed as mean  $\pm$  S.D. or S.E.M., when indicated. Differences between experimental conditions were analyzed using two-tailed Student's t-test. *P* value  $<0.05$  was considered statistically significant.

## Results

### Nestin-positive cells are present in rat leptomeninges during development up to adulthood

Immunofluorescence confocal microscopy with anti-nestin antibodies was used to identify potential stem cell sites in coronal sections of rat brain cortex at different stages of development. Figure 1 shows that nestin-positive cells were present in peripheral cell layers of the parietal cortex of brains obtained from embryos (embryonic day 20 [E20]), animals at different post-natal days (P1, P8, P15) and in adulthood. The distribution of nestin-positive cells within the cortical layers decreased over time but persisted in the superficial layer covering the cortex up to adulthood. As previously described by several groups, nestin-positive cells were present at high density in the SVZ at all the developmental stages and

in adults [32] (positive control). Interestingly nestin-positive cells were also present in choroid plexus.

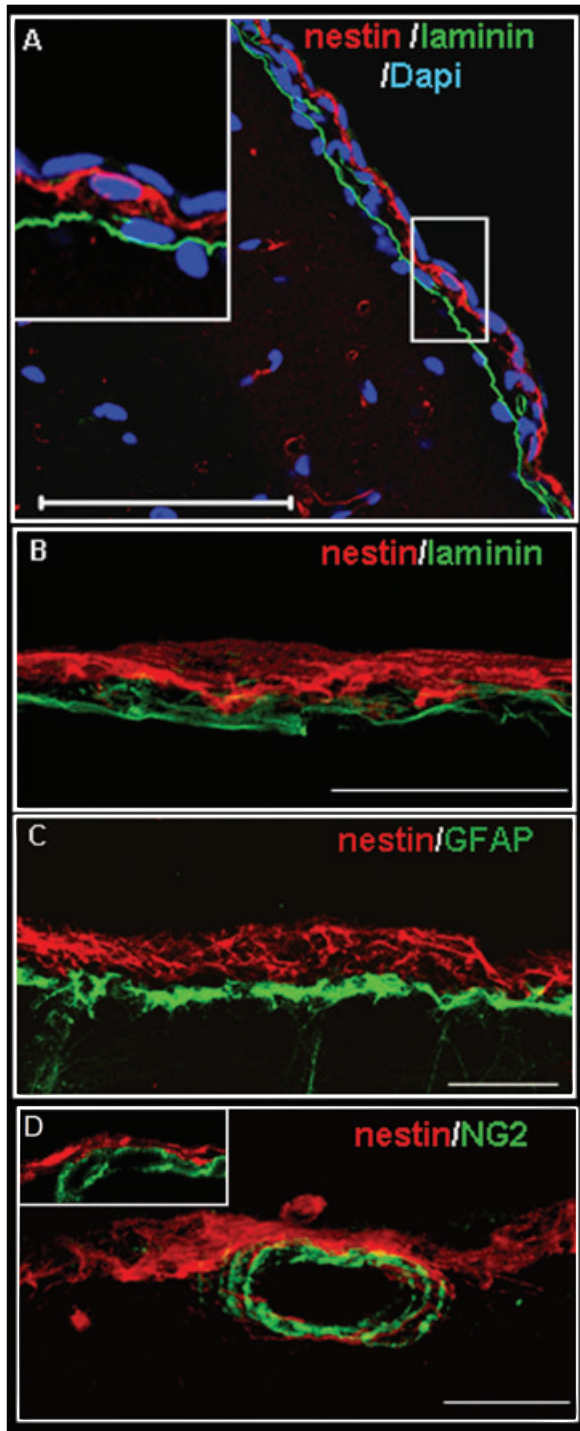
To better define and characterize this population of nestin-positive cells, we first analyzed the distribution of this marker in comparison with that of laminin (a specific marker of pia mater) [19] and glial fibrillary acidic proteins (GFAP, a marker of glial cells). DAPI staining was used to visualize cell nuclei. Figure 2 was obtained from P15 rat brains and indicates that the nestin-positive tissue was a layer of densely packed nucleated cells residing outside the pia mater boundary of the cortex (as shown in details in Fig. 2A and B) in the leptomeninges. This cell population was distinct from astrocytes (Fig. 2C).

We then assessed whether the nestin-positive cell population included vascular cells. To this aim, we used antibodies against NG2 (integral membrane chondroitin sulphate proteoglycan, Cspg4) a marker of smooth muscle cells [33] and of microvascular pericytes [34]. As shown in Fig. 2D, the nestin-positive cell population did not include NG2-positive vascular cells.

### *In vitro* expansion of the nestin-positive cell population derived from leptomeninges

Leptomeninges were stripped from the brain surface of P15 rats ( $n = 6$  experiments, 10 animals each); the stripped samples also included the outermost layers of the cortex (Fig. 3A). Mechanically





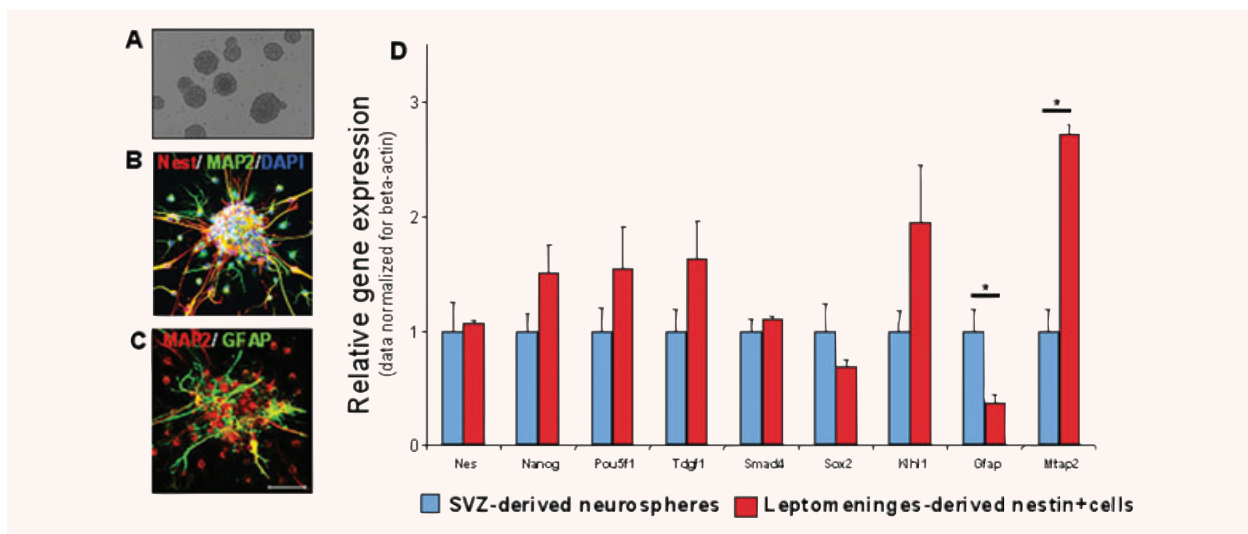
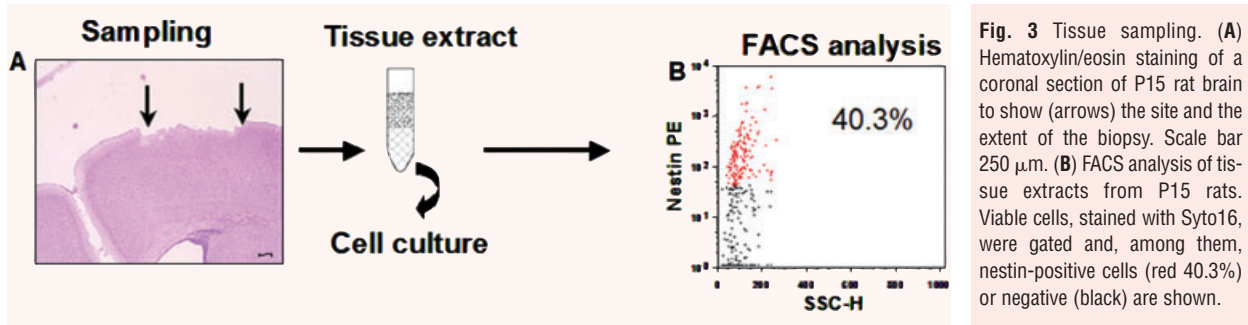
**Fig. 2** Localization of nestin-positive cells in leptomeningeal tissue. (A) Coronal section of the parietal cortex of P15 rat, stained with DAPI (blue), anti-laminin (green) and anti-nestin (red) antibodies. Scale bar 50  $\mu\text{m}$ . (B, C, D) Z-stack reconstruction assembled from 10 serial 2  $\mu\text{m}$  confocal sections. Sections were stained in red with anti-nestin antibodies and in green with either anti-laminin (B) or -GFAP (C) or -NG2 (D) antibodies; high magnification. None of these markers co-localizes with nestin. Scale bars 50  $\mu\text{m}$ .

dissociated tissue extracts contained living nestin-positive cells (40.3%), as shown by FACS analysis (Fig. 3B).

**Standard NSC growth condition.** To assess whether cells with neural differentiation potential were present in the tissue extracts from this region, we first cultured these cells in the same conditions used to expand SVZ-NSCs. In NSC medium (see Materials and Methods), cells from tissue extract generated floating neurospheres in 9–11 days (Fig. 4A). Neurospheres could be expanded *in vitro* up to several months (data not shown). Similarly to SVZ-derived neurospheres [35], leptomeninges-derived neurospheres consisted of different cell types, including nestin-positive cells, neuronal MAP2-positive cells and glial GFAP-positive [36] (Fig. 4B and C). Quantitative gene expression assay was performed to analyze self-renewal regulators (Pou5f1/Oct4 [37], Nanog [38], Sox2 [39]), genes related to the undifferentiated state maintenance (Tdgf1/Cripto-1, Smad4, and Nestin) [40, 41] and genes related to neural differentiation (Klhl1, Gfap, Mtap2) [42]. Comparison of gene expression between SVZ- and leptomeninges-derived neurospheres were shown in Fig. 4D. Data were normalized for beta-actin expression level and were expressed as ratios between the two populations. A tight correlation between the gene expression pattern of SVZ-NSC-derived neurosphere and the leptomeninges-derived neurospheres (Fig. 4D) was observed. Statistically significant differences ( $P < 0.05$ ) were observed for differentiation genes Gfap and Mtap2 only (Fig. 4D).

**Adherent stem cell growth condition.** We tried to overcome the complexity [36, 43, 44] of neurosphere assay by modifying the culture conditions. The whole tissue extract was plated in flasks with growing medium (see Materials and Methods). After 48 hrs, non-adherent cells were removed and the medium completely changed. In 1–3 weeks, cell colonies could be recognized. Only a minority of cells from the whole tissue extract adhered and gave rise to CFUs. Non-adherent cells underwent apoptosis or were removed by medium change. All the adherent cells proliferating after several days derived from CFU and displayed a homogeneous immunophenotype, as shown in Fig. 5A and E. This cell population was expanded for several passages and was clonogenic, since CFU were present at each passage (Fig. 5B). Time course experiments were performed to assess whether a cell population of nestin+/GFAP-/NG2-cells (similar to what found *in vivo* in the leptomeninges) was present in the culture. As shown in Fig. 5A, nestin+/NG2- cells were present as early as 5 hrs after plating (approximately 90% of attached cells). Similar results were obtained with GFAP staining (data not shown). From day 8, nestin+/GFAP-/NG2- cell colonies are detectable.

FACS analysis showed that *in vitro* expanded cells always retained nestin positivity, did not express markers of either neuronal (MAP2)/glial (GFAP) or hematopoietic (CD45, CD34)



[45]/endothelial (CD105, CD31, CD106) [46] lineages and were positive for CD90, a non-specific marker of neural cells and stromal cells [47] (Fig. 5E). Immunofluorescence microscopy confirmed that expanded cells were a homogeneous population of nestin-positive cells (Fig. 5D) that did not express neuronal (MAP2), glial (GFAP), oligodendrocytic and pericytic markers (NG2) [48] (data not shown). Interestingly, a similar population of nestin-positive cells could be extracted and *in vitro* expanded also from choroid plexi, a tissue devoid of neural elements (data not shown). We studied by quantitative RT-PCR the expression of different MSC markers (see Table 1); we found that nestin-positive cells from leptomeninges displayed a different pattern of expression as compared with MSC. In addition, no evidence of adipocyte and osteocyte differentiation was achieved with the standard protocols used for MSC (data not shown).

Gene expression analysis was performed to compare the stemness molecular signature and neural differentiation pattern of leptomeningeal nestin-positive cells with that of other known stem cell types derived from P15 rats, such as NSC from SVZ and bone marrow mesenchymal stem cell (BM-MS). All these stem cell types were cultured in the same condition. Adherent/expanded leptomeninges-derived cells showed higher stemness-related gene expression, such as Pou5f1/Oct4, TdGF1 (Cripto-1), Nanog, ( $P < 0.01$ ) as compared with adherent cultured SVZ-NSC. By contrast, adherent/expanded leptomeningeal cells expressed lower neural differentiation genes, such as MtAp2 (MAP2) and Gfap as compared with adherent cultured SVZ-NSC (Fig. 5F). These findings were in line with data obtained by FACS and immunofluorescence, and they suggest a homogeneous phenotype of adherent/expanded leptomeningeal cells.

### ***In vitro* neuronal differentiation of expanded leptomeninges-derived cells**

To determine whether the leptomeningeal adherent/expanded cells had neural differentiation potential, they were cultured with differentiating medium for at least 1 month and then analyzed for the presence of the neural marker MAP2. Real-time gene expression analysis of leptomeninges-derived cells in basal condition and after differentiation showed a statistically significant increase in *Mtap2* (MAP2) and decrease in *nestin* expression levels ( $P < 0.01$ ). Immunofluorescence analysis showed that cells differentiated in cultures into MAP2-positive neurons with high efficiency (30–50% of cells,  $n = 6$  experiments; Fig. 6B–D). Most of MAP2-negative cells were *nestin*-positive. Some GFAP-positive astrocytes and rare NG2- or O4-positive oligodendrocytic precursors were also found in the differentiated cultures (data not shown). Moreover, MAP2-positive cells showed other features of neuronal phenotype, including distinct neuritic arborization, dendritic spines (arrows in Fig. 6C) and the expression of presynaptic protein synaptophysin (Fig. 6E). A fraction of the differentiated MAP2-positive cells also expressed the GluR2 sub-unit of the ionotropic AMPA-glutamate receptor (Fig. 6G) and the glutamate decarboxylase (GAD67), marker of GABAergic neurons (Fig. 6F).

These terminally differentiated cells were not residual adult neuronal cells from tissue extract. This was demonstrated by incubating *nestin*-positive cultures with BrdU for 9 hrs before inducing differentiation. As shown in Fig. 6D, MAP2-positive cells were also labelled by the anti-BrdU antibody, thus indicating that these neurons derived from replicating cells.

Responses to depolarizing agents were analyzed by calcium imaging after Fura-2 loading. Stimulation with 55 mM KCl caused increase of the intracellular calcium concentration indicated by the shift of the 340/380 ratio. Fast application of 55 mM KCl (approximately 40 sec.) produced a significant response in 77% of the cells studied ( $n = 36$ ). Response reached a peak level after  $9.7 \pm 1.9$  sec. of KCl application and was sustained and reversible (Fig. 7A). Similar data were obtained from primary neuronal culture (Fig. 7B). These data suggest that leptomeningeal cells *in vitro* differentiated into neurones are excitable and express functional voltage-dependent calcium channels.

### ***In vivo* neuronal differentiation of leptomeninges-derived cells**

Transplantation studies were performed to determine whether adherent/expanded cells derived from leptomeninges of P15 rats could generate neural cell types *in vivo*. To recognize injected cells, expanded leptomeningeal *nestin*-positive cells were derived from EGFP-transgenic rats [29]. Cells were stereotaxically injected into the hippocampus of adult rats ( $n = 8$ ). Immunofluorescence analysis with anti-EGFP antibody revealed the presence of EGFP+ cells in hippocampus 4–8 weeks after transplantation (Fig. 8). EGFP+ cells were not detected in the control rat (non-injected

adult rats or rats injected with vehicle only). Real-time PCR confirmed that EGFP expression was restricted to injected rats (data not shown). To evaluate the differentiation of engrafted cells, 30- $\mu$ m-thick sections were analyzed by confocal microscopy following immunostaining with anti-EGFP antibody and markers for either undifferentiated cells (*nestin*) or neurons (MAP2) or astrocytes (GFAP) or oligodendrocyte precursors (NG2). The injection needle track was recognizable in the CA2-CA3 hippocampal region (Fig. 8A). EGFP+ cells close to the injection point were surrounded by abundant GFAP-expressing astrocytes (Fig. 8B). Most of the transplanted cells at the injection site expressed *nestin* (Fig. 8C); EGFP+/GFAP+ and EGFP+/NG2+ double positive cells were rarely detected (Fig. 8D and E).

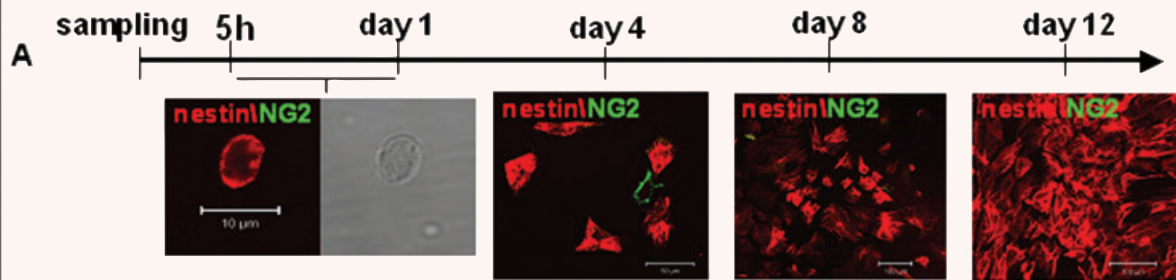
Sixty days after injection,  $49.8 \pm 17.9\%$  of the EGFP+ cells found in the hippocampus were also MAP2-positive ( $n = 4$ , see Materials and Methods). Most EGFP+/MAP2- cells were *nestin*-positive, undifferentiated cells. Most of the EGFP+ cells were located in the CA1 region (Fig. 9A–D). In this site, some of the grafted cells displayed complex morphology resembling the pyramidal neurons of the hippocampus (Fig. 9B'). EGFP+/MAP2+ engrafted cells were detected in the pyramidal layer and in the stratum oriens (Fig. 9C). Not all the EGFP+ cells observed in this layer also expressed MAP2, even if they displayed neuronal morphology and appeared to be well integrated within the CA1 (Fig. 9D and E). Transplanted cells displaying distinct neuronal morphology and MAP2 expression were also found in the hilus and in the sub-granular zone (SGZ) of the dentate gyrus (Fig. 9F).

## **Discussion**

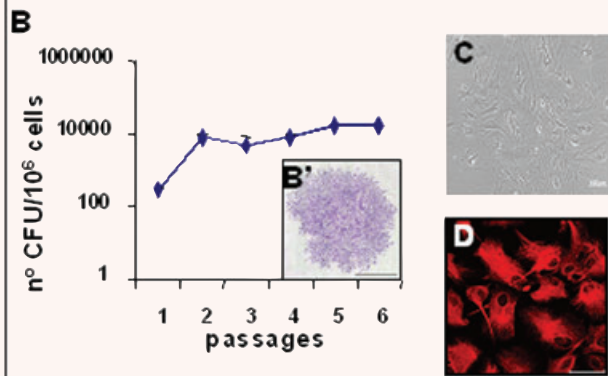
In this work, we analyzed the leptomeningeal compartment of the rat brain to assess whether a stem cell population with neuronal differentiation potential is present in this structure. Indeed, we found that (i) *nestin*-positive cells are present in the leptomeningeal compartment at the embryonic stages and persist up to adulthood, (ii) leptomeningeal *nestin*-positive cells can be extracted and cultured as neurospheres with features similar to the NSC-derived neurospheres, (iii) leptomeningeal *nestin*-positive cells can be cultured as adherent cells and expanded *in vitro* as homogeneous population of *nestin*-positive cells that highly express many of the stemness-related genes, (iv) expanded *nestin*-positive cells can be induced to differentiate *in vitro* with high efficiency to generate excitable neurons and (v) expanded cells can differentiate into neurons when injected into brains of living rats.

Previous studies described the distribution of stem cell markers inside the brain [49], but there was no evidence so far that leptomeninges could be a stem cell niche. *Nestin*-positive cells were seen in the most superficial portion of the rat brains in embryos and post-natal puppies. These cells were neither glial nor muscle cells or pericytes. Number and distribution of the *nestin*-positive cells declined during development and become restricted to the leptomeninges from post-natal day 15. At this age, *nestin*-positive

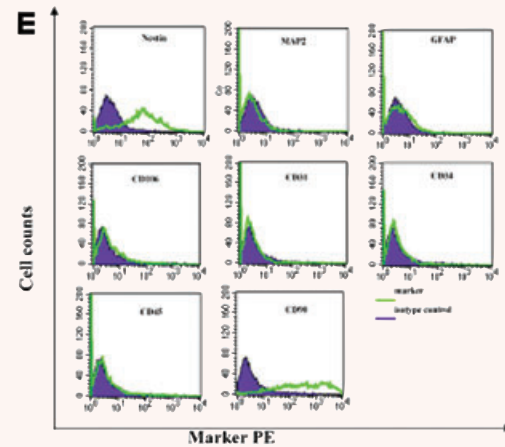
## Time course experiment



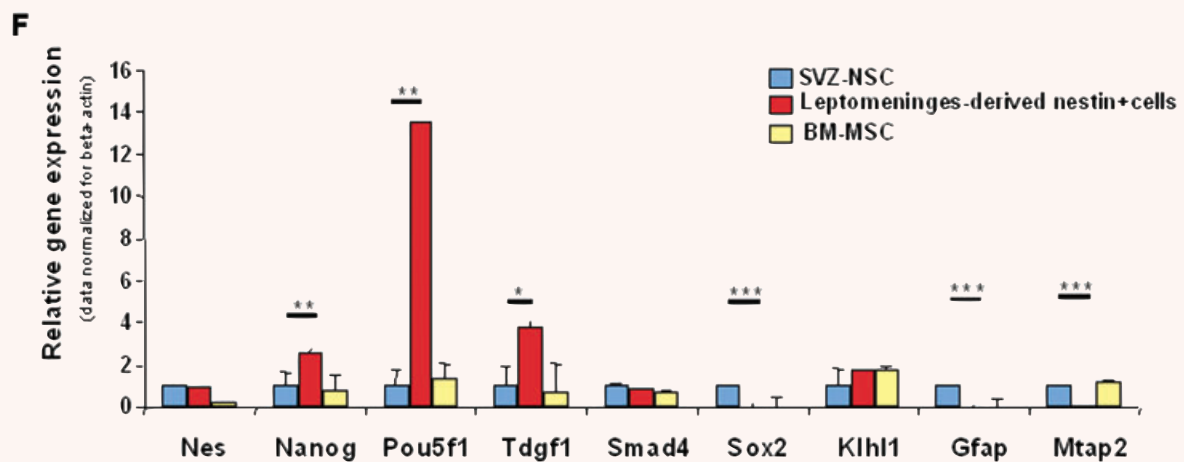
## In vitro expansion and self-renewal



## Immunophenotype



## Real time gene expression analysis





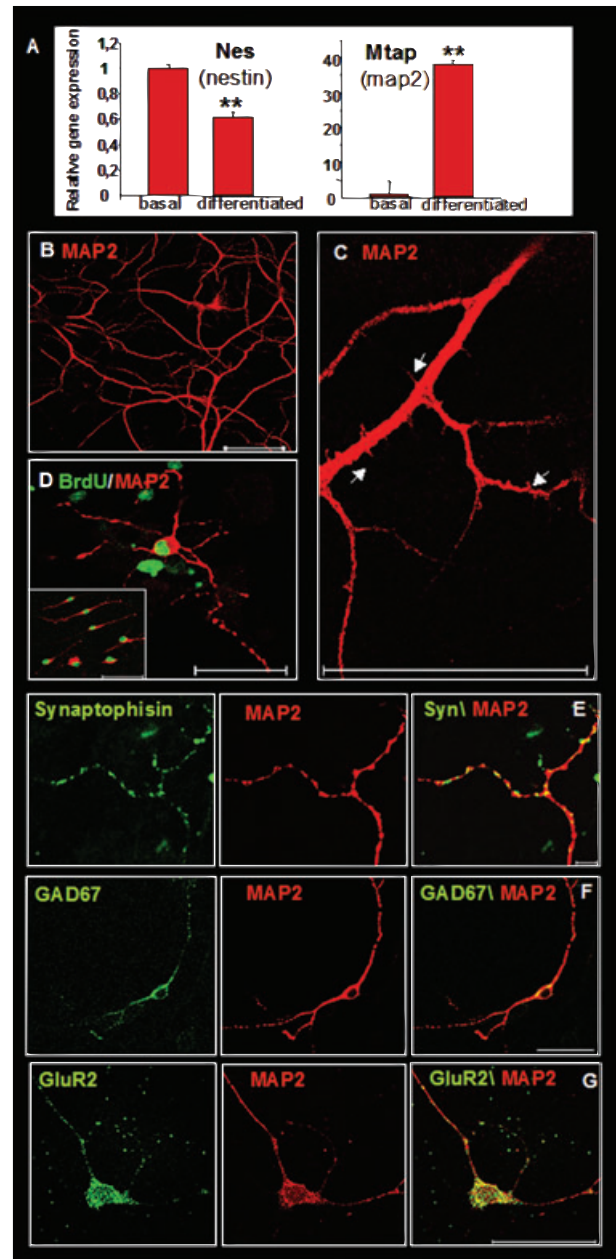


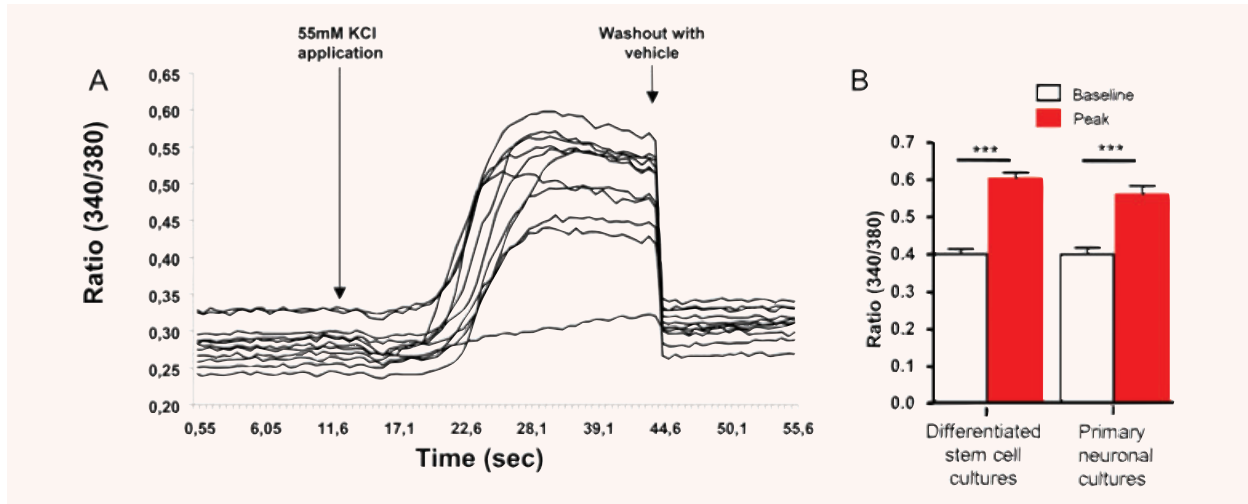
**Fig. 5** *In vitro* expansion of the tissue extract in adherent cells culture conditions. **(A)** Time course experiment. Confocal images of nestin+ / NG2- cells in adherent culture at 5 hrs, 1, 4, 8 and 12 days after plating. **(B)** CFU derived from leptomenigeal cells at different passages. Bars represent the CFU number/million of plated cells  $\pm$  S.D. **(B')** May–Grunwald Giemsa staining of a single colony of leptomenigeal adherent cells. **(C)** Transmitted light and **(D)** immunofluorescence, nestin (red), of adherent leptomenigeal cells. **(E)** FACS analysis, carried out on expanded cells obtained from adult rats, shows high expression of nestin and CD90. *In vitro* expanded cell population does not express neuro-glial (MAP2, GFAP and O4), leukocyte (CD45), endothelial (CD31, CD106) and haematopoietic stem cell markers (CD34). **(F)** Relative gene expression analysis. Leptomenigeal-derived nestin+ cells and BM-MSCs *fold changes* in transcription, normalized to actin mRNA, have been *shown compared to SVZ-NSCs*.

**Table 1** Assessment by quantitative RT-PCR of MSC marker expression by leptomenigeal nestin-positive cells

Protein	Gene	Leptomenigeal nestin-positive cells/BM-MSC fold change	P
Nestin	Nes	4.9	<0.01
Collagen type1a	Col1a1	0.58	<0.01
NG2	Cspg4	0.64	<0.01
CD34	Cd34	0.2	<0.01
Hyaluronate binding protein (CD44)	Cd44	1.16	0.16
Stem cell growth factor receptor kit (C-Kit)	Kit	3.06	<0.01
Endoglin (CD105)	Eng	1.24	<0.01
Fibroblast growth factor receptor 1	Fgfr1	0.017	<0.01
Fibroblast growth factor receptor 2	Fgfr2	1.93	<0.01
Fibroblast growth factor receptor 3	Fgfr3	26.93	<0.01
Cripto-1	Tdgf1	5.48	0.06
Oct4	Pou5f1	9.86	<0.05
Homeobox transcription factor Nanog	Nanog	3.34	<0.05

**Fig. 6** *In vitro* neural differentiation of leptomenigeal cells after adherent culture expansion **(A)** Real-time gene expression analysis of Nestin and Mtap2 (MAP2) in leptomeninges-derived cells in basal condition and after differentiation. The bars show folds change  $\pm$  S.D. of normalized mRNA expression levels measured before and after differentiation ( $P < 0.01$ ). **(B, C, D)** Differentiated cells, stained with antibodies against MAP2 (red). Arrows in **(C)** indicate dendritic spines. **(D)** BrdU staining (green) indicates that the MAP2-positive cells (red) derived from replicating cells. MAP2-positive cells also expressed components of the synaptic apparatus, including the presynaptic marker synaptophysin **(E)**, the glutamate ionotropic receptor sub-unit GluR2 **(G)** and glutamate decarboxylase (GAD67) **(F)**. Scale bars 50  $\mu$ m.





**Fig. 7** Calcium imaging analysis of *in vitro* neural differentiated leptomeningeal cells. **(A)** Changes in intracellular free calcium are indicated by variation of 340 nm/380 nm fluorescence ratio in Fura-2-loaded cells. Depolarization induced by 55 mM KCl led to increase of 340/380 ratio in most of the cells present in the field. **(B)** Average responses (mean  $\pm$  S.E.M.) expressed as peak and baseline values in differentiated cells and in primary neuronal cultures; \*\*\* $P < 0.001$ .

cells appeared as a layer associated to the leptomeninges and in contact but distinct from the pia mater. Other nestin-positive cells were abundant at known loci of neurogenesis, that is, SVZ, dentate gyrus and olfactory bulb [6]. Starting from these morphological observations, we decided to analyze leptomeninges as a potential source of nestin-positive stem/progenitor cells.

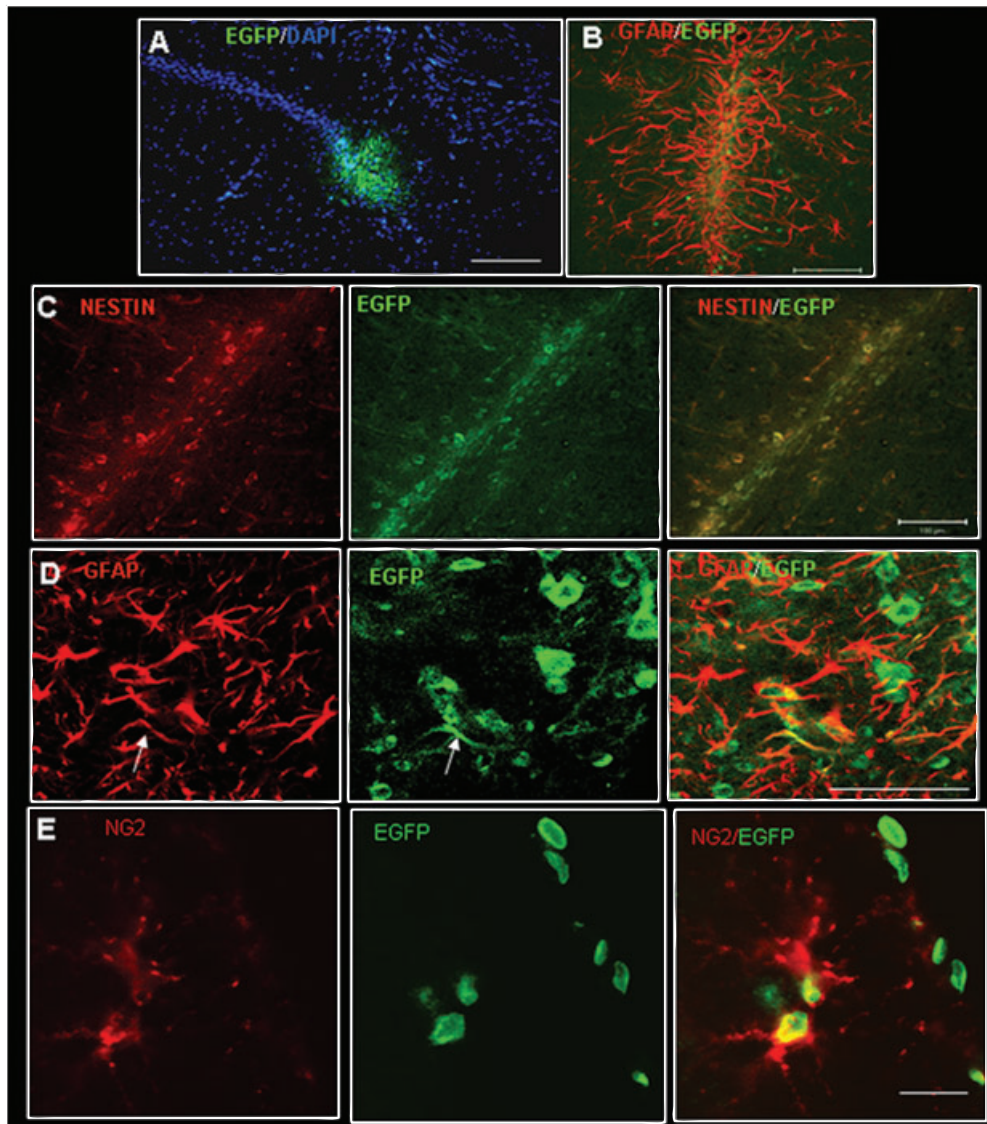
In this study, we report data from post-natal P15 rats. Preliminary data indicate that extraction efficiency is higher in younger animals and embryos. On the other hand, at P15 the cortical development is complete. This limits possible contamination by other potential sources of stem cells. This was confirmed by morphological examination of the stripped brains showing that SVZ, dentate gyrus and olfactory bulb remained intact. It is interesting to note that a similar population of nestin-positive cells could be extracted from choroid plexi, a site free of neural cells further confirming that the newly identified population of cultured nestin-positive cells did not originate from brain parenchyma. In addition, the number of cells that could be obtained from the biopsies was large enough to conduct extensive characterization of the leptomeningeal cells.

The nestin-positive cell population grown *in vitro* are likely to originate from the nestin-positive cells present in the stripped tissue and not from a process of transformation occurring *in vitro*. This was indicated by the presence of the nestin signal in cells as early as the cells become adherent to the flask following extraction. Previous work had shown that cells extracted from the whole brain express both NG2 and nestin antigens *in vitro*; these cells are endowed of neural differentiation potential [48]. The newly identified population of nestin-positive cells extracted from the leptomeninges were neither NG2- nor GFAP-positive cells, both *in vivo* and after expansion *in vitro*.

The extracted cells could be expanded as neurospheres or adherent cultures. Real-time PCR was used to show that leptomeninges-derived neurospheres had gene expression and multipotent differentiation potential comparable to those of SVZ-NSCs. Floating neurospheres are heterogeneous cultures containing multipotent stem cells, cell progenitors and many neuro-glial differentiating cells [35, 36]. We have used a different culturing protocol and extracted cells were grown as an adherent layer. At difference from the floating neurospheres, cells grew as a homogeneous cell population of nestin-positive/lineage-negative cells, characterized by clonogenicity and differentiation potential. This cell population showed high levels of stemness-related genes [40], such as Oct4, Nanog, Cripto-1 and Nestin, but it did not express differentiated neural genes. This procedure allowed a clear-cut differentiation between proliferative and differentiative stages of the cultured cells.

Leptomeninges-derived cells can be expanded and maintained in undifferentiated stage for up to several months; by switching medium, the cells can then be induced to differentiate *in vitro* with high efficiency into neuronal lineage. In 1 month, up to half of the cell population differentiated into MAP2-positive cells showing many features of terminally differentiated neurons, including dendritic spines, presynaptic proteins, receptors for neurotransmitters, enzymes involved in neurotransmitter synthesis and depolarization-induced changes of  $[Ca^{2+}]_i$  [50]. In conclusion, the nestin-positive stem/progenitor cells extracted from leptomeninges and grown *in vitro* have the potential to generate cells with several of the properties that identify mature and functional neurons.

As a further step of characterization of the newly identified cell population, we tested their neural differentiation potential *in vivo*.



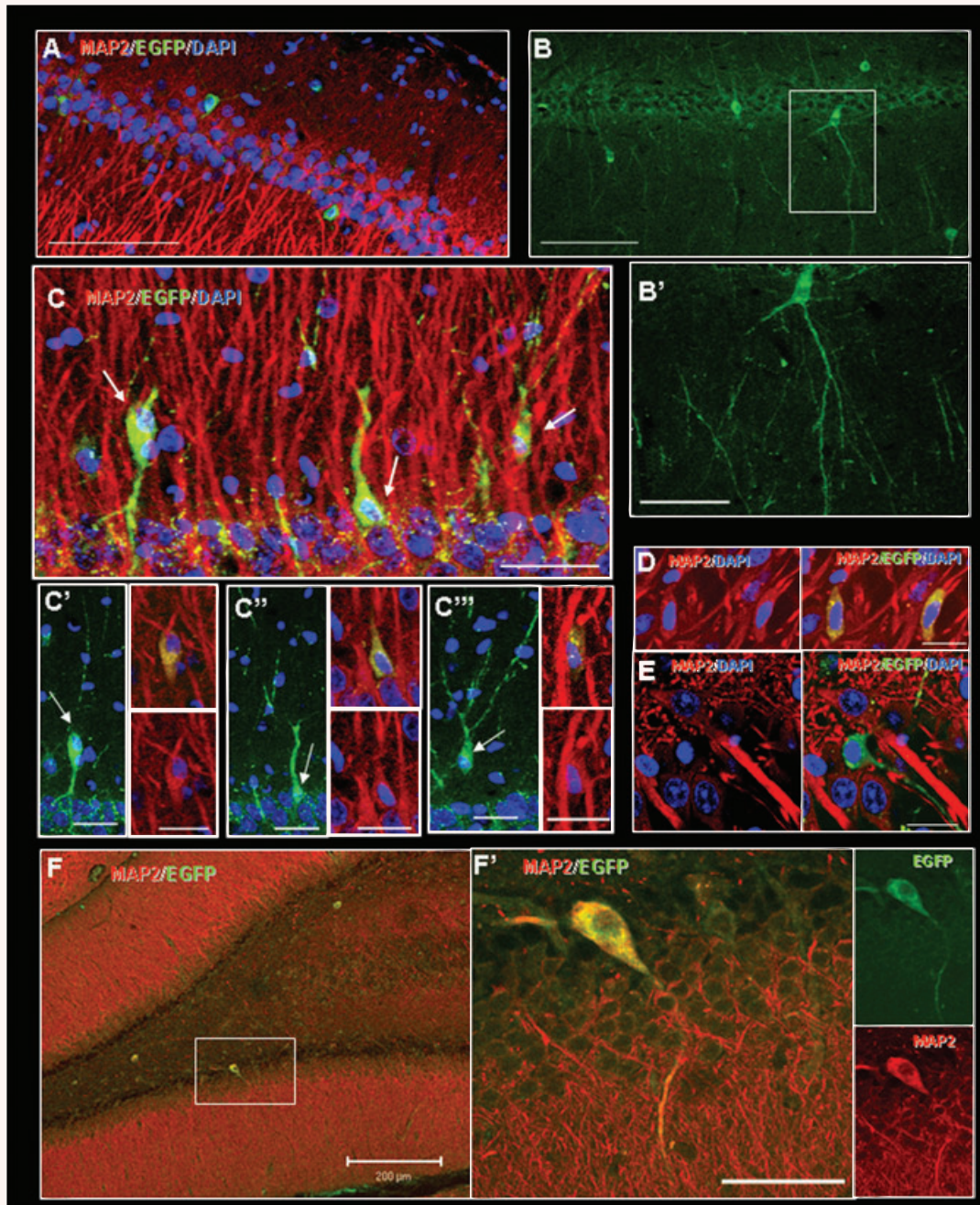
**Fig. 8** Confocal images of injection site of transplanted leptomeningeal cells. (A) GFP-positive cells (green) into the injection site in CA2 hippocampal region stained with nuclear marker DAPI (blue), scale bar 200  $\mu\text{m}$ ; (B) GFP-positive cells in the injection site surrounded by glia (GFAP red signal), scale bar 100  $\mu\text{m}$ ; (C) Colocalization of GFP-positive cells with nestin in the injection site (yellow signal), scale bar 100  $\mu\text{m}$ ; (D) Colocalization of GFP-positive cells with GFAP (yellow signal) near the injection site, scale bar 50  $\mu\text{m}$ ; (E) Colocalization of GFP-positive cells with NG2 (yellow signal), scale bar 50  $\mu\text{m}$ .

This was done by injecting expanded nestin-positive cells in hippocampus and following their fate for up to 60 days. To distinguish injected from resident cells, leptomeningeal cells were extracted and expanded from enhanced green fluorescent protein (EGFP) transgenic rats [29]. Following intra-brain transplant, the EGFP-positive cells persisted for up to 60 days into the hippocampus when approximately half of the transplanted cells in the hippocampus were MAP2-positive and had the morphological aspect of differentiated neurons. We cannot exclude that the EGFP+ engrafted cells may fuse with resident neurons. Fusion of stem cells with resident neurons has been shown to occur in some experimental setting when *in vivo* differentiation is rare [51]. This does not appear to be the case for leptomeningeal cells because

up to 50% the engrafted cells express the neuronal MAP2 antigen; in addition, none of these cells appeared to be multi-nucleated. In conclusion, the data suggest that nestin-positive stem/progenitor cells extracted from leptomeninges may have capability of neural differentiation also *in vivo*.

Somatic stem cells have been described in many adult organs and are presumably responsible of the maintenance of tissue homeostasis [3]. Stem/progenitor cells normally reside *in vivo* in special microenvironments capable of regulating their persistence and differentiation [4]. The discovery that stem/progenitor cells are present in leptomeninges is new but not surprising. Indeed, this region is strictly associated to pia mater cells that secrete important chemotactic factors, such as SDF-1 [19], as well as several





**Fig. 9** Confocal images of *in vivo* leptomeningeal cells neural differentiation. **(A)** Transplanted EGFP+ cells (green) are localized in CA1 region, scale bar 100  $\mu\text{m}$ . **(B)** EGFP+ cells (green) showed complex phenotypes mimicking the pyramidal neurons of the hippocampus (CA1 pyramidal cell layer), scale bar 100  $\mu\text{m}$ ; **(B')** high magnification, scale bar 20  $\mu\text{m}$ . **(C)** Z-stack reconstruction assembled from 10 serial 1.63- $\mu\text{m}$  confocal sections. EGFP (green)/MAP2 (red)/DAPI (blue)-positive cells in CA1 pyramidal layer, scale bar 50  $\mu\text{m}$ . **(C', C'', C''')** confocal images pointing single cell (arrowhead) co-localizations (yellow) of EGFP (green) and MAP2 (red), scale bars 20  $\mu\text{m}$ . EGFP+ cells co-localizing with MAP2 (yellow) **(D)** or not **(E)**, scale bars 20  $\mu\text{m}$ . Transplanted EGFP+ cells (green) co-localizing with MAP2 (yellow) in sub-granular zone (SGZ) and hilus of the dentatus gyrus, scale bar 200  $\mu\text{m}$  **(F)**. **(F')** High magnification, scale bar 50  $\mu\text{m}$ .



extracellular matrix components endowed of trophic functions [52]. In addition, it may be in contact/communication with other known NSC niches such as the ventricular zone mediated by the cerebrospinal fluid. Thus, this structure is a potentially favourable microenvironment, or niche, capable of hosting stem/precursor cells.

Other somatic stem cells displaying some of the characteristics of leptomeningeal nestin-positive cells, that is formation of neurospheres in liquid culture and neuronal differentiation, and not related to NSC have been previously described [53–58]. A transient wave of MSC originates during embryogenesis from Sox1<sup>+</sup> neuroepithelium [59]. In adulthood, MSC appears to reside in multiple human organs as perivascular cells [60]. The origin and identity of the stem/progenitor cells we described is unknown; however, our data show that leptomeningeal nestin-positive cells are different from NG2-positive perivascular MSC and have different localization, as compared with all the other known somatic stem cells.

Further studies are needed to establish the *in vivo* role of leptomeningeal nestin-positive cells in embryonic, post-natal and adult CNS. As leptomeninges cover the entire CNS surface and also follow vessels into the brain mass, it is possible that the adult CNS has the potential to host regenerative stem cells over a large proportion of its volume. Finding factors that may expand migration, replication and differentiation properties of the resident population of stem cells may be relevant for the improvement of regenerative therapies for brain diseases.

Because of the persistence of these cells up to adulthood, their proliferation capability *in vitro*, and their differentiation potential into neuronal cells *in vitro* and *in vivo*, we suggest to name them *leptomeningeal stem/progenitor cells* (LeSC) as a new entity. Further characterization of LeSCs in normal and pathological conditions may help to better understand the cortical neurogenesis as well as their potential usefulness in the treatment of neurological diseases when used as source of NSC-like population.

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