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CHARACTERIZATION OF NOVEL MONOCLONAL ANTIBODIES ABLE TO IDENTIFY NEUROGENIC NICHES AND ARREST NEUROSPHERE PROLIFERATION AND DIFFERENTIATION


Abstract—Two monoclonal antibodies (Nilo1 and Nilo2) were generated after immunization of hamsters with E13.5 olfactory bulb-derived mouse neuospheres. They are highly specific for neural stem cell and early progenitor cell surface antigens. Nilo positive cells present in the adult mouse subventricular zone (SVZ) were able to initiate primary neural stem cell cultures. Moreover, these antibodies added to neuosphere culture induced proliferation arrest and interfered with their differentiation. In the lateral ventricles of adult mice, Nilo1 stained a subpopulation lining the ventricle and cells located in the SVZ, whereas Nilo2 stained a small population associated with the anterior horn of the SVZ at the beginning of the rostral migratory stream. Co-staining of Nilo1 or Nilo2 and neural markers demonstrated that Nilo1 identifies an early neural precursor subpopulation, whereas Nilo2 detects more differentiated neural progenitors. Thus, these antibodies identify distinct neurogenic populations within the SVZ of the lateral ventricle. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: neural stem cell markers, early progenitor cell markers, adult neural stem cells, embryonic neural stem cells, neuospheres.

Adult stem cells are defined by their ability to proliferate undergoing self-renewal and generating a differentiated progeny specific of the organ from which they derive. In adult animals, stem cells are likely to compensate cell loss by physiological turnover, or pathological conditions, such as injury or degenerative diseases. The mammalian brain was considered to have a poor neuronal regenerative capacity and a very low cellular turnover. It is clearly established by now, however, that neurogenesis is maintained in discrete brain regions during mammal’s lifespan (Altman and Das, 1965; Alvarez-Buylla et al., 2000; Lennington et al., 2003; Bedard and Parent, 2004; Bonnert et al., 2006). Neurogenesis in mammals primarily occurs in two areas of the adult brain, the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. In the DG, neo-generated neuronal cells of the subgranular zone (SGZ) migrate to the granular cell layer, where they project axons to the CA3 area of Ammon’s horn (Cameron et al., 1993; van Praag et al., 2002). The main source of adult neural stem cells in rodents is, however, the telencephalic SVZ, a thin layer of cells lining the wall of the lateral ventricles (Doetsch et al., 1999) actively proliferating throughout adulthood. In rodents, postnatal neurogenesis in the SVZ is characterized by the division of multipotent glial cells (astrocyte type-B cells), generating neuroblasts (type-A cells), through a transit-amplifying cell population (type-C cells). These newly generated neuroblasts migrate to the olfactory bulb (OB) through the rostral-migratory stream (RMS), where they home as inhibitory interneurons (Alvarez-Buylla et al., 2000; Dutton and Bartlett, 2000; Pencea et al., 2001; Bedard et al., 2002; Doetsch et al., 2002). Neural progenitor cells, isolated from neurogenic regions, are able to grow in vitro either as monolayers on substrate-coated tissue plates, or as free-floating tridimensional spheres (known as neuospheres) in uncoated tissue plates. Neural stem cells proliferate and preserve their self-renewal capacity in vitro, when grown in appropriate media supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Withdrawal of these trophic factors triggers differentiation of the culture into the three major cell types of the CNS, neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1992; Gage et al., 1995).

In the SVZ of the adult mouse, the neural differentiation stages comprised between a quiescent neural progenitor and a fully differentiated neuron or glia cell, are defined by combinations of different markers primarily recognizing intracellular antigens, including nestin (Lendahl et al., 1990), vimentin (Coehard and Paulin, 1984; Sancho-Tello et al., 1995), glial fibrillary acidic protein (GFAP) (Doetsch et al., 1997) and Sox2 (Brasz et al., 2005), which identify highly
undifferentiated neural precursors. Conversely, combinations of doublecortin (DCX) (Yang et al., 2004), polysialic-acid neural cell adhesion molecule (PSA-NCAM) (Doetsch et al., 1997) and Tuj-1 (Moody et al., 1989; Membre and Hall, 1995) identify more differentiated neural precursors, such as the migrating neuroblasts. These markers are not exclusive of neural stem cells, since GFAP is expressed outside the CNS and thus, in order to identify the astrocyte-like neural stem cells in the adult SVZ, nestin (a neuroepithelial marker) must be co-expressed.

Due to the lack of an antigenic surface signature for neural stem cells, the identification of novel specific markers represents a valuable experimental tool. Our work describes the generation of Nilo (Neural Identification Lineage from Olfactory bulb) monoclonal antibodies that recognize surface antigens from mouse neurospheres. Nilo1 and Nilo2 antibodies labeled not only neurospheres, but also specific neurogenic areas on the adult mouse SVZ. Co-staining with various neural markers demonstrated that Nilo1 identified early progenitor cells (Sox2+/vimentin+/H9252/H11001), whereas Nilo2 identified more differentiated neural progenitor cells, committed to the neuroblast pathway (Tuj-1+/PSA-NCAM+/DCX+).

Finally, the antigens recognized by Nilo1 and Nilo2 antibodies are highly relevant on neural stem/early progenitor cell biology, since these antibodies were able, not only to arrest neurosphere proliferation in vitro, but also interfered with their differentiation into mature neural cells.

**EXPERIMENTAL PROCEDURES**

**Animals**

Armenian hamsters were purchased from Cytogen Research and Development (MA, USA) and housed in our animal facility. FVB mice (originally obtained from IFFA-Credo, France), were bred and housed under standard conditions in our animal facility. The CSIC Committee of Animal Experimentation approved animal manipulation and experimental methods. Efforts were made to minimize the number of animals and their suffering. All experiments described were performed in compliance with the European Union (Council Directive 86/609/EEC) and Spanish laws on care for experimentation animals.

**Antibodies and reagents**

Matrigel Basement Membrane Matrix Growth Factor Reduced (BD Pharmingen, Erembodegem, Belgium, #356230) was used 1:20 in culture medium. Nilo1 and Nilo2 antibodies for labeling were used at a 1:2 dilution from hybridoma supernatant. Protein-A sepharose-purified antibodies were labeled either with fluorescein isothiocyanate (FITC) or phycocerythrin (PE) (Immunostep Inc., Salamanca, Spain). The following commercial primary antibodies were used: GFAP (1:200 LabVision, Thermo Fisher Scientific Inc., Fremont, CA, USA), nestin (1:100 Chemicon, Millipore, Molsheim, France), Sox2 (1:400 Chemicon), JIV-tubulin (1:400 Abcam), PSA-NCAM (1:400 Chemicon), Tuj1 (1:2000 Covance), DCX (1:100 Santa Cruz), Kd67 (1:200 LabVision), vimentin (1:200 AbCam, Cambridge, UK), EGFR (1:100 Santa Cruz Biotechnology Inc., Heidelberg, Germany) and O4 (20 μg/ml Chemicon). The following secondary antibodies were used: FITC-conjugated anti-Armenian and Syrian hamster IgG cocktail (1:100 BD Pharmingen), Cy3-conjugated anti-rabbit IgG (1:200 Jackson Immunoresearch, West Grove, PA, USA), Texas Red-conjugated anti-mouse IgG (1:200 Mol. Probes, Invitrogen, Carlsbad, CA, USA), and biotinylated anti-hamster IgG antibody cocktail (1:100 BD Pharmingen). Incubation with the biotinylated antibody was followed by avidin-horseradish peroxidase (Vectorstain ABC kit, Vector Laboratories Inc., Burlingame, CA, USA) and developed with 3,3’-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, USA) and H2O2.

**Generation of hamster-derived monoclonal antibodies**

Spleen cells from two male Armenian hamsters, immunized with 30×10^6 embryo-derived OB neurospheres, were fused with P3X63Ag8.6.5 mouse myeloma cells using 50% (w/w) PEG (Sanchez-Madrid et al., 1983, Sanchez-Madrid and Springer, 1986). Hybridomas were selected, expanded, cloned by limiting dilution, and tested for specific reactivity against mouse neurosphere surface cell epitopes. Hybridomas were subsequently maintained on RPMI 10% fetal calf serum (FCS), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

**Culture of neural stem cells**

Olfactory bulb stem cells were obtained from E13.5 FVB mouse embryos (Vicario-Abejon et al., 2003). Briefly, OB was dissected and mechanically disrupted to obtain a single cell suspension. Adult-derived neurospheres were prepared from micro-dissected SVZ of 6 to 8 weeks-old FVB mice (Bonilla et al., 2005). Cells were resuspended in DMEM/F12 medium, digested with papain 1 mg/ml (Worthington DBA, NJ, USA) for 30 min at 37 °C, followed by mechanical dissociation. Single cell suspensions were seeded at 5000 cells/cm^2 in six well plates in neurosphere complete medium (DMEM/F12 containing 0.6% glucose, 0.1% NalHCO3, 5 mM Hepes, 25 μg/ml insulin, 1 mg/ml apotransferrin, 96 ng/ml putrescine, 0.2 μM progesterone and 0.3 μM sodium selenite (all from Sigma), supplemented with 20 ng/ml EGF, 20 ng/ml bFGF and 0.7 U/ml heparin). Cells were incubated at 37 °C, 5% CO2 and 95% humidity and cultured until neurosphere formation was observed (4–7 days). Unless otherwise indicated, neurospheres from passages 6 to 8 were used, to ensure homogeneity of the cultures.

For proliferation assays, single cells from SVZ neurospheres (10,000 cells/cm^2) were incubated in the presence of Nilo antibodies at different concentrations (ranging from 0.5 to 0.125 mg/ml) in 96-well plates during 24–72 h. The assays were performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA, Cat. No. G3580), according to manufacturer’s instructions. This assay measures the reduction from MTS to formazan by metabolically active cells and the number of living cells in the culture is estimated by the value of A570nm/600nm.

For differentiation assays, neurospheres were mechanically dissociated and plated onto glass coverslips in 24-well plates at a density of 5×10^4 cells/cm^2 (in neurosphere complete medium containing 0.5% FCS devoid of EGF and bFGF), and incubated for 7 days at 37 °C, 5% CO2 and 95% humidity.

**Immunocytochemistry**

Neurospheres or single cells were transferred onto 24-well culture plates containing Matrigel-coated glass coverslips (diluted 1:20) in neurosphere complete medium supplemented with EGF and bFGF. Neurospheres were attached for 15 min and fixed in 4% paraformaldehyde (PF), phosphate buffer saline (PBS) for 20 min at room temperature. Disaggregated neurospheres were cultured at 37 °C, 5% CO2 and 95% humidity for 48 h prior to fixation. After blocking in 10% FCS in PBS, cells were incubated overnight at 4 °C with Nilo1 or Nilo2 antibodies, washed with PBS and incubated with FITC-conjugated anti-hamster IgG antibody for 1 h at...
room temperature (RT). Cells were then washed and treated with 10% FCS in PBS containing 0.05% (v/v) Triton X-100, stained with antibodies recognizing GFAP or nestin, followed by incubation with the corresponding secondary antibodies for 1 h at RT. Double labeling of differentiated neural stem cells with Nilo2 antibody in combination with GFAP, TuJ-1 or O4 was performed according to the previous protocol. Simultaneous labeling with both Nilo antibodies were performed using purified directly labeled Nilo1-FITC (1:100) and Nilo2-PE (1:1000) in PBS-10% FCS for 1 h at 37 °C. Coverslips were mounted either in Mowiol or Mowiol/Dapi. Controls were performed to confirm primary and secondary antibody specificities. Images were acquired with either a Nikon Eclipse 80i fluorescent microscope or a Leica TCS-SP2-AOBS-UV confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

**Immunohistochemistry**

Six to eight weeks-old FVB mice were deeply anesthetized and perfused transcardially with 0.1 M phosphate buffer (PB), followed by 4% PF in 0.1 M PB. Dissected brains were post-fixed for 24 h in 4% PF in 0.1 M PB, cryoprotected in 30% sucrose for 24–48 h, and subsequently frozen at −80 °C for cryostat sectioning. Immunostaining was performed in 25 μm brain coronal free-floating sections. Briefly, sections were blocked with 10% FCS in PBS containing 0.1% Triton X-100 and incubated overnight at 4 °C with Nilo1 or Nilo2 antibodies. Sections were then washed and incubated with the corresponding anti-hamster-FITC secondary antibody for 2 h at 37 °C. After exhaustive washing and re-blocking, samples were incubated either with GFAP, vimentin, Sox2, DCX, PSA-NCAM, Tuj-1, IV-tubulin, EGFR or Ki67 primary antibodies, followed by the appropriate secondary antibodies. Incubation with anti-hamster biotinylated antibody was followed by avidin-horseradish peroxidase incubation, and developed with DAB and H2O2. Antibody specificity was confirmed; sections were transferred to poly-lysine-coated glass slides, mounted with Mowiol/DABCO, and analyzed with a Leica TCS-SP2-SP2-UV confocal microscope.

**Immunoprecipitation assays**

Disaggregated neurosphere cells (30×10^6), derived from mouse OB embryo (E13.5) were incubated with Nilo1 or Nilo2 antibodies (50 μl of hybridoma supernatant per 1×10^6 cells) for 1 h at 4 °C. After two washes with PBS, cells were incubated in lysis buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.025% sodium azide, 1% Brij 35, 1% BSA and protease inhibitors) for 1 h on ice, and centrifuged for 15 min at 15,000 g (4 °C). The supernatant was incubated with Sepharose-coupled Protein G beads for 3 h at 4 °C, and thoroughly washed with lysis buffer. The eluted proteins were separated by SDS-PAGE, transferred to PVDF membranes (Millipore) and analyzed by Western blotting with the corresponding Nilo antibodies followed by HRP conjugated anti-hamster IgG secondary antibody, and finally revealed using electrochemical luminescence (ECL) (Amersham Biosciences, GE Healthcare Europe GmbH, Barcelona, Spain) reagent. A hamster antibody negative for neurosphere cell labeling (irrelevant antibody) was used as an immunoprecipitation control. When N-linked carbohydrates were removed, SVZ neurosphere cell lysates were immunoprecipitated with Nilo1 or Nilo2 antibodies. Eluted proteins after boiling in 2% SDS were incubated for 3 h with PNGaseF according to the manufacturer’s instructions (New England Biolabs Inc., Ipswich, MA, USA). Proteins were separated and analyzed with western blot.

**Flow cytometry**

Analyses were performed using single cell suspensions obtained from adult SVZ neurospheres, embryo-derived OB neurospheres or freshly isolated thymocytes. Cells were labeled with Nilo mAbs in PBS supplemented with 10% mouse serum, 3% BSA and 0.025% Sodium azide, for 1 h at 4 °C, washed, and subsequently incubated with anti-hamster-FITC secondary antibody for 45 min at 4 °C. Cells incubated with secondary antibody in the absence of primary antibody were used as control. Double labeling of neurosphere cells with Nilo1-FITC and Nilo2-PE antibodies was performed during 1 h at 4 °C. Prior to analysis, propidium iodide (4 μg/ml) was added to each sample to gate on viable cells. Cells were analyzed with a Coulter analyzer flow cytometer Epics XL (EXPO 32 software), collecting at least 10,000 events per sample.

**Neurosphere-generating cell assays**

Single cell suspensions obtained from the SVZ of adult FVB mice were stained either with Nilo1-PE (1:100) or Nilo2-PE (1:1000) antibodies in PBS, 3% BSA, 0.025% Sodium azide for 1 h at 4 °C. After washing in PBS, cells were sorted by flow cytometry (FACS Vantage, BD). Nilo1 and Nilo2, or Nilo1 and Nilo2 cell populations were plated at different concentrations in 96 well/plates to evaluate the frequency of neurosphere-forming precursors. Cells were cultured in neurosphere complete medium supplemented with EGF, bFGF and heparin, fed weekly, and wells were scored for neurosphere growth 7–14 days later. Linear regression analysis of the proportion of negative wells for each cell concentration was used to determine the frequency of neurosphere-forming cells.

**RESULTS**

**Nilo1 and Nilo2 monoclonal antibodies recognize mouse neural stem cell antigens**

New cell surface antigens of neural stem cells were identified with a panel of monoclonal antibodies generated against mouse embryo (E13.5) olfactory bulb-derived neurospheres. Neural stem cells isolated from OB are well characterized, representing an excellent source of neural stem cells, in particular since there is a neurogenesis peak at the developmental stage used (Vicario-Abejon et al., 2003). The supernatants from the resulting hybridomas (more than 100) recognized cell surface antigens on neural stem cells, as demonstrated by flow cytometry analyses, and the antibody panel was denominated Nilo. Here we describe two of these monoclonal antibodies (Nilo1 and Nilo2), which were selected on the basis of their high specificity against neurosphere-derived cells from mouse embryo OB and adult mouse SVZ. As measured by flow cytometry, Nilo1 recognized 60–70% of total viable cells obtained from primary cultures of embryo OB and adult SVZ-derived neurospheres, whereas Nilo2 recognized more than 90%. Moreover, freshly isolated thymocytes and neural tissue-unrelated cell lines were not stained with these antibodies (Fig. 1A and data not shown). Immunoprecipitation of protein extracts from neurosphere primary cultures demonstrated that whereas Nilo1 immunoprecipitated a major protein band of about 40 kDa, Nilo2 immunoprecipitated a protein doublet of approximately 150–170 kDa (Fig. 1B). Thus, indicating that Nilo1 and Nilo2 identified different surface antigens. Furthermore treatment of neurosphere cell lysates with an endoglycosidase dase indicated that the antigens recognized by these antibodies were N-glicosylated proteins (Fig. 1C) as demonstrated by their decrease in molecular weight. Nilo1 and Nilo2 antibodies stained a low percentage...
(4–8.6%) of cells from freshly disaggregated adult mouse SVZ, although Nilo1 cells increased up to 60–70%, and Nilo2 cells rose to 95% in neurospheres after 15 days in culture (Fig. 1D).

Fig. 1. Characterization of Nilo1 and Nilo2 monoclonal antibodies (A) FACS analyses of Nilo1 and Nilo2 expression in neurospheres obtained from adult mouse SVZ or from embryonic OB and in thymocytes (grey). Cells labeled with secondary antibody were included as controls (white). (B, C) Immunoprecipitation with Nilo1 and Nilo2 of membrane protein extracts from neurosphere cells (B) and from SVZ neurosphere cell lysates (C). In (C) eluted proteins were incubated (+) or not (−) with endoglycosidase (PNGaseF) to remove N-linked carbohydrates. In (B, C) western blotting was performed with corresponding antibodies (Nilo1 or Nilo2), HRP labeled anti-hamster IgG antibody, and detected by enhanced chemoluminiscence (ECL). An irrelevant hamster antibody was used as control (irrelevant). (D) Time course analysis of SVZ neurosphere-derived single cell suspensions labeled with Nilo1 or Nilo2. FACS data is represented as mean of positive cells ±SD n=3.
Single cell suspensions from freshly isolated and disaggregated SVZ tissue were used to purify Nilo1 or Nilo2 positive and negative cells by flow cytometry cell sorting (FACS) using the corresponding antibodies. The sorted cells were directly plated on 96 well plates at different cell densities, and the ability of each positive and negative population to generate primary neurospheres was determined by limiting dilution assays. The resulting data indicated that both Nilo1+ and Nilo2+ cells efficiently generated neurospheres (40/20,000 for Nilo1+ and 24/20,000 for Nilo2- cells), whereas the negative populations were unable to do so (<1/20,000 for Nilo1- and <2/20,000 for Nilo2- cells).

Neurosphere labeling with Nilo1 or Nilo2 antibodies

Cultured neurospheres were co-stained with Nilo1 or Nilo2 antibodies and other markers that label neural stem cells (nestin and GFAP). Similarly than for Nilo1+ cells, the majority of Nilo2+ cells were also nestin positive (Fig. 2A, Suppl. Fig. 1). Nilo1+/GFAP+ and Nilo2+/GFAP+ cells were also detected, suggesting that Nilo1 and Nilo2 antibodies identified early neural precursor cells. Moreover, in neurosphere-derived single cell suspensions cultured on Matrigel™, we observed a lack of co-localization for GFAP and nestin with either Nilo1 or Nilo2 on double positive cells, corroborating the different subcellular localization of these markers (membrane antigens for Nilo1 and Nilo2; intracellular for GFAP and nestin) (Suppl. Fig. 2). Although FACS analyses demonstrated that in neurosphere cultures there was a high fraction of Nilo1+ Nilo2+ double positive cells (Fig. 2B), these antigens did not co-localize in the cells grown on Matrigel™ (Fig. 2C), corroborating that these monoclonal antibodies recognized different cell surface antigens.

Neurospheres were maintained in a proliferating self-renewal state when cultured in media supplemented with EGF and bFGF. Factor withdrawal, together with FCS addition, induce plastic adherence of the cells and differentiation into the three major neural lineages (neurons, oligodendrocytes and astrocytes). We observed that under these differentiation conditions, Nilo1 expression was lost early, indicating that the antigen recognized by Nilo1 antibody was selectively expressed by undifferentiated neural stem cells. In contrast, a small percentage of cells retained Nilo2 labeling for several days after the onset of differentiation (Fig. 3A). Some Nilo2+ cells were also positive for the early neuronal marker Tuj-1 (Fig. 3B), but were negative for the oligodendrocyte marker O4 (Fig. 3C). Moreover, we identified a low number of cells double positive for Nilo2 and the astrocyte marker GFAP (data not shown).

Nilo antibodies arrest proliferation and block differentiation of neural stem cells

To evaluate the functional effect of Nilo antibodies, mechanically disaggregated cells from adult mice-derived neurospheres were cultured in the presence of various concentrations of purified Nilo1 and Nilo2 antibodies, under proliferation or differentiation conditions. As shown in Fig. 4A, both Nilo1 and Nilo2 monoclonal antibodies efficiently inhibited cell proliferation, although comparatively, Nilo1 antibody had a weaker effect. As control, the same concentrations of an irrelevant hamster monoclonal antibody had no significant effect. Microscopic examination confirmed that the addition of the Nilo antibodies led to a reduction on the number and size of neurospheres (Fig. 4B), although the few generated harbored an undifferentiated phenotype since they remained nestin positive.

Nilo antibodies also had an effect interfering with neurosphere differentiation (Fig. 4C). Indeed, differentiation conditions triggered attachment to the plastic and the cells underwent differentiation. When neurosphere-derived cells were put in these conditions, but in the presence of Nilo antibodies, differentiation was abrogated and the cells remained nestin positive. As control, cells treated with similar concentrations of an irrelevant monoclonal antibody were used. Nilo2 inhibited differentiation of neurosphere cells with a faster kinetics than Nilo1, being observed as early as 48 h (data not shown) after the addition to the primary cell culture, with a highly significant effect after 4 days. These results indicated that Nilo antibodies recognized cell surface molecules involved in the proliferation and differentiation processes of neural stem cells.

Nilo1 and Nilo2 antibodies identify distinct progenitor populations within the adult mouse SVZ

Expression of the antigens recognized by Nilo antibodies in the mouse adult brain was further analyzed in the major neurogenic regions (SVZ of the lateral ventricles, OB and DG of the hippocampus). We observed that in the SVZ, Nilo1 mAb recognized a small cell population lining the ventricle (Fig. 5A). However, Nilo2+ cells formed a thin layer in the periventricular area of the SVZ, located inside the anterior SVZ (SVZA), at the beginning of the RMS (Fig. 5B). These data suggest that Nilo1 and Nilo2 antibodies identified distinct progenitor populations inside the SVZ. Interestingly, the expression of both antibodies in the adult olfactory bulb was restricted to the ependymal and subependymal layers, located in the core of the adult olfactory bulb, a site previously described to hold neural stem cells (Gritt et al., 2002) (Fig. 5C, D). The DG of the hippocampus was negative for these antibodies (Fig. 5E, F). Interestingly, Nilo1 stains patches of cells throughout the periventricular area and the anterior horn of the ventricles, some of them with ependymal localization while others were subependimal (Fig. 5A, G). Within the subependymal Nilo1+ cells, some were also positive for GFAP and nestin (Fig. 5H–K) corresponding to early neural precursors.

Early neural precursors have been defined by the expression of GFAP, EGFR, vimentin and Sox2. Although individually, none of them fulfill the criteria for early precursor cells, it is generally accepted that cells co-expressing these markers represent bona fide neural precursors (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). In adult SVZ tissue sections, the cells stained with Nilo1 mAb were, independent of their ependymal or subependymal location, positive for Sox2 (Fig. 6A), and most of them also positive for GFAP (Fig. 6B), although a small proportion of Nilo1+GFAP+ cells were identified. In addition, we...
Fig. 2. Neurosphere staining with Nilo mAbs. (A) SVZ-derived neurospheres were double stained with Nilo1 or Nilo2 antibodies and either nestin or GFAP antibodies. Orthogonal projections of these data are shown in Suppl. Fig. 1. (B) FACS analysis of a neurosphere-derived single cell suspension labeled with Nilo1-FITC and Nilo2-PE antibodies. (C) Neurosphere-derived single cell suspensions seeded in Matrigel® were fixed and stained with Nilo1-FITC and Nilo2-PE antibodies and analyzed by confocal microscopy. Magnification: 100x.
found Nilo$^{+}$ cells lining the lateral ventricle that were vimentin$^+$ (Fig. 6C) and β-IV tubulin$^+$ (Fig. 6D). There was, however, a population of subependymal Nilo$^{+}$ cells that were β-IV tubulin$^+$ (Fig. 6D). Interestingly, only a small fraction of dividing cells (positive for the nuclear marker Ki67) were included within the Nilo$^{+}$ population (Fig. 6E), suggesting that Nilo$^{+}$ cells were quiescent. Furthermore, Nilo$^{+}$ cells were EGFR positive (Fig. 6F), but negative for more differentiated progenitor markers such as PSA-NCAM, DCX, or Tuj-1 (see Suppl. Fig. 3). Thus, these results indicated that Nilo1 antibody identified an early neural progenitor cell population within the SVZ.

Conversely, Nilo2$^{+}$ cells co-expressed neuroblast markers (DCX, PSA-NCAM, and Tuj-1), indicating that Nilo2 antibody labeled more differentiated neural progenitor cells (Fig. 7A–C). Moreover, only a very low percentage of Nilo2$^{+}$ cells were positive for the early progenitor neural markers (Sox-2, GFAP or vimentin), and none expressed the ependymal marker β-IV tubulin (Suppl. Fig. 4). Finally, many Nilo2$^{+}$ cells within the anterior horn co-expressed the proliferation marker Ki67 (Fig. 7D).

Taken together, these data suggested that Nilo1 antibody labeled quiescent neural progenitor cells, whereas Nilo2 antibody detected post-mitotic neuronal precursors, such as type 1 neuroblasts, and therefore, these antibodies identified distinct progenitor populations within the adult mouse SVZ.

**DISCUSSION**

Neural development is mainly embryonic, however, tissue-specific neural stem cells support low-rate neurogenesis throughout the individual’s life. In the adult brain, neural stem cells are restricted to specific areas, where they can be isolated and expanded *in vitro* as neurospheres. Neurospheres were used as immunogen to generate a panel of monoclonal antibodies referred to as Nilo, here we describe two of them (Nilo1 and Nilo2). These antibodies recognize surface antigens in neurosphere-derived single cell suspensions. Available antibodies specifically recognizing cell surface antigens in neural stem cells are scarce. These include the mAb directed against CD133, a surface
molecule that successfully identifies neurosphere-generating single cells (Uchida et al., 2000), as well as hematopoietic cell precursors used for transplantation experiments (Spangrude et al., 1991). However, the identity and function of CD133/H11002 expressing cells in the adult mouse SVZ remains controversial (Pfenninger et al., 2007; Coskun et al., 2008).

Nilo1 and Nilo2 antibodies were selected for their ability to recognize surface molecules in neurospheres derived from adult mice SVZ and from embryonic olfactory bulb. As expected, and in agreement with the low number of neural progenitor cells within the adult brain, Nilo1 and Nilo2 antibodies recognized a cell fraction in the adult SVZ, which is the major neurogenic region in mice (Lois and Alvarez-Buylla, 1994). Despite the technical limitations in calculating total cell numbers following enzymatic tissue digestion, we consistently detected a higher neurosphere-generating frequency within the Nilo1 and Nilo2 positive cells (40/20,000 for Nilo1/H11001 cells; 24/20,000 for Nilo2/H11001 cells), as compared to Nilo1/H1102 or Nilo2/H1102 populations (<2/20,000, detection limit of the assay). These results indicate that Nilo antibodies were able to efficiently select the neurosphere-initiator cells.

Nilo1 and Nilo2 identified different cell surface antigens, as demonstrated by (i) double staining experiments, where single stained cells were detected; (ii) the different sub-cellular localization of the antigens recognized by Nilo1 and Nilo2 as determined by confocal microscopy; (iii) the different mass of the immunoprecipitated antigens (a 40 kDa protein immunoprecipitated by Nilo1 and a 150 kDa protein immunoprecipitated by Nilo2).

Fig. 4. Nilo antibodies arrest neurosphere proliferation and block cell differentiation. A single cell suspension of SVZ-derived neurospheres (10,000 cell/cm²) was incubated with different concentrations of Nilo1 or Nilo2 antibodies (0–0.5 μg/μL) under proliferating conditions. (A) Cell proliferation and viability was measured 24, 48 and 72 h using a colorimetric MTS assay. The value of the absorbance at 490 nm correlates with the number of viable cells. (B) Bright field microscopy of cell cultures described in (A) with 0.25 μg/μL antibody (either Nilo1, Nilo2 or irrelevant) after 72 h incubation. (C) Bright field microscopy of neurospheres under differentiation conditions 4 d after treatment with 0.25 μg/μL antibody (either Nilo1, Nilo2 or irrelevant). Insets in (B, C) show Nestin staining of cells after treatment with Nilo antibodies. Magnification: 40X. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
170 kDa protein doublet immunoprecipitated by Nilo2); and (iv) the differences shown in immunohistochemistry analyses, not only with respect to different localization of *Nilo1* and *Nilo2* cells, but also on co-expression of other known markers of undifferentiated cells. Work is currently being performed to further characterize and identify the antigens recognized by *Nilo1* or *Nilo2*. These antibodies recognized functionally relevant molecules in neurospheres, since they were able not only to arrest their proliferation but also to block neurosphere differentiation in a dose dependent manner. The signaling pathways altered by *Nilo1* and *Nilo2 mAbs* still remain unknown, however, the higher efficiency of *Nilo2* on inhibiting neurosphere proliferation, together with the faster kinetics inhibiting dif-

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**Fig. 5.** Neurogenic regions labeled with *Nilo1* or *Nilo2* antibodies. (A–F) adult coronal brain sections from SVZ (A, B), olfactory bulb (C, D) or hippocampus DG (E, F) labeled with *Nilo1* (A, C, E, G–K) or *Nilo2* (B, D, F) antibodies and developed with DAB. Magnification: 20X. A schematic representation of the brain area analyzed for each region is shown (right). Black arrows indicate positive staining with *Nilo1* and *Nilo2* antibodies. (G) Low magnification confocal microscopy of a SVZ brain tissue section stained with *Nilo1* showing the whole ventricle. (H–K) higher magnification of the rectangle shown in G stained with *Nilo1* (green) alone (H) or in combination with anti-Nestin (I, K) (magenta) and anti-GFAP (red) (J, K) antibodies. Nuclei were stained with DAPI (blue) (H–J). White arrows indicate triple positive (*Nilo1*/*Nestin*/GFAP) cells.
differentiation would be consistent with a model where the effect takes place on type I neuroblasts, which represent cells more advanced in the differentiation pathway than the ones identified by Nilo1.

Fig. 6. Nilo1 labeling is associated with early progenitor markers in the adult SVZ. Coronal sections were double-labeled with Nilo1 antibody and antibodies recognizing Sox2 (A), GFAP (B), vimentin (C), β-IV tubulin (D), Ki67 (E) or EGFR (F). Merge images include nuclear DAPI labeling (blue). Arrows indicate co-expression. Insets show higher magnification images. Scale bars 25 μm (A, F) and 10 μm (B–E). A scheme of the brain area analyzed is shown. Orthogonal projections of the data are shown in the right panels.
Immunohistochemistry analyses indicated that Nilo1 and Nilo2 antibodies identified distinct progenitor populations in the adult mouse brain. Indeed, Nilo1 labeled a small cell population lining the lateral ventricles and a small fraction of cells in the anterior SVZ, displaying phenotypic features of early neural progenitors, as demonstrated by the concomitant expression of GFAP, Sox2 and vimentin (Reynolds and Weiss, 1992; Morshead et al., 1994; Gritti et al., 1996; Chiasson et al., 1999), whereas they were negative for the Ki67 proliferation antigen (Platel et al., 2008). This conclusion is further sustained by the finding that Nilo1+ cells were EGFR+, since both neural stem cells and transit amplifying cells have been described as EGFR+, whereas neuroblasts were described as either EGFRlow or EGFR− (Doetsch et al., 2002; Cesetti et al., 2009; Kim et al., 2009; Pastrana et al., 2009). Conversely, Nilo2 antibody identified a cell population co-expressing DCX, PSA-NCAM and Tuj-1 restricted to the anterior horn of the lateral ventricles, where proliferating neuroblast type I progenitors, committed to become olfactory bulb neurons have been previously described (Luskin, 1993; Lois and Alvarez-Buylla, 1994). Thus, two independent areas, which most likely represent different neural differentiation stages, could be identified within the adult mouse SVZ with Nilo1 and Nilo2 antibodies. In addition, Nilo1 and Nilo2 expression in the adult olfactory bulb was restricted to the ependymal and subependymal layers, an area reported...
to contain neural progenitors (Gritti et al., 2002; Liu and Martin, 2003). However, the DG of the hippocampus was not labeled with the Nilo antibodies.

Thus, considering the need for reliable surface markers of neural stem cells and early progenitors, the identification of these novel markers might represent a valuable contribution to enable identification and isolation of neural stem cells from adult or embryonic brain, as well as to perform tracing in vivo experiments with the labeled antibodies, to ascertain the migration patterns of these cells.

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REFERENCES


APPENDIX

Supplementary data