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

I. DEL VALLE,[a1] G. ELVIRA,[a1] L. GARCIA-BENZAQUEN,[a] A. ARMESILLA-DIAZ,[a] L. KREMER,[b] J. A. GARCIA-SANZ,[a] S. MARTINEZ[c] AND A. SILVA[a]*

[a]*Department of Cellular and Molecular Physiopathology, Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain*

[b]*Department of Immunology and Oncology, Centro Nacional de Biotecnología, CSIC, Darwin 3, 28039 Madrid, Spain*

[c]*Institute of Neurosciences CSIC-UMH, Avda. Ramon y Cajal s/n, 03550 San Juan de Alicante, Alicante, Spain*

Abstract—Two monoclonal antibodies (Nilo1 and Nilo2) were generated after immunization of hamsters with E13.5 olfactory bulb-derived mouse neurospheres. They are highly specific for neural stem 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 neurosphere cultures induced proliferation arrest and interfered with their differentiation. In the lateral ventricles of adult mice, Nilo1 stained a cell 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.

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

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 neurospheres) 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 (Cochard and Paulin, 1984; Sancho-Tello et al., 1995), glial fibrillary acidic protein (GFAP) (Doetsch et al., 1997) and Sox2 (Brazel et al., 2005), which identify highly

[1] Both authors contributed equally to this work and should be regarded as joint first authors.

*Corresponding author. Tel: +34-918373112; fax: +34-915360432.
E-mail address: asilva@cib.csic.es (A. Silva).

Abbreviations: bFGF, basic fibroblast growth factor (or fibroblast growth factor type II); DAB, 3,3′-diaminobenzidine; DCX, doublecortin; DG, dentate gyrus; DMEM/F12, Dulbecco's modified minimal essential media: F12 media (1:1, v/v); ECL, electrochemical luminescence; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FACS, fluorescence activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; Ig, immunoglobulin; mAb, monoclonal antibody; Nilo, neural identification lineage from olfactory bulb; OB, olfactory bulb; PB, phosphate buffer; PBS, phosphate buffer saline; PE, phycoerythrin; PEG, polyethylene glycol; PF, paraformaldehyde; PSA-NCAM, polysialic-acid neural cell adhesion molecule; RMS, rostral-migratory stream; RT, room temperature; SGZ, subgranular zone; SVZ, subventricular zone.

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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; Memberg 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 neurosphere cells, but also specific neurogenic areas on the adult mouse SVZ. Co-staining with various neural markers demonstrated that Nilo1 identified early progenitor cells ($Sox2^+$, Epidermal growth factor receptor or $EGFR^+$, $GFAP^+$ and $vimentin^+$), whereas Nilo2 identified more differentiated neural progenitor cells, committed to the neuroblast pathway ($Tuj\text{-}1^+$, $PSA\text{-}NCAM^+$ and 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 phycoerythrin (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), βIV-tubulin (1:400 Abcam), PSA-NCAM (1:400 Chemicon), Tuj1 (1:2000 Covance), DCX (1:100 Santa Cruz), Ki67 (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), Cy-3-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 (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA, USA) and developed with 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, USA) and H_2O_2.

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 microdissected 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% NaHCO3, 5 mM Hepes, 25 μg/ml insulin, 1 mg/ml apotransferrin, 96 mg/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% CO_2 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 A_{490nm}.

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% CO_2 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% CO_2 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