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Emx2 regulates the proliferation of stem cells of the adult mammalian central nervous system

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SUMMARY

The appropriate control of proliferation of neural precursors has fundamental implications for the development of the central nervous system and for cell homeostasis/replacement within specific brain regions throughout adulthood. The role of genetic determinants in this process is largely unknown.

We report the expression of the homeobox transcription factor *Emx2* within the periventricular region of the adult telencephalon. This neurogenetic area displays a large number of multipotent stem cells. Adult neural stem cells isolated from this region do express *Emx2* and down-regulate it significantly upon differentiation into neurons and glia. Abolishing or, increasing *Emx2* expression in adult neural stem cells greatly enhances or reduces their rate of proliferation, respectively. We determined that altering the expression of *Emx2* affects neither the cell cycle length of adult neural stem cells nor their ability to generate neurons and glia. Rather, when *Emx2* expression is abolished, the frequency of symmetric divisions that generate two stem cells increases, whereas it decreases when *Emx2* expression is enhanced.

Key words: CNS stem cell, *Emx2*, Cell proliferation, Mouse

INTRODUCTION

Soon after birth, neurogenesis is completed within most brain regions. Notable exceptions are found within germinative areas that persist in restricted CNS regions into adulthood. These include the dentate gyrus of the hippocampus and, particularly, an extensive forebrain periventricular region (PVR) (for reviews, see Peretto et al., 1999; Temple and Alvarez-Buylla, 1999; Gage, 2000; Alvarez-Buylla et al., 2001), which contains multipotent precursors that generate both neurons and glia.

In rodents, type-B subventricular progenitors from this region, which are immunoreactive for glial fibrillary acidic protein (GFAP), give rise to neuroblasts and glial cells in vivo (Doetsch et al., 1999). The latter form tube-like scaffolding through which chains of neuroblasts migrate rostrally to reach the olfactory bulb, where they give rise to olfactory bulb interneurons (granule and periglomerular GABAergic cells) (Wichterle et al., 1997). Some type B subventricular (Doetsch et al., 1999) cells and cells derived from the ventricular ependymal layer (Johansson et al., 1999) display stem cell properties, including self-maintenance and multipotentiality (the ability to generate the three major CNS lineages, i.e., neurons, astrocytes and oligodendrocytes).

Since their initial isolation (Reynolds and Weiss, 1992), several extra-cellular signals have been identified that regulate proliferation and differentiation of adult neural stem cells (ANSCs). Epidermal growth factor (EGF) (Craig et al., 1996; Gritti et al., 1995; Gritti et al., 1996; Gritti et al., 1999), type-2 fibroblast growth factor (FGF2) (Gritti et al., 1995; Gritti et al., 1996; Gritti et al., 1999) and α-transforming growth-factor (TGFα) (Weickert and Blum, 1995; Tropepe et al., 1997) can function as mitogens, both in vivo and in vitro, while ciliary neurotrophic factor (CNTF) (Johe et al., 1996), platelet derived growth factor (PDGF) (Johe et al., 1996), bone morphogenetic protein 2 (BMP2) (Gross et al., 1996) and brain derived neurotrophic factor (BDNF) (Benoit et al., 2001) have been implicated in fate selection and differentiation of NSC progeny (for a review, see McKay, 1997).

During telencephalic development, several genes have been identified that regulate specification, proliferation and differentiation of progenitors in the dentate gyrus and olfactory bulbs (Bulfone et al., 1998; Wilson and Rubenstein, 2000; Pleasure et al., 2000; Galceran et al., 2000). Notably, the analysis of *Hes1* knocked-out animals during CNS development has revealed that this gene might regulate the proliferation and commitment toward the neuronal lineage of embryonic precursors (Nakamura et al., 2000). Also, loss of

the basic helix-loop-helix gene *Mash1* (*Ascl1*) may reduce the number of embryonic neuronal progenitors in the ventral telencephalic subventricular zone (Casarosa et al., 1999; Torii et al., 1999) suggesting that it depicts the early step of differentiation of neuronal precursors. Very recently, a well-known tumor suppressor gene, *Pten* has also been shown to negatively regulate the proliferation of developing neural precursors (Groszer et al., 2001).

Conversely, very little is known of the genetic cues that regulate the function of the adult PVR stem cells and our understanding of the role of specific genes and their target cell in the mature CNS is very poor.

Emx2 – one of the two mammalian homologues of the *Drosophila* gene *empty spiracles* (*ems*) (Simeone et al., 1992; Gulisano et al., 1996) – plays a prominent role in the regulation of neurogenesis in the developing telencephalon. Phenotypic alterations in mutant animals (Pellegrini et al., 1996; Yoshida et al., 1997; Mallamaci et al., 1998) (for a review, see Cecchi and Boncinelli, 2000) suggest that the gene is required for the timely formation of the dentate gyrus, although it is unnecessary for the specification of the dentate field identity. Also, the maturation of the medial limbic cortex and the size of the olfactory bulbs are negatively affected by the absence of the gene (Pellegrini et al., 1996; Yoshida et al., 1997). Thus, *Emx2* might be relevant in controlling stem cell functional attributes such as proliferation, self-renewal and differentiation potential (Cecchi and Boncinelli, 2000).

Therefore, we investigated its expression and possible function in precursor cells from the PVR of the adult forebrain, wherein neurogenesis persists throughout life. We found that *Emx2* is expressed within the PVR and in its rostral extension to the olfactory bulb. By perturbing *Emx2* expression in cultured PVR precursors, we found that *Emx2* regulates the proliferation of multipotent ANSCs in a negative fashion, probably by diminishing their inherent capacity for self-maintenance.

MATERIAL AND METHODS

In situ hybridization

In situ hybridization (ISH) on paraffin sections was performed according to the method of Neubuser et al. (Neubuser et al., 1995), with minor modifications. 10 μm thick paraffin sections of adult mouse brain were hybridized overnight at 65°C in a solution containing the *Emx2* riboprobe (10 ng/μl). Slides were then incubated overnight at 4°C with an anti-digoxigenin-AP conjugated antibody (Roche, Basel, Switzerland) and stained with BM purple AP substrate (Roche). In situ hybridization on cells was performed as follows. Cells were plated (Gritti et al., 1996; Gritti et al., 1999) on Matrigel-coated 4-well glass chamber slides (75,000 cells/well) and fixed with 4% paraformaldehyde at the appropriate time. Following dehydration in methanol, cells were hybridized overnight at 70°C in a solution containing the *Emx2* riboprobe (1 ng/μl). The remainder of the assay was performed as described above for in situ hybridization on sections.

Cell culturing, propagation, cloning and population analysis

Adult mouse neural stem cell primary cultures were established according to the method of Lim and Alvarez-Buylla (Lim and Alvarez-Buylla, 1999). Briefly, PVR tissue was dissected and then digested with papain for 45 minutes at 37°C. For cell fractionation, the cell suspension was plated on PDK-treated dishes and cells were allowed to adhere for 4-6 hours. Afterwards, by using a pipette, basal medium (without growth factors) was streamed over the plate. The first fraction collected was mainly composed of type A cells. Following collection of the second fraction, the plate was incubated with 0.25% trypsin, 1 mM EDTA (GIBCO) at 37°C. The trypsin-treated cells (mainly type B/C cells) were plated on Matrigel-coated 4-well glass chamber slides and processed for in situ hybridization as well as cultured in the presence of mitogens.

Adult neural stem cell cultures were established as described previously (Reynolds et al., 1992; Gritti et al., 1996; Gritti et al., 1999). Neural stem cell cultures were established from the forebrain periventricular zone of wild-type and knocked-out P0 embryos. Tissues were dissociated by triturating with a fire-polished Pasteur pipette and separate cell line(s) were established from single embryos. These were genotyped by DNA-PCR. For population analyses, cells were plated at 8,000 cells/cm^2, and the resulting spheres were collected every 3-5 days. The total number of viable cells was assessed at each passage by Trypan Blue (Sigma, St. Louis, MO, USA) exclusion. For clonogenic assays, cells derived from the dissociation of clonal single neurospheres were seeded in 48-well plates and the number of secondary spheres generated was assessed after 8-10 days. In order to avoid including the colonies that may have been formed by transiently amplifying cells in these cultures, only secondary spheres that exceeded 120-150 μm in diameter were counted. To confirm that the latter were, indeed, formed by stem cells, at least 15 individual secondary spheres were randomly selected and subjected to further, long-term (2 months) propagation in each subcloning experiment. As expected, all of these spheres invariably gave rise to steadily expanding cultures that could be differentiated into neurons and both type of glia.

For the [^{3}H]thymidine incorporation assay, cells were plated onto polyornithine-coated 96-well plates (10,000 cells/well) and 1 μCi/well of [methyl-^{3}H]thymidine (74 GBq/mmol; Amersham, Uppsala, Sweden) was added to the medium. After 48 hours, cells were harvested and, following lysis with distilled water, DNA was blotted onto nitrocellulose paper filters. Filters were transferred into plastic vials containing 4 ml of scintillation medium and the incorporation of [methyl-^{3}H]thymidine was assayed in a β-scintillation counter.

Differentiation of stem cell progeny and immunocytochemistry

Multiple immunofluorescence assays were performed as described previously (Gritti et al., 1996). Cells plated onto Matrigel-coated glass coverslips were fixed (20 minutes) with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) and processed for the detection of neural antigens.

Molecular analysis

Total RNA from neural stem cell lines was extracted using the RNeasy Mini kit (Qiagen). cDNA was obtained using Superscript RNase H$^-$ Reverse Transcriptase (Gibco, Rockville, Maryland, USA). For analysis of gene expression, the following primers were used:

Emx1:
5′-AATCACTACGTGGTGGGAGC-3′
5′-CCCTTCCTCTTCCAGCTTCT-3′
Emx2:
5′-CCCAGCTTTTAAGGCTAGAG-3′
5′-CTCCGGTTCTGAAACCATAC-3′
Otx1:
5′-GTTCGCAAAGACTCGCTACC-3′
5′-CGGTTCTTGAACCAAACCTG-3′
Otx2:
5′-CAGAGAGTGGAACAAGTGGC-3′
5′-GTGGAAAGAGAAGCTGGGGACTG-3′

Mash1:
5′-CTCTTAGCCCAGAGGAAC-3′
5′-GGTGAAGGACACTTGCAC-3′
GAPDH:
5′-CGGAGTCAACGGATTTGGTCGTAT-3′
5′-AGCCTTCTCCATGGTGGTGAAGAC-3′
RT-PCR amplification consisted in 35 cycles with primer annealing at 60°C.

Retroviral infection

A replication-deficient retroviral vector (pLNCX2; Clontech, Palo Alto, CA, USA), was engineered to introduce the mouse *Emx2* full-length sequence containing a C-terminal epitope tag from the human influenza hemagglutinin protein (HA), as well as the *Emx2* antisense sequence into the genome of undifferentiated multipotent neural stem cells. Following transfection, the supernatant of confluent GP+env E86 ecotropic packaging cell line was harvested after 24 hours at 37°C, 0.45-μm filtered, supplemented with 8 μg/ml polybrene and employed for stem cell transduction. Neurospheres were collected by mild centrifugation, dissociated to single cells and seeded into the conditioned medium at a plating density of 40,000 cells/cm^2. After 24 hours, virus-containing supernatant was replaced with fresh medium. Three days following infection, cells were exposed to 250 μg/ml G418 (Sigma) and analyzed for nuclear expression of HA by means of a monoclonal anti-HA antibody (1:1,000; BabCo, Richmond, CA, USA).

Generation and molecular characterization of *Emx2* overexpressing transgenic mice and ANSCs

A cassette containing the chicken β-actin promoter (Balling et al., 1989), the *Emx2* coding sequence and the SV40 poly(A) was generated, purified and injected in pronuclei of F_1 C57Bl6/CBA × C57Bl6/CBA mouse zygotes, according to standard techniques. Six transgenic lines were established. Two of these lines, tested for transgene expression in embryonic cortex by RNAse protection assay, were further propagated on C57Bl6 background and employed in this analysis. RNA was extracted from freshly dissected telencephalon of E19 mouse embryos, according to standard procedures (Sambrook et al., 1989). The RNAse protection assay was performed as described by Simeone et al. (Simeone et al., 1990), with minor modifications; 30 μg of RNA per each sample were used. For RT-PCR/Southern blotting assay, total RNA was extracted from freshly dissected PVR and from ANSCs of adult age-matched wild-type and transgenic mice by means of the RNeasy Mini kit (Qiagen). One hundred ng of each sample were reverse transcribed in a final volume of 20 μl, in the presence of 2 pM βact/RT3- and RT-specific oligos, by 200 U of Superscript II enzyme (Gibco), according to manufacturer's instructions. One μl of RT product was used as substrate in each PCR.

PCRs were performed in a final volume of 50 μl, in the presence of each primer (1 μM), of 1.5 mM $MgCl_2$ and of 2.5U of Taq polymerase enzyme (Promega, USA), according to manufacturer's instructions. Oligos βact/RT1 and βact/RT2 were used to amplify the endogenous β-*act* gene cDNA; oligos PF and PR were used to amplify the transgene cDNA. PCRs were run by hot start according to the following protocol: 98°C for 5 minutes, 98°C for 1 minute, 65°C for 1 minute, 72°C for 1 minute (5 cycles); 95°C for 1 minute, 65°C for 1 minute, 72°C for 1 minute (30 cycles); 72°C for 10 minutes. Half of each amplification product was run on TBE1X – 1.6% agarose, and transferred onto Hybond-N$^+$ nylon membranes. Membranes were hybridized with 10^6 dpm/ml of the two α-^{32}P-labelled DNA probes, βact (corresponding to the amplified β-actin fragment) and SP (corresponding to the first 200 bp of the *Emx2* 3′UTR). Membranes were then washed and further processed for autoradiography, according to standard protocols (Sambrook et al., 1989).

Primers:
βact/RT3: 5′-TTGCAGTACATAATTTACACAG-3′
RT: 5′-CAATAAACAAGTTAACAACAACAATTGC-3′
βact/RT1: 5′-AGTATCCATGAAATAAGTGGTTACAGG-3′
βact/RT2: 5′-GGGGTGGACTCAGGGCATGGAC-3′
PF: 5′-TTCCAAGGGAACGACACAAGTCC-3′
PR: 5′-CAACAATTGCATTCATTTTATGTTTCAGGTTCAG-3′

Determination of cell-cycle length by BrdU incorporation

Wild-type as well as mutant-derived stem cells or infected ANSCs were plated at a cell density of 30,000 cells/cm^2 on 10 mm Matrigel-coated glass coverslips in NS-A medium containing 1 μM BrdU (Roche) for up to 96 hours. Cells were fixed in 4% paraformaldehyde and stained with a monoclonal anti-BrdU antibody (ready to use; Amersham). Nuclei were labeled with DAPI. One thousand cells (identified by DAPI staining) were counted per coverslip. The relative labeling index (LI) was plotted against the BrdU labeling time to obtain an S-phase cumulative labeling curve (Tang et al., 2000). LI(t) and the correlation index *r* were determined in the linearity interval by linear regression methods. Using the formula y=ax+b, we determined Tc=1/LI(t).

Determination of cell viability by MTT assay

Cells were plated onto Matrigel-coated 96-well plates (10,000 cells/well). At every time point, one hour before collection, the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (5 mg/ml in PBS; Sigma) was added to the medium (final dilution: 500 μg/ml). The pale yellow redox indicator MTT is reduced to a dark blue end product, MTT-formazan, by the mitochondrial dehydrogenases of living cells. Following 1 hour incubation at 37°C, the medium was discarded and cells were lysed by adding 50 μl of DMSO. After 15 minutes at room temperature, MTT reduction was measured spectrophotometrically at a wavelength of 550 nm.

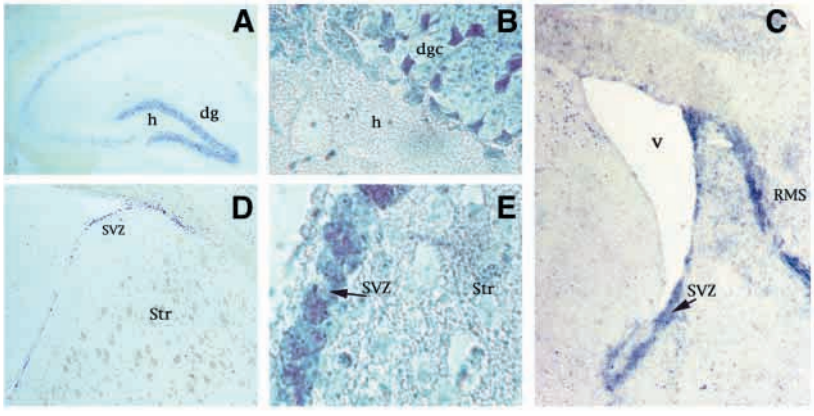

Fig. 1. *Emx2* is expressed in progenitors of both the adult dentate gyrus of the hippocampus and the forebrain subventricular zone (SVZ). (A,B) *Emx2* mRNA is present in (A) the adult hippocampus and (B) the dentate gyrus. (C) Strong expression of the gene could be observed along the rostral migratory stream (RMS) that extends toward the two olfactory bulbs and (D) in the periventricular region that lines the lateral ventricles. (E) Cells expressing *Emx2* messenger appear to be localized in the subventricular layer (arrow). V, ventricle; Str, striatum; SVZ, subventricular zone; dg, dentate gyrus; h, hilus; dgc, dentate granule cells.

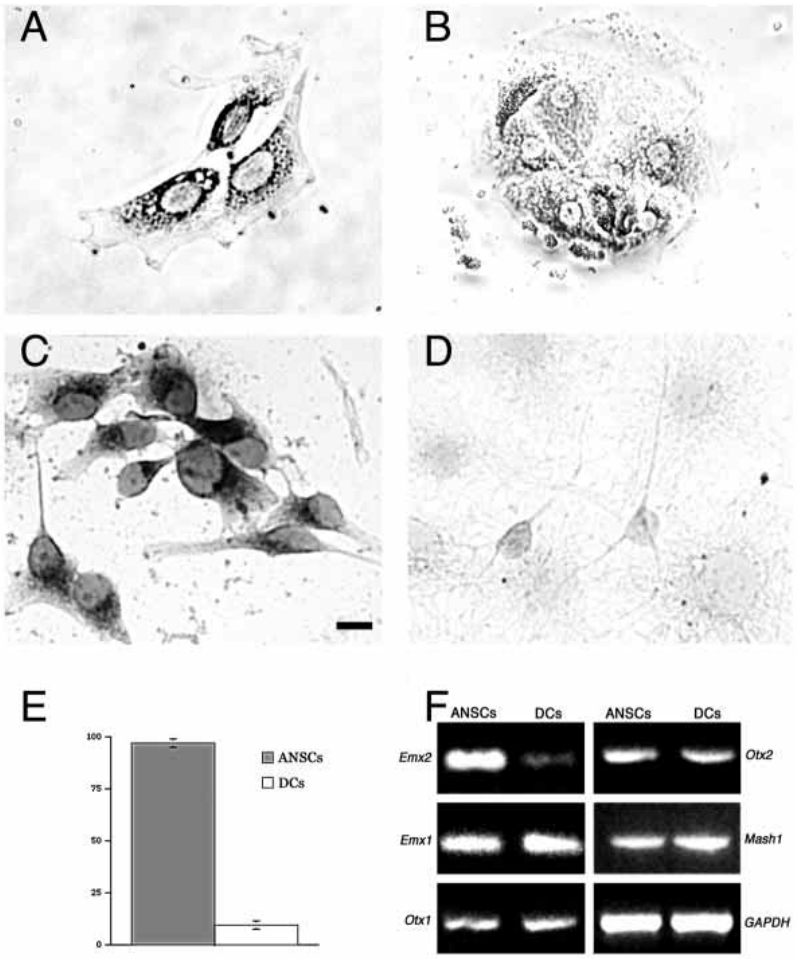

Fig. 2. *Emx2* is expressed in acutely isolated stem cells and in ANSCs and is down regulated during differentiation. (A,B) Primary cultures from the adult PVR were obtained by differential adhesion on PDK-treated plastic (Lim and Alvarez-Buylla, 1999) in order to establish two distinct cell fractions, the type B/C cell fraction and the PSA-NCAM-positive fraction, called type A. By means of in situ hybridization, *Emx2* was shown to be expressed in (A) type B and (B) type A cells. (C) High levels of *Emx2* expression were also detected in undifferentiated adult CNS stem/progenitors. (D) A faint signal was observed in the terminally differentiated progeny of ANSCs, restricted to cells morphologically identifiable as immature neurons. Bar in C, 15 μm. (E) A high frequency of cells expressing *Emx2* messenger can be observed when cells are highly undifferentiated. The onset of differentiation and maturation is coincident with a decrease in the number of *Emx2*-positive cells that drops to 8% of the total cell number by 7 days following induction of differentiation. (F) Semi-quantitative RT-PCR shows high levels of *Emx2* messenger in undifferentiated multipotent neural stem cells (ANSCs), while in stem cell-derived terminally differentiated progeny (DCs) lower expression could be detected. No such change was observed in the level of the *Emx2* paralog *Emx1*, or of *Otx1*, *Otx2* and *Mash1*. Quantitative comparison was made with the housekeeper gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

RESULTS

Restricted expression of *Emx2* in progenitors of the adult mouse forebrain

Expression of *Emx2* mRNA and protein has been reported within primary germinative areas of the embryonic cerebral cortex, as well as in primary and secondary germinative regions of the hippocampus (Simeone et al., 1992; Gulisano et al., 1996; Mallamaci et al., 1998) during embryonic and early post-natal life. Since neurogenesis persists through adulthood in specific, tertiary germinative areas (Peretto et al., 1999; Temple and Alvarez-Buylla, 1999; Gage, 2000; Alvarez-Buylla et al., 2001), we investigated the distribution of *Emx2* on coronal sections of the adult forebrain by in situ hybridization (Fig. 1). High levels of *Emx2* expression were detected in the adult hippocampus (Fig. 1A), particularly in the dentate gyrus (Fig. 1B), where granule cells continue to be generated postnatally (Kaplan and Bell, 1984).

Of note, expression of the gene was observed in the region of the SVZa, defined by Luskin (Luskin, 1993) and in its extension, the rostral migratory stream (RMS) (Fig. 1C). *Emx2* transcript was also detected in the periventricular region of the dorsal striatum, and in the striato-cortical angle (Fig. 1D). Higher magnification showed that *Emx2* expression seems to be restricted to cells located in the subventricular zone (Fig. 1E).

Regulation of *Emx2* gene expression in adult neural stem cells (ANSCs)

The pattern of *Emx2* as detected by in situ hybridization strongly suggests its expression in SVZ precursor cells and implicates it in the regulation of neurogenesis within tertiary germinative regions. We focused our efforts on determining if one of the cell types expressing *Emx2* could be identified as a multipotent adult neural stem cell. To begin addressing this issue, primary cultures from the adult PVR were fractionated by differential adhesion on PDK-treated plastic (Lim and Alvarez-Buylla, 1999) in order to establish two distinct populations: (1) the type B/C cell fraction (Fig. 2A) and (2) the PSA-NCAM-positive fraction (Fig. 2B), which was consistently composed of Tuj1-positive cells. While the latter is known to be composed mainly of type A cells – that is neuronal precursors that do not form colonies in vitro – the former fraction is known to contain the clonogenic cells, likely the type B cells, that give rise to neural stem cell in culture. We confirmed these findings showing that, under our conditions, no clonogenic stem cells that give rise to the so called neurospheres could be detected in the type A fraction, whereas neurosphere-forming activity could be found in the type B/C fraction. These neurospheres were confirmed to derive from stem cells contained in the type B/C fraction by the same clonogenic and sub-cloning assays and population analysis (data not shown) used throughout this study (see below).

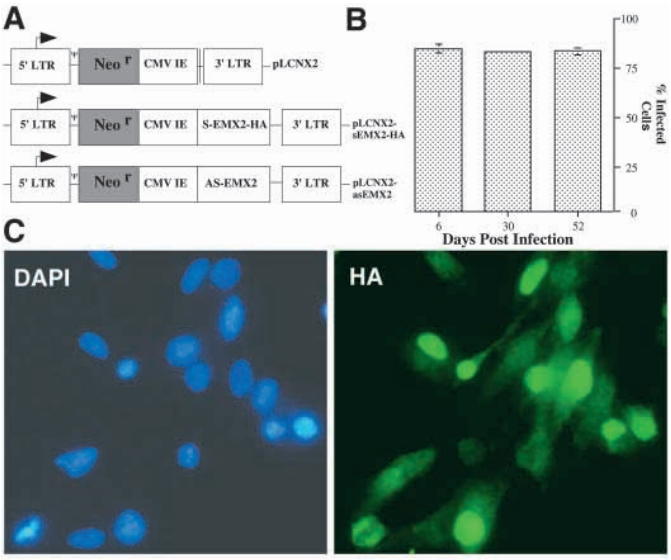

Fig. 3. Generation and characterization of retroviral vectors. (A) The structure of the retroviral constructs generated for ANSC infection. (B) *Emx2* overespression in ANSCs is stable for up to 3 months in culture as shown by quantitative analysis of HA immunoreactivity. (C) As shown by immunofluorescence, following exposure to G418, 70-80% of the cells displayed nuclear HA immunoreactivity (right panel). (Left panel) DAPI staining of nuclei to determine total cell numbers. Bar: 10 μm.

When we carried out in situ hybridization, all the cells in the type B/C fraction (including the stem cells) invariably expressed significant levels of *Emx2* (Fig. 2A). Also type A cells (Fig. 2B) were shown to express *Emx2*.

To confirm that *Emx2* was indeed expressed in cells endowed with stem cell potential, we established cultures from the periventricular region under conditions in which only a minor fraction of the total cells proliferate, and all the others die (Reynolds and Weiss, 1992; Gritti et al., 1996; Gritti et al., 1999). All of the former did express *Emx2* and were shown to possess features of ANSC such as self-maintenance and multipotentiality, as expected on the basis of previous findings (Gritti et al., 1996, Gritti et al., 1999) (data not shown). From these cells, we further established two types of cultures. (1) A cell population in which virtually all the cells express the neuroepithelial marker nestin, incorporate S-phase markers such as BrdU and [^{3}H]thymidine, are immunoreactive for proliferating cellular nuclear antigen (PCNA) and in a serial sub-cloning assay are clonogenic, self-renewing and multipotent (Gritti et al., 1996; Gritti et al., 1999; Morrison et al., 1999) (ANSCs). (2) The differentiated progeny of ANSCs, consisting of mature cells expressing either neuronal or glial markers (differentiated cells, DCs) (Gritti et al., 1996; Gritti et al., 1999).

RNA in situ hybridization on the two cell preparations confirmed the specific expression of *Emx2* in all ANSCs (Fig. 2C), whereas only a faint signal could be observed in few DCs (Fig. 2D). There, gene expression was restricted to a subset of mainly bipolar, ovoidal neuron-like cells. Quantification of this phenomenon showed that the number of *Emx2*-expressing cells diminished tenfold 7 days after differentiation of ANSCs was induced (Fig. 2E). Thus, *Emx2* mRNA is present in undifferentiated ANSCs of the adult SVZ and its expression is heavily down-regulated upon their differentiation into neurons and glia.

When the same two cell preparations were subjected to semi-quantitative RT-PCR analysis (Fig. 2F), *Emx2* transcript was detected at high levels in ANSCs but its expression was almost abolished in DCs. It is worthwhile noting that expression of the *Emx2* paralog of *ems*, *Emx1*, was equivalent in the two different cell populations. The same observation applied to other transcription factors such as *Otx1*, *Otx2* and *Mash1*.

Perturbation of *Emx2* expression alters the proliferation of ANSCs

We then tried to identify the potential role of *Emx2* in regulating the differentiation and proliferation capacity of ANSCs. We perturbed the expression of *Emx2* in ANSCs by retrovirus-mediated infection. A retroviral construct, containing the mouse full-length sequence of *Emx2* under the transcriptional control of the CMV IE promoter and the resistance gene *neo* driven by the 5′ viral LTR was used to

Table 1. The differentiation potential of ANSCs does not change following modifications in *Emx2* expression

Cell type	MAP2-IR neurons (%)	GFAP-IR astrocytes (%)	GalC-IR oligodendrocytes (%)
Wild-type ANSCs passage 4	8.9±2.0	54.0±1. 2	3.0±0.05
Emx2 gain-of-function ANSCs passage 4	10.1±0.9	51.0±2. 5	2.3±0.9
Wild-type ANSCs passage 12	12.5±1.9	49.7±1.1	2.9±0.1
Emx2 gain-of-function ANSCs passage 12	10.0±2.1	50.8±0.6	1.9±1.0
Mock-infected ANSCs passage 4	9.1±2.8	48.7±0.9	2.6±0.01
Emx2-infected ANSCs Passage 4	10.5±1.2	50.1±1.7	3.1±0.04
Mock-infected ANSCs passage 12	10.1±0.8	45.7±2.7	3.0±0.1
Emx2-infected ANSCs passage 12	12.5±2.4	48.1±2.1	2.4±0. 9
Wild-type ANSCs passage 4	12.0±0.4	56.0±2.3	3.0±0.02
Emx2 ko ANSCs passage 4	13.0±1.1	58.0±3.1	2.5±0.1
Wild-type ANSCs passage 12	12.2±0.1	55.9±2.0	2.8±0.9
Emx2 ko ANSCs passage 12	10.4±1.8	52.7±0.9	2.1±0.7

Upon removal of mitogens, NSCs from *Emx2* gain- and loss-of-function mice as well as ANSCs infected with *Emx2* gave rise to neurons (MAP2-positive), astrocytes (GFAP-positive) and oligodendrocytes (GalC-positive). Their ability to differentiate was neither affected by changes in *Emx2* expression, nor by long term culturing. Values are expressed as percentages of immunoreactive (–IR) cells over total cell number, as determined by DAPI staining. Data are the means±s.e.m. of 3 experiments; 3 coverslips/condition/experiment were analyzed. Between 1500 and 2000 cells were counted on each coverslip.

Table 2. Analysis of proliferation and evaluation of *Emx2* effect on the mode of stem cell division in gain- and loss-of-function NSCs

	Wild-type ANSCs	Emx2 gain-of-function ANSCs	Mock-infected ANSCs	Emx2-infected ANSCs	Wild-type ANSCs	Emx2 ko ANSCs
[^{3}H]thymidine incorporation (counts per minute)	3467±213	2047±245	2958±792	1580±159	3825±302	7192±278
*P**	<0.005		<0.005		<0.005	
Cloning efficiency (% of total cell number)	6.1±0.43	3.4±0.66	8.0±2.00	5.6±1.50	5.9±1.43	21.5±2.23
*P**	<0.01		<0.005		<0.001	

Values are mean±s.e.m.
**P*, Student's *t*-test.
n=8 for all samples.

infect ANSCs (Fig. 3A). In order to detect EMX2 protein, the sense sequence of *Emx2* was fused with the hemagglutinin (HA) epitope tag, which allowed subsequent identification by means of a specific anti-HA antibody. As controls, the empty vector and the same vector containing the antisense sequence of *Emx2* were used for infection (Fig. 3A).

Three to four days following incubation in the medium conditioned by the virus-producing ecotropic packaging cells, ANSCs were exposed to a sub-lethal concentration of neomycin (G418; 250 μg/ml). It should be noted that the steady expression of the *Emx2* construct was assessed and confirmed in both short- and long-term cultures (cell lines) at every sub-culturing step throughout the whole study. This was demonstrated by the fact that mock- and *Emx2*-infected cells were able to expand in culture in a constant fashion for over 2 months when grown in the continuous presence of sub-lethal concentration of neomycin. While this observation clearly shows that infected cells continuously expressed neomycin resistance, the finding that, at each sub-culturing stage, over 95% of the total cells (Fig. 3B) in the same cultures displayed nuclear immunoreactivity for the HA epitope tag (Fig. 3C, right panel), as compared to DAPI staining (Fig. 3C, left panel) confirmed the long-term sustained expression of *Emx2* and its protein in ANSCs cultures.

Neither construct elicited any appreciable effect on the known capacity of ANSCs to spontaneously differentiate into neurons and glia upon growth factor removal (Gritti et al., 1996; Gritti et al., 1999) (Table 1). Conversely, a significant negative effect on ANSC proliferation rate was observed in cells infected with the sense construct, as measured by a 50% decrease in the ability of ANSCs to incorporate [^{3}H]thymidine (Table 2). Although an increase in [^{3}H]thymidine incorporation was observed using the vector encoding the *Emx2* antisense sequence, this did not reach a statistically significant level (not shown).

In order to confirm that *Emx2* influences the proliferation of ANSCs, we began by establishing six *Emx2* gain-of-function mouse lines in which the *Emx2* coding sequence and the SV40 poly(A) were expressed under the transcriptional control of the promoter of the ubiquitous gene β-actin (Fig. 4A) (Balling et

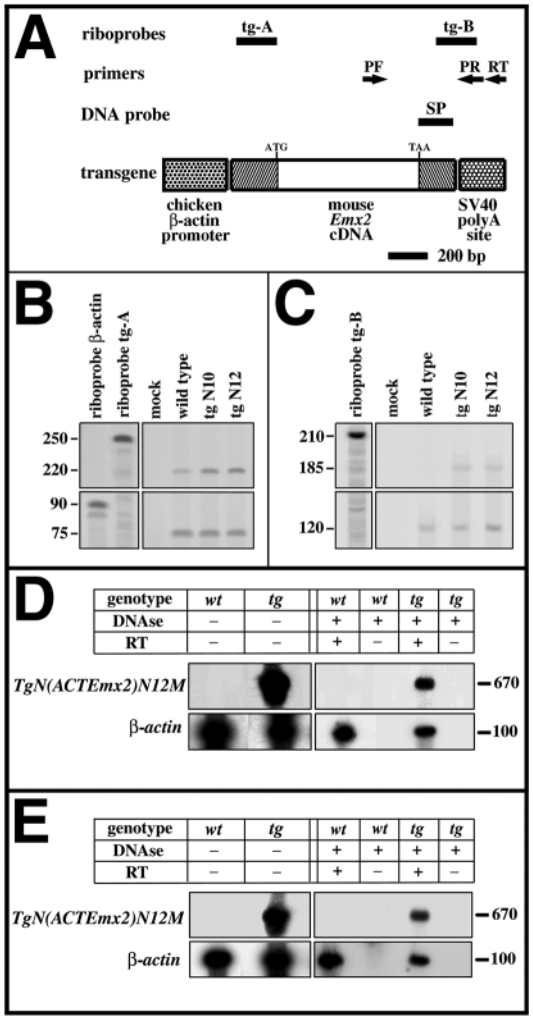

Fig. 4. Generation and characterization of transgenic mice and ANSCs. (A) The structure of the transgene driving ectopic-heterochronic expression of *Emx2* and the location of both oligos and probes employed for the analysis of transgene expression. These include the two riboprobes, tg-A and tg-B, used for the RNAse protection assay; the three primers, RT, PF and PR, used for the RT-PCR assay; the *Emx2* DNA probe, SP, hybridized to RT-PCR products to confirm their specificity. (B,C) Expression of *Emx2* in embryonic telencephalon of wild-type and transgenic mice, as measured by the RNAse protection assay. (D) Expression of the *Emx2* transgene in the forebrain periventricular tissue dissected from adult wild-type and mutant mice belonging to one of the two lines used, as assayed by RT-PCR-Southern blotting assay. Similar results were obtained for the other transgenic line (not shown). (E) Expression of the *Emx2* transgene in ANSCs isolated and propagated from the same tissues described in D is shown.

al., 1989). Initially, transgene expression in the embryonic telencephalon of two of these lines was confirmed by RNAse protection assay (Fig. 4B,C) prior to their propagation on a C57Bl6 background. Furthermore, by means of RT-PCR using a oligonucleotide primer spanning the SV40 region in the transgene cassette, followed by Southern blot analysis, we were able to show that the transgene was expressed not only in the periventricular tissue dissected from the adult brain (Fig. 4D), but also in ANSCs isolated from this region and propagated (Fig. 4E). Cells from these two transgenic mouse lines were used in this study and, in agreement with the retroviral infection experiments, comparable percentages of neurons, astrocytes and oligodendrocytes were found to be generated by stem cells isolated from both wild-type or gain-of-function mice (Table 1). Yet, the same cells displayed an approximate 30% decrease in their rate of [^{3}H]thymidine incorporation, suggesting a significant reduction in their proliferation ability (Table 2).

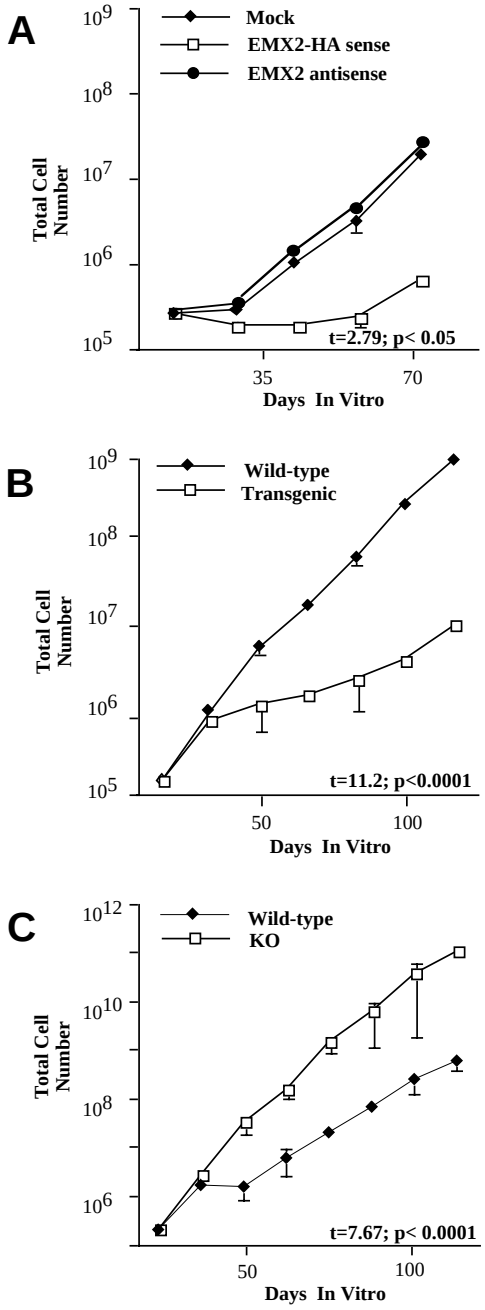

Fig. 5. Modulation of *Emx2* expression influences growth capacity of neural stem cells. Growth profiles of (A) *Emx2*-infected ANSCs, (B) *Emx2*-overexpressing transgenic ANSCs and (C) *Emx2* KO-derived stem cell lines as compared to their wild-type controls. Enforced gene expression always gives rise to severe growth impairment, whereas absence of the gene enhances neural stem cell growth. Logarithmic scale on y-axis. Statistical analysis was performed by means of the Bonferroni test. Means ± s.e.m., n=6 for infected ANSCs, n=4 for gain-of-function ANSCs and n=12 for loss-of-function ANSCs.

These observations found support in similar experiments performed using ANSCs isolated from *Emx2* null mutant mice. *Emx2* knocked-out (KO) mice die soon after birth (Pellegrini et al., 1996; Yoshida et al., 1997), therefore ANSCs were isolated from these animals at post-natal day 0. At P0 there is a clearly defined subventricular zone, and neuronal neogenesis and migration to the olfactory bulb are established at this age. These progenitors may therefore be considered comparable to those isolated from the adult periventricular region (Law et al., 1999). Once again, no difference in the capacity to give rise to neurons and glia could be observed between wild-type and KO cells (Table 1). Yet, as opposed to the *Emx2* gain-of-function counterpart, these precursors incorporated twice the amount of [^{3}H]thymidine as wild-type cells (Table 2). Altogether, these findings indicate that *Emx2* negatively regulates the proliferation of precursor cells of the post-natal/adult rodent forebrain.

Given the above results, we investigated whether ANSCs may represent the more prominent, though not necessarily exclusive, target cell type of *Emx2*. To this end, we analyzed the effect of *Emx2* on long-term cultures of ANSCs. By virtue of the culture system adopted, the vast majority of the adult brain cells died soon after plating while only ANSCs survived (Gritti et al., 2001). As expected for stem cells cultured under appropriate growth conditions, ANSCs adopt a proliferation mode by which their overall number is not only maintained but also expands over serial subculturing (Potten and Loeffler, 1990; Loeffler and Potten, 1996; Loeffler et al., 1997). We previously showed that this process, called self-renewal, entails the continuous generation and amplification of cells identical to the original clonogenic stem cell (Gritti et al., 1996; Gritti et al., 1999). Thus, stable cell lines are generated that bear steady functional features identical to those of the founder cell and in which ANSCs are responsible for the expansion of the overall cell number (Reynolds and Weiss, 1996; Gritti et al., 1999). In such a system, genetic alterations that impinge on the growth capacity of the founder stem cell will also be reflected in a modification of the overall growth rate of the derived lines.

Thus, we analyzed the growth rate of wild-type lines as compared to cells over-expressing *Emx2*, either by means of retroviral infection or by isolation and expansion from gain-of-function models, as well as to cells isolated from loss-of-function mice (Fig. 5). We followed the growth rate of all cell lines for 3-4 months, looking for stable alterations in their growth profiles.

In agreement with [^{3}H]thymidine incorporation studies, we found that either cells infected with *Emx2* (Fig. 5A) or isolated

Table 3. Analysis of cell viability by MTT assay

Hours after plating	Wild-type ANSCs	Emx2 gain-of-function ANSCs	Mock-infected ANSCs	Emx2-infected ANSCs	Wild-type ANSCs	Emx2 ko ANSCs
0	0.085±0.009	0.075±0.009	0.109±0.005	0.094±0.010	0.065±0.005	0.073±0.004
8	0.089±0.005	0.080±0.005	0.106±0.004	0.097±0.008	0.088±0.005	0.087±0.004
24	0.092±0.010	0.086±0.004	0.094±0.010	0.080±0.010	0.162±0.005	0.167±0.005
30	0.114±0.003	0.102±0.010	0.102±0.009	0.113±0.007	0.152±0.006	0.160±0.009

Cells viability was assayed in experiments aiming at determining the overall frequency of symmetric proliferative cycles in ANSCs in which *Emx2* expression was perturbed. Cell viability was measured by reduction of the tetrazolium dye MTT at 0, 8, 24 and 30 hours after plating. Statistically significant differences could not be detected at any of the time points considered (Student's *t*-test).
n=6 for all samples.

from *Emx2* gain-of-function mice (Fig. 5B) exhibited a steady, significantly lower rate of amplification compared to wild-type cells. The opposite effect was observed in cells derived from *Emx2* null mutants (Fig. 5C). As expected, when either type of *Emx2* mutant ANSCs was induced to differentiate, no alterations in their ability to give rise to neurons, astrocytes and oligodendrocytes could be observed, irrespective of the stage of serial subculturing (up to four months) (Table 1). Taken together, these findings point to a role for *Emx2* as a genetic, negative regulator of ANSC proliferation that affects their long-term ability to expand ex-vivo.

Emx2 affects proliferation of ANSCs by regulating their overall symmetry of division

Two mechanisms, acting alone or combined, may account for the alteration in the long-term growth rate observed in *Emx2* mutant cells, provided that no significant alteration in the rate of cell death is involved: (i) modifications of the overall speed of progression through the cell cycle; (ii) alteration of the relative frequency of symmetric divisions yielding two stem cells (symmetric proliferative cycles) with respect to symmetric cycles yielding differentiated cells, and to asymmetric divisions generating one stem cell and a more differentiated progeny (Morrison et al., 1999; Potten and Loeffler, 1990; Loeffler and Potten, 1996; Loeffler et al., 1997). We define this parameter as "symmetry of division" within the ANSC population.

To verify if cell cycle alterations might be responsible for the modification in the rate of growth in mutant cell lines, we performed cumulative BrdU labeling experiments (Tang et al., 2000) and estimated the cell cycle duration (Tc) of both control and mutant cell cultures. The cells were pulsed with BrdU for up to 120 hours, when all of the cells had incorporated the S-phase marker. Calculated Tcs (see Materials and Methods) show that neural stem cells isolated from null mutant mice display a Tc of 62.5±7.9 hours whereas control cells had a Tc of 64.7±7.7 hours (mean±s.e.m., *n*=3). Accordingly, cells isolated from gain-of-function animals displayed a Tc of 87±3.4 hours as compared to their wild-type counterpart (85±5.5 hours, mean±s.e.m., *n*=3). Similarly, no difference in the cell cycle time could be detected between *Emx2*-infected and mock ANSCs (92±7.5 hours for *Emx2* over-expressing cells versus 96±3.1 hours for control cells, mean±s.e.m., *n*=3).

In none of these experiments was any difference in cell viability detected, according to MTT viability assay (Table 3).

In order to analyze the influence of *Emx2* on the symmetry of division in ANSCs, we performed serial sub-cloning experiments (see Fig. 6 for explanation). In these assays, the number of secondary ANSCs in a given ANSC-derived clone was expressed as a percentage of the total cell number in the clone (Loeffler and Potten, 1996; Gritti et al., 1999). This analysis returned an estimate of the relative frequencies of symmetric proliferative (two ANSCs generated at each cycle) and symmetric differentiative division (two differentiated and/or dead cells generated after cell division). Modification of the frequency of the asymmetric stem cell cycles generating one stem and one more differentiated cell cannot affect the outcome of this assay (see also the discussion) (Loeffler and Potten, 1996), and thus this type of mitotic division is not taken into account here.

In this analysis, we compared wild-type ANSCs with cells isolated from genetically modified mice as well as to cells transduced with retroviral vectors carrying the mouse *Emx2* sense sequence. As shown in Table 2, infection by *Emx2* sense encoding retrovirus resulted in a significant decrease in the frequency of proliferative symmetric divisions with respect to mock-infected cells. Accordingly, a similar phenomenon was observed in ANSCs from gain-of-function mutants, in which primary pβ-actin-*Emx2* clonogenic founders gave rise to secondary clonogenic cells with a much lower frequency than observed in wild-type cells (Table 2).

Finally, when the same assay was carried out comparing wild-type and *Emx2*$^{-/-}$ stem cells, a very significant increase in the frequency of symmetric proliferative divisions was detected at all time points tested (Table 2).

In conclusion, *Emx2* affects the proliferation of ANSCs by regulating the frequency of symmetric divisions that generate two stem cells within the ANSC population. Over-expression of this gene decreases this frequency, whereas deletion of the gene elicits the opposite effect.

DISCUSSION

Given the relatively recent identification of residual neurogenic regions in the mature brain, the role and identity of the genetic determinants involved in post-natal/adult neural stem cell regulation is largely undocumented. We report that the transcription factor *Emx2* is expressed in neural progenitors found in neurogenetic regions of the adult brain, such as the dentate gyrus of the hippocampus and, particularly, the forebrain periventricular area. When cells from PVR are placed in culture (Gritti et al., 2002), expression of *Emx2* is retrieved in various neural precursors, including those that are known to be the source of multipotent cells that display critical stem cell features upon exposure to growth factors (Potten and Loeffler, 1990; Loeffler and Potten, 1996; Loeffler et al., 1997). Such expression is almost abolished when these stem cells give rise

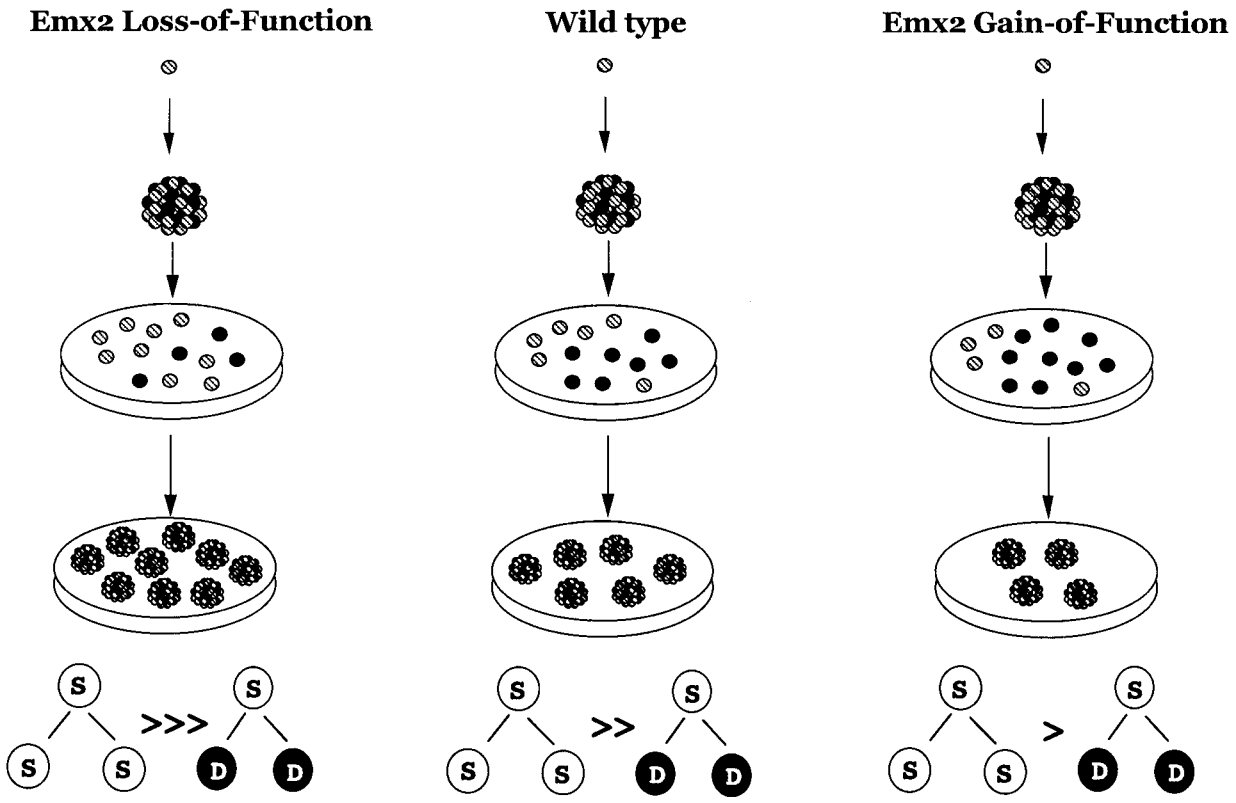

Fig. 6. The schematic illustrates the assay by which the frequency of symmetric proliferative cycles that give rise to two stem cells was evaluated in a given ANSCs population. *Emx2* loss-of-function ANSCs were established from animals in which *Emx2* expression was knocked out; conversely, *Emx2* gain-of-function ANSCs were obtained by isolation and expansion from mice constitutively overexpressing *Emx2*, as well as by infection with a retrovirus carrying the *Emx2* sense sequence. Both types of ANSCs were compared with their controls. Single clones were subjected to a clonogenic assay in which the number of secondary clonogenic cells was expressed as percentage of the total cell number in the clone. In the absence of differential cell death – which was not observed in any of these cultures (Table 3) – this value indicates the ratio between symmetric proliferative cycles (generating two stem cells) and symmetric differentiative cycles (generating two more differentiated cells) that contributes to the formation of the clone. If the frequency of the proliferative symmetric divisions exceeds that of the differentiative ones, the number of stem cells in the clone is greater than one and the stem cell population increases. As expected in this culture system, this was observed under all the conditions tested. However, in the *Emx2* KO-derived cells, the relative frequency of symmetric proliferative divisions was far greater than in wild-type animals, as shown by the higher frequency of secondary clones generated, whereas this frequency decreased in gain-of-function cells. Asymmetric cycles that may also have occurred during clone formation generated a stem and a more differentiated cell at each division. Thus, alterations in their frequency did not influence the outcome of this assay.

to neurons and glia. Experimental down- or, alternatively, up-regulation of *Emx2* expression in ANSCs determines an increase or, decrease, respectively, in their proliferation rate while their differentiation capacity remains unchanged. We show that *Emx2* elicits this effect by acting as a negative regulator of proliferation of stem cells. At the level of overall cell population, the gene is likely to regulate the balance between symmetric cell divisions yielding two stem cells, and symmetric cycles, generating two more differentiated progeny.

During telencephalic development, *Emx2* is expressed in the germinative neuroepithelium and in the Cajal-Retzius cells of the marginal zone (Gulisano et al., 1996; Pellegrini et al., 1996; Yoshida et al., 1997). Its localization (Simeone et al., 1992; Gulisano et al., 1996; Mallamaci et al., 1998), its graded posteroanterior distribution throughout the maturing cortex (Cecchi and Boncinelli, 2000), the phenotype of *Emx2* null mutants (Pellegrini et al., 1996; Yoshida et al., 1997) and the notion that *Emx2* is required for the timely formation of the dentate gyrus and for the maturation of medial limbic cortex progenitors (Tole et al., 2000) point to a critical role for this gene in neural precursors. They also indicate its pleiotropic function in neurogenesis and likely activation in distinct neural precursor cell types. Our finding shows that *Emx2* is expressed in neural precursors of the PVR (Fig. 2A,B), and that a subset of these cells retains significant *Emx2* expression upon removal from their brain environment (Fig. 2C,D). Such expression, which appears to be regulated at the transcriptional level, continues throughout extensive proliferation, serial subculturing and exponential cell expansion for over six months, during which cells display stem cell characteristics such as multipotentiality and steady self-maintenance capacity. Therefore, one of the cell types from the PVR that expresses *Emx2* may be considered as a kind of neural precursors that displays bona fide stem cell characteristics in vitro. Nevertheless, based on its expression in the majority of the cells in the PVR and in the RMS in vivo, in primary type A neuroblast cells from the adult SVZ and in a small fraction of the differentiated progeny of ANSCs, it should not be ruled out

that neural progenitors other than ANSCs might also express *Emx2*.

Little is known about the function(s) of *Emx2* in specific neural precursors types. The abnormalities initially detected in *Emx2* null mutants – the missing dentate gyrus and the greatly reduced size of medial limbic cortex and olfactory bulbs – have suggested a possible loss of proliferation capacity of the neural precursors responsible for the formation of these regions (Pellegrini et al., 1996; Yoshida et al., 1997). Yet, the more recent observation of a significant enlargement of the proliferative ventricular and subventricular zones in null mutant cortex (Tole et al., 2000; Mallamaci et al., 2000) has raised the hypothesis of a delay in maturation and a consequent increased proliferation of neural precursors in *Emx2* knocked-out. A number of our findings support this view and show that one of the post-natal forebrain cell types in which *Emx2* may operates as a negative regulator of proliferation displays neural stem cell potential, in vitro.

Over-expression of *Emx2* in cultured post-natal ANSCs, either by viral infection or by culturing of ANSCs from gain-of-function mice, reproducibly resulted in loss of proliferation capacity as measured by [^{3}H]thymidine incorporation. Conversely, analysis of ANSCs from *Emx2* null mutant mice showed a two-fold increase in the same parameter.

However, although the culture conditions adopted in this study are known to positively select for ANSCs, it is also clear that precursors dividing only transiently (the so called transit proliferating/amplifying cells) (Potten and Loeffler, 1990) are also generated in this system as the time from the last subculturing step increases (Vescovi et al., 1993; Reynolds and Weiss, 1996; Gritti et al., 1996; Gritti et al., 1999). Since the [^{3}H]thymidine incorporation cannot discriminate between the NSC and the transit progenitor pools, it is difficult to allocate the effect of *Emx2* on cell proliferation specifically to ANSCs.

In this view, in order to identify NSCs as the cells affected by *Emx2* perturbation, we exploited the negative selection of the transit progenitors that occurs in our system after subculturing. By this procedure, ANSCs are positively selected at each passage and the expansion in cell number observed in our cultures is a direct consequence of stem cell proliferation (Loeffler and Potten, 1996). Owing to their self-maintenance capacity, ANSCs ought to retain unchanged functional features over time. Therefore, if *Emx2* acts as a genetic determinant of ANSCs proliferation, its perturbation ought to affect the overall growth rate in our cultures. This is what we observed by extended (three-month long) population analysis in PVR cultures, in which the rate of growth was strongly enhanced or, alternatively, diminished in *Emx2* null or gain-of-function mutant mice, respectively, as compared to wild-type cells. This indicates that *Emx2* elicits a negative effect on the rate of proliferation and expansion of cultured bona fide post-natal/adult neural stem cells.

Further investigation allowed us to identify the prospective mechanism by which *Emx2* may affect ANSC proliferation. In a stem cell pool the overall rate of cell expansion results from three phenomena: (i) cell death; (ii) cell cycle length and (iii) the relative frequencies between symmetric divisions that yield two stem cells (proliferative), symmetric cycles generating differentiated/dead cells (differentiative) and asymmetric divisions giving rise to one stem and one differentiated cell (Loeffler and Potten, 1996).

We analyzed the three parameters and found that no alterations could be detected in either cell death or the cell cycle time in ANSCs from *Emx2* loss- or gain-of-function mice. Conversely, serial sub-cloning assays, in addition to proving the actual stem cell identity of the precursors analyzed, revealed an alteration in the frequency of the proliferative symmetric cycles within the NSC population. In fact, in cultures from *Emx2* null mutants (expanding faster) the frequency of symmetric cycles yielding two stem cells was significantly higher than in control cells (Table 2). Accordingly, the frequency of symmetric proliferative cycles was considerably down-regulated in cells infected with a viral vector carrying the *Emx2* codifying sequence as well as in slow-growing cells from gain-of-function mice (Table 2).

It should be emphasized that, given the current lack of stem cell-specific markers, the assessment of the frequency of the various kinds of symmetric divisions and asymmetric cycles occurring in a ANSC clone can only be carried out indirectly. This is done on a functional basis, using an assay that determines the percentage of secondary clonogenic cells in a clone with respect to the overall cell number. While this assay allowed us to show that *Emx2* may increase the frequency of symmetric divisions yielding two stem cells, there is currently no method to establish whether the frequency of asymmetric cycles is also affected by *Emx2*. The imminent development of antibodies that may discriminate between stem elements and other cells may help to unravel this issue in the future (Temple, 2001).

All throughout this study, the capacity of NSCs to generate mature progeny was also thoroughly assessed. Under all the conditions and time points tested, we could not detect any noticeable alteration in the capacity of mutant or infected ANSCs to originate the three major neural cell types, namely neurons, astrocytes and oligodendrocytes. This seems to reinforce the idea that the most prominent function of *Emx2* in ANSCs is the regulation of precursor cell division which is achieved by the gene varying the frequency at which proliferative cell divisions take place within the overall stem cell population.

The analysis of the regulation of adult neural precursors is a rapidly expanding area of investigation with implications for the understanding of the basic mechanisms that underlie adult neurogenesis. These findings may also impact on the development of novel therapeutic techniques relying on the ex-vivo expansion and transplantation, or in vivo recruitment of stem cells for the cure of neuro-degenerative disorders.

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