Intrinsic fate determinants of neural and multipotent CNS precursor cells

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Dissertation der Fakultät für Biologie der Ludwig-Maximilians-Universität München

2004

Content

Date of the oral examination:

München, 8. Juni 2004

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, daß ich die vorgelegte Dissertation mit dem Titel „***Intrinsic fate determinants of neural and multipotent CNS precursor cells***“ selbständig und ohne unerlaubte Hilfe angefertigt habe und daß ich die Arbeit noch keinem anderen Fachbereich bzw. noch keiner anderen Fakultät vorgelegt habe.

Göteborg, den 06. Januar 2003.

Summary

The cells of the mammalian central nervous system (CNS) arise from multipotential precursor cells. The mechanisms that drive precursor cells toward a distinct cell fate are not well understood. Since transcription factors are known to control fate decisions, I attempted to determine the role of transcription factors Emx1, Emx2 and Pax6 that are particularly interesting since they specify area identities in the mouse telencephalon. To analyze their roles in precursor cells I chose gain-of-function experiments. Overexpression of these transcription factors showed that Emx2, Emx1 and Pax6 affect precursor cells in a region-specific manner. Emx2 transduction increases proliferation by promoting symmetric cell divisions, whereas blockade of endogenous Emx2 by antisense Emx2 mRNA limits the number and fate of progenitors generated by an individual cortical precursor cell. In the $Emx2^{-/-}$ asymmetrical cell divisions are increased in the cerebral cortex *in vivo*. In contrast to Emx2 Pax6 decreases proliferation. Pax6 deficient cells show more symmetrical cell divisions while Pax6 promotes asymmetric cell divisions *in vitro*. Emx2 endows *in vitro* cortical precursor cells with the capacity to generate multiple cell types, including neurons, astrocytes and oligodendrocytes. Emx1 keeps cells in an undifferentiated cell type, while Pax6 increases the proportion of neurons and can also convert astrocytes to neurons. The bHLH transcription factors Olig2 and Mash1 are up-regulated upon Emx2-transduction whereas Pax6 negatively influences those transcription factors and specifically up-regulates Ngn2. Thus, Emx2 is the first cell-intrinsic determinant able to instruct CNS precursors towards a multipotential fate. These results demonstrated an important role of Pax6 as intrinsic fate determinant of the neurogenic potential of glial cells. Taken together, Emx2 and Pax6 have opposing roles in cell proliferation, mode of cell division and cell fate.

Zusammenfassung

Die molekularen Vorgänge, wie sich aus relativ undifferenzierten Zellen des Zentralen Nervensystems hochspezialisierten Zelltypen entwickeln sind weitgehend unbekannt. Eine zentrale Rolle bei diesen Vorgängen spielen Transkriptionsfaktoren, die zeitlich und räumlich begrenzt gebildet werden und im Zusammenspiel miteinander die Prozesse auslösen, die letztlich zur Bildung von unterschiedlichen Zelltypen führen. Um die Rolle der Transkriptionsfaktoren Emx1, Emx2 und Pax6 in der Vorderhirnentwicklung der Maus zu anlysieren, sind diese Fakoren jeweils in einzelnen Vorläuferzellen überexprimiert worden. Alle Transkriptionsfaktoren zeigten regionsspezifische Aktivität, wobei Emx2-Überexpression zu verstärkter Proliferation durch Zunahme von symmetrischer Zellteilung sowie Zunahme multipotenter Zellen fuehrte. Emx1 zeigte keinen Einfluss auf die Proliferation, erhöhte jedoch die Zellzahl undifferenzierter Zellen. Dagegen reduzierte Pax6 Proliferation und induzierte Neurogenese sogar in adulten Astrocyten.

Diese Ergebnisse belegen, dass Emx2 und Pax6 in Proliferation, Zellteilungsmodus und Zellschicksal gegensätzliche Rollen ausueben.

Abbreviations

bFGF	Basic fibroblast growth factor
bHLH	basic helix-loop-helix
bp	Base pairs
BrdU	5-bromo-2'deoxy-uridine
cDNA	Complementary DNA
CFU	Colony forming unit
ctx	Cortex
DNA	Desoxyribonucleic acid
CNS	Central nervous system
div	Days *in vitro*
DMEM	Dulbecco's modified eagle medium
E 14	Embryonic day 14
ECM	Extracellular matrix
EGF	Epidermal growth factor
FCS	Fetal calf serum
GE	Ganglionic eminence
Gof	gain-of-function
IRES	Internal ribosome entry site
Lof	loss-of-function
mRNA	Messenger ribonucleid acid
P 5	Postnatal day 5
PBS	Phosphate buffered saline
PDGF	Plateled derived growth factor
PDL	poly-D-lysine
RNA	Ribonucleic acid
RT	Room temperature
PCR	Polymerase chain reaction
SEM	Standard error of the mean
SVZ	Subventricular zone
VZ	Ventricular zone
WT	Wildtype

Introduction

The cells of the mammalian central nervous system (CNS) are thought to arise from multipotential precursor cells whose developmental potential becomes progressively restricted (Anderson, 1989; Lilien, 1997). The central question in the specification of distinct cell fates is the interaction between two sets of determinative factors: extrinsic components like secreted or transmembrane factors present in the local environment of a cell and intrinsic signals that operate in a cell-autonomous manner, like transcription factors. However, the nature of these extrinsic extracellular mechanisms that drive precursor cells toward a distinct cell fate and the intrinsic intracellular mechanisms by which these cues promote such a fate are not well understood (Anderson, 1989; Lillien, 1997). In the cerebral cortex, for example, precursor cells are multipotential at early stages of development prior to neurogenesis (Williams and Price, 1995; Qian et al., 1998; Shen et al., 1998; Qian et al., 2000). Their potential then decreases during neurogenesis when most precursor cells become specified to generate a single cell type (Grove et al., 1993; Reid et al., 1995) (Fig. 1). During development specific subsets differ in their proliferative and phenotypic potentials and vary in their amounts (William and Price, 1995; Kilpatrick and Bartlett, 1995). Their relative representation tends to reflect the cell types generated at specific stages of development. For example, at earlier embryonic stages, such as embryonic day 14 (E 14), when more neurons are generated, progenitor cells that are restricted to a neuronal fate are more abundant. In contrast at later stages around E 17, when more glial cells begin to develop, progenitor cells that are restricted to a glial fate are more abundant. That means there are important intrinsic cues necessary to maintain this restriction (Qian et al., 2000).

These distinct lineages have also been observed under isolated *in vitro* conditions (Luskin et al., 1988; Williams et al., 1991; Williams and Price, 1995) and exhibit characteristic stereotyped lineage trees (Qian et al., 1998, 2000), implying cell-intrinsic determinants of the fate of distinct precursors (Fig. 1).

Since transcription factors are known to control fate decisions (Enver, 1999; Watt and Hogan, 2000), I attempted to determine the role of transcription factors that are expressed in subsets of cortical precursor cells in the mouse telencephalon. Moreover these transcription factors show region-specific expression pattern (Gulisano et al., 1996; Götz et al., 1998; Toresson et al., 2000; Hartfuss et al., 2001) and therefore their

analysis is necessary to understand the molecular mechanisms of cell fate determination.

The genes Emx1, Emx2 and Pax6 are particularly interesting since they specify area identities (Mallamaci et al., 2000b; Bishop et al., 2000; Muzio et al., 2002a, b). In the case of of Emx1 or Emx2 this function is well conserved, since members of this gene family (orthodenticle (otd) and empty spiracles (ems)) have a distinct function in head formation of the Drosophila brain. Their functional ablation causes the loss of specific brain segments in Drosophila (Dalton et al., 1989). In vertebrates the two of four related genes Emx1 and Emx2 show nested expression pattern in the fore- and midbrain at embryonic stages (Simeone et al., 1992a; Simeone et al., 1992b). They are, together with Pax6, among the earliest transcription factors specifying the dorsal region of the telencephalon (see Fig. 4), the prospective cerebral cortex, and are absent from the ventral region, the ganglionic eminence (GE) (Walther and Gruss, 1991; Simeone et al. 1992; Gulisano et al., 1996). Emx2 and Emx1 are expressed in the presumptive cerebral cortex in a developmental period (E 8 – E 17) corresponding to the major events in cortical neurogenesis (Simeone et al., 1992b). The expression of Emx2 starts prior to neurogenesis at E 8 in the mouse and peaks already at early stages of neurogenesis (E 12), where it is exclusively expressed in precursor cells, while it is down regulated thereafter. Emx1 expression begins later around E 9.5 and occurs in both proliferating and postmitotic cells (Simeone et al., 1992b).

Mice with a deletion of the Emx2 gene point to an important function of this gene in corticogenesis (Pellegrini et al., 1996; Yoshida et al., 1997; Bishop et al., 2000; Mallamaci et al., 2000a, b; Tole et al., 2000; Shinozaki et al., 2002). The earliest defect of the telencephalon of $Emx2^{-/-}$ mice is a prominent size reduction of the cerebral cortex, the olfactory bulb and parts of the hippocampus. The thinner cortex in the Emx2-mutant, showing also a cortical plate smaller than normal (Tole et al., 2000), could be a phenotype due to either defects in proliferation, neuronal migration or differentiation. At later stages further aberrations can be observed in Emx2-mutant mice: reelin-expressing cells in the marginal zone are lost and concomitantly defects in neuronal migration develop (Mallamaci et al., 2000a). Furthermore, areas with high Emx2-expression shrink at the expense of an area with usually low Emx2 contents in the absence of Emx2 protein (Bishop et al., 2000; Mallamaci et al., 2000b; Muzio et al., 2002).

In the *Emx1* single mutant the phenotype in neocorticogenesis is more subtle than in the *Emx2* single mutant (Yoshida et al., 1997). Homozygote mice with the functional deletion of the Emx1 gene are in contrast to the Emx2 homozygotes viable and exhibit no obvious morphological or behavioral defects (Qiu et al., 1996).

These defects are more severe in the *Emx1/Emx2* knock-out where the homozygote embryos exhibit prominent neocortical defects. The thickness of the cerebral wall is diminished with a decrease in cell number. Moreover their analysis suggested that *Emx1* and *Emx2* cooperate to promote cell migration from the ventral telencephalon and cooperate in the development of Cajal-Retzius cells and subplate neurons (Shinozaki et al., 2002).

Furthermore it was demonstrated that Emx2 and Pax6 have opposing roles in area specification (Bishop et al., 2000). However, while the region-specific role of Emx2 in the dorsal telencephalon is clear, its cellular function is still not understood, partially due to the difficulties of separating secondary from primary defects in the mutant analysis. In case of Emx1 neither the region-specific role nor the cellular function is understood.

The transcription factor Pax6 belongs to an evolutionarily conserved transcription factor family called paired class of homeobox transcription factors that plays a crucial role in organogenesis. During telencephalic development, Pax6 is expressed only in a subpopulation of precursor cells in the cortex, in cells that express the main marker for radial glial cells RC2, and acts as a crucial determinant of those cells during neurogenesis (Götz et al., 1998). In the absence of functional Pax6 proteins, in the Sey/Sey mutant, radial glial cells of the cerebral cortex have a distorted morphology, change their expression of cell surface and extracellular matrix (ECM) molecules and their cell cycle properties (Götz et al., 1998; Stoykova et al., 1997). These defects are restricted to the phase of neurogenesis while astrocyte precursors are not affected consistent with the reduction of Pax6 expression in radial glial cells when they transform into astrocytes (Götz et al., 1998; Stoykova et al., 1997). These data therefore suggest that Pax6 might play a role in the recently discovered neurogenic lineage of radial glia in rodent cortex (Malatesta et al., 2000; Noctor et al., 2001; Miyata et al., 2001).

In the previously generated Emx2/Pax6 knock-out the regionalization of the telencephalic hemispheres was dramatically altered (Muzio et al., 2002).

In this thesis I analyzed whether these patterning transcription factors (see Fig. 4) have also direct effects on cell fate, with focus on the neuronal-glial fate decision. These transcription factors were choosen since their functional ablation generates defects that seem to originate in the germinal ventricular zone (VZ) of the cerebral cortex, where these genes are expressed (Simeone et al., 1992; Stoykova et al., 1996). To elucidate the role of Emx1, Emx2 and Pax6 in cortical precursor cells I chose a combination of cell- and time-specific gain-of-function experiments. I used replication-incompetent retroviral vectors to express Emx2, Emx1 or Pax6 in few precursor cells within a wild type environment (see Fig. 2). In the case of Emx2 the retroviral vectors contain the Emx2 full length cDNA in sense orientation which is followed by an internal ribosome entry site sequence (IRES) and the lacZ gene (Fig. 2, 5 C). As control constructs I used BAG, a vector that contains the NEO resistance gene and the lacZ gene connected via a SV40 promoter (Fig. 2, 5 A; Price et al., 1987) or 1726 (Fig. 2, 5 B), the same construct I used for the Emx2- and Pax6-co-expression virus containing the NEO resistance gene instead of Emx2. To block gene expression I used a construct with Emx2- cDNA in antisense orientation. The approach of using retroviral vectors allows the examination of the cell autonomous effects of each gene in individual precursor cells and all their progeny, i.e. in a clonal analysis (see Fig. 3). Mainly I used a previously established culture system that mimics the lineage of cortical precursor cells *in vivo* (Luskin et al., 1988; Williams and Price, 1991; Grove et al., 1993; Götz et al., 1995; Reid et al., 1995; Lavdas et al., 1996). In this culture system, like *in vivo* (Price and Thurlow, 1988; Grove et al., 1993; Reid et al., 1995), most cortical precursors generate only a single cell type and most of them are exclusively neuronal. The same pattern of lineage is observed in single cell cultures without addition of growth factors (Qian et al., 1998; Shen et al., 1998). These results suggest that already early in cortical development, most cortical precursors are intrinsically restricted in their potential to the generation of a single cell type and that these *in vitro* systems provide a suitable model to investigate the lineage of cortical precursors. Indeed, the most recent evidence for a close correspondence of cell lineage in dissociated cortical cells *in vitro* and *in vivo* is the neurogenic role of radial glial cells. The neuron-restricted lineage of radial glial cells was first discovered *in vitro* using the culture conditions of this study (Malatesta et al., 2000a) and then in slice cultures and *in vivo* lineage tracing (Noctor et al., 2001). Thus, high-density dissociated cell cultures provide a good model system to manipulate gene expression

in order to examine the molecular mechanisms influencing lineage restriction. These *in vitro* findings were in the case of Emx2 and Pax6 then compared to the behavior of cortical precursors in *Emx2*$^{-/-}$ or *Sey/Sey* mice *in vivo* (Hill et al., 1991; Pellegrini et al., 1996). To analyze directly a cell-autonomous role of Emx2, we used loss-of-function experiments in a wildtype environment by Emx2 antisense transduction in precursor cells. Observations of these in vitro studies were then compared with the *in vivo* situation of the Emx2 knock-out.

In vivo observations of functional interactions between Emx2 and Pax6 were recently examined in a knock-out for both genes (Muzio et al., 2002). In these embryos, the conversion of the cerebral cortex into basal ganglia takes place. However, to prove a direct influence of Emx2 on corticogenesis *in vivo* we performed additional experiments to overexpress it *in vivo*. These *in vivo* studies were compared to the *in vitro* studies to relate the gain-of-function experiments to the role of endogenous Emx2 or Pax6 proteins in cortical development.

Experimental Methods

Animals

The Pax6 mutant mouse used in some experiments carries the Sey allele on a C56BL6/6J x DBA/2J background. This naturally occurring point mutation leads to non-functional truncated Pax6 protein (Hill et al., 1991). Heterozygous Sey-mice were recognized by their eye-phenotype and were crossed to obtain homozygous and WT mice in a litter. WT and *Sey/Sey* mice were mainly used in the experiments. Sey homozygote embryos were recognized by the lack of eyes. Heterozygote mice were recognized in their reduced eye size (Hill et al., 1991).

Emx2 mice

The Emx2 mutant mice (Pellegrini et al., 1996) used in the experiments were obtained by genotyping of mice by polymerase chain reaction (PCR) on genomic DNA extracted from tails (Laird et al., 1991). Genotyping of the Emx2 allele was performed with primers situated in the upper (CGT-AAG-ACT-GAG-ACT-GTG-AGC-C) and lower (CCA-GTC-ATA-GCC-GAA-TAG-CC) strands of the Emx2 sequence giving rise to a 169 base pairs (bp) fragment. The neomycin insert was detected using an upper primer in the 5'-region of the neo insert (CCA-GTC-ATA-GCC-GAA-TAG-CC) that amplifies together with the 3'-Emx2 primer a fragment of the size 294 bp. The reaction mix of the PCR: 1.5 µl genomic DNA, 0.3 µl Taq polymerase (Perkin Elmer), 3 µl dNTPs (2 mM stock), 3 µl buffer (Perkin Elmer), 0.7 µl 5'-neo primer (10 µM), 0.7 µl 3'-Emx2 primer (10 µM), 1.4 µl 5'-Emx2 primer (10 µM) and 19.4 µl H_2O.

PCR conditions were 30 cycles of 94°C for 1 min; 65°C for 1 min; 72°C for 1 min.

The day of vaginal plug was considered as embryonic day 0 (E 0), the day of birth as postnatal day 0 (P 0). $Emx2^{+/-}$ mice (Pellegrini et al., 1996; Tole et al., 2000) were maintained on a C57BL/6J background.

Emx2-genotyping was performed by PCR on tail DNA to recognize the homozygote embryos of a littermate.

Dissociated Cell Cultures

Embryos (E 12– E 18) were removed by cesarean section from time-pregnant mice (Lewis, plug date regarded as E 0) anesthetized by an overdose of diethylether. The meninges were removed, the telencephalic hemispheres separated, the hippocampi and the olfactoric bulbi removed. The Cortex (ctx) and the GE were dissected and collected in separate Falcon tubes under sterile conditions in HBSS containing 10 mM HEPES on ice (Götz et al., 1998). The tissue was then allowed to settle, and the balanced salt solution was removed and replaced by trypsin-EDTA (0.05 % [w/v] in HBSS (without Ca^{2+}, Mg^{2+} with 0.02 [w/v] EDTA (GIBCO)) and incubated for 15 min at 37°C. The enzyme activity was stopped by the addition of Dulbecco's modified eagle medium (DMEM; GIBCO) supplemented with 10 % fetal calf serum (FCS; Sigma) and penicillin/streptomycin (100 units/ml penicillin, 100 units/ml streptomycin [GIBCO]. Afterwards there was added twice the volume of DMEM with 10 % FCS. The tissues were mechanically dissociated with a fire-polished Pasteur pipette coated with serum, twice pelleted for 5 min at 172 x g and resuspended in FCS containing medium. Cells were plated at 10^6 cells/ml in DMEM/FCS (0,5ml/well) in a 24-well plate on poly-D-lysine (PDL) coated cover slips and incubated at 37°C and 5 % CO_2. After one day in culture a volume of chemically defined SATO-medium was added (Götz et al., 1998). Cultures from $Emx2^{+/-}$ crosses were separately prepared from each embryo and genotyping was performed on tail DNA. In some experiments 5-bromo-2'deoxyuridine (Sigma) was added to the culture medium at the final concentration of 10 µM, 16 hours prior to fixation. After 2–10 days *in vitro* (div), cells were fixed with 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.4) for 15 min at room temperature (RT).

Astrocyte preparation

Brains were isolated from postnatal mice at day 3 to 5 (P 3–5) and the cortex was dissected as described for the dissociated cell preparation. The cortices of 3–5 animals were collected in HBSS with 10 mM HEPES on ice and cut up into small pieces. The tissue was then mechanically dissociated into single cells with a plastic pipette. After centrifugation for 5 min at 172 x g the pellet was resuspended in 10 % DMEM/FCS (1 ml medium per brain). Cells were afterwards cultured in 75 cm^2 tissue culture

flasks (Corning) at 37°C and 5 % CO_2. After 3 div half of the medium was replaced by fresh 10 % DMEM/FCS medium. Every 7 div the cells were passaged and after 3 passages hardly any oligodendrocytes were present. The cells were plated at a density of $1.7 \cdot 10^5$ cells/ml onto tissue culture petri dishes infected with retroviral vectors after two hours and fixed after further 3–5 div with 4 % PFA for 15 min at RT. After 3 washes with PBS the cells were processed for immunocytochemistry (Hartfuss et al. 2001).

Embryonic Neurosphere cultures

Embryonic neurosphere cultures were preparated from E 14 telencephali as described for the dissociated cell preparation with the exception that these cells were mechanically dissociated without enzymatic digestion. The dissociation process was done by careful tituration with a serum coated fire-polished Pasteur pipette. The cells were afterwards collected by centrifugation at 172 x g for 10 min and resuspended in DMEM medium/Ham's F-12 (DMEM/F-12). The basic medium contained 10 % FCS, penicillin/streptomycin (each 100 units/ml), B-27 supplement (concentration 1/50 in F-12 medium; GIBCO), 20 ng/ml bFGF (Sigma), 20ng/ml EGF (Sigma) and additional 3.5 mM glucose. Two-thousand cells/cm^2 were plated into 75 cm^2 plastic culture flasks (Corning) in serum-free medium containing FGF-2 (human recombinant, 20 ng/ml; Boehringer) and EGF (human recombinant, 20 ng/ml; Boehringer). The cells were continuously cultured for 5 to 6 days at 37°C and 5 % CO_2. By 5–6 div individual proliferating cells had generated floating clustered clones of undifferentiated cells (primary neurospheres; Reynolds and Weiss, 1992). These cells were collected by centrifugation (10 min at 172 x g), mechanically dissociated to give a suspension of single cells and replated in the continuous presence of EGF and bFGF to generate new clones. This procedure was repeated up to 6 times, producing an increase in the number of undifferentiated bFGF and EGF-responsive precursor cells.

In vivo injection

Viral supernatants were injected in the brain lateral vesicles of E 16 rat embryos as described (Price, J. and Thurlow, 1988). Pregnant mice on day E 14 of pregnancy were anaesthetized Serial coronal sections of postnatal day (P) 0 and P 21 brains were cut at 100 mm by a vibratome and β-galactosidase reporter activity was detected by X-gal staining as described in (Price et al., 1987).

X-Gal histochemistry

Dissociated cell cultures were fixed for 15 min at RT or 30 min at 4°C. The fixative contained 0.5 % Glutaraldehyde, 2 mM $MgCl_2$ and 1.25 mM EGTA. The cells were washed twice in PBS and incubated for 1 hour at 37°C in X-Gal staining solution, containing 4 mM $K_3Fe(CN)_6$, 4 mM K_4 $Fe(CN)_6$, 1 mM $MgCl_2$, 0.01 % sodium desoxycholate and 0.02 % NP-40. The X-Gal (5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) was added to a final concentration of 0.04 mg/ml in PBS.

Plasmid preparation and *in vitro* transcription

Plasmids were transduced in XL-1 Blue E. coli bacteria by electroporation as described in the Maniatis protocol with following parameters: 0.2 mm electroporation cuvettes, 2.2 kV, 25 µF and 400 Ω. After recovering of the bacteria for 1 hour in the incubator, 50 µl were plated on ampicillin containing (50 µg/ml) LB-agar plates. The next day one colony was picked and grown for around 4 hours in 3 ml LB-ampicillin medium. This preculture was given to 100 ml LB-ampicillin and shuttled over night at 37°C. Following the Quiagen midiprep protocol the plasmid was harvested through a midi Tip 100 column. The DNA pellet was dissolved in 200 µl ddH_2O. 20 µg plasmid was linearized with the appropriate enzyme (40 units) in a total volume of 50 to 60 µl for 2.5 hours at 37°C. The plasmid was afterwards phenol extracted.

Retroviral vectors and cell lineage analysis

The 1300 bp fragment containing the entire coding sequence of Emx2 was inserted either in sense or antisense orientation in the BglII unique restriction site of the 1704

retroviral vector between the upstream LTR and the EMC IRES sequence (kind gift of J.E. Majors; Ghattas et al., 1991). For Pax6 a 783 bp fragment containing the entire coding sequence of Pax6 was inserted in sense orientation in the BglII unique restriction site of the 1704 retroviral vector between the upstream LTR and the EMC IRES sequence (kind gift of J.E. Majors; Ghattas et al., 1991). BOSC 23 helper-free packaging cells were used for viral production as described by Pear et al. (1993). The titer ranged from $0.5 \cdot 10^5$ CFUs (colony forming units) to $0.5 \cdot 10^6$ CFUs.

Primary cells from the cortex or the GE were infected 2 hours after plating at a concentration giving not more than 25 clones per coverslip. The probability of superimposition of clones is 2.8 % as determined by the calculation described in Williams et al. (1991) for the average diameter (300 µm) of the control and Emx2-, Emx1- or Pax6-transduced clones. However, in the case of the Emx2-sense transduced clones which contained often more than 100 cells and covered a larger area, the biggest clone of about 250 cells extended over a distance of 800 µm. Assuming this most extreme clone diameter as the average, 13 % of clones could be superimposed. Thus even an extreme overestimation of clonal superimposition does not come close to the increase in the number of mixed clones observed with the Emx2-sense virus (35 %). To evaluate the overexpression of Pax6 or Emx2 protein in the transduced cells, digital pictures of the Emx2 or Pax6-immunoreactivity were obtained with an Axiocam color camera and analyzed with the software ImageJ 1.23y (NIH, USA). The intensity of the signals of β-galactosidase-positive cells was measured with respect to the Emx2-or Pax6-intensity of β-galactosidase-negative cells in the same cultures.

Immunocytochemistry

For precursor cells we used as cell type-specific antibodies the monoclonal antibody (mAb) directed against nestin (Rat 401, IgG1, 1:4, Developmental Studies Hybridoma Bank), for astrocytes the mAb directed against GFAP (IgG1, 1:200; Sigma), the mAb against β-tubulin-III we used for neurons (IgG2b; 1:100, Sigma) and for oligodendrocytes mAb against O4 (IgM, 1:100 and mAb against BrdU (IgG1, 1:10, Bioscience Switzerland)). A polyclonal antibody specific for β-galactosidase (O3; Williams and Price, 1995) was used to detect retrovirally labeled cells and a

polyclonal antibody directed against caspase-3 to detect apoptotic cells. Incubation in primary antisera was performed at 4°C overnight. For Emx2 staining the cells were fixed in a 50 % methanol / 50 % acetone solution for 5 minutes at –20°C. After three washes in PBS, cells were incubated in PBS containing 10 % FCS and 0.1 % Triton. The polyclonal Emx2 antibody was used 1:600 for 1 hour at RT (Mallamaci et al., 1998). Pretreatment for BrdU-detection and secondary antibody incubations were performed as described in Hartfuss et al. (2001). Some cultures were double-stained with DAPI (4'-6'Diamidino-2-phenylindole, Sigma, final concentration 0.1 µg/ml, incubation for 10 minutes).

BrdU dilution assay

In order to distinguish between clones generated by purely symmetric cell divisions and clones generated by at least some rounds of asymmetric division we used a previously established assay (Malatesta et al., 2000b). As described by Malatesta et al. (2000b), cells were incubated for 36 hours in BrdU-containing medium, replated and fixed after additional 5-7 div. Digital microphotographs of the clones identified by β-galactosidase immunoreactivity were taken using a MicroMAX CCD camera (Visitron System) mounted on an Axiovert 10 microscope (Zeiss). MetaView Imaging Software was used to quantify the relative intensities of BrdU-label. Within a clone, we measured the BrdU-immunoreactivity of all nuclei. The highest value (MAX) is the BrdU-content of the strongest labeled cell. This is the cell that stopped to divide as the first in each respective clone. The cell that contained the weakest labeled nucleus (MIN) is the cell that underwent the most rounds of divisions in the clone. We then classified each clone based on the ratio in percentage between MAX and MIN (MIN/MAXx100). Since clones containing only one or two cells could not dilute their label for several generations and reliable differences in BrdU-content can not be measured if the BrdU is too dilute, i.e. if too many rounds of cell divisions were performed, we restricted this analysis to clones containing 3–16 cells.

Histological analysis of cell division

Brains of embryos from crosses of $Emx2^{+/-}$ mice were isolated and genotyping of the embryos was performed on tail DNA as already described. Brains were frozen in Tissue Tek and frontal sections were cut at a thickness of 20 µm using a cryostat. Sections were fixed in 4 % PFA for 10 min at RT and stained with DAPI as described above. Analysis was performed blind, i.e. the evaluator did not know the genotype of the embryos from which the sections were derived.

Data analysis

Since our data are not normally distributed, the non-parametric test of the median was used to calculate a significant difference if $p < 0.001$. For cell fate analysis, the proportion of neuronal, mixed and non-neuronal clones was analyzed for each coverslip and means were calculated from different coverslips. Data are derived from 1–15 different experimental batches containing at least 12 different coverslips.

Results

Emx2

Cell autonomous function of the transcription factor Emx2

When cells from the cortex isolated at E 14, the peak of neurogenesis, were infected with the Emx2-sense virus and analyzed after 3 days *in vitro* (div), the β-galactosidase immunoreactive cells also exhibited strong Emx2-immunoreactivity in the nucleus (Fig. 5 E, F). The nuclear immunoreactivity of infected cells appears to be stronger than the endogenous Emx2 expression. The endogenous expression consists in cortical precursor cells mostly of cytoplasmic Emx2-immunoreactivity. Those cells do not contain β-galactosidase (see asterisks in Fig. 5 E, F). Since relative amounts of Emx2 proteins have been shown to be relevant for area specification in the cerebral cortex (Bishop et al., 2000; Mallamaci et al., 2000b), I measured the relative amounts of endogenous and virally-expressed Emx2 by image analysis. This analysis revealed 3.6-fold higher fluorescence intensity in Emx2-transduced cells compared to the endogenous Emx2-immunoreactive fluorescence in cortical cells (data not shown). The fluorescence measurement was normalized to the immuno-negative cells from the GE that not contain Emx2. All (n = 53) β-galactosidase-immunoreactive cells in cultures infected with the Emx2-virus were strongly Emx2-immunoreactive, confirming the previous data that the IRES-co-expression construct leads to reliable co-expression of the β-galactosidase protein and the protein encoded by the first gene, i.e. Emx2 (see also Ghattas et al., 1991; Hojo et al., 2000). However, I also detected some cells (two cells of 72) that expressed a high amount of Emx2 characteristic for virally-directed expression but failed to express β-galactosidase. These cells do not affect my analysis that is based on the β-galactosidase marker protein.

The use of these replication-incompetent retroviral vectors allows examination of the progeny generated by a single precursor cell that is defined as a clone (Fig. 3, 5 G). These vectors have the same integration mechanism of naturally-occurring retroviruses producing a single copy of the viral genome that is stably integrated into the host chromosome. Those that we use for lineage analysis have been modified so that they are replication incompetent and thus cannot spread from one infected cell to another. They are however passed on to all daughter cells of the originally infected

progenitor cell, making them an ideal tool for lineage analysis. When analyzing the cell types that result from the marking of a single progenitor cell that was infected by a retroviral vector one can gain insight into whether the progenitor was committed to the production of one or multiple cell types. If multiple cell types are found in a clone, one can conclude that the progenitor that gave rise to these cells was not restricted to the production of only one cell type. We infected with a low titer of retrovirus (see Experimental Methods) only a few cultured cells gave rise to distinct clusters of β-galactosidase immunoreactive cells. Those cells were surrounded by immuno-negative cells (e.g. Fig. 5 H, I). These clusters were considered as individual clones derived from single infected precursor cells, if they were separated by at least 300 μm from a neighboring cluster (see Experimental Methods).

Clone size

A difference between the clones derived from E 14 cortical cells infected with control (Fig. 2 II, B, 5 A, B) or Emx2-sense (Fig. 2 III, 5 C) virus after 7 div was immediately obvious (Fig. 6). Clusters of β-galactosidase-positive cells infected with the control virus BAG (Fig. 5 A) or 1726 (Fig. 5 B) were mostly small and rarely contained more than 10 cells, consistent with previous results (Williams et al., 1991; Williams and Price, 1995; Götz et al., 1995; Fig. 6). Both control constructs were comparable as control vectors and showed the same clonal size after 7div (BAG: 5.2; n: 61; 1726: 4.9; n: 46). These controls further indicate that even if the first gene is favored (in case of BAG NEO) the clone size will not be affected. In contrast, cells infected with the Emx2-sense virus frequently generated large clones containing more than 20 cells (Fig. 6). This prominent phenotype is reflected in a significant (see Experimental Methods) increase of the mean size of Emx2-expressing clones compared to control clones (Fig. 6 A). Notably, a change in the size of clones was not observed in Emx2-sense infected cells of the GE, the later basal ganglia that do not contain endogenous Emx2. Thus, Emx2-sense transduction affects cells in a region-specific manner.

Since Emx2 is expressed in a graded manner (caudal: high, rostral: low) in the developing cerebral cortex (Gulisano et al., 1996; Mallamaci et al., 1998; Bishop et al., 2000; Mallamaci et al., 2000b), I separately examined the effects of Emx2 transduction in rostral and caudal cortex isolated at E 14. In both regions, Emx2

transduction resulted in a significant increase in the size of clones, but the effect was much more pronounced in cortical cells from rostral regions (Fig. 6 B). This result demonstrated a greater effect of Emx2 transduction in the cortical region that contains less endogenous Emx2. I also observed a slightly larger size of clones infected with the control virus from caudal cortex compared to rostral cortex, consistent with the higher amounts of endogenous Emx2 in caudal cortex. These data support a role of Emx2 in regulating clone size in the developing cortex and demonstrate that cortical cells of all regions are able to respond to Emx2, while cells from the GE are not.

While the endogenous Emx2 levels decrease significantly during development (Gulisano et al., 1996; Mallamaci et al., 1998), I next established a developmental series of the effect of Emx2 transduction. When precursor cells were infected at E 12, the onset of neurogenesis in the cortex (Bayer and Altman, 1991), the mean size of Emx2-overexpressing clones was double the value observed after infection with control virus (Fig. 6 C). Analysis at later stages (E 16) showed an even larger, 4-fold, increase of the mean size of Emx2-overexpressing clones compared to the size of the control clones (Fig. 6 C). Fig. 6 C shows that the size of the control clones decreases during development, which is correlated to a decrease in endogenous Emx2 expression. In contrast, Emx2 transduction generates the same clone size independent of the age of the cortical cells. While cortical precursors cease to divide with increasing age, Emx2-overexpressing cells continue to divide and often generate very large clones, even if cells were isolated at the end of neurogenesis (E 16). To further test whether this effect is still present in astrocytes from postnatal cortex (see Experimental Methods), I infected them with control or Emx2-sense virus. After 5 div, no difference in the clone size (ctrl: 2.1 ± 0.3; Emx2: 2.0 ± 0.7) could be detected between control and Emx2-sense transduced clones, indicating that astrocytes from postnatal cortex are not amenable to effects mediated by Emx2-sense transduction.

I next asked whether the increase in clone size after Emx2 transduction during neurogenesis is due to a general shift towards larger clones, or selectively affects clones of a particular size. The composition of clones infected with the Emx2-sense virus revealed a particular strong increase in the number of large clones composed of more than 15-20 cells after 7 div (Fig. 6 D). The increase in large clones mostly occurred at the expense of small clones containing 1-5 cells. Single cell clones are the

most frequent size of clones in the cortex *in vivo* (Price and Thurlow, 1988; Grove et al., 1993) and in cultures infected with the control virus (Williams and Price, 1991; Götz et al., 1995; Qian et al., 1998; Hajihosseini and Dickson, 1999). This population was reduced from 40 % in control cultures to 13 % in the Emx2-sense-transduced clones with a corresponding increase in clones containing more than 15 cells (Fig. 6 D). The shift from single-cell clones to large clones resulting from Emx2 transduction could be due to a decrease in cell death, changes in the mode of cell division or an increase in the number of dividing cells. These parameters were examined in the following experiments.

Cell death

Increased survival of Emx2-transduced precursor cells might explain the large clones after Emx2-sense transduction (see e.g. Hajihosseini and Dickson, 1999). To test this possibility I analyzed clones for the presence of apoptotic cells by DAPI-staining, each day after viral infection. At all time points, dead cells were rare and constituted 7.6 % of all β-galactosidase-positive cells with a mean of 1.4 dead cells per clone. There was not any significant difference between the percentage of dead cells infected with the control (8.3 %; n: 63) or Emx2-sense (7.3 %; n: 83) virus. Similar results were also obtained by labeling dying cells with the caspase-3 antibody. I therefore conclude that selective survival does not contribute to the increased clone size seen after Emx2-sense transduction.

Number of mitotically active precursor cells

Emx2 overexpression might also result in an enlarged clone size by increasing the number of proliferating cells since its normal expression is restricted to precursor cells. I examined the number of dividing cells by addition of 5-bromo-2'deoxy-uridine (BrdU), a DNA-base analogon that is incorporated into the DNA during the S-phase of the cell cycle (Nowakowski et al., 1989), 16 hours prior to fixation (see Experimental Methods, Fig. 7). When cells were labeled with BrdU after 3 div, most β-galactosidase-positive cells still incorporated BrdU, both in the control and after Emx2-sense transduction (Fig. 7). However, the Emx2-sense transduced cells already contained a higher number of proliferating cells compared to control infected cells and this difference further increased during the time in culture. After 7 div, only 23 %

of cells infected with the control virus incorporated BrdU, i.e. most cells had differentiated and ceased to divide (Fig. 7). In contrast, half of the Emx2-transduced cells (46 %, Fig. 7) were still dividing at this time. These data suggest that Emx2 prolonged the precursor status of cortical cells thereby increasing the size of Emx2-sense transduced clones.

Mode of cell division

The very large clones, consisting of more than 20 cells, however, are difficult to explain simply by an increased number of precursor cells. Rather, an increase in symmetric cell divisions could account for some clones consisting of up to hundred cells. To test this suggestion, I used a previously established assay to distinguish between symmetric and asymmetric lineages based on the dilution of `5-bromo-2'deoxy-uridine (Malatesta et al., 2000b, see Materials and Methods for details). Retroviral infected cells were exposed to a prolonged pulse of BrdU (36 hours) to ensure incorporation of BrdU by all dividing precursors. Cells were then fixed 5–7 days after removal of BrdU thereby allowing enough time for dilution of the BrdU-label by several rounds of division. Residual BrdU-levels in the cells of each clone were quantified and distinct levels of BrdU-content were observed as described previously (Malatesta et al., 2000b). As depicted schematically in Fig. 8 A, D cells dilute the BrdU-label to half the amount in each cell division. When a cell stops to divide one cycle earlier than other cells in a clone, it will maintain double the amount of BrdU than its sister cells. Therefore differences in BrdU-contents close to 50 % were considered as one cell cycle of difference (see Materials and Methods for details). For each clone we considered the ratio in percentage between the amount of BrdU of the strongest labeled cell (MAX) and the weakest labeled cell (MIN). The clones with a ratio MIN/MAX higher than 50 % contained cells that underwent the same number of cell divisions. An example of such a clone is given in Fig. 8 B, C. All the cells in this clone contain similar levels of BrdU and thus had the same numbers of divisions. Accordingly, clones with a ratio MIN/MAX lower than 50 % contain at least one cell that divided one time more than the strongest labeled cell in the clone. Clones with a ratio less than 25 % contain at least one cell that divided 2 rounds more. In analogy every halving of the ratio MIN/MAX shows one round of cell division more between the strongest and the weakest labeled cell of the clone. Since this assay

is based on enough time for cells within a clone to divide several times, only clones containing more than 2 cells are included in this analysis.

While more than half (53 %) of the control clones showed a difference of two or more rounds of cell divisions among their cells, none of the Emx2 transduced clones presented such difference (Fig. 8). The data are in good agreement with previous observations of asymmetric cell divisions in living cortical slices (Chenn and McConnell, 1995). Indeed the majority (57 %) of the Emx2 transduced clones was composed by cells that divided the same number of times, i.e. they were generated by pure symmetrical divisions (Fig. 8). Please note that we excluded the clones with one single cycle difference from our conclusions since this difference could also arise by an asynchronous cell division. A difference of two generations in BrdU-content, however, must have included at least one round of asymmetric cell division, because one of the asymmetric generated cells is postmitotic and keeps its BrdU-intensity whereas the other cell continues to divide. Altogether, this analysis revealed a reduction in the number of asymmetrically dividing clones and a corresponding increase in the number of symmetrically dividing clones after Emx2-sense transduction (Fig. 8). It is important to realize, however, that the Emx2-transduced clones contained a higher number of large clones than the control clones. We therefore also compared clones of the same size (5-10 cells) infected with the control virus or the Emx2-sense virus, and we observed the same difference in the mode of division. While 68 % of the control clones containing 5-10 cells show a difference of at least two rounds of division among their cells, none of the Emx2-sense transduced clones shows such difference. To sum up, these results strongly suggest an influence of Emx2 on the mode of cell division in cortical precursor cells, favoring the symmetric mode of cell division even in smaller clones.

Cell fate

The experiments described above implicate Emx2 in the regulation of the mode of division and cell proliferation. I next analyzed the fate of individual Emx2-sense transduced cells to evaluate whether Emx2 is also directly able to affect fate decisions. The differentiation of Emx2-sense transduced cells was examined by double-staining with cell type-specific antisera directed against β-tubulin-III for postmitotic neurons (Lee et al., 1990), nestin for precursor cells (Frederiksen and

McKay, 1988), GFAP for astrocytes (Bignami et al., 1972) and O4 for oligodendrocytes (Bansal et al., 1989). Fig. 9 depicts examples of control and Emx2-sense transduced clones labeled with neuronal, astroglial, oligodendroglial and precursor cell markers. As control-infected cells, Emx2-sense transduced cells were found to co-localize with β-tubulin-III or GFAP, nestin or O4 (Fig. 9), suggesting that they differentiate into all cell types of the CNS. Moreover, Emx2-sense transduced neurons, astrocytes and oligodendrocytes exhibit a normal morphology compared to the control cells (Fig. 9). Thus, Emx2 does not block a particular differentiation pathway.

Quantitative analysis of the clone types revealed, however, a significant increase in clones containing different cell types after Emx2-sense transduction (Fig. 9). I classified clones in 'pure neuronal' (clones composed exclusively by β-tubulin-III-positive cells, Fig. 9 Q), 'pure non-neuronal' (clones containing exclusively nestin-, GFAP- or O4-positive cells, but not β-tubulin-III-positive cells, Fig. 9 Q) and 'mixed' (clones containing β-tubulin-III-positive neurons and other cell types, i.e. nestin-, GFAP- or O4-positive cells, Fig. 9). As depicted in Fig. 9 Q, the proportion of mixed clones was dramatically increased by Emx2-sense transduction in E 14 cortical precursor cells, while there was a complementary decrease in the number of pure neuronal clones. These data are in good agreement with the results of the clone size analysis, since most 1-3 cell clones are pure neuronal clones and most large clones are mixed and contain different cell types (see also Price and Thurlow, 1988; Williams et al., 1991; Grove et al., 1993; Götz et al., 1995; Reid et al., 1995). Thus, the reduced number of small clones is reflected in a reduced number of pure neuronal clones and the increase in clones containing more than 15 cells is reflected in an increased number of mixed clones.

The increased number of precursor cells after Emx2-sense transduction (see Fig. 7) might explain some 'mixed' clones that contain neurons and precursor cells instead of only neurons as in control clones. I therefore used triple-immunocytochemistry to examine the composition of the large, mixed clones more closely. I noted that most of the large clones containing more than 20 cells are indeed composed by β-tubulin-III-immunoreactive neurons and nestin-positive precursor cells (Fig. 10). Only 20 % of

the large control clones contained neurons and differentiated glial cells (i.e. β-tubulin-III- and GFAP- or O4-immunopositive cells), consistent with the small number of bi- or multipotential cells at this stage (Williams and Price, 1995). I could not observe any large clones (20 cells and more) containing only a single cell type. While in most cases the Emx2-transduced large clones were also composed of neurons and precursor cells, the proportion of clones containing neurons and differentiated glial cells nearly doubled (40 %; Fig. 10 H) in comparison to the control clones. Thus, the true bi- or multipotential precursors giving rise to neurons and glial cells were increased by Emx2 transduction. Consequently, Emx2 is the first transcription factor to promote a multipotential precursor fate at least *in vitro*.

Emx2-antisense transduction

These data raise the possibility that Emx2 might be involved in the instruction or maintenance of multipotential precursor cells in the cerebral cortex. However, Emx2 overexpression might force cells towards a particular fate different from the role of endogenous Emx2. To determine the role of endogenous Emx2, I used a retroviral construct containing Emx2 in antisense orientation (Fig. 2 IV; see e.g. Galileo et al., 1992). Emx2-antisense-transduced cells had a normal morphology and also differentiated in the major CNS cell types (data not shown), suggesting that antisense expression is not harmful to the cells. However I attempted to reveal a reduction of the endogenous Emx2 protein after retroviral transduction of telencephalic cells and by antibody staining could not show a decrease of the endogenous Emx2 protein levels. Contrary to the effect of Emx2-sense transduction, Emx2-antisense resulted in a significant decrease of the size of clones generated by E 14 cortex cells after 7 days *in vitro* (Fig. 11). Interestingly, after Emx2-antisense transduction hardly any clones containing more than 10 cells could be detected (Fig. 11). While large clones were reduced, clones consisting of 2-4 cells increased after infection with the Emx2-antisense virus (Fig. 11). Thus, the exact opposing effects were observed by Emx2-sense transduction, namely an increased number of large clones and furthermore Emx2-antisense transduction that lead to a decreased number of large clones. Interestingly also the progeny generated by infected precursor cells was affected in the opposite way after Emx2-antisense transduction compared to the results obtained with Emx2-sense virus. The use of the Emx2-antisense virus resulted in a decrease of

mixed clones from 35 % in the control infections of these experimental batches to 12 % (Fig. 11). Conversely, the frequency of pure non-neuronal clones and also pure neuronal clones increased after Emx2-antisense transduction. Thus, endogenous Emx2 seems to favor and increase the generation of multiple cell types by cortical precursors.

Analysis of Emx2$^{-/-}$ cortex

The observations of the reduced clonal size after Emx2-antisense transduction demonstrated that the blockade of endogeneous Emx2 by antisense limits the number of progenitors generated by an individual cortical precursor cell. To further characterize the cellular defects of Emx2-deficient cortical cells, I examined the clone size *in vitro* in Emx2-homozygous mutant cortical precursor cells (Pellegrini et al., 1996). Fig. 12 depicts the distribution of the size of clones generated by wildtype (WT) and Emx2$^{-/-}$ cortical cells isolated at E 14 and cultured for 7 div. Interestingly, the proportion of clones composed of only 1-3 cells is increased in the Emx2$^{-/-}$ cultures compared to WT cultures (WT: 46 %, n = 276; Emx2$^{-/-}$: 58 %, n = 113). In contrast the 4-6 cell clones diminished in the Emx2-mutant cortical cultures (WT: 29 %; Emx2$^{-/-}$: 17 %). Surprisingly, however, there is no significant difference in the number of larger clones between WT and Emx2-mutant cultures.

To evaluate whether endogenous Emx2 has similar effects *in vivo* as those obtained by Emx2-overexpression, I examined whether the mode of cell division is affected in Emx2-homozygous mutant cortex (Pellegrini et al., 1996; Bishop et al., 2000; Mallamaci et al., 2000a, b). It has previously been demonstrated that cell divisions orienting parallel to the ventricular surface (see e.g. arrow in Fig. 13) result in the generation of two differently behaving cells and are therefore considered as asymmetric cell divisions (Chenn and McConnell, 1995; Huttner and Brand, 1997). Time-lapse video microscopy revealed that the upper, basal daughter cell of such an asymmetric cell division migrates away and therefore most likely differentiates into a neuron, while the lower, apical daughter remains in the VZ most likely as a precursor cell (Chenn and McConnell, 1995). In contrast, the daughter cells of a division orienting vertically to the ventricular surface both remained in the VZ, suggesting that this orientation of cell division represents a symmetric division producing two identical daughter cells (Chenn and McConnell, 1995). I therefore analyzed the

orientation of cell division stained as a measure of the mode of division. Sections of WT and Emx2$^{-/-}$ mice were stained with DAPI to visualize cells in late anaphase and telophase when condensed chromosomes are separating and quantified the angle of cell division as depicted in Fig. 13. A cell division was considered as symmetrical when DAPI stained metaphase chromosomes were oriented 60° to 120° to the ventricular surface and as asymmetrical with –30° to 30° orientation to the ventricular surface. The remainder was classified as oblique. Interestingly, symmetric cell divisions were strongly reduced and a prominent increase in asymmetric cell divisions was observed in Emx2$^{-/-}$ cortex compared to WT (Fig. 13). Thus, the opposite phenotype in the mode of cell division was observed in Emx2-overexpression experiments (increase in symmetric cell division) and Emx2-null mice (decrease in symmetric cell division). Notably the proportion of oblique cell divisions was not affected, suggesting that the absence of Emx2 results in the transformation of symmetrically to asymmetrically dividing precursors.

In vivo Emx2 overexpression of cortical precursors

To test the *in vivo* Emx2 overexpression of cortical precursors I transduced Emx2 in individual cortical precursor cells of E 14 mouse embryos *in vivo* by means of replication-incompetent retroviral vectors (see Experimental Methods). A comparison of the cell type by morphology revealed a difference between control- and Emx2 injected brains. As an example is shown only the Emx2 injected brain (Fig. 14). I could not detect the presence of large clones in this single experiment. This could mean that the proliferation effect of Emx2 within the VZ *in vivo* is not as strong compared to the *in vitro* experiments. But most exciting, overexpression of Emx2 caused the predominant appearance of a cell type with long processes (Fig. 14). Such long cells could be radial glial cells. These observations suggest a distinct effect of Emx2 cortical precursors to develop the radial glial like shape, although a more detailed analysis is required to dissect the exact cell type.

Emx2 regulates gene expression of the bHLH genes Mash1 and Olig2

The strong effect of Emx2 observed in gain-of-function experiments raises the question how Emx2 regulates other transcription factors of the basic helix-loop-helix (bHLH) family that have been shown to play important roles in the neuronal versus

glial fate decisions (Nieto et al., 2001; Sun et al., 2001; Parras et al., 2002; Zhou and Anderson, 2002). The bHLH transcription factors Mash1, Neurogenin1 (Ngn1), Neurogenin2 (Ngn2) and Olig2 are expressed in the developing telencephalon. We examined Olig2 and Mash1 in the Emx2-gain-of-function condition (Fig. 15). Similar to Mash1, the bHLH gene Olig2 is strongly expressed in precursor cells of the GE (Lu et al., 2000; Takebayashi et al., 2000**;** Fig. 22 A, C). In contrast only a few cells of the cortex express Olig2 (Takebayashi et al., 2000). Interestingly, Olig2 is highly expressed in embryonic neural stem cells (neurospheres), both of the GE and the cerebral cortex (Hack et al., 2004). Since Emx2 is also enriched in those stem cells (Gangemi et al., 2001; Muzio et al., 2002), it is likely to act in the same signaling cascade as Olig2 even though they are expressed in different areas in the telencephalon. Two days after Emx2 transduction I found a significant up-regulation of Olig2 (ctrl: 8.3 ± 2.1; n: 336; Emx2: 18 ± 4.2; n: 172) and Mash1 (ctrl: 14.2 ± 2.2; n: 82; Emx2: 21.7 ± 1.9; n: 132) in cells transduced with the Emx2 virus compared to control infected cells (Fig. 15). In contrast to the pronounced effect on Olig2 and Mash1, the number of Ngn2-immunoreactive cells was not significantly affected after Emx2 transduction (ctrl: 5.5 ± 1.4; Emx2: 4.7 ± 2.4). Overall, these results suggest that Emx2 acts positively on Mash1 and Olig2 in embryonic cortical precursors, while there is no regulation of Ngn2 in embryonic cortical precursors.

Emx2 overexpression interferes with EGF and FGF2 signaling

The findings that Emx2 seems to promote a multipotential precursor fate at least *in vitro* could link to epidermal and fibroblast growth factor signaling. Both, fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF), are known to behave as mitogenic regulators for multipotent stem cell populations in the forebrain (Gritti et al., 1999). In order to examine the potential cross regulation by Emx2 and those growth factors, I treated cultures infected with Emx2 and the control viruses with EGF and FGF2. I found that after 7 div the clones infected with the control virus were significantly larger in presence of EGF or FGF2 than those of untreated cortex (Fig. 16 A). In contrast after growth factor treatment, the mean clone size of Emx2 transduced cells was reduced to values similar to untreated control cultures (Fig. 16 A). These observations suggest the possibility that Emx2 and both, FGF2 and EGF, oppose each other in function. This might explain discrepancies between our (Heins et

al., 2001) and other (Galli et al., 2002) data (see Discussion). Furthermore, in presence of EGF or FGF2 the expression of the bHLH transcription factor Mash1 (EGF: 29 %, n: 89, FGF2: 36 %, n: 129) was significantly increased compared to the control without growth factors (Mash1: 10 %, n: 567; Fig. 16 B). In contrast the proportion of Olig2 positive cells was only increased by FGF2 (31 %, n: 135), whereas EGF had no influence on Olig2 (10 %, n: 58) compared to the untreated control (11 %, n: 337; Fig.16 C). These data show that EGF influences a different population of cells than FGF2, since Olig2-positive cells do not respond to EGF.

Emx1

Cell autonomous function of the transcription factor Emx1

Like Emx2 Emx1 is primarily expressed in the developing cerebral cortex. There it is expressed in both proliferating and postmitotic cells (Simeone et al., 1992b). Thus Emx1 could be interesting as a potential regulator of proliferation and neuronal differentiation. In order to determine the cell-autonomous function of Emx1 in cortical precursor cells I used replication-incompetent retroviral vectors containing the Emx1 full length cDNA in sense orientation, followed by an IRES sequence and the lacZ gene (Fig. 2 V). I infected cells from the cortex isolated at E 14 with the control and Emx1 virus (Fig. 2 V, see Experimental Methods) and analyzed them after 7 days *in vitro*.

Clone size after Emx1 overepression

In contrast to the Emx2 data there was not any difference in the clone size with Emx1 overexpression vector (Fig. 17) or control vector (Fig. 17) after seven days *in vitro*. Also no change in the size of clones, i.e. the number of cells per clone, was observed in Emx1-sense infected cells of the GE, where Emx1 is normally not expressed (Simeone et al., 1992). The comparison of the size and composition of the clones (Fig. 17) revealed two clear differences between Emx1 and control clones. Although Emx1 did not have a general effect on the clonal size (Fig. 17), it caused the appearance of some unusually densely packed large clones, typically containing between 18 and 28 cells. Even though a few of these were found to contain some neurons, cells of the remainder that were immunopositive for nestin showed an

undifferentiated character. Such large undifferentiated clones have never been found among the control clones. The prominent phenotype of large undifferentiated clones is reflected in a significant increase (see Experimental Methods) in the proportion of nestin positive cells generated during Emx1 transduction compared to control clones. In correlation to this, cultures overexpressing Emx1 significantly yielded less neuronal or glial clones compared to the control. These data suggest that Emx1 appears to keep a subpopulation of precursor cells in an undifferentiated state. A similar phenomenon of a reduced neuronal lineage and increased precursor cell pool was found in the Pax6 loss-of function.

Pax6

Pax6 transduction in cells from WT cortex and GE

The reduced neuronal lineage in the Pax6-loss-of-function (Heins et al., 2002) implies that Pax6 is necessary at least for part of the neuroblasts in the cortex. Since Pax6 expression starts early in cortical development, it is conceivable that changes in the progeny of Pax6-deficient cells might be secondary to earlier defects in the *Sey/Sey* cortex (Stoykova et al., 1996; Stoykova et al., 2000; Toresson et al., 2000). To evaluate the direct role of Pax6 on the neuronal cell lineage, I performed gain-of-function experiments using replication-incompetent retroviral vectors containing the Pax6 full length cDNA, followed by an IRES sequence and the lacZ gene (Fig. 2 VI and 18 A). First I examined the reliability of the co-expression of Pax6 and the marker molecule β-galactosidase after retroviral transduction of telencephalic cells. Double-immunocytochemistry with antisera directed against Pax6 and β-galactosidase confirmed reliable co-expression of the marker molecule and the Pax6 protein (Fig. 2 B–D). Further analysis of more than 50 clones revealed co-expression of the Pax6 protein and β-galactosidase in more than 95 % of the clones. Since Pax6 dosage is known to be important (Schedl et al., 1996), I determined the increase in Pax6-immunoreactivity and found that virally transduced cells of the cortex showed a 3.6x stronger Pax6-immunoreactivity compared to endogenous Pax6 levels which is consistent with the expression level found in the Emx2 (4x stronger) transduced cells when those where compared to the endogenous Emx2 expression (see Experimental Methods). The vast majority of Pax6-transduced cells were neurons with a typical

neuronal morphology containing either β-tubulin-III or NeuN (Fig. 18 D, E and F) with a significant increase in pure neuronal clones after Pax6 transduction (70 % versus control: 54 %) and a corresponding decrease in the number of pure non-neuronal clones (5 % versus control: 17 %). Interestingly, the mixed clones were not affected in their frequency, but in their composition: They contained more neurons after Pax6 transduction (60 % versus control: 40 %). Indeed, the overall proportion of neurons amongst all cells increased by 25 % while neuronal clones only increased by 15 % after Pax6 transduction. The neurogenic effect of Pax6 transduction was accompanied by a reduction in proliferation indicated by a reduced size of clones (Fig. 19, Table 1 A) that was mostly due to a prominent increase in the 1-2 cell clones after Pax6 transduction (Fig. 19: 72 % compared to 47 % in the control infected WT cells). Indeed, single cell clones are typically neurons, consistent with the increase in pure neuronal clones after Pax6 overexpression. In contrast, the mean clone size of cells from the *Sey/Sey* cortex infected with the control vector was significantly larger than the clone size of the WT cortex (Fig. 19, Table 1 A). However, Pax6 transduction of *Sey/Sey* cortex cells reduced the clone size to values similar to WT cortex cells transduced with Pax6 (Table 1 A). Pax6 transduction is therefore able to affect cortical progenitors that do not express endogenous Pax6. By contrast, no effect of Pax6 transduction on the clone size of GE cells was observed (Table 1 A) and these cells were also not affected in their ability to generate neurons (data not shown). In summary, the clone size and the cell types generated are affected in opposite ways by Pax6-loss-of-function and Pax6-gain-of-function and support a neurogenic role of Pax6.

Mode of cell division

Cells of the Sey-mutant infected with the control virus generated less than half the number of single cell clones observed in control virus infected cells of WT cortex and instead of that generated larger clones (data not shown). Thus, as for the neurogenic role of Pax6, exactly the opposite effects were observed in cortical precursor cells after Pax6 transduction and in the Pax6-loss-of-function in the Sey/Sey mutant cortex. These data are consistent with an effect of Pax6 on the mode of cell division, predicting an increase in symmetric cell divisions in the Sey/Sey mutant and a corresponding increase in asymmetric cell divisions after Pax6 transduction. The

increase in symmetrical divisions in absence of functional Pax6 proteins is controversial since recent observations suggest the opposite, namely an increase in the proportions of asymmetrical divisions from E 12.5 to E 15.5. Our findings of the increased clonal size and higher presence of even numbered clones (Fig. 19, Table 1) in the Pax6-mutant Sey are difficult to explain simply by an increased number of precursor cells. Rather, an increase in symmetric cell divisions could account for clones consisting of more than 12 cells. To test this suggestion, I used the same assay based on the dilution of BrdU in dividing cells that I used for the analysis of the Emx2 overexpressing cells (see Experimental Methods; Malatesta et al., 2000b). This analysis revealed a completely different distribution of control and Pax6 mutant clones (Fig. 20). The majority of control clones showed cells with different BrdU-content, consistent with a predominantly asymmetric mode of division. Indeed, my data are in good agreement with previous observations of asymmetric cell divisions in living cortical slices (Chenn and McConnell, 1995). In contrast, 31 % of Pax6 mutant cells within a clone did not show any strong difference in their BrdU-content. Thus, most cells having a non-functional Pax6 protein completed the same number of divisions and thus divided symmetrically. The same effect was also observed for medium-sized clones containing 5-10 cells. While 73 % of the control clones with 5-10 cells show a difference of at least two rounds of division among their cells, 11 % of the Pax6 mutant clones show such a difference. Taken together, these results suggest a positive influence of Pax6 on the asymmetric mode of cell division in cortical precursor cells, i. e. the opposite effect observed with Emx2.

Pax6 induces neurogenesis in astrocytes from postnatal cerebral cortex

As Pax6 expression strongly decreases when radial glial cells transform into astrocytes (Walther and Gruss, 1991; Götz et al., 1998), I was interested in testing whether astrocytes that do not express endogenous Pax6 (Fig. 21) might be induced to generate neurons when forced to express Pax6. Astrocytes isolated from postnatal cortex were propagated in fetal calf serum (FCS) containing medium (FM), infected with control and Pax6-sense virus and switched to chemically defined medium one day after infection to allow neuronal differentiation. To examine the purity of these

cultures I performed double-stainings with the GFAP-, O4-, Fibronectin- (FN) and β-tubulin-III-antisera and DAPI to reveal all cells. This analysis showed that 90 ± 2 % (n = 235) cells were GFAP-positive, 2 ± 2 % (n = 235) β-tubulin-III-immunoreactive, 3 ± 3 % (n = 150) O4-positive and 4 ± 4 % (n = 26) were FN-immunoreactive. Most GFAP-, O4- and FN-positive cells proliferated and incorporated BrdU in the culture medium or were Ki67-immunoreactive (Gerdes et al., 1997). When I checked the identity of retrovirally infected cells two days after infection, at the earliest time β-galactosidase levels is high enough for detection, almost all clones were GFAP-positive (ctrl: 99 ± 1 %, n = 131). Interestingly, clones infected with the Pax6-virus already after 2 days contained fewer GFAP-positive cells (Pax6: 86 ± 1 %, n = 161). When clones were examined 7 days after infection, a minor fraction of GFAP-positive cells infected with the control virus had turned into pure neuronal clones (12 %, see Fig. 21), consistent with previous results (Hildebrand et al., 1997; Laywell et al., 2000). In contrast, GFAP-positive cells infected with Pax6-encoding virus generated 67 ± 12 % clones containing exclusively β-tubulin-III-positive neurons (Fig. 21). The β-tubulin-III-positive cells also contained NeuN, another neuron-specific protein (Mullen et al., 1992) and some were GABA- or glutamate-immunoreactive, suggesting that these cells are indeed neurons. As observed with cells from embryonic cortex, Pax6 transduction of astrocytes also resulted in a reduced clone size due to an increase in the number of single cell clones (Fig. 19, Table 1 B). Despite the large number of neuronal clones, some astrocytes (30 %) do not generate neurons after Pax6 transduction. Pax6-immunoreactivity showed that cells of the β-tubulin-III-negative clones also contained high amounts of Pax6 protein, suggesting that the lack of response is not due to the loss of the Pax6 protein. Interestingly most of these cells did not contain GFAP despite their astroglial morphology and thus might be in the process of conversion to a neuronal fate.

Changes in bHLH transcription factors in the Pax6-loss-of-function and -gain-of-function condition

The prominent neurogenic effect of Pax6 observed in loss-of-function and gain-of-function experiments raises the question how Pax6 interacts with transcription factors of the bHLH family that have been shown to play important roles in the neuronal versus glial fate decisions (Nieto et al., 2001; Sun et al., 2001). I therefore examined

Neurogenin (Ngn) 1 and 2, Mash1 and Olig2 in the Pax6-loss-of-function and -gain-of-function condition (Fig. 22). Consistent with previous results obtained by in-situ hybridization (Stoykova et al., 2000; Toresson et al., 2000), I found a marked reduction of Ngn2 (and less pronounced of Ngn1, data not shown) in the *Sey/Sey* cortex by immunohistochemistry (Fig. 22 A, B). Similar to Mash1, Olig2 is restricted in its expression to the GE in WT mice (Takebayashi et al., 2001) and expands ectopically into the cortex in *Sey/Sey* mutants (Fig. 22 C, D). Conversely, a significant up-regulation of Ngn2 (21.1 ± 3.5 %, n = 1394 versus control: 9.8 ± 1.2 %, n = 1083) and down-regulation of Mash1 (1.2 ± 0.5 %, n = 1406 versus control: 7.3 ± 1.2 %, n = 832) was found in cells transduced with the Pax6 virus compared to control infected cells 2 days after infection (Fig. 22 E). Less than half of the control-infected Ngn2-containing clones were pure and only contained Ngn2-positive cells (46 %) while this proportion increased to 58 % after Pax6-overexpression. In contrast to the pronounced effect on Ngn2, the number of Ngn1-immunoreactive cells was not significantly affected after Pax6 transduction (4.7 ± 2.4 %, n = 801 versus control: 5.5 ± 1.4 %, n = 463). Cortical cells also express Mash1 *in vitro* (Fig. 22 E), consistent with previous *in vivo* data (Fode et al., 2000), and these were significantly reduced after Pax6-transduction (Fig. 22 E). In these experiments Olig2-immunoreactive cells could not be detected in control or Pax6-infected cortical cells. Astrocytes from postnatal cortex differed in their bHLH expression pattern in that most of them contained Mash1 and Olig2, but Ngn2- (or Ngn1-) immunoreactivity could not be detected (Fig. 22 F). Double-staining with GFAP confirmed that Mash1 and Olig2 are expressed in GFAP-positive cells *in vitro*. Interestingly both transcription factors were down-regulated upon Pax6-transduction (Fig. 22 F), but Ngn2- (or Ngn1-) immunoreactivity was not induced. Taken together, these results suggest that Pax6 negatively acts on Mash1 and Olig2 in embryonic and postnatal cortical precursors, while the positive regulation of Ngn2 is restricted to embryonic cortical precursors.

The Pax6 mutant Sey generates more neural multipotent precursor cells

The prominent increase of mitotically active precursor cells in the *Sey/Sey* mutant (Götz et al., 1996), the expansion of Olig2 in the *Sey/Sey* telencephalon and the

promotion of symmetric divisions raise the possibility that deficiency of Pax6 impedes differentiation maintaining a precursor cell type that can form neurospheres. Indeed while neuronal progenitors were reduced in the absence of functional Pax6 proteins, we observed a converse increase in non-neuronal progenitors that did not generate GFAP-positive astrocytes, but rather remained as immature precursors in the cell culture without the addition of growth factors (data not shown). In addition in neurosphere cultures Pax6-mutant cortex cells generate more neurospheres than WT cortex (Fig. 23 B), suggesting an increase in multipotent precursors in the absence of Pax6. These *in vitro* data therefore suggest that Pax6 seems to affect the transition of an immature precursor to a neuron-restricted progenitor rather than acting as a neuron-glia fate switch (Fig. 24).

Discussion

The control of proliferation of telencephalic precursor cells has fundamental implications for the embryonic development of the central nervous system. The role of intrinsic genetic determinants in this process is largely unknown. In this thesis I analyzed which aspects of the precursor cells in the telencephalon are affected by the transcription factors Emx1, Emx2 and Pax6. In the first part of this work I examined the role of Emx2 in telencephalic precursor cells in a combination of gain-of-function and loss-of-function approaches *in vitro* and *in vivo*. In this context I made the following five observations:

First I found that Emx2, Emx1 and Pax6 transduction are able to affect precursor cells in a region-specific way from the cerebral cortex, i.e. the dorsal, but not the ventral telencephalon.

Second, Emx2 transduction increases the number of cells generated by a single progenitor. Furthermore, inhibition of endogenous Emx2 by antisense Emx2 mRNA limits the number and fate of progenitors generated by an individual cortical precursor cell. In contrast to Emx2, Pax6 decreases proliferation, while Pax6 deficient cells generate larger clones *in vitro*.

Third, Emx2 promotes the symmetric mode of cell division. In the Emx2-loss-of-function condition asymmetrical cell divisions increased in the cerebral cortex *in vivo*, while symmetrical cell divisions are increased *in vitro* after Emx2 transduction *in vitro*. Pax6 deficient cells show more symmetrical cell divisions while Pax6 promotes asymmetric cell divisions *in vitro*. This shows the antagonistic nature of Emx2 and Pax6 which is also reflected in their expression areas.

The fourth observation is that Emx2 *in vitro* endows cortical precursor cells with the capacity to generate multiple cell types, including neurons, astrocytes and oligodendrocytes. Emx1 keeps cells in an undifferentiated state but does not have any general influence on proliferation, while Pax6 increases the proportion of neurons and can also convert astrocytes to neurons.

The fifth point is the up-regulation of the bHLH transcription factors Olig2 and Mash1 upon Emx2-transduction. In contrast to this, Pax6 negatively influences those transcription factors and specifically up-regulates Ngn2.

Taken together, Emx2 and Pax6 have opposing roles in cell proliferation, mode of cell division and cell fate.

Technical considerations

Most methods for blocking gene expression *in vivo* affect many cells in the tissue. Thereby this can affect the phenotype of individual cells in indirect ways. The retroviral system that we have chosen in our experiments circumvents this limitation by generating small clones of transgenic cells in a WT environment. This allows the analysis of direct, cell autonomous effects after introduction of a gene. However, the mechanism of post transcriptional gene silencing is not understood well. RNA silencing is shown to be part of an adaptive antiviral defense in animal cells. RNA silencing processes result in the sequence-specific degradation of RNA and post-transcriptional gene silencing (Li et al., 2002). Unfortunately we could not show that the presence of antisense sequences decreased Emx2 levels, since there were no reliable antibodies available. Also western blot analysis was ruled out due to the transduction efficiency of the retroviral vectors.

Proliferative role of Emx2

During neurogenesis in the cerebral cortex from E 12 – E 18, the most numerous clones are single-cell neuronal clones (Price and Thurlow, 1988; Grove et al., 1993; Williams et al., 1995; Qian et al., 1998; Shen et al., 1998; Hajihosseini and Dickson, 1999). This clone type *in vitro* is dramatically reduced by overexpression of Emx2 and replaced by larger clones, mostly containing more than 15-20 cells. Since we could not detect any differences in cell survival, these data suggest that Emx2 endows cortical precursor cells with a large proliferative potential. Two mechanisms seem to account for this: Emx2-sense transduced precursor cells continue to divide longer, i.e.

complete more rounds of cell divisions and perform a larger number of symmetrical divisions.

Cortical precursor cells normally stop proliferating after a few cell divisions *in vivo* (Grove et al., 1993; Reid et al., 1996; Takahashi et al., 1999) and *in vitro* (Williams and Price, 1991; Götz et al., 1995; Williams et al., 1995; Hajihosseini and Dickson, 1999). The suggestion that cortical precursors complete a precisely limited number of cell cycles (Takahashi et al., 1999) is further supported by our observation that precursors isolated at increasingly later stages generate fewer descendants. The mean clone size decreases from about 8 cells, if cells are isolated at E 12, to 4 cells if they are isolated at E 16. These data are consistent with an intrinsic timer limiting the total proliferative capacity of cells (Durand and Raff, 2000). It might be that Emx2-sense transduction can somehow reset this timer, since the clones generated by Emx2-transduced cells exhibit the same large size. This is independent of the time of their isolation at early (E 12) or late (E 16) stages of development (see Fig. 6). A potential implication of Emx2 in the regulation of cell division in the cortex is further consistent with the gradual decrease in Emx2-expression after E 12 in the cortical VZ (Gulisano et al., 1996). Moreover, the reduced thickness of the Emx2-mutant cortex might result from a reduced number of cell divisions (Mallamaci et al., 2000; Tole et al., 2000). The cellular mechanisms that underlie the decreased size of the Emx2 mutant medial cortex and of the cerebral hemispheres in general could be explained by our findings. Our data support a simple explanation that Emx2 step by step might regulate the differentiation of a multipotent precursor cell to a more committed precursor cell. In the absence of Emx2, proliferation is reduced as the antisense and clonal analysis of the Emx2 homozygotes embryos indicated.

For example, smaller cerebral hemispheres, a sign of reduced proliferation are also seen in mice, where the winged helix transcription factor BF-1 is knocked-out (Xuan et al., 1995). In these mice cerebral hypoplasia results from reduced cell proliferation together with impaired differentiation that depletes the precursor pool. All in all, these results implicate Emx2 in the regulation of the number of precursor cell divisions in the cerebral cortex.

In contrast to BF-1 and other factors that influence proliferation, Emx2 does not simply induce proliferation of precursor cells but also allows the differentiation of some descendants along the neuronal, astroglial and oligodendroglial lineage

(Fig. 9, 24). This effect is notably different from the effects of growth factors, like e.g. EGF and FGF2, which instruct proliferation of precursor cells (Reynolds and Weiss, 1992; Johe et al., 1996; Qian et al., 1998; Hajihosseini and Dickson, 1999). Differentiation is blocked by the presence of these factors; their withdrawal is required to allow differentiation. Actually a dramatic proliferation decrease is taking place in cultures treated with either of these factors and transduced by Emx2 retroviral vectors. This is particularly interesting since each of those factors increase proliferation and only their interaction abolishes this effect. Therefore the anti-proliferating effect of Emx2 in the presence of EGF and FGF2 can directly be related to one of these factors and is supported obviously by data from Gangemi et al., 2001, since they found Emx2 to be a stimulus of an asymmetric division for neural stem cells. Since those cells were treated with EGF and FGF2, this could be caused by the same mechanism shown in our work.

Rather there is the FGF2 that could act on a broader spectrum of cells, whereas EGF influence only cells that upregulate Olig2 but did not express Olig2 before. It has been reported that FGF-8b beads suppress Emx2 expression (Crossley et al., 2002). More recently it was found that that *in vivo* overexpressing of Emx2 altered the area map, but only when ectopic Emx2 overlapped the FGF8 source (Fukuchi-Shimogori and Grove, 2003). Furthermore, they found that FGF8 levels were decreased by excess Emx2, and increased in mice lacking Emx2. This shows that Emx2 patterns the neocortex by regulating FGF positional signaling. Therefore Emx2, as intrinsic fate determinant, is directly involved in the interaction between extrinsic signals like FGF-8b and probably other growth factors. In this, Emx2 interaction shows a dual role: It not only influences proliferation but it seems to orchestrate both, the proliferation and the subsequent differentiation. As Emx2 Emx1-transduction only influences precursor cells region-specifically of the cortical area, while cells from the GE do not respond to ectopic Emx1 expression. Most surprising, however, is the finding that cortical cells seem to respond to Emx1 overexpression with an increase of precursor cell clones but without increasing proliferation by an increase of the clonal size. This result is consistent with the fact that Emx1-knock-out animals do not have particular proliferation defects (Guo et al., 2000). Probably in those animals endogenous Emx2 can rescue the function of Emx1. Emx1 could have a function for a distinct precursor cell type, since its gain-of-function data increase large unspecified precursor cell clones that also increased after Emx2-antisense transduction. This suggests that Emx2

can not mask Emx1 function. These experiments therefore suggest that Emx1 instructs cells of the cerebral cortex to maintain an un-restricted precursor cell fate without having an influence on proliferation like Emx2. Conversely Emx1 is also expressed in postmitotic neurons. It is possible that Emx1 protein in those cells is inactive, due to factors blocking the function or by location of the transcription factor in the cytoplasm.
Overall our data indicate that Emx2 seems to endow cortical precursor cells with a large proliferative potential.

Emx2 promotes symmetric cell divisions

The maintenance of a proliferative status in cortical precursor cells is not sufficient to explain the generation of more than 20 descendants of a single precursor cell during 7 div, assuming an average the cell cycle length between 12-15 hours (e.g. Takahashi et al., 1993; Cai et al., 1997; Haydar et al., 1999). There can be at least four additional reasons to explain these proliferation phenomena: reduced apoptosis, prolonged proliferation, an alteration of the cell cycle length and more symmetrical cell divisions. It is well known that apoptosis is of great importance for the development of the telencephalon. For example clonal lineage analysis revealed that nearly 60 % of the constitutively proliferating precursor cells undergo cell death at E 14 (Morshead et al., 1998). Our data could not reveal an influence of Emx2 on cell death. The second reason appears to be a mechanism that accounts for the strong influence on proliferation: Emx2-sense transduced precursor cells continue to divide longer, i.e. complete more rounds of cell divisions. The third possibility that the cell cycle length is responsible for the proliferation phenomena, can also be ruled out, since previous studies indicated that the length of the cell cycle is not altered in the Emx2-mutant cortex (Mallamaci et al., 2000b). Thus in the large clones generated by Emx2-transduced precursor cells, some cells should have gone through rounds of symmetrical divisions. Indeed, a quantitative assay for symmetric and asymmetric cell division (Malatesta et al., 2000) showed that the majority of cortical precursors *in vitro* divide asymmetrically. In contrast after Emx2-transduction most cells divide symmetrically. Moreover, the lack of clones containing more than 10 cells after Emx2-antisense transduction implies that in the regulation of the mode of cell division endogenous Emx2 is necessary. Consistent with these *in vitro* data, the loss of Emx2

in the cortex *in vivo* results in the increase of the number of cells dividing parallel to the ventricular surface, i.e. in a supposed asymmetrical mode (Chenn and McConnell, 1995).

These data are in good agreement with the earliest defects observed in the Emx2-mutant mice. A reduction in size of the cortical hemispheres was already observed at E 13 (Bishop et al., 2000; Mallamaci et al., 2000; Tole et al., 2000). This parameter is thought to be influenced by symmetrically dividing early precursor cells, the so-called 'founder cells', which determine the size of the progenitor pool (Rakic, 1995; Chenn and McConnell, 1995; Vaccarino et al., 1999). The cellular mechanisms that underlie the decreased size of the Emx2 mutant medial cortex and of the cerebral hemispheres in general, could be explained by our findings. Our data support a simple explanation that Emx2 might step by step regulate the differentiation from a multipotent precursor cell to a more committed precursor cell. In absence of Emx2, proliferation is reduced as our antisense approach and also the clonal analysis of the Emx2 homozygote embryos indicated. A similar phenotype as the Emx2 homozygotes brains is also found in the knock-out mice, where the winged helix transcription factor BF-1 is knocked-out (Xuan et al., 1995). These *in vivo* results therefore point to a similar role of Emx2 in early proliferative symmetric divisions of cortical precursors, as determined in our gain-of-function and loss-of-function *in vitro* experiments. All in all, these data suggest that endogenous Emx2 plays a role in the regulation of the mode of cell division, *in vivo* favoring symmetrical divisions oriented vertically to the ventricular surface.

Emx2 instructs a broader potential in cortical precursor cells

Emx2 does not simply induce proliferation of precursor cells but also allows the differentiation of some descendants along the neuronal, astroglial and oligo-dendroglial lineage. While precursor cells prior to or during early stages of neurogenesis in the cerebral cortex have the potential to generate neurons and glial cells, most precursor cells during neurogenesis are restricted to the generation of a single cell type (Krushel et al., 1993). Emx2-sense transduction, however, resulted in an increased number of clones containing neurons and glial cells even in cultures from

cortex at late stages of neurogenesis. Thus, the transduction with viral vectors containing the Emx2-sense construct increased the potential of cortical precursor cells, while transduction with the Emx2-antisense construct restricted their potential since it decreased the number of mixed clones. These results are highly relevant for the attempts to expand cells of a high proliferative potential *in vitro*. Indeed, Emx2 expression was detected in neurosphere cells, an *in vitro* model of multipotential stem cells (Gangemi et al., 2001). Furthermore strong Emx2 expression was observed in two regions in the adult brain, where neurogenesis occurs, namely in stem cells located in the dentate gyrus (Mallamaci et al., 1998) and in the adult subventricular zone (SVZ) (Gangemi et al., 2001). The presence of Emx2 protein in neurosphere cells and in the SVZ implies a role of endogeneous Emx2 in the regulation of both the proliferative and the cell fate potential of cortical precursor cells. Interestingly, Emx2 expression is induced in stem cells from areas which never express Emx2 like the spinal cord. Furthermore we observed that the majority of cell in neurospheres express the bHLH transcription factor Olig2 (Hack et al., 2004). In the spinal cord Olig2 is specifically expressed in zones of neuroepithelium from which oligodendrocyte precursors emerge, as well as in those precursors themselves (Takebayashi et al., 2000). Moreover, it couples there neuronal and glial subtype specification together with proneural genes to comprise a combinatorial code of transcription factors, necessary for the specification of neurons, astrocytes, and oligodendrocytes (Zhou and Anderson, 2002). The up-regulation of Olig2 that we found in neural precursor cells after Emx2 transduction also indicates the close relationship of those transcription factors in regard to their function.

These findings also relate to the question whether a broad potential of precursor cells is simply due to the absence of restrictive cues or to positive signals that instruct and/or maintain a large potential. There is the possibility that the progenitor heterogeneity, that can represent cells of different competence, can either respond to Emx2 overexpression having the right cues leading to proliferation or being just uninfluenced. At E 14 in the cortex most cells are competent to respond to high doses of Emx2 protein. Therefore many cells can keep a multipotential state. For example Notch functions as an instructor and/or preserver of a large potential. The Notch target Hes1 has been implicated in the maintenance of multipotential stem cells in the CNS (Nakamura et al., 2000). This supports the view that instruction of an ‘immature’ precursor state is accompanied by a multipotential precursor state. Originally

discovered in Drosophila (Artavanis-Tsakonas et al., 1999), Notch signaling has been shown to be involved in the differentiation of both neurons and glia in the vertebrate retina and in the developing telencephalon. Notch activation also supports differentiation along the glial lineage (Dorsky et al., 1995; Bao, Z. Z. and Cepko, C. L., 1997; Furukawa et al., 2000; Gaiano et al., 2000; Hojo et al., 2000; Morrison et al., 2000). In particular, Notch activation in the developing cortex promotes radial glia and astroglial differentiation (Gaiano et al., 2000). Previously it was shown that radial glia cells have the potential to generate neurons, precursor cells and astrocytes (Malatesta et al., 2000a). Preliminary observations of the Emx2 *in vivo* injections indicate that there are more radial glia like cells after Emx2 transduction. These results are clearly different from the phenotype induced by Emx2 manipulation of precursor cells *in vitro*. Emx2 rather inhibits the differentiation of pure glial clones, as concluded from the Emx2-antisense experiments, and favors the differentiation of cortical precursors along multiple lineages. In addition, Emx2 enhances the symmetric mode of cell division, thus combining traits of early neuroepithelial precursor cells that exhibit a broad fate and a large proliferative potential. Therefore our results provide the first evidence of a single transcription factor as a unique positively instructive cue for precursor cells with a large proliferative and differentiative potential in the developing cortex. Emx2 could be required to enable cortical precursor and adult subventricular cells (Gangemi et al., 2001) to divide symmetrically and could provide a cell the cue to stay in an uncommitted state. Whereas Emx2 favors symmetric cell divisions maintaining cells in an undifferentiated state, Pax6 exerts exactly the opposite effect, i.e. it promotes asymmetric cell divisions and induces differentiation.

Pax6 is required for one of two distinct neurogenic lineages in the cerebral cortex

Our results demonstrate that Pax6 directs precursor cells from the embryonic and postnatal cerebral cortex *in vitro* towards neurogenesis. In embryonic cortex the Pax6-dependent neurogenic lineage (Heins et al., 2002) most likely corresponds to the recently discovered neurogenic population of radial glial cells (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001). Recent experiments showed that Pax6 is localized in cells with radial glial properties such as RC2- and GLAST-

immunoreactivity and hGFAP-GFP-expression (Götz et al., 1998; Heins et al., 2002). Furthermore our experiments clearly indicated that the only precursor cell population affected by the Pax6-loss-of-function shows the characteristics of radial glial cells. These data are also in line with previous results on cell-autonomous defects in radial glia morphology and cell cycle in the Pax6-mutant cortex (Götz et al., 1998; see also: Caric et al., 1997).

Pax6 is a potent neurogenic gene and regulates bHLH transcription factors

Consistent with its dorsally restricted expression in the telencephalon (Stoykova et al., 2000), the gain-of-function experiments showed a region-specific neurogenic role of Pax6 in cells from its expression territory, but had no effect on cells from the ventral telencephalon. This implies the lack or distinct regulation of essential co-factors or target genes of Pax6 in the developing GE and further supports the region-specific function of Pax6 in the dorsal telencephalon in the same way like Emx1 and Emx2. Surprisingly the gain-of-function experiments show a prominent neurogenic effect even in astrocytes from postnatal cortex. That effect could not be detected by over-expression of Emx2 (data not shown).

After Pax6-transduction more than half of all infected GFAP-positive cells generate neurons, clearly demonstrating that astrocyte precursors from postnatal cortex, i.e. normally non-neurogenic precursors, have been converted to a neurogenic fate by Pax6 transduction. Extrinsic stimuli exert only a weak neurogenic effect on astrocytes, consistent with a low degree of contamination of stem cells from the adult subventricular zone (Hildebrand et al., 1997; Doetsch et al., 1999; Laywell et al., 2000). The transduction of astrocytes with transcription factors known for their neurogenic role in development, namely members of the bHLH proneural transcription factor family, achieves hardly any or no neurogenesis (Farah et al., 2000; Sun et al., 2001). For example the Emx2-sense transduction did not have any effect on astrocyte precursor cells of the postnatal cerebral cortex. Recent experiments revealed that the bHLH transcription factor Ngn1 inhibits glial and promotes neuronal fate by two molecularly distinct mechanisms (Sun et al., 2001). But while Ngn1 is able to induce a neurogenic fate in precursors from the embryonic cortex, it only influenced the GFAP-levels and morphology in astrocytes (Sun et al., 2001). Thus both Pax6 and

Ngn1 enhance neurogenesis in embryonic precursors, but only Pax6 is able to initiate the complete neurogenic program in astrocytes.

Ngn1 might be responsible for the neurogenic lineage generated by non-radial glial precursors not affected in the Pax6-mutant cortex. This would be consistent with the failure of Pax6 to regulate Ngn1 in gain-of-function experiments and the residual Ngn1-expression in the Pax6-mutant cortex. In contrast, Pax6 positively regulates Ngn2 [see also (Marquardt et al., 2001; Scardigli et al., 2001)], but negatively affects Mash1 in cortical embryonic precursor cells and astrocyte precursors. Since Mash1 is not able to rescue a decrease of neurogenesis in the embryonic cortex (Fode et al., 2000, and our data), it might play other than positive neurogenic roles in the cortex. Interestingly Mash1 is up-regulated by Pax6 in the retina where Pax6 instructs multipotent fate (Marquardt et al., 2001). This could explain the opposite phenotype of Pax6-mutation in the retina (loss of multipotent precursors and decreased proliferation) and in the cortex (increased proliferation (Götz et al. 1998) and multipotent precursors; Fig. 22). Thus, the combinatorial network of bHLH transcription factors might implement the close link between patterning and cell fate in vertebrate CNS (Götz and Campbell, 2002).

It has recently been demonstrated that the transcription factor genes Emx2 and Pax6, that are expressed in the developing cerebral cortex along two complementary gradients, are essential for the shaping of the cortical area at late developmental ages, when cortical neurogenesis is almost completed (Bishop et al., 2000). It also has recently been shown that Emx2 and Pax6 are necessary for the establishment of their own specific expression pattern and are able to down-regulate each other (Muzio et al., 2002). These results suggest that pre-neurogenic cortical regionalization may rely on reciprocal interactions between these two transcription factors. Furthermore an intrinsic factor COUP-TFI has been identified as a regulatory factor for Pax6 and Emx2 that is important for early neocortical regionalization (Zhou et al., 2001). This could be an essential mediator between both intrinsic factors Pax6 and Emx2.

Pax6 is required for the transition from multipotent to neuronal precursors

While neuronal progenitors are reduced in the absence of Pax6, we observed a converse increase in non-neuronal progenitors that do not generate GFAP-positive

astrocytes, but remain as immature precursors in dissociated cell cultures without the addition of growth factors (Heins et al., 2002). Previous results on cell-autonomous defects in radial glia morphology and cell cycle in the Pax6-mutant cortex are also in line with these experiments (Götz et al., 1998; see also: Caric et al., 1997). Here it was reported that short pulse of BrdU given to mice aged between E 13.5 and E 16.5 seems to label more cells in the Pax6-deficient cortex than in WT cortex. This could be sign of a generally increased precursor pool, a shortened S-phase of the cell cycle or could be due to the presence of a special precursor cell type.

Indeed in neurosphere cultures Pax6-mutant cortical cells generate more neurospheres than the WT cortex (Fig. 23), implying an increase in multipotent precursors in the absence of Pax6. These *in vitro* data therefore suggest that Pax6 affects the transition of an immature precursor to a neuron-restricted progenitor rather than acting as a neuron-glia fate switch (Fig. 24). Indeed, in contrast to the premature astrocyte differentiation in the cortex of *Ngn2;Mash1* double mutant mice (Nieto et al., 2001), astrocyte differentiation is normal in the *Sey/Sey* cortex as assessed by the markers GFAP, TN-C, S100β and synemin (data not shown, see also: Stoykova et al., 1997). Interestingly the two mutants share the phenotype of an early increase in subventricular zone (SVZ) precursors, i.e. cells that undergo cytokinesis in the parenchyma (Schmahl et al., 1993; Caric et al., 1997; Stoykova et al., 1997). SVZ precursors do not contain the radial glial marker GLAST (Malatesta et al., 2003). Therefore the enlarged SVZ of *Sey/Sey* mice should contain the increased population of GFP- and GLAST-negative precursors comprising the abnormal potentially multipotent subpopulation that is increased in the Pax6-mutant. Indeed alterations in gene expression occur in the Pax6-mutant SVZ (Tarabykin et al., 2001), one of the regions where stem cells are located. The SVZ phenotype of Pax6-mutant mice is particularly intriguing in the light of the SVZ location of stem cells at later developmental stages and implies Pax6 as a simultaneous negative regulator of stem cell and SVZ fate (see Fig. 24).

Implications of this work for neural stem cell studies

The gain-of-function studies showed that the three transcription factors Emx1, Emx2 and Pax6 are important intrinsic fate determinants in precursor cells of the developing

telencephalon. In the developing telencephalon they are expressed in more than half of the precursor cell population in the VZ. Emx2 region-specifically controls the differentiation process of a multipotent precursor whereas Pax6 region-specifically induces the following decisions to enter the neuronal lineage, even in astrocytes. These roles of Emx2 and Pax6 as important intrinsic fate determinants for the regulation of cell differentiation and development could have implications for stem cell based therapies. In this regard especially the neurogenic function of Pax6 illustrates interesting implications of our results. In the cerebral cortex, Pax6 seems to function primarily in cells with astroglial traits during development and postnatal stages. Besides radial glial cells generating neurons (Malatesta et al., 2000; Noctor et al., 2001), cells with astroglial traits are responsible for the neurogenesis in the two regions of the adult mammalian CNS that continue to generate neurons (Doetsch et al., 1999; Seri et al., 2001). Moreover, expanded glial cultures from the embryonic GE generate region-specific neuronal subtypes (Skogh et al., 2001). This evidence suggests a general role of cells with astroglial features as neuronal precursor cells and my results imply Pax6 as a critical neurogenic determinant in these cells. Since Pax6-transduced astrocytes *in vitro* are able to generate neurons, the loss of Pax6 expression in most astrocytes of the adult CNS might be one reason for the loss of their neurogenic lineage. Therefore manipulation of Pax6 within the neural stem cells could increase the ability of these cells to generate large numbers of neurons (Hack et al., 2004) that might be useful for treating neurodegenerative disorders or repairing the injured nervous system. Emx2 could be an essential fate determinant of stem cells without restricting their potential. Thus these data are an important step in the identification of the molecular signals able to reinitiate the former neurogenic potential in astrocytes and should ultimately help to utilize the most abundant cell type in the mammalian CNS for the replacement of degenerating neurons. Recent findings show that human embryonic stem cells (hES) cells have great potential to become an unlimited cell source for neurons in culture (Rathjen et al., 2002; Bibel et al., 2004). Then these cells may be used in transplantation therapies for neural pathologies.

Acknowledgements

I would like to thank to:

Magdalena Götz, my supervisor. She tempted me with the project, making me curious to find more, pushed and encouraged me. I thank her for sharing her knowledge, creativity and open ears all the time and for creating a great research environment.
Muecilla Öcallan for great technique assistance.
Prisca Chapouton, Julia von Frowein, Eva Hartfuss and Nicole Haubst for being there and help out when last minute panic set in.

Paolo Malatesta, that helped in establishing new protocols as the AEZ technique and introduced new fishing techniques in Germany and Sweden.
Michael Hack that was a great partner in the primer design, the splitting of neurospheres and the MFF4all.

Finally thanks to Simone and Stefan Bartels helping in corrections and lay-out. My parents that pushed me forward to finish the writing and made it at the end possible to finish.

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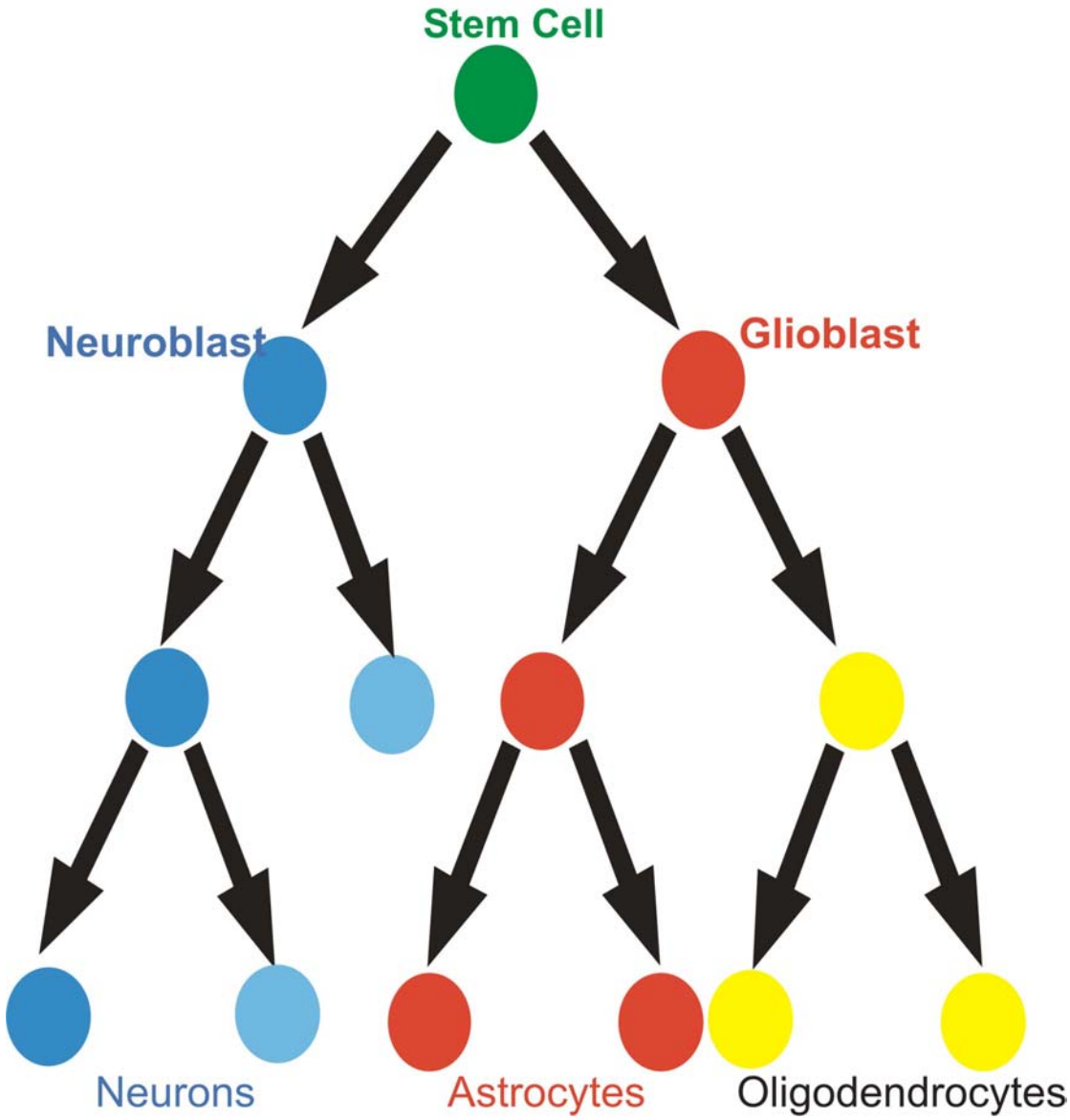


Fig. 1 Lineage tree of neural development. In the cerebral cortex a multipotent stem cell (green) can generate cells of the glial lineage or the neuroblast lineage. The glial lineage subdivides into astrocytes (red) and Oligodendrocytes (yellow), the neuroblast precursor cell (dark blue) generates by asymmetric divisions daughter neuroblast s and neurons (light blue).

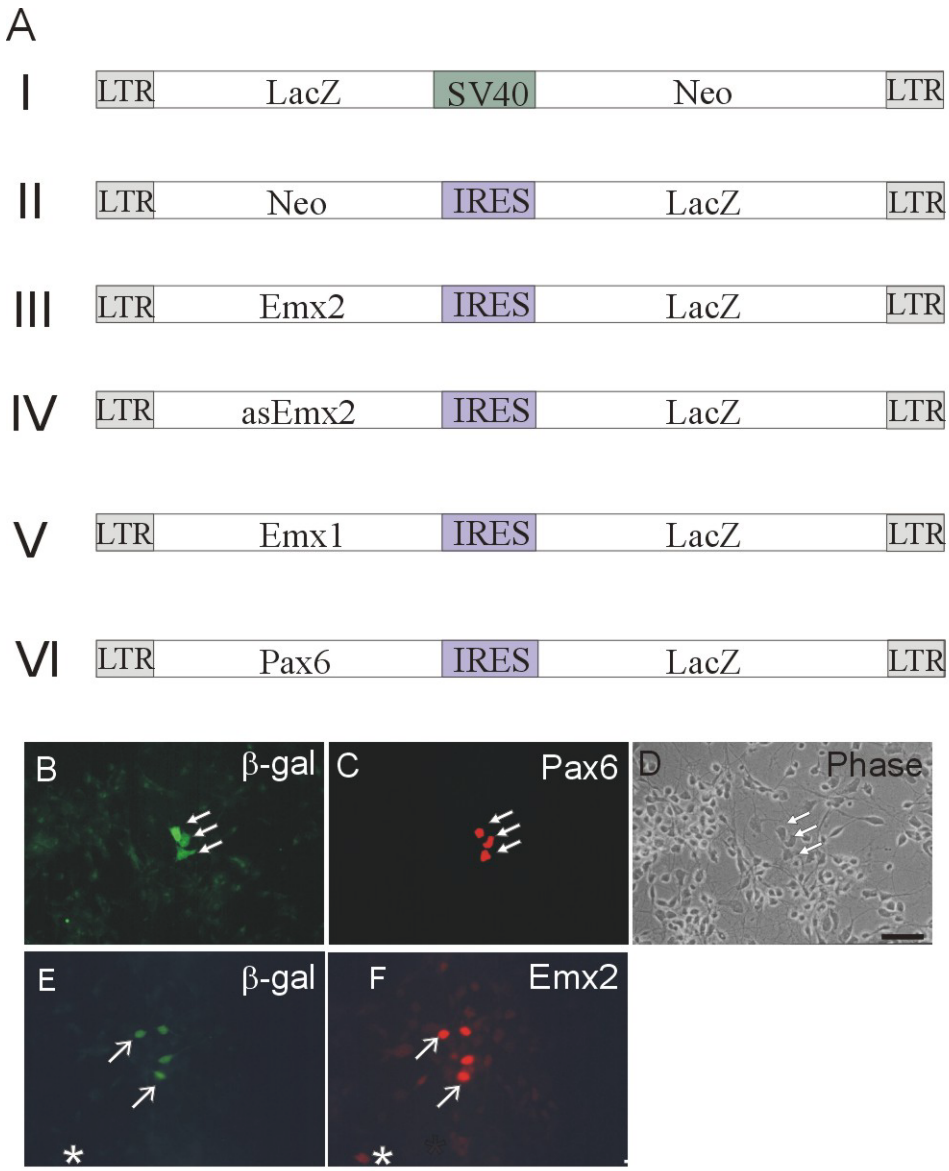


Fig. 2 Schematic view of the co-expression vectors use . Arrows depict double-positive cells; scale bar: 12.5 μm. The star ind and examples of their co-expression. The control vectors BAG (Price et al., 1987) and 1726 (Ghattas et al., 1991) are depicted in I and II, respectively. The viral vectors in II-VI have a picornaviral internal ribosome entry site (IRES), which allows efficient co-expression of both the LacZ gene and the neomycin resistence (I) with the inserted cDNA. Construct (III) contains the Emx2-sense cDNA, (IV) shows the Emx2 antisense construct, (V) the Emx1-sense and (VI) the Pax6-sense cDNA insertion. Examples of their co-expression are depicted in the corresponding fluorescent micrographs (B-F The micrographs show β-galactosidase immunoreactive cluster of four cells (F), double-stained with Emx2-antiserum (Mallamaci et al., 1998) or double-stained with Pax6-antiserum (C). Compare with the corresponding phase contrast micrograph in (D). Cells were isolated from the cortex at E 14 and analyzed indicates an example of the weaker endogenous Emx2-immunoreactivity in cortical cells (F).

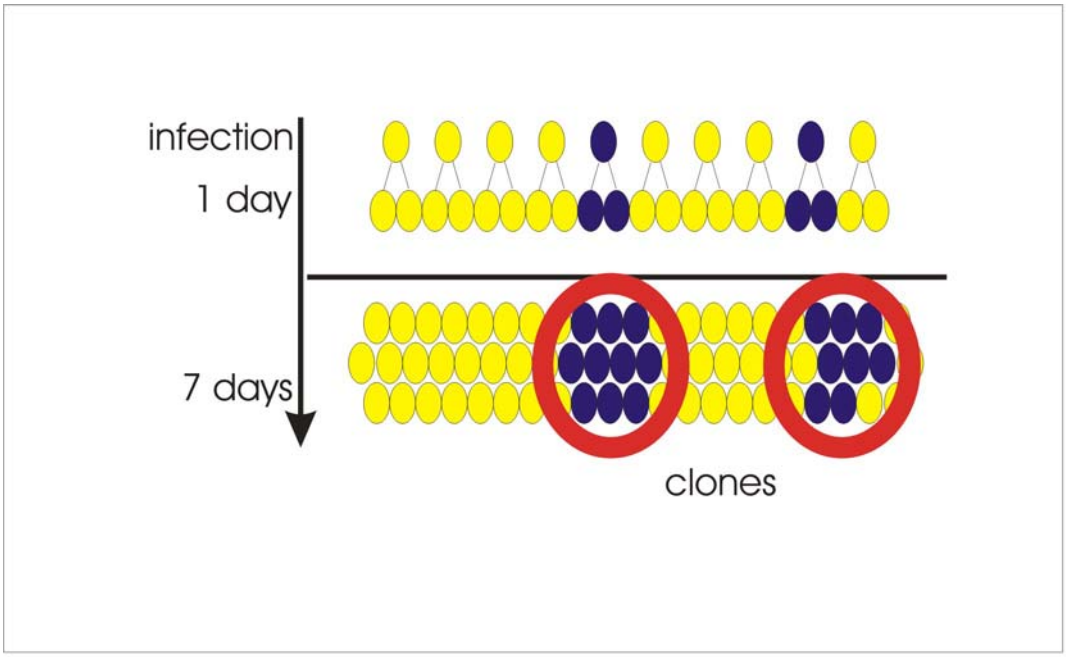


Fig. 3 Schematic generation of a clone. A single retrovirally labeled cell can generate a group of genetically identical cells that is called a clone.

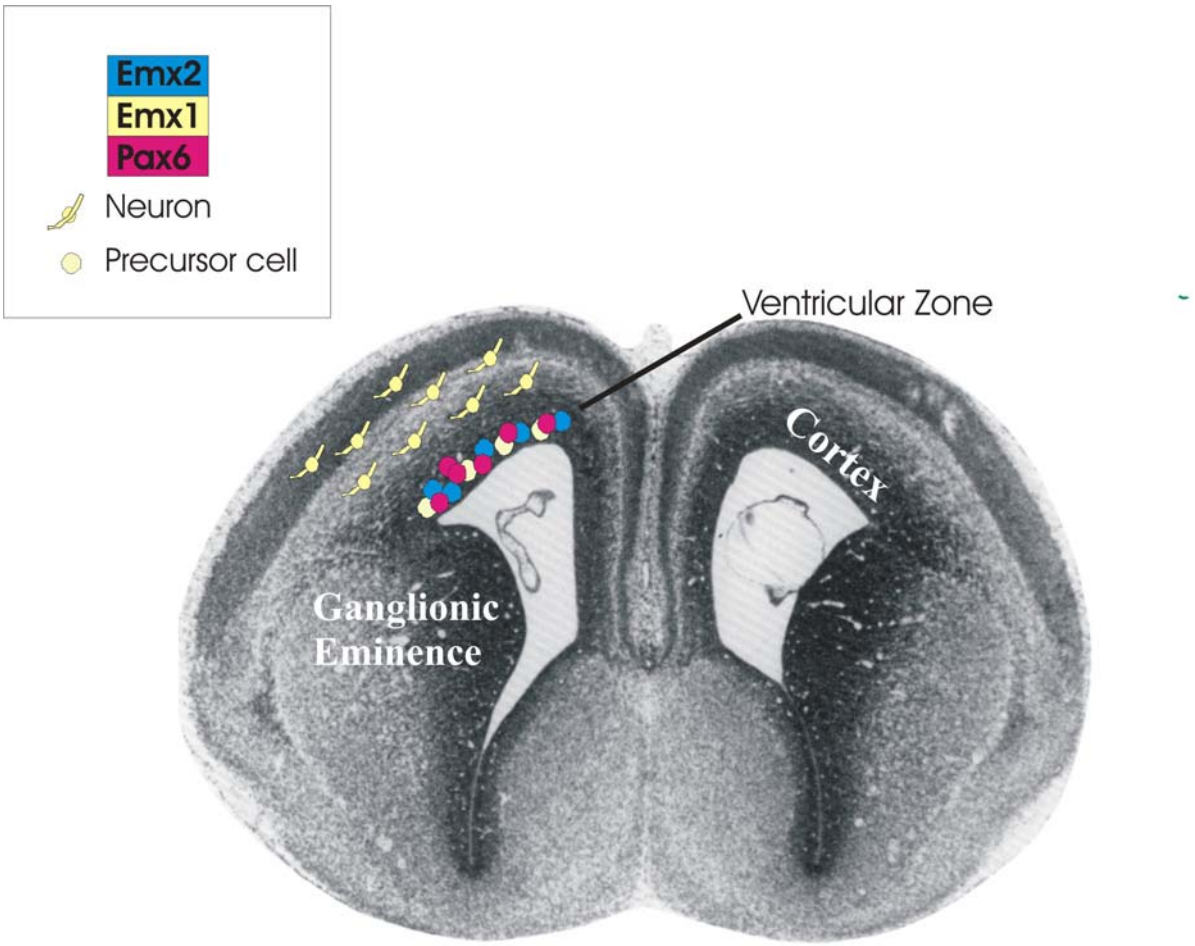


Fig. 4 Region specific expression of the three transcription factors. Schematic view of the regionalization in the developing mouse telencephalon at E 14. Emx2, Emx1 and Pax6 are expressed in precursor cells of the ventricular zone in the cortex (ctx) while Emx1 is also expressed in non-dividing neurons.

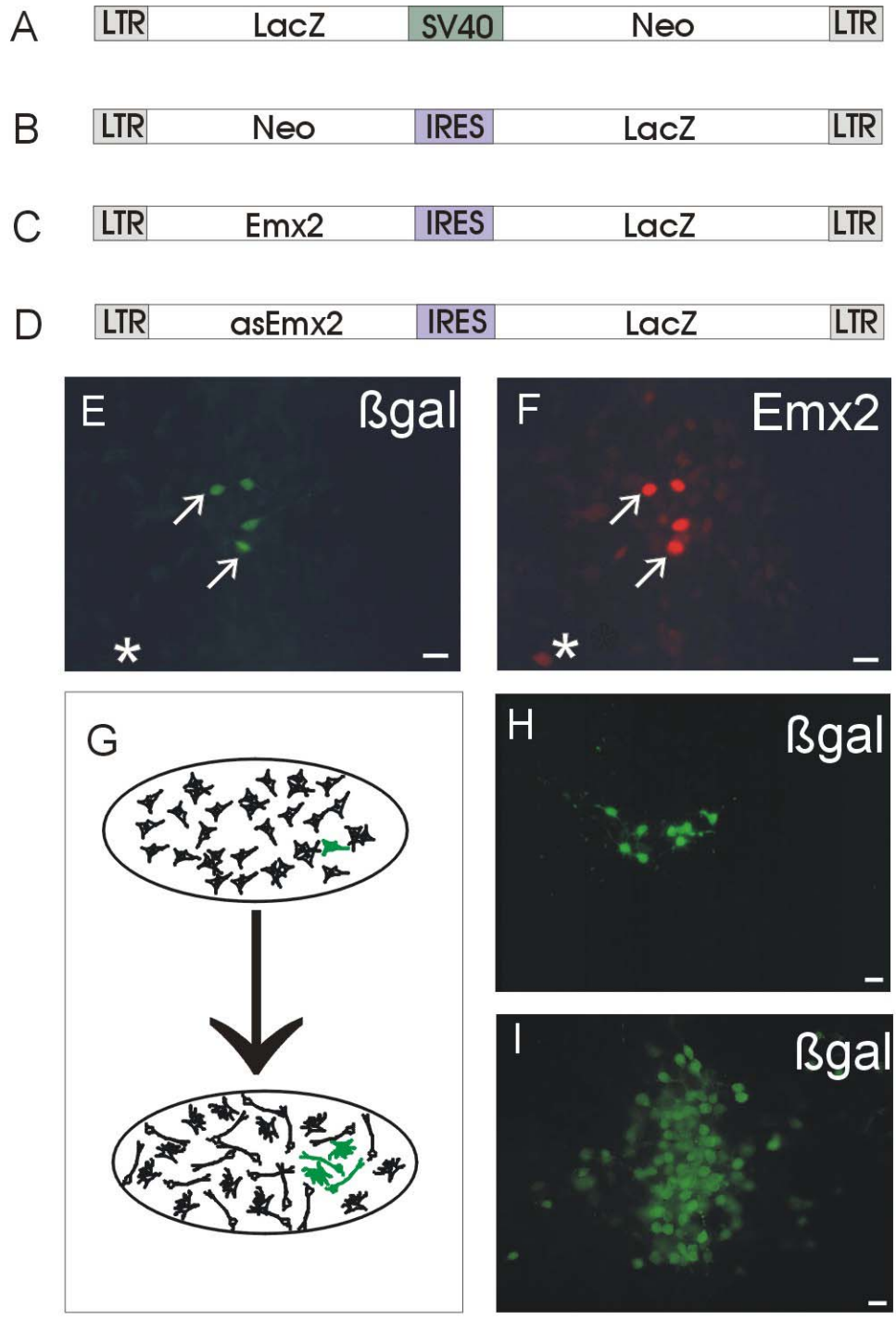


Fig. 5 Retroviral vectors. The control vectors BAG (Price et al., 1987) and 1726 (Ghattas et al., 1991) are depicted in A and B, respectively. The viral vectors in B, C and D have a picornaviral internal ribosome entry site (IRES), which allows efficient co-expression of both the LacZ gene and the neomycin resistence (B) and Emx2-sense cDNA (C), respectively. Corresponding fluorescent micrographs of a β-galactosidase immunoreactive cluster of four cells (E), double-stained with Emx2-antiserum (Mallamaci et al., 1998) depicted in (F). Cells were isolated from the cortex at E 14 and analyzed 3 days after infection with the Emx2-sense virus (C). Arrows depict double-positive cells; the star indicates an example of the weaker endogenous Emx2-immunoreactivity in cortical cells. (G) Schematic drawing illustrating the generation of a clone, the group of green cells depicted in the lower coverslip in G, generated by infection of a single cell depicted in green on the upper coverslip in G. (H, I) Micrographs of β-galactosidase-immunoreactive clusters of cells, isolated from the cortex at embryonic day 14 (E14), 7 days after infection with the control vector (A), depicted in H, or the Emx2-sense vector (C), depicted in I. Scale bar, 25 μm.

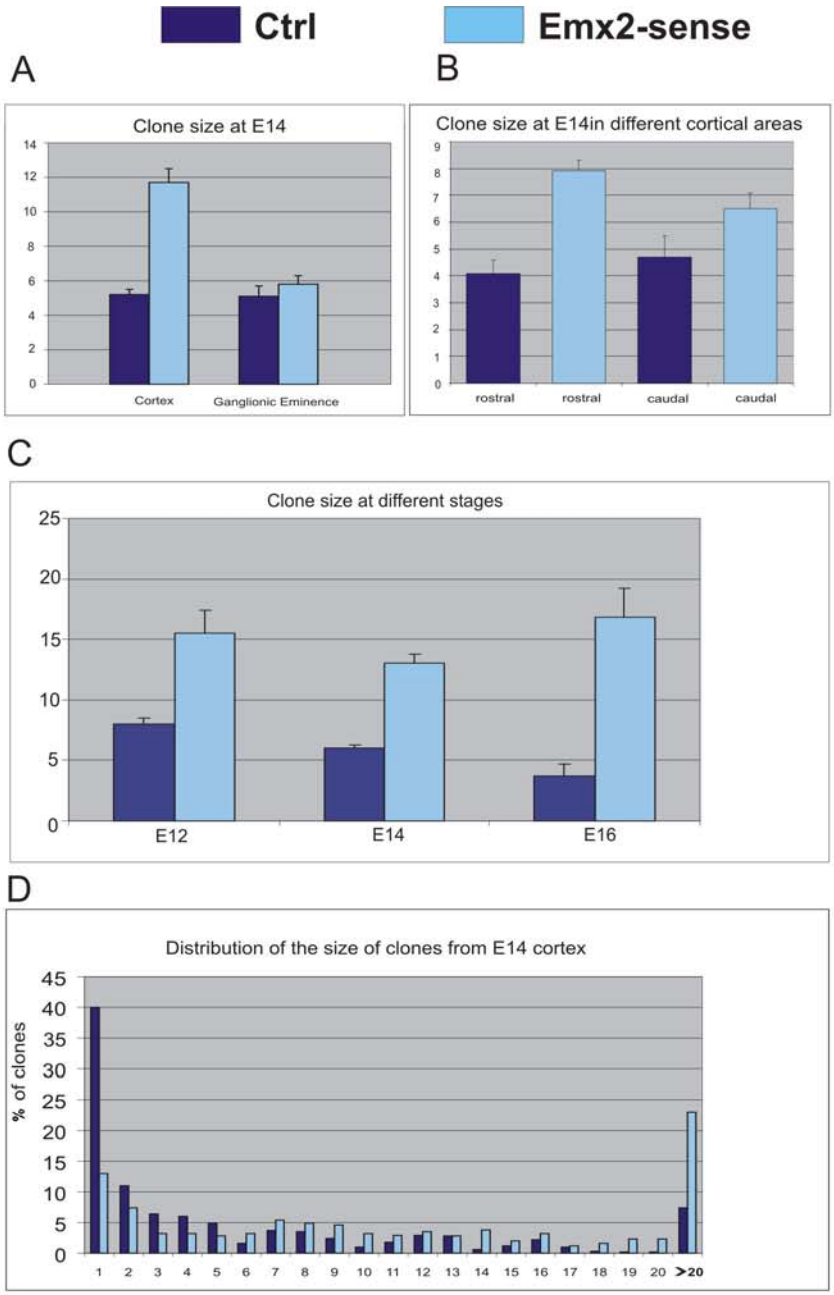


Fig. 6 Transduction of Emx2-sense vector increases the clone size of cortical, but not striatal precursors. (A) The histogram shows the mean size of clones (number of cells per clone, y-axis) generated by precursor cells from E 14 cortex and GE after 7 div. Cells were infected either with the control vectors (dark bars) or the Emx2-sense vector (light bars) as described in Fig. 3. Please note the significant increase in the size of clones generated by Emx2-sense-transduced cells from the cortex, but there is no effect in cells from the GE (number of clones analyzed (n): cortex, control: 714; Emx2: 1021; GE, control: 631, Emx2: 748). (B) The histogram shows the mean clone size generated by precursor cells of different cortical areas after 7 div (n: rostral cortex, control: 78, Emx2: 142; caudal cortex, control: 61, Emx2: 119). (C) The histogram depicts the mean size of clones of cortical cells from different embryonic stages after 5-7 div (n: E 12: control: 219, Emx2: 380; E 14: control, 1075; Emx2: 1282; E 16: control, 248; Emx2: 423). (D) Size distribution of clones generated by precursor cells from E 14 cortex after 5-7 div. The x-axis indicates the clone size as numbers of cells per clone and the y-axis the frequency of clones in percentage. Please note the strong increase in the number of large clones (>20 cells) at the expense of small clones after Emx2 transduction (n: control: 1075, Emx2: 1282). The error bars in all histograms indicate the standard error of the mean (SEM).

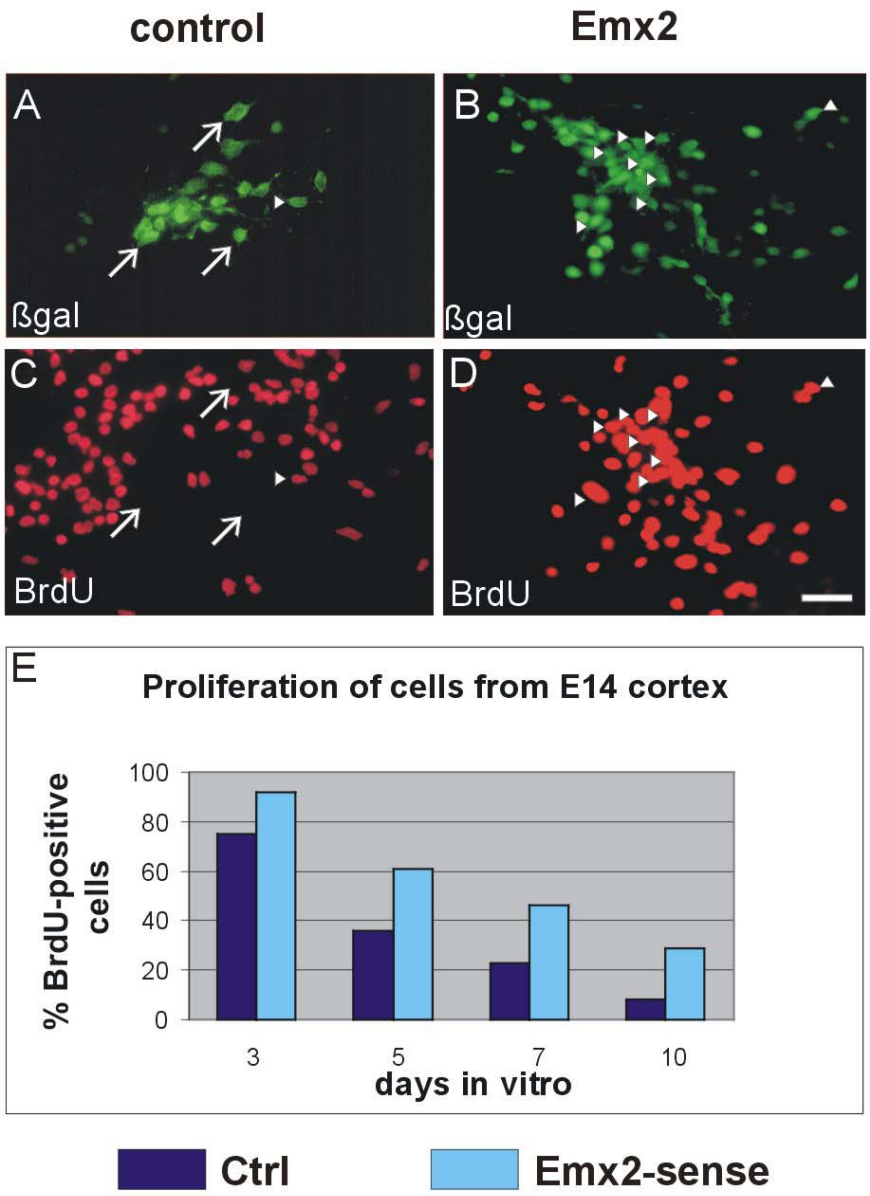


Fig. 7 Increase in proliferation of Emx2-transduced cells. (A-D) Corresponding fluorescent micrographs (A, C, B, D) of β -galactosidaseimmunoreactive (A, B) and BrdU-positive (C, D) cells from E 14 cortex after 7 div. BrdU was added 16 h prior to fixation. Arrowheads indicate examples of double-positive cells; arrows mark β-galactosidase-positive and BrdU-negative cells. A and C show a large clone (> 15 cells) infected with the control virus BAG. B and D depict a large clone generated by Emx2-sense transduced cells. Please note that many Emx2-transduced cells still proliferate and incorporate BrdU after 6-7 div, whereas most of the control infected cells do not incorporate BrdU. Scale bar, 25 μm. (E) The histogram depicts the proportion of BrdU-immunoreactive cells transduced with the control (dark bars) or Emx2-sense (light bars) virus labeled at different div (x-axis) with a 16-h BrdU-pulse prior to fixation. Please note that the number of dividing cells incorporating BrdU decreases during the time in vitro. This decrease is slower for Emx2-sense transduced cells that maintain a higher number of BrdU-positive cells than the control-infected cells (n: day 3: control: 160, Emx2: 258; day 5: control: 141, Emx2: 281; day 7: control: 142, Emx2: 192; day 10: control: 68, Emx2: 92).

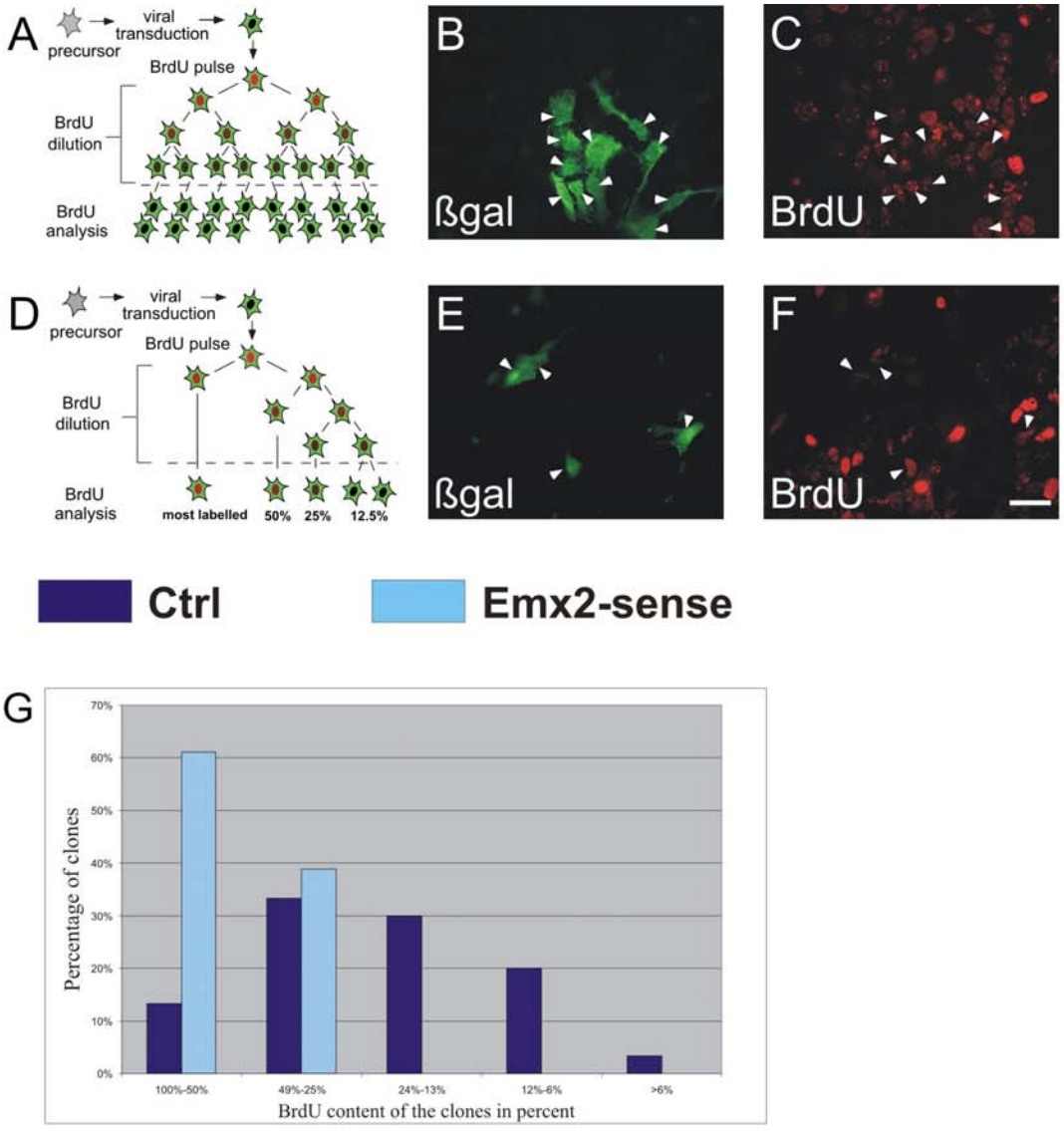


Fig. 8 Emx2-sense transduction promotes a symmetric mode of cell division. (A) and (D) depict schematically the dilution of BrdU-label (red nucleus) in clones dividing exclusively symmetrically (A) or asymmetrically (D). A symmetrical mode of division leads to similar amounts of BrdU-label in all cells of a clone (A). An example of such a clone is shown in (B, C). Corresponding fluorescent micrographs show an 11-cell clone generated by cortical precursors from E 14 after 5 div infected with Emx2-sense virus. The arrowheads point to cells labeled with β-galactosidase antiserum (B) and BrdU (C) that all exhibit a similar level of BrdU-staining. In contrast, if cells divide asymmetrically (D), some cells stop to divide earlier than others, thereby causing differences in the BrdU-content between members of the same clone. (E, F) An asymmetrically dividing 4-cell clone from E 14 cortex after 5 div infected with control viruses shown in (E) and (F). Please note the difference in BrdU-immunoreactivity (F) between the lowest and the two uppermost cells. The histogram in (G) shows the classification of the BrdU content of all clones analyzed (30 control, 18 Emx2-sense). Note that most control infected clones contain cells with different BrdU-content, while the majority of Emx2-transduced clones contain cells with similar BrdU-content. Scale bar, 25 μm.

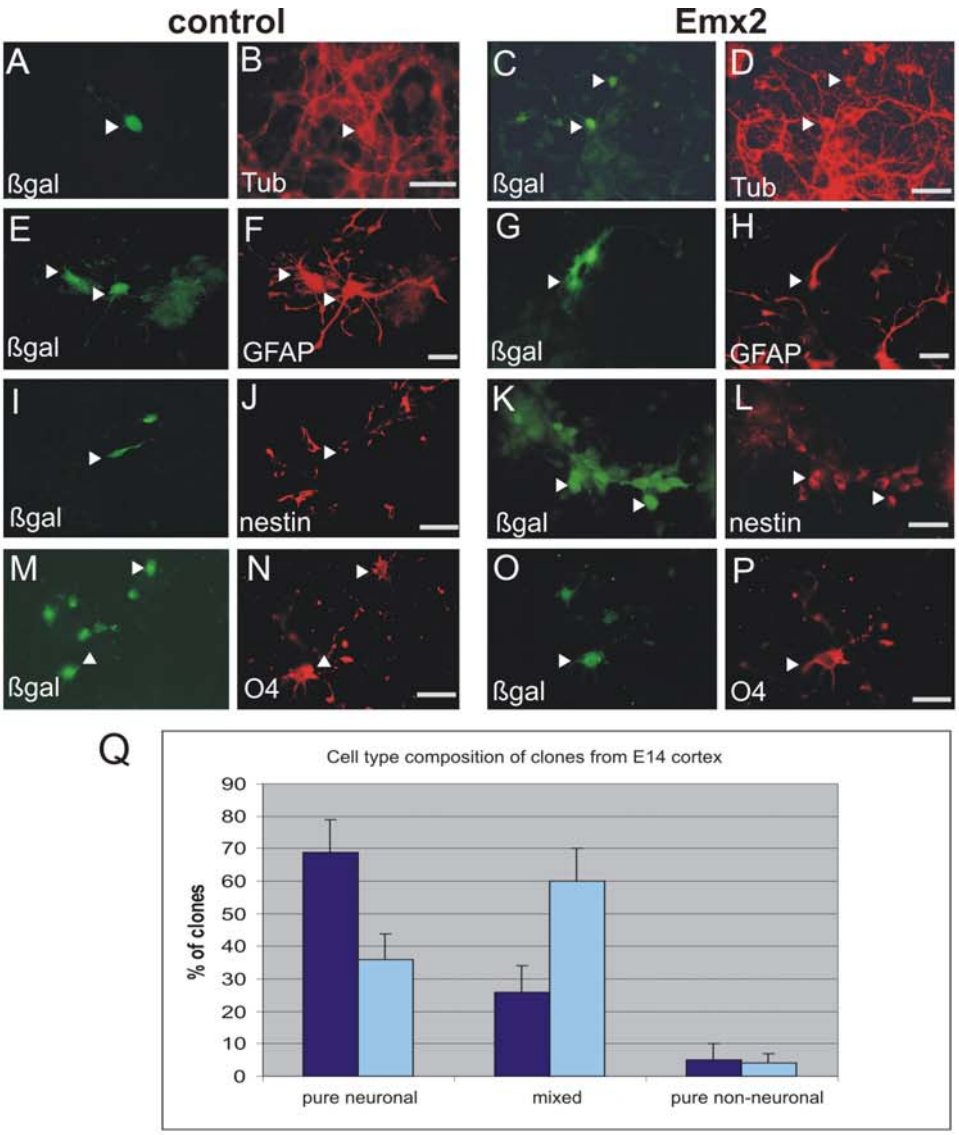


Fig. 9 Emx2-sense transduced cells differentiate into all CNS cell types. Corresponding fluorescent micrographs show β-galactosidase-positive cells transduced with the control vector or the Emx2-sense vectors as indicated in the figure, double-stained with cell-type specific markers. Arrowheads depict double-positive cells. (A-D) show examples of neuronal clones containing β-tubulin-III-immunoreactive cells while (E-H) show examples of astroglial clones immunoreactive for GFAP; (I-L) examples of clones containing nestin-immunoreactive precursor cells; (M-P) clones containing O4-positive oligodendrocytes. Note that Emx2-transduced cells generate all main cell types of the CNS, neurons, astrocytes, oligodendrocytes and precursor cells. Scale bar, 25 μ m. (Q) Cell type analysis of clones from E 14 cortex infected with the control virus BAG (dark bars) or the Emx2-sense virus (light bars) after 7 div. Clones were classified as pure neuronal based on the content of only β-tubulin-III-positive cells, as mixed (neuronal and nonneuronal) when they contained both β-tubulin-III-positive and -negative cells and as pure nonneuronal when they contained no β-tubulin-III-positive cells. The β-tubulin-III-negative cells were either nestin-, GFAP-, or O4-positive cells (see also Fig. 6). Note the increase in mixed clones at the expense of pure neuronal clones after Emx2-sense construct transduction (n: control: 298, Emx2: 417).

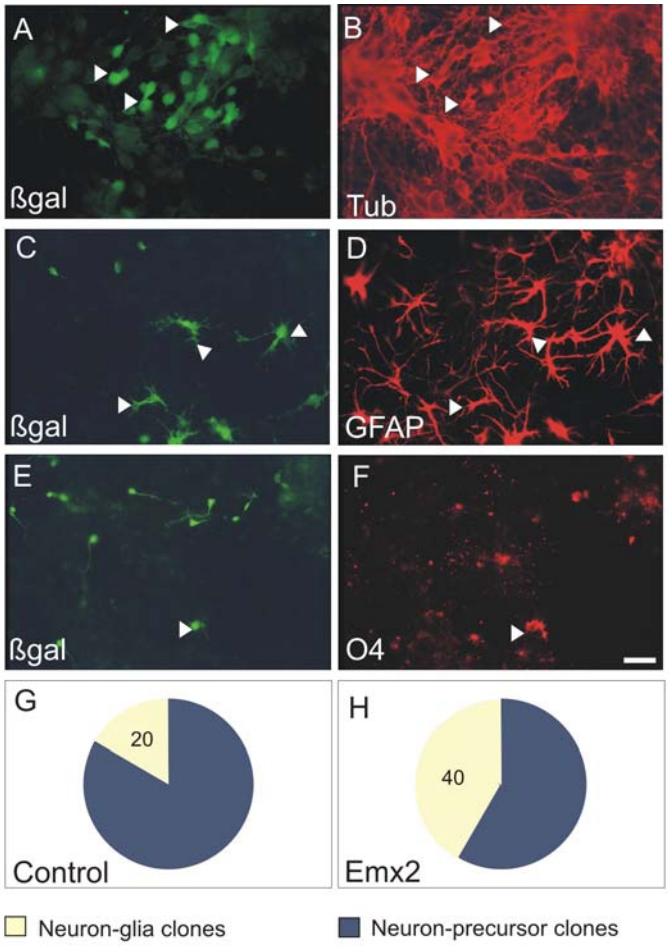


Fig. 10 Large Emx2-sense transduced clones contain neurons, astrocytes and oligodendrocytes. (A-F) Examples of cell types contained in large (>20 cells) Emx2-sense transduced clones from E 14 cortex after 7 div. Some β-galactosidase-positive cells of large clones (see Fig. 2 D) are depicted in corresponding micrographs showing colocalization with the cell-type specific antibodies as indicated in the panels. Arrowheads depict examples of double-positive cells. Note that large clones are not only composed of undifferentiated precursor cells, but contain diverse differentiated cells including neurons, astrocytes and oligodendrocytes. Scale bar, 25 μ m. (G, H) The pie diagrams depict the cell type analysis of large clones. Note the increased proportion of large clones containing neurons and differentiated glial cells after Emx2 transduction (control: 21, Emx2: 41).

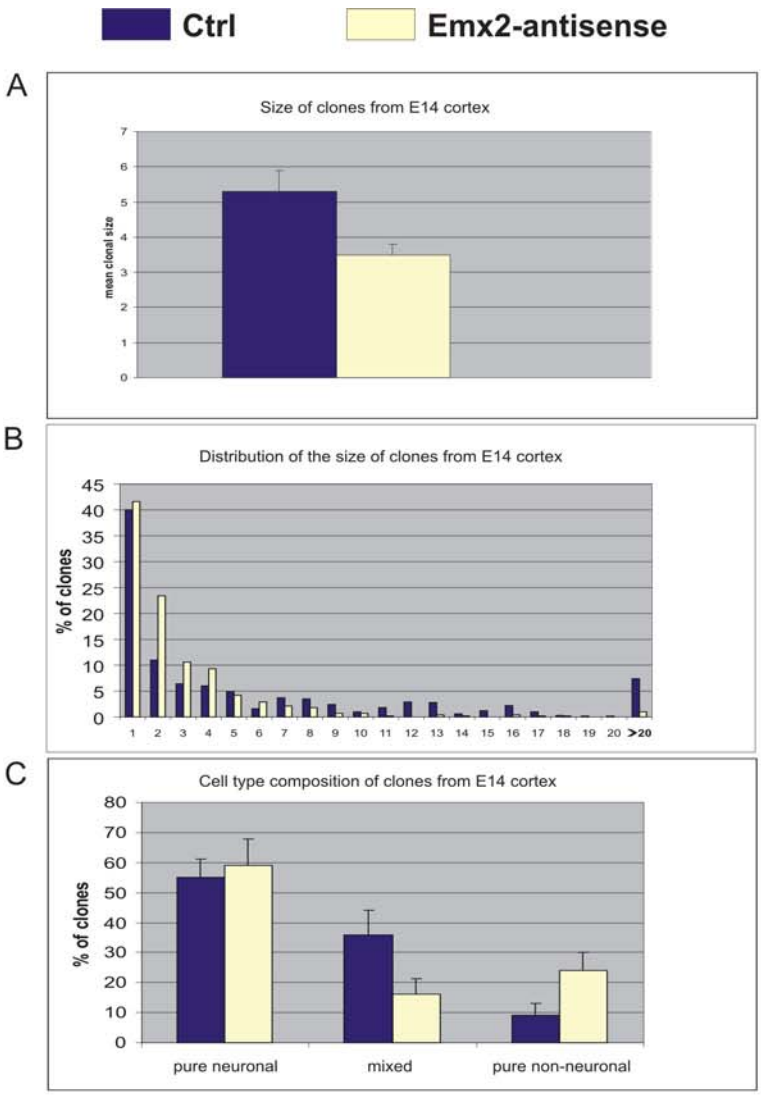


Fig. 11 Transduction of Emx2-antisense vector decreases the clone size of cortical precursor cells. (A) The histogram shows the mean size of clones (number of cells per clone, y-axis) generated by precursor cells from E 14 cortex after 7 div. Cells were infected either with the control vectors (blue bars) or the Emx2-antisense vector (yellow bars) as described in Fig. 3. Note the significant decrease in the size of clones generated by Emx2-antisense-transduced cells from the cortex (number of clones analyzed (n): control: 1075, Emx2as: 452). (B) Size distribution of clones generated by precursor cells from E 14 cortex after 7 div. The x-axis indicates the clone size as numbers of cells per clone and the y-axis the frequency of clones in percentage. Note the strong increase in the number of small clones (4 = cells) at the expense of large clones (20 = cells after Emx2-antisense transduction (n: control: 41, Emx2: 11). (C) Cell type analysis of clones from E 14 cortex infected with the control virus BAG (dark bars) or the Emx2-antisense virus (light bars) after 7 div. Clones were classified as pure neuronal based on the content of only β-tubulin-III-positive cells, as mixed (neuronal and non-neuronal) when they contained both β -tubulin-III-positive and -negative cells and as pure non-neuronal when they contained no β-tubulin-III-positive cells. The β-tubulin-III-negative cells were either nestin-, GFAP-, or O4-positive cells. Note the increase in non-neuronal clones at the expense of mixed clones after Emx2-antisense construct transduction (n: control: 1075, Emx2as: 452). The error bars in all histograms indicate the SEM.

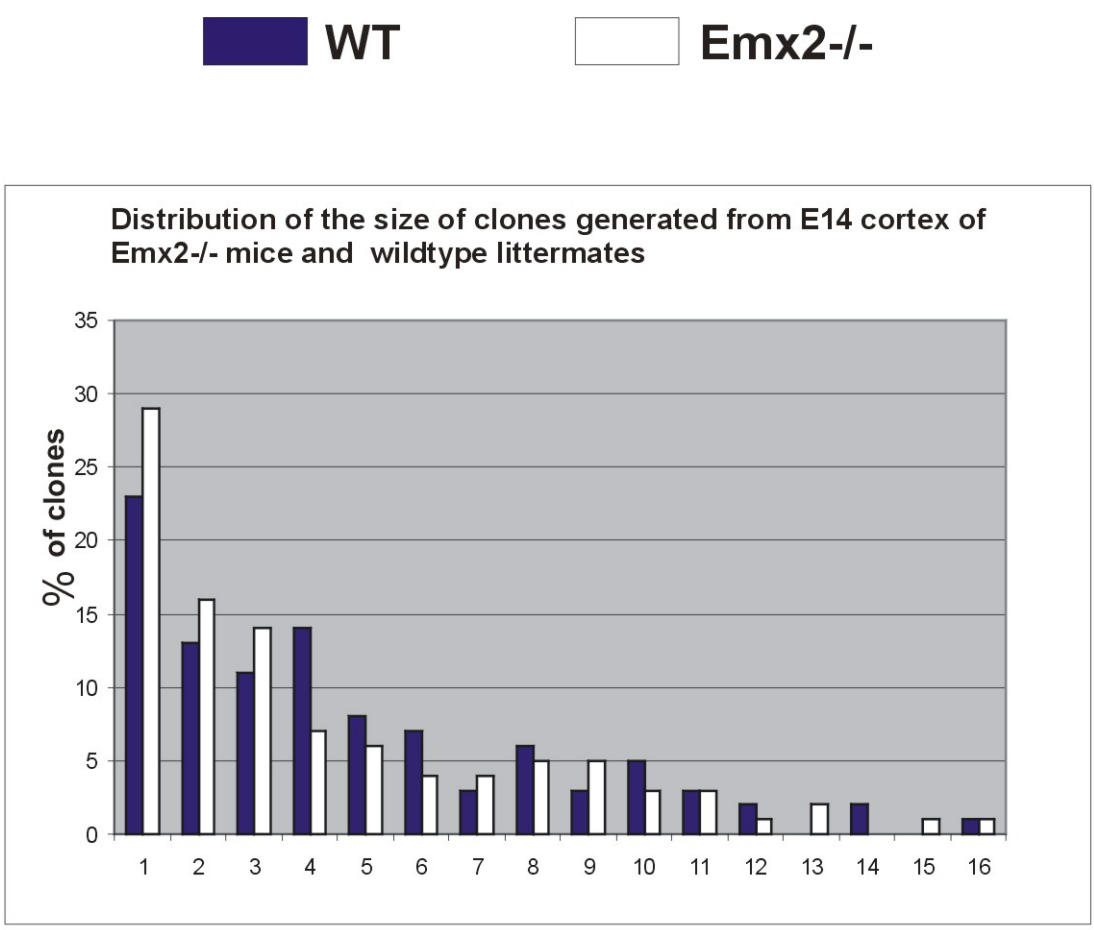
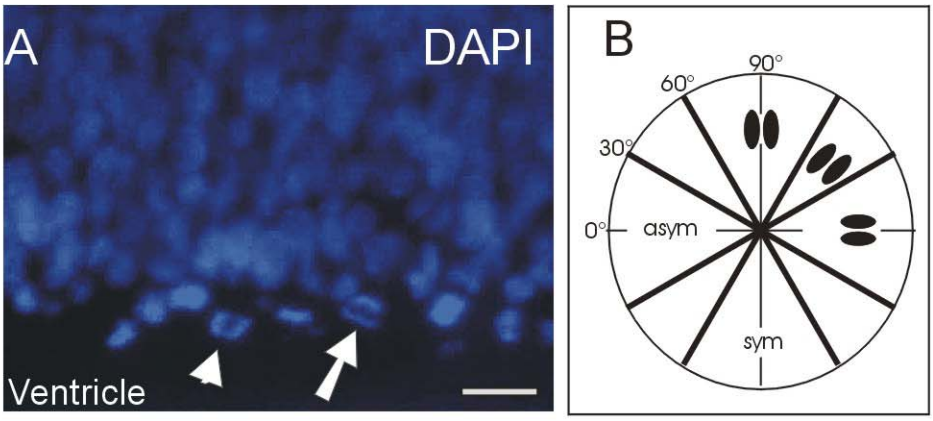


Fig. 12 Clone size of Emx2-deficient cortical precursors in vitro. The histogram shows the distribution of clones generated by control infected cortical precursor cells from E 14 WT (dark bars) or Emx2 $^{-/-}$ cortex (white bars) after 5-7 days in vitro. The x-axis indicates the clone size as numbers of cells per clone and the y-axis the frequency of clones in percent. Note the increase in the number of 1 to 3 cell clones in homozygous Emx2-mutant cortical cells while the clones from 4 to 6 cells are diminished (n: WT: 276; Emx2 -/-: 113).



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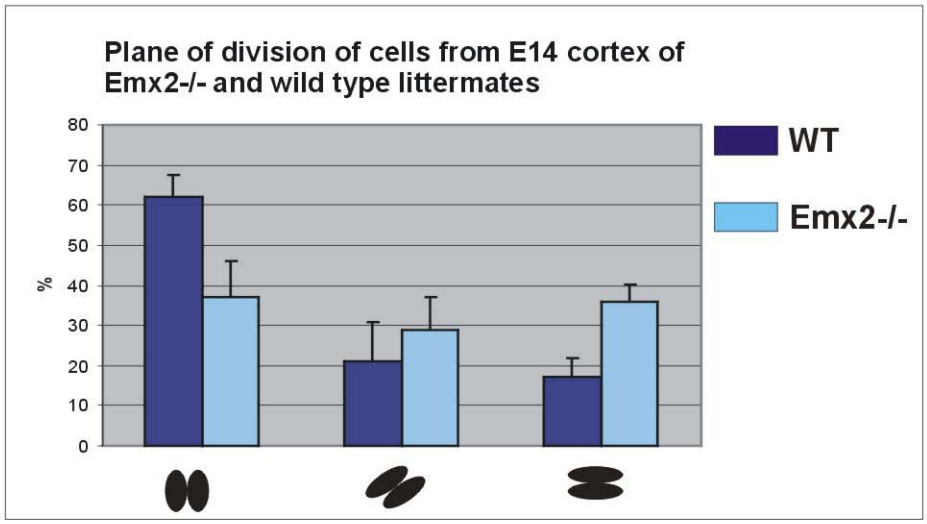


Fig. 13 Orientation of the cell divisions in Emx2 -/- cortex. (A) Fluorescent micrograph of a frontal section of the cortex of Emx2 -/- mice at E 14. Cell nuclei were stained with DAPI allowing the analysis of cell division at the ventricular surface (VS). Scale bar, 15 μ m. (B) Schematic drawing showing the analysis of the orientation of the plane of cell division relative to the VS. Angles from 230° to 30° are interpreted as an asymmetric division, (arrow in A), from 30° to 60° as oblique division, and from 60° to 120° as symmetric division (arrowhead in A; see results for details). (C) The histogram shows the percentage of the plane of division in sections at comparable rostrocaudal

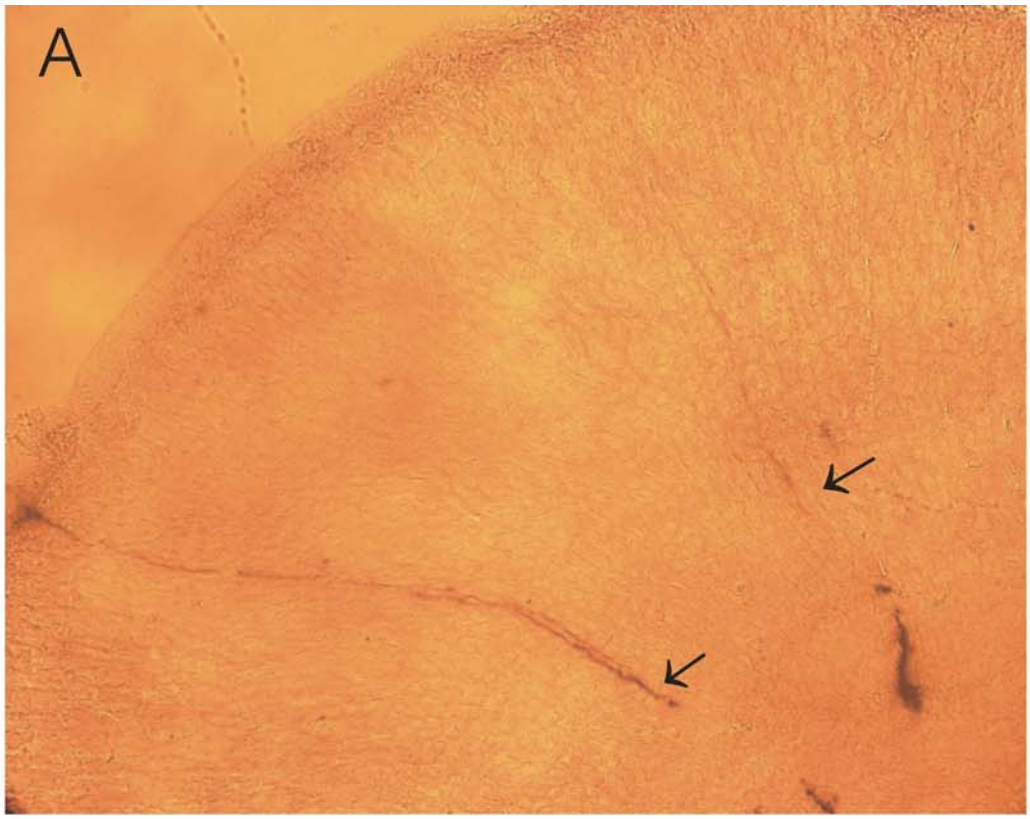


Fig. 14 Analysis of cortical transduced cells at P 3. This figure shows cells overexpressing Emx2 stained with X-gal. Black arrows point to cells still close to the ventricle that have long radial processes.

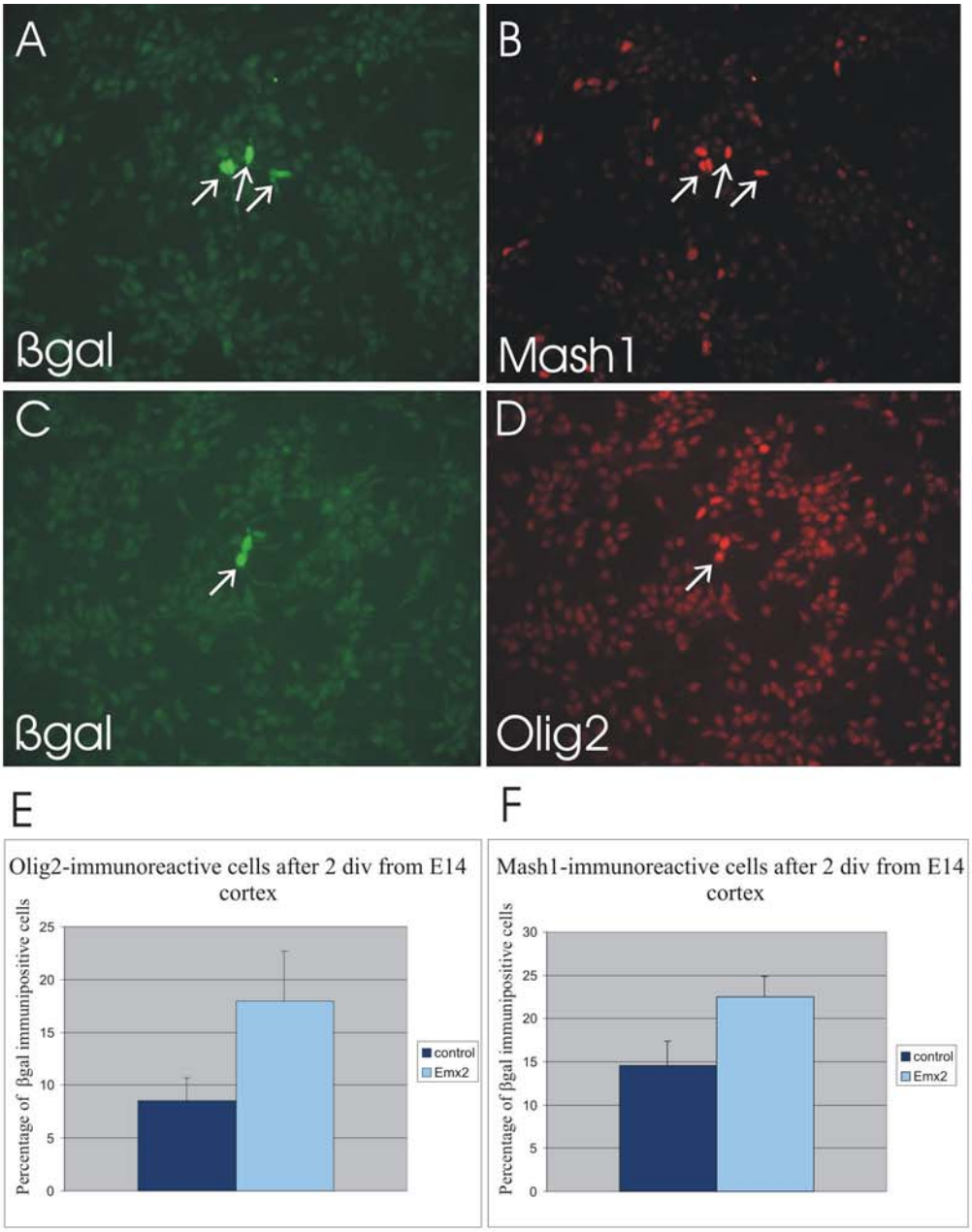


Fig. 15 Emx2 activates the bHLH transcription factors Mash1 and Olig2. Cultures were fixed 2 days after infection. β-galactosidase-positive cells in dissociated cell cultures from E 14 cortex transduced with Emx2 viral vectors at the time of plating (A, C) are immunopositive for the bHLH transcription factors Mash1 (B) or Olig2 (D) as indicated by arrowheads. The histograms in (E and F) show that overexpression of Emx2 (light blue bars) has regulatory effects, namely upregulation of Mash1 or Olig2 in comparison to the control (dark bars). Number of clones analyzed (A-F), Mash1, 82 (BAG), 132 (Emx2); Olig2, 336 (BAG), 172 (Emx2). **, $p < 0.005$.

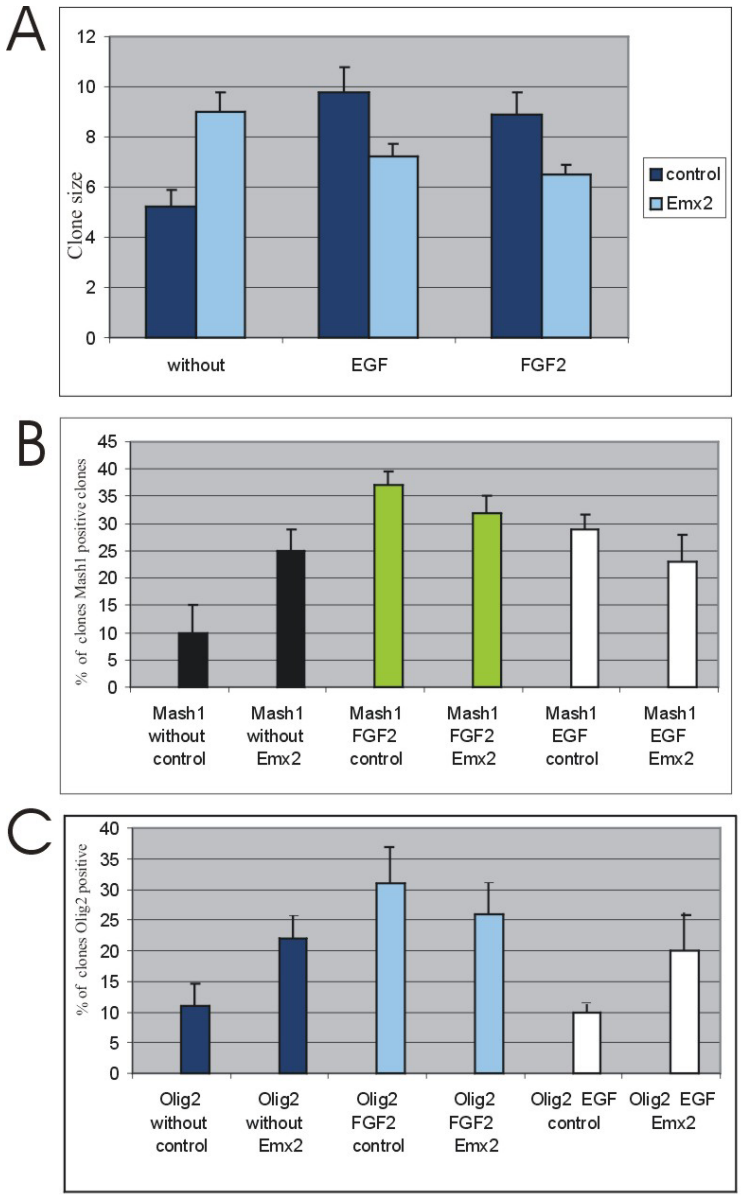


Fig. 16 EGF and FGF2 interact with Emx2. After infection with Emx2 or control viruses the cultures were treated with the growth factors EGF, FGF2 or non-treated. The histogram in (A) shows the clone size of cultures infected with the control (dark blue) and Emx2 (light blue) viruses after 7 div. Note that the mean clone size of Emx2 transduced cells after treatment with either FGF2 or EGF is reduced to values similar to untreated control cultures. In (B) non-treated (black), FGF2 (green) or EGF (white) treated cultures were fixed 2 days after infection and stained for Mash1 (B). In (C) non-treated (dark blue), FGF2 (blue) or EGF (light blue) treated cultures were fixed 2 days after infection and stained for Olig2 (C). The error bars in all histograms indicate the standard error of the mean (SEM). (A, n: 904 (control), n: 623 (Emx2), n: 103 (EGF), n: 244 (EGF Emx2), n: 172 (FGF2), n: 194 (FGF2 Emx2); B, Mash1, n: 567 (control), Mash1, n: 382 (Emx2), Mash1, n: 129 (FGF2), Mash1, n: 203 (FGF2 Emx2), Mash1, n: 89 (EGF), Mash1, n: 77 (EGF Emx2); C, Olig2, n: 337 (control), Olig2, n: 241(Emx2), Olig2, n: 135 (FGF2), Olig2, n:183 (FGF2 Emx2), Olig2, n: 58 (EGF), Olig2, n: 42 (EGF Emx2). Note that the controls in C and D present the data of two experiments under different growth factor conditions whereas the controls in figure 15 E and F present the pooled data of all experiments.

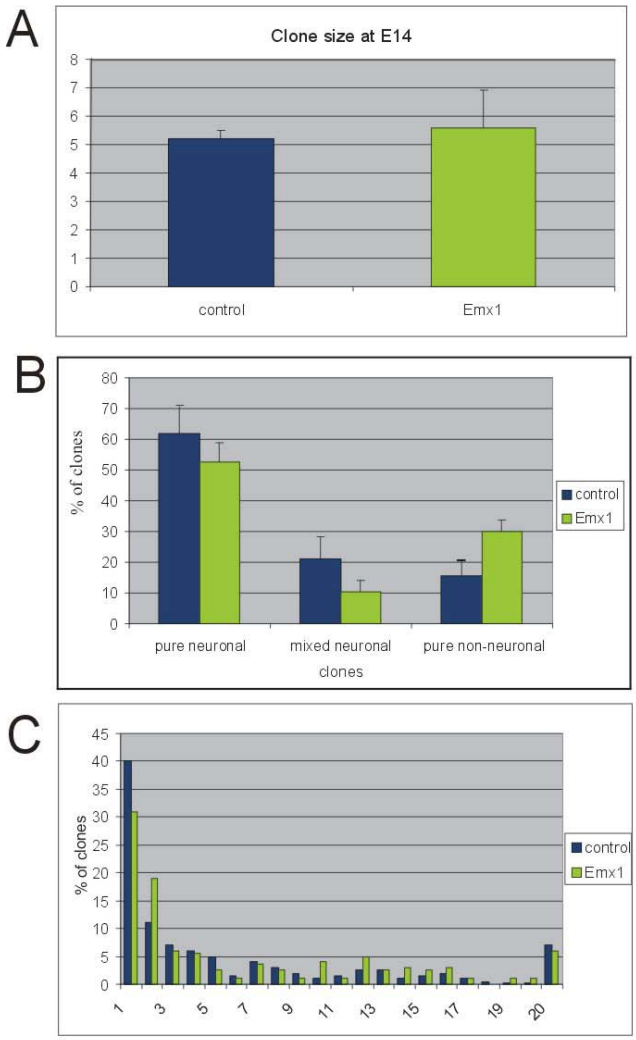


Fig. 17 Transduction of Emx1-sense vector has no influence on the clone size of cortical, and striatal precursors. (A) The histogram shows the mean size of clones (number of cells per clone, y-axis) generated by precursor cells from E 14 cortex after 7 div. Cells were infected either with the control vectors (dark bars) or the Emx1-sense vector (green bars) as described in Fig. 2. (n): cortex, control: 714; Emx1: 311. (B) Cell type analysis of clones from E 14 cortex infected with the control virus BAG (dark bars) or the Emx1-sense virus (green bars) after 7 div. Clones were classified as pure neuronal based on the content of only β-tubulin-III-positive cells, as mixed (neuronal and non-neuronal) when they contained both β-tubulin-III-positive and -negative cells and as pure non-neuronal when they contained no β-tubulin-III-positive cells. The β-tubulin-III-negative cells were either nestin-, GFAP-, or O4-positive cells. Note the increase in non-neuronal clones at the expense of mixed clones after Emx1-sense construct transduction (n: control: 298, Emx1: 107). (C) Size distribution of clones generated by precursor cells from E 14 cortex after 7 div. The x-axis indicates the clone size as numbers of cells per clone and the y-axis the frequency of clones in percentage. Note the increase in the number of small clones that contain around 3 to 4 cells) after Emx1-sense transduction (n: control: 714, Emx1: 311). The error bars in all histograms indicate the SEM.

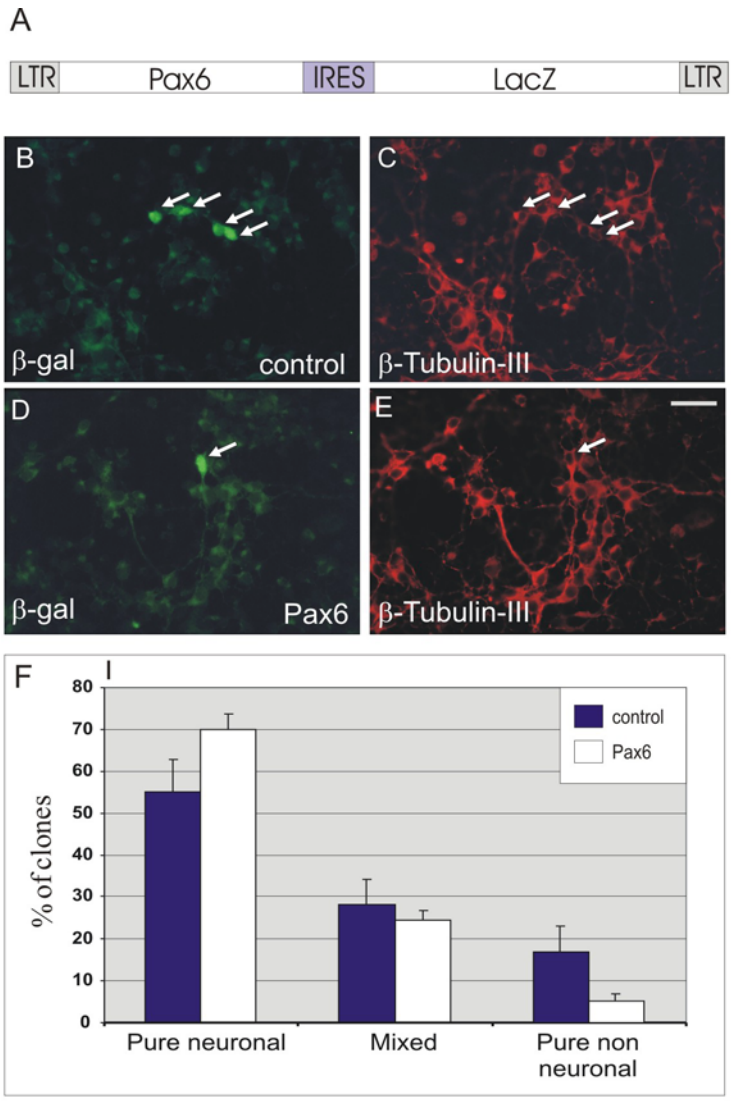


Fig. 18 Pax6 transduction increases neurogenesis in precursors from embryonic cortex. The retroviral vector (A) allows co-expression of lacZ and Pax6 cDNA. (B-E) Corresponding fluorescence micrographs show β-galactosidase-positive cells (E, G) from E 14 WT cortex transduced with control (B, C) or Pax6 (D, E) virus double stained with the neuron-specific antibody against β-tubulin-III (F, H) after 7 div. Arrows, double-positive cells; scale bar: 12.5 μm. (F) Histogram of cell type analysis of clones from E14 WT cortex infected with the control (dark bars) or Pax6 virus (light bars) after 7 div (y-axis indicates the percentage of clones). Note that pure neuronal clones increase at the expense of pure non-neuronal clones after Pax6 transduction. Number of clones analyzed: 461 (control); 1,016 (Pax6). *, $p < 0.01$; **, $p < 0.005$.

A

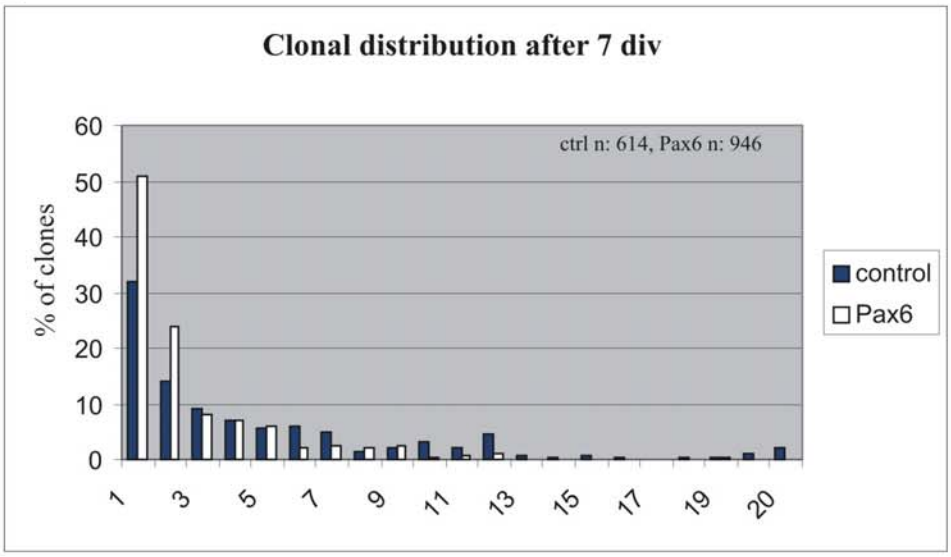


Table 1

A

Cell type (all E 14)	Virus	Clone size	SEM	Number of clones
WT ctx	Control	5.8	0.6	811
WT ctx	Pax6	3.0 **	0.3	983
Sey/Sey ctx	Control	7.4 *	0.7	520
Sey/Sey ctx	Pax6	2.7 **	0.1	159
WT GE	Control	5.2	0.8	514
WT GE	Pax6	5.0	0.9	226

B

Cell type (all P 3-11)	Virus	Clone size	SEM	Number of clones
WT ctx Astrocytes	Control	2.7	0.25	36
WT ctx Astrocytes	Pax6	1.8 **	0.17	30

Fig. 19 (A) Size distribution of clones generated by control (dark bars) and Pax6 (light bars) infected cortical precursor cells from E 14 WT after 5-7 div. The x-axis indicates the clone size as numbers of cells per clone and the y-axis the frequency of clones in percent. Note the strong increase in the number of single cell clones at the expense of larger clones after Pax6 transduction. Number of clones analyzed: 811 (control), 983 (Pax6). Table 1 Mean size of clones from Pax6- and control transduced. A significant decrease occurs in the size of clones generated by Pax6-transduced cells from the cortex of both WT and Sey/Sey and by Pax6-transduced postnatal astrocytes, but there is no effect in cells from the GE. In contrast, the clone size of Pax6-deficient Sey/Sey cortical precursors increased compared to cells from the WT cortex. Significant differences to WT cortex infected with control virus are indicated (*, $p < 0.01$; **, $p < 0.005$).

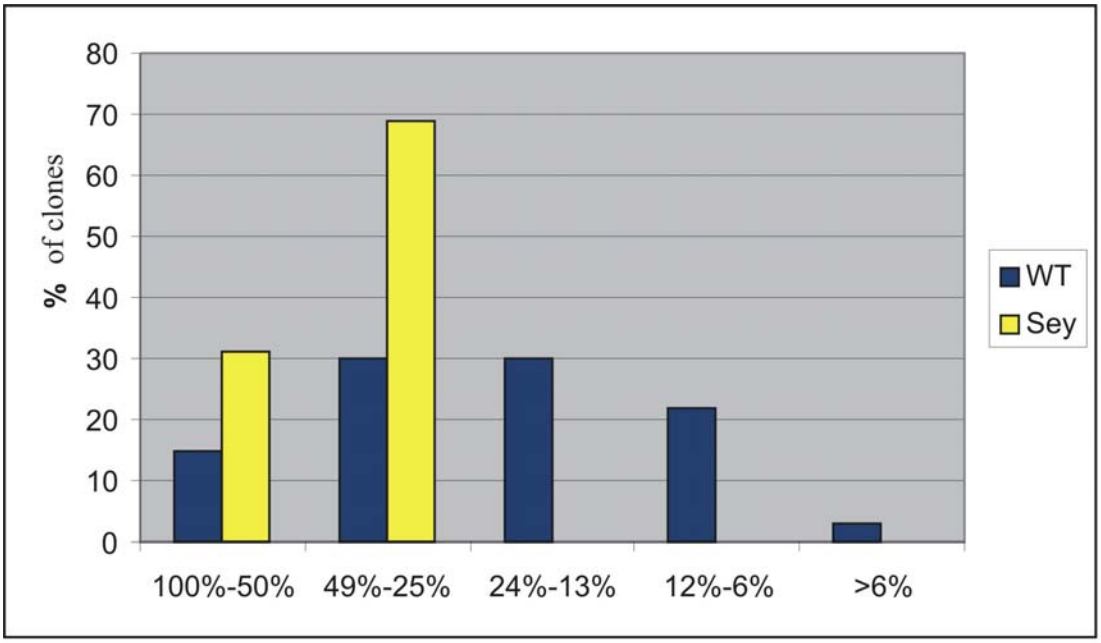


Fig. 20 Loss of functional Pax6 protein promotes a symmetric mode of cell division. A symmetric mode of division leads to similar amounts of BrdU-label in all members of a clone (for more details see Fig. 8 or Methods). The histogram shows the classification of the BrdU content of all clones analyzed (30 WT, 61 Sey/Sey). Note that most of the WT clones contain cells with different BrdU-content, while the majority of the Sey/Sey clones contain cells with similar BrdU-content.

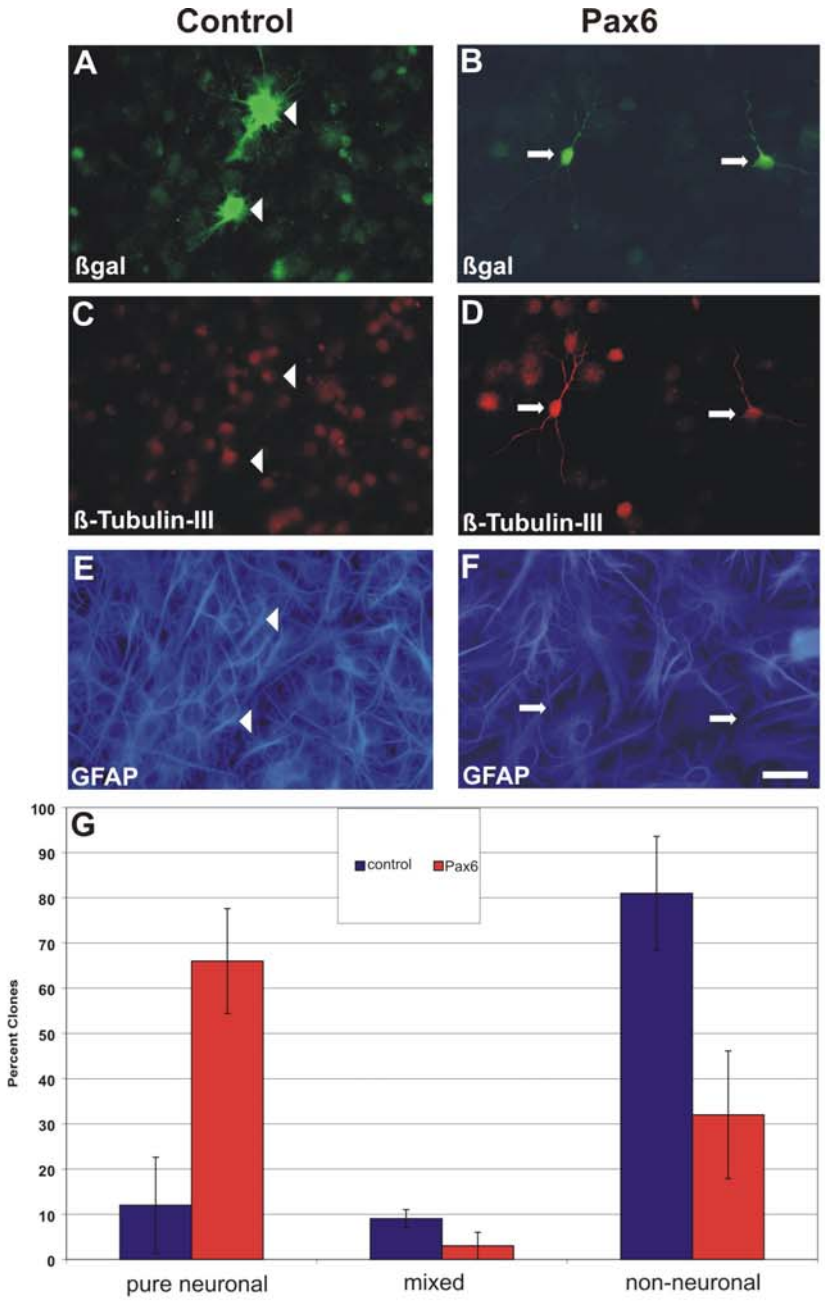


Fig. 21 Pax6 directs astrocytes towards neurogenesis. (A- F) Corresponding fluorescence micrographs (A, C, E; B, D, F) show β-galactosidase-positive cells (A, B) after 7 div in chemically defined medium generated from astrocytes of postnatal cortex transduced with the control (A, C, E) or Pax6 virus (B, D, F). Cells were double stained with cell-type specific markers for neurons (C, D) or astrocytes (E, F). Arrowheads depict an example of an astroglial clone (A, C, E) and arrows (B, D, F) depict an example of a two-cell neuronal clone generated by astrocytes infected with the Pax6 virus. (G) Histogram depicts the quantification of clone types generated by astrocytes from postnatal cortex infected with the control virus BAG (blue bars) or the Pax6 virus (red bars) after 7 div. Pure neuronal clones increase at the expense of pure non-neuronal clones after Pax6 transduction. Number of clones analyzed: 324 (control), 67 (Pax6). **, $p < 0.005$. Scale bar, 25 μm.

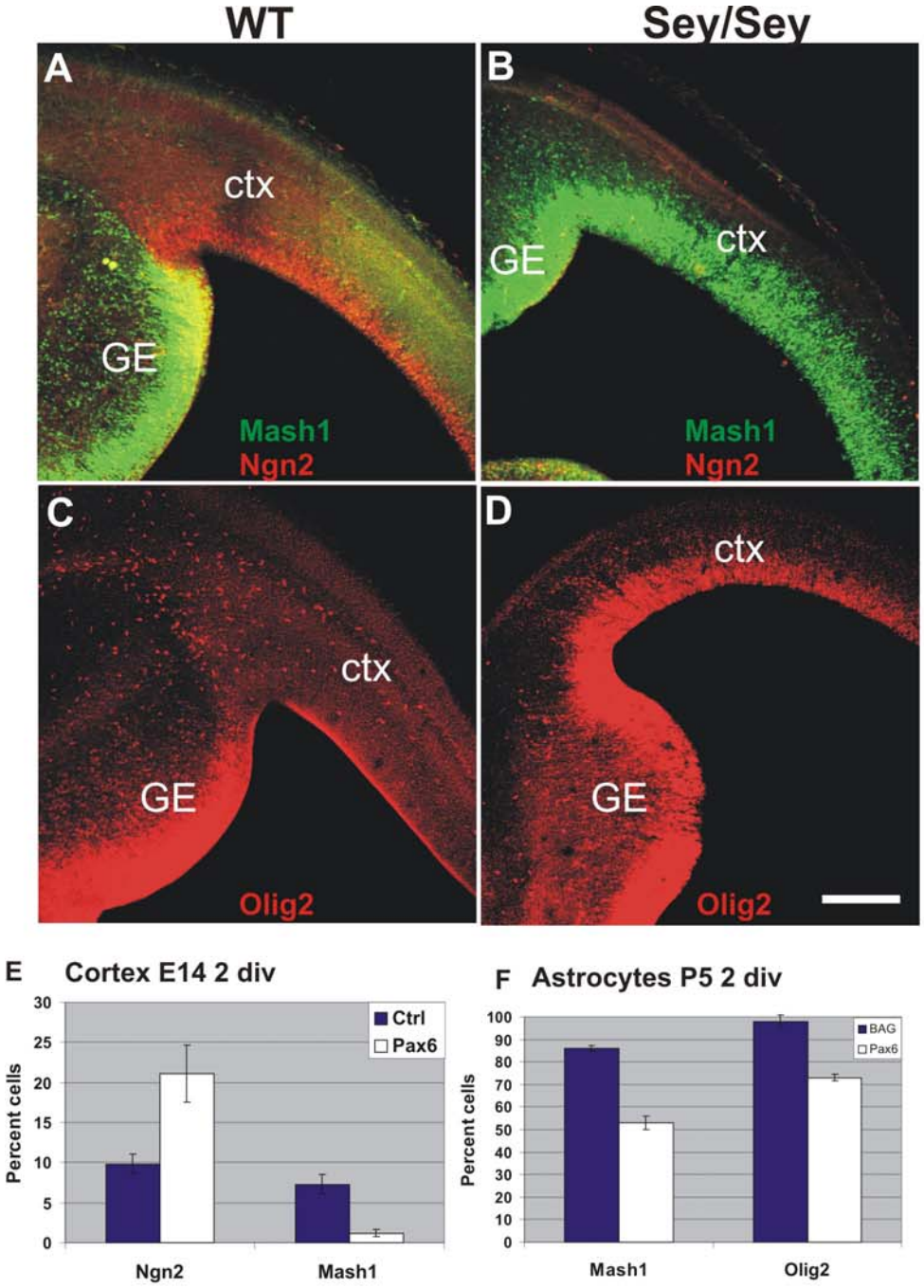
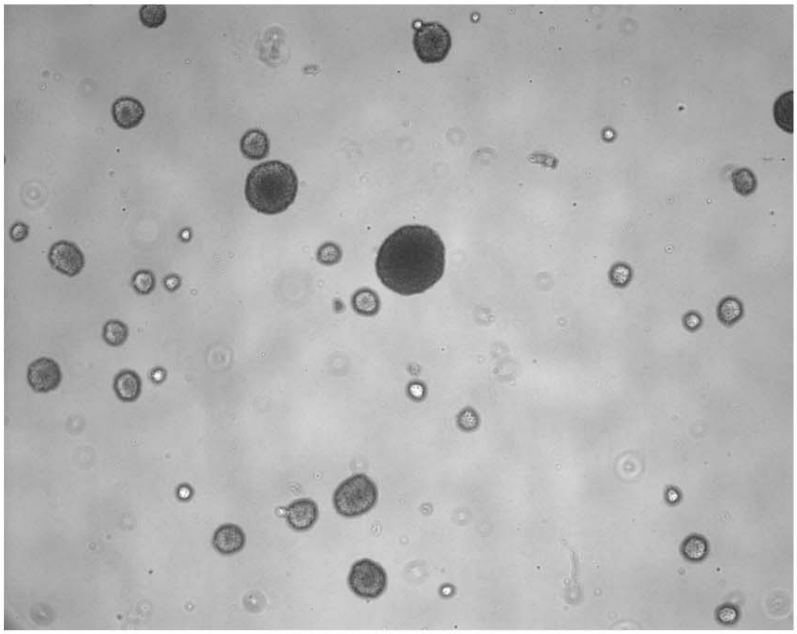


Fig. 22 Pax6 regulates bHLH transcription factors. (A-D) show micrographs of sections from E 14 WT; A, C) and Sey/Sey (B, D) telencephalon stained with the antisera indicated. Ngn2 immunoreactivity is lost, whereas Mash1 and Olig2 staining is increased in the Sey/Sey cortex. Scale bar, 200 μm. (E, F) Percentage of β-galactosidase-positive cells in dissociated cell cultures from E 14 cortex infected at the time of plating (E) or P 5astrocyte cultures infected after the first passage (F) that are immunopositive for the bHLH transcription factors are indicated in the histogram. Cultures were fixed 2 days after infection with the control (dark bars) or the Pax6 virus (light bars). Overexpression of Pax6 has regulatory effects opposite to those of the loss of Pax6 function in Sey/Sey cortex, namely upregulation of Ngn2 and downregulation of Mash1 or Olig2. Number of clones analyzed (E, F), Mash1, 82 (BAG), 132 (Pax6); Olig2, 336 (BAG), 172 (Pax6). **, $p < 0.005$.

A



B

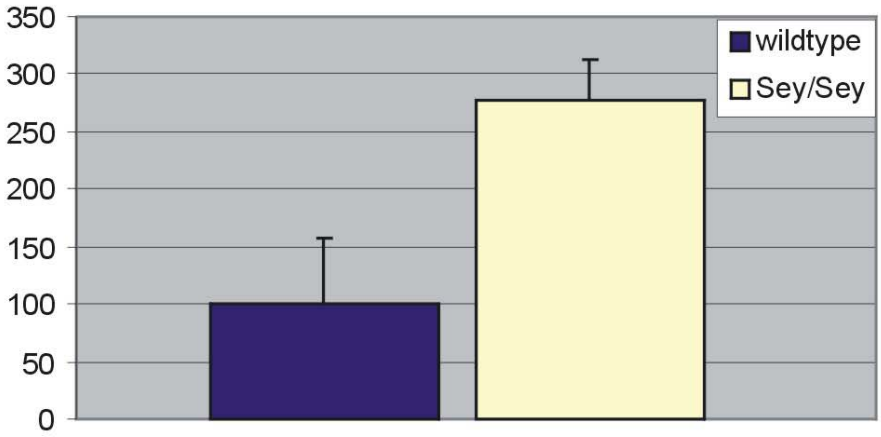


Fig. 23 Increase in neurosphere forming cells from Sey/Sey telencephali (A) Micrograph shows a neurosphere culture derived from E 14 WT cortex after 3 passages prepared as described by Gritti et al., (1999). The number of neurospheres generated by the same number of cells from E 14 WT and Sey/Sey cortex was evaluated after 1-5 passages and is depicted in the histogram in (B). Note that Pax6 deficient precursors generate more than double the number of neurospheres than WT cortical precursors (data were normalized to the number of neurospheres generated from WT cells). Scale bar: 600 μm.

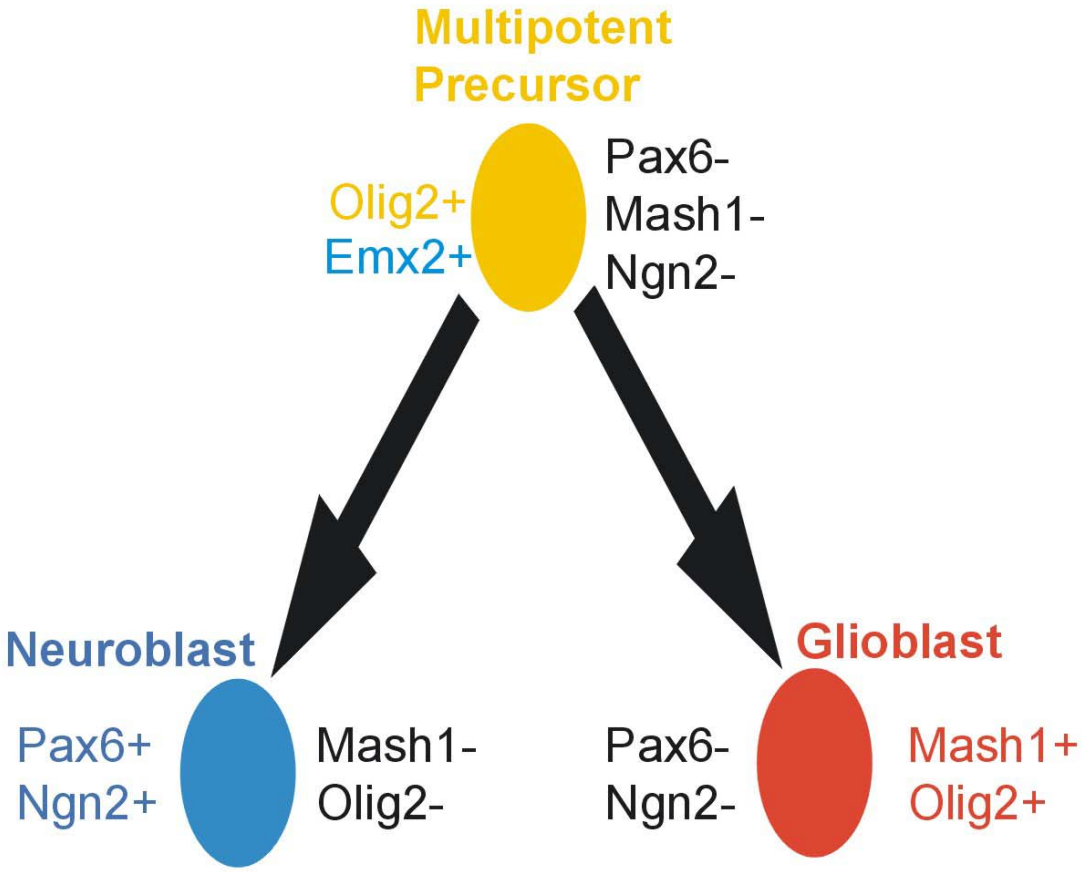


Fig. 24 Sequential expression of transcription factors in a multipotent precursor cell specifies the cell fate. A multipotential precursor cell (yellow) of the cerebral cortex initially expresses Olig2 and Emx2. The expression of Pax6 and Ngn2 drives the cell towards a neuroblast (blue). The glial cell fate (red) is characterized by the expression of Mash1 and Olig2.

Curiculum Vitae

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