Conditional activation of Pax6 in the developing cortex of transgenic mice causes progenitor apoptosis

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During development, Pax6 is expressed in a rostral-lateral-high to caudomedial-low gradient in the majority of the cortical radial glial progenitors and endows them with neurogenic properties. Using a Cre/loxP-based approach, we studied the effect of conditional activation of two Pax6 isoforms, Pax6 and Pax6-5a, on the corticogenesis of transgenic mice. We found that activation of either Pax6 or Pax6-5a inhibits progenitor proliferation in the developing cortex. Upon activation of transgenic Pax6, specific progenitor pools with distinct endogenous Pax6 expression levels at different developmental stages show defects in cell cycle progression and in the acquisition of apoptotic or neuronal cell fate. The results provide new evidence for the complex role of Pax6 in mammalian corticogenesis.

KEY WORDS: Corticogenesis, Cre/loxP, Overexpression, Pax6, Pax6-5a, Progenitor, Apoptosis, Mouse

INTRODUCTION

Pax6 is an evolutionarily conserved transcription factor with pivotal roles in the morphogenesis of the eye, pancreas and brain. In the brain, Pax6 acts as a pattern formation gene, involved in the regional specification of the telencephalon. As early as E8.5, the expression of Pax6 is confined to the neuroepithelium of the dorsal part of the telencephalic primordium (Walther and Gruss, 1991), outlining the anlage of the future cortex, while cross-repressive interactions between Pax6 and Gsh2 establish the pallial-subpallial boundary (Toresson et al., 2000). In developing cortex, Pax6 is expressed in a prominent rostromedial-high to mediolateral-low gradient, with expression lost progressively after E15.5 (Muzio et al., 2002a; Stoykova et al., 1997; Stoykova et al., 2000). In the Pax6 loss-of-function (LOF) mouse Small eye (Sey) (Hill et al., 1991), the cortical progenitors acquire ventral molecular identity starting at the ventral pallium (VP) and lateral pallium (LP), and subsequently extending dorsally and medially (Kroll and O’Leary, 2005; Muzio et al., 2002b; Stoykova et al., 1996; Stoykova et al., 2000; Torresson et al., 2000; Yun et al., 2001). Recent data indicate that the different levels of Pax6 expression in cortical progenitors along the anterior-posterior axis play a role in progenitor regionalization. Thus, in homozygous Sey/Sey embryos, the rostromedial-lateral regions of the cortex, where Pax6 is normally expressed at the highest level, are reduced, whereas the caudomedial cortical domains, which normally show low Pax6 expression, are expanded (Bishop et al., 2000). Similarly, the high expression level of Pax6 in the progenitors of the rostral VP and LP appears essential for the specification of distinct amygudal nuclei (Tole et al., 2005).

The expression of Pax6 specifies the majority of the cortical progenitors, namely the RC2-positive radial glial (RG) cells (Götz et al., 1998). These cells have been shown to act as pluripotent progenitors, generating both neuronal and glial cells (Heins et al., 2002; Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2004). At birth, the cortical plate (CP) of Sey/Sey mice is hypocellular, with overgrown ventricular and subventricular zones (V.Z. and S.V.Z., respectively) (Schmahl et al., 1993; Stoykova et al., 1996; Stoykova et al., 2000), and the RG progenitors show defects in their mitotic cycle (Estivill-Torrus et al., 2002; Götz et al., 1998), migratory and adhesive properties (Caric et al., 1997; Hartfuss et al., 2001; Nomura and Osumi, 2004; Stoykova et al., 1997), boundary formation (Hartfuss et al., 2001; Stoykova et al., 1996) and differentiation (Göt z et al., 1998; Warren et al., 1999).

In vertebrates, alternative splicing generates two different Pax6 protein isoforms, Pax6 and Pax6-5a, possibly having different sets of targets (Czerny et al., 1993; Epstein et al., 1994b; Kozmik et al., 1997). Dorsoventral patterning and boundary formation seem to be mediated exclusively by the Pax6 isoform, whereas progenitor proliferation is influenced by both isoforms (Haubst et al., 2004). Retrovirus-mediated overexpression of Pax6 in RG progenitors in vitro and cell lineage experiments indicated a neurogenic activity of Pax6 (HACK et al., 2004; HEINS et al., 2002; Haubst et al., 2004).

Using a Cre/loxP-based recombination approach we have developed an in vivo system for conditional Pax6 gain-of-function (GOF) expression in transgenic mice and studied the effects of the activation of the two Pax6 isoforms in different progenitors during corticogenesis. We found that ectopic activation or overexpression of the two isoforms, Pax6 and Pax6-5a, inhibits the proliferation of cortical progenitors. Furthermore, activation of transgenic Pax6 in vivo causes misregulation of the mitotic cycle, premature neurogenesis, and massive apoptosis in different progenitor pools, which seems to depend on the distinct spatiotemporal sensitivities of the cortical progenitors containing different levels of endogenous Pax6.

MATERIALS AND METHODS

Construction of plasmids and generation of transgenic mice

A loxP-egr-polyA cassette was cloned into the EcoRI site of pCAGGS (Niwa et al., 1991) followed by a loxP-(XhoI)-IRES-lacZ-polyA cassette. The coding sequence of Pax6 or Pax6-5a was inserted into the XhoI site resulting in pJoP6 and pJoP6-5a, respectively. The plasmids were used to generate JoP6 and JoP6-5a mice by pronuclear microinjection. Transgenic

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Table 1. Primers used in this study

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mice were identified by GFP fluorescence. Genomic PCR was performed with the primers JoP6F, JoP6R and JoP6SαR (Table 1). pPD and pHD were generated by cloning four PD- and five HD-consensus sequences into pGL3 (Promega) (Epstein et al., 1994a).

Immunohistochemistry

Immunohistochemistry was carried out as described (Ashery-Padan et al., 2000) and in situ hybridization analysis was according to Moorman et al. (Moorman et al., 2001). We used mouse monoclonal antibodies against Pax6 (Babco), 5-bromo-2-deoxyuridine (BrdU; Roche), nestin (Chemicon) and neuron-specific class III β-tubulin (Tuji; Babco), and rabbit polyclonal antisera against pHH3 (Biomol), β-actin (Abcam), and activated caspase3 (Cell Signaling Technology) on 8 μm cryostat sections (5 μm for BrdU labeling). Secondary antibodies were from Molexular Probes (1:500). For TUNEL labeling, the ApopTag kit was used (Intergen, Purchase, NY). The BrdU labeling index was estimated as the percentage of the total cell number of BrdU+ cells on DAPI-stained sections in equally sized areas in medial pallium (MP) and dorsal pallium (DP) from cortex of both genotypes. pHH3+ cells were counted and compared over the whole cortex in equally sized units of control and double-transgenic apical VZ surface. Data are shown as means±s.d. P values were derived using Student’s t-test.

Luciferase reporter assay

Transient transfection of SAOS2 (ATC# HTB-85) and HeLa cells were performed using Lipofectamine2000 (Invitrogen) and 3 μg of the following plasmids: pCMV and pPax6 (Maulebecker and Gruss, 1993), p53Luc (Stuart et al., 1995), pmRb-pJ3Omega15R0X-p (Bernards et al., 1989), and pJoP6-5a. For in vitro overexpression of Pax6, 16 μg of pPax6 was used (indicated by Pax6++ in Fig. 7). Cells were subjected to the Dual-Luciferase Reporter Assay System (Promega) after 48 hours. The assay was performed in triplicate and luciferase activities were normalized to internal control activities (data shown as means±s.d.).

q-PCR analysis

Total RNA isolated from cortex was quantified by optical density and used for q-PCR with the QuantiTect Rev. Transcription Kit and the QuantiTect SYBR Green PCR Kit (Qiagen). Assays were performed in triplicate and normalized to internal 18S RNA (data as means±s.d.). Primer sequences are listed in Table 1.

RESULTS

The system for conditional activation of Pax6 in vivo

For conditional activation of Pax6 we generated a construct (pJoP6) that contains a floxed gfp-stop cassette under control of the β-actin/CMV fusion promoter (Niwa et al., 1991), driving ubiquitous expression of the gfp reporter gene (Fig. 1). Upon Cre recombination the gfp-stop cassette is excised, leading to simultaneous expression of Pax6 and a second reporter, lacZ, via an IRES sequence. The transgenic mouse line generated with this construct was named JoP6.

In order to test the recombination of the integrated construct, JoP6 males were crossed with female Emx1IREScre mice directing recombination in most of the pallial progenitors (Gorski et al., 2002). Recombination in the JoP6:Emx1IREScre mice was monitored by PCR with the primers JoP6F and JoP6R (arrows in Fig. 1), which bind 5’ and 3’ of the floxed gfp-stop cassette, respectively. Genomic PCR with DNA isolated from the cortex of JoP6 brains resulted in a 1831 bp DNA fragment, whereas after successful recombination an additional 261 bp DNA fragment was detected using DNA isolated from JoP6:Emx1IREScre double-transgenic cortex (Fig. 2A). Sections of JoP6 control cortex exhibited widespread GFP fluorescence in the entire brain, which

Fig. 1. The construct for transgenic activation of Pax6. The construct pJoP6 contains the β-actin/CMV fusion promoter and a loxP-flanked gfp-stop cassette placed in front of the Pax6 and lacZ coding sequences. After Cre-mediated recombination, the gfp-stop cassette is eliminated allowing simultaneous expression of transgenic Pax6 and lacZ via an IRES sequence from the resulting construct pJoP6rec. Arrows indicate the annealing sites of the primers JoP6F and JoP6R.
was switched off in most of the cortical cells in the VZ and CP of JoP6;Emx1<sup>irescre</sup> transgenic mice, indicating successful excision of the <sup>gfp</sup>-stop cassette (Fig. 2B, B'). Remaining GFP-positive cells might correspond to GABAergic interneurons (Gorski et al., 2002). The function of the second reporter, lacZ, was tested with isolated brains after whole-mount staining for -galactosidase (β-gal). The JoP6 brain showed only non-specific staining of the CP and the striatum (str). (B, B') Abundant GFP fluorescence is detected on coronal sections of E14.5 JoP6 cortex, whereas the majority of the cells in the VZ and SVZ of the JoP6;Emx1<sup>irescre</sup> cortex are GFP-negative. Arrows indicate preserved GFP+ cells in the VZ. Note the preserved fluorescence in the thin CP and the striatum (str). (C) Whole-mount staining for β-gal indicates specific expression of lacZ in the telencephalon (tel), olfactory bulb (ob) and scattered cells in the diencephalon (di) of E15.5 JoP6;Emx1<sup>irescre</sup> cortex (bottom), whereas the mesencephalon (mes) and the metencephalon (mt), as well as the JoP6 control brain (top), are unstained. (D) Western blot confirms higher level of Pax6 in the E15.5 JoP6;Nex-Cre as compared with the JoP6 cortex. (E) E12.5 section of JoP6;Emx1<sup>irescre</sup> reveals weak β-gal staining of individual or aggregated (arrows and inset) progenitors. (F) At E15.5, β-gal+ cells are within LV/VP, IZ and the lower part of the CP. (G) Strong β-gal staining is seen at E18.5 in late progenitors as well as postmitotic cells of the CP and (H) at P28 throughout the whole cortex. (I, I') Pax6 immunohistochemistry on sections of E18.5 JoP6 (l) and JoP6;Emx1<sup>irescre</sup> (l') cortex, showing ectopic Pax6 expression in postmitotic cells in the IZ and the lower part of the CP. The magnifications of the framed area in I' demonstrate Hoechst and Pax6 co-labeling. (J, K) q-PCRs with RNA isolated from cortex of JoP6 control, JoP6;Emx1<sup>irescre</sup>, and JoP6;Nex-Cre normalized to 18S RNA indicate upregulation of Pax6 in the double-transgenic cortices (J) and specific elevation of the level of Pax6 transcripts in the JoP6;Emx1<sup>irescre</sup> cortex, whereas the level of Pax6-Sa in is fact diminished, as compared with the controls (K). (L) SAOS2 cells were transiently co-transfected with a luciferase reporter construct containing either the HD (blue) or the PD (red) domain and either pJoP6 or pJoP6rec, which express Pax6. Luciferase activities are shown as relative values compared with the activity measured in lysates from cells co-transfected with the JoP6 control plasmid. Co-transfection of pJoP6rec with either the HD- or the PD-containing reporter construct induced an increase in luciferase activity, indicating functionality of transgenic Pax6. Error bars indicate s.d.

In contrast to the endogenous Pax6 expression, which was confined to VZ progenitors of the JoP6 control cortex, the JoP6;Emx1<sup>irescre</sup> cortex exhibited – similar to the β-gal staining –
ectopic Pax6 immunoreactivity in the IZ and CP (Fig. 2L'), indicating ectopic expression. Co-labeling with Pax6 antibody and Hoechst staining confirmed Pax6 immunoreactivity exclusively in the cell nuclei (Fig. 2I'). The higher level of Pax6 as compared with controls was demonstrated for the JoP6;Emx1<sup>IREScre</sup> cortices by q-PCR and additionally for JoP6;Nex-Cre by western blotting (Fig. 2J,D, arrow). Recent data indicate that the introduction of constructs expressing only one isoform (Pax6 or Pax6-5a) in Neuro2A and NIH3T3 cells in vitro increases the cellular levels of not only that isoform, but also of the other, indicating that positive autoregulation of the endogenous Pax6 locus occurs (Pinson et al., 2006). Interestingly, however, the results from the q-PCR assay revealed that in the cortex of the double-transgenic JoP6;Emx1<sup>IREScre</sup> mice, only the level of the Pax6 transcript was elevated, whereas the level of Pax6-5a was in fact diminished (Fig. 2K). The functionality of the transgenic Pax6 was indicated by co-transfection experiments demonstrating the binding activity of Pax6 to its target consensus sequences (Fig. 2L).

**Activation of transgenic Pax6 misregulates the cell cycle of early cortical progenitors**

Examination of Hematoxylin and Eosin (HE) -stained histological sections from adult P23 brains revealed that the thickness of the JoP6;Emx1<sup>IREScre</sup> cortex was significantly reduced (by 43±3%) as compared with the control (P<0.001, n=12; Fig. 3A,A'). Despite the prominent hypopcellularity, the correct positioning of the layers was not affected, as indicated by layer-specific markers [Clim1a (Llh2), Rorβ and Cux2 (Cul2); not shown]. This phenotype strongly suggests that conditional activation of transgenic Pax6 might affect proliferation and/or cell survival of the cortical progenitors. Therefore, proliferation was examined at stage E11.0, when the vast majority of the cells are still proliferating. To label S-phase nuclei and determine the BrdU labeling index (percentage of BrdU<sup>+</sup> cells from the total number of DAPI<sup>+</sup> cells), a short, 30-minute pulse of BrdU was used. Significant reduction in proliferation, by 14±6% and 16±7%, was found in the medial pallium (MP) and dorsal pallium (DP) of JoP6;Emx1<sup>IREScre</sup> mice, respectively (P<0.001, n=5 for MP and n=6 for DP; Fig. 3B,B'), two regions where recombination was strong.

During neurogenesis, proliferating nuclei follow interkinetic nuclear migration, entering into S phase at the basal surface of VZ and progressively moving to the apical VZ surface, where they enter into M phase (Takahashi et al., 1993). After 90 minutes of BrdU incorporation, most of the progenitors in the control JoP6 cortex had intensively stained (S-phase) nuclei, which were still located predominantly within the basal region of the VZ (Fig. 3C). Some nuclei with dilute BrdU content (that were at the very end of their S phase, when the BrdU pulse started) were seen at or near to the apical surface of the control VZ (arrows in Fig. 3C). In the JoP6;Emx1<sup>IREScre</sup> cortex, the S-phase labeled progenitor nuclei appeared to be distributed throughout the VZ, and only a few cells with dilute BrdU content were seen at the apical VZ (Fig. 3'C'). In addition, faintly stained aggregates of cells were visible in the mutant VZ (arrowheads in Fig. 3'C'). After 6 hours of BrdU labeling, the intensively stained progenitor nuclei of the control VZ reached the apical VZ, whereas in the JoP6;Emx1<sup>IREScre</sup> VZ these nuclei were retained at the basal VZ (Fig. 3D and arrows in D') indicating cell cycle arrest or extended S phase. Quantitation of equally sized areas of VZ with phospho-histone H3 (pHH3) at E14.5 revealed 42±6% fewer mitotic cells at the apical VZ surface of JoP6;Emx1<sup>IREScre</sup> as compared with JoP6 mice (P<0.001, n=14; Fig. 3E,E'). Taken together, these results indicate that in vivo activation
DEVELOPMENT

of transgenic Pax6 in the early cortical progenitors leads to a defect of interkinetic nuclear migration, a reduction in progenitor proliferation, and cortical hypocellularity.

**Conditional Pax6 activation enhances neurogenesis and changes cell adhesive properties**

Given the neurogenic activity of Pax6 for the RG progenitors (Hack et al., 2005; Heins et al., 2002), we studied the effect of Pax6 GOF on neuronal differentiation in vivo by immunostaining with monoclonal antibody (TuJ1) against neuron-specific class III β-tubulin. Despite the 21±2% reduction in JoP6;Emx1IREScre cortical thickness compared with JoP6 (P<0.001, n=12) mice, the thickness of the TuJ1+ mantle zone of LP at E13.5 showed no significant difference between genotypes, suggesting enhanced neuronal differentiation in JoP6;Emx1IREScre mice (Fig. 4A,A’). Furthermore, ectopic TuJ1+ cells were detected in the apical VZ regions of the MP at E11.5 (Fig. 4C,C’), as well as in the LP and VP at E14.5 (Fig. 4B,B’), suggesting premature neurogenesis in subsets of cortical progenitors. In addition, the neuronal bHLH transcription factor gene Nex (Neurod6 – Mouse Genome Informatics) showed a stronger in situ hybridization signal in the VP and LP of the E11.5 JoP6;Emx1IREScre cortex as compared with control JoP6 cortex at E11.5.

**Fig. 4. Enhanced neurogenesis and altered adhesive properties after activation of transgenic Pax6 in developing cortex.** (A,A’) The E13.5 JoP6;Emx1IREScre VZ is significantly diminished as compared with JoP6, but the size of the TuJ1+ mantle zone (M) is unchanged. Also, premature differentiated TuJ1+ cells are found ectopically in the VZ of JoP6;Emx1IREScre at E14.5 (B,B’) and E11.5 (C,C’). (D) q-PCR of total RNA from control and JoP6;Emx1IREScre cortices normalized to 18S RNA. N-CAM and R-cadherin are upregulated, whereas Pan, Tnc, Itgb3 and Itga5 are downregulated. Error bars indicate s.d.

**Fig. 5. Pax6 GOF at early corticogenesis causes apoptosis.** (A-B’) TUNEL assay on sections from JoP6 (A,B) and JoP6;Emx1IREScre (A’,B’) embryos at stages E11.5 (A,A’ with higher magnification) and E14.5 (B,B’) illustrates apoptosis of JoP6;Emx1IREScre cortical progenitors, more severe at E11.5. (C,C’) Immunolabeling against activated Casp3 confirms enhanced apoptosis in the JoP6;Emx1IREScre cortex. (D-D’) Double immunostaining of E13.5 JoP6;Emx1IREScre cortex against nestin (D) and activated Casp3 (D’) illustrates that many of the apoptotic cells are nestin+ progenitors (D’ and magnifications counterstained with Hoechst). (E) At stage E18.5, apoptosis is no longer detected in JoP6;Emx1IREScre. (F) In situ hybridization with ephrin A5 reveals that the apoptotic cell aggregates of the JoP6;Emx1IREScre cortex are positive for ephrin A5 (arrows).
with the control (see Fig. S1D,D' in the supplementary material). Together, these results suggest that upon Pax6 activation in vivo, a subset of cortical progenitors, mainly those within the VP and LP, undergoes premature differentiation.

Because of the cell aggregation detected after activation of transgenic Pax6 (Fig. 2E), we further analyzed the expression of genes involved in cell adhesion and cell signaling. q-PCRs performed with RNA extracted from E12.5 JoP6;Emx1^{IREScre} cortex showed an increase in the expression of genes encoding the cell adhesion molecules N-CAM (Ncam1 – Mouse Genome Informatics) and R-cadherin (Cdh4 – Mouse Genome Informatics) as compared with the controls, whereas the expression of genes involved in cell-cell interaction and signaling such as paxillin (Pax), tenascin C (Tnc), integrin beta 3 (Igb3) and integrin alpha 5 (Igta5) were reduced (Fig. 4D). The change in the expression of these genes strongly suggests their involvement in the aggregation of cortical cells after activation of transgenic Pax6. However, further experiments are necessary to elucidate their individual roles.

**Activation of transgenic Pax6 in the developing cortex causes apoptosis**

In order to study whether the cortical hypoplasia seen in Pax6 GOF in vivo might also involve an increase in cell death, we performed TUNEL reactions. At stage E11.5, massive apoptosis could be detected in the proliferating VZ of the JoP6;Emx1^{IREScre} cortex, in contrast to the JoP6 control (Fig. 5A,A'). Double immunolabeling against nestin, a marker for VZ progenitors associated with the cell membrane, and cytoplasmatic activated caspase 3 (Casp3) showed colocalization in many cases suggesting that the cell death is confined to progenitors (Fig. 5D-D'). At stage E14.5, apoptosis was still detectable in progenitors of the JoP6;Emx1^{IREScre} cortex, although at a lower level (Fig. 4B,B'). Labeling with antibodies against activated Casp3 implicated the caspase-dependent pathway as causing apoptosis (Fig. 4C,C'). At stage E18.5, the activation of transgenic Pax6 expression was still maintained, as judged by the expression of the lacZ reporter in the VZ and CP (see Fig. 2G); however, TUNEL reaction revealed no further apoptosis (Fig. 4E). Recent evidence has indicated that overexpression of ephrin A5 in vivo causes progenitor apoptosis in the embryonic cortex (Depaep et al., 2005). Interestingly, both aggregates and single TUNEL⁺ cells in the JoP6;Emx1^{IREScre} cortex show enhanced ephrin A5 expression, suggesting that Pax6 might act in an ephrin-A5-dependent manner (Fig. 4F).

When we examined whether Pax6 overexpression affects the early patterning of the cortical primordium, thereby possibly initiating progenitor apoptosis, we found that neither the dorsoventral nor the medioventral patterning in the E11.5 JoP6;Emx1^{IREScre} cortex was affected, as indicated by the normal expression of corresponding markers (e.g. Dlx1, Wnt3a, Emx2; see Fig. S1 in the supplementary material).

**Pax6 GOF induces apoptosis in specific cortical progenitor pools**

Several types of progenitors contribute to neurogenesis in the vertebrate cortex: neuroepithelial cells (at the beginning of neurogenesis, E11) and RG cells (after E13), which can be divided into Ngn2/Pax6⁺, RC2/Pax6⁺ and RC2/Pax6⁻ (Guillenot, 2005; Hartfuss et al., 2001) (Ngn2 is also known as Neurog2, and RC2 as Ifaprc2 – Mouse Genome Informatics). By utilizing mouse lines with distinct spatiotemporal activation of the Cre recombinase, we analyzed the effect of transgenic Pax6 activation in different subsets of progenitors.

Recombination directed by Emx1^{IREScre} starts at E9.5 and initially proceeds at highest level in the MP progenitors (Li et al., 2003). Because the expression of Pax6 in MP at this early stage is at the in situ hybridization detection limit (Mazor et al., 2002a), the massive apoptosis detected in the JoP6;Emx1^{IREScre} MP at E11.0 involves a set of progenitors that either express endogenous Pax6 at extremely low level or are Pax6-negative (Fig. 6A,B). The results suggest a high sensitivity of the early progenitors of the MP to ectopic expression or enhancement of the Pax6 expression level.

To gain insight into the consequences of overexpression of Pax6 specifically in Pax6-positive RG progenitors, we used the hGFAP-cre line (Zhuo et al., 2001). This line promotes activation of Cre recombinase in the majority of the RC2/Pax6⁺ radial glial progenitors as early as E13.5 (Götz et al., 1998; Heins et al., 2002). After overexpression of Pax6 in the JoP6;hGFAP-cre cortex, as indicated by β-gal staining (Fig. 6C), massive apoptosis was detected in single progenitors as well as in cell aggregates (Fig. 6D,D'). Therefore, we conclude that overexpression of Pax6 in the midgestation cortical progenitors expressing Pax6 at a moderate level also leads to cell death.

Interestingly, in the VP of JoP6;hGFAP-cre, where endogenous Pax6 and its target Ngn2 are expressed at a very high levels, apoptosis is seen only rarely (Fig. 6D'). To specifically examine the effect of Pax6 overexpression in the VP and LP progenitors, we crossed JoP6 mice with the E1-Ngn2/Cre line, in which Cre recombinase is directed by the E1 enhancer element of the gene encoding transcription factor Ngn2 (Berger et al., 2004). Despite significant recombination in the VP (as detected by β-gal staining), neither significantly increased apoptosis nor cell aggregates were seen at stage E11.5 or E14.5 in the JoP6;E1-Ngn2/Cre double-transgenic cortex as compared with the JoP6 control (Fig. 6E-F' and data not shown). Together, these data indicate that at the onset of neurogenesis, early cortical progenitors show differential sensitivity towards the elevation of the Pax6 expression level, which correlates inversely with their endogenous Pax6 expression level: the highly Pax6-positive progenitors of the rostral VP appear to be more resistant to Pax6 GOF, whereas the Pax6-negative progenitors, or progenitors that are expressing Pax6 at extremely low level (e.g. in the MP), undergo apoptosis.

**Pax6 GOF in postmitotic cells has no effect on cell survival**

Although recombination and thus activation of transgenic Pax6 is still detectable at E18.5 in postmitotic cells of the CP of JoP6;Emx1^{IREScre} mice, these cells do not undergo apoptosis (Fig. 5G). In order to directly assess the specific effect of the activation of transgenic Pax6 expression in newly born neurons, we used the Nex-Cre mouse line, which induces Cre recombinase activity in postmitotic neurons after their exit from the mitotic cycle (Schwab et al., 2000). In the double-transgenic JoP6;Nex-Cre mice cortex, where the level of Pax6 expression is higher than in the controls (see Fig. 2L), no enhancement of apoptosis was detected at E14.5 and P21 (Fig. 6H,H' and data not shown). Thus, transgenic activation of Pax6 in vivo specifically induces cortical progenitor apoptosis, whereas the fate of the postmitotic neurons is not affected.

**Pax6-induced apoptosis does not involve transcriptional activation of p53**

Deregulation of proliferation and apoptosis is assumed to involve both the p53 (Trp53) and pRb (Rhi1)-dependent pathways, where pRb prevents the induction of apoptosis through transcriptional repression of p53 (Ookawa et al., 1997). Previous evidence indicated that Pax6
binds to the human P53 (TP53) promoter with low affinity, although the effect on p53 gene activity has not been assessed so far (Stuart et al., 1995). Remarkably, Pax6 also binds directly to pRB (Cvekl et al., 2004), suggesting the existence of a possible relationship between Pax6, pRB- and p53-dependent apoptosis. In an attempt to address this issue, we studied the effect of Pax6 on the activity of the P53 promoter in a human osteosarcoma cell line (SAOS2) lacking endogenous pRB and p53. The reporter plasmid p53Luc containing the P53 promoter followed by the luciferase gene was co-transfected with pPax6 or pPax6-5a expression plasmids in the absence and presence of a mouse pRB expression plasmid. As illustrated in Fig. 7, expression of Pax6 barely influenced luciferase activation via the P53 promoter, whereas upon strong overexpression of Pax6 the P53 promoter activity decreased. pRB was not able to significantly enhance the effect of Pax6. Similar results were obtained with the cell lines NIH-2H3, HeLa and HelaTAT (data not shown). Pax6-5a expression showed no effect on the P53 promoter. Together, these results suggest that the apoptosis induced by transgenic Pax6 in vivo is due neither to activation of the cell death pathway through a direct transcriptional activation of p53, nor by abolishment of the pRb-dependent active repression of p53 activity.

**In vivo overexpression of Pax6-5a moderately inhibits progenitor proliferation**

Previous GOF experiments in primary cortical cultures indicated that retrovirus-mediated overexpression of Pax6 and Pax6-5a leads to inhibition of cell proliferation (Haubst et al., 2004). To study the in vivo activation of Pax6-5a, we generated the transgenic line JoP6-5a based on the same principles as JoP6 (see Materials and methods). Upon crossing with the Emx1IREScre line, the functionality of the JoP6-5a line was tested: PCR with primers JoP6F and JoP65aR detected recombination of genomic DNA (1955 bp and 394 bp fragments); expression of the two reporter genes gfp and lacZ was monitored by GFP fluorescence and β-gal staining, respectively; and Pax6 immunostaining indicated the expression of the JoP6-5a (Fig. 8A-D). Co-transfection experiments of pJoP6-5aR with pHD and pPD indicated the functionality of transgenic Pax6-5a (Fig. 8E). q-PCR showed enhancement of Pax6-5a expression in the JoP6-5aEmx1IREScre cortex, which was much weaker compared with the Pax6 enhancement in the JoP6Emx1IREScre cortex (Fig. 8F). Statistical analysis of the apoptotic pattern of JoP6-5aEmx1IREScre and control cortices at stages E11.5 and E13.5 revealed no difference, and no cell aggregates were formed (n=5; Fig. 8G,G’). Nevertheless, estimation of the BrdU index in the E11.5 DP after 30 minutes labeling revealed a mild but significant reduction (by 10±2%) in progenitor proliferation (P<0.001, n=4; Fig. 8D,D’). Taken together, these results suggest a difference in the biological activity of the two Pax6 isoforms in vivo.

**DISCUSSION**

To study the in vivo function of Pax6 during corticogenesis we developed a conditional GOF approach that allows activation of either the Pax6 or the Pax6-5a isoform. In this work, we provide evidence for a differential spatiotemporal sensitivity of cortical progenitors towards Pax6 GOF in vivo. We found that different levels of Pax6 play an essential role in the regulation of cortical growth by controlling progenitor proliferation, cell cycle...
progression, the acquisition of progenitor apoptotic fate and induction of neurogenesis. A similar role in controlling the proliferation of cortical progenitors was found for the Pax6-5a transcript. We show that the induced progenitor cell death evident in our assays, is unlikely to be due to a direct transcriptional regulation of the anti-tumor gene p53 by Pax6.

**Pax6 controls cortical progenitor proliferation and cell cycle progression**

We found that the adult cortex is significantly reduced in size (by 43±3%) after transgenic Pax6 activation, having a preserved cortical layering and dorsoventral patterning. Subsequent analysis of progenitor proliferation at E11.0 revealed a significant reduction of the BrdU labeling index as compared with the controls: by 14±6% and 43±3% after transgenic Pax6-5a, indicated by ++ does inhibit the Pax3 promoter activity. By contrast, Pax6-5a shows no significant effect. Normalized luciferase activity in the presence or absence of functional pRb is indicated; error bars indicate s.d. The luciferase activity of the ‘empty’ vector pCMV without pRb was given an arbitrary value of 100. Note that pRb slightly increases the Pax6 inhibitory effect on p53.

Experiments involving Pax6 transduction in RG cell cultures and adult neurospheres (Hack et al., 2004; Heins et al., 2002) as well as in HeLa cells (Cartier et al., 2006) have revealed premature neuronal differentiation. Owing to the massive apoptosis in the JoP6;Emx1<sup>RES</sup> mice during early corticogenesis, a quantitative estimation of possible premature neurogenesis is difficult. However, we found that although the JoP6;Emx1<sup>RES</sup> cortical thickness at E13.5 is significantly reduced (by 21±2%, as compared with the control), the thickness of the Tuj1<sup>+</sup> mantle layer appeared unchanged. Furthermore, the ectopically located Tuj1<sup>+</sup> cells in the VZ, and the enhanced Nex in situ hybridization signal in the E11.5 CP of JoP6;Emx1<sup>RES</sup> mice, suggest enhanced neurogenesis in the Pax6 GOF condition in vivo. Thus, subpopulations of early progenitors of the JoP6;Emx1<sup>RES</sup> cortex seem to exit prematurely from the mitotic cycle and differentiate, which would diminish the cortical progenitor pool early in development and contribute to the severe hypocellularity of the adult JoP6;Emx1<sup>RES</sup> cortex. It would also be of interest to test the expression of the potential Pax6 downstream target Fapb7, recently reported to be involved in maintenance of proliferation versus neuronal differentiation in cortical progenitors (Arai et al., 2005).

In addition to misregulation of the mitotic cycle, we found that transgenic Pax6 activation causes aggregation of cortical progenitors. Previously, we reported on changes in the adhesive properties of isolated cortical progenitors from Sey/Sey cortex and reduced expression of R-cadherin (Stoykova et al., 1997). After Pax6 GOF in vivo we found strong enhancement of the expression of N-CAM, which encodes a cell-cell adhesion molecule positively regulated by Pax6 (Holst et al., 1998; Yamaoka et al., 2000), as well as increased expression of R-cadherin. The latter result further supports the idea of R-cadherin mediating Pax6-dependent function in cell adhesion (Andrews and Mastick, 2003), possibly by direct genetic interaction of these two genes. We also found Pax6-mediated inhibition of the expression of integrin alpha 5, which contains Pax6-binding sites in its promoter (Duncan et al., 2000), as well as a decrease in the expression levels of integrin beta 3, paxillin and tenasin C, molecules involved in cell-cell interaction. Given the complex pathways in which these proteins participate, further detailed analysis is required to dissect the specific role of Pax6 in these processes. However, the system for conditional activation of Pax6 described here seems to be a reliable tool for such studies in vivo.

**Pax6 GOF in vivo induces apoptosis in specific sets of cortical progenitors**

TUNEL assays and double immunohistochemistry with antibodies against activated Casp3 and nestin revealed abundant apoptosis in the cortical progenitors of E11.5 JoP6;Emx1<sup>RES</sup> mice. When Pax6 GOF was directed into postmitotic cells by the Nex-Cre mouse line
(Schwab et al., 2000), no apoptosis could be detected. These results provide the first evidence that Pax6 is involved in progenitor apoptosis during mammalian corticogenesis.

Given that apoptosis is a very fast process, with dead cell bodies cleared out of the rat cortex in 2 hours 20 minutes (Thomaidou et al., 1997), the apoptosis at the beginning of neurogenesis in JoP6-5a;Emx1IREScre mice is massive. Although extensive apoptosis is apparent at E11.5, it weakens by E14.5, and at E18.5 no increased cell death appears to occur. In parallel, a limited number of /H9252-gal+ cells, marking recombined survived progenitors, are found at E12.5, whereas at E15.5 and E18.5 the /β-gal staining is progressively spreading into the IZ and CP, respectively, and at P28 the entire depth of the cortex is populated by /β-gal+ cells. We assume, therefore, that a significant part of the early (E9.5-E14.5) cortical progenitors, where transgenic Pax6 is induced, undergo rapid apoptosis and are removed from the cortex before they have accumulated enough /H9252-gal to be confidently registered on thin sections, whereas the later progenitors are affected much less by Pax6 GOF.

Subpopulations of cortical progenitors survive Pax6 GOF and are monitored as /β-gal+ cells. By using different mouse lines for regionalized recombination, we provide evidence that cortical progenitors in vivo have spatiotemporal differences in their sensitivity towards Pax6 GOF. In the JoP6-5a;Emx1IREScre cortex, activation of transgenic Pax6 is initiated at E9.5 predominantly in the MP, where endogenous Pax6 is only barely expressed, if at all (Muzio et al., 2002b). Thereafter, Pax6 GOF progressively spreads to the majority of the glutamatergic cortical progenitors (Li et al., 2003). Therefore,
the observed massive apoptosis in the MP of JoP6:Emx1\textsuperscript{CRE/lo}\ mice appears to be the result of either ectopic expression of Pax6 in the early Pax6-negative progenitor pool (neuroepithelial cells at E9.5-E12 in MP and Pax6-RG progenitors), or overexpression of Pax6 in RG cells expressing Pax6 only faintly. Also, in the JoP6:βGFAP-cre cortex, where transgenic Pax6 becomes activated after E13.5 exclusively in the RC2/Pax6\textsuperscript{+} radial glial cells (Malatesta et al., 2003; Zhuo et al., 2001), extensive apoptosis was detected, indicating that the overexpression of Pax6 in the Pax6\textsuperscript{+} midgestation cortical progenitors leads to apoptosis as well. In order to study the effect of Pax6 overexpression in the early VP progenitors, where the endogenous Pax6 expression level is at its highest (Stoykova et al., 1997), we used the E1-Ngn2/Cre line (Berger et al., 2004). This line drives recombination directed by the E1-Ngn2 enhancer that is activated only by a high dosage of Pax6 (Marquardt et al., 2001; Scardigli et al., 2003). No significant enhancement of apoptosis was observed in the JoP6:E1-Ngn2/Cre cortex, suggesting that the VP and LP progenitors with the highest level of endogenous Pax6 are resistant to further elevation of Pax6. Collectively, these in vivo results demonstrate that the cortical progenitors have different sensitivity towards the Pax6 GOF condition, which is probably dependent upon the endogenous Pax6 expression level.

The tumor suppressor gene p53 is involved in the control of cell cycle arrest and apoptosis, inducing cell death upon activation (Hickman et al., 2002). In an attempt to study the molecular mechanism of the induced apoptosis in the JoP6:Emx1\textsuperscript{CRE/lo} cortex, we tested the effect of Pax6 expression on the p53 promoter, which contains Pax6 target sequences (Stuart et al., 1995). Pax6 protein binds to hypophosphorylated pRb (Cvekl et al., 2004), whereas pRb allows the formation of pRb-E2F complexes, which actively repress the transcription of E2F-responsive promoters, including the pro-apoptotic gene p53 (Ookawa et al., 1997; Sellers et al., 1995). Therefore, we also tested whether, upon overexpression, Pax6 could sequester pRb and abrogate pRb-E2F-dependent repression of the p53 promoter, thereby inducing apoptotic fate. We found that Pax6 is unable to trigger p53 activation in vitro, but, by contrast, inhibits p53 transcription upon strong overexpression and independently of pRB. Therefore, although the underlying molecular mechanism of the Pax6-induced apoptosis in vivo is still unclear, our results indicate that this phenomenon is not likely to be a consequence of p53 pathway activation. It is interesting to note that after overexpression of Pax6, we detected a strong enhancement of ephrin A5 expression in apoptotic cortical progenitors. Most intriguingly, a similar apoptotic phenotype of early cortical progenitors has recently been discovered in GOF experiments for ephrin A5 in transgenic mice in vivo (Depaepe et al., 2005), raising the possibility of genetic interplay between the Pax6- and ephrin-A5-dependent pathways in the control of cortical progenitor cell death.

Accumulating evidence supports the view that Pax6 is involved in tissue growth, not only by modulating progenitor proliferation and cell cycle progression, but possibly also by the involvement of apoptosis. Evidence has been presented that a high copy-number of transgenic Pax6 leads to microphthalmia in mice (Schedl et al., 1996). Pax6 overexpression in cultivated corneal epithelial cells slows down cell cycle progression and causes apoptosis (Ouyang et al., 2006). Ectopic activation of Pax6 in undifferentiated and mature pancreatic β-cells of transgenic mice inhibits progenitor proliferation and leads to apoptosis (Yamaoka et al., 2000), and activation of Pax6 suppresses tumorigenicity of glioblastoma cells inducing apoptosis as well (Zhou et al., 2005). In addition, the Casp3 target gene Purp acts as a regulator of Pax6 expression in neuroretina (Plaza et al., 1999) and, in developing Xenopus, expression of Pax6 at the neural-fold stage overlaps with TUNEL-positive cells (Hensey and Gautier, 1998). In the Pax6 LOF mutant Sey/Sey, the failure in the transition of the nasal ectoderm into nasal placode has been attributed to abnormal apoptosis (Fukuda et al., 2000), but no enhanced apoptosis is detected in the developing cortex (Grindley et al., 1995) (data not shown). Therefore, further research is required to reveal the biological significance of the apoptosis induced by Pax6 GOF in vivo in different cellular and experimental contexts.

We were unable to detect apoptosis in the cortical progenitors of JoP6-5a:Emx1\textsuperscript{CRE/lo} mice. It should be noted, however, that in contrast to the substantial elevation of the level of Pax6 transcripts in JoP6:Emx1\textsuperscript{CRE/lo} mice (3.8-fold higher, compared with the controls), the increase in the level of Pax6-5a in JoP6-5a:Emx1\textsuperscript{CRE/lo} mice was much less evident (1.2-fold, as compared with the controls). The possibility remains that the Pax6-5a level necessary to induce apoptosis was not achieved in this assay. Therefore, presently, it cannot be stated whether the detected progenitor apoptosis is a specific feature of the in vivo elevation of the Pax6 isoform only. However, in agreement with results from Pax6 and Pax6-5a GOF experiments in vitro (Haubst et al., 2004), we find that the two Pax6 isoforms successfully repress progenitor proliferation in the developing cortex.

Taken together with all the evidence available so far, the results presented in this study support the view that during corticogenesis, the modulation of Pax6 expression levels is crucial for progenitor cell fate acquisition as this influences cell proliferation, differentiation and apoptosis. Using the Pax6 GOF approach described here, we provide new in vivo evidence for a complex role of the Pax6 gene during multiple phases of mammalian corticogenesis.

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Supplementary material
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