

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 rostralateral-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 rostralateral-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 rostralateral 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 amygdalar 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-Torres 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 (Götz 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). Dorsal-ventral 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-egfp-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

Target gene	Name	Primer sequence (5' to 3') or Qiagen catalog number
<i>Pax6</i>	F	CCTGGTTGGTATCCCGGGA
	R	CCGCTTCAGCTGAAGTCGCA
<i>Pax6-5a</i>	F	CACAGCGGAGTGAATCAGCTT
	R	GCACTGGACTTTTGCATCTG
<i>N-CAM</i>	F	ATTGTCATCTTCGTCCTGCTCC
	R	TCCTCCATGTCTTTGCCCTTG
<i>Itga5</i>	F	GGATTCTGGAGTCTCACTGTA
	R	GTACTCCAGAATCCGGGTGAA
R-cadherin	F	CTATGGTGC GGCTGCTGGTA
	R	GGAGTTCTCGGGAACGTTGAT
<i>Pxn</i>	F	CCGGCTGTTACTGGAGCTGAA
	R	GGAACCTCCAGTTCATCCAAGA
<i>Tnc</i>	F	GCAACCAAGGACAATGTGTGTA
	R	CTCCCGAATGGTGGTGGATGT
18S	F	QT01036875
	R	
<i>Itgb3</i>	F	QT00128849
	R	
	JoP6F	CCTGGGCAACGTGCTGGTTATTGTGCTG
	JoP6R	CACAACCGTTGGATACCTGCAGAATTCGG
	JoP65aR	GTCGCCACTCTTGCTTACTCCCTCCG

mice were identified by GFP fluorescence. Genomic PCR was performed with the primers JoP6F, JoP6R and JoP65aR (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 (Tuj1; Babco), and rabbit polyclonal antisera against pHH3 (Biomol), β -actin (Abcam), and activated caspase3 (Cell Signaling Technology) on 8 μ m cryostat sections (5 μ m for BrdU labeling). Secondary antibodies were from Molecular 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 \pm 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 μ g of the following plasmids: *pCMV* and *pPax6* (Maulbecker and Gruss, 1993), *p53Luc* (Stuart et al., 1995), *pmRb-pJ3Omega115ROX-p* (Bernards et al., 1989), and *pJoP6-5a*. For in vitro overexpression of *Pax6*, 16 μ g 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 \pm 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 \pm 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 *Emx1^{IREScree}* mice directing recombination in most of the pallial progenitors (Gorski et al., 2002). Recombination in the *JoP6;Emx1^{IREScree}* 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;Emx1^{IREScree}* double-transgenic cortex (Fig. 2A). Sections of *JoP6* control cortex exhibited widespread GFP fluorescence in the entire brain, which

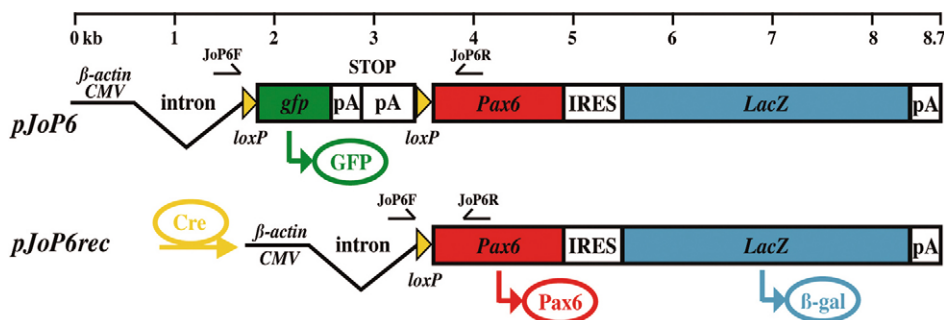


Fig. 1. The construct for transgenic activation of Pax6. The construct *pJoP6* contains the β -actin/CMV fusion promoter and a loxP-flanked *gfp*-stop cassette placed in front of the *Pax6* and *lacZ* coding sequences. After Cre-mediated recombination, the *gfp*-stop cassette is eliminated allowing simultaneous 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.

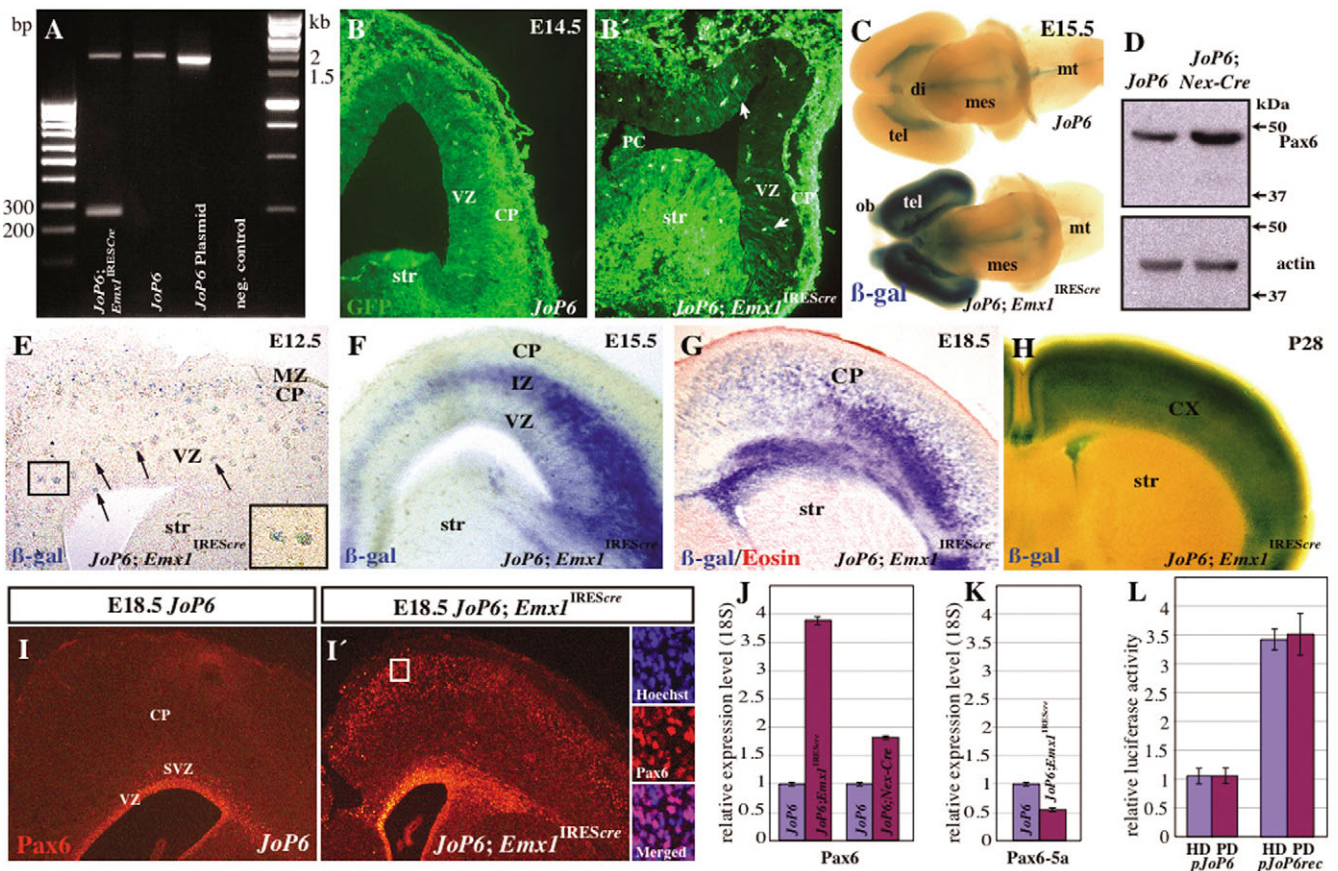


Fig. 2. Efficiency of recombination in *JoP6* mice. (A) Genomic PCR was performed with the primers *JoP6F* and *JoP6R* on DNA isolated from the *pJoP6* plasmid and cortices from wild-type (negative control), *JoP6* and *JoP6;Emx1^{IREScree}* embryos. The PCR amplifies a 1831 bp fragment from unrecombined DNA templates and from *pJoP6* plasmid and *JoP6* cortex, whereas an additional 261 bp DNA band is seen with the DNA sample from the *JoP6;Emx1^{IREScree}* cortex. (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^{IREScree}* 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^{IREScree}* 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^{IREScree}* cortex reveals weak β -gal staining of individual or aggregated (arrows and inset) progenitors. (F) At E15.5, β -gal⁺ cells are within LPVP, 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* (I) and *JoP6;Emx1^{IREScree}* (I') 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^{IREScree}*, 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^{IREScree}* cortex, whereas the level of Pax6-5a is in 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 *pJoP6* 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.

was switched off in most of the cortical cells in the VZ and CP of *JoP6;Emx1^{IREScree}* transgenic mice, indicating successful excision of the *gfp*-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 β -galactosidase (β -gal). The *JoP6* brain showed only non-specific staining of the choroid plexus, whereas the *JoP6;Emx1^{IREScree}* cortex, which is smaller than in the control, was intensively stained, indicating activation of transgenic Pax6. We further followed the expression of the *lacZ* reporter throughout brain development. At E12.5, β -gal⁺ aggregates as well as some individual cells were detected in

the VZ and in the thin CP (Fig. 2E). Later, at E15.5, *lacZ* expression was apparent in both VZ progenitors and cells migrating throughout the intermediate zone (IZ) towards the CP, invading its lower part (Fig. 2F). At E18.5, the late cortical progenitors showed strong β -gal staining and the CP was massively populated by β -gal⁺ postmitotic cells (Fig. 2G), whereas at P28 the whole cortex was extensively stained for β -gal (Fig. 2H), similar to the recombination pattern in the adult *Emx1^{IREScree}* brain (Gorski et al., 2002).

In contrast to the endogenous Pax6 expression, which was confined to VZ progenitors of the *JoP6* control cortex, the *JoP6;Emx1^{IREScree}* cortex exhibited – similar to the β -gal staining –

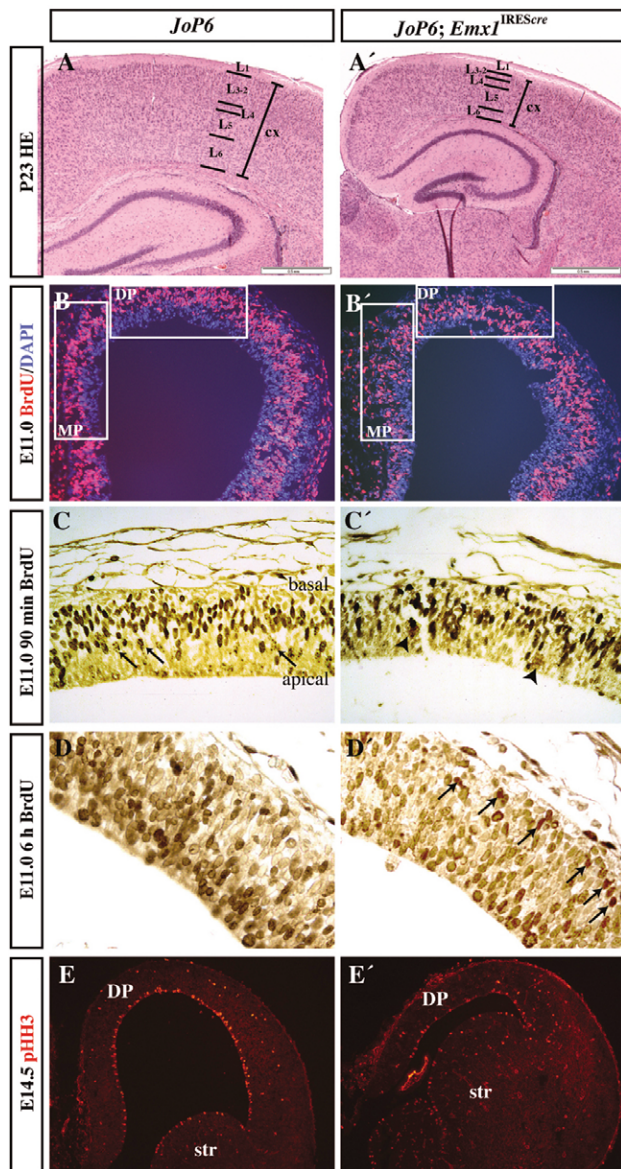


Fig. 3. Activation of transgenic *Pax6* disturbs the mitotic cell cycle. (A,A') HE staining of P23 cross-sections of *JoP6* control and *JoP6;Emx1^{IREScree}* cortex (cx). The size of the *JoP6;Emx1^{IREScree}* cortex is significantly diminished, but cortical layering is preserved. Scale bars: 0.5 mm. (B,B') Coronal 5 μ m sections of E11.0 *JoP6* control and *JoP6;Emx1^{IREScree}* cortex immunostained for BrdU and DAPI after a 30-minute BrdU pulse. Estimated BrdU labeling indices reveal a significant inhibition of the proliferation rate in the MP and DP of the *JoP6;Emx1^{IREScree}* cortex as compared with the *JoP6* control. The frames indicate the equally sized areas used for determination of the BrdU labeling index of the genotypes compared. (C-D') Patterns of BrdU pulse labeling of cortical progenitors at stage E11.0 for 90 minutes (C,C') and 6 hours (D,D') in *JoP6* control (C,D) and *JoP6;Emx1^{IREScree}* mice (C',D'). The arrows in C point to nuclei in the control cortex that were labeled at the end of their S phase and underwent mitosis thereafter, as indicated by their diluted content of BrdU. Nuclei of such appearance are scarce in the *JoP6;Emx1^{IREScree}* cortex (C'); arrowheads point to cell aggregates). Note the severe distortion of the interkinetic nuclear migration in the *JoP6;Emx1^{IREScree}* cortex, in which, even after a 6-hour BrdU pulse, many nuclei are still in the basal VZ (arrows in D'). (E,E') Immunolabeling of pHH3 at stage E14.5 reveals significantly fewer mitotic cells at the apical surface of the VZ in *JoP6;Emx1^{IREScree}* as compared with *JoP6* mice.

ectopic *Pax6* immunoreactivity in the IZ and CP (Fig. 2I,I'), 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^{IREScree}* 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^{IREScree}* 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^{IREScree}* cortex was significantly reduced (by $43\pm 3\%$) as compared with the control ($P < 0.001$, $n = 12$; Fig. 3A,A'). Despite the prominent hypocellularity, the correct positioning of the layers was not affected, as indicated by layer-specific markers [*Clim1a* (*Ldb2*), *Rorb* and *Cux2* (*Cutl2*); 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⁺ cells from the total number of DAPI⁺ cells), a short, 30-minute pulse of BrdU was used. Significant reduction in proliferation, by $14\pm 6\%$ and $16\pm 7\%$, was found in the medial pallium (MP) and dorsal pallium (DP) of *JoP6;Emx1^{IREScree}* 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 diluted 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^{IREScree}* cortex, the S-phase labeled progenitor nuclei appeared to be distributed throughout the VZ, and only a few cells with diluted BrdU content were seen at the apical VZ (Fig. 3C'). In addition, faintly stained aggregates of cells were visible in the mutant VZ (arrowheads in Fig. 3C'). After 6 hours of BrdU labeling, the intensively stained progenitor nuclei of the control VZ reached the apical VZ, whereas in the *JoP6;Emx1^{IREScree}* 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 $42\pm 6\%$ fewer mitotic cells at the apical VZ surface of *JoP6;Emx1^{IREScree}* as compared with *JoP6* mice ($P < 0.001$, $n = 14$; Fig. 3E,E'). Taken together, these results indicate that in vivo activation

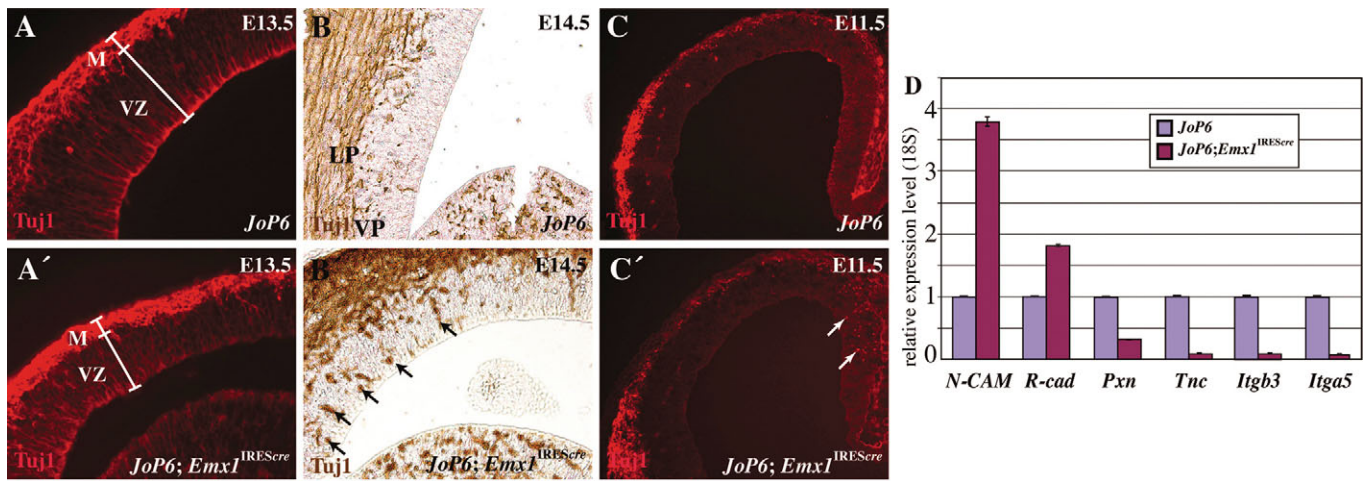


Fig. 4. Enhanced neurogenesis and altered adhesive properties after activation of transgenic Pax6 in developing cortex. (A,A') The E13.5 *JoP6;Emx1^{IREScree}* VZ is significantly diminished as compared with *JoP6*, but the size of the Tuj1⁺ mantle zone (M) is unchanged. Also, premature differentiated Tuj1⁺ cells are found ectopically in the VZ of *JoP6;Emx1^{IREScree}* at E14.5 (B,B') and E11.5 (C,C'). (D) q-PCR of total RNA from control and *JoP6;Emx1^{IREScree}* cortices normalized to 18S RNA. *N-CAM* and R-cadherin are upregulated, whereas *Pxn*, *Tnc*, *Itgb3* and *Itga5* are downregulated. Error bars indicate s.d.

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;Emx1^{IREScree}* 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;Emx1^{IREScree}* 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;Emx1^{IREScree}* cortex as compared

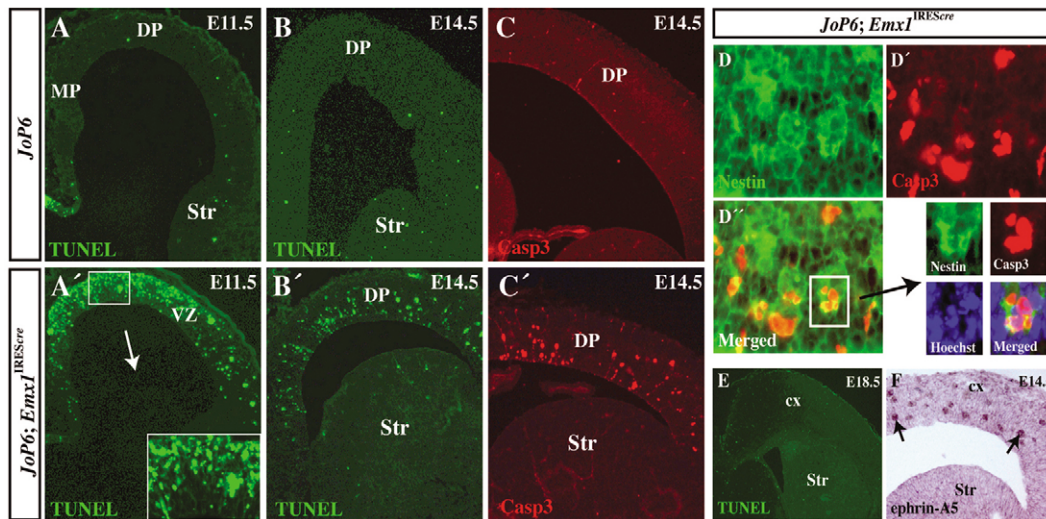


Fig. 5. Pax6 GOF at early corticogenesis causes apoptosis. (A-B') TUNEL assay on sections from *JoP6* (A,B) and *JoP6;Emx1^{IREScree}* (A',B') embryos at stages E11.5 (A,A' with higher magnification) and E14.5 (B,B') illustrates apoptosis of *JoP6;Emx1^{IREScree}* cortical progenitors, more severe at E11.5. (C,C') Immunolabeling against activated Casp3 confirms enhanced apoptosis in the *JoP6;Emx1^{IREScree}* cortex. (D-D'') Double immunostaining of E13.5 *JoP6;Emx1^{IREScree}* 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;Emx1^{IREScree}*. (F) In situ hybridization with ephrin A5 reveals that the apoptotic cell aggregates of the *JoP6;Emx1^{IREScree}* 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^{IREScree}* 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 (*Pxn*), tenascin C (*Tnc*), integrin beta 3 (*Itgb3*) and integrin alpha 5 (*Itga5*) 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^{IREScree}* 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 cytoplasmic 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^{IREScree}* 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 (Depaepe et al., 2005). Interestingly, both aggregates and single TUNEL⁺ cells in the *JoP6;Emx1^{IREScree}* 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 mediolateral patterning in the E11.5 *JoP6;Emx1^{IREScree}* 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⁻* (Guillemot, 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^{IREScree}* 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 (Muzio et al., 2002a), the massive apoptosis detected in the *JoP6;Emx1^{IREScree}* 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^{IREScree}* 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. 2I,J), 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* (*Rb1*)-dependent pathways, where pRb prevents the induction of apoptosis through transcriptional repression of *p53* (Ookawa et al., 1997). Previous evidence indicated that *Pax6*

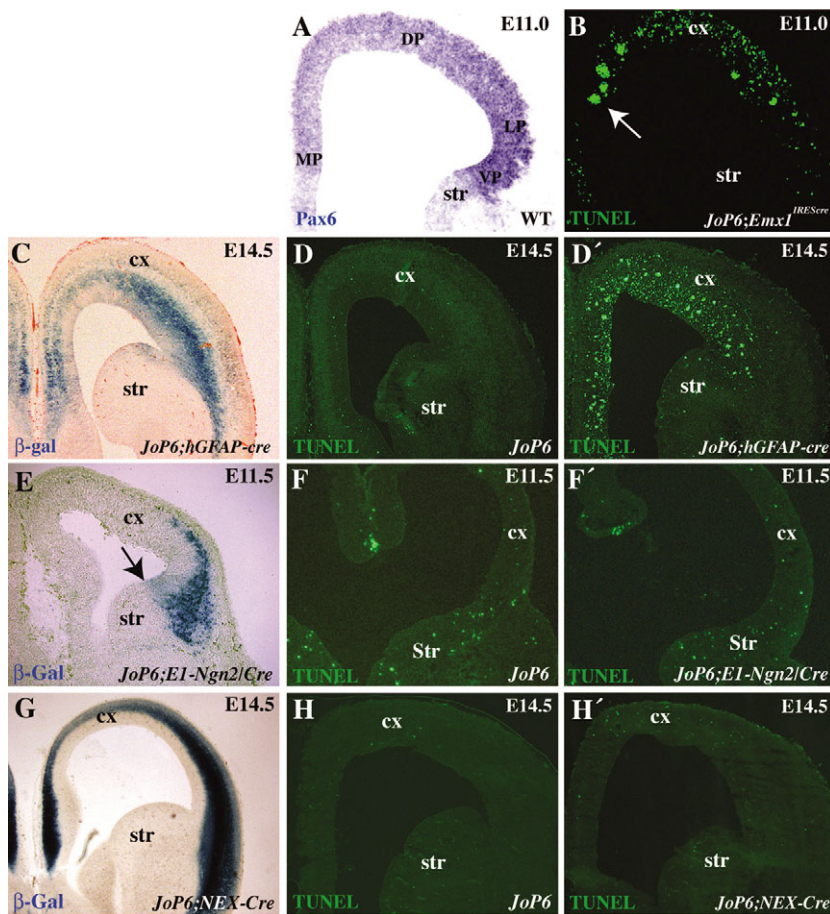


Fig. 6. Activation of transgenic Pax6 leads to progenitor apoptosis depending on the endogenous Pax6 expression level. (A) Pax6 in situ hybridization on E11.0 wild-type section demonstrates extremely faint endogenous Pax6 expression in the MP and (B) TUNEL assay on E11.0 *JoP6;Emx1^{IREScree}* reveals massive apoptosis within the same region (arrow). (C) β -gal staining of section at E14.5 indicates successful recombination in the *JoP6;hGFAP-cre* cortex and (D, D') TUNEL assay on *JoP6* and *JoP6;hGFAP-cre* shows extensive apoptosis in the Pax6⁺ radial glial progenitors. (E) β -gal staining of an E11.5 *JoP6;E1-Ngn2/Cre* embryo indicates recombination in the LP and VP (arrow indicates corticostriatal border). (F, F') No significant difference in apoptosis is detected via TUNEL assay on sections of E11.5 *JoP6* (F) and *JoP6;E1-Ngn2/Cre* (F') embryos. (G) E14.5 section of *JoP6;Nex-Cre* cortex stained for β -gal indicates recombination in postmitotic neurons in the dorsal telencephalon. (H, H') TUNEL assay did not detect additional apoptosis in the cortex of the *JoP6;Nex-Cre* embryos (H') as compared with the control (H).

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 both 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 *Emx1^{IREScree}* 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 transgenic Pax6-5a (Fig. 8A-D'). Co-transfection experiments of *pJoP6-5a* with *pHD* and *pPD* indicated the functionality of transgenic Pax6-5a (Fig. 8E). q-PCR showed enhancement of Pax6-5a expression in the *JoP6-5a;Emx1^{IREScree}* cortex, which was much weaker compared with the Pax6 enhancement in the *JoP6;Emx1^{IREScree}* cortex (Fig. 8F). Statistical analysis of the apoptotic pattern of *JoP6-5a;Emx1^{IREScree}* 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 $10\pm 2\%$) 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

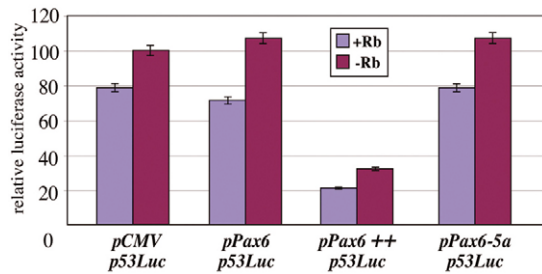


Fig. 7. Effect of Pax6 protein on activity of the P53 promoter.

Pax6 protein (3 μ g of *pPax6*) does not positively regulate activity of the human *P53* promoter in transiently transfected SAOS2 cells, but upon overexpression (16 μ g *pPax6*, indicated by ++) does inhibit the *P53* 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*.

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 $43\pm 3\%$) 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 $14\pm 6\%$ and $16\pm 7\%$ for the MP and DP, respectively. Despite the much lower level of transgenic *Pax6-5a* expression, the proliferation rate of cortical progenitors in *JoP6-5a;Emx1^{IREScree}* mice was inhibited as well (by $10\pm 2\%$). These results are in line with in vitro experiments in which *Pax6* transduction leads to the inhibition of progenitor proliferation (Hack et al., 2004; Heins et al., 2002; Cartier et al., 2006). Given the enhanced progenitor-proliferation rate found in the *Pax6* LOF *Sey/Sey* cortex at E10.5 (Warren et al., 1999) and E14.5–E16.5 (Götz et al., 1998), these findings confirm the pivotal role of both *Pax6* and *Pax6-5a* in controlling cortical progenitor proliferation. Remarkably, in different environmental contexts, the two Pax6 isoforms exert different control functions on progenitor proliferation. *Pax6* enhances proliferation in the neural retina of vertebrates (Marquardt et al., 2001) and *Drosophila* (Dominguez et al., 2004), but reduces proliferation of human glioblastoma cells (Zhou et al., 2005), cultivated corneal epithelial cells (Ouyang et al., 2006), and cortical progenitors in primary cell cultures (Haubst et al., 2004; Heins et al., 2002) as well as in vivo (this work). Similarly, although *Pax6-5a* seems to promote proliferation in the developing eye (Singh et al., 2002), it inhibits proliferation of cortical progenitors in vitro (Haubst et al., 2004) and in vivo (this study).

The majority of the S-phase and many of the M-phase progenitors express *Pax6*, suggesting that *Pax6* regulates cell cycle progression (Haydar et al., 2000). During the transition from S to M phase, the nuclei of the progenitors migrate from the basal to the apical surface of the VZ, a process termed interkinetic nuclear migration. Previous results from our laboratory (Götz et al., 1998) and from other groups

(Warren et al., 1999) indicated that in *Pax6* LOF, the interkinetic nuclear movement of cortical progenitors is impaired, with more cells found in the S phase as a result of a shorter cell cycle (Estivill-Torrus et al., 2002). Here we provide evidence that overexpression of *Pax6* in vivo leads to defects of mitotic cycle progression such that many cells seem to be stuck or prolonged in S phase and a significantly smaller proportion of progenitors undergo mitosis. In support of this conclusion, recent results from quantitative FACS analysis demonstrate that activation of *Pax6* in HeLa cells strongly reduces the number of cells in S and G2–M phases, indicating cell cycle arrest (Cartier et al., 2006). Similarly, overexpression of *Pax6* in corneal epithelial cell lines and primary cell culture causes inhibition of cell proliferation and retardation of the cell cycle (Ouyang et al., 2006).

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^{IREScree}* mice during early corticogenesis, a quantitative estimation of possible premature neurogenesis is difficult. However, we found that although the *JoP6;Emx1^{IREScree}* cortical thickness at E13.5 is significantly reduced (by $21\pm 2\%$, as compared with the control), the thickness of the Tuj1⁺ mantle layer appeared unchanged. Furthermore, the ectopically located Tuj1⁺ cells in the VZ, and the enhanced *Nex* in situ hybridization signal in the E11.5 CP of *JoP6;Emx1^{IREScree}* mice, suggest enhanced neurogenesis in the *Pax6* GOF condition in vivo. Thus, subpopulations of early progenitors of the *JoP6;Emx1^{IREScree}* 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^{IREScree}* cortex. It would also be of interest to test the expression of the potential *Pax6* downstream target *Fabp7*, 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 tenascin 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^{IREScree}* mice. When *Pax6* GOF was directed into postmitotic cells by the *Nex-Cre* mouse line

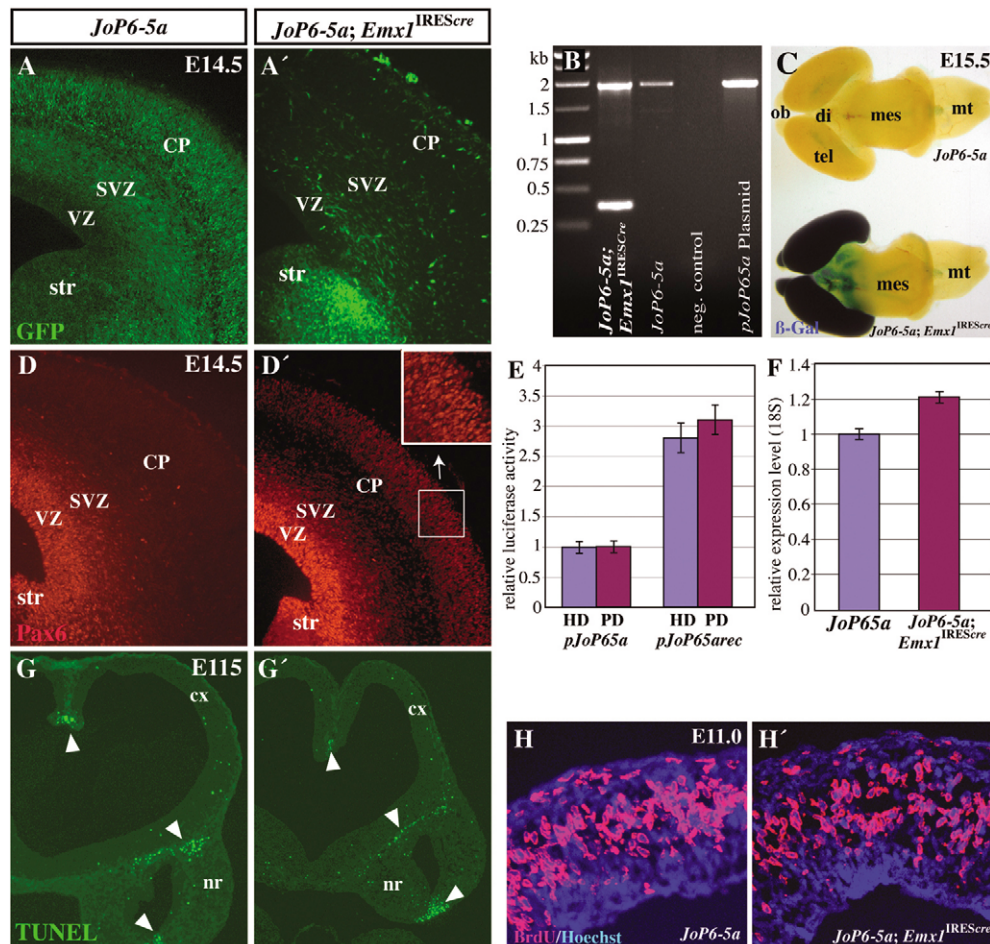


Fig. 8. Conditional transgenic activation of Pax6-5a via the JoP6-5a mouse line. (A,A') GFP fluorescence on sections of E14.5 cortex of *JoP6-5a* (A) is partially lost in *JoP6-5a;Emx1^{IREScree}* (A'). **(B)** Two products generated by genomic PCR with the primers JoP6F and JoP65aR (1955 bp and 394 bp) confirm successful recombination in the *JoP6-5a;Emx1^{IREScree}* cortex, whereas the *JoP6* cortex and the *pJoP6-5a* plasmid show no recombination. **(C)** Whole-mount β -gal staining of E15.5 *JoP6-5a* and *JoP6-5a;Emx1^{IREScree}* brains shows strong activation of the *lacZ* reporter in the *JoP6-5a;Emx1^{IREScree}* telencephalon and olfactory bulb. **(D,D')** with inset) Immunostaining for Pax6 on E14.5 *JoP6-5a* and *JoP6-5a;Emx1^{IREScree}* cortices shows ectopic antibody staining indicating Cre-induced activation of Pax6-5a in the *JoP6-5a;Emx1^{IREScree}* CP. **(E)** SAOS2 cells were transiently co-transfected with a luciferase reporter construct containing either the HD (blue) or the PD (red) domain and either *pJoP65a* or *pJoP65arec*, which express Pax6-5a. Luciferase activities were normalized to co-transfections with the *pJoP65a* control plasmid. Co-transfection of *pJoP65arec* with either the HD- or the PD-containing reporter construct induced an increase in luciferase activity, indicating functionality of transgenic Pax6-5a. **(F)** q-PCRs with RNA isolated from E12.5 cortex of *JoP6-5a* control and *JoP6;Emx1^{IREScree}* cortex, normalized to 18S RNA, indicate only a slight enhancement of the Pax6-5a expression level in the *JoP6-5a;Emx1^{IREScree}* telencephalon and olfactory bulb. Error bars indicate s.d.. **(G,G')** TUNEL assay on E11.5 brain sections of both genotypes reveals no significant difference in apoptosis (arrowheads point to endogenous apoptosis). **(H,H')** BrdU immunohistochemistry after a 30-minute BrdU pulse on cross-sections of E11.0 *JoP6-5a* and *JoP6-5a;Emx1^{IREScree}* cortices reveals a significant inhibition of the proliferation rate in the *JoP6-5a;Emx1^{IREScree}* DP.

(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;Emx1^{IREScree}* 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 β -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 β -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;Emx1^{IREScree}* 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^{IRESCre}* 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;hGFAP-cre* cortex, where transgenic *Pax6* becomes activated after E13.5 exclusively in the RC2⁺/*Pax6*⁺ radial glial cells (Malatesta et al., 2003; Zhuo et al., 2001), extensive apoptosis was detected, indicating that the overexpression of *Pax6* in the *Pax6*⁺ 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^{IRESCre}* 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 micropthalmia 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 *Parp* 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^{IRESCre}* mice. It should be noted, however, that in contrast to the substantial elevation of the level of *Pax6* transcripts in *JoP6;Emx1^{IRESCre}* mice (3.8-fold higher, compared with the controls), the increase in the level of *Pax6-5a* in *JoP6-5a;Emx1^{IRESCre}* 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

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/7/1311/DC1>

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