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Is Pax6 Critical for Neurogenesis in the Human Fetal Brain?

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Transcription factor Pax6 plays an important role in fate determination of neural progenitor cells in animal models, yet, its distribution and role in the human developing brain have not been reported. Here we demonstrated that Pax6 was strongly expressed in dorsal and ventral proliferative zones, mainly in proliferating radial glia (RG) cells, some neuronal and intermediate progenitors, and sporadic deep cortical plate neurons. In contrast to reports in rodents, Pax6 in the human fetal brain occasionally colocalized with ventral transcription factor Olig2 in progenitor cells. Transfection with short interfering RNA abolished Pax6 expression in the cell cultures of human fetal RG, and significantly decreased the number of neurons generated from Pax6 knock-down cells. Hence, Pax6 has a critical role in neurogenic regulation of RG cells in the human forebrain, similar to reports in rodents. What is different in human forebrain is that Pax6 seems to regulate not only the genesis of cortical pyramidal neurons, but also a subpopulation of interneurons from both dorsal and ventral sources. Thus, regional distribution, colocalization with Olig2, and the role of Pax6 in neurogenesis of both projection and interneurons, suggest that developmental regulation by transcription factors may differ in primates and nonprimate mammals.

Keywords: cerebral cortex development, human fetal cell cultures, interneurons, Olig2, LeX immunopanning, siRNA

Introduction

The telencephalon in mammals consists of two main parts, ventrally positioned subpallium, called also basal or ventral telencephalon, which consists mainly of ganglionic eminence (GE), and dorsally positioned pallium, or dorsal telencephalon. This ventro-dorsal subdivision is based on the differential expression of homeobox transcription factors, extensively studied in rodents (e.g., Puelles and Rubenstein 2003), and to lesser extent in human embryonic brain (Lindsay et al. 2005). In the mouse brain, the transcription factors Pax6 (paired box 6) and Emx1 are expressed in dorsal regions (Puelles et al. 2000; Stoykova et al. 2000; Yun et al. 2001), whereas Olig1,2 (oligodendrocyte lineage gene 1,2) are found in ventral regions (e.g., Ross et al. 2003). In the cortico-striatal border (CSB) Pax6 expressing cells might control ventro-dorsal migration from the GE to the dorsally positioned cerebral cortex (Stoykova et al. 1996, 1997; Chapouton et al. 1999; Kim et al. 2001; Carney et al. 2006). Pax6 expressed by radial glia (RG) cells in the mouse dorsal telencephalon has a role in determining the neurogenetic fate of these cells (Götz et al. 1998). This finding has been confirmed in a mutant Sey mouse where a nonfunctional Pax6 molecule results in a 50% reduction of the number both RG cells and cortical neurons (Heins et al. 2002). Accordingly, the overexpression of Pax6 in vitro can instruct the neuronal fate of all progenitors in either embryonic or adult neurosphere preparation (Hack et al. 2004). In rodents, Pax6 is downregulated as RG cells differentiate into the intermediate progenitors specified by the transcription factor Tbr-2 (T-brain), and subsequently into young neurons of the cortical plate (CP), which express Tbr-1 (Hevner et al. 2001; Englund et al. 2005).

The role of Pax6 in central nervous system (CNS) development was mainly studied in rodents, whereas rare reports in humans are focused on Pax6 expression in the developing eye (Glaser et al. 1994; reviewed in Chi and Epstein 2002), and in neuroblasts of the adult human subventricular zone (SVZ) (Baer et al. 2007).

In this study, we demonstrate Pax6 in the human fetal forebrain both in dorsal cortical ventricular zone (VZ)/SVZ and in ventrally positioned GE. In addition to dividing RG cells, a subpopulation of neuronal progenitor cells and young neurons also expressed Pax6. Interestingly, numerous cells in cortical VZ/SVZ and in GE at midgestation coexpressed both Pax6 and another transcription factor, Olig2. RG cells isolated at midgestation from either cortical VZ/SVZ or the GE and transfected with Pax6 short interfering RNA (siRNA), lost their ability to express Pax6 and consequently to proliferate and generate either intermediate progenitor cells or neurons. Moreover, Pax6 knock-down reduced the number of both projection neurons and interneurons regardless of the brain region. This suggests that the Pax6 role in RG neurogenesis is well maintained from rodents to humans. However, the neurogenetic potential of RG in the human brain is not limited to the dorsal telencephalon or to the projection neurons.

Materials and Methods

Human Fetal Brain Tissue and Cell Culture

Human fetuses ($n = 6$), ranging in age from 14 to 23 gestational weeks, were obtained from the Tissue Repository of The Albert Einstein College of Medicine (Bronx, NY). Tissue was collected with proper parental consent and the approval of the Ethics Committees of the University of Connecticut and The Albert Einstein College of Medicine. Ultrasonic and neuropathological examinations found no evidence of disease or abnormalities. The postmortem delay was on average 15 min. Brain tissue was collected in oxygenized Hank's Balanced Salt Solution containing 0.75% D-glucose, and transported on ice to our lab. All procedures were performed under sterile conditions.

Dissociated cell cultures were prepared from the VZ/SVZ of the fetal forebrain, dissected from the frontally cut hemispheres as a tissue band approximately 2000 μm high from the VZ surface (Zecevic et al. 2005). Tissue was dissociated with 0.05% trypsin–0.02% ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, CA) and triturated through a fire-polished pipette. Cells were resuspended in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen) and subjected to immunopanning.

Real Time Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated using Trizol reagent (Invitrogen) according to the protocol provided by the company. Real-time reverse transcription PCR (RT-PCR) analysis was performed starting with 1 μg of reverse transcribed total RNA, with a 200 nM concentration of both forward and reverse primers in a final volume of 25 μL, using the Sybr Green PCR core reagents and the BioRad iCycler detection System (Bio-Rad, Hercules, CA). Messenger RNA (mRNA) levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The sequence of primers is *Pax6*: forward: 5′-gaatcaga gaagacaggcca-3′, reverse: 5′-gtgtaggtatcataactccg-3′; *GAPDH*: forward, 5′ggtgaaggtcg gagtcaacgga3′, reverse: 5′tcttccaggagcgagatccctc3′. Experiments were repeated three times. The data were presented as means ± standard error of the means (SEMs) and analyzed using Student's *t* tests. The criterion for significance was set at $P \leqslant 0.05$.

Immunopanning and Cell Culture

To isolate neural progenitor cells, we used immunopanning with a surface marker LeX, according to a procedure described earlier (Mo et al. 2007). LeX^+ cells were first cultured in DMEM/F12/B27 supplemented with 10 ng/mL fibroblast growth factor 2 (FGF2), which increased their proliferation (Capela and Temple 2006). Later in the text we refer to this medium as the expansion medium. Subsequently, the amount of FGF2 was reduced to 1 ng/mL facilitating cell differentiation, hence we refer to this medium as differentiation medium. Immunolabeling with LeX antibody (1:100, Lab Vision, Fremont, CA) determined that the purity of immunopanned cells was 95%.

Pax6 siRNA and LeX^+ Cell Transfection

A 60-bp oligonucleotide containing *Bgl*II and *Hin*dIII restriction sites was inserted into the multiple cloning sites of pSuper-enhanced green fluorescent protein (EGFP) (Oligoengine, Seattle, WA). The following sequence is unique to the human Pax6 mRNA (accession number: A56674) encoding paired box domain (bold): 5′-gatcccc**agtcacagcg-gagtgaatc**ttcaagaga**gattcactccgctgtgact**ttttta-3′. As a control, a scrambled version of the above oligonucleotide was designed, containing the following sequence: 5′-gatcccc**aggcacatcggagtgactc**ttcaagaga**gagt-cactccgatgtgcct**ttttta-3′. This sequence cannot recognize any coding regions in the human genome. The oligonucleotide strands were purchased from IDT Genosys, (Coralville, IA) and were annealed before cloning into pSuper-EGFP. LeX^+ cells were cultured onto 12-mm coverslips (Carolina Biological Supply, Burlington, NC) in expansion medium for 7 div and were transfected with plasmid using lipofectamine (Invitrogen) according to the standard protocol provided by the manufacturer. The transfection efficiency was about 10%.

Proliferation Assay

Bromodeoxyuridine (BrdU; 20 μM, Sigma, St Louis, MO) was added to the cell cultures kept in a expansion medium for the last 6 h before immunostaining. Thymidine analog, BrdU, incorporates into DNA of dividing cells and could then be detected by immunocytochemistry. Briefly, cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min, treated with 2 N HCl for 10 min at room temperature, neutralized by rinsing in 100 mM boric acid (pH 8.5, Sigma) for 10 min followed by incubation in a blocking solution containing 3% bovine serum albumin (BSA; Sigma) in Tris buffered saline (TBS; 10 mM Tris; 150 mM NaCl; 2 mM $MgCl_2$) for 1 h. Primary antibody, monoclonal anti-BrdU (1:100; Sigma) was applied overnight at 4 °C. After washing in TBS for 5 min, 3 times, the cells were incubated with the secondary antibody.

Terminal Uridine Deoxynucleotidyl transferase dUTP Nick end Labeling Method

Apoptosis in cell cultures was determined by "In Situ Cell Death Detection kit," according to manufacturer's instructions (Roche, Germany). Identification of apoptotic nuclei was done using terminal deoxynucleotidyltransferase enzymatic reaction for the incorporation of rhodamine-labeled nucleotides into DNA strand breaks in situ.

Immunostaining

For cryosections, frozen brain blocks were serially sectioned in the coronal plane at 15-μm thickness. Sections were incubated in a blocking solution (1% BSA [Sigma], 5% normal goat serum [Vector, Burlingame, CA], and 0.5% Tween-20 in PBS) for 30 min. Primary antibodies were applied overnight at 4 °C, whereas corresponding secondary antibodies were subsequently applied for 1 h. A short incubation in bisbenzamide was used to reveal the cell nuclei.

Cell cultures were fixed with 4% paraformaldehyde for 10 min, washed with PBS at room temperature, and incubated with primary and secondary antibodies, as described above. The specificity of primary antibodies was tested with corresponding isotype controls (mouse IgG1, IgG2a, or IgG2b, or rabbit serum). The specificity of secondary antibodies was tested by omitting the primary antibodies from the protocol. Both tests resulted in a lack of immune reaction.

Cell Counting and Statistical Analysis

Cells stained with nuclear stain bisbenzamide and various cellular markers were visualized with a Zeiss Axivision fluorescence microscope and photographed with a digital camera. Before quantification 10 predesignated adjacent optical fields of view were selected in each culture and examined at magnification 10× (one field = surface area 1 mm^2) or 20× (0.25 mm^2 surface area). The percentage of immunolabeled cells of total bisbenzamide or GFP positive cells was calculated. The data were expressed as means ± SEMs and analyzed using Student's *t* tests. The criterion for significance was set at $P \leqslant 0.05$.

Results

Distribution of Pax6 Transcription Factor in the Human Fetal Forebrain

At 8–9 gestation weeks, the earliest case studied here, Pax6 was expressed in the VZ/SVZ of the lateral telencephalic wall, with sharp increase in immunofluorescence at the CSB. In contrast, in the ventral telencephalon, including the GE, Pax6 immunoreactivity was not present at this developmental age (Fig. 1*A–B*).

Almost all VZ cells of the lateral telencephalic wall strongly expressed Pax6 (Fig. 1*C,C′*), whereas individual, dispersed cells in the upper regions of the wall, including layer I and the thin CP, showed weak Pax6 immunofluorescence (Fig. 1*D*). In contrast, a much thinner medial telencephalic wall, which at this age consisted only of the VZ and the premordial plexiform layer (Marin-Padilla 1983), contained a sparse population of $Pax6^+$ cells throughout the width of the wall (Fig. 1*E*). Occasionally, $Pax6^+$ cells on the ventricular surface look like dividing, which is consistent with their progenitor status (Fig. 1*E*, arrow, and inset).

At 15 g.w., the next stage studied here, Pax6 was expressed very strongly throughout the entire cortical VZ (Fig. 1*F–G*). In the medial cortex, $Pax6^+$ nuclei in the SVZ were often adjacent to each other, resembling symmetric divisions of the intermediate progenitors (Fig. 1*G,G′*-arrow).

As in the previous stage of development, at 15 g.w. the immunoreactivity for Pax6 remained strong at the CSB (Fig. 1*I*), and numerous $Pax6^+$ cells were spreading laterally and ventrally. In the CSB many $Pax6^+$ cells were proliferating, as seen with double labeling with an active cell cycle marker, Ki67 (Fig. 1*J*). Even Pax6 cells that seem to be migrating away from the VZ, were colabeled with proliferation marker, Ki67 (Fig. 1*K*). In the cortical SVZ a number of Pax6+ cells were proliferating, but this number was far smaller than in the VZ (Supplemental Fig. 1).

The expression of Pax6 expanded at a 15 g.w. brain into the ventral telencephalon, in a characteristic region-specific

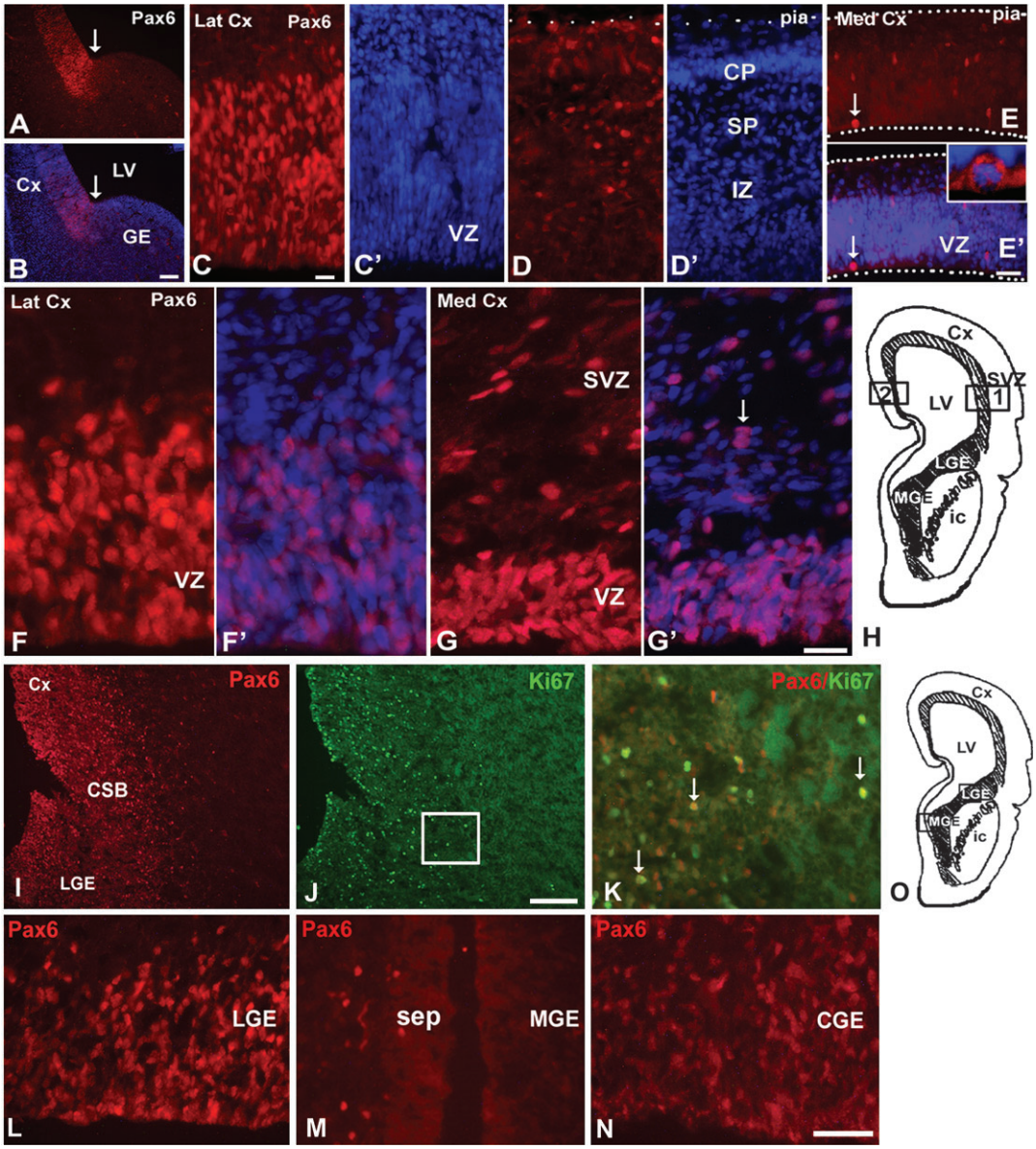

Figure 1. The expression of Pax6 on frontally cut cryosections of the human fetal forebrain. (*A*, *B*) At 8–9 g.w., Pax6 (red) is expressed in the VZ and particularly at the CSB (arrow). Blue, bisbenzamide (BB)–labeled cell nuclei. (*C*) In the lateral cerebral cortex, Pax6 expression in the VZ; (*C′*) the same section stained with BB. (*D*, *D′*) In the upper levels of the same section, Pax6 is expressed also in the emerging CP. Dash line marks pia position. (*E*, *E′*) Medial cerebral cortex at 8–9 g.w. is much thinner and still without the CP. Pax6^{+} cells are seen through the whole width of the wall, occasionally proliferating at the VZ surface (arrow). Inset shows a higher magnification of cell labeled with the arrow. (*F*–*H*) At 15 g.w., Pax6 is very strongly expressed in the VZ of the (*F*–*F′* lateral and *G*–*G′*) the medial cerebral cortex; note the expression of Pax6 "double" nuclei (arrows) in the SVZ. (*F′*) and (*G′*) are parts of the same sections counterstained with nuclear stain BB. The expression of Pax6 in CSB and the ventral telencephalon. (*A*) Pax6 in the CSB region, (*B*) the same section double stained with proliferation marker, Ki67. Boxed area shown on (*K*) higher magnification displays merged image of Pax6 (red) and Ki67 (green) with a number of double-labeled cells (arrows). (*L*) Pax6 in the VZ of the lateral GE, but not (*M*) in MGE; immunoreactive nuclei are, however, present in the septum (sep). (*N*) In the caudal GE less packed Pax6 nuclei. LV, lateral ventricle. Scale bars (*A*, *B*) (*I*–*J*): 100 μm; C–E′: 50 μm F–G′, K–N′ 20/μm (*H*) Drawing of the frontal section with boxed area 1, lateral cortex and 2, medial cortex, where pictures were taken. LV, lateral ventricle; Cx, cerebral cortex; ic, internal capsule.

manner. Pax6 was demonstrated in the VZ of the lateral (LGE) and in the VZ and SVZ of the caudal GE (CGE), whereas no immunofluorescence was observed in the medial GE (MGE) (Fig. 2*L*–*N*). At the ventricular surface of LGE, Pax6^{+} cells were proliferating as determined by Ki67 labeling (not shown). To further examine this regional distribution we used real-time RT-PCR to detect Pax6 mRNA. The specificity of the real-time RT-PCR product following gel electrophoreses was confirmed by the presence of a single product of the expected length (302 bp, not shown). Similar levels of Pax6 mRNA expression were seen in cortical VZ/SVZ, LGE, and CGE. In contrast, in the MGE Pax6 expression was almost 250-fold lower, consistent with our immunolabeling results.

Pax6 is Expressed in Various Cell Types of the Human Forebrain

To study whether in the human brain Pax6 is expressed by RG cells as was reported in rodents, we double labeled cryosections of the 15 g.w. forebrain with antibody to Pax6 and markers of human RG, BLBP (brain lipid binding protein) and GFAP (glial fibrilary acidic protein). Colocalization of these

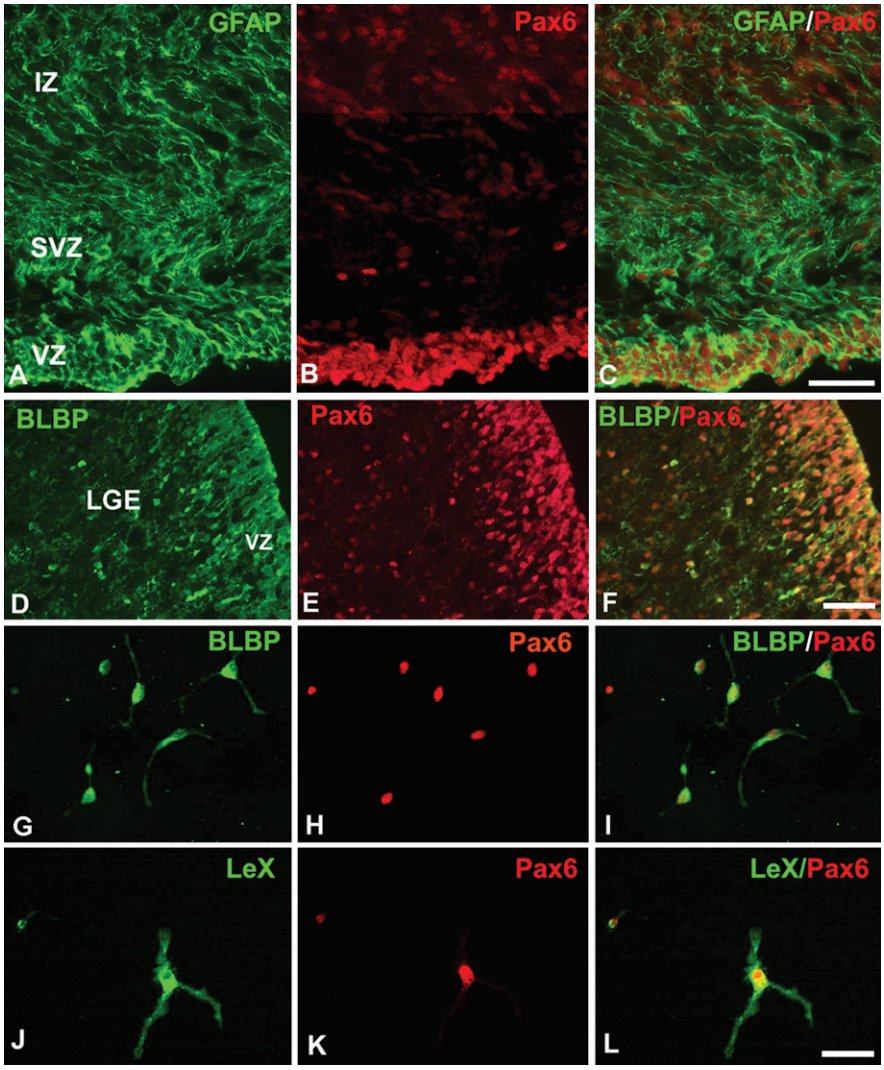

Figure 2. Colocalization of Pax6 and RG markers. (*A–C*) Proliferative VZ/SVZ region of the lateral cerebral cortex at 15 g.w. fetus double labeled with (*A*) GFAP and (*B*) Pax6, shows a well-localized immunoreaction in the cortical VZ. (*C*) Colocalization of these two markers is seen in the VZ and in rare SVZ cells. (*D–F*) Lateral GE at 15 g.w.: (*D*) BLBP immunoreaction in the VZ/SVZ, (*E*) Pax6 is localized mainly to VZ, and (*F*) colocalization in a majority of cells. (*G–L*) In cell culture obtained from 16 g.w. fetal cortical VZ/SVZ and kept for 12 h in expansion medium, $Pax6^{+}$ cells (*H*, *K*) are colabeled with (*G*, *I*) BLBP (arrows) and (*J–L*) LeX (arrows). Scale bars: (*A–C*) 50 μm, (*G–L*) 20 μm.

markers was demonstrated in the majority of cells in the cortical VZ/SVZ (Fig. 2*A–C*) and LGE (Fig. 2*D–F*). Consistent with our previous study that 95% of isolated LeX^{+} cells represent RG cells (Mo et al. 2007), here we show that in cell cultures ($n = 6$, 16–22 g.w.) obtained from cortical VZ/SVZ and maintained in expansion medium for 12 h, 96 ± 4.2% and 94 ± 3.8% of $Pax6^{+}$ cells can be colabeled with BLBP (Fig. 2*G–I*) and LeX antibody, respectively (Fig. 2*J–L*). Similar results were obtained in LGE cultures.

Further analysis of double-labeled cryosections demonstrated that Pax6 could be expressed in a subset of neuronal progenitors, immature cortical neurons, and interneurons. Both transcription factors Tbr-1 (T-brain) and Tbr-2 can be demonstrated in $Pax6^{+}$ cells. Colocalization of Tbr-2, a marker of intermediate progenitors, and Pax6 was demonstrated in the VZ and SVZ (Supplemental Fig. 2). Doublecortin (DCX), a marker of migrating neurons occasionally colocalized with Pax6 in the VZ/SVZ zones (not shown). Another marker of immature neurons, β-III-tubulin, was rarely demonstrated in $Pax6^{+}$ cells on cryosection, but was often seen in vitro (Fig. 3*A–C*). A marker of deep cortical neurons, the transcription factor Tbr-1, colocalized with Pax6 in a number of cells at the border of CP and subplate (Fig. 3*D–F*) and in cell bands streaming from the VZ/SVZ toward the overlying cortex (not shown). Calretinin (CalR), one of interneuron markers, can also be found in $Pax6^{+}$ cells located in the cortical VZ (Fig. 3*G–I*).

These results, obtained on cryosections, suggest a possible role of Pax6 in regulation of human forebrain neurogenesis. To further study this role, we knocked down the expression of Pax6 in vitro by creating a siRNA (see Methods).

Characteristics of Pax6 Knock-Down Cells

Isolated human fetal LeX^{+} RG cells were transfected with either Pax6 siRNA or scrambled RNA (cRNA), which served as a control. The efficacy of transfection was around 10%. Three days after transfection control transfected cells kept in the expansion medium could be colabeled with Pax6 and BLBP

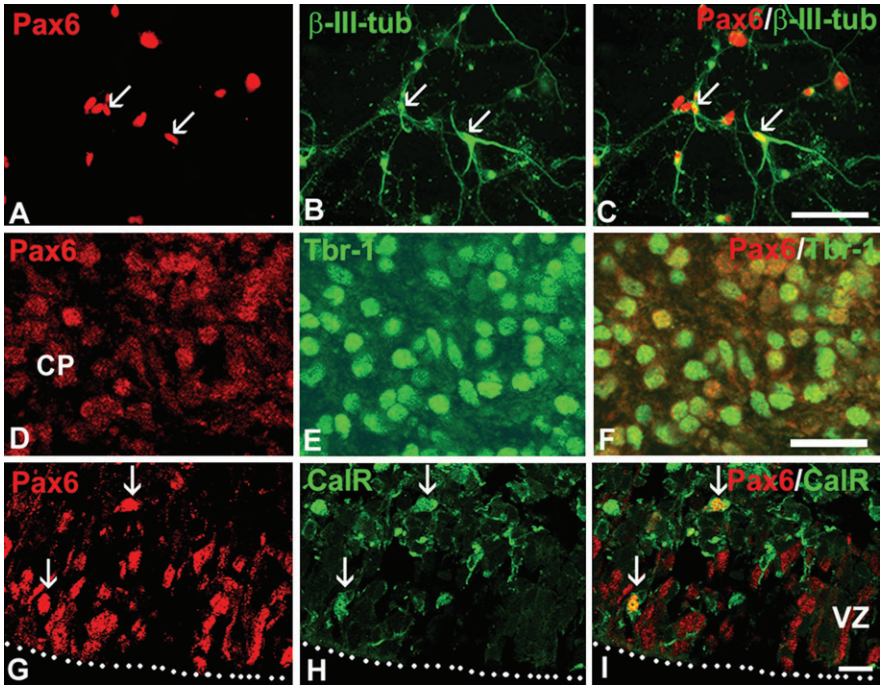

Figure 3. Pax6 and neuronal markers. (*A–C*) Coexpression of Pax6 and neuronal marker β-III-tubulin in a number of cells (arrows) in vitro, after 3 div. (*D–F*) On cryosections, Pax6 is coexpressed with Tbr-1 (arrows) in the deep CP at 15 g.w. (*G–I*) In the VZ, subset of $Pax6^+$ cells are colabeled with interneuron marker CalR (arrows). Dash line marks ventricular surface. Scale bars: (*A–C*) 30 μm; (*D–F*) 20 μm; (*G–I*) 10 μm.

(Fig. 4*A–D*), indicating that the Pax6 expression was maintained in these cells. In contrast, in cells transfected with siRNA, Pax6 was not detectable by immunolabeling, suggesting a successful knock-down of Pax6 protein expression (Fig. 4*E–F*). The expression of BLBP, however, was unaffected in the Pax6 knock-down cells (Fig. 4*G–H*), confirming that these cells still have RG identity. This finding was consistent with previous report in rodents (Götz et al. 1998). Transfected cells looked morphologically normal, and were not selectively dying as confirmed by the terminal uridine deoxynucleotidyl transferase dUTP nick end labeling method (not shown). The proliferation rate of Pax6 siRNA transfected RG cells, however, was reduced as determined by BrdU staining. Notably, almost one half (49.2 ± 4.6%) of the cells transfected with control RNA incorporated BrdU (Fig. 4*I–L*), in contrast to only 11.3 ± 2.3% of Pax6 siRNA cells (Fig. 4*M–P*). Most transfected cells that were proliferating were $BLBP^+$ cells (Fig. 4*I–P*). This result suggests that blocking Pax6 expression reduces the proliferation of RG cells.

To investigate whether Pax6 affects generation of the intermediate progenitors, which are the immediate downstream cells from RG, we applied the Tbr2 antibody, shown in rodents to label this cell type (Englund et al. 2005). At 3 div after transfection, Tbr2 immunoreactivity was present in 27.1 ± 3.6% of control transfected cells, but very rarely (<2%) in siRNA transfected cells (Fig. 5*A–F*). This result is consistent with the idea that Pax6 is needed for the next step in RG differentiation into $Tbr2^+$ intermediate progenitor cells.

Progeny of Pax6 Knock-Down Cells

To trace the progeny of Pax6 siRNA transfected cells, we cultured them for up to 3 weeks (21 div) in differentiation medium, and then immunostained cultures with various neuronal and glia markers.

At the initial point of the study, after 3 div in the expansion medium, about 11% of control transfected cells were colabeled with β-III-tubulin and 67% with GFAP. Interestingly, similar results were obtained from Pax6 siRNA transfected cells (Fig. 6*A*). This suggests that siRNA transfection did not affect the number of already specified $\beta\text{-III-tub}^+$ or $GFAP^+$ cells.

After 5 div in differentiation medium, however, only 15% of the Pax6 knock-down cells colabeled with β-III-tubulin, compared with almost one third (28%, $P < 0.05$) of control transfected cells. In contrast, the number of GFAP colabeled cells was significantly higher (82%, $P < 0.05$) in the knock-down group than in the control group (62%) (Fig. 6*B*). This result is consistent with the idea that Pax6 specifically regulates neurogenic fate of RG cells. Accordingly, the number of migrating neurons labeled with DCX^+ in the siRNA transfected group was significantly lower compared with controls (not shown). Moreover, the marker of mature neurons, NeuN, was not demonstrated in siRNA transfected cells, even after a prolong time in differentiation medium. In contrast, NeuN labeled 2.2 ± 0.3% of control transfected cells (Fig. 7).

It is noteworthy that human fetal RG without any transfection differentiated in vitro into neurons and glia, in a similar way as cRNA transfected cells (Mo et al. 2007), indicating that cRNA transfection did not interfere with RG fate determination.

Neuronal Cell Types and Region Specificity of RG Progeny

To investigate whether RG obtained from different forebrain regions show similar neurogenic properties related to Pax6 expression, we established RG cultures from cortical VZ/SVZ and LGE and studied them as untransfected, control transfected (with cRNA) or transfected with Pax6 siRNA. We used SMI-32 antibody, demonstrated to label a subtype of cortical pyramidal neurons in primates (Campbell and Morrison 1989; Del Rio and

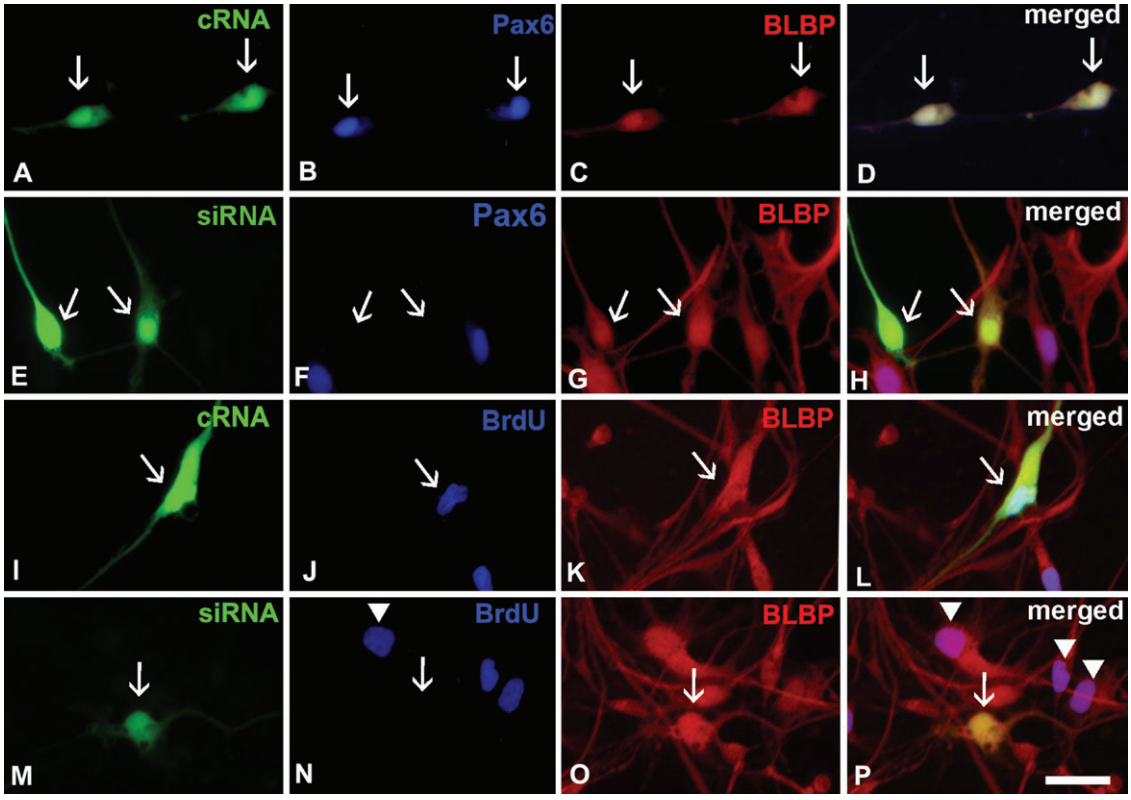

Figure 4. Molecular characteristics of Pax6 knock-down cells. 3 div after transfection, (*A*) control transfected cells (cRNA, green) can be colabeled with both (*B*) Pax6 (blue) and (*C*) BLBP (red, arrows). (*D*) Merged image. (*E–H*) In cells transfected with siRNA (green), (*F*) Pax6 expression is not detectable (arrows), but (*G*) transfected cells still express BLBP (red). (*H*) Merged image. (*I–L*) The control transfected cells (cRNA) labeled with (*J*) BrdU (blue) and (*K*) BLBP (red). (*L*) Merged image shows that control transfected cell divides and is $BLBP^+$ (arrow). (*M–P*) In contrast, a Pax6 knock-down cell (siRNA) is not labeled with (*N*) BrdU (blue), but (*O*) expresses BLBP (red). (*P*) Merged image shows that a cell coexpressing BLBP and siRNA is not dividing (arrow), whereas the surrounding $BLBP^+$ cells that were not transfected are dividing (arrowheads). Scale bars: 20 μm.

DeFilipe 1994; our unpublished results) and one of the interneuron markers, CalR, to study different neuronal subtypes that are generated in these cultures.

After 10 div in differentiation medium, nonoverlapping cell populations were labeled with SMI-32 and CalR (not shown). Both untransfected and control transfected cells from the cortical VZ/SVZ generated around 12% $SMI\text{-}32^+$ cells (Fig. 8*A–C,G*). This result shows that transfection does not influence the genesis of neurons. In contrast to these controls, only 2.2% $SMI\text{-}32^+$ cells were generated from Pax6 knock-down cells (Fig. 8*G*). The capacity of Pax6 knock-down cells to generate $CalR^+$ neurons in culture was also decreased. Only 1.5% of Pax6 knock-down cells were labeled with CalR (Fig. 8*G*) compared with 7% of control RNA transfected cells (Fig. 8*D–G*). In general, more interneurons were generated in LGE cultures than in cortical cultures under any conditions (Fig. 8*G*), but it is important that RG cells could generate interneurons regardless of the forebrain region of their origin. In the LGE cultures, transfection with siRNA reduced the generation of both $SMI\text{-}32^+$ neurons and $CalR^+$ interneurons, similar to findings in cortical cultures (Fig. 8*G*).

Colocalization of Pax6 and Olig2

In the cryosections, Pax6 and Olig2 transcription factors were colocalized in the same regions, and even in the same cell nuclei, from the earliest age examined here (8–9 g.w.) (Fig. 9*A–C*). Colocalization of Olig2 and Pax6 was seen in the CSB, and in lateral and medial cerebral cortex (compare Fig. 1*A–B* with Fig. 9*A–D*). Occasionally, this colocalization was seen in dividing progenitor cells on ventricular surface (Fig. 9*A*, arrow).

In the fetal stages (15, 17, 20 g.w.) a subpopulation of cells coexpressed Pax6 and Olig2 in the LGE and caudal GE region (Fig. 9*E–G*). Moreover, in vitro (8 and 14 div cell culture from the 21 g.w. fetal VZ/SVZ) Olig2 and Pax6 were occasionally coexpressed in dividing cells (Fig. 9*H–J*). To further investigate the correlation between the expression of Olig2 and Pax6, we immunolabeled Pax6 siRNA transfected cells from cortical VZ/SVZ with Olig2 antibody 5 div after transfection. The results obtained in transfected and control cultures were comparable: from all Pax6 siRNA transfected cells, 18.2 ± 2.6% were colabeled with Olig2, compared with 21.3 ± 3.2% ($P > 0.05$) in the control group. Similar results were obtained from the LGE cells (not shown). This suggests that Olig2 expression is independent of Pax6, but that their combined action is probably important for keeping the common neuron/OPC cells.

Discussion

Our study showed that Pax6 expression is critical for neurogenetic capabilities of human RG, similar to what has been reported in rodents (e.g., Götz et al. 1998; Heins et al. 2002; Hack et al. 2004). In human forebrain, however, Pax6 regulates not only the genesis of cortical pyramidal neurons, but also a subpopulation of interneurons from both dorsal and ventral sources. Regarding the Pax6 distribution in the human fetal

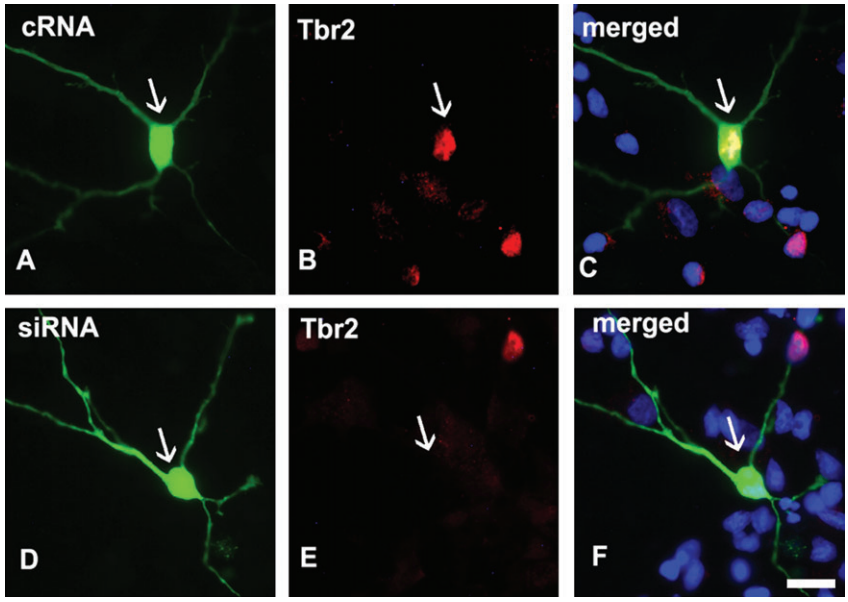

Figure 5. Pax6 is required for the generation of intermediate progenitors labeled with Tbr2. (*A–C*) Control transfected cell (cRNA) (*B*) expresses Tbr2 (red, arrow). (*C*) Merged image. (*D–F*) In contrast (*D*) in knock-down (siRNA) transfected cells (*E*) Tbr2 is not detectable (red, arrow). (*F*) Merged image (arrow). Scale bars: 10 μm.

forebrain, two findings were unexpected. First, Pax6 was expressed both in the dorsal and ventral telencephalon (GE) in age-related manner, and second, Pax6 and Olig2 colocalized in the same cells, in contrast to what has been reported so far in rodents.

Pax6 Expression in the Human Fetal Forebrain

The distribution and cell-specific type of Pax6 expression in the human forebrain varied greatly with the stage of development and the region studied. In the early fetal forebrain (8–9 g.w.) Pax6 was expressed dorsally, in the proliferative zone (VZ/SVZ) of the cerebral cortex. At midgestation, however, Pax6 generally described as a dorsal transcription factor (e.g. Götz et al. 1998; Heins et al. 2002), spreads to more ventral regions of the GE. Moreover, $Pax6^+$ progenitor cells in the LGE are proliferating, suggesting their local origin. Similar to this finding in human, a number of reports in rodents suggest that Pax6 spreads to the border region between the ventral pallium (cerebral cortex) and the subpallium (LGE) (Puelles et al. 2000; Stoykova et al. 2000; Yun et al. 2001) or even into the LGE (Carney et al. 2006).

Notably, we demonstrated Pax6 in the LGE and CGE, but not in the MGE, at least not at the stages of development studied here. This is consistent with the idea that the progenitor population varies with stage of development and region of the brain. For example, the MGE, is a well-known source of cortical interneurons (reviewed in Wonders and Anderson 2006), and early transitory oligodendrocyte progenitors (OPCs), which later are replaced by OPCs generated in the LGE and CGE, and postnatally by OPCs from the dorsal telencephalon (Kessaris et al. 2006).

Another unexpected finding in our study was a colocalization of Pax6 and Olig2 in the same brain regions and occasionally in the same cells. The transcription factor Olig2 (oligodendrocyte lineage gene 2) belongs to a basic helix–loop–helix group of transcription factors, described in common progenitors for motor neurons (MN) and oligodendrocytes in the ventral pMN region of the spinal cord (Lu et al. 2000, 2002; Takebayashi et al. 2000; Zhou et al. 2000). In the human fetal brain, we reported Olig2 expression in a subset of neural progenitor cells, immature neurons, and oligodendrocytes (Jakovcevski and Zecevic 2005). In the present study $Pax6^+/Olig2^+$ cells showed age and region-dependent distribution. At early fetal stages (8–9 g.w.) double-labeled cells were seen in the entire telencephalic wall, whereas at 15–20 g.w. they were demonstrated mainly in the CSB and in the LGE and CGE. In addition, in our primary cultures of cortical VZ/SVZ, Olig2 and Pax6 were coexpressed in dividing progenitor cells. The knock-down of Pax6 did not affect the expression of Olig2, which advocates that the cell fate determination may depend on a combination of several transcription factors.

The presence of $Pax6^+/Olig2^+$ cells in the human forebrain suggests a common neuron/OPC, as reported earlier (Jakovcevski and Zecevic 2005). These progenitors are the topic of a separate study (Mo et al. in preparation).

In contrast to these findings, Olig2 and Pax6 transcription factors in the mouse brain have inverse patterns of expression (Heins et al. 2002; Hack et al. 2005). The overexpression of Pax6 resulted in downregulation of Olig2, whereas in Pax6–/– mutants, Olig2 had increased expression and expanded from GE to the cortical VZ/SVZ (Heins et al. 2002). This difference in results points to species specificities in regulation of developmental transcription factors.

Other examples of "misplaced" transcription factors have been reported, mainly in the human brain. For example, the two ventral transcription factors, NKx2.1 and Dlx, were expressed in proliferating cells in the dorsal cortical VZ/SVZ (Rakic and Zecevic 2003; Zecevic et al. 2005). The Tbr1, another transcription factor expressed in rodents in the dorsal telencephalon (Englund et al. 2005; Hevner et al. 2006) is present in the ventral telencephalon and diencephalon in human embryos (Bystron et al. 2006). Similar to these findings in humans, dorsal and ventral transcription factors were reported to be coexpressed in the same cells in rodents. For

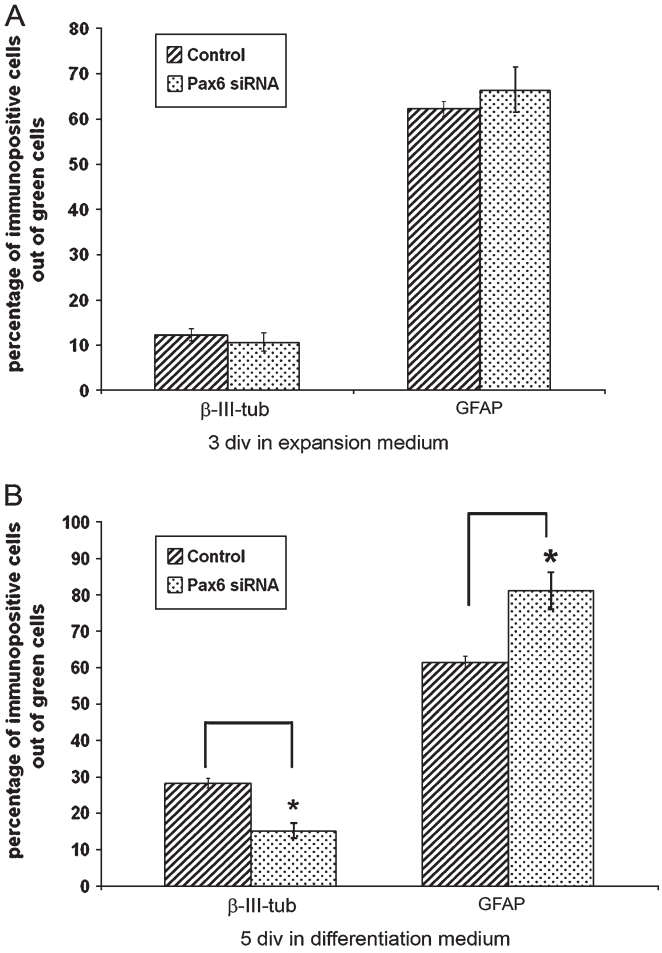

Figure 6. Quantification of the progeny of Pax6 knock-down cells. (*A*) LeX$^+$ cells cultured in the expansion medium ($n = 3$). 3 div after transfection with either control (cRNA) or siRNA Pax6, double-labeling experiments show the same number of β-III-tubulin$^+$ neurons and GFAP$^+$ astroglia regardless of transfection. (*B*) When these cells were kept in differentiation medium for 5 div more neurons are generated from control (cRNA) than from siRNA transfected cells (*$P < 0.05$). In contrast, more GFAP cells were generated from siRNA transfected RG cells ($P < 0.05$).

example, cells double labeled with Dlx2 (ventral marker) and Pax6 (dorsal marker) were reported in the LGE and in the lateral cortical stream (Carney et al. 2006). These findings suggest that the distribution of regional transcription factors may vary with developmental stage and different species.

Neurogenic Capabilities of RG are Determined by Pax6 Expression

Double immunolabeling of cryosections of the human fetal brain demonstrated Pax6 expression mainly in RG cells, similar to previous reports in animal models (Stoykova et al. 1996, 1997; Götz et al. 1998; Englund et al. 2005). In addition, our in vivo and in vitro experiments showed Pax6 also in a subpopulation of Tbr2$^+$ intermediate progenitors, restricted neuronal progenitors (β-III-tubulin$^+$), migrating cortical neurons (DCX$^+$), deep CP neurons (Tbr-1$^+$, SMI-32$^+$), and a subpopulation of interneurons (CalR$^+$). The fact that similar results with these markers were obtained in vivo and in vitro, indicates that Pax6 specification is maintained in primary cultures. Colocalization of neuronal markers and Pax6 may be explained by slow downregulation of Pax6 in a process of RG differentiation into cortical neurons (Mo et al. 2007). This is also in accord with report on the embryonic mouse VZ/SVZ cell cultures (Kawaguchi et al. 2004).

The neurogenic role of Pax6 was more directly demonstrated when Pax6 expression was abolished by siRNA transfection. This method allowed us to study, for the first time in the human brain, more directly the effects of Pax6 on neurogenesis. The Pax6 siRNA treated RG cells had reduced proliferation measured by BrdU incorporation, and generated far less intermediate progenitor cells, as well as neurons. The intermediate progenitors, identified by Tbr2 expression in rodents, are the first step in RG differentiation into cortical neurons (Noctor et al. 2004; Englund et al. 2005; Hevner et al. 2006; Martinez-Cerdeno et al. 2006). In addition, Pax6 knock-down cells were unable to differentiate into neurons in the appropriate differentiation medium. Preliminary electrophysiological measurements showed that none of the Pax6 knock-down cells had neuronal characteristics, but instead

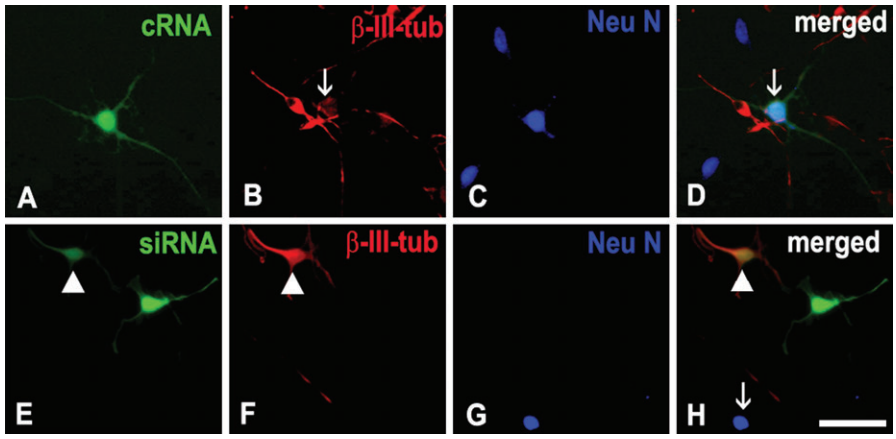

Figure 7. Pax6 is required for neuronal maturation. After 21 div in culture (*A–D*) control transfected cells (cRNA) express a very low level of (*B*) β-III-tubulin (arrow), but (*C*) mature neuron marker NeuN (arrow) labels transfected cell. (*D*) Merged image (arrow). (*E–H*) In contrast, siRNA transfected cell (*E*) can be labeled with (*F*) β-III-tub (arrowhead), but not with (*G*) NeuN (blue, arrow). (*H*) Merged image-arrow points to NeuN$^+$ cells that is not transfected, arrowhead to transfected cell colabeled with β-III-tubulin. Scale bars: 20 μm.

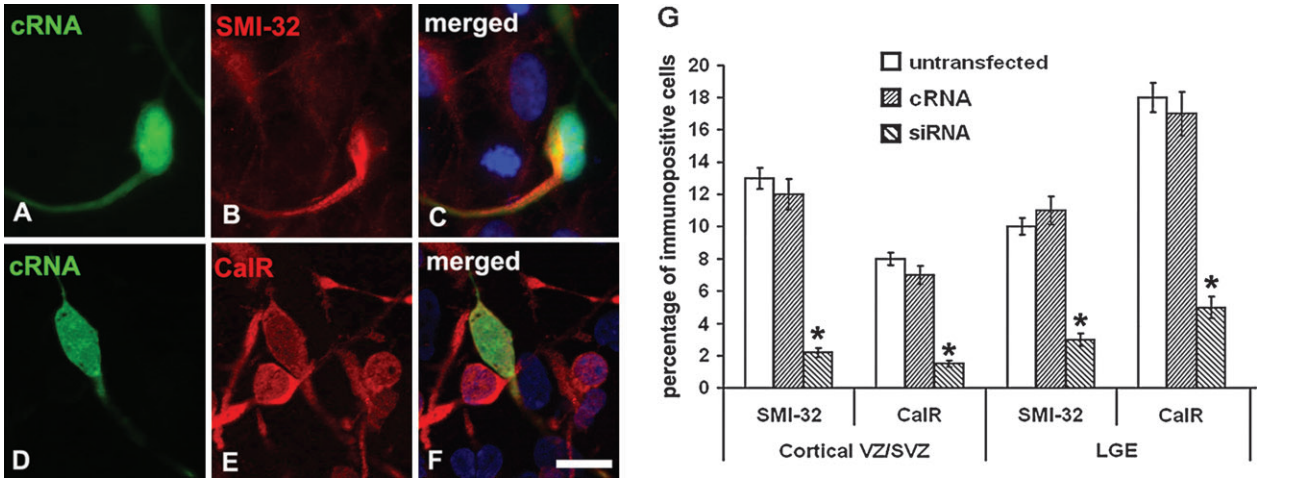

Figure 8. Cell type and region specificity of RG progeny. (*A*) Control transfected cells (cRNA) give rise to (*B*, *C*) SMI-32$^+$ cells and (*D–F*) CalR$^+$ cells. (*G*) Quantification of two neuronal cell types in untransfected, control transfected (cRNA), and siRNA transfected cultures from cortical VZ/SVZ or LGE. In untransfected cultures, the number of SMI 32$^+$ or CalR$^+$ cells is calculated as a percentage from all cells in the culture; in transfected cultures, the number of SMI 32$^+$ or CalR$^+$ cells is calculated as a percentage of transfected (green) cells ($n = 3$). Scale bars: 10 μm.

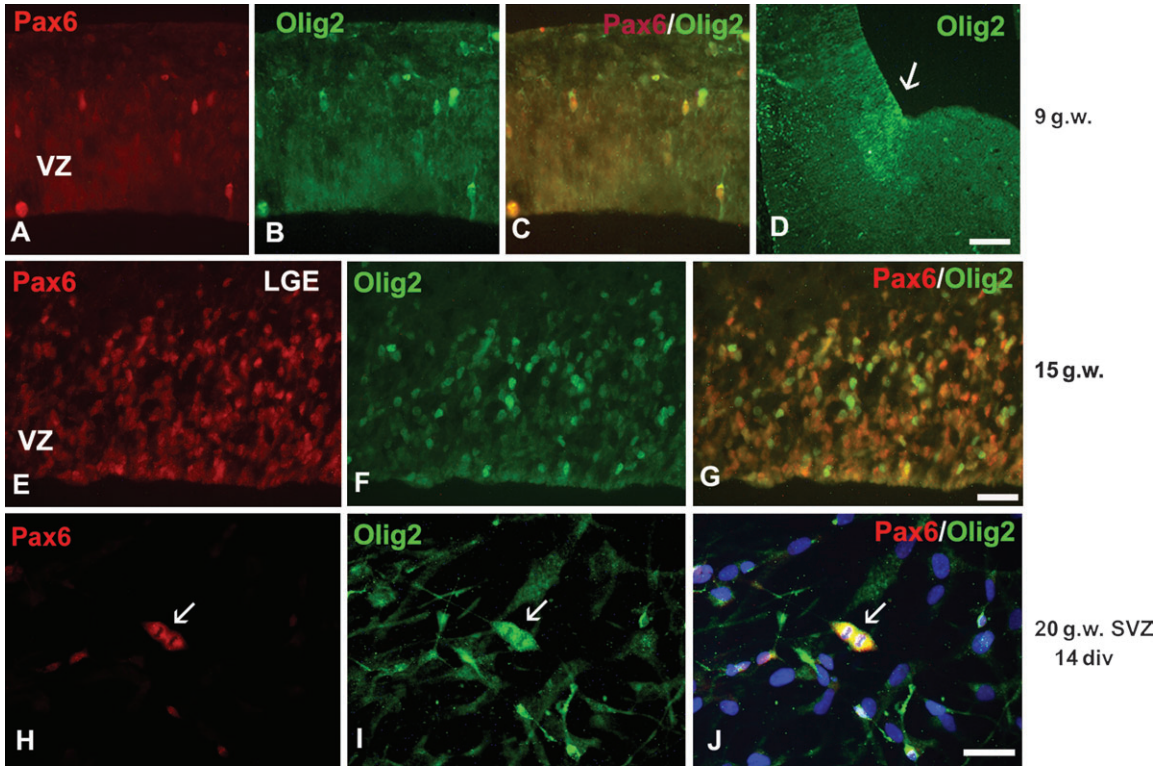

Figure 9. Coexpression of Pax6 and Olig2 transcription factors. (*A–D*) At 8–9 g.w. on the cryosections the two transcription factors are coexpressed in the same cells (arrows) of medial cerebral cortex (compare with Fig. 1*E*) and (*D*) the CSB (compare with Fig. 1*A*). (*E–G*) At 15 g.w., this colocalization is demonstrated in the LGE. (*H–J*) At 20 g.w. in the SVZ mixed cell culture after 14 div, proliferating cells occasionally coexpress Pax6 and Olig2 (arrow). Scale bars: (*A–D*), 50 μm; (*E–G*), 25 μm; (*H–J*), 20 μm.

were displaying glia electrical characteristics (unpublished data). These findings strengthen the notion that in the human forebrain, the expression of Pax6 in RG cells has a crucial role in production and maintenance of neuronal progenitors. Similar role for Pax6 in RG neurogenesis has been reported in animal models (Götz et al. 1998; Heins et al. 2002; Hack et al. 2004).

Regional Characteristics of RG

Experiments on the mouse led to a conclusion that neurogenic capabilities of RG are specific for dorsal telencephalon (Götz et al. 1998; Heins et al. 2002; Malatesta et al. 2003). Consistent with this, the loss of Pax6 reduces the number of neurogenic RG cells and neurons in the cerebral cortex (Heins et al. 2002). Cortical RG in Pax6-mutant mice seem to predominantly

generate glial cells and only few neurons (Malatesta et al. 2003). In the ventral telencephalon, few neurons generated by RG are specific types of interneurons that migrate to the olfactory bulb (Malatesta et al. 2003). These data suggest that RG cells are also involved in instructing regionalization of the developing CNS (Campbell and Götz 2002).

Conversely, a number of reports from animal studies (Anthony et al. 2004; Casper and McCarthy 2006) as well as our previous (Mo et al. 2007) and present results, demonstrate that neurogenetic potential of RG is not limited to the dorsal telencephalon. Using Cre/loxP fate mapping with either promoter of human GFAP (Casper and McCarthy 2006) or BLBP (Anthony et al. 2005), it has been shown that both $hGFAP^+$ and $BLBP^+$ RG cells generate neurons throughout the mouse brain. Here we confirmed that in the human fetal brain RG generate neurons independent of region of origin, but the subtype of generated neurons might be influenced by the forebrain region. Thus, more $CalR^+$ interneurons (50%) were generated from LeX^+ cells cocultured with the GE than cocultured with cortical VZ/SVZ (20%) (Mo et al. 2007). We now extended this finding to demonstrate that Pax6 has a critical role in the generation of both $SMI\text{-}32^+$ projection neurons and $CalR^+$ interneurons from RG cells isolated from either the cortical or GE proliferative zone. The generation of interneurons from cortical RG was in line with reports that in the human brain a substantial proportion of cortical interneurons have cortical origin (Letinic et al. 2002; Rakic and Zecevic 2003).

In summary, results obtained in this study suggest a critical role for Pax6 in RG-based neurogenesis in the human fetal brain, similar to what has been reported in rodents. However, Pax6 expression in the GE, its role in generation of interneurons, as well as colocalization of Pax6 and Olig2 in a subset of neural progenitors, suggest that important differences may exist between human and rodent brains in regulation of transcription factors related to neurogenesis. The fact that regional transcription factors may change their expression with stage of development, region of the brain, and species, should be taken into consideration when extrapolating results from animal models to human brain.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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