Paracrine Pax6 activity regulates oligodendrocyte precursor cell migration in the chick embryonic neural tube

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SUMMARY
Homeoprotein transcription factors play fundamental roles in development, ranging from embryonic polarity to cell differentiation and migration. Research in recent years has underscored the physiological importance of homeoprotein intercellular transfer in eye field development, axon guidance and retinotectal patterning, and visual cortex plasticity. Here, we have used the embryonic chick neural tube to investigate a possible role for homeoprotein Pax6 transfer in oligodendrocyte precursor cell (OPC) migration. We report the extracellular expression of Pax6 and the effects of gain and loss of extracellular Pax6 activity on OPCs. Open book cultures with recombinant Pax6 protein or Pax6 blocking antibodies, as well as in ovo gene transfer experiments involving expression of secreted Pax6 protein or secreted Pax6 antibodies, provide converging evidences that OPC migration is promoted by extracellular Pax6. The paracrine effect of Pax6 on OPC migration is thus a new example of direct non-cell autonomous homeoprotein activity.

KEY WORDS: Oligodendrocyte precursor cells, Migration, Homeoprotein transfer, Pax6, Spinal cord, Chick

INTRODUCTION
The homeoproteins (HPs) are transcription factors characterized by the helix-turn-helix structure of their DNA-binding domain, the homeodomain (Gehring et al., 1994). They are implicated in a plethora of developmental functions ranging from embryonic polarity to cell differentiation and migration, as illustrated for the paired homeodomain transcription factor Pax6 (Chi and Epstein, 2002; Pichaud and Desplan, 2002). As all bona fide transcription factors, HPs exert their action by binding to DNA in a sequence-specific manner, activating and/or repressing downstream effector genes. HPs can also be secreted and internalized owing to the presence of specific secretion and internalization sequences within the highly conserved homeodomain (Joliot and Prochiantz, 2004). This extracellular HP activity is implicated in a number of processes (Brunet et al., 2007; Prochiantz and Joliot, 2003). In the context of migration, an important example is provided by the extracellular HP Engrailed (Engrailed 1 and Engrailed 2), which participates in the guidance of retinal growth cones and in retinotectal patterning (Brunet et al., 2005; Wizenmann et al., 2009).

Based on these findings, and on similarities between cell and growth cone guidance, we postulated that extracellular HPs may regulate cell migration in the neural tube. To explore this hypothesis, we focused on the oligodendrocyte precursor cells (OPCs), highly migratory cells that populate the developing grey matter and the future white matter in the embryonic CNS (Tsai et al., 2002). OPCs mainly arise from ventricular progenitors expressing the b-HLH transcription factor Olig2 and localized between two other ventricular domains expressing Nkx2.2 ventrally and Pax6 dorsally (Lu et al., 2000; Zhou et al., 2001). These transcription factors participate in the patterning of the ventricular neuroepithelium in response to Sonic hedgehog (Briscoe et al., 2000; Ericson et al., 1997), regulate the expression of Olig2 and contribute to determine the ventricular identity of the oligodendrogenic domain (Sun et al., 2003). In addition, although Nkx2.2 regulates the differentiation of OPCs (Zhou et al., 2001), Pax6 influences the early steps of oligodendrogenesis. Indeed, in Pax6 mutants, the oligodendrocyte precursors are shifted dorsally and their specification is delayed (Sun et al., 1998).

Pax6 displays a non-cell autonomous activity in the developing eye anlagen (Lesaffre et al., 2007) and is thus a valuable candidate to investigate a paracrine effect of HPs in early oligodendrogenesis. We used the embryonic chick spinal cord, where gliogenesis has been extensively described (Agius et al., 2004; Danesin et al., 2006; Fu et al., 2002; Gotoh et al., 2011; Ono et al., 1995; Soula et al., 2001), to verify an extracellular expression of Pax6 and to evaluate how manipulating extracellular Pax6 modifies the OPC migration pattern. We find that OPCs migrate close to Pax6-expressing neurons in the ventrolateral domain of the spinal cord and that extracellular Pax6 available in their environment promotes their migration. This finding suggests a new HP direct non-cell autonomous role to refine the migratory behavior of precursor cells in the neural parenchyma.

MATERIALS AND METHODS
Reagents
Antibodies used were: anti-Pax6 (polyclonal rabbit, 1/500, Covance), anti-Olig2 (polyclonal rabbit, 1/200, Millipore), anti-Nkx2.2 (monoclonal mouse, 1/10, DSHB), anti-NeuN (mouse, 1/1000, Millipore), mouse anti-MNR2 (1/100, DSHB), anti-RHOA (monoclonal mouse, 1/500, SANTA-CRUZ, sc-418), anti-NCAM (polyclonal rabbit, 1/4000, provided by Dr C. Goridis (IBENS, Paris)), anti-Phospho-Histone 3 (mouse, 1/500, Cell Signaling) and anti-HA (rat, 1/100, Roche). Secondary antibodies were HRP-conjugated anti-rabbit (1/5000), anti-mouse (1/5000), or anti-rat...
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(1/5000) (Amersham Lifescience). Peroxidase activity was revealed using ECL (Amersham). Secondary antibodies were either Alexa Fluor 594 or Alexa Fluor 488 immunoglobulin (Molecular Probes).

The blocking antibodies used were polyclonal anti-Pax6 (rabbit; 10 μg/ml Covance), monoclonal anti-Pax6 (mouse; 100 μg/ml, DSHB), monoclonal anti-Engrailed (mouse; 100 μg/ml; DSHB), anti-DCC (polyclonal; goat; 20 μg/ml, R&D Systems) and anti-netrin (polyclonal, goat; 20 μg/ml, R&D Systems). Recombinant Pax6 and En-2 proteins (100ng/ml) were produced in isopropyl-β-d-thiogalactoside (IPTG)-inducible bacteria using published methods (Montesinos et al., 2001). Anti-sense Pdgfra riboprobe was synthesized from quail Pdgfra cDNA, as reported by Perez-Villegas (Perez-Villegas, 1999).

Cell surface biotinylation and proteinase K treatments

Biotinylation on open-book cultures was conducted according to PIERCE kit (ref: 89881). Hindbrains and spinal cords of E5 chick were cultured for 1 day on tissue culture inserts 0.4 μm pore size (Millipore). For PK treatments, neural tube explants from E5 chick embryo were cultured for 1 day at 37°C and treated with PK (Fermentas) at 1 mg/ml for 10 minutes at 4°C. PK activity was stopped with PMSF. Proteins were extracted, loaded on streptavidin beads and eluted. For quantification, explants were lyzed in 500 μl of lysis buffer and 50 μl of total lysis extract was kept as input. Input (10 μl) was separated on a SDS page gel and further processed for western blot detection. Biotinylated proteins were eluted in 400 μl of elution buffer. Eluted protein (100 μl) was precipitated and separated on a SDS gel in 25 μl of Laemmli solution and further processed for western blot detection. Intensities of bands were calculated with MULTI GAUGE from Fuji and normalized with respect to the initial amount of protein in the initial neural tube total extract.

Constructs

All gain- and loss-of-function constructs for electroporation are based on pCAGGS expression vector (Niwa et al., 1991) containing the composite CMV/chicken β-Actin promoter, engineered for bicistronic expression of GFP (pCAGGS-ires-GFP). For loss-of-function experiments, single-chain antibody (scFv) sequences myc-tagged at their C terminus were taken from plasmids previously described (Lesaffre et al., 2007), and inserted into pCAGGS-ires-GFP to give non-secreted or secreted versions of the anti-Pax6 scFv (pcGsalP6GFP, coding for P6SalGFP, coding for P6Sal, respectively), and the secreted version of the anti-Engrailed scFv (pCGs4G11GFP, coding for S4G11). A mutant form of the secreted anti-Pax6 (C43S) was constructed by oligonucleotide insertion (pCGs4G11iGFP, coding for S4G11). A mutant form of the secreted SaP6, respectively), and the secreted version of the anti-Engrailed scFv into pCAGGS-IRES-GFP to give non-secreted or secreted versions of the construct. Constructs from Fuji and normalized with respect to the initial amount of protein in the initial neural tube total extract.

In situ hybridization

In situ hybridization using digoxigenin (DIG)-labeled cRNA antisense probes (Boehringer Mannheim, Mannheim, Germany) were performed on coronal sections of thoracic spinal cord of chick embryos. In situ hybridization was performed as previously described (Spassky et al., 2001). Sections were examined under a fluorescence microscope (Zeiss). Open-books were mounted with Fluoromount and analyzed with confocal microscopy (SP5 Nikon). The monoclonal antibodies developed by A. Kawakami (Pax6), and T. M. Jessell and S. Brenner-Morton (Nkx2.2) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242.

RESULTS

Pax6 extracellular localization and intercellular transfer in the chick neural tube

In the embryonic spinal cord, the Olig2 and Pax6 transcription factors are expressed by adjacent domains of ventricular progenitors (Fig. 1A). Between E5 and E7, numerous Pax6-expressing cells migrate away from the ventricular domain and invade the ventral-lateral domain of the neural tube where they intermingle with Olig2-expressing cells that correspond to OPCs based on PDGFRα co-expression (Fig. 1A,B). At E6.5, around 85% (two embryos, four sections per embryo with approximately 30 migrating cells/section) of Pax6-positive migrating cells express the neuronal marker NeuN (Fig. 1C). Moreover some Pax6+ cells are PSA-NCAM, DCX and TuJ1 positive (data not shown).

The presence of Pax6- and Olig2-expressing cells in the same region of the developing neural tube suggested that extracellular Pax6 may influence OPC migration. A starting point in evaluating
This hypothesis was to verify the extracellular localization of Pax6 in the developing chick neural tube. To that end, we used the same procedure developed for Engrailed 1 and Engrailed 2 (Wizenmann et al., 2009). E5 chick neural tubes were placed in culture in the open-book configuration and after 1 day in vitro (DIV), extracellular proteins were biotinylated and purified on streptavidin beads. Extracellular and intracellular protein localizations were further verified by their sensitivity to proteinase K. Fig. 1D compares the behavior of NCAM and RhoA taken as typical extracellular and intracellular proteins, respectively. RhoA was not accessible to biotinylation, and thus not retained on streaptavidin beads and or detected in the eluates. Intracellular localization was further confirmed by resistance to proteinase degradation. NCAM was entirely degraded by proteinase K and biotinylated NCAM (retained on streptavidin beads) was lost in the presence of PK. Pax6 presented a mixed behavior with 95% of the protein behaving like RhoA and 5% as NCAM.

This result demonstrates that, as already shown for Engrailed, 5% of the transcription factor shows an extracellular localization, the other 95% being intracellular. Because this small amount of protein did not allow us to visualize with certainty low levels of Pax6, in particular extracellular Pax6, we electroporated the neural tube (at E3) with a pcDNA expression vector encoding a Pax6 bearing a N-terminal HA-tag (to discriminate from endogenous Pax6) and a GFP, separated with a IRES from the Pax6 sequence. Fig. 1E illustrates the presence of HA-Pax6 (red) in many cells and territories abutting GFP-positive Pax6-expressing cells (green), demonstrating that the protein can be secreted and internalized in vivo.

**Extracellular Pax6 neutralization prevents OPC dispersal**

We then designed a series of experiments to examine the effects of antagonizing the extracellular component of Pax6 on oligodendroglial cells. In a first set of experiments, we used plasmids encoding Pax6 single chain antibodies (scFv) exhibiting a signal peptide for secretion at their N terminus (SaP6). This antibody, which does not gain access to the intracellular compartment (Lesaffre et al., 2007), is secreted and hence only neutralizes extracellular Pax6. For control experiments, we developed a number of other constructs depicted in Fig. 2A. These included: (1) a secreted antibody directed against another HP, Engrailed (SaEN); (2) a single-chain anti-Pax6 antibody to which a signal peptide was not added (aP6) and is thus with intracellular expression; (3) an inactive SaP6, following a mutation replacing a cysteine necessary for disulfide bond formation by a serine (SaP6-CS). All plasmids contained an IRES GFP that allowed for observation of plasmid expression. These plasmids were electroporated at E3 and Olig2+ cells were revealed by IHC and counted 3 days after
electroporation. OPCs were detected by immunolabeling with anti-Olig2 antibodies or by in situ hybridization with an Pdgfrα antisense riboprobe. Fig. 2B illustrates the decrease in the number of Olig2+ migrating cells on the side expressing the secreted anti-Pax6 single-chain antibody, which is significant, as indicated by the quantifications shown in Fig. 2D. The number of Pdgfrα-expressing cells similarly decreased after SaP6 electroporation (Fig. 2C). Although SaP6 does not localize to the nucleus of transfected cells in culture (Lesaffre et al., 2007) and thus very likely does not antagonize intrinsic Pax6 proteins, one cannot fully rule out the possibility that a small amount of SaP6 escapes the secretion pathway, as well as escaping denaturation by reduction of disulfide bonds by the intracellular reducing milieu. The absence of activity of intracellular aP6 (Fig. 2D), best explained by its intracellular instability (Auf der Maur et al., 2004; Strube and Chen, 2004), precludes that small amounts of SaP6 escaping the secretion pathway could be at the origin of the observed phenotype. The importance of proper antibody folding was further demonstrated by the absence of activity of the SaP6-CS in which a cystein was modified into a serine, thus preventing the formation of disulfide bounds. Finally the secreted anti-Engrailed, although active in other developmental situations (Wizenmann et al., 2009), was inefficient in regulating OPC distribution.

To block unambiguously the extracellular Pax6 content, we directly added anti-Pax6 antibodies to spinal cord explants. The neural tube was dissected at E5, opened dorsally and cultured in open-books for 1 day in the presence of the reagents. At this stage of development, oligodendroglial cells have been specified (Soula et al., 2001) and antibodies against Pax6 should primarily interfere with the migration and/or differentiation of OPCs (see below). Fig. 3A shows Olig2 labeling for open-book neural tubes placed in culture. The ventricular zone is observed as a dense population of cells spanning from the most anterior regions to the posterior tube and migrating cells are observed migrating dorsally towards the exterior of the open book preparation and ventrally towards the floor plate. Fig. 3B illustrates and quantifies, for a designated region corresponding to rhombomeres 7 and 8 (r7 and r8), the percentage of cells located in either a region of migration or in the ventricular zone. The ratio of migrating versus non-migrating cells is quantified as a function of fluorescence intensity in respective areas. We validated the open book culture model with a well-established OPC guidance molecule, netrin. Netrin is described in the literature as a repulsive cue for migrating OPCs in the embryonic spinal cord; this has been shown with the use of a chemotaxis assay (Jarjour et al., 2003; Tsai et al., 2003). Furthermore, blocking the netrin receptor DCC blocks the repulsive signal in the chemotaxis assay and inhibits dispersal of OPCs in
Extracellular Pax6 regulates OPC

**Fig. 3. Pax6-blocking antibodies decrease Olig2+ cell dispersal.** (A) Open book of neural tube immunolabeled with anti-Olig2 antibody. Open books are derived from the most posterior part of hindbrain of E5 chick embryos and cultured for 24 hours in DMEM alone (control). Open books were fixed in 2% PAF for 30 minutes and immunolabeling for Olig2 was performed. (B) Migrating Olig2+ cells and ventricular Olig2+ progenitors detected on open books were quantified using integrated intensity plot analysis to count the percentage of migrating Olig2+ cells outside PMN domain; the left-hand arrowhead indicates a region of ventral migration and the right-hand arrowhead indicates a region of dorsal migration. (C) To validate the method, open books were cultivated with netrin-blocking antibodies and DCC-blocking antibodies. The percentage of migrating Olig2+ cells when the open books were cultured with DMEM or anti-Netrin or anti-DCC. (D) The percentage of Olig2+ migrating cells when open books were cultured with DMEM, rabbit polyclonal anti-Pax6 (pk-anti-Pax6, recognizing the NTer part of Pax6 protein), pk-anti-Pax6 + its specific blocking peptide (i.p), i.p alone, and pk-anti-Pax6 + a scrambled peptide of same amino-acid composition to the i.p (sc.p). DMEM, n (embryos)=12; pk-anti-Pax6, n=15; pk-anti-Pax6 + i.p, n=12; i.p, n=6; pk-anti-Pax6 + sc.p, n=6. Data are the mean of three independent experiments. (E) The percentage of Olig2+ migrating cells present upon addition of an anti-Pax6 monoclonal antibody (mk-anti-Pax6) or an anti-Engrailed monoclonal antibody (mk-anti-EN). DMEM, n (embryos)=10; mk-anti-Pax6, n=7; mk-anti-EN, n=5. Representative results from three independent experiments. Bars indicate s.e.m. P values were calculated using unpaired t-tests. The variation in DMEM base line between C, D and E occurs because it is impossible to maintain the same conditions between independent experiments.
chick spinal cord sections (Tsai et al., 2003). We tested this system on open book cultures and observed a significant decrease in Olig2+ cell dispersal after co-culture with netrin-blocking antibodies and DCC-blocking antibodies (Fig. 3C). This result is in agreement with the reduced migration of OPCs reported previously (Jarjour et al., 2003; Tsai et al., 2003).

Once validated, the open-book cultures were performed in the presence of Pax6-blocking antibodies. Fig. 3D quantifies the inhibition of Olig2+ cell dispersal in the presence of an anti-Pax6 polyclonal antibody (recognizing C terminal of Pax6). This decrease is not observed when the antibody is pre-incubated with an antibody inhibiting peptide (the peptide against which the antibody is directed). However, pre-incubation with a scramble peptide with the same amino acid composition has no effect on the inhibition of Olig2+ cell dispersal. The experiment was repeated using an anti-Pax6 monoclonal antibody (recognizing the Pax6 N terminus). The neutralization of extracellular Pax6 with the monoclonal antibody also led to a strong bilateral decrease in Olig2+ cell dispersal, an effect that was not observed with a monoclonal anti-Engrailed antibody (Fig. 3E).

**Extracellular Pax6 gain of function induces OPC dispersal**

We then turned to gain-of-function experiments. In a first series of experiments, we electroporated a Pax6 construct (secPax6) to which a signal peptide was added, in order to eliminate cell autonomous activities. We verified that conventional secretion did not modify the size of the protein nor induced its glycosylation (Fig. 4A). Then electroporations were carried out at E3. The embryos were left to develop for 3 days in ovo, and the transfer of Pax6 into non-electroporated cells, including Olig2+ cells was verified (Fig. 4B, left panel). For control experiments, we electroporated a mutated version of Pax6 in which the WF doublet of the homeodomain is replaced by a SR doublet (Pax6SR) that, in addition toyielding a transcription-deadan HP, prevents its internalization (Derossi et al., 1996; Derossi et al., 1994; Joliot et al., 1998; Joliot and Prochiantz, 2004). This absence of translocation is illustrated in Fig. 4B (right panel). Fig. 4C,D illustrates and quantifies a significant increase in Olig2+ cell dispersal on the side electroporated with the secPax6, but not with secPax6-SR. We next used open-book spinal cord cultures to analyze the response of migrating Olig2+ cells to an environment enhanced with a purified recombinant Pax6 protein (rPax6). Neural tubes were dissected at E5 and incubated for 1 day with rPax6 or recombinant Engrailed-1 protein (rEN). As shown in Fig. 4E,F, recombinant Pax6 strongly enhances the number of ventral and dorsal migrating Olig2+ cells, an effect not induced by Engrailed 1, a homeoprotein used as an internal control.

**Specificity of the regulation of OPC dispersal by extracellular Pax6**

Because anti-netrin and anti-DCC antibodies antagonize the dispersion of Olig2+ cells (Fig. 3), we wanted to know whether the two pathways were independent. To achieve this, we compared the effect of recombinant Pax6 alone and that of rPax6 and anti-netrin. As quantified in the graphs in Fig. 4G, the effect of the HP is blocked in absence of active netrin. This demonstrates that the two pathways are not independent. This finding is reminiscent of the interaction of extracellular Engrailed signaling with the Ephrin/Eph chick in retinal axon guidance (Wizenmann et al., 2009) and Drosophila anterior cross vein formation (Layalle et al., 2011).

We next questioned whether extracellular Pax6 had a specific effect on OPCs or whether it could also influence the behavior of other cell types in the neural tube. As shown in Fig. 5A-D, no significant change was found in the number of Mnr2+ or Nx2.2+ cells in the neural parenchyma following electroporation of both the Sap6 and secPax6 (loss and gain of function, respectively), rather suggesting that extracellular Pax6 acts specifically on OPCs in the spinal cord at this stage of development.

Given the increase in the number of Olig2+ and Pdgfra-expressing cells in gain-of-function experiments we examined if the mitotic index of Olig2+ cells was modified in gain-of-function (recombinant Pax6 added) or loss-of-function (monoclonal and polyclonal anti-Pax6) conditions. Fig. 6 shows that, in all the experimental conditions tested, the mitotic behavior of Olig2+ cells was similar, which diminishes the possibility of a primary trophic effect of extracellular Pax6 on OPCs.

**Specification (or re-specification) versus migration**

As already mentioned, the open-book experiments were carried out at E5, when OPC specification is achieved (Soula et al., 2001), making it unlikely that the changes in the number of migrating OPC could only result from an earlier progenitor re-programming. In addition, antagonistic to this idea is the interaction between Pax6 and netrin signaling (Fig. 4G) as netrin has no known function in OPC specification. However, to better evaluate this possibility, we did the open-book experiment at E6, plus 1 day in presence of the monoclonal anti-Pax6 antibody or of the anti-DCC. Fig. 7A,B illustrates and quantifies the decrease in OPC migration when either antibody is added to the open book.

Another way of asserting a change in migration not dependent, at least for its largest component, on specification changes, was to electroporate at E3 several constructs encoding wild-type and mutated Pax6 forms. Fig. 7 describes the latter Pax6 forms (Fig. 7C) and quantifies (Fig. 7D) the effect of their expression on OPC migration. Secreted Pax6 induces migration, as already shown and the internalization mutant (secPax6-SR) has no activity. Furthermore, abolishing Paired-domain-associated transcription (Haubst et al., 2004) by deleting exon 6 in the paired domain (secPax6-mp) did not alter the effect on cell migration (unless the protein was also inactivated for its internalization), making it unlikely that extracellular Pax6 is primarily acting, at this stage at least, at the level of lineage decisions.

Altogether, this series of gain- and loss-of-function experiments bring together converging results indicating that direct Pax6 non-cell autonomous signaling can specifically act on OPCs and influence their early migration in the developing spinal cord.

**DISCUSSION**

Our cell surface biotinylation experiments clearly demonstrate the presence of an extracellular pool of Pax6 in the neural tube and thus raise the issue of Pax6 secretion function and physiological importance in vivo. The observation of a population of migrating Pax6-positive cells and the proximity of these cells to early migrating OPCs led us to disrupt or antagonize extracellular Pax6 by neutralization with a blocking antibody and to observe any subsequent effects on OPC migration. When we neutralized extracellular Pax6 by expressing a fully characterized and neutralizing secreted single chain antibody (Lesaffre et al., 2007), we observed a decrease in the number of OPCs, identified by expression of Olig2 and Pdgfra, emanating from the ventricular zone of the neural tube. Importantly, OPC dispersal was not
Fig. 4. In ovo and in vitro gain of function of extracellular Pax6 enhances Olig2+ cell dispersal. (A) secPax6-expressing plasmids were transfected in HEK293 cells. After 24 hours in culture, protein extracts were prepared and treated (+) or not (–) with PNGaseF. Proteins were separated on SDS-PAGE and analyzed by western blot with anti-HA antibody. No difference was observed between the treated and nontreated protein extracts. (B) Chick embryos were electroporated at E3 with cDNA of either secPax6 (left) or mutated secreted Pax6 (secPax6-SR) (right) and analyzed at E6 by immunolabeling of spinal cord cryosections with anti-HA and anti-Olig2 antibodies. The GFP-positive side is the electroporated side. Arrows indicate GFP+/luminal Pax6-expressing cells; stars indicate examples of transfer of HA-Pax6 protein into GFP-negative non-electroporated cells. (C,D) Chick embryos were electroporated at E3 with cDNA of either secPax6 or mutated Pax6 (secPax6-SR) and analyzed at E6 by immunolabeling of spinal cord cryosections with anti-Olig2 antibody. (C) Olig2 immunolabeling following electroporation of secPax6 shown in green. (D) The ratio of migrating Olig2+ cells on the electroporated side versus the non-electroporated side for secPax6-SR and secPax6. Ten to 15 Olig2+ cells were counted on one side of one section on at least four sections along the thoracic level of the spinal cord; hence, the ratio was calculated with at least 40 Olig2-positive cells per side. secPax6-SR, n (embryos)=7; secPax6, n=7 of at least three independent experiments. (E,F) Open books of E5 neural tube are cultured with the recombinant proteins Pax6 (rPax6) or Engrailed (rEN) for 24 hours. (E) Open books immunolabeled with anti-Olig2 antibody. (F) The percentage of migrating cells for open books cultured with DMEM, rPax6 or rEN. DMEM, n (embryos)=15; rPax6, n=10; rEN, n=3. Mean of two independent experiments. (G) The percentage of Olig2+ migrating cells in open book cultures following co-culture with DMEM, rPax6 or rPax6 + anti-netrin. Pax6-dependent increase in Olig2+ cell migration was abolished upon netrin neutralization. DMEM, n (embryos)=6; rPax6, n=7; rPax6 + anti-netrin, n=8. Mean of two experiments. (H) In situ hybridization illustrating PDGFRα at E6 following electroporation of secPax6 at E3. Ratio of PDGFRα+ cells on the electroporated side versus the non-electroporated side for secPax6. secPax6, n=8 chicks; secPax6-SR, n=5 chicks. Black flash in H indicates the electroporated side. Bars indicate the s.e.m. For EP experiments, unpaired t-tests were used to detect significant differences between the ratios of EP side versus non-EP side for different constructions. For open book experiments, unpaired t-tests were used to detect significant differences between the control condition versus treated conditions.
modified when we electroporated an antibody containing a cysteine mutation that renders the antibody inactive. The electroporation of a neutralizing secreted single-chain anti-Engrailed antibody (Lesaffre et al., 2007) failed to affect Olig2+ cell pattern, and, conversely, the extracellular Pax6 neutralization or gain of function had no effect on the number of MNR2- or Nkx2.2-expressing cells. These results strongly suggest that extracellular Pax6 is actively and specifically implicated in the dispersal of OPCs.

The electroporation of anti-Pax6 constructs at stage E3 may have primary effects on ventricular progenitors, prior to the onset of oligodendrocytes, which starts between E5 and E5.5 in the chick spinal cord (Soula et al., 2001). If so, these effects are not massive as migration is still observed with a Pax6 construct encoding a protein mutated in the paired domain and inactive for OPC specification (Haubst et al., 2004). Furthermore, to manipulate extracellular Pax6 selectively during the period of OPC migration, we applied anti-Pax6 antibodies directly on E5 and E6 neural tube explants. We used the model of open-book cultures that we validated by reproducing results previously obtained on neural tube sections, i.e. the blocking of OPC dispersal by neutralizing netrin or its receptor DCC (Tsai et al., 2003). In this context, the fact that neutralizing either extracellular Pax6 or netrin antagonized OPC dispersion suggests that, at physiological concentrations of the proteins, these signals may cooperate in regulating OPC migration. This calls to mind the cooperation of EphrinA5 and extracellular Engrailed in the induction of temporal retinal ganglion cell growth cone collapse (Wizenmann et al., 2009).

The open book culture model leaves no room for ambiguity concerning the extracellular activity of the blocking antibody, as it is simply added to the culture medium. The use of this second model not only confirmed a role for extracellular Pax6 in OPC dispersal but also enabled us to add a purified protein to the medium, thus permitting us to enhance extracellular Pax6. Pax6, when added to the culture medium enhances OPC dispersal; an effect mirrored by the electroporation studies with the secreted protein. It should be noted that the electroporated mutated Pax6 (Pax6SR) protein lacks the tryptophan in position 50 (W50) of the homeodomain that plays an important role in HP internalization (Derossi et al., 1996). However, because W50 is also indispensable for transcription regulation (Gehring et al., 1994), this result does not fully demonstrate that Pax6 effects on OPC dispersal requires its internalization (following secretion). Although unlikely, the
possibility remains that Pax6 acts extracellularly and that the WF doublet in position 48 and 49 is necessary for such extracellular activity, e.g. involved in the recognition of a binding site or receptor. However, based on previous results (Brunet et al., 2005), we favor the idea that non-autonomous Pax6 activity requires its internalization and that our loss-of-function protocols act through a decrease in the amount of protein transferred.

Immunohistochemistry following electroporation of secreted HA-Pax6 protein suggests that Pax6 can be transferred into cells that do not express Pax6, from neighboring cells expressing Pax6 following electroporation, and producing the secreted HA-Pax6 protein. The HA-Pax6 protein also seems to be localized in the cytoplasm, supporting a translation regulatory hypothesis. Such a mechanism is not foreign to homeoprotein signaling and has been previously described for Engrailed in the retino-tectal system (Brunet et al., 2005; Wizenmann et al., 2009). This does not allow us to eliminate an extracellular activity completely or to distinguish, following internalization, between a regulation at the transcription or translation level, or both. However, if transcription is required in the recipient cells it does not involve the paired domain, because a transducing Pax6 with a deletion of exon 6 (Haubst et al., 2004) still induces OPC migration. This point (translation and/or transcription) could not be investigated in our experimental models, as migration analysis requires a minimum period in culture, a duration that forbids the use of transcription and translation inhibitors. In the case of Engrailed, in vitro studies showed that in order to elicit a turning response or a collapse, the factor must be captured by the growth cones and imported into the cytosol where it regulates protein synthesis through the mTOR pathway (eIF4E-dependent capped mRNA translation initiation) (Wizenmann et al., 2009). We have recently obtained evidence of the interaction between Pax6 and eIF4E in immunoprecipitation experiments, thus suggesting a possible role for Pax6 in translation regulation, a phenomenon already reported for several HPs that regulate translation (Gingras et al., 1999; Nedelec et al., 2004; Topisirovic and Borden, 2005).

Fig. 6. Extracellular Pax6 does not modulate neural precursor cell proliferation. (A) Coronal sections of spinal cord showing immunostaining of phospho-histone 3 (PH3) after electroporation of SaP6 or secPax6 constructs at E3 + 3DIO. (B) The ratio of PH3 cells in the electroporated side versus the non-electroporated side for SaP6 and secPax6; SaP6, n (embryos)=6; secPax6, n=7, three independent experiments. PH3-positive cells were counted in the ventricular region. Bars indicate the s.e.m. Paired t-tests were used to detect significant differences between the EP side versus the non-EP side. (C) To analyze mitotic index of Olig2+ cells, co-immunostaining of Olig2 and PH3 was performed on open-book preparations. (D) Area under the curve of Intensity plot of Olig2+/PH3+ and Olig2+ images was calculated using Prism software. Area Value of Olig2+/PH3+ image was measured with respect to value of Olig2+ image to obtain the mitotic index of Olig2+ cells. (E) Mitotic index of Olig2+ cells cultured with DMEM or in the presence of recombinant Pax6 protein. (F) Loss-of-function experiments (E5 + 1DIV). Graph shows the mitotic index of Olig2+ cells in the presence of pk-anti-Pax6. Bars indicate the s.e.m. Unpaired t-tests were used to detect significant differences between the control condition versus treated conditions.
Another question that our results raise is with regards to the specificity of the action of extracellular Pax6. Both antagonism and enhancement of extracellular Pax6 produced a significant change in the number of migrating OPCs. As already mentioned, this was not observed with other cell types, thus denoting specificity for OPCs. Although HPs are capable of being internalized by most if not all cell types in vitro (Joliot and Prochiantz, 2004), the question of their specificity in vivo remains largely unknown. In the visual cortex, the HP Otx2 is specifically internalized by a particular subset of interneurons containing the Ca2+-binding protein parvalbumin (Sugiyama et al., 2008). It would thus be interesting to investigate whether Pax6 is specifically internalized by specific subsets of cells, notably OPCs. If Pax6 is captured by all cell types, this does not preclude specificity. For example, in the case of retinal axon guidance by Engrailed, nasal axons are attracted and temporal repelled (Brunet et al., 2005), establishing that the receiving entity participates in the specificity of the response.

We can conclude that extracellular Pax6 is indeed present in the developing neural tube and is actively implicated in the dispersal of migrating OPCs, most probably via an internalization-dependant mechanism and the regulation of translation and/or transcription. More generally, should the possibility that local HP intercellular transfer regulates migration, in cooperation with other signaling entities, their position-dependent expression would provide a parsimonious explanation for the precision and specificity of cell and growth cone guidance during development.

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Competing interests statement
The authors declare no competing financial interests.

References
Extracellular Pax6 regulates OPC


