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The transcription factors Emx1 and Emx2 suppress choroid plexus development and promote neuroepithelial cell fate

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Abstract

The transcription factors Emx1 and Emx2 exert important functions during development of the cerebral cortex, including its arealization. Here, we addressed their role in development of the derivatives of the midline region in the telencephalon. The center of the midline region differentiates into the choroid plexus, but little is known about its molecular specification. As we noted a lack of Emx1 or 2 expression in the midline region early in development, we interfered by misexpressing Emx1 and/or Emx2 in this region of the chick telencephalon. Ectopic expression of either Emx1 or Emx2 prior to HH 13 instructed a neuroepithelial identity in the previous midline region instead of a choroidal fate. Thus, Gli3 and Lhx2 normally restricted to the neuroepithelium expanded into the Emx misexpressing region. This was accompanied by down-regulation of Otx2 and BMP7, which implicates that these factors are essential for choroid plexus specification and differentiation. Interestingly, the region next to the ectopic Emx-misexpression then acquired a hybrid identity with some choroidal features such as Bmp7, Otx2 and Ttr gene expression, as well as some neuroepithelial features. These observations indicate that the expression levels of Emx1 and/or Emx2 restrict the prospective choroid plexus territory, a novel role of these transcription factors.
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Keywords: Telencephalon; Cortical hem; Midline; Roof plate; Patterning; Emx1; Emx2

Introduction

Patterning mechanisms are crucial to establish regional differences within the neuroepithelium, yet less is known about the mechanisms delineating neuroepithelial cells from the non-neural choroid plexus (ChP) epithelium, a secretory epithelium that differentiates from the roof of the neural tube in all brain ventricles. In the telencephalon, the roof plate undergoes a dramatic morphological change at midneurogenesis stages in rodents and birds: it invaginates and becomes buried between the two cerebral hemispheres. Concomitant with this invagination, the roof plate differentiates into the non-neural secretory ChP (Dziegielewska et al., 2001; Sturrock, 1979; Zaki, 1981). This differentiation is first detectable by the cessation of proliferation and the expression of Transthyretin (Ttr), an indicator of secretory epithelial differentiation (Currle et al., 2005; Furuta et al., 1997).

Between the ChP and the hippocampal region of the cortex, an intermediate multilayered epithelium forms a Wnt-rich signaling center, known as the cortical hem (Grove et al., 1998), that may partially derive from the roof plate (Currle et al., 2005). Due to its position as a signaling center between ChP and hippocampal anlage, the cortical hem might influence both sides. Indeed, mice lacking the region of the hem, usually also lack the ChP and hippocampus, which is the case in Emx1 and Emx2-double knockout mice (Shinozaki et al., 2004), Gli3-mutants (Grove et al., 1998; Theil et al., 1999) and the Lhx5−/− mice (Zhao et al., 1999). Mice displaying partial defects in the hem-region like Emx2−/− (Tole et al., 2000a; Yoshida et al., 1997) and Wnt3a−/− (Lee et al., 2000) also exhibit reductions in the ChP and hippocampal regions. Conversely, deletion of the Lhx2 gene leads to a significant enlargement of the cortical hem region accompanied by an enlargement of the ChP (Bulchand et

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al., 2001; Monuki et al., 2001). These dates suggest that Wnt-signaling from the cortical hem is important for the development of the ChP and seems to regulate its extension.

Members of the bone morphogenic proteins (Bmps) are crucial for ChP differentiation as conditional deletion of the Bmp receptor1a (BmpR1a) in the telencephalon interrupts ChP differentiation (Hebert et al., 2002). High levels of Bmps are expressed in the telencephalic roof plate region (Hebert et al., 2003; Jones et al., 1991) and contribute to the characteristic thin morphology of the differentiating ChP epithelium by instructing apoptosis and differentiation (Panchision et al., 2001; Solloway and Robertson, 1999). Indeed, Bmps seemingly act by an autocrine mechanism as the BMP-expressing cells themselves differentiate into the ChP as recently demonstrated in elegant fate mapping as well as cytotoxic deletion experiments (Currle et al., 2005; Monuki et al., 2001).

While the importance of Wnt and Bmp signaling for ChP specification and differentiation has been demonstrated, it is not known how the border between the neuroepithelium and the secretory epithelium is established and maintained. Wnt signaling affects both development of the neuroepithelial hippocampus as well as the secretory epithelium of the ChP. The molecular cues that instruct the hippocampal anlage to become or remain neuroepithelial tissue upon Wnt signaling, while the region medially adjacent to the hem differentiates into secretory epithelium upon Wnt signaling remain unknown. Here, we examined whether the transcription factors Emx1 and Emx2 may exert such a role, as they are expressed at particularly high levels in the medial telencephalon (hippocampal anlage and hem), but are absent from the roof plate and ChP region in the developing telencephalon of both mice and chick (Fernandez et al., 1998; Mallamaci et al., 1998; Muzio and Mallamaci, 2003, Shinozaki et al., 2004). In contrast, other transcription factors present in the neuroepithelium, such as Pax6, are also expressed in the chick ChP (data not shown). Here, we examined the role of the gap of Emx1 and Emx2 expression in the roof plate and ChP region by ectopic expression of these transcription factors in early development in the chick telencephalon (HH 9–14). Our results demonstrate a key role for Emx1 and Emx2 in instructing neuroepithelial identity, as misexpression of Emx1 and Emx2 was sufficient to convert non-neuronal ChP tissue into neuroepithelium. These data therefore imply that the limits of Emx1 and Emx2 expression determine the border between the neural and secretory epithelium.

Materials and methods

Electroporation

Fertilized eggs were windowed with scissors at embryonic day (E) 2. Electroporation was performed between HH 9 and HH 14, staged according to Hamburger and Hamilton (1951). The control plasmids pCAG containing either GFP or mRFP (kind gift of J. Guilford) and pCAX containing GFP, or the pMES plasmid (Swartz et al., 2001) containing either IRES-EGFP only, Emx1-IRES-EGFP or Emx2-IRES-EGFP (2–5 μg/μl) were injected into the forebrain and electroporated into the tissue (1–5 pulses, 50 ms, 20–25 V) with electrodes placed left and right of the forebrain. 70 μl of Penicillin/Streptomycin (GIBCO) was applied onto the embryo and the egg was sealed with tape. The embryos were harvested at E4 or E6 and the heads were fixed for 2–4 h at 4°C in 4% paraformaldehyde in phosphate buffered saline (PBS) and cryoprotected in 20% sucrose in PBS at 4°C over night. Coronal cryostat sections (20 μm) were collected on Superfrost Plus slides (Menzel).

Plasmid constructs for electroporation

Emx1 and Emx2 mouse cDNAs (kind gift of Antonio Simeone) containing parts of the 3′ and 5′ UTR were cloned into the multiple cloning site of the expression plasmid pMES containing an IRES-EGFP sequence that allows reliable coexpression of EGFP and the gene of interest (gift of Cathrin Krull) (Swartz et al., 2001). The expression of ectopic mouse Emx1 and Emx2 was confirmed by in situ hybridization (Supplementary Fig. 1) with a mouse-specific antisense mRNA probe (Emx1, Emx2) (Simeone et al., 1992a,b) and by immunostaining for Emx1 (rabbit, 1:1000, G. Corte) or Emx2 (rabbit, 1:4000, O. Hatano) and GFP (Supplementary Fig. 1).

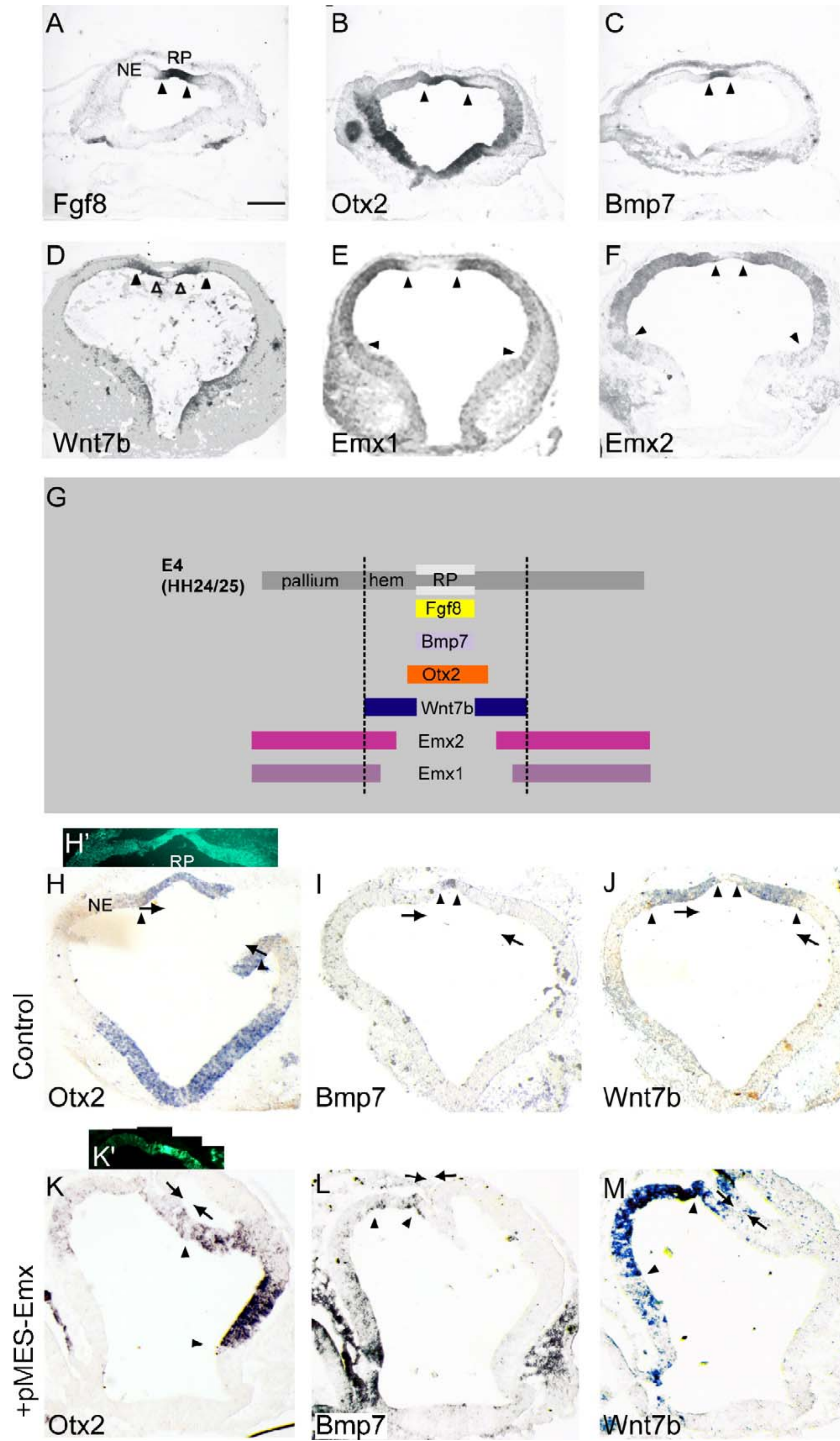
Immunohistochemistry and RNA in situ hybridization

Sections were stained as described previously (Hartfuss et al., 2001), using primary antibodies against the phosphorylated form of Histon H3 (PH3, rabbit, Biomol, 1:200), MAP2 (mouse IgG1, 1:500, Sigma), Pax6 (rabbit, 1:300, Babco or Chemicon) and EGFP (rabbit, 1:500, RDI; or mouse IgG1, 1:300, Chemicon). Secondary antibodies were purchased from Jackson ImmunoResearch and Southern Biotechnology Associates. DAPI (4′,6-diamidino-2-phenylindole, Pierce) was used as a nuclear stain. Plasmid templates were used to generate digoxygenin antisense riboprobes: Otx2 (Wassef et al., 1987), Wnt7b (J. McMahon) (Hollyday et al., 1995), Bmp7 (A. Graham) (Begbie et al., 1999), Gli3 (C. Tabin) (Schweitzer et al., 2000), Ttr (M. Wassef) (Duan et al., 1991), Emx1, Emx2 (E. Bell) (Bell et al., 2001), Lhx2a (S. Richter) and in situ hybridization was performed as previously described (Chapouton et al., 2001).

Analysis of tissue thickness and proliferation

Cell proliferation (PH3+ cells) was quantified in single optical sections as the percentage of GFP-positive cells at E4 (electroporated with the control plasmid pMES or the pMES-Emx1 and/or Emx2-EGFP plasmid). This analysis was performed in a defined square, covering the entire neuroepithelium from the ventricular zone to the pial surface. Alternatively, quantification was performed per area in the electroporated region mostly at later stages (E6), since the GFP-signal was starting to weaken and individual GFP-positive cells were sometimes difficult to discern. Quantification was performed separately in the ChP region, the hem and the dorsal telencephalon in a radial stripe covering 200 μm of the ventricular surface (about 50 cell diameters). In these regions, we also counted the number of DAPI-positive nuclei located in a radial stripe from the ventricular to the pial surface to assess the radial thickness of the respective tissue. In cases, in which the identity of the tissue was not easily discernible by morphology any more (after ectopic expression of Emx1 and/or Emx2), the ‘midline’ of the dorsal telencephalon was defined as located at half the extension of the dorsal telencephalon. Error bars indicate the standard error of the mean (SEM) and the unpaired Student’s *t* test was used to test for significance.

Fig. 1. Gene expression changes upon ectopic expression of Emx1 and/or Emx2 at E4. In situ hybridization was performed in frontal sections for the genes indicated in the panels. Filled arrowheads show the extension of expression of the indicated genes, while the open arrowheads in panel D indicate also the region lacking expression around the dorsal midline. (A–F) Expression patterns in the telencephalon at E4 indicate the regional specification of the ChP (A–C), the cortical hem (D) and dorsal pallium (E, F). (G) Schematic summary of the region-specific expression pattern. (H–M) Frontal sections of E4 telencephali electroporated either with control plasmid (H–J) or the Emx1- and/or Emx2-containing plasmids (K–M). (H′, K′) GFP immunostaining indicates the electroporated region, the extent of which is indicted by arrows in panels H–M. Note the expression of Otx2, Bmp7 and Wnt7b in the midline region of control electroporated brains (H–J), while these mRNAs are down-regulated, respectively shifted laterally to the region of ectopic Emx1 and/or Emx2 expression (K–M). NE, neuroepithelium; RP, roof plate. Scale bar: 250 μm.


A
NE
RP
Fgf8
B
Otx2
C
Bmp7
D
Wnt7b
E
Emx1
F
Emx2
G
E4
(HH24/25)
pallium
hem
RP
Fgf8
Bmp7
Otx2
Wnt7b
Emx2
Emx1
H'
RP
H
NE
Control
Otx2
I
Bmp7
J
Wnt7b
K'
K
+pMES-Emx
Otx2
L
Bmp7
M
Wnt7b

Results

Morphology and gene expression at the telencephalic midline

In order to examine the genetic mechanisms regulating ChP specification, we examined gene expression in the roof plate of the dorsal telencephalon (also referred to as pallium) at embryonic day (E) 4. At this time, the first signs of morphological differences of the roof plate and the dorsal telencephalon became apparent (Fig. 1 or Supplementary Fig. 2; see also Crossley et al., 2001). The roof plate showed a reduced tissue thickness (2.8 cells) compared to the dorsal telencephalon (9.1 cells), a reduced rate of proliferation as detected immunocytochemically by the phosphorylated form of Histone 3 (PH3) present in G2- and M-phase of the cell cycle (Hendzel et al., 1997) (5 PH3+/200 μm in the roof plate compared to 20.3 PH3+ cells/200 μm in the dorsal telencephalon; see Materials and methods for quantification) and a lack of neurons as detected by immunoreactivity for MAP2 (Supplementary Fig. 2). In contrast, neurons just started to appear in the lateral most part of the dorsal telencephalon, while they were more frequent in the ventral telencephalon consistent with the ventral-to-dorsal and lateral-to-medial gradient of neurogenesis (Bayer and Altmann, 1991). Moreover, expression of transcription factors and signaling molecules discriminated the roof plate from the remainder of the dorsal telencephalon (Fig. 1). Expression of Otx2, Bmp7 and Fgf8 was prominent in the midline region, with Fgf8 more rostrally and Otx2 and Bmp7 further caudally (Figs. 1A–C). Wnt7b expression flanked the expression domain of Otx2 on both sides (Figs. 1D, G), reminiscent of the Wnt-rich cortical hem in the mouse telencephalon intercalated between the ChP and the hippocampal anlage (Grove et al., 1998). Further comparable to the mouse telencephalon was the expression of the transcription factors Emx1 and 2 expressed in the dorsal telencephalon excluding the midline region (see also Bell et al., 2001), with Emx2 extending into the Wnt7b-positive region (Figs. 1F, G) while Emx1 expression stopped laterally to the Wnt7b-positive region (Figs. 1E, G).

These gene expression patterns were maintained at later stages (E6) when Otx2 and BMP7 still marked the most medial region of the telencephalon that has by then undergone invagination (Figs. 2D, G). This region now also expressed Ttr (Fig. 2P) and acquired cell biological characteristics of the ChP as described below (Fig. 3B). At E6, Otx2 was still flanked by Wnt7b and Emx1 and 2 expression (Figs. 2D, J and Supplementary Fig. 3). At this stage, Emx1 and 2 were mostly coexpressed and little difference in the medial extension of either mRNA was visible (Supplementary Figs. 3A, B). This expression analysis in agreement with previous analyses in the mouse telencephalon (Tole et al., 2000a; Yoshida et al., 1997) prompted the idea that the complementary expression of Emx1 and/or Emx2 and Otx2 may delineate the midline region differentiating into the ChP.

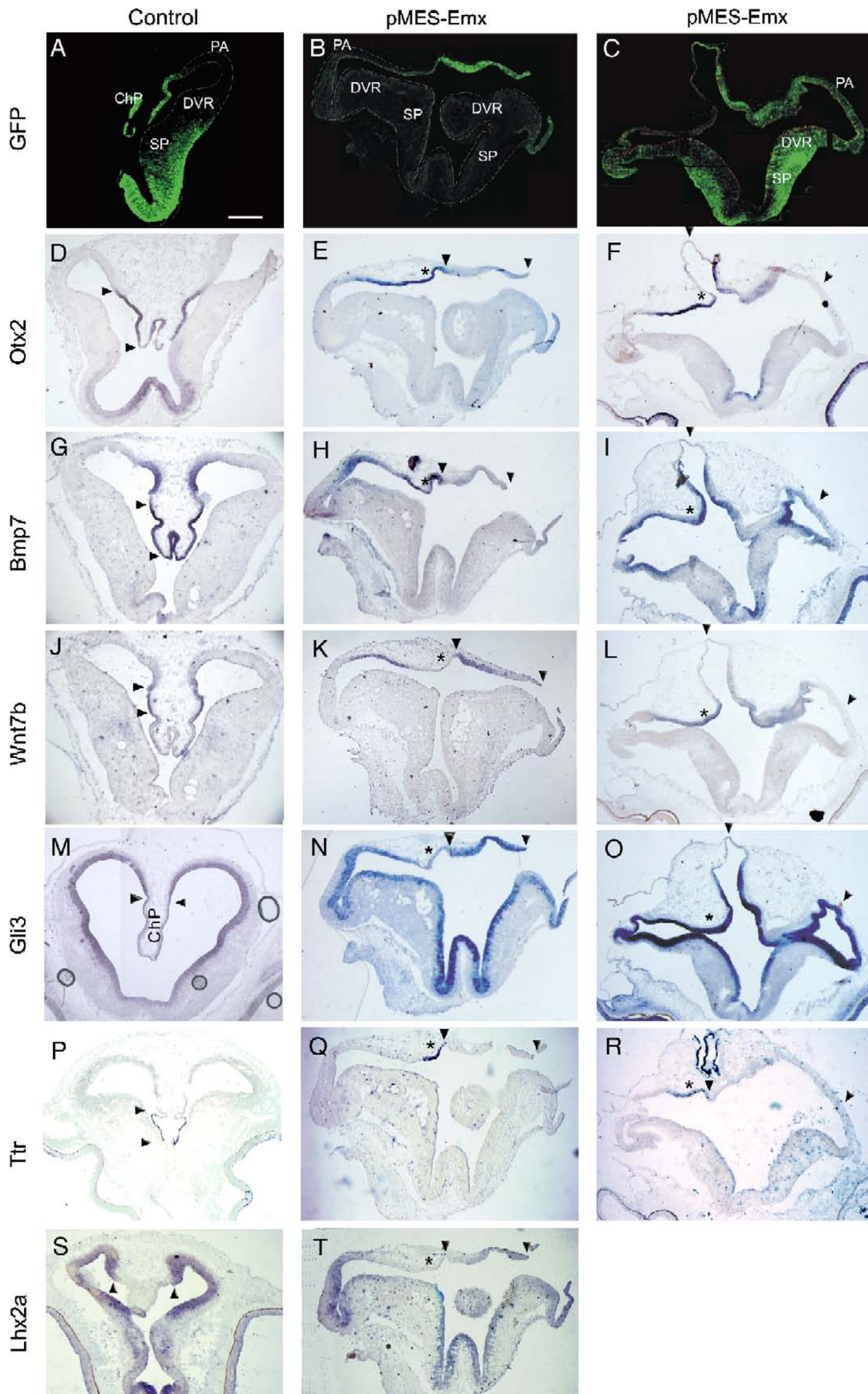
Ectopic expression of Emx1 and 2 in the telencephalic midline

As the roof plate region is normally devoid of Emx1 and Emx2, we examined the effect of misexpressing these transcription factors in the dorsal midline region by electroporation. Plasmids containing either Emx1 or Emx2 and IRES-GFP (pMES-Emx1, pMES-Emx2) or the control plasmids (pMES or pCAX) carrying only GFP or mRFP were injected into the telencephalic ventricle of chick embryos at E2 (HH 9–12, and some at HH 13/14) and electroporated into the dorsal midline as well as in adjacent neuroepithelial regions. The efficiency of electroporation was monitored by GFP-immunostaining and the dorsal midline was targeted effectively in most cases. For example, GFP labeling at E4 revealed expression covering the entire midline in 7 out of 12 brains, while large parts of the midline were GFP-positive in the remaining 5 brains. In situ hybridization for mouse Emx1 or Emx2 mRNA or immunostaining for the respective proteins confirmed the colocalization of Emx1 or 2 and GFP (Supplementary Fig. 1). As Emx1 and Emx2 were largely coexpressed as described above, we also coelectroporated pMES-Emx1 and pMES-Emx2 in most cases (n = 10). However, no difference in phenotype was observed, when only pMES-Emx1 (n = 3) or pMES-Emx2 (n = 3) was electroporated alone. Hence, we consider all Emx1 and/or Emx2 electroporations together (8 for E4 and 8 for E6 analysis = 16 at HH 9–12; in addition 6 at HH 13–14).

After electroporation with the control plasmids neither Otx2, Bmp7 nor Wnt7b expression was affected in the GFP-positive electroporated cells and the normal expression pattern was unchanged at E4 (Figs. 1H–J; n = 3) and at E6 (data not shown). Moreover, no morphological alterations were observed in the telencephali after control vector electroporation at E4 (Figs. 1H–J) or E6 (Figs. 2A, D) compared to brains of embryos that had not been injected and left undisturbed in the egg (Figs. 1A–F). In contrast, when pMES-Emx1 and/or pMES-Emx2 were electroporated, Otx2 expression was strongly reduced in the GFP-positive midline region in 7 out of 8 cases analyzed at E4 (Fig. 1K). In 4 of these cases, Otx2 was suppressed mostly in rostral or caudal sections of the ectopic Emx1- and/or Emx2-expressing region. While Otx2 was reduced in the electroporated midline,

Fig. 2. Midline defects caused by ectopic expression of Emx1 and/or Emx2 at E6. In situ hybridization was performed in frontal sections for the genes indicated in the panels. Panels D, G, J, M, P and S depict the normal expression pattern in the dorsomedial region at E6. The expression of Bmp7 (G), Otx2 (D) and Ttr (P) delineate the ChP. Wnt7b (J) is expressed in the cortical hem. Gli3 (M) is expressed in the telencephalic neuroepithelium including the hem but not in the ChP (indicated). (A–F, H, I, K, L, N, O, Q, R, T) Frontal sections of telencephali that were electroporated either with the control plasmid (A, D) or with Emx1 and/or Emx2 containing plasmids (B, C, E, F, H, I, K, L, N, O, Q, R, T). GFP-immunoreactivity in panels A–C indicates the electroporated region. Panels E and H show that Otx2 and Bmp7 were down-regulated in the midline region ectopically expressing Emx-GFP, while in panels F and I, only Otx2 expression is weakened and Bmp7 is still detectable. Wnt7b, Gli3 and Lhx2a (K, L, N, O, T) were ectopically expressed in the GFP-positive region. Note, the laterally shifted expression domain of Otx2 and Bmp7, which also expresses Ttr (Q, R). In all panels to the left (A, D, G, M, P), arrowheads show the extension of expression of the indicated genes, or the extension into the hem region (M, S), while in the right two panels, the arrowheads indicate the extent of the Emx1/2 misexpression. ChP, choroid plexus; DVR, dorsal ventricular ridge; PA, pallium; SP, subpallium. Scale bar: 250 μm.

its expression shifted laterally, adjacent to the Emx-misexpressing territory (Fig. 1K, black arrowheads). Moreover, ectopic expression of Emx1 and/or Emx2 in the midline also induced down-regulation of BMP7 and Wnt7b (Figs. 1L, M; in 5 of 8 brains analyzed at E4 along the entire anterior–posterior axis; in 3 of 8 brains, expression of Wnt7b was not reduced caudally



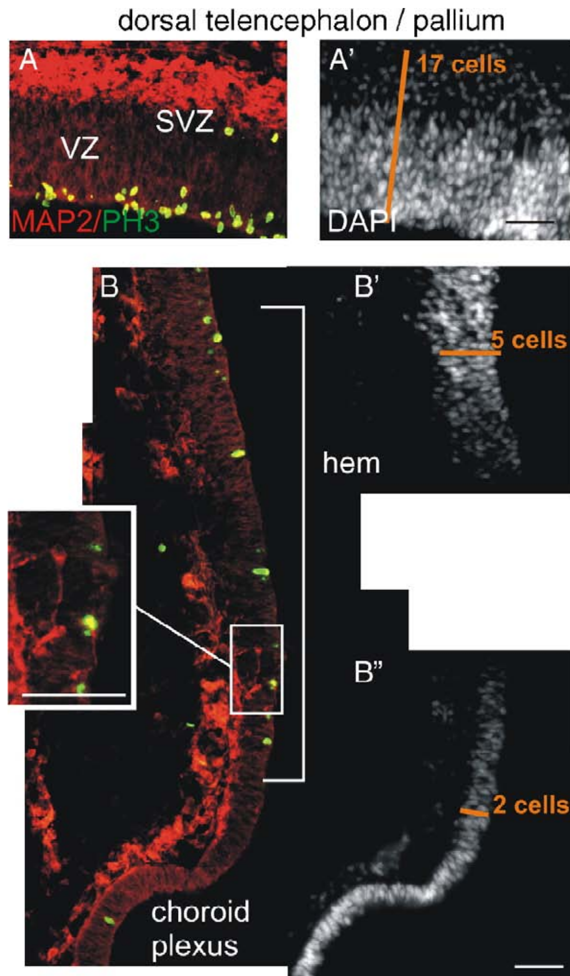


Fig. 3. Cell biological characterization of telencephalic midline region at E6. Frontal sections of E6 telencephali were stained with MAP2 (red, A, B), PH3 (green; A, B) and DAPI (A′, B′, B″). Note the few cell layers (orange, radial line in panels A′, B′, B″) and the lack of proliferation in the choroid plexus region (B, B″) compared to the hem (B, B′) and dorsal neuroepithelium (pallium, A, A′). The inset in panel B indicates MAP2-positive neurons in the region of the hem. SVZ, subventricular zone; VZ, ventricular zone. Scale bars: 25 µm.

and in 1 of 8 brains Bmp7 was still expressed caudally). Thus, misexpression of Emx1 and/or Emx2 in the dorsal midline of the telencephalon led to a strong reduction in midline-specific gene expression at E4, although no changes in the morphology were apparent at this time.

Misexpression of Emx1 and Emx2 in the telencephalic midline causes malformations at E6

To investigate if these early induced changes of the expression pattern influence the development of the midline region, we examined the effects of Emx1 and/or Emx2 misexpression in the midline 2 days later at E6, when the ChP region has further differentiated. The dorsomedial region is now folded to the inside, bifurcating the forebrain into two hemispheres with a thin layer of ChP tissue located most medially and the hem region located laterally and dorsally (Figs. 2A, D, G, J, M, P, S). This morphology was not altered in brains electroporated with control plasmids (n = 5, Figs. 2A, D). In contrast, upon electroporation of the pMES-Emx1 and/or pMES-Emx2 constructs into the dorsal midline of the telencephalon at HH 9–12, the morphology of the dorsal midline was strongly abnormal in all cases analyzed at E6 (Figs. 2B, C; n = 8). The manipulated brains exhibited different deformations ranging from weaker deformations with a certain degree of midline invagination persisting (3/8), a flat midline region (Figs. 2B, E, H, K, N, Q, T; 1/8) to some brains where the medial region evaginated (Figs. 2C, F, I, L, O, R; 4/8). Thus, ectopic expression of the transcription factors Emx1 and Emx2 covering the dorsal telencephalic midline resulted in severe aberrations of midline formation in all cases analyzed.

Molecular characterization of the territory misexpressing Emx1 and Emx2

Given the severe morphological aberrations in the midline upon Emx1 and/or Emx2 transduction, we determined the regional identity of the area of forced Emx1 and/or Emx2 expression. Notably, upon Emx1 and/or Emx2 electroporation, the normal ChP identity, positive for Otx2 and BMP7, but negative for Wnt7b and Gli3, was never observed in the transfected territory (n = 8). In only 1 out of 8 brains, Otx2 and BMP7 were still expressed in the area transduced with Emx1 and/or Emx2, but this region then also acquired the expression of the hem marker Wnt7b. In 7 out of 8 Emx1 and/ or Emx2-electroporated brains, BMP7 was down-regulated in the GFP-positive region (Figs. 2H), and in 5 of these brains Otx2 (Figs. 2E, F) was absent as well. Thus, from 8 brains electroporated between HH 9 and HH 12, the majority (5/8) had lost both Otx2 and BMP7 expression in the former midline. This complete loss of ChP identity was confirmed by the absence of Ttr mRNA, a marker for the differentiating ChP (Duan et al., 1991), in all cases analyzed with this probe (Figs. 2Q, R; 4/4). This Otx2-, Bmp7- and Ttr-negative region seemingly had neuroepithelial features as it also expressed Gli3 (Figs. 2N, O; 4/8), Wnt7b (Figs. 2K, L; 3/8) and Lhx2 (3/ 3, Figs. 2S, T). Taken together, these data demonstrate that, within the Emx-GFP-transduced area, Otx2, BMP7 and Ttr expression was consistently absent, while this was never the case in the midline region targeted with control vectors despite the reliable successful targeting of the midline by electroporation in all cases analyzed (see above). As the loss of molecular markers for ChP fate occurs within the misexpression domain of Emx1 and Emx2, we conclude that these transcription factors interfere with the specification of the telencephalic midline towards a ChP fate and rather promote the acquisition or maintenance of a neuroepithelial identity in most cases.

We also investigated whether the extent of midline evagination versus invagination would correlate with specific marker expression. Otx2 and BMP7 were down-regulated in the midline of brains with evaginated (2/4: Otx2-negative; 3/ 4: BMP7-negative) or invaginated morphology (2/3: Otx2-negative; 2/3: BMP7-negative). The telencephali with evaginated morphology all expressed Wnt7b (Fig. 2L; 4/4), as did the only one telencephalon with a flat morphology (Fig. 2K). In contrast, only 1 out of 3 telencephali with invaginated

midline expressed Wnt7b. This suggests that Wnt7b expression may mediate excessive proliferation interfering with the invaginated morphology and rather causing evagination (n = 4) or flattening (n = 1) of the midline (see also Discussion). Thus, the observed gene expression changes correlate with cell biological changes that we further examined in regard to neurogenesis and cell proliferation.

Cell biological characterization of territory misexpressing Emx1 and Emx2

To verify the alterations in cell fate suggested by the molecular analysis in the region misexpressing Emx1 and/or Emx2, we also examined the cell biological characteristics of this region. Neuroepithelium is characterized by neurogenesis giving rise to a pronounced band of MAP2-positive neurons in the mantle zone at this stage (Fig. 3A). In contrast, MAP2-positive cells were completely absent in the ChP region and only few neurons could be detected in the hem (Fig. 3B). These tissues also differed in their radial thickness: the ChP region was only about 2 cells thick (Fig. 3B″, Table 1), while the neuroepithelium then further increased in its radial extension ranging from a thickness of 5 cells in the hem (Fig. 3B′, Table 1) to 17 cells in the dorsal telencephalon (Fig. 3A′, Table 1). Accordingly, the rate of proliferation increased from the ChP to the pallial region. Precursors undergo M-phase (PH3-positive cells) at the apical side with higher rates in the neuroepithelium (pallium, 14 PH3+ cells/200 μm, Fig. 3A, Table 2), lower rates in the hem (5 PH3+ cells/200 μm; Fig. 3B, Table 2) and drastically reduced rates in the ChP (2 PH3+ cells/200 μm, Fig. 3B, Table 2; see also Hebert et al., 2002).

Consistent with the absence of molecular ChP characteristics, the cellular analysis confirmed the absence of ChP tissue characteristics in the territory of Emx1 and/or Emx2 misexpression. The radial thickness of the Emx-transduced midline region, which was Otx2-negative, was significantly increased to an average of 9 cells (Fig. 4B2 and Table 1), a value normally observed in the neuroepithelium (P < 0.005). In pronounced contrast to the low proliferation rate in differentiating ChP tissue (2 PH3+ cells/200 μm), the number of PH3+ cells was significantly increased in the region of strong Emx1- and/or Emx2-GFP expression (P < 0.005; 12 PH3+ cells/200 μm; Fig. 4C2, Table 2) to values exceeding not only the normal rate of proliferation in the ChP but also the hem tissue (Table 2). This result further supports a neuroepithelial identity of the

Table 1
Thickness of tissue

	Control electroporation			Emx1/2 electroporation of midline	
	Dorsal telencephalic	Hem	ChP	Gfp+/Otx−	Gfp−/Otx+
	17.2	5.3	2.4	9.4	7.9
Average SEM	0.7	0.3	0.1	1.5	0.1

Quantification of thickness at E6 of control (pMES, pCAX or mRFP) and Emx1 and/or Emx2 electroporated dorsomedial telencephalon. The thickness was analyzed by counting DAPI-positive cells in a radial line covering the entire thickness of the tissue from ventricular to pial surface.

Table 2
Proliferation rate of tissue

	Control electroporation			Emx1/2 electroporation of midline	
	Dorsal telencephalic	Hem	ChP	Gfp+/Otx−	Gfp−/Otx+
	13.7	4.6	1.9	11.8	9.4
Average SEM	2.3	0.3	0.4	2	2.4

Quantification of proliferation at E6 of control (pMES, pCAX or mRFP) and Emx1 and/or Emx2 electroporated dorsomedial telencephalon. Proliferation was analyzed by counting PH3-positive cells in an area of 200 μm width at the ventricular surface comprising the total area above it.

ectopically Emx1 and/or Emx2 expressing region. However, there were fewer neurons observed in this respecified region than in the normal hippocampal or cortical neuroepithelium (Fig. 4D2), suggesting that ectopic Emx induces the ectopic formation of neuroepithelium but also restricts the process of neuronal maturation due to the enhanced proliferation (see Discussion).

Proliferation could not only be drastically enhanced by misexpression in the dorsomedial region, but also increased 2.5 times in more lateral regions of the dorsal telencephalon that overexpressed Emx1 and/or Emx2 (normal situation in dorsal telencephalon: 14 PH3-positive cells/200 μm; dorsal telencephalon (except midline) transfected with Emx1 and/or Emx2 (n = 3): 35 PH3-positive cells/200 μm). At E4, when cells still expressed higher levels of the constructs, we quantified the percentage of PH3-positive cells amongst the GFP-positive cells (see Materials and methods). The transduction with the control plasmid showed 5.3% PH3-positive cells (n = 9), while electroporation of Emx1 and/or 2 enhanced proliferation to 9.3% (n = 16; P < 0.005). As expected due to the increase in proliferation, the normally continuous band of postmitotic MAP2-positive neurons in the mantel zone appeared disrupted, suggesting that the increase in proliferation elicited by Emx1 and/or Emx2 overexpression occurs at the expense of neurogenesis (data not shown). This result confirms the effect of ectopic Emx1 and/or Emx2 observed in the dorsomedial region, where these genes also enhanced proliferation and blocked differentiation. In contrast, misexpression of these constructs in the ventral telencephalon did not result in significant alterations of the proliferation rate (normal situation in the ventral telencephalon: 8%, n = 2; ventral telencephalon transfected with Emx1 and/or Emx2: 6%, n = 4), consistent with previous data, demonstrating that Emx2 expression had no effect in precursors of the ventral telencephalon in vitro (Heins et al., 2001).

Respecification of the tissue adjacent to the region of Emx1 and/or Emx2 misexpression

When we noted the loss of Otx2 expression in the Emx1 and/or Emx2 misexpressing region, we also observed its expression in an adjacent region in 6 out of 8 embryos (see examples in Figs. 2E, F). In all cases (6/6), the Otx2 expressing region next to the GFP-positive territory also coexpressed Wnt7b, in 3 of these 6 cases, BMP7 was

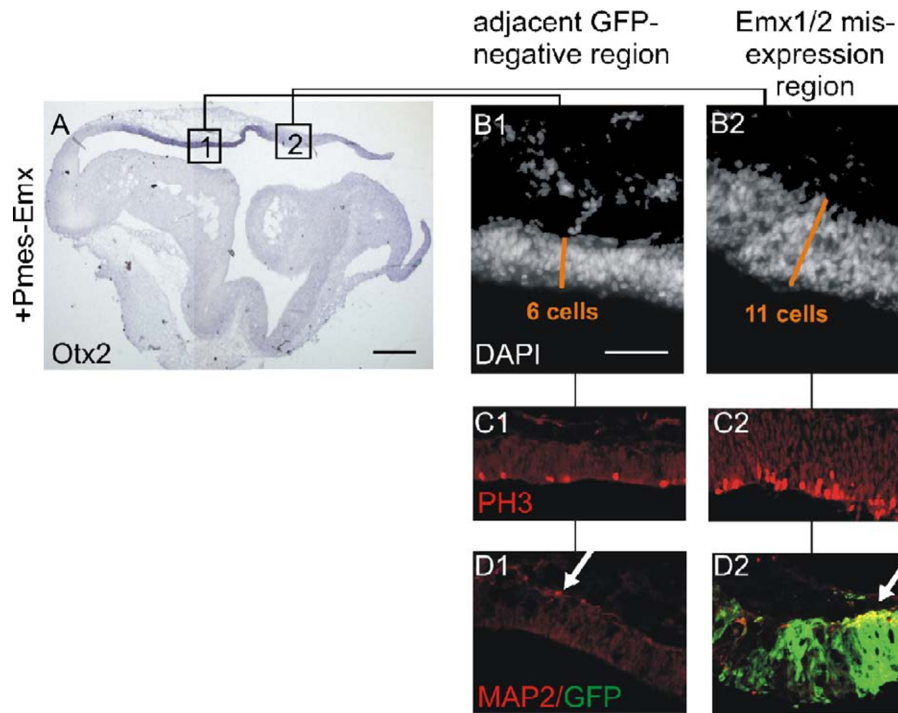


Fig. 4. Cell biological analysis of Emx1 and/or Emx2 electroporated brains at E6. The two regions that were analyzed are indicated in the frontal sections in panel A. The panels to the right (B2, C2, D2) depict region 2 in panel A, which misexpresses Emx1 and/or Emx2 (for region of misexpression see Fig. 2B). The panels to the left (B1, C1, D1) depict the laterally shifted Otx2-expressing territory (region 1 in panel A). The radial thickness of the tissue was quantified by counting the number of DAPI-positive cells in a radial line as indicated in panels B1 and B2. Panels C1 and C2 show proliferating cells labeled with an antibody against PH3. In panels D1 and D2, neurons were visualized with an antibody against MAP2 (arrows indicate neurons). Note the higher rate of proliferation and neurogenesis in the midline region with ectopic expression of Emx1 and/or Emx2 (see also Tables 1 and 2). DMR, dorsomedial region, PA, pallium; SP, subpallium. Scale bars (A): 250 μm.

expressed and in 3 of 4 cases even Ttr expression was observed in the area adjacent to the Emx transduced, GFP-positive region (see asterisk in Figs. 2Q, R). Notably, most Ttr-positive cells were detected where Bmp7 and Otx2 were coexpressed (3/4; Figs. 2E, H, Q and Figs. 2F, I, R). However, besides the expression of genes characteristic for ChP, this region also expressed genes normally detected only in the neuroepithelium, such as Gli3 (see asterisk in Fig. 2O) or Lhx2 (1/3, data not shown). In 2 of 3 cases, Lhx2 expression was reduced or absent in this region (Figs. 2S, T).

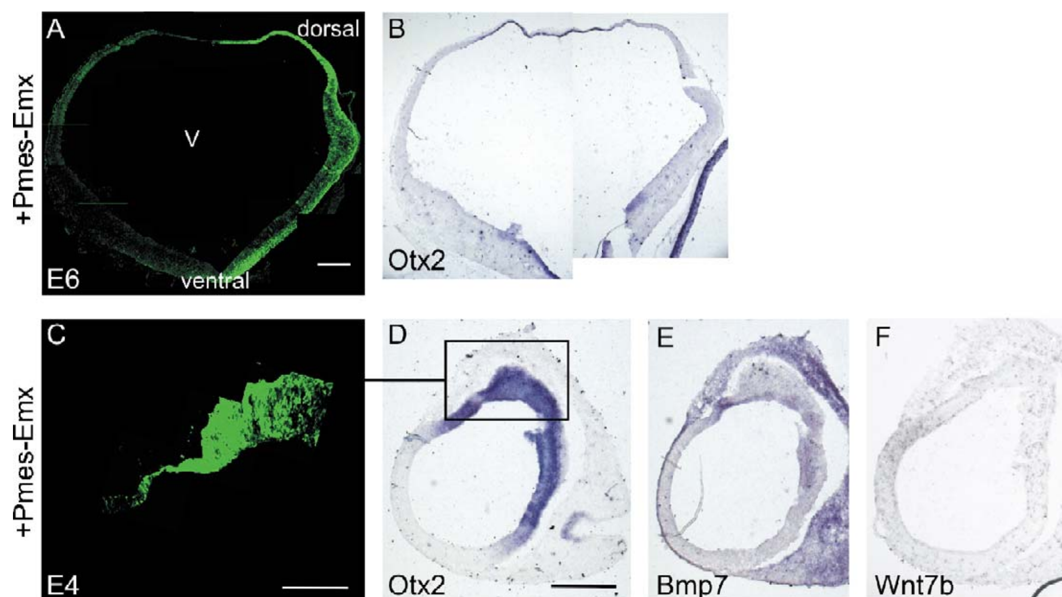


Fig. 5. Electroporation of Emx1/2 at HH 13/14 analyzed at E6 (A, B) and at E4 (C, D, E, F). Frontal sections of the telencephalon were stained for GFP (A, C) or Otx2 (B, D), Bmp7 (E) and Wnt7b (F) mRNA. Note that the expression of Otx2 and BMP7 was not affected by Emx1/2 electroporation into the midline at HH 13/14. V, ventricle; RP, roof plate; PA, pallium; SP, subpallium. Scale bars: 250 μm.

Taken together, these data show that the region adjacent to the ectopic Emx1 and/or Emx2 adopts a hybrid fate with the partial expression of ChP and neuroepithelial molecular traits.

We also examined the cell biological parameters in the area adjacent to the GFP-positive territory in order to confirm the fate changes suggested by the molecular analyses. The Otx2-positive region adjacent to the electroporate area contained a mean of 9 PH3-positive cells/200 μm (n = 5; Fig. 4C1), an obviously higher rate of proliferation than in a normal ChP region (2 PH3+ cells/200 μm; Fig. 3B), but lower than in normal neuroepithelial tissue. A very similar phenotype was observed when the thickness of this area was examined. Normally, the Otx2-positive ChP exhibits a thickness of 2 cells. In the cases were the territory adjacent to the Emx1 and/or Emx2 overexpression expressed Otx2, the mean thickness was 8 cells, a value lower than in the normal cortical neuroepithelium but exceeding the normal thickness of the Otx2-positive ChP (Fig. 4B1; Table 2). Thus, both the molecular and cell biological analysis demonstrated that the former neuroepithelial tissue adjacent to the Emx1 and/or Emx2 misexpression domain gradually lost its neuroepithelial properties and acquired several ChP features. Notably, however, in 3 out of 4 cases examined, MAP2-positive cells were still present in this region, suggesting that this region also maintains neuroepithelial features (Fig. 4D1).

Stage dependency of gene regulation

After we observed these profound changes upon Emx1 and/or Emx2 transduction, we were interested to determine the time window during which Emx genes are sufficient to instruct a neuroepithelial identity in the midline region. When Emx1 and/or Emx2 electroporation was performed after HH 12, at HH 13 or HH 14, it never (6/6) resulted in a down-regulation of Otx2 in the region of Emx1 and/or Emx2-misexpression, neither at E6 (3/3; Fig. 5A) nor at E4 (3/3; Figs. 5C, D). The telencephali analyzed at E6 developed a normal ChP identity with thin tissue (1.8 ± 0.2), Otx2 expression and a low rate of proliferation (2.5 ± 0.3) despite Emx1 and/or Emx2 transduction of this tissue. However, the medial region did not invaginate although Otx2-expression was not altered. When embryos transduced with Emx1 and/or Emx2 in the midline at HH 13–14 (n = 3) were analyzed at E4, they also showed normal Otx2 and Bmp7 expression (Figs. 5C–E). In contrast, Wnt7b was down-regulated (Fig. 5F). These data indicate that the midline region becomes irreversibly specified to express Otx2 and develop into a thin non-neuronal tissue around HH 13. Taken together, Emx1 and/or Emx2 are sufficient to direct cells towards a neuroepithelial fate by suppressing the development of non-neuronal epithelial ChP tissue during a specific time window.

Discussion

Here, we show a novel role for Emx transcription factors, namely to delineate the anlage of the ChP epithelium. While the midline region that normally develops into ChP is devoid of Emx1 and/or Emx2 expression, misexpression of either of these transcription factors between HH 9 and HH 12 in the midline region interferes with ChP differentiation and rather instructs neuroepithelial characteristics. Emx1 and/or 2 misexpression leads to down-regulation of Otx2, BMP7 and Ttr and further inhibits the acquisition of cell biological ChP characteristics, such as the low rate of proliferation and the differentiation into a thin epithelial tissue. Rather, the Emx-misexpressing midline region up-regulates genes characteristic for the dorsal neuroepithelium, such as Lhx2 or Gli3. Moreover, this region continues to proliferate and increase in thickness, comparable to the adjacent neuroepithelium and even generates neurons in few cases. Thus, both Emx1 and Emx2 transcription factors are sufficient to interfere with ChP differentiation and maintain a neuroepithelial identity, suggesting that they normally limit the ChP territory by their adjacent expression domain.

Specification of the choroid plexus in the telencephalon

The roof plate is known as a crucial signaling center (Altmann and Brivanlou, 2001; Furuta et al., 1997; Lee and Jessell, 1999; Wilson and Rubenstein, 2000), yet the specification of its later main derivative, the ChP (Currle et al., 2005), is not well understood, despite its crucial role for the function of the brain (Dziegielewska et al., 2001; Emerich et al., 2005). Some of the earliest hallmarks of ChP differentiation is an early stop of proliferation, discriminating it already at E4 in the chick telencephalon from the surrounding neuroepithelial tissue. This stage is followed by morphological changes with the invagination of the ChP anlage, which is associated with interaction with the mesenchyme and – in the mouse – tropomyosin expression (Nicholson-Flynn et al., 1996). Further differentiation is then achieved by an increased rate of apoptosis and expression of ChP-specific genes such as Ttr, an indicator of secretory epithelial differentiation (Currle et al., 2005; Furuta et al., 1997). While the later events of ChP differentiation are mediated by BMP-signaling (Hebert et al., 2002) and also require Wnt-signaling from the neighboring cortical hem (see Introduction), much less is known about the earlier events determining the region and size of the ChP anlage. Here, we showed that already at E4 (HH 24/25) when the forebrain still consists of a small vesicle the midline region is devoid of both Emx1 or Emx2 expression, while it expresses Bmp7 and Otx2 (and rostrally also FGF8). The expression of Otx2 and BMP7 and the lack of Emx1 or Emx2 transcription factors are maintained in the region that differentiates morphologically into ChP at E6. At this time, the definite ChP marker Ttr begins to be expressed. The territory that we consider the ChP anlage at E4 is flanked already at this stage by a region that coexpresses Emx1, Emx2 and Wnt7b, reminiscent of the cortical hem region, a Wnt-rich signaling center intercalated between the neuroepithelium and the ChP in the mouse telencephalon (Grove et al., 1998).

Upon misexpression of Emx1 and/or Emx2 at HH 9–12 covering the medial part of the telencephalon, the midline-specific expression of Otx2 and BMP7 was severely reduced in most cases already at E4. These changes elicited by Emx1 and/or Emx2 misexpression were more dramatic at E6, when

ChP differentiation normally became obvious at the morphological level. Invagination of the ChP tissue was abolished, proliferation still continued in this region and genes normally exclusively expressed in the adjacent neuroepithelium, such as Gli3 and Lhx2, were present throughout the Emx-misexpressing territory (Fig. 6). Moreover, the proliferation rate and tissue thickness resembled the cortical neuroepithelium (Fig. 6), consistent with a maintenance and further development along a neuroepithelial fate rather than a ChP or hem identity. Taken together, our data suggest that the transcription factors Emx1 and/or Emx2 are sufficient to suppress the acquisition of ChP identity and induce or maintain neuroepithelial fate (Fig. 6).

Emx1 and Emx2 enhance the rate of proliferation

The increase in proliferation and thickness of the original ChP anlage, which is normally low in number of proliferating cells compared to the neighboring Emx positive neuroepithelium, is caused by the effect of Emx on cell proliferation. A more general role of Emx genes in promoting cell division is supported by the reduced proliferation and lengthening of the cell cycle in the Emx2−/− cortex (Acampora et al., 1995; Boyl et al., 2001; Heins et al., 2001; Mallamaci et al., 2000a,b; Muzio et al., 2005; Simeone and Acampora, 2001), see, however, Bishop et al. (2003). The promotion of cell proliferation by Emx genes is also consistent with previous in vitro data (Heins et al., 2001), but in contrast to Emx 2 overexpression in vivo (Hamasaki et al., 2004). Interestingly, cells in the ventral telencephalon do not respond to the proliferative effects of neither Emx genes as observed in our electroporations of ventral telencephalon regions at E2, again consistent with previous in vitro data (Heins et al., 2001). This regional difference in Emx-responsiveness may also explain why adult neural stem cells that may at least in part originate in the ventral telencephalon (Merkle et al., 2004) react differently to Emx2 expression, namely by a reduction of proliferation (Galli et al., 2002; Gangemi et al., 2001).

Interesting candidates to mediate the proliferation instructed by Emx-mediated transcription in the dorsal but not ventral telencephalon may be the Wnt signaling molecules. Emx gene expression was previously shown to positively regulate Wnt expression and canonical Wnt-signaling positively regulates Emx2 expression (Backman et al., 2005; Shimogori et al., 2004; Theil et al., 2002). Moreover, Wnt signaling often acts to promote cell proliferation (Machon et al., 2003; Megason and McMahon, 2002; Yun et al., 2002). This is consistent with the scenario of Wnt signaling acting as effector of the

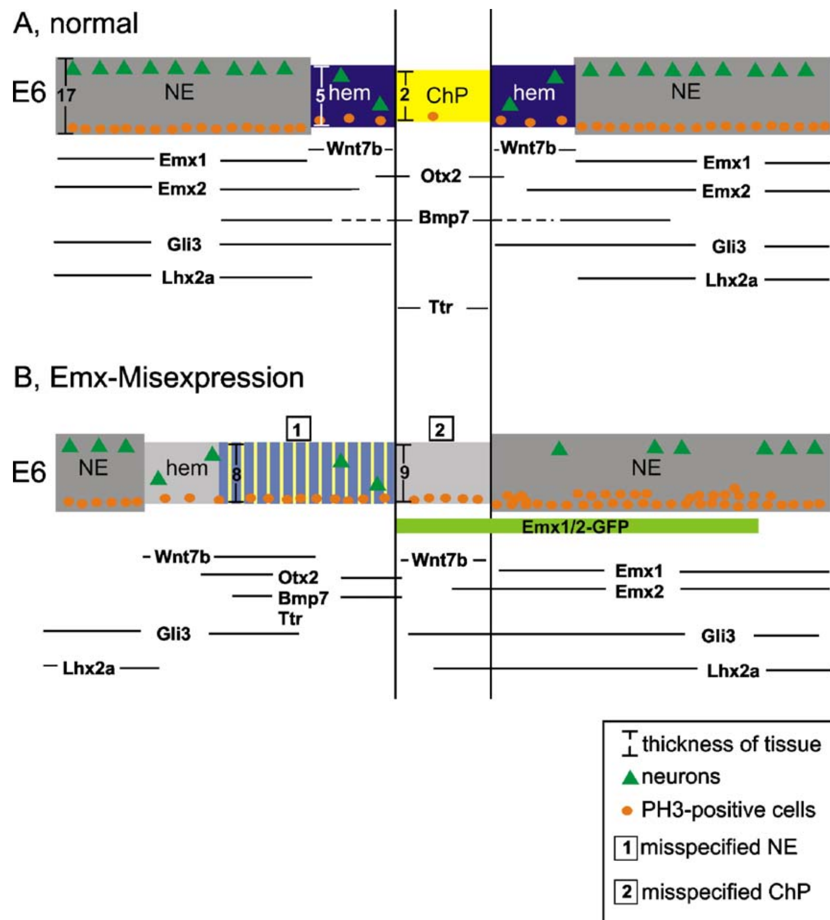


Fig. 6. Summary. The schematic drawing summarizes the cellular and molecular characteristics of the dorsal telencephalic midline regions at E6 (A) and the alterations elicited by ectopic expression of Emx1 and/or Emx2 at HH 9–12 in this region (B). For details see text.

proliferative role of Emx transcription factors. Indeed, the phenotype of the dorsal telencephalon overexpressing Emx1 and/or Emx2 with tissue folds due to over-proliferation (see Fig. 2C) resembles the phenotype of expression of constitutively active β-catenin (Chenn and Walsh, 2003). The enhanced proliferation we observed upon Emx misexpression often occurred at the expense of neuronal differentiation. Thus, compared to the wild type or control electroporated embryos, the Emx misexpressing cortices displayed only few MAP2 positive cells instead of a band in the mantle zone. This effect is most likely due to the down-regulation of the transcription factor Pax6, a key neurogenic fate determinant in the dorsal telencephalon (Hack et al., 2005; Haubst et al., 2004; Heins et al., 2001). We find that only 7% of the Emx electroporated cortical cells were Pax6-immunopositive compared to 66% of the cells electroporated with the control plasmid, suggesting that suppression of Pax6 may be a further reason why Emx-expressing cells continue to proliferate and fail to differentiate (data not shown; Heins et al., 2002; Tole et al., 2000a; Yoshida et al., 1997). Indeed, the antagonistic role of Emx2 and Pax6 is also responsible for area specification during mammalian cortex development (Bishop et al., 2002; Hamasaki et al., 2004; Mallamaci et al., 2000b; Muzio and Mallamaci, 2003), consistent with a general antagonism between these transcription factors in the developing telencephalon.

Mechanism of Emx function—cell fate changes

Given the potent effect of Emx1 and/or Emx2 on cell proliferation, it is important to consider whether the changes observed in midline development may simply be due to overproliferation of the Emx1 and/or Emx2 transduced area. Some of the genes altered in their expression may be linked to the higher proliferation of the Emx-transduced tissue, such as Wnt7b or Lhx2. However, others, such as Otx2, are already expressed in early highly proliferating tissue, at very early stages even together with Emx2 (e.g Bell et al., 2001). Thus, Otx2 down-regulation in the tissue misexpressing Emx1 and/or Emx2 in the midline region is rather linked to alteration of cell fate than the higher proliferation rate. Moreover, we think we can exclude the possibility that overproliferation of Emx1 and/or 2 transduced neuroepithelium may solely cause the loss of ChP tissue. Since we targeted successfully in all electroporations either all or part of the ChP region (Fig. 6), we should detect ChP markers, such as Otx2 or Ttr, in at least a part of the GFP-positive, transduced region. Indeed, upon electroporation with control constructs, we always observed GFP-positive cells expressing ChP markers (Bmp7, Otx2 and Ttr), while no such cells were observed within the GFP-positive region after electroporation of the Emx-IRES-GFP constructs. This strongly indicates that ChP markers were not pushed to one side of the transduced region, as expected if Emx misexpression would only elicit changes in proliferation, but were completely absent from the GFP-positive territory. Therefore, we conclude that GFP-positive tissue transduced with Emx changed its fate as well as increasing proliferation rate.

Interestingly, however, some ChP markers, such as Bmp7, Otx2 and Ttr, were observed outside and adjacent to the Emx-IRES-GFP-transduced region. Since Emx-IRES-GFP was not expressed in the entire midline region in 40% of all electroporations, the regions expressing these genes might be derived from parts of the roof plate that were not targeted by our electroporation. In these cases, the former roof plate region partially maintained its previous fate as indicated by Otx2, BMP7 and even Ttr expression. However, this region also expressed genes characteristic of the neuroepithelium, such as Gli3 and rarely also Lhx2. Thus, this area also underwent fate changes, independent of whether it is derived from midline tissue (in which case it up-regulated neuroepithelial markers) or from neuroepithelial tissue (in which case it up-regulated ChP markers, as depicted in Fig. 6). As we did not yet observe Otx2 expression next to the GFP-positive area at E4, this rather suggests a neuroepithelial origin of the hybrid area next to the Emx1 and/or Emx2 misexpressing region. In any case, these fate changes in the area adjacent to the GFP-positive, electroporated region are particularly remarkable, as they are obviously not elicited directly by Emx transduction.

A possible mechanism that could explain how neuroepithelial tissue adjacent to the Emx misexpressing midline acquires partial ChP identity, may be the relative expression levels of Emx genes for positioning the ChP. Different Emx expression levels are usually just adjacent to the ChP anlage in the cortical hem (Figs. 1E, F; Supplementary Fig. 3). Upon electroporation in the midline, the highest Emx1 and/or Emx2 expression levels are in the midline. This high level of Emx expression may then shift Otx2 expression eventually (not yet at E4) to the adjacent region with relatively lower endogenous Emx expression levels. However, the shift of Otx2, Bmp7 and even some Ttr expression is obviously not sufficient to respecify a former neuroepithelial tissue into a full ChP fate, given the maintenance of Gli3 expression, a rate of proliferation similar to the cortex and also the presence of some MAP2 positive neurons. Nevertheless, the Otx2 expressing region was clearly thinner than the normal neuroepithelium at this stage, possibly due to the BMP expression inducing higher levels of apoptosis (Hebert et al., 2002; Panchision et al., 2001). These results suggest that high levels of Emx transcription factors at ectopic position in the midline region are not only sufficient to affect cell fate within the region of Emx misexpression but also in the adjacent region that acquires an identity hybrid between ChP and neuroepithelium.

A further important factor to consider is time or rather the developmental stages when alterations occur. For example, the shift in Otx2 and BMP7 expression next to the Emx transduced region was not yet apparent at E4, suggesting that it may have occurred too late to instruct complete fate changes. In contrast, the loss of Otx2 expression within the midline region misexpressing Emx1 and/or Emx2 was already apparent at E4. However, also the fate change of the midline region was stage-dependent and most changes could not be elicited any more when Emx1 and/or Emx2 were electroporated after HH 12. While Wnt7b expression could still be modulated, Otx2 and Bmp7 expression was not changed upon Emx1 and/or Emx2

electroporation after HH 12. These data further suggest that down-regulation of Otx2 and Bmp7 is a key mechanism by which Emx1 and/or Emx2 misexpression interferes with ChP specification and differentiation.

Molecular mechanisms of midline respecification

The above considerations raise the issue of the molecular mediators of the potent respecification achieved by Emx misexpression in the midline. Suppression of BMP expression is certainly an important step in this process as it was previously shown to be essential for differentiation of ChP tissue (Hebert et al., 2002). Moreover, the transcription factor Otx2 that is expressed specifically in the differentiating ChP tissue, while it is absent from the surrounding neuroepithelial tissue in the developing telencephalon of mice and chick (Kimura et al., 2005), may be key to regulate other ChP properties that fail to be initiated by suppression of Otx2 in the Emx-misexpressing region. Our results further show that Ttr expression, which indicates a differentiated ChP fate, develops only in Bmp7- and Otx2-expressing regions. These data suggest that an interplay of both genes is necessary for ChP development. Thus, the failure of ChP development upon Emx1 and/or Emx2 misexpression is due to the interruption of either of these key factors for ChP differentiation. In contrast, Wnt7b was still expressed at E6 adjacent to or within the Emx1 and/or Emx2 transduced region, suggesting that the absence of Wnt expression cannot be the reason for the failure of ChP differentiation as is the case in the Emx1/Emx2−/−, Gli3−/− and Lhx5−/− mice (Grove et al., 1998; Shinozaki et al., 2004; Zhao et al., 1999).

The down-regulation of Otx2 and Bmp7 allows the ectopic expression of neuroepithelial genes like Lhx2 and Gli3 that may well contribute to further suppress ChP development. Lhx2 has been suggested as direct repressor of hem and ChP, since in the Lhx2 −/− mutant both structures are enlarged (Bulchand et al., 2001; Monuki et al., 2001). As Lhx2 is positioned adjacent to the hem within the neuroepithelium, it seems to restrict the expansion of the hem territory, consistent with the enlargement of the hem in the Lhx2−/− mice (Bulchand et al., 2001; Monuki et al., 2001). An enlarged hem is always associated with an enlarged ChP territory, supposedly due to the increase in Wnt signaling (see also introduction). From these data, we would conclude that Lhx2 acts rather indirectly on the ChP territory—via positioning the hem. However, the border between hem and neuroepithelium is still present in the Lhx2−/− telencephalon (Bulchand et al., 2001; Monuki et al., 2001). Thus, the respective proliferation of the hem region versus neuroepithelial tissue may be affected in the absence of Lhx2, rather than patterning itself. In accordance with this suggestion, over-expression experiments in zebrafish suggest that Lhx2 regulates cellular proliferation in the developing forebrain without major influences on patterning (Ando et al., 2005).

As Emx1, Emx2 and Lhx2 are expressed in the dorsal neuroepithelium, this raises the question of whether these transcription factors regulate each other. In Emx1 and/or Emx2 mutants Lhx2 is still expressed (Shinozaki et al., 2004), suggesting that Lhx2 expression does not require Emx-mediated transcription. Nevertheless, both the hem and the ChP are missing in the Emx1/2-double mutant mice (Shinozaki et al., 2004), suggesting that Emx1 and Emx2 are necessary for the formation of these regions earlier in development. Consistent with this, ectopic expression of Emx1 and/or Emx2 was sufficient to induce ectopic Lhx2 expression in the former midline region. Thus, Emx1 and Emx2 play a potent role to position the neuroepithelial territory and are sufficient to instruct LHX-expression -directly or indirectly.

Thus, on the one hand, Emx gene function is required for the formation of the cortical hem and thereby necessary for ChP development that fails in the telencephalon of Emx1 and/or Emx2 double mutant mice and is impaired in Emx2−/− mice (Shinozaki et al., 2004; Tole et al., 2000b; Yoshida et al., 1997). On the other hand, the absence of Emx gene expression in the medial part of the telencephalic roof is also an essential prerequisite for ChP differentiation. Taken together, Emx transcription factors delineate the ChP territory by their own expression and simultaneously instruct an important signaling center at this border, the Wnt-rich cortical hem, that is also required for ChP differentiation. These data therefore show that transcription factors of the Emx family act on various levels to promote cell proliferation and instruct or maintain neuroepithelial cell fate. They are therefore crucial players the in development of telencephalic midline structures.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.04.461.

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