Sp8 and COUP-TF1 Reciprocally Regulate Patterning and Fgf Signaling in Cortical Progenitors

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To gain new insights into the transcriptional regulation of cortical development, we examined the role of the transcription factor Sp8, which is downstream of Fgf8 signaling and known to promote rostral cortical development. We have used a binary transgenic system to express Sp8 throughout the mouse telencephalon in a temporally restricted manner. Our results show that misexpression of Sp8 throughout the telencephalon, at early but not late embryonic stages, results in cortical hypoplasia, which is accompanied by increased cell death, reduced proliferation, and precocious neuronal differentiation. Misexpression of Sp8 at early developmental stages represses COUP-TF1 expression, a negative effector of Fgf signaling and a key promoter of posterior cortical identity, while ablation of Sp8 has the opposite effect. In addition, transgenic misexpression of COUP-TF1 resulted in downregulation of Sp8, indicating a reciprocal cross-regulation between these 2 transcription factors. Although Sp8 has been suggested to induce and/or maintain Fgf8 expression in the embryonic telencephalon, neither Fgf8 nor Fgf15 was upregulated using our gain-of-function approach. However, misexpression of Sp8 greatly increased the expression of Fgf target molecules, suggesting enhanced Fgf signaling. Thus, we propose that Sp8 promotes rostral and dorsomedial cortical development by repressing COUP-TF1 and promoting Fgf signaling in pallial progenitors.

Keywords: corticogenesis, Fgf signaling, neurogenesis, patterning, proliferation, Sp8

Introduction

The complexity of functions characteristic of the vertebrate cerebral cortex are a consequence of the formation of specialized cortical areas, which are established during development. Secreted molecules expressed at patterning centers establish a graded expression of transcription factors, which initiate the formation of these functional areas of the cerebral cortex (Grove and Fukuchi-Shimogori 2003; O’Leary et al. 2007). Fgf8 and 17 signaling promote the formation of rostral cortical regions (Fukuchi-Shimogori and Grove 2001; Chalfin and Rubenstein 2007). Downstream of Fgf signaling, rostral versus caudal cortical identity is regulated by the transcription factors Pax6 and Emx2, respectively (Bishop et al. 2000, 2002; Hamasaki et al. 2004).

The COUP-TF1 (Nr2f1) transcription factor is enriched in caudalventral portions of the developing cerebral cortex (Liu et al. 2000). Conditional COUP-TF1 knockouts are known to have reduced caudal cortical areas and a concomitant expansion of rostral areas (Armentano et al. 2007). Additionally, COUP-TF1 overexpression under the D6 promoter results in reduced levels of Pax6 along the dorsovenal axis (Faedo et al. 2008). There is evidence that COUP-TF1 regulates cortical patterning by repressing MAPK/ERK signaling, which is likely downstream of Fgf signaling (Faedo et al. 2008).

The zinc finger transcription factor Sp8 is expressed in a complementary pattern to COUP-TF1 with high levels in the rostromedial cortical regions (Sahara et al. 2007). Accordingly, loss-of-function studies have indicated that Sp8 is required for the specification of rostral cortical identity (Sahara et al. 2007; Zembrzycki et al. 2007). Sp8 appears to be induced by Fgf signaling and has been suggested to subsequently maintain Fgf8 expression within the rostral cortical signaling center (Sahara et al. 2007). Thus, it remains unclear whether Sp8 plays a direct role in the specification of rostral cortical identity or if its role is indirect, though the regulation of Fgf expression. If it is direct, the complementary expression of Sp8 and COUP-TF1 might indicate a cross-repressive mechanism intrinsic to the cortical progenitors.

To gain further insights into the molecular mechanisms underlying the patterning and growth of the cerebral cortex, we focused our studies on Sp8. Using a binary transgenic approach to misexpress Sp8 temporally throughout the telencephalon, we examined the role of this transcription factor in rostrocaudal and dorsoventral patterning of the developing cortex. Moreover, our gain-of-function approach allowed us to examine the role of Sp8 in the proliferation, survival, and differentiation of telencephalic progenitors and, importantly, its relationship to Fgf signaling, allowing us to identify a novel molecular mechanism that couples patterning and growth of the developing cortex.

Materials and Methods

Animals

To generate the tetO-Sp8-ires-EGFP (J2) mice a 1.3-kb cDNA PCR fragment, containing the entire coding sequence of Sp8, was cloned into the Bam HI site of the ptetO-IRES-EGFP vector using compatible Bgl II ends that were designed into the primers used to produce the Sp8 amiplicon. The 3.7-kb TetO-Sp8-IRES-EGFP fragment was released from the construct with Abhd and Ascl and gel purified. Pronuclear injections were done at the transgenic core at Cincinnati...
Children’s Hospital Medical Center (CCHMC) and produced 3 founder lines. Two of these founder lines robustly expressed Sp8 and EGFP throughout the developing telencephalon when crossed to Foxg1fl/fl mice (Hanashima et al. 2002). The third founder also expressed Sp8 and EGFP but not as robustly as the other 2 lines. For experiments in this article, we have used the lines that showed strongest and most consistent expression of the Sp8 and EGFP. We used the Foxg1fl/fl and the tetO- Sp8-IE single transgenic embryos as controls because they did not show any obvious patternning defects. Moreover, these 2 single transgenic lines were no different from wild-type embryos. To repress transgene expression in Foxg1fl/fl, tetO- Sp8-IE embryos, doxycycline hyclate (Dox; Sigma) was administered to the pregnant mouse, at 0.02 mg/ml in the drinking water, between embryonic day E8–E11. This represents a slight modification from our previous studies in which Dox was added from E7 to E9, and transgene expression was delayed until approximately E13 (Waclaw et al. 2009; Pei et al. 2011).

D6/COUP-TFI mice were maintained and genotyped as described (Faedo et al. 2008). Foxg1fl/fl mice were genotyped as described (Waclaw et al. 2009). Foxg1fl/fl (Hébert and McConnell 2000), EnlxIII (Gorski et al. 2002), and Sp8fl/fl mice (Waclaw et al. 2006) were maintained and genotyped as described (Waclaw et al., 2006, 2009, 2010). To account for the observed haploinsufficiency of the Foxg1fl/fl line (Eagleson et al. 2007; Shen et al. 2006; Storm et al. 2006; Siegenthaler et al. 2006), we used Foxg1fl/fl, Sp8fl/fl mice as a control for Foxg1fl/fl, Sp8fl/fl embryos. Mouse colonies were maintained at the University of California, San Francisco (UCSF) or CCHMC, in accordance with National Institutes of Health, CCHMC, and UCSF guidelines.

**Histology**

Pregnant females were euthanized with CO2 followed by cervical dislocation. Noon on the day of the vaginal plug was considered as E0.5. The embryos were dissected and fixed overnight by immersion in a 4% PFA in phosphate-buffered saline (PBS) at 4°C. The tissue was cryoprotected by immersion in 30% sucrose/PBS, embedded in OCT (Tissue-Tek), and cryostat sectioned (10–20 μm).

In situ RNA hybridization on cryostat sections was performed as previously described (Borello et al. 2008). cRNA Probes (sources in parentheses) against COUP-TFI (M-J Tsai), Enlx2 (A. Simeone), Erm (A. Chotteau-Lelievre), Fgf8 (G. Martin), Flg15 (J.R. McWhirter), Lhx2 (S. Rétaux), Mst (R. Livesey) Pax6 (P. Gruss), Sp8 (S. Bell), and Spry2 (G. Martin).

**COUP-TFI** in situ hybridization signal (Supplementary Fig. S5) was quantified as described by Faedo et al. (2008).

Immunofluorescence on cryostat sections was performed as previously described (Borello et al. 2008; Waclaw et al. 2009). The antibodies used were as follows: mouse anti-JHII-tubulin (1:1000, Covance); rabbit anti-Foxg1 (NCAB) (1:500, NeuroCell), rabbit anti-phospho-Histone H3 (1:500, Millipore), goat anti-Sp8 (1:7000, Santa Cruz), mouse anti-COUPTFI (1:5000, R&D Systems), rabbit anti-GFP-488 (1:500, Invitrogen), rabbit anti-Pax6 (1:1000, Covance), rabbit anti-pErik1/2 (1:500, Cell Signaling), and rabbit anti-Cleaved Caspase-3 (Asp175) (1:200, Cell Signaling). For the pErik1/2 and COUP-TFI staining, an antigen retrieval step with citrate buffer was added at the beginning of our immunostaining protocol. The secondary antibodies for fluorescent staining were as follows: donkey anti-goat antibodies conjugated to Cy2, Cy3, or Cy5 (Jackson Immunoresearch), donkey anti-mouse antibodies conjugated to Cy3 or Cy5 (Jackson Immunoresearch), and donkey anti-rabbit antibodies conjugated to Cy3 (Jackson Immunoresearch) or AlexaFluor 488 (Invitrogen) were used at a dilution of 1:200.

Comparisons of gene or protein expression patterns between brains of different genotypes were performed by matching the planes of sections, using multiple anatomical features. Whenever possible, this was performed for multiple planes of section for each gene, and from at least 3 brains for each genotype.

Cortical thickness of E12.5 embryos was measured using ImageJ software. Thickness of the ventrolateral and dorsomedial pallium were measured in double transgenic and control embryos as shown in Fig. 2C,D. Measurements were made from both hemispheres of matching midlevel sections from at least 3 animals of each genotype. Student’s t-test was performed to determine statistical significance.

Cell counts for apoptosis and cell proliferation were performed by counting the total number of positive cells from designated levels (rostal areas were designated as those that only had the septum and lateral ganglionic eminence, midlevel sections were designated as those that had the lateral and medial ganglionic eminence or caudal levels were defined as those that had the caudal ganglionic eminence and within designated areas in the section—subpallium and lateral pallium). Caspase-3- or pH3-positive cells were counted separately from 2 hemispheres of 3 animals each for each genotype (tetO- Sp8-IE or Foxg1fl/fl, tetO- Sp8-IE). Student’s t-test was performed to assess statistical significance.

**Results**

**Reciprocal Expression of Sp8 and COUP-TFI in the Pallium**

Toward understanding the role of Sp8 in the early phases of mouse cortical development, we have analyzed its expression along the rostrocaudal and dorsoventral axes between E9.5 and E12.5. We assessed these axes as many of the transcription factors that regulate cortical patterning are expressed in bidimensional gradients along these axes.

As shown for Sp8 RNA (Sahara et al. 2007), Sp8 protein is expressed in a gradient of high rostral to low caudal at E9.5 (Fig. 1A). Along the dorsoventral axis of the pallium, Sp8 is expressed in a high-dorsal to low-ventral gradient, which is particularly obvious at E12.5 (Fig. 1B). Sp8 expression is complementary to that of COUP-TFI on both the rostrocaudal and dorsoventral axis (Fig. 1A,B). These expression gradients are maintained until at least E14.5, when the level of pallial Sp8 expression is greatly decreased (Waclaw et al. 2006). The complementary expression patterns of Sp8 and COUP-TFI suggest cross-repressive interactions.

**Early Loss of Sp8 Function in the Nascent Cortex Leads to Increased COUP-TFI Expression in the Rostral Dorso medial Pallium**

To examine whether the complementary expression of Sp8 and COUP-TFI in pallial progenitors reflects a cross-repressive interaction, we studied COUP-TFI expression in conditional Sp8 mouse mutants. First, we inactivated Sp8 at the earliest stages of telencephalic development (e.g., E8.5) using Foxg1£££££££ mice (Hébert and McConnell 2000). COUP-TFI is normally expressed at high levels in the caudal and ventrolateral pallium (Fig. 1C,E), however, in the Foxg1£££££££;Sp8fl/fl mutants COUP-TFI expression is expanded rostrally and dorsomedially within the pallium (Fig. 1D,F), supporting the hypothesis that Sp8 represses COUP-TFI in the rostral and dorsomedial pallium. These findings are consistent with those of Zembrzycki et al. (2007) showing that dorsomedial telencephalic patterning is disrupted in Sp8 mutants.

Next, to test whether this cross-repression also functions at later telencephalic stages, we inactivated Sp8 using the EnlxIII allele (Gorski et al. 2002), which begins to delete the floxed Sp8 allele around E10.5 (data not shown). We verified that the mutant lacked dorsal telencephalic Sp8 expression at E12.5 (Supplementary Fig. S1 A,B). Interestingly, COUP-TFI expression at E12.5 in these conditional mutants (Fig. 1H) appeared normal when compared with the control (Fig. 1G). Therefore, unlike the Foxg1£££££££;Sp8fl/fl mutants, COUP-TFI expression in the dorsomedial pallium of the EnlxIII;Sp8fl/fl mutants was similar to the controls. Thus, it appears that Sp8 regulates cortical patterning.
represents COUP-TF1 pallial expression prior to E10.5. To explore this hypothesis, we made use of a doxycycline-regulated binary transgenic mouse model to spatially and temporally control Sp8 expression within the embryonic cerebral cortex (Hanashima et al. 2002; Waclaw et al. 2009).

**Misexpression of Sp8 Throughout the Developing Telencephalon**

To misexpress Sp8 in the developing telencephalon, we generated a mouse line containing a tetO-Sp8-IRES-EGFP (IE) transgene and crossed it with Foxg1TAA/- mice (Hanashima et al. 2002) to induce Sp8 and EGFP expression throughout the embryonic telencephalon (Fig. 2). To temporally repress transgene-derived Sp8 and EGFP expression during specific intervals of embryonic development, doxycycline (Dox) was administered to the pregnant mothers between E8 and E11 (Fig. 2A,B).

Transgene (i.e., Sp8 and EGFP) expression was detected in the pallium of Foxg1TAA/-,tetO-Sp8-IE embryos as early as E10 (Supplementary Fig. S1C,C); between E9.5 and E10.5, transgene expression was observed only in the basal ganglia (data not shown). We carried out the phenotypic analysis in double transgenic embryos at E11.5, E12.5, E14.5, E16.5, and E18.5 (see experimental design in Fig. 2B). The double transgenic embryos showed a uniform expression of Sp8 and EGFP along the dorsoventral axis, excluding the cortical hem (Fig. 2D,E). This expression was distinct from the tetO-Sp8-IE or Foxg1TAA/- controls, which showed Sp8 in the dorsomedial pallium and dlGE (Fig. 2C and data not shown). Additionally, transgene expression along the rostrocaudal axis of the cortex in double transgenic embryos appeared uniform (Fig. 2G, inset Fig. 2E, and data not shown). Using this system, we found that Sp8 is overexpressed by about 46-fold over that observed in the control cortex (data not shown). Misexpression of Sp8 throughout the telencephalon beginning E9.5 reduced telencephalic surface area and thickness, particularly affecting the subpallium (Fig. 2D,E,G), as compared to the controls (Fig. 2C,F and data not shown). Quantification of the ventrolateral and dorsomedial pallial thickness at E12.5 showed that ventrolateral pallial thickness was reduced by ∼20% (P < 0.05; n = 3) in the double transgenic (cyan dashed lines in Fig. 2D) compared with similar regions of control animals (cyan dashed lines in Fig. 2C). On the other hand, no significant change was detected in dorsomedial pallial thickness (yellow dashed lines in 2C,D).

**Misexpression of Sp8 Alters Dorsoventral Patterning in the Pallium**

Although the highest levels of Sp8 and COUP-TF1 in the pallium reside at the rostral and caudal extremes, at midtelenecphalic levels, these 2 factors are enriched in the dorsal and ventral portions of the pallium, respectively (e.g., Fig. 1B). To gain insights into the role of Sp8 in dorsoventral patterning of the pallium, we examined the expression of genes that mark defined progenitor zones within the dorsal and ventral pallium.

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To misexpress Sp8 throughout the telencephalon, Foxg1\(^{TA/+}\) males were bred to tetO-Sp8-IE females to yield Foxg1\(^{TA/+}\); tetO-Sp8-IE progeny (A). Foxg1\(^{TA/+}\); tetO-Sp8-IE animals overexpress Sp8 and EGFP starting at E9.5, and this expression can be delayed by 5 days by administering doxycycline (Dox) to the mother between Days E8 and E11 (B). Foxg1\(^{TA/+}\); tetO-Sp8-IE animals express the transgenes uniformly through the rostrocaudal extent of the telencephalon. Inset in E, shows EGFP expression in a sagittal section of an E11.5 animal, where the olfactory epithelium (oe) marks the rostral portion of the animals head and the diencephalon (di) indicates the caudal regions. Double transgenic animals also show uniform overexpression of Sp8 and EGFP along the dorsoventral (D/V) axis of the telencephalon (D and E, respectively, arrow marks the D/V border) as compared to tetO-Sp8-IE controls (C) that only express Sp8 in the medial pallium (MP) and the subventricular zone (arrow) of the LGE. Misexpression of Sp8 leads to reduction in ventrolateral pallial thickness at E12.5 (cyan line in D) compared with control animals (cyan line in C); the thickness of the dorso medial pallium (yellow lines in C and D) shows no significant changes. This phenotype is amplified by E18.5 resulting in a severe hypoplasia of the cortex (Ctx) in Foxg1\(^{TA/+}\); tetO-Sp8-IE animals (G) as compared to control tetO-Sp8-IE animals (F). Insets show Nissl-stained cross sections of the brains in Foxg1\(^{TA/+}\); tetO-Sp8-IE (G) and control tetO-Sp8-IE animals (F) at the level indicated by the white line. The cortex of double transgenic animals (G) has large ventricles, which lack a clear laminar organization compared with the brains of control animals (F). LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MP, medial pallium; Ctx, cortex; Mb, midbrain; oe, olfactory epithelium; di, diencephalon.

Figure 2. To misexpress Sp8 throughout the telencephalon, Foxg1\(^{TA/+}\) males were bred to tetO-Sp8-IE females to yield Foxg1\(^{TA/+}\); tetO-Sp8-IE progeny (A). Foxg1\(^{TA/+}\); tetO-Sp8-IE animals overexpress Sp8 and EGFP starting at E9.5, and this expression can be delayed by 5 days by administering doxycycline (Dox) to the mother between Days E8 and E11 (B). Foxg1\(^{TA/+}\); tetO-Sp8-IE animals express the transgenes uniformly through the rostrocaudal extent of the telencephalon. Inset in E, shows EGFP expression in a sagittal section of an E11.5 animal, where the olfactory epithelium (oe) marks the rostral portion of the animals head and the diencephalon (di) indicates the caudal regions. Double transgenic animals also show uniform overexpression of Sp8 and EGFP along the dorsoventral (D/V) axis of the telencephalon (D and E, respectively, arrow marks the D/V border) as compared to tetO-Sp8-IE controls (C) that only express Sp8 in the medial pallium (MP) and the subventricular zone (arrow) of the LGE. Misexpression of Sp8 leads to reduction in ventrolateral pallial thickness at E12.5 (cyan line in D) compared with control animals (cyan line in C); the thickness of the dorso medial pallium (yellow lines in C and D) shows no significant changes. This phenotype is amplified by E18.5 resulting in a severe hypoplasia of the cortex (Ctx) in Foxg1\(^{TA/+}\); tetO-Sp8-IE animals (G) as compared to control tetO-Sp8-IE animals (F). Insets show Nissl-stained cross sections of the brains in Foxg1\(^{TA/+}\); tetO-Sp8-IE (G) and control tetO-Sp8-IE animals (F) at the level indicated by the white line. The cortex of double transgenic animals (G) has large ventricles, which lack a clear laminar organization compared with the brains of control animals (F). LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MP, medial pallium; Ctx, cortex; Mb, midbrain; oe, olfactory epithelium; di, diencephalon.
We first analyzed the expression of Foxg1 to examine whether cortical progenitors in the double transgenic embryos maintain telencephalic fate. Foxg1 protein expression persisted in the double transgenic embryos (Fig. 3B), albeit at a reduced level than in the controls (Fig. 3A), demonstrating that this feature of telencephalic fate was not significantly modified by the misexpression of Sp8.

To analyze dorsoventral patterning of the pallium, we looked at the expression of Coup-TF1 and Pax6, markers that are expressed highly in the ventral portion the pallium (Fig. 3C, E), unlike Sp8 (Fig. 1B). The earliest observed change in gene expression was the loss of Coup-TF1 expression in the ventrolateral pallium at E11.5 (Fig. 3D). Coup-TF1 is normally expressed in both progenitors and ventrolaterally.
derived neurons. Thus, misexpression of Sp8 in the ventrolateral portions of the pallium appeared to alter the identity of both progenitors and newborn neurons of this region. While Pax6 expression was still present in the double transgenic pallium at E11.5 (data not shown), it was severely reduced by E12.5 (Fig. 3F). By contrast, expression of Emx2, a marker that is expressed highly in the dorsomedial cortical neuroepithelium, was maintained; its levels appeared to be slightly increased in ventrolateral pallium (cf. Fig. 3G and 3H). Unlike Emx2, Lhx2 (which shows a similar dorsoventral gradient of pallial expression; Fig. 3J), was severely downregulated in the dorsomedial region of embryos misexpressing Sp8 (Fig. 3J). To analyze the properties of the dorsomedial pallium in the double transgenic embryos, we studied the expression of Wnt8b, a marker of the medial pallium and cortical hem. Wnt8b expression in this portion of the pallium appeared normal (cf. Fig. 3K and 3L), suggesting that some aspects of the molecular identity of these dorsal-most pallial regions remained intact in the embryos misexpressing Sp8. However, the repression of Lhx2 in the dorsomedial pallium provides evidence that Sp8 has specific effects in that cortical domain.

Thus, telencephalic misexpression of Sp8 induces molecular patterning defects in the pallium. Initially it leads to a downregulation of COUP-TF1 followed by other alterations in pallial gene expression, which disrupts ventrolateral pallium patterning. These changes in pallial gene expression were similar when analyzed at different positions along the rostrocaudal axis (data not shown), likely reflecting the fact that Foxg1Tα expression is fairly uniform along this dimension.

Although telencephalic size appeared normal at E11.5 in double transgenic embryos (data not shown), by E12.5 it was reduced (data not shown and Fig. 2), suggesting that COUP-TF1 downregulation was not a secondary effect of the reduced cortical surface, and that by this stage the observed molecular patterning changes reduce progenitor maintenance and/or expansion. To test this idea, we performed in situ hybridization for Hes5 at E12.5, to study expression of this marker of neuroepithelial progenitors and Notch signaling. Importantly, Hes5 expression was reduced in the double transgenic embryos (Fig. 3N) as compared to controls (Fig. 3M), providing evidence that Sp8 overexpression caused telencephalic hypoplasia by repressing neuroepithelial properties. Below, we show that the hypoplasia was also mediated by premature neurogenesis, reduced proliferation, and increased apoptosis. These data together demonstrate that Sp8 misexpression throughout the dorsal telencephalon suppresses the molecular identity of the ventrolateral pallium, while maintaining aspects of dorsomedial pallial identity.

Pax6 is required for dorsoventral patterning in the telencephalon, where it has a critical role in the ventrolateral pallium (Stoykova et al. 2000; Toresson et al. 2000; Yun et al. 2001). The loss of Pax6 results in the dorsal expansion of subpallial markers such as Gsx2 and Dlx proteins. However, this is not the case when Sp8 is overexpressed in the double transgenic embryos, as expression of Gsx2 and Dlx were severely reduced (Fig. 3P and data not shown), as compared to controls (Fig. 3O and data not shown), even though Pax6 expression was suppressed (Fig. 3E,F). Despite the loss of ventral telencephalic markers, the molecular identity of the ventral portion of the double transgenic telencephalon does not appear to fully convert to pallial fates, because the pallial marker Tbr1 does not extend into the ventral-most regions (Supplementary Fig. S2).

**Effects of Delayed Misexpression of Sp8**

As mentioned earlier, Sp8 is only expressed within pallial progenitors until around E15 (Waclaw et al. 2006). Thus, we wondered if the phenotype described above would be evident in pallial progenitors misexpressing Sp8 after E15. To test this, we delayed the overexpression of Sp8 by administering Dox to the pregnant mice between E8 and E11 (Fig. 2B). This Dox treatment schedule delayed transgene activation until approximately E15 (data not shown). In contrast to the non-Dox-treated Sp8 misexpressing embryos, the morphology of the Dox-treated double transgenic embryos appeared similar to controls (cf. Fig. 4A and 4B). Moreover, COUP-TF1 expression in the Dox-treated double transgenic embryos (Fig. 4D) appeared unchanged, similar to that in the control (Fig. 4C). These results indicate that delaying misexpression of Sp8 until E15 had no obvious effects on cortical development and supports an early role for Sp8 between E8 and E15 in dorsomedial pallial development.

**Effect of COUP-TF1 Misexpression on Sp8 Expression**

Our findings show that Sp8 negatively regulates the expression of the COUP-TF1 and Pax6 transcription factors that are expressed in ventrodorsal gradients of the pallium. Given that Sp8 and COUP-TF1 exhibit reciprocal pallial expression patterns (Fig. 1A,B), we wondered if COUP-TF1 might also function as a negative regulator of pallial Sp8.
Misexpression of Sp8 Alters Survival, Proliferation and Differentiation of Cortical Progenitors

To understand the cellular mechanisms underlying the hypoplasia, resulting from Sp8 gain of function, we examined apoptosis, differentiation, and cell proliferation in the double transgenic at E11.5 and E14.5. Sp8 misexpression increased activated Caspase3 staining in both the lateral pallium and the subpallium of double transgenic embryos at E11.5 (Fig. 6B,R). There were no significant differences in the rostral to caudal distribution of these apoptotic cells (Fig. 6Q). By E14.5, activated Caspase3 staining was greatly increased in region of the ventral telencephalon; increased staining was also apparent in the lateral pallium of embryos misexpressing Sp8 as compared to control animals (cf. Fig. 6C and 6D). Thus, misexpression of Sp8 throughout the telencephalon increased apoptosis, which at later stages is particularly enhanced in the ventral telencephalon. This increase in apoptosis was, however, not due to a general cytotoxic effect of overexpressing Sp8 in telencephalic progenitors. When Sp8 overexpression in double transgenic embryos was delayed until E15 by Dox treatment between E8 and E11, there was no increase in cell death observed either at E16.5 or at E18.5 (i.e., 3–4 days of overexpression) when compared with control embryos (Fig. 6E,F,G,H respectively), despite robust transgene expression (see insets in Fig. 6F,H). Thus, the increased cell death after misexpression of Sp8 is specific to early stages (i.e., before E15).

In addition to apoptosis, Sp8 misexpression promoted precocious neuronal differentiation. There was a robust increase in βIII-tubulin expression throughout the neuroepithelial wall as early as E11.5 in the double transgenic embryos (Fig. 6F). At E14.5, expression of βIII-tubulin is evident within the ventricular zone region of the lateral pallium (arrows in Fig. 6L), whereas in controls staining is excluded from the ventricular zone, and is restricted to the mantle zone (Fig. 6K). Thus, misexpression of Sp8 leads to precocious neuronal differentiation of ventricular zone progenitors, particularly within the ventrolateral portion of the pallium. In conjunction, we observed a disruption of apical–basal polarity of ventricular zone progenitors within this portion of the pallium, based on β-catenin staining in the double transgenic embryos (data not shown).

Finally, we examined cell division by staining with phospho-Histone 3 (pH3), which marks cells in M-phase. Misexpression of Sp8 led to a reduction in pH3* cells in the double transgenic telencephalon already at E11.5 (Fig. 6N) as compared to control animals (Fig. 6M). Along the rostrocaudal axis, proliferation was most significantly decreased in the mid and caudal levels, with reductions of 70% and 40%, respectively (Fig. 6S). Similarly, along the dorsoventral axis, proliferation was decreased by 63% in the subpallium and 42% in the dorsal pallium (Fig. 6T). The reduction in M-phase cells became more severe over time. By E14.5, the lateral portion of telencephalon in embryos misexpressing Sp8 was nearly devoid of pH3-expressing cells (Fig. 6P). Taken together, these results indicate that the misexpression of Sp8 leads to increased cell death, precocious neurogenesis, and reduced proliferation of telencephalic progenitors. These results are consistent with the reduction in Hes5 expression in double transgenic animals (see Fig. 3M,N), suggesting that reduced Notch signaling may contribute to the reduced proliferation and precocious neuronal differentiation.
Figure 6. Misexpression of Sp8 alters cell death, proliferation, and differentiation of telencephalic progenitors. Cell death is strongly increased in the ventrolateral telencephalon of Foxg1\textsuperscript{TA/+}:tetO-\textit{Sp8}-IE animals (B) compared with tetO-\textit{Sp8}-IE controls (A) as early as E11.5. These apoptotic cells are concentrated ventrally in the telencephalon (in the subpallium and ventrolateral pallium) (R); on the other hand, they appear to be evenly distributed along the rostrocaudal axis (Q). Cell death in these regions is further increased by E14.5 (C and D). Green arrows in B and D indicate the approximate position of the pallial/subpallial boundary; the white arrows indicate the ventrolateral pallium. However, no increase in apoptosis is observed in E16.5 or E18.5 Dox-treated animals, in which \textit{Sp8} misexpression is delayed until E15 (cf. E with F and G with H). Insets in F and H show EGFP and Sp8 overexpression throughout the telencephalon at E16.5 and E18, after Dox treatment between E8 and E11. Another early effect of Sp8 overexpression is an increase in neuronal differentiation, as marked by βIII-tubulin. As early as E11.5, βIII-tubulin is increased in Foxg1\textsuperscript{TA/+}:tetO-\textit{Sp8}-IE animals (J) compared with control tetO-\textit{Sp8}-IE animals (I). βIII-tubulin immunoreactivity becomes even more pronounced by E14.5 (K and L) when the ventricular zone in lateral regions (arrows in L) of Foxg1\textsuperscript{TA/+}:tetO-\textit{Sp8}-IE cortex shows precocious neuronal differentiation compared with tetO-\textit{Sp8}-IE control (K). Cell proliferation, as indicated by phospho-Histone 3 (pH3), can be seen along the ventricular surface of control brains (M and N and O and P, E11.5 and E14.5, respectively) but is severely reduced in the lateral pallium (arrows in N and P) and subpallium of Foxg1\textsuperscript{TA/+}:tetO-\textit{Sp8}-IE animals. The effects of Sp8 misexpression on cell proliferation are more pronounced at E14.5 (O and P) than at E11.5 (M and N). Proliferation is strongly reduced at mid and caudal levels of the telencephalon as shown in S; the reduction is not limited to either the lateral or ventral part of telencephalon (T). LGE, lateral ganglionic eminence. *P < 0.01.
Expression of Fgfs and Downstream Effectors in Embryos Misexpressing Sp8

A previous gain-of-function study using Sp8 electroporation (Sahara et al. 2007) suggested that Sp8 is sufficient to induce and/or maintain Fgf8 expression within the telencephalon. However, Sp8 loss-of-function studies show that, although reduced, Fgf8 remains expressed in the Sp8 null mutant telencephalon (Bell et al. 2003; Zembrzycki et al. 2007). To determine whether misexpression of Sp8 alters the expression domain of Fgf genes, we examined the expression of Fgfl5 and Fgf15. In the double transgenic telencephalon, Fgf8 expression was confined to the rostroventral midline (Fig. 7B), similar to that in the control (Fig. 7A). On the other hand, Fgf15 expression was present in much of the ventral/subpallial portion of the double transgenic telencephalon; however, it was not ectopically expressed in the pallial domain of Sp8 misexpression (Fig. 7D). Therefore, misexpression of Sp8 using our binary transgenic approach did not induce Fgf8 or Fgf15 expression in pallial progenitors.

Sp8 expression is downstream of Fgf8 and Fgf17 function based on loss-of-function (Storm et al. 2006; Cholfin and Rubenstein 2008) and gain-of-function (Sahara et al. 2007) experiments. Therefore, we reasoned that Sp8 may participate in transducing Fgf signaling. To examine this possibility, we analyzed the expression of the Fgf effectors like Spry2 and Spry2

Discussion

In this study, we have examined the role of the transcription factor Sp8 on cortical patterning and differentiation. While the role of Sp8 on cortical arealization has been reported in other studies (Sahara et al. 2007; Zembrzycki et al. 2007), here we focus on the earlier cortical functions of Sp8. We concentrated on the early phenotype of Sp8 gain-of-function mutants to identify the first changes in gene expression and cell behavior (e.g., survival, proliferation, and differentiation) due to Sp8 misexpression.

Using a unique and powerful binary transgenic approach to misexpress Sp8 temporally throughout the telencephalon, we examined the role of this transcription factor in rostro-caudal and dorsoventral patterning of the developing cortex. Moreover, our gain-of-function approach allowed us to examine the role of Sp8 in the proliferation, survival, and differentiation of telencephalic progenitors. In our study, we found that Sp8 exerts its activity on regional patterning and progenitor cell differentiation and survival during a precise time window in the embryonic cortical anlage. We also provide evidence that Sp8 regulates these processes by promoting Fgf signaling. Because of the severe effects of Sp8 misexpression on early cortical development, analysis of later cortical properties, such as arealization, was not possible.

Our findings provide evidence that Sp8 and another transcription factor, COUP-TF1, mutually repress each other's cortical neuroepithelial expression along the anterior–posterior and dorsoventral axes. The expression of these factors is downstream of Fgf signaling: Sp8 is positively regulated by Fgf8 (Storm et al. 2006; Sahara et al. 2007), whereas COUP-TF1 is repressed (Garel et al. 2003; Storm et al. 2006; Borello et al. 2008). Our data suggest that Sp8 mediates its effects, at least in part, by potentiating Fgf signaling, whereas previously COUP-TF1 was proposed to repress Fgf signaling. Therefore, we propose a novel molecular mechanism that couples cortical patterning and growth through a regulatory circuit consisting of a feedback loop between Fgf signaling and its transcription factor effectors Sp8 and COUP-TF1.
Sp8 Regulates Cortical Patterning

To analyze the role of Sp8 during forebrain development, we focused on the effects of cortical Sp8 gain of function, using a method that allows for temporal and spatial control of Sp8 expression. Early misexpression of Sp8 throughout the telencephalon (from E8.5 onward) using the Foxg1tTA driver mice, causes severe telencephalic hypoplasia. Additionally, this misexpression alters the expression gradients of cortical patterning genes. We observed the most severe alterations in ventrolateral pallial identity, with the earliest change being a loss of COUP-TF1 followed by a loss of Pax6 expression. Medial pallium identity was less affected by Sp8 misexpression; expression of Emx2 and Wnt8b was maintained, while there was a clear reduction in Lhx2 expression. It is interesting to note that reduced Lhx2 may contribute to forebrain hypoplasia (Porter et al. 1997). Taken together, Sp8 appears to promote medial pallial identity through the repression of dorsolateral patterning, notably via the downregulation of COUP-TF1 and Pax6.

Sp8 is also expressed in a rostral high/caudal low gradient (e.g., Fig. 1A,C) and its expression is downstream of the Fgf8 signaling pathway driven from the rostral patterning center (Storm et al. 2006). This suggests that Sp8 has a role on rostrocaudal as well as dorsoventral patterning of the developing cortex. Indeed, by repressing COUP-TF1 expression and by promoting Fgf signaling, we propose that Sp8 promotes rostral properties.

Patterning and growth are generally linked. During corticogenesis, COUP-TF1 induces neuronal differentiation and downregulates progenitor markers such as Pax6 (Faedo et al. 2008). One would, therefore, predict that the reduction of COUP-TF1 expression in the double transgenic embryos misexpressing Sp8 would repress neurogenesis. However, this was not the case; on the contrary, we observed precocious neuronal differentiation in the cortex of these embryos. Thus, despite their opposing roles in ventrolateral and dorsomedial cortical patterning, COUP-TF1 and Sp8 both appear to promote neurogenesis, at least in overexpression transgenic
experiments. Thus, in synchrony with their distinct roles in cortical regional specification, Sp8 might promote the generation of neurons with dorsomedial pallial properties, whereas COUP-TFI might promote the generation of neurons with ventrolateral pallial properties.

Finally, to gain insights into the temporal requirement of Sp8 activity during corticogenesis, we took advantage of our binary genetic system. We used Dox treatment to delay the misexpression of Sp8 in double transgenic embryos until roughly E15, and found no obvious effect on cortical development. Thus, cortical progenitors were only sensitive to Sp8 misexpression between E10 and E15.

Sp8 Modulates the Balance Between Proliferation and Differentiation of Cortical Progenitor Cells

By misexpressing Sp8, we provide evidence that this transcription factor promotes neuronal differentiation of cortical progenitor cells, particularly when ectopic in the dorsolateral pallium. One hypothesis is that precocious neuronal differentiation could be attributed to the loss of apical polarity within the epithelium of the cortical VZ, as demonstrated by the loss of the apical protein β-catenin (data not shown). Indeed, previous studies have shown that disruptions in apical–basal polarity promote neurogenic divisions (Gotz and Huttner 2005).

The early neural differentiation of the Sp8 overexpressing progenitor cells was accompanied by a strong reduction in proliferation. This correlates well with evidence that there was reduced Notch signaling, based on the reduced Hes5 expression. Therefore, an increase in Sp8 dosage shifts the proliferation/differentiation balance toward neural differentiation. This Sp8-induced alteration in the balance of proliferation/differentiation leads to a depletion of the early cortical progenitor cell pool, which we propose is the main cause of the cortical hypoplasia observed after Sp8 misexpression. The loss of progenitors would also deplete radial glia; this may underlie the observed lamination defect (Supplementary Fig. S4).

Sp8 Modulates Cell Survival

Sp8 misexpression induced apoptosis in the ventrolateral pallium and subpallium; higher levels of apoptosis were observed with the subpallium. In the wild-type subpallium, Sp8 is expressed in the SVZ and not the VZ of the dLGE (Waclaw et al. 2006). When Sp8 is misexpressed throughout the SVZ of the subpallium (including both the MGE and LGE), cell death is not increased (Madhavan and Campbell unpublished results). Moreover, Sp8 misexpression in telencephalic progenitors is not generally toxic because Dox-delayed misexpression of Sp8 between E15 and E18 did not increase cell death above levels seen in the controls. Therefore, the proapoptotic effect of high levels of Sp8 depends on regional, cell-type, and temporal variables. The role of Sp8 on progenitor cell survival will be the focus of future studies. It is worth noting, however, that Sp8 exerts its proapoptotic effect when Sp8 protein levels are increased as well as when they are decreased (Zembrzycki et al. 2007). This same trend for apoptosis and other effects, observed during corticogenesis in the 2 opposite conditions of the gain and loss of Sp8 function, suggests that Sp8 is a key molecule of a basic gene network regulating corticogenesis. When Sp8 protein concentration changes in progenitor cells, the main protein interactions of this network are not established properly and cortical patterning and growth are severely affected.

Sp8/Fgf Signaling Feedback Loop

Our study provides evidence that Sp8 misexpression promotes Fgf signaling in cortical progenitor cells. This could be through directly activating Fgf signaling in the cells and/or through increasing the receptivity to Fgf signaling.

Sp8 has been implicated in the induction and maintenance of Fgf8 expression in the developing limb (Bell et al. 2003). Although Fgf8 expression is present in the telencephalic midline of Sp8 mutants at early stages (i.e., E9.5 and E10.5), it appears to be lost by E12.5 (Bell et al. 2003; Zembrzycki et al. 2007). This suggests that Sp8 is required for the maintenance but not the induction of Fgf8 expression in the telencephalic midline. In contrast, a previous study showed that electroporation of Sp8 in the lateral telencephalon resulted in ectopic expression of Fgf8 (Sahara et al. 2007). We did not observe a similar induction of Fgf8 in our Sp8 misexpression system. In fact, the expression domains of both Fgf8 and Fgf15 appeared similar to those in controls. The discrepancy in these results may come from the fact that the electroporation experiments may drive high levels of the Sp8 expression in the SVZ and mantle zone (with lower levels in the VZ) of the lateral telencephalon, whereas our transgenic system drives high levels of Sp8 throughout the telencephalic VZ. Thus, in line with the loss-of-function findings (Bell et al. 2003; Zembrzycki et al. 2007), it appears that Sp8 does not induce Fgf8 expression in VZ progenitors, but rather appears to be important for the maintenance of its expression within the telencephalic midline.

Despite the observation that Sp8 does not increase Fgf gene expression, it appears that it increases the level of Fgf signaling, particularly in ventrolateral pallial progenitors. Indeed, we found that downstream effectors of Fgf signaling such as Spry and pERK were upregulated in the lateral pallium of embryos misexpressing Sp8.

Fgf signaling is most commonly associated with proliferation and patterning of cortical progenitors, however, it also plays a role in neuronal differentiation (Maric et al. 2007; Iwata and Hevner 2009). Our data suggest that Sp8, which is regulated in a dose-dependant manner by Fgf8 (Storm et al. 2006), enhances the differentiative effects of Fgfs in ventrolateral pallial progenitors. The increased Fgf signaling in this context promotes neural differentiation and represses proliferation. It is possible, however, that Sp8 misexpression sensitizes ventrolateral pallial cells to additional signals, because Spry and pERK are also downstream of other factors such as EGF and Ras (Mason et al. 2006). The increase in Fgf signaling could be a direct transcriptional effect of Sp8, and/or could be through Sp8-inducing molecules that increase the cell’s sensitivity to Fgf signals. In either case, our findings have led us to propose a model for Sp8 function in cortical patterning. We suggest a feedback loop between Fgf signaling and Sp8 for regulating rostral and dorsomedial cortical patterning (Fig. 9). This feedback loop could be at the core of how Sp8 regulates the balance between cortical progenitor proliferation, survival, and differentiation.

During cortical development, Sp8 is expressed in the dorsomedial pallium together with Emx2 and the 2 have been

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shown to physically interact (Zembrzycki et al. 2007). Emx2 keeps progenitor cells in an undifferentiated state and enlarges the pool of cortical progenitor cells by promoting symmetric cell division (Heins et al. 2001), whereas Sp8 induces differentiation of cortical progenitor cells. In fact, Sp8 expression is increased in the dorsomedial domain of the Emx2 mutant cortex (Cholfin and Rubenstein 2008). Emx2 mutant mice also have increased expression of the neuronal marker βIII-tubulin (Mallamaci et al. 2000) and the neurogenesis-promoting transcription factor Ngn2 (Muzio et al. 2005). Therefore, one role for Emx2 may be to counteract and/or delay the neuronal differentiation effects of Sp8. When we overexpress Sp8 in the medial pallium (which still expresses high levels of Emx2), the balance between proliferation and differentiation is largely unaffected. However, in the lateral pallium, which has lower levels of Emx2, misexpression of Sp8 at high levels shifts the balance toward precocious neurogenesis. A similar scenario was proposed for COUP-TFI and Pax6 in the ventrolateral pallium, where COUP-TFI promotes neuronal differentiation, while Pax6 promotes progenitor cell proliferation (Faedo et al. 2008).

In summary, we propose a novel molecular mechanism to explain the link between patterning and growth of the cortex with the identification of a new regulative module consisting of feedback loops between the Fgf signaling and its effectors Sp8 and COUP-TFI, which together coordinates patterning and differentiation of the cerebral cortex (Fig. 9).

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

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