PATTERNS & PHENOTYPES

Differential Requirements for Fgf3 and Fgf8 During Mouse Forebrain Development

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Multiple *Fgfs* are expressed during formation and patterning of the telencephalon in vertebrates. *Fgf8* has been shown to control the size of the telencephalon and the development of signaling centers in zebrafish and mouse. Next to *Fgf8*, *Fgf3* also influences telencephalic gene expression in the zebrafish. Moreover, *Fgf3* and *Fgf8* have been shown to have combinatorial functions during forebrain development in this species. Here, we have examined telencephalic development in *Fgf3* null mouse mutants and embryos that lack both *Fgf3* and *Fgf8* in their forebrain. In contrast to zebrafish, *Fgf3* mutants show normal forebrain development and expression of telencephalic marker genes. Although double mutants for *Fgf3* and *Fgf8* show a further reduction of forebrain size no additional changes of telencephalic gene expression are observed compared with *Fgf8* mutants. Therefore unlike in zebrafish, *Fgf3* is not required for mouse forebrain development whereas *Fgf8* has a central role during this process. *Developmental Dynamics 00:000–000, 2008.*

Key words: telencephalon; fibroblast growth factor; conditional knockout

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INTRODUCTION

During development of the telencephalon signaling molecules establish the expression of transcription factors that define and maintain the identity of the ventral and dorsal telencephalon. In the early telencephalon, midline cells that split the telencephalon into two hemispheres generate signaling centers by the expression of secreted factors (Hebert, 2005; O'Leary et al., 2007). Three different signaling centers have been identified, including the dorsal midline that expresses Bmps (Furuta et al., 1997) and Wnts (Grove et al., 1998; Lee et al., 2000), the rostral midline expressing Fgfs (Crossley and Martin, 1995; McWhirter et al., 1997; Maruoka et al., 1998; Walshe and Mason, 2003) and the ventral midline characterized by Shh expression (Ericson et al., 1995). Bmps and Wnts have been shown to act antagonistically to Fgf and Shh (Ohkubo et al., 2002; Kuschel et al., 2003; Storm et al., 2003; Shimogori et al., 2004). Modifications in the relative strength of these signaling centers have thus profound effects on the size and nature of telencephalic subdivisions by regulating the expression of transcription factors, such as *Nkx2.1*, *Emx2*, and *Pax6* (Sussel et al., 1999; Stoykova et al., 2000; Muzio et al., 2002; Theil et al., 2002; Fuccillo et al., 2004). These transcription factors are required for correct specification of the telencephalon and regulate expression of region-specific genes that control neurogenesis, such as *Ngn2* (Fode et al., 2000).

The rostral midline, termed also commissural plate, is derived from the anterior neural ridge (ANR) and both are characterized by the expression of several *Fgfs*, including *Fgf3*, *Fgf8*, *Fgf15*, *Fgf17*, and *Fgf18* (Crossley and Martin, 1995; McWhirter et al., 1997; Maruoka et al., 1998; Bachler and Neubuser, 2001; Shinya et al., 2001; Walshe and Mason, 2003; Cholfin and Rubenstein, 2007, 2008). Amongst these *Fgfs*, *Fgf8* has been shown to be required for the patterning of the ros-

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tral telencephalon in the mouse and zebrafish (Fukuchi-Shimogori and Grove, 2001; Shinya et al., 2001; Garel et al., 2003; Walshe and Mason, 2003; Hebert, 2005; Mason, 2007; O'Leary et al., 2007). *Fgf8* expression is first detected at embryonic day (E) 8 in the ANR and in the rostral midline between E9 and E12.5 (Crossley and Martin, 1995). Telencephalic conditional *Fgf8* mouse mutants ($Fgf8^{TelKO}$) show hypoplasia of rostral telencephalic structures and an increase in *Bmp4* and *Wnt8b* in the dorsal midline and a rostral expansion of *Emx2* in the neocortex, whereas *Pax6* expression expands ventrally (Storm et al., 2003; Storm et al., 2006). On the other hand, analysis of $Fgf8^{TelKO}$ mutants also shows that *Fgf8* is essential for establishing normal expression patterns of *Shh* and the transcription factors *Nkx2.1* and *Dlx2* in the rostroventral telencephalon (Storm et al., 2006).

In the zebrafish, *fgf8* mutants or morphants shown an expansion of *emx1*, a transcription factor that is closely related to *emx2*, in the subpallial telencephalon and a reduced expression of *shh* and *nkx2.1* in the ventral telencephalon (Shanmugalingam et al., 2000; Shinya et al., 2001; Walshe and Mason, 2003). Interestingly, in *fgf3* morphants expression of these genes are also affected and some markers like, for example, *dlx2* appear even more dependent on *fgf3* (Shinya et al., 2001; Walshe and Mason, 2003). Lastly, *fgf3* and *fgf8* also show combinatorial effects on the expression of *emx1*, *shh*, and *nkx2.1* (Shinya et al., 2001; Walshe and Mason, 2003). These data thus demonstrated that Fgf3 shows unique and redundant functions together with Fgf8 during forebrain development in zebrafish and prompted us to examine the function of Fgf3 during development of the murine telencephalon. Our analysis shows that *Fgf3* expression is initiated at E.7.75 in the ANR and is later detected in the rostral midline and the lateral telencephalon. However, null mutants for *Fgf3* show no defects in the formation of signaling centers and regionalization of the forebrain. Double mutants lacking both *Fgf3* and *Fgf8* during forebrain development show a reduced size of their forebrain compared with $Fgf8^{TelKO}$ mutants. Moreover, defects in dorsal and ventral patterning of the telencephalon are observed in *Fgf3*/$Fgf8^{TelKO}$ animals. However, these defects are similarly observed in $Fgf8^{TelKO}$ mutants.

RESULTS

Fgf3 Expression During Forebrain Development

To monitor *Fgf3* expression during forebrain development in the mouse embryo between E7.5 until E12.5 we used a *Fgf3*/*lacZ* reporter transgene previously described (Powles et al., 2004a). *Fgf3* expression is first detected at E7.75 in the ANR (Fig. 1A). This expression domain expands along the rostral edge of the neural plate bilaterally at E8.5 and upon fusion of the rostral edges at E9 includes the rostral midline (Fig. 1B,C; see also Bachler and Neubuser, 2001). *Fgf3* expression in the forebrain is down-regulated at E10 and absent around E10.5 (data not shown; Powles et al. 2004; Bachler and Neubuser; 2001). At E11.5 and E12.5 a novel domain of *Fgf3* expression is observed in the lateral telencephalon (Fig. 1D and Supp. Fig. S1, which is available online).

Requirements for *Fgf3* During Murine Forebrain Development

Due to the restricted *Fgf3* expression pattern and its previously described role in zebrafish forebrain development we became interested in investigating its functions during murine forebrain development. To this end, we used *Fgf3* knock-out mice in which the *Fgf3* coding region has been deleted (Alvarez et al., 2003a). Using in situ hybridization on coronal sections of the E11.5 telencephalon, we investigated the expression of several transcription factor genes which are characteristic for the major subdivisions of the telencephalon. *Emx2* and *Pax6* are expressed in the dorsal telencephalon in opposing gradients and have been implicated in various aspects of cortical development (Bishop et al., 2000; Bishop et al., 2002; Muzio et al., 2002) However, the expression of both genes is unaltered in the $Fgf3^{-/-}$ telencephalon (Fig. 2A–D). The *Dlx2* homeobox gene is expressed in the medial (MGE) and lateral ganglionic eminences (LGE) within the ventral telencephalon while *Nkx2.1* expression is confined to the MGE only (Fig. 2E,F; Anderson et al., 1997; Sussel et al., 1999). In the *Fgf3* mutant, these expression patterns are maintained. Whole mount in situ hybridization at E9.5 and E11.5 did not reveal differences in the expression pattern of these transcription factors either (Supp. Fig. S2, and data not shown). In addition, the expression of several signaling molecules including *Shh*, *Fgf8* and *Wnt3a* was not affected by the *Fgf3* mutation (Supp. Fig. S2 and data not shown). Taken together, these unaltered expression patterns suggest that *Fgf3* is dispensible for regionalization of the telencephalon in the mouse in contrast to its role in the zebrafish.

Fgf3 Does Not Cooperate With *Fgf8* During Forebrain Patterning in the Mouse

Given the previously described genetic interaction between *Fgf3* and *Fgf8* during zebrafish forebrain development (Shinya et al., 2001; Walshe and Mason, 2003) we hypothesized that a similar interaction might occur between the corresponding mouse genes and that a redundancy between these genes might obscure a role for *Fgf3* in forebrain development. To test for this, we crossed *Fgf3* and $Fgf8^{TelKO}$ mutant mice (see Experimental Procedures; Storm et al., 2003) and started to analyze brain development in double mutant embryos. $Fgf8^{TelKO}$ mutants have previously been reported to have smaller telencephalic vesicles (Storm et al., 2006). Morphological inspection of E10.5 and E12.5 embryos confirmed this finding and also showed a further size reduction of the forebrain in $Fgf3^{-/-}$; $Fgf8^{TelKO}$ embryos (n = 15), suggesting an interaction between *Fgf3* and *Fgf8* in determining forebrain size (Fig. 3). To examine potential causes for the size reduction of the telencephalic vesicle in $Fgf3^{-/-}$;$Fgf8^{TelKO}$ cell proliferation and cell death was evaluated on sections of the rostroventral telencephalon in E9.5 embryos (Supp. Figs. S3 and S4).Staining with antibodies directed against the cell cycle

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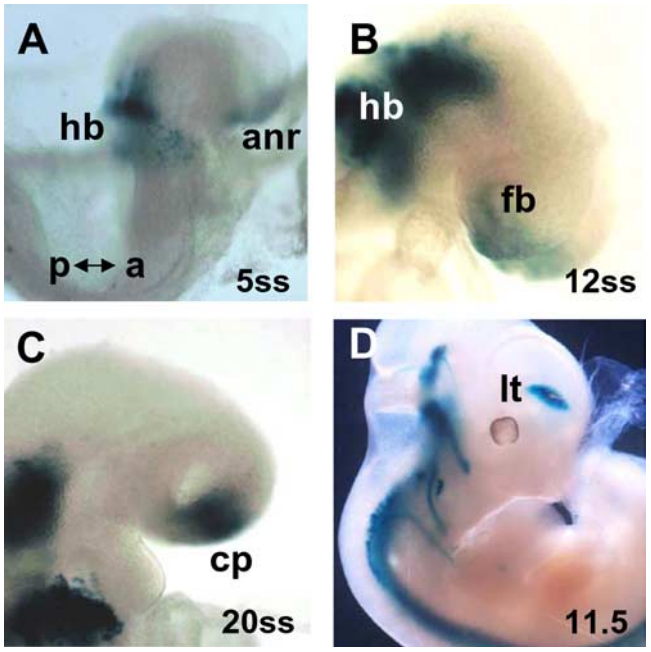

Fig. 1. *Fgf3* expression in the developing forebrain. *Fgf3* expression was monitored using a *Fgf3/lacZ* transgene at the indicated embryonic stages. **A:** *Fgf3* expression is observed in the anterior neural ridge (anr) and hindbrain (hb) at embryonic day (E) 7.75. The orientation of the embryo along the anterior (a) to posterior (p) axis is indicated. **B,C:** Expression then further expands in the rostral forebrain (fb) including the commissural plate (cp). **D:** At E11.5 *Fgf3* expression is observed in the lateral telencephalon (lt). ss; somite stage.

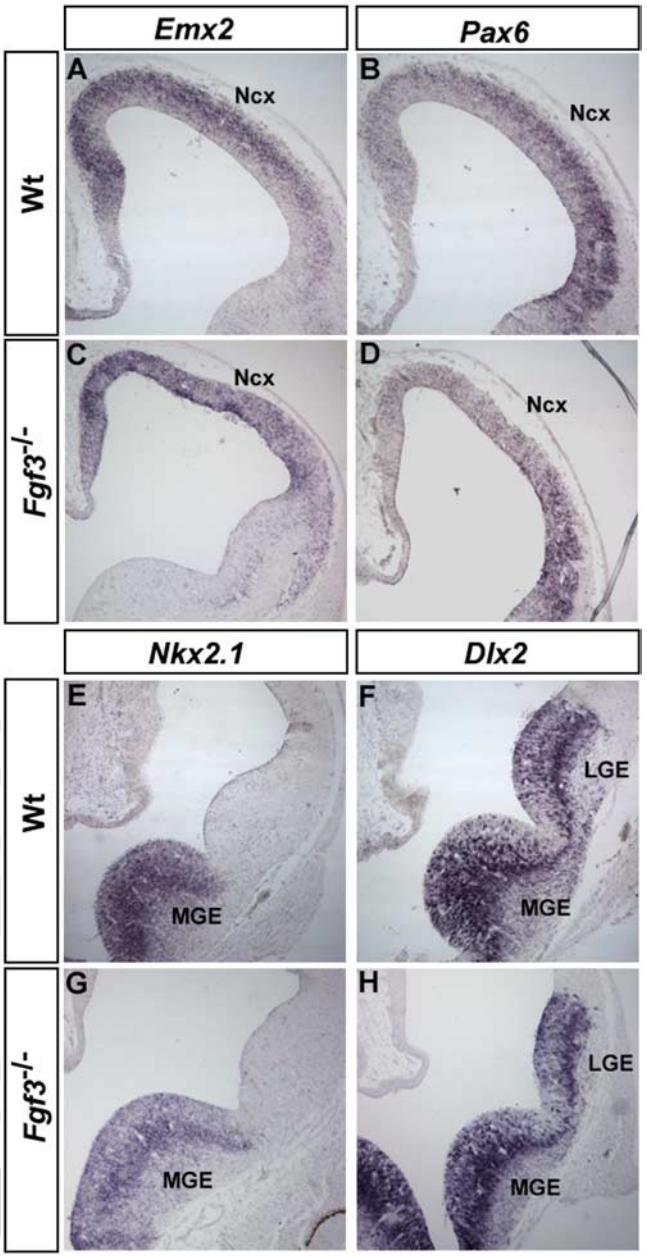

Fig. 2. Expression of dorsal and ventral specific markers in the *Fgf3* mutant telencephalon. A–H: In situ hybridization analysis on coronal sections through the telencephalon of embryonic day (E) 11.5 wild-type (Wt; A,B,E,F) and *Fgf3*$^{-/-}$ (C,D,G,H) embryos using the indicated probes. **A–D:** The expression of the neocortex (Ncx) markers *Emx2* and *Pax6* is unaltered in the mutant. **E–H:** The expression of *Nkx2.1* and *Dlx2* is restricted to the medial (MGE) or medial and lateral (LGE) ganglionic eminences, respectively, in both wild-type and mutant embryos.

marker phosphorylated histone H3 (pH3) was performed and revealed no apparent differences between the wild-type and mutant embryos upon visual inspection (Supp. Fig. S3). However, counting of pH3-labeled cells revealed that wild-type embryos had roughly a twofold higher mitotic index than *Fgf3*$^{-/-}$;*Fgf8*^{TelKO}mutants (7 ± 0.8 vs. 3.7 ± 1.5; $P < 0.01$; n = 4, see the Experimental Procedures section). Apoptosis was examined using the TUNEL assay. In this case *Fgf3*$^{-/-}$; *Fgf8*^{TelKO}mutants showed a dramatic increase of apoptotic cells (Supp. Fig. S4).

In addition, while the isthmus that expresses both *Fgf3* (Powles et al., 2004) and *Fgf8* (Crossley et al., 1995) next to *Foxg1*-Cre, (Hebert and McConnell, 2000) is reduced but still present in the *Fgf3*$^{-/-}$and *Fgf8*^{TelKO} single mutants, it does not form in the *Fgf3*$^{-/-}$;*Fgf8*^{TelKO} double mutant indicating a requirement for *Fgf3* and *Fgf8* for establishment or maintenance of the isthmus (Fig. 3D and Supp. Fig. S4).

To analyze whether patterning of the telencephalon is affected in mutants lacking telencephalic *Fgf3* and *Fgf8* expression we performed in situ hybridizations on coronal sections of the E10.5 telencephalon of wild-type, *Fgf8*^{TelKO} and *Fgf3*$^{-/-}$;*Fgf8*^{TelKO} embryos. First, we analyzed whether the *Fgf* mutations affected the expression of signaling molecules in the telencephalon. *Bmp4* and *Wnt3a* are expressed in the dorsal midline of the telencephalon and specify dorsal telencephalic cell fates (Furuta et al., 1997; Grove et al., 1998; Ohkubo et al., 2002; Shimogori et al., 2004). In the *Fgf8*^{TelKO} as well as in the *Fgf3*$^{-/-}$; *Fgf8*^{TelKO} double mutant,

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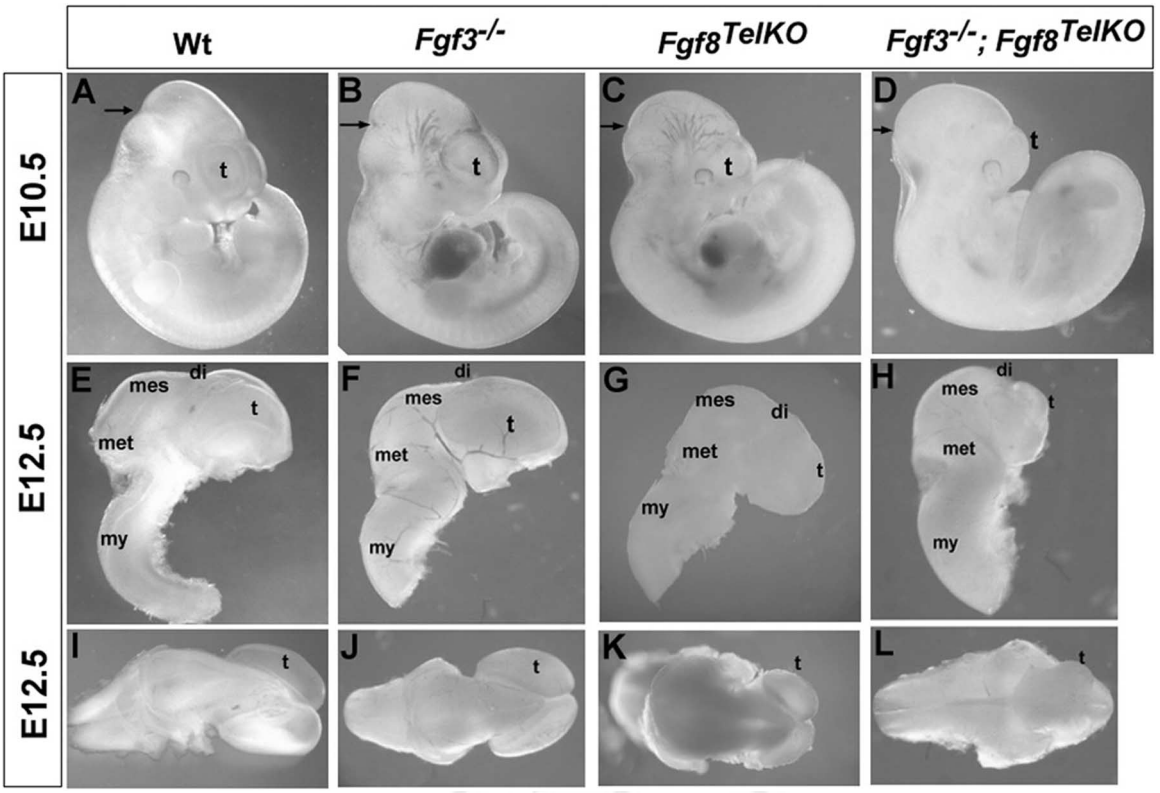

Fig. 3. A–L: Morphological appearance of embryonic day (E) 10.5 (A–D) and E12.5 (E–L) wild-type, $Fgf3^{-/-}$, $Fgf8^{TelKO}$ single mutant and of $Fgf3^{-/-}$;$Fgf8^{TelKO}$ double mutant embryos. **A–D:** At E10.5, the $Fgf8^{TelKO}$ and $Fgf3^{-/-}$;$Fgf8^{TelKO}$ mutant telencephali (t) are smaller. The arrow points at the isthmus, which is morphologically not visible in the double mutant embryos (D). **E–L:** Dissected E12.5 brains. Note the severe size reduction of the telencephalon and diencephalon (di) in the $Fgf8^{TelKO}$ and $Fgf3^{-/-}$;$Fgf8^{TelKO}$ mutant embryos, while the size of the midbrain and hindbrain is unaltered. mes, mesencephalon; met, metencephalon; my, myencephalon.

both genes show slightly expanded expression domains in the dorsal telencephalon (Fig. 4A–F). Ventral telencephalic cell fates are determined by *Shh* which is expressed in the MGE and in the hypothalamus (HT; Ericson et al., 1995). *Shh* expression is specifically lost in the MGE but not in the HT of both $Fgf8^{TelKO}$ single and $Fgf3^{-/-}$; $Fgf8^{TelKO}$ double mutants (Fig. 4H,I) consistent with the requirement for *Fgf8* in the maintenance of *Shh* expression in the ventral telencephalon (Ohkubo et al., 2002; Storm et al., 2006). Collectively, these data suggest that the dorsal and ventral signaling centers in $Fgf8^{TelKO}$ and $Fgf3^{-/-}$;$Fgf8^{TelKO}$ mutants are affected to similar degrees and that the *Fgf3* mutation does not increase the severity of these defects.

We next analyzed whether these alterations in signaling centers affect dorsal/ventral patterning of the telencephalon. In wild-type embryos, *Nkx2.1* and *Dlx2* are expressed in the ventral telencephalon with *Nkx2.1* expression being restricted to the MGE (Fig. 5A,D; Sussel et al., 1999; Anderson et al., 1997). In the $Fgf8^{TelKO}$ mutant, the expression of both markers is lost from the telencephalon except for a weak expression in the HT (Fig. 5B,E; Storm et al., 2006). A similar loss of *Nkx2.1* and *Dlx2* expression was observed in the $Fgf3^{-/-}$;$Fgf8^{TelKO}$ double mutant (Fig. 5C,F). The dorsal telencephalon is marked by *Ngn2* expression which shows a lateral to medial expression gradient in E10.5 wild-type embryos (Fig. 5G). In contrast, in both $Fgf8^{TelKO}$ and $Fgf3^{-/-}$; $Fgf8^{TelKO}$ mutants there is *Ngn2* expression throughout the dorsal and ventral telencephalon and the *Ngn2* expression gradient is lost (Fig. 5H,I). Thus, the loss of either *Fgf8* or of both, *Fgf3* and *Fgf8*, results in a dorsalization of the ventral telencephalon. However, we found no evidence that the concomitant loss of *Fgf3* and *Fgf8* increases the patterning defects observed upon *Fgf8* loss only.

DISCUSSION

Fgf3 and *Fgf8* both initiate their expression in the mouse forebrain around E8 (see Fig. 1A; Crossley et al., 1995). However, *Fgf3* expression is lost at around E10.5, whereas *Fgf8* is maintained in the rostral midline until at least E12.5 (Crossley et al., 1995). Of interest, we have also observed a novel domain of *Fgf3* expression in the lateral telencephalon between E11.5 and E12.5. Thereafter *Fgf3* expression has also been described in the basal ganglia at E14.5, where also Fgf7 has been detected (Mason et al., 1994; Yaylaoglu et al., 2005).

In contrast to zebrafish morphants created by *fgf3* morpholinos we have observed no defects during forebrain development in *Fgf3* knockout mouse mutants. However, it is worthwhile mentioning that ENU-induced *lia/fgf3* mutants that are considered null mutants do not show the expansion of *emx1* and the reduction of *nkx2.1* de-

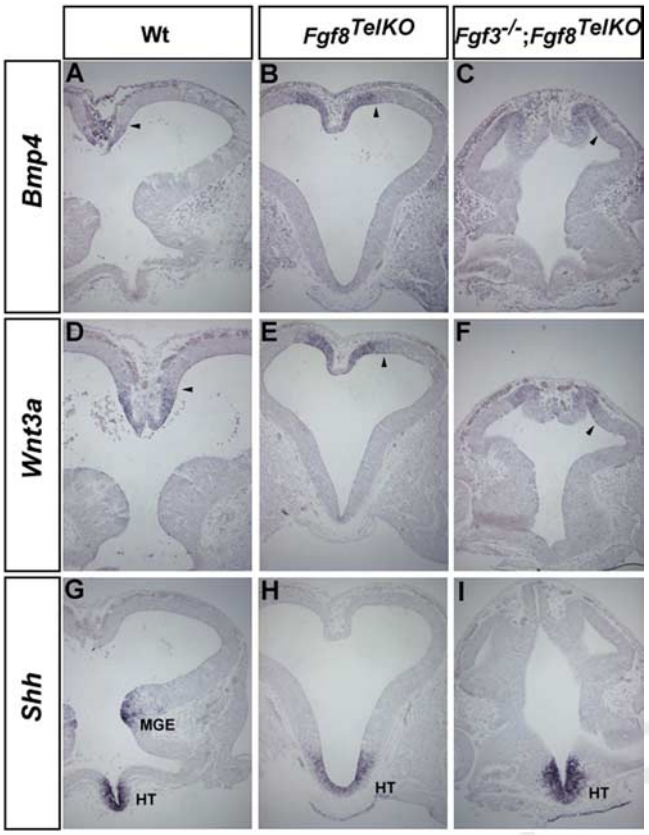

Fig. 4. Telencephalic patterning centers are affected in *Fgf8*^{TelKO} and *Fgf3*$^{-/-}$;*Fgf8*^{TelKO} double mutant embryos. Coronal sections of the E10.5 telencephalon hybridized with the indicated probes. **A–F:** *Bmp4* and *Wnt3a* expression are confined to the dorsomedial telencephalon of wild-type embryos but slightly expand in more lateral regions in the *Fgf* mutants (arrowheads). **G–I:** *Shh* expression is detected in the MGE and in the hypothalamus (HT) of the wild-type embryo. While this latter expression domain is still present in the *Fgf* mutants *Shh* expression is lost in the ventral telencephalon. Please note that, due to the reduced size and altered morphology of the telencephalon in *Fgf8*^{TelKO} and especially the *Fgf3*$^{-/-}$;*Fgf8*^{TelKO} embryos, it is very difficult to match sections exactly along the anteroposterior axis.

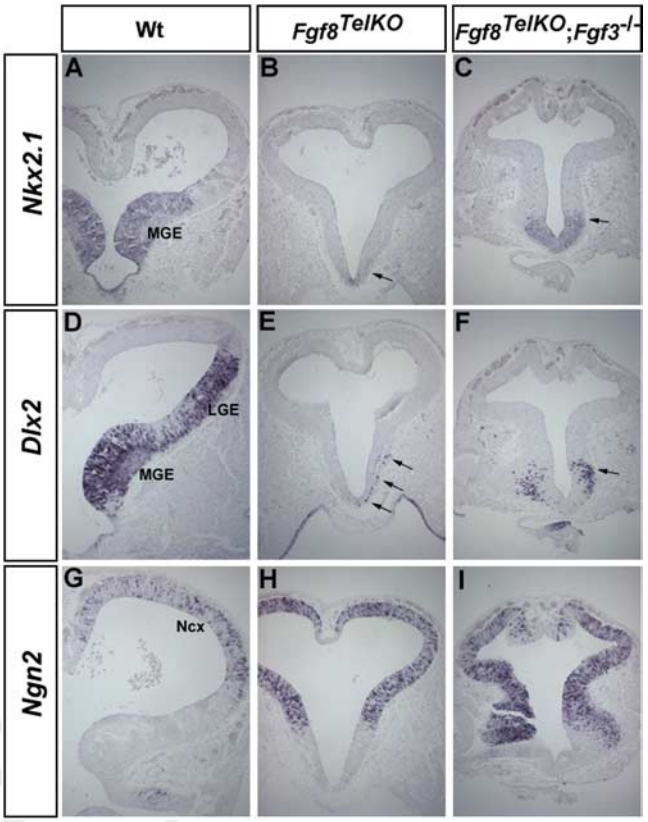

Fig. 5. Dorsal/ventral patterning in the telencephalon of *Fgf8*^{TelKO} and *Fgf3*$^{-/-}$;*Fgf8*^{TelKO} double mutant embryos. Coronal sections hybridized with the indicated probes. **A–F:** *Nkx2.1* and *Dlx2* expression are characteristic of the wild-type ventral telencephalon. In the *Fgf* mutants, the expression of both markers is lost except for expression in the POA (arrows). **G,H:** *Ngn2* expression in the neocortex (Ncx) expands into the ventral telencephalon in *Fgf* mutants. Please note that, due to the reduced size and altered morphology of the telencephalon in *Fgf8*^{TelKO} and especially the *Fgf3*$^{-/-}$;*Fgf8*^{TelKO} embryos, it is very difficult to match sections exactly along the anteroposterior axis.

scribed during forebrain development in *fgf3* morpholino morphants (Shinya et al., 2001; Walshe and Mason, 2003; Herzog et al., 2004). Therefore further analysis of the role of Fgf3 during zebrafish forebrain development is required.

Our present results suggest that in the mouse Fgf8 has a dominant role during development of the forebrain where it most likely acts by means of Fgf receptor 1 (Fgfr1) and Fgfr2 (Gutin et al., 2006). Double mutants that lack *Fgf3* and *Fgf8* in the telencephalon show a reduced size compared with *Fgf8*^{TelKO} mutants. Examination of mitosis and apoptosis suggest that increased cell death is likely to be the major underlying cause for the decreased size of the telencephalon of *Fgf3*$^{-/-}$;*Fgf8*^{TelKO} mutants. We observed no difference in the expression patterns of signaling molecules and region-specific transcription factors in the forebrain between these *Fgf8*^{TelKO} and *Fgf3*$^{-/-}$;*Fgf8*^{TelKO} mutants. Thus, unlike in the zebrafish, we have found no evidence for a cooperation between Fgf3 and Fgf8 during forebrain patterning in the mouse. However, it is worthwhile mentioning that the restricted tissue- and stage-specific inactivation of *Fgf8* mediated by the *Foxg1*Cre transgene in the mouse versus the global down-regulation mediated by *fgf8* morpholinos in the zebrafish may influence the different phenotypes obtained. Additionally, other Fgf family members may show unique or redundant functions together with Fgf8 and Fgf3 during this process. Fgf17, which belongs to the Fgf8 subfamily, has been shown to control dorsal forebrain patterning in the mouse (Cholfin and Rubenstein, 2007, 2008). Next to Fgf8 and Fgf17, other candidates that may participate in telencephalic patterning are Fgf15 and Fgf18 (Rash and Grove, 2007). Interestingly in zebrafish Fgf19, the ortholog of Fgf15 in the mouse, has recently also been shown to be involved in forebrain development (Miyake et al., 2005). Similar to *fgf3* morphants, *fgf19* morphants show expansion of *emx1* and reduction of *dlx2* during forebrain development (Shinya et al., 2001; Walshe and Mason, 2003). Additionally, ectopic expression of *fgf3* and *fgf8* are observed

in *fgf19* morphants indicating further crossregulation between different FGF family members. Knockdown of *fgf3* does not affect *fgf8* expression but in *fgf8* morphants *fgf3* expression is up-regulated (Walshe and Mason, 2003). In contrast in the present study, we found no evidence for a change in *Fgf8* expression in *Fgf3* mouse mutants (Supp. Fig. S2). It will therefore be necessary to further define the unique and redundant functions of FGFs in different species to fully understand their conserved and species-specific roles during forebrain development.

EXPERIMENTAL PROCEDURES

Transgenic Mice

The following mouse lines used in this study have been described previously: $Fgf3^{-/-}$ knockout mutants (Alvarez et al., 2003b), mutants carrying a conditional ($Fgf8^{flox}$) or a null allele ($Fgf8^{d2,3}$) for *Fgf8* (Meyers et al., 1998), mouse lines in which *cre* has been targeted to the *Foxg1* locus (*BF-1;* (Hebert and McConnell, 2000), and transgenic mice which express *lacZ* under the control of *Fgf3* regulatory sequences (Powles et al., 2004b). To obtain mouse mutants that lacked *Fgf8* expression in the telencephalon ($Fgf8^{TelKO}$), we crossed animals carrying a *Fgf8* null allele and the *Foxg1*-Cre transgene ($Fgf8^{d2,3/+}$;$Foxg1^{Cre/+}$) with animals carrying a conditional *Fgf8* allele ($Fgf8^{flox/flox}$) to obtain $Fgf8^{flox/d2,3}$;$Foxg1^{Cre/+}$ ($Fgf8^{TelKO}$) mutants as previously described (Storm et al., 2003). To obtain $Fgf3^{-/-}$/ $Fgf8^{flox/d2,3}$; $Foxg1^{Cre/+}$ ($Fgf3^{-/-}$/$Fgf8^{TelKO}$) mutants we crossed $Fgf3^{+/-}$/$Fgf8^{flox/d2,3}$;$Foxg1^{Cre/+}$ animals with $Fgf3^{-/-}$/$Fgf8^{flox/flox}$ mice, as described previously (Zelarayan et al., 2007).

RNA In Situ Hybridization and β-Galactosidase Staining

RNA whole-mount in situ hybridization and sectioning, β-galactosidase staining, preparation of histological sections and riboprobes used have been described or referred to previously (Crossley and Martin, 1995; Theil et al., 1999, 2002; Alvarez et al., 2003a).

Detection of Proliferating Cells and Cell Death

Detection of cell proliferation insections was performed by immunohistochemistry using the anti-phosphorylated histone H3 antibody (rabbit polyclonal Phospho H3 from Upstate Biotechnology, USA) diluted at 1/100. Sections were counterstained with eosin. The number of pH3-positive cells was counted in the rostroventral telencephalon corresponding to the boxed area indicated in Supp. Fig. S3A. TUNEL analysis was performed using the Apotag kit following the manufacturer's recommendations (Intergen).

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