Fgf8 expression defines a morphogenetic center required for olfactory neurogenesis and nasal cavity development in the mouse

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Summary

In vertebrate olfactory epithelium (OE), neurogenesis proceeds continuously, suggesting that endogenous signals support survival and proliferation of stem and progenitor cells. We used a genetic approach to test the hypothesis that *Fgf8* plays such a role in developing OE. In young embryos, *Fgf8* RNA is expressed in the rim of the invaginating nasal pit (NP), in a small domain of cells that overlaps partially with that of putative OE neural stem cells later in gestation. In mutant mice in which the *Fgf8* gene is inactivated in anterior neural structures, FGF-mediated signaling is strongly downregulated in both OE proper and underlying mesenchyme by day 10 of gestation. Mutants survive gestation but die at birth, lacking OE, vomeronasal organ (VNO), nasal cavity, forebrain, lower jaw, eyelids and pinnae. Analysis of mutants indicates that although initial NP formation is grossly normal, cells in the *Fgf8*-expressing domain undergo high levels of apoptosis, resulting in cessation of nasal cavity invagination and loss of virtually all OE neuronal cell types. These findings demonstrate that *Fgf8* is crucial for proper development of the OE, nasal cavity and VNO, as well as maintenance of OE neurogenesis during prenatal development. The data suggest a model in which *Fgf8* expression defines an anterior morphogenetic center, which is required not only for the sustenance and continued production of primary olfactory (OE and VNO) neural stem and progenitor cells, but also for proper morphogenesis of the entire nasal cavity.

Key words: FGF, Vomeronasal organ, Neurogenesis, Olfactory epithelium, Nasal cavity, Stem cell, Apoptosis, Cre recombinase, *Fgf8*, *Foxg1*, *Sox2*, *Pax6*, *Mash1*, Neurogenin 1, *Ncam1*, *Pyst1*, *Shh*, *Dlx5*, Neuronal progenitor, Mouse mutant

Introduction

Members of the fibroblast growth factor (FGF) superfamily of signaling molecules have important effects on cell proliferation, developmental patterning, cell growth and homeostasis in virtually all tissues in higher vertebrates (Ornitz, 2000). In the nervous system, studies in vitro and in vivo have shown that FGFs promote proliferation, differentiation and survival of most neural cell types, including stem cells and neuronal progenitors (DeHamer et al., 1994; Eckenstein, 1994; Ford-Perriss et al., 2001; Reuss and von Bohlen und Halbach, 2003; Temple and Qian, 1995). FGF8 in particular appears to play an important role in developing nervous system, although the mechanism(s) by which it acts have not been elucidated fully. FGF8 was first identified as a mitogen (Tanaka et al., 1992), and some data support a mitogenic role for FGF8 in neural tissues (Xu et al., 2000; Lee et al., 1997). However, a number of studies indicate that FGF8 acts as a neural morphogen, which regulates expression of downstream genes that control neural patterning (Fukuchi-Shimogori and Grove, 2001; Fukuchi-Shimogori and Grove, 2003; Garel et al., 2003; Grove and Fukuchi-Shimogori, 2003; Irving and Mason, 2000; Ohkubo et al., 2002; Trainor et al., 2002). More recently, the idea has emerged that FGF8 is involved in survival and/or maintenance of specific developing cell populations that ultimately give rise to particular components of the nervous system (Chi et al., 2003; Mathis et al., 2001; Storm et al., 2003).

Mouse olfactory epithelium (OE) provides a useful model system with which to understand how neurogenesis is regulated at the cellular and molecular levels. Studies in vitro and in vivo have demonstrated four distinct developmental stages in the neuronal lineage of established OE: neural stem cells, which express the transcription factor Sox2; *Mash1* (*Ascl1* – Mouse Genome Informatics)-expressing committed neuronal progenitors, the progeny of the stem cells; *Ngn1*-expressing immediate neuronal precursors (INPs), the progeny of *Mash1*+ progenitors; and olfactory receptor neurons (ORNs), which differentiate from daughter cells of INP divisions and can be identified by expression of the neural cell adhesion molecule, NCAM1 (Beites et al., 2005; Calof et al.,

2002; Kawauchi et al., 2004). Interestingly, the OE is one of the few regions of the nervous system in which neurogenesis and nerve cell renewal take place throughout life (Murray and Calof, 1999). This capacity for continual neurogenesis suggests that cells within the OE produce signals that stimulate this process.

In a previous study, we have shown that several FGFs, particularly FGF2, are potent stimulators of neurogenesis in cultured OE, where they promote divisions of OE neuronal transit amplifying progenitors and maintain the stem cells that give rise to these progenitors (DeHamer et al., 1994). Moreover, a number of FGFs, including FGF2, are expressed in and around OE at various stages of development (DeHamer et al., 1994; Hsu et al., 2001; Kawauchi et al., 2004; Key et al., 1996). However, two observations argue that FGF2 is unlikely to be a crucial regulator of developmental neurogenesis in the OE. First, mice with targeted inactivation of the *Fgf2* gene show few if any defects in developmental neurogenesis (Dono et al., 1998; Ortega et al., 1998). Second, *Fgf2* is not highly expressed in mouse OE until postnatal ages (Hsu et al., 2001; Kawauchi et al., 2004) (S.K. and A.L.C., unpublished). Because *Fgf8* has been reported to be expressed in the frontonasal region near the olfactory placodes (Bachler and Neubuser, 2001; Crossley and Martin, 1995; Mahmood et al., 1995), we hypothesized that it may serve as a signal promoting neurogenesis during early OE development.

To test this hypothesis, we analyzed expression of *Fgf8* and its actions on OE neurogenesis in vivo, using a conditional genetic approach in which the *Fgf8* gene was inactivated in mouse OE from the earliest stages of OE development. Tissue culture assays were also used to investigate effects of recombinant FGF8 on OE neurogenesis. Expression analysis indicated that *Fgf8* is initially transcribed in a small domain – which we have termed the morphogenetic center – at the rim of the invaginating neural pit. These *Fgf8*-expressing cells give rise to new cells that both contain *Fgf8* mRNA and express markers of OE neural stem cells. Analysis of FGF8 signaling and cell death demonstrate that, as a consequence of *Fgf8* inactivation, cells within the morphogenetic center and in adjacent developing neuroepithelium undergo apoptosis. As a consequence, although initial invagination of the nasal pit (NP) and initiation of the OE neuronal lineage take place, both NP morphogenesis and OE neurogenesis halt shortly thereafter. This in turn results in a failure of development of definitive OE, vomeronasal organ (VNO) – the pheromone-sensing component of the primary olfactory system (also derived from the olfactory placode) (Farbman, 1992) – and the nasal cavity as a whole. Thus, *Fgf8* is required for the survival of cells in a crucial anterior morphogenetic center, which is responsible not only for nasal cavity and OE development, but also for the generation and survival of the stem cells that ultimately generate all cell types in the OE neuronal lineage and endow this neuroepithelium with its capacity for neuronal regeneration.

Materials and methods

Animals

Mice were naturally mated with the appearance of a vaginal plug designated as embryonic day 0.5 (E0.5). The *Fgf8* allelogenic line was described previously (Meyers et al., 1998). $Fgf8^{flox/flox}$ animals were maintained as homozygotes. Offspring were genotyped using primers to differentiate between *flox* and wild-type alleles by amplicon size (300 bp and 200 bp, respectively; F1 forward primer, 5′-CTTAGGGCTATCCAACCCATC-3′; F2 reverse primer, 5′-GGTCTCCACAATGAGCTTC-3′). The $Fgf8^{d2,3}$ (null for *Fgf8* function) (Meyers et al., 1998) and *Foxg1-Cre* (Hebert and McConnell, 2000) lines were maintained as hemizygotes (termed $Fgf8^{d2,3/+}$ and $Foxg1^{Cre/+}$) on an outbred Swiss Webster (Charles River) background and genotyped with specific primers: (1) $Fgf8^{d2,3/+}$ (200 bp amplicon), F1 forward primer; F3 reverse primer, 5′-AGCTCCCGCTGGATTCCTC-3′; (2) $Foxg1^{Cre}$ (200 bp amplicon), C1 forward primer, 5′-GCACTGATTTCGACCAGGTT-3′; C2 reverse primer, 5′-GCTAACCAGCGTTTTCGTTC-3′. R26R mice were maintained as homozygotes ($R26R^{lacZ/lacZ}$) and genotyped using *lacZ*-(5′-CCAACTGGTCTGAGGAC-3′ and 5′ACCACCGCACGATAGAGATT-3′) or *R26R* locus-specific primers (Soriano, 1999).

Detection of gene expression

Whole-mount X-gal staining of mouse embryos was performed as described (Murray et al., 2003). For RT-PCR, E10.5 frontonasal tissue (including forebrain and olfactory pit) RNA was purified using Trizol (Invitrogen). PCR primers were set to detect cDNA coding for *Fgf8* exon 2 and exon 3 (forward, 5′-GTGGAGACCGATACTTTTGG-3′; reverse, 5′-GCCCAAGTCCTCTGGCTGCC-3′). Cycling parameters were denaturation at 96°C for 20 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 1 minute, for 35 cycles.

Section in situ hybridization for E8.5-E17.5 embryos was performed as described (Murray et al., 2003). For two-color in situ, one probe was synthesized using fluorescein-12-UTP and detected using AP-conjugated anti-fluorescein Fab fragments from sheep with INT/BCIP as the chromagen/substrate mix (Roche). For whole-mount in situ hybridization, E9.5-10.5 embryos were fixed overnight in 4% paraformaldehyde in PBS with 2.5 mM EGTA at 4°C and processed as described (Kawauchi et al., 1999). Probes used in this study were: ORF of mouse *Fgf8* (GenBank Accession Number MMU18673); 463 bp of mouse *Fgf18* (520-983 bp of GenBank #AF075291); 748 bp of mouse *Sox2* (1281bp-2029 bp of GenBank Accession Number X94127); and mouse *Pyst1* (*Dusp6* – Mouse Genome Informatics) (Dickinson et al., 2002). Unless otherwise indicated, *Fgf8* expression was detected using a probe generated from the full-length *Fgf8* ORF cDNA (GenBank Accession Number MMU18673) (Mahmood et al., 1995). *Fgf8 ex1*, *ex2,3* and *int* probes were generated in our laboratory by PCR amplification of genomic DNA or cDNA. The *int* probe consists of bp 2464-3142 of the 3274 bp intron sequence between exons 3 and 4 (Ensemble #ENSMUST00000026241). *Mash1*, *NgnI*, *Gdf11* and *Ncam1* probes were described previously (Murray et al., 2003; Wu et al., 2003).

Immunostaining and TUNEL assays

Cells in M-phase were detected by immunostaining using polyclonal rabbit anti-phospho-histone H3 (Upstate Biotechnology, Cat. No. 06-570) at 1:200 dilution, visualized with Alexa Red-conjugated goat anti-rabbit-IgG (1:1000 dilution; Invitrogen). Cells in S-phase were detected as follows: 1 hour before sacrifice, timed-pregnant dams were given a single intraperitoneal injection of 5-bromo-2′-deoxyuridine (BrdU; 50 μg/gm body weight). Tissue was fixed and sectioned as for in situ hybridization, and immunostaining for BrdU was performed as described (Murray et al., 2003). TdT-mediated dUTP nick end-labeling (TUNEL) staining to detect DNA fragmentation in apoptotic cells was performed as described (Murray et al., 2003), except that 20 μm cryosections of paraformaldehyde-fixed tissue were used and incubated with four changes of 10 mM citric acid (70°C; 15 minutes per wash) following permeabilization and prior to the TdT reaction. Texas Red-conjugated NeutrAvidin (1:200 dilution; Invitrogen) was used to detect incorporated biotin-16-dUTP (Roche).

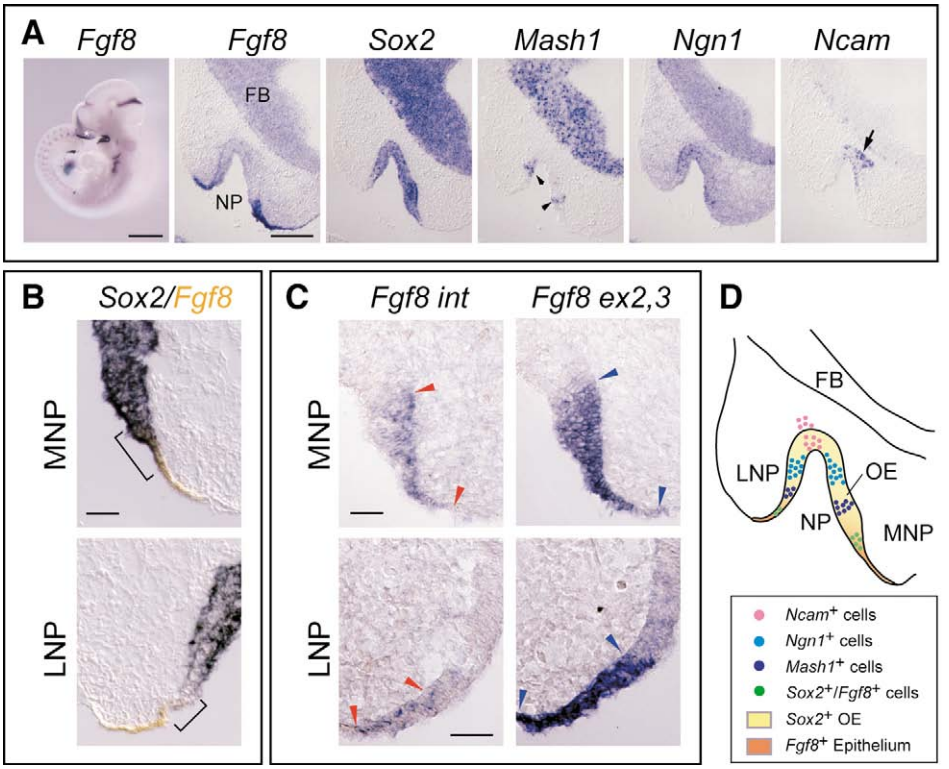

Fig. 1. Expression of *Fgf8* and neuronal cell markers in developing OE. (A) Five successive images show in situ hybridization for *Fgf8* (full-length ORF probe) and OE neuronal lineage markers in invaginating nasal pit (NP) at E10.5. In whole-mount in situ hybridization, *Fgf8* is detected in commissural plate and olfactory placode (white asterisk), branchial arches, mid-hindbrain junction, and limb and tail buds. Scale bar: 1 mm. In serial sections, locations of neuronal lineage markers within the OE are shown: arrowheads indicate *Mash1*-expressing cells, arrow indicates *Ncam1*-expressing neurons. Scale bar: 200 μm. (B) Double label in situ hybridization for *Fgf8* (full-length ORF probe, orange) and *Sox2* (blue) demonstrates overlap of the two markers in a small rim of surface ectoderm and adjacent invaginating neuroepithelium (brackets). Scale bar: 50 μm. (C) In situ hybridization for unprocessed, intronic RNA (*Fgf8int* probe) expression and processed *Fgf8* mRNA (*Fgf8ex2,3* probe) expression. Red and blue arrowheads indicate the basolateral extent of intronic RNA versus processed mRNA expression, respectively. The domain of mRNA expression subsumes that of intronic expression. Scale bars: 50 μm. LNP, lateral nasal process; MNP, medial nasal process. (D) Model of peripheral-to-central process of neuronal differentiation in developing OE and origin of *Sox2*-expressing neural stem cells from *Fgf8*-expressing ectoderm.

Results

Fgf8 is expressed in a neurogenic domain of invaginating nasal epithelium

To understand how *Fgf8* might act to regulate OE neurogenesis, we first performed in situ hybridization for *Fgf8* [using a probe encompassing the entire open reading frame (ORF) of the cDNA; see Materials and methods] and OE neuronal lineage markers on serial sections of invaginating NPs at day 10 of gestation (E10.5). As shown in Fig. 1A, *Fgf8* is expressed in cells within a domain that encompasses a ring of ectodermal epithelium at the rim of the invaginating NP, as well as adjacent neuroepithelial cells inside the pit. *Sox2* expression is observed throughout the entire neuroepithelium of the invaginating NP, and defines the OE at this early stage. Interestingly, expression of different OE neuronal cell type-specific markers occurs in an outside-in pattern that reflects the stage of each expressing cell in the neuronal lineage: cells expressing *Mash1*, which marks the earliest committed neuronal progenitors, are present next to the *Fgf8*-expressing cells at the inner rim of the NP; adjacent to *Mash1*-expressing cells are *Ngn1*-expressing INPs; and in the center of the pit lie cells that are positive for *Ncam1* (expressed by postmitotic ORNs).

Because the data in Fig. 1A suggest an overlap in the expression domains of *Fgf8* and *Sox2*, which marks many early neuroepithelial stem cells as well as OE neural stem cells once the definitive OE structure has been established (Beites et al., 2005; Ellis et al., 2004; Graham et al., 2003; Kawauchi et al., 2004; Wood and Episkopou, 1999), we performed two sets of experiments to try to determine if a subpopulation of the *Fgf8*-expressing cells in the NP are early OE neural stem cells. In the first experiment, we used double-label in situ hybridization for *Fgf8* (ORF probe) and *Sox2* on the same sections. The results, shown in Fig. 1B, indicate that many *Fgf8*-expressing cells that lie within the neuroepithelium of the invaginating NP also express *Sox2*. These cells also express *Pax6* and *Dlx5*, other markers of definitive OE at this stage (see Fig. S1 in the supplementary material). Thus, by the criterion of *Sox2* expression, a subpopulation of *Fgf8*-expressing cells can be considered to be early OE neural stem cells. The presence of cells that co-express both *Fgf8* and *Sox2* suggested to us that *Fgf8* expression defines a region from which neural stem cells emerge and enter the invaginating olfactory neuroepithelium. Moreover, the sequential appearance of cells expressing markers of successively more differentiated stages in the OE neuronal lineage, in NP regions that are further and further from the *Fgf8*-expressing domain, is consistent with the idea that OE morphogenesis and initiation of the OE neuronal lineage proceed in an outside-in fashion, with early stem cells at the periphery and terminally differentiated ORNs in the center of the NP (cf. Cau et al., 1997).

In a second set of experiments, we investigated the origin of *Fgf8*-expressing cells in the OE neuroepithelium, using a technique similar to that of Dubrulle and colleagues (Dubrulle and Pourquie, 2004). In situ hybridization was performed on adjacent sections of invaginating NP, in one case using a probe to intron sequences to determine the location of cells that initially transcribe unprocessed *Fgf8*

RNA (‘*Fgf8 int*’ probe), and in the second case a probe for exons 2 and 3 of the processed mRNA (‘*Fgf8 ex2,3*’ probe). The results, shown in Fig. 1C, demonstrate that the cells which initially transcribe *Fgf8* form a subset of all cells that actually contain *Fgf8* RNA in this region. These *Fgf8*-transcribing cells appear to be located preferentially at the rim of the NP and in the basal region of the invaginating neuroepithelium. By contrast, cells that contain processed mRNA for *Fgf8* (positive for the *Fgf8 ex2,3* probe) are found more extensively both within invaginating neuroepithelium and in the ectoderm surrounding the NP. As cells that are *Fgf8 ex2,3* positive must be the progeny of the *Fgf8 int*-positive cells (Dubrulle and Pourquie, 2004), these findings suggest a lineal relationship between the *Fgf8*-expressing ectodermal cells that define the rim of the invaginating NP and the neuroepithelial cells that express both *Fgf8* mRNA and *Sox2*. Thus, at least some of the *Sox2*-expressing neural stem cells in the developing OE must be derived from *Fgf8*-expressing ectodermal cells. Altogether, these observations suggest that *Fgf8* expression defines a morphogenetic center that gives rise to at least some of the *Sox2*-expressing neural stem cells of the OE, and that the earliest of these neural stem cells themselves transcribe *Fgf8*. These ideas are depicted in the cartoon shown in Fig. 1D.

At later stages (beyond E12-13), OE stem and progenitor cells take on a more restricted location, and come to lie in a compartment adjacent to the basal lamina of the epithelium (Kawauchi et al., 2004). Analyses by in situ hybridization and RT-PCR indicate that *Fgf8* continues to be expressed in scattered cells located in the basal compartment of OE at E14.5 (see Fig. S2 in the supplementary material). These data suggest that *Fgf8* continues to be expressed in or near stem and progenitor cells of the OE. Moreover, in vitro assays show that recombinant FGF8 is capable of stimulating development of neural stem cells and proliferation of neuronal progenitors in cultures taken from E14.5 OE (see Fig. S3 in the supplementary material), demonstrating that OE progenitors are responsive to FGF8 at this stage of development.

Localization of *Foxg1*-driven Cre activity and *Fgf8* conditional knockout strategy

To examine the functional importance of our observations from in situ hybridization and tissue-culture studies, we used a conditional *Fgf8* knockout model, using Cre-*LoxP* tissue-specific gene disruption to determine the role of FGF8 signaling in OE development from E10.5 to birth [complete loss of *Fgf8* results in early embryonic lethality (Meyers et al., 1998; Sun et al., 1999)]. A transgenic mouse line in which Cre recombinase expression is controlled by *Foxg1* (forkhead box G1, also known as brain factor 1; *Foxg1*-Cre line) regulatory elements was chosen, as mice carrying this allele have been shown to express Cre in anterior neural structures, including developing OE and forebrain (Hebert and McConnell, 2000). To confirm Cre activity in vivo, we crossed ROSA26 reporter mice [R26R (Soriano, 1999)] with *Foxg1-Cre* mice and analyzed resulting progeny by X-gal staining. Clear staining was observed in the anterior neural ridge at E8.5, prior to the time that olfactory placodes form (data not shown). A day later, at E9.5, we observed staining in olfactory placodes and OE, as well as forebrain neuroepithelium and branchial arches; OE and forebrain structures continued to be stained at later times in development (Fig. 2A). These data indicate that the *Foxg1-Cre* line is suitable for driving tissue-specific expression of Cre in olfactory neuroepithelium from the earliest time at which it can be observed to be differentiating from head ectoderm.

The strategy for generating conditional mutants is outlined in Fig. 2B. $Fgf8^{flox/flox};Foxg1^{+/+}$ females were crossed with $Fgf8^{d2,3/+};Foxg1^{Cre/+}$ males, and ~25% of embryos generated were $Fgf8^{flox/d2,3};Foxg1^{Cre/+}$ animals (hereafter referred to as ‘mutant’ animals). Mutant animals were detected at all embryonic stages and in newborn litters, but died shortly after birth owing to multiple defects (see below).

Mutant embryos have severe defects in frontonasal structures

Intact mutant embryos were examined from E9.5 to birth (Fig. 3A). Defects are evident as early as E9.5, primarily as a reduction in the size of the forebrain and frontonasal structures.

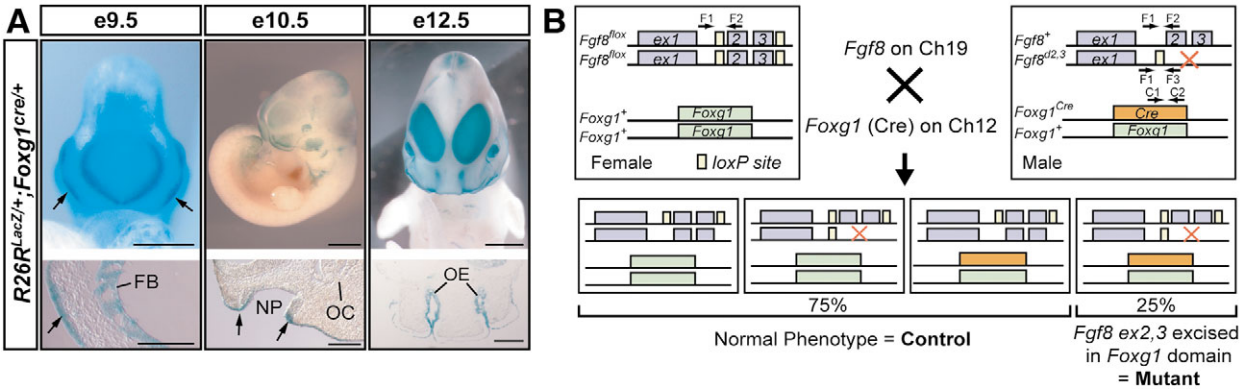

Fig. 2. Localization of *Foxg1*-driven Cre expression and strategy for generating *Fgf8* conditional knockouts. (A) X-gal staining of $R26R^{lacZ/+};Foxg1^{Cre/+}$ embryos at E9.5, E10.5 and E12.5. Top panels show whole-mount images and bottom panels show corresponding sections from embryos processed as whole mounts. At E9.5, staining is detected in forebrain (FB) and olfactory placode (arrows). At E10.5, staining is observed at rim of invaginating nasal pit (NP; arrows). OC, optic cup. At E12.5, prominent staining is observed in OE. Scale bars: 500 μm in the top panels; 200 μm in the bottom panels. (B) $Fgf8^{flox/flox},Foxg1^{+/+}$ females were crossed with $Fgf8^{d2,3/+};Foxg1^{Cre/+}$ males and embryos genotyped with three different primer sets to detect different alleles: *Fgf8-flox* and wild-type (F1 and F2), *Fgf8-d2,3* (F1 and F3) and *Cre* (C1 and C2). One-quarter of offspring had the $Fgf8^{d2,3/flox};Foxg1^{Cre/+}$ (mutant) genotype.

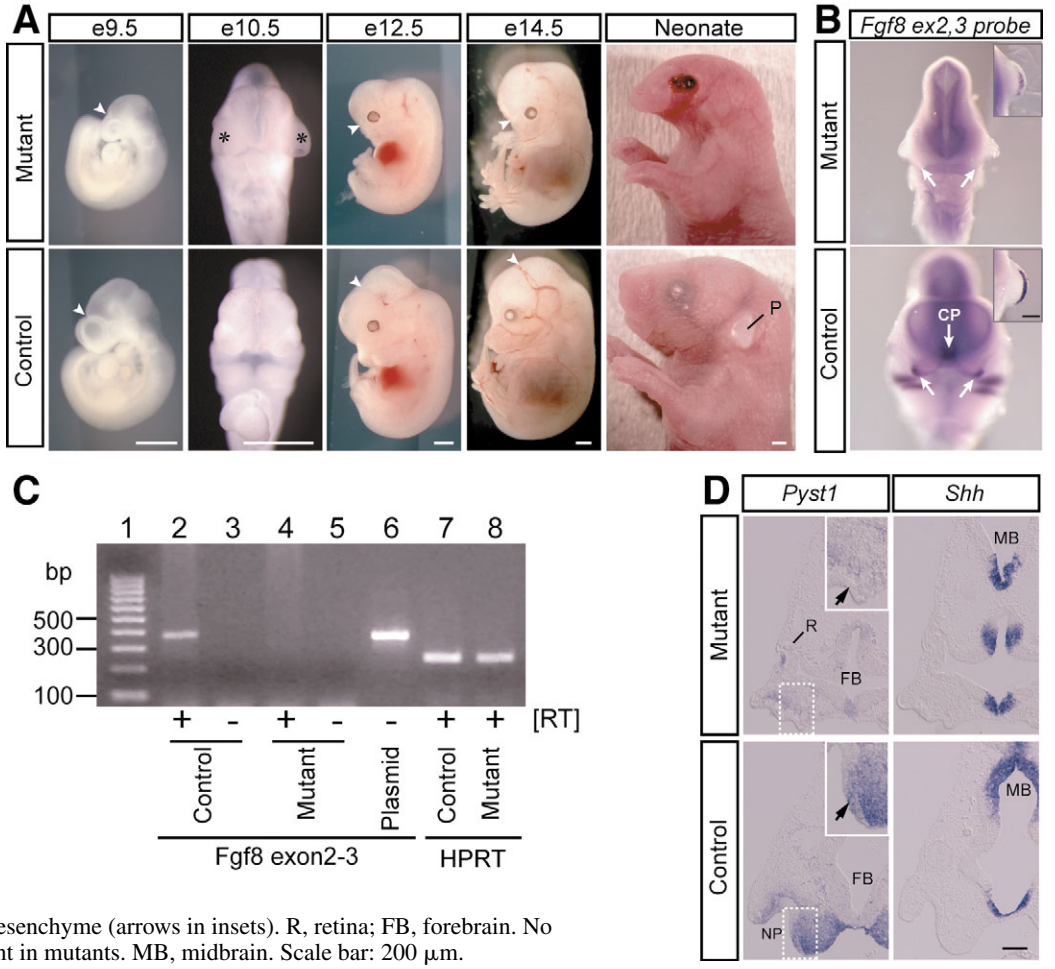

Fig. 3. Deficits in anterior neural structures and FGF-mediated signaling in mutant embryos. (A) Pictures of mutant animals and control littermates from E9.5 to birth. White arrowheads indicate forebrain-midbrain boundary. Black asterisks indicate nasal pits in E10.5 mutants. P, pinna. Scale bars: 1 mm. (B) Whole-mount in situ hybridization with *Fgf8 exon2,3* probe in E10.5 embryos. White arrows indicate nasal pits. CP, commissural plate. Insets show forelimbs of embryos hybridized with *Fgf8 ex2,3* probe. Scale bar: 0.5 mm. (C) RT-PCR for *Fgf8* exons 2 and 3 using cDNA from E10.5 forebrain/frontonasal tissue of control and mutant animals. Lane 1, 100 bp marker; lane 2, control tissue with *Fgf8 ex2,3* primers; lane 3, control tissue and no RT; lane 4, mutant tissue with *Fgf8 ex2,3* primers; lane 5, mutant tissue with no RT; lane 6, *Fgf8 exon2,3* plasmid control; lane 7, control cDNA and *Hprt* primers; lane 8, mutant cDNA with *Hprt* primers. (D) *Pyst1* expression is reduced in OE and underlying mesenchyme (arrows in insets). R, retina; FB, forebrain. No change in *Shh* expression is apparent in mutants. MB, midbrain. Scale bar: 200 μm.

Strikingly, all mutant animals have at least a rudimentary olfactory pit at E10.5; however, by this time, defects in medial nasal process development are often apparent, with this region being flattened and smaller than in controls. From E12.5 onwards, the forebrains of mutant embryos show dramatic reductions in size compared with control littermates; limbs and other posterior structures appear grossly normal, however. At birth, mutants have a small short snout, and the lower jaw, pinnae (outer ears) and eyelids are either reduced in size or absent. The size and gross structure of the eye itself appear to be unaffected in mutants (data not shown). Littermate embryos with genotypes other than $Fgf8^{flox/d2,3};Foxg1^{Cre/+}$ showed normal development and survived to adulthood.

To confirm that the phenotype we observed is due to absence of *Fgf8*, we performed whole-mount in situ hybridization (*Fgf8 ex2,3* probe) and RT-PCR at E10.5 to ensure that mutant embryos no longer express exons 2 and 3 of *Fgf8*, the regions of the gene that should be excised by Cre recombinase in mutant animals. In controls, *Fgf8 ex2,3* expression was detected in olfactory pits, commissural plate and branchial arches, whereas no signal was detected in these regions in mutants (Fig. 3B). Importantly, the *Fgf8 ex2,3* probe could detect normal *Fgf8* expression in the apical ectodermal ridge of the limb in both mutant and control embryos (Fig. 3B, insets). To ensure that no residual active *Fgf8* mRNA is produced in mutant animals, we also performed RT-PCR on frontonasal tissue of E10.5 embryos using primers specific for the exon 2,3-coding region. Expression was readily detectable in tissue from control animals, but was undetectable in mutants (Fig. 3C). Together, these data confirm that mutants express no detectable *Fgf8* mRNA in the frontonasal region.

FGF signaling is downregulated in *Fgf8* mutant embryos

Experiments described above demonstrate that *Fgf8* gene expression is effectively eliminated in the OE of mutant animals. However, it might still be the case that FGF8-mediated signaling could somehow be compensated for in mutants, thus complicating analysis of phenotypes. To test this hypothesis, we examined expression of *Pyst1/Mkp3* in the frontonasal region of E10.5 mutant and control littermate embryos. *Pyst1* encodes a tyrosine phosphatase that is an FGF-inducible antagonist of FGF signaling (Eblaghie et al., 2003), and is one of a number of genes in the FGF 'synexpression' group, i.e. genes expressed in the same temporal and spatial pattern when FGFs initiate signaling (Niehrs and Meinhardt, 2002). *Pyst1* is expressed in many known sites of FGF signaling in mouse embryos, including the olfactory system (Dickinson et al., 2002), making its expression useful as a read-out for active FGF signaling. As shown in Fig. 3D, *Pyst1* is normally expressed in the rim of the invaginating olfactory pit (where *Fgf8* itself is expressed) and in the mesenchyme

throughout the medial nasal process. In mutant embryos, expression of *Pyst1* is strongly downregulated in both domains (insets), confirming that loss of *Fgf8* leads to severe decrements in FGF signaling in the developing olfactory region.

We also examined expression of *Shh*, which, like *Fgf8*, is a key signaling molecule in limb and telencephalon (Ohkubo et al., 2002; Panman and Zeller, 2003), and which evidence suggests may be positively regulated by FGFs (Niswander et al., 1994; Zuniga et al., 1999). As shown in Fig. 3D, *Shh* is not expressed in OE at E10.5, but instead is expressed in the ventral wall of the telencephalon next to the medial nasal process and forebrain commissural plate. In mutants, the basic pattern of *Shh* expression appears unaffected. Expression of the SHH receptor patched, which is autoregulated through SHH signaling in overlapping and complementary patterns, also appeared unaffected in mutants (data not shown) (Drossopoulou et al., 2000; Marigo and Tabin, 1996; Platt et al., 1997). These observations indicate that effects on neurogenesis observed in mutants are unlikely to be mediated indirectly via *Shh* signaling (LaMantia et al., 2000).

Severe reduction or absence of olfactory structures in mutant embryos

To evaluate OE formation and growth in the absence of *Fgf8*, we analyzed sections of mutant and control animals from E10.5 to E17.5 (a minimum of four mutant animals were examined at each age, with a total of 31 analyzed). Normal OE development is shown in Fig. 4A. At E10.5, some olfactory placode/nasal pit structure was observed in all animals, even mutants; however, the OE and nasal cavity were severely reduced in size or absent in all mutant embryos by E11.5 (Fig. 4B). On the basis of histology, mutants were placed into one of two categories: Type A (aplastic) mutants had essentially no nasal cavity or OE detectable from E11.5 onward; Type B (hypoplastic) mutants retained a vestige of nasal cavity, usually present as an S-shaped tubular structure lined with an epithelium, at E11.5 and after. The numbers of each type of mutant found at each age are given in Table 1. Interestingly, at E10.5 some mutant embryos already displayed a phenotype, in that they had extremely small placodes and obvious reductions in the sizes of the lateral and medial nasal processes (Fig. 4B). We surmise that such embryos would exhibit the more severe, aplastic OE phenotype at later stages. Nasal bone structures are present in hypoplastic (Type B) mutants, and closely surround any remnant of OE, suggesting that bone formation in this region is at least partially dependent on OE development, possibly through an inductive signal derived from OE tissue. Of significance also is the fact that no vomeronasal organ (VNO) structure was observed in any mutant animals, whether these exhibited aplastic or hypoplastic phenotypes. As the VNO is derived from the olfactory placode, and starts to develop during the period when olfactory pits are invaginating (around E11.5) (Farbman, 1992), this observation indicates an absolute requirement for *Fgf8* in VNO formation.

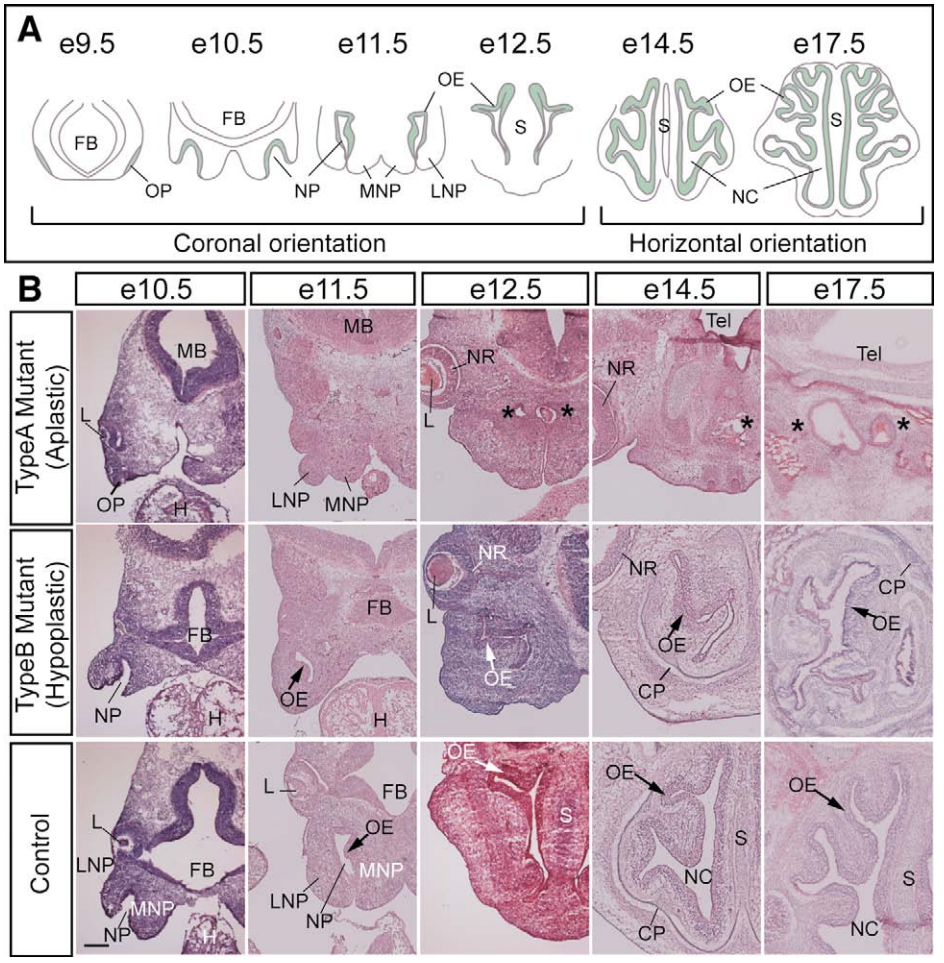

Fig. 4. Histological analysis of mutant OE. (A) Schematic model of mouse OE development. OP, olfactory placode; NP, nasal pit; FB, forebrain; MNP, medial nasal process; LNP, lateral nasal process; S, nasal septum; NC, nasal cavity. (B) Hematoxylin-eosin staining was performed on 20 μm cryosections through the entire frontonasal region of control and mutant embryos from E10.5-E17.5. Scale bar: 200 μm. CP, cartilage primordium of nasal capsule; H, heart; L, lens; MB, midbrain; NR, neural retina; Tel, telencephalon.

Neuronal cells form initially in mutant OE but fail to increase in number

The phenotypes observed in mutant animals suggested that *Fgf8* would be likely to affect development of cells in the OE neuronal lineage. To test this, we used in situ hybridization to compare expression of neuronal lineage markers in the OE of mutants and controls at four ages spanning the extent of prenatal OE development. Fig. 5A shows that at E10.5, mutant OE, which does not express functional *Fgf8*, continues to express *Pax6*, a gene known to be important in early determination of the OE

Table 1. Phenotypes of *Fgf8* conditional mutants

Gestational age	Type A Aplastic OE (% of total embryos)	Type B Hypoplastic OE (% of total embryos)	Total embryos examined
E10.5	0 (0%)	7 (100%)	7
E11.5	2 (40%)	3 (60%)	5
E12.5	2 (29%)	5 (71%)	7
E14.5	3 (37%)	5 (63%)	8
E17.5	3 (75%)	1 (25%)	4

Each embryo was cryosectioned (20 μm) throughout the entire frontonasal region and sections stained with Hematoxylin-eosin. Criteria for categorization of mutants is discussed in the text.

Table 2. Deficits in neuronal cell types in the OE of *Fgf8* mutants at E10.5

Neuronal lineage marker	Mean number of cells/ 0.03 mm^2 area (s.e.m.)		*n* (nasal pits evaluated)	*P* (*t*-test)
	Mutant	Control		
Mash1	4.45 (1.16)	23.30 (3.47)	Mutant=4 Control=4	0.002
Ngn1	33.37 (3.02)	48.57 (1.28)	Mutant=6 Control=3	0.012
Ncam1	7.29 (2.58)	23.95 (3.98)	Mutant=3 Control=3	0.025

In situ hybridization for each neuronal marker was performed on serial sections (20 μm) through the full extent of nasal pits in e10.5 mutants and littermate controls (20-25 sections in controls, 15-20 sections in mutants). The number of cells expressing a given marker and total OE area were measured for all sections encompassed by a given nasal pit. For comparison, cell counts were normalized to an area of 0.03 mm^2, the average area of OE in a single section.

(Grindley et al., 1995). In addition, neuronal lineage markers in E10.5 mutants are expressed in a pattern generally similar to that seen in controls: the early progenitor marker *Mash1* is expressed peripherally, with *Ngn1* expressed central to *Mash1* and *Ncam1* expressed in the deepest recess of the invaginating pit (Fig. 5A). We were surprised at this finding, as the OE is so severely affected in mutants at ages greater than E10.5. To determine if some defect in neurogenesis is already present at E10.5, we counted cells expressing each lineage marker throughout the entire extent of the nasal pit in mutant embryos and control littermates at this age. The data are shown in Table 2. Interestingly, every neuronal cell type is affected, with the largest reduction in *Mash1*-expressing cells [the first committed progenitor cells in the neuronal lineage (Calof et al., 2002)]: a greater than fivefold reduction was seen in the number of these cells in mutant OE, compared with controls. Thus, despite the fact that all cell types in the OE neuronal lineage initially form, major deficits in the numbers of these

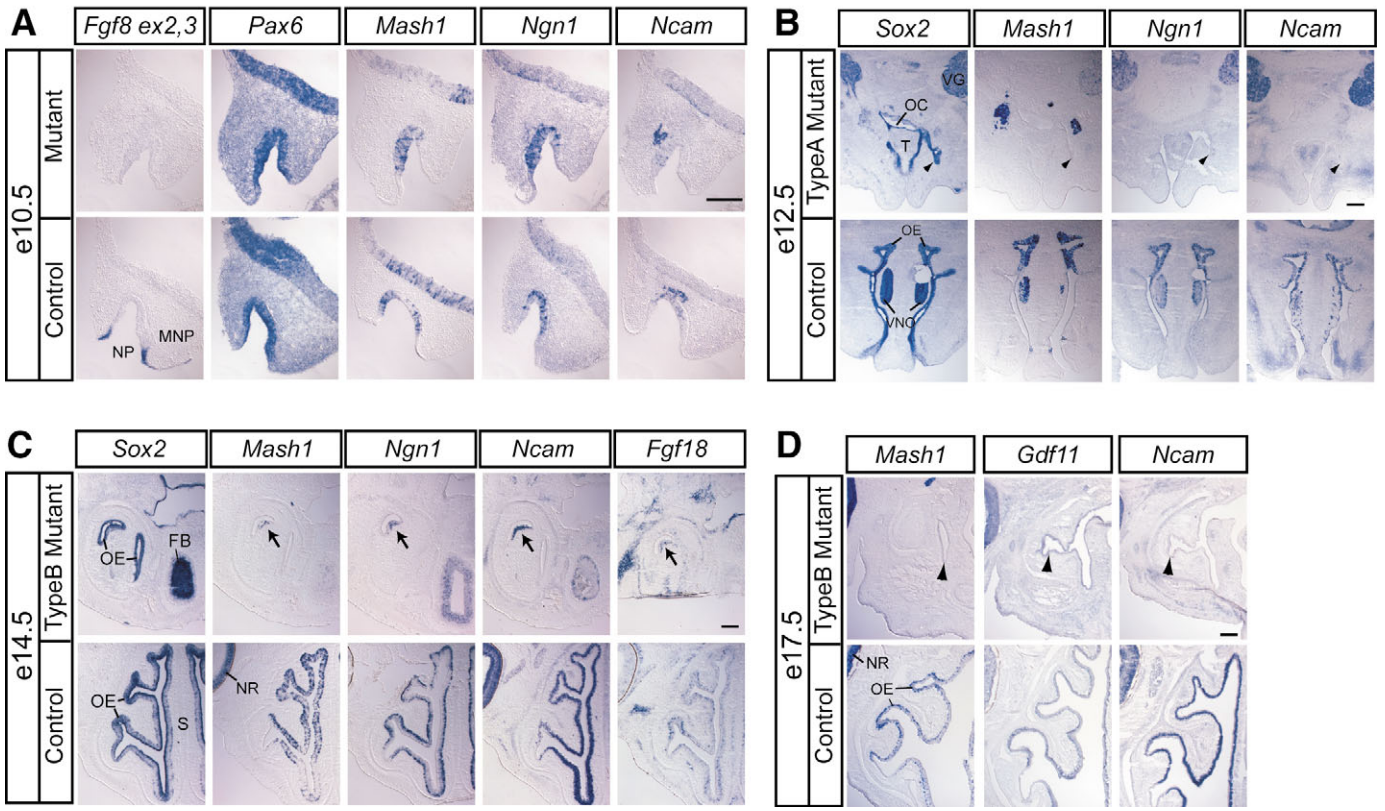

Fig. 5. Cessation of neuronal lineage development in mutant OE. (A) In situ hybridization on serial coronal sections through E10.5 nasal pit. No *Fgf8 ex2,3* is detected in mutant. (B) In situ hybridization on serial coronal sections of E12.5 Type A mutant. Arrowhead indicates region of *Sox2*-expressing epithelium in sections hybridized with other probes. (C) In situ hybridization on horizontal serial sections of E14.5 Type B mutant. Arrow indicates apparent OE remnant. (D) In situ hybridization on horizontal sections of E17.5 Type B mutant. Arrowheads indicate presumptive remnant of OE. NP, nasal pit; MNP, medial nasal process; VG, Vth ganglion; OC, oral cavity; T, tongue; VNO, vomeronasal organ; FB, (presumptive) forebrain; NR, neural retina; S, nasal septum. Scale bars: 200 μm.

cells are already apparent at E10.5, indicating that OE neurogenesis is already severely affected by absence of *Fgf8* at this age.

In analyses of older animals, we used an in situ hybridization probe for *Sox2* to delineate potential remnants of OE in the frontonasal regions of mutants. An example of a Type A (aplastic) mutant at E12.5 is shown in Fig. 5B. In Type A mutants, frontonasal structures are drastically reduced in size, resulting in compression of dorsal and ventral structures such that no obvious nasal cavity can be seen. In this particular embryo, a region of *Sox2*-expressing epithelium (Fig. 5B, arrowhead) was observed to bud off from the oral cavity (OC), suggesting that this might be a remnant of nasal cavity and contain OE neuronal cells. However, as shown in adjacent sections hybridized with probes for specific lineage markers, no neuronal cells were present in this region [the patches of ectopic *Mash1*-expressing cells in Fig. 5B (mutant) are not within the *Sox2*-expressing epithelium, and are probably sections through cranial ganglia]. Thus, by E12.5, neuronal development in the OE appears to have ceased altogether in Type A mutants.

Fig. 5C,D show Type B (hypoplastic) mutants, which retain small vestiges of OE. Even in mutants with this less severe phenotype, almost no OE neuronal cells remain, and the OE itself is very small compared with that of controls. Interestingly, we detected expression of *Fgf18*, which is closely related to *Fgf8* in structure and function (Maruoka et al., 1998; Ornitz and Itoh, 2001), in the OE remnant present in an E14.5 Type B mutant (Fig. 5C, arrows). This observation suggests that, in Type B mutants, there may be some compensation for loss of *Fgf8* function by *Fgf18*, provided that the OE is able to develop to the stage when *Fgf18* normally starts to be expressed in this region (about E12.5) (S. Kawauchi, data not shown) (see Bachler and Neubuser, 2001; Xu et al., 2000). Fig. 5D illustrates that, in older Type B mutants (E17.5), even when a relatively large remnant of nasal cavity persists (arrowheads), the epithelium that lines it is essentially devoid of cells expressing genes specific to OE neuronal progenitors and ORNs (*Mash1*, *Gdf11*, *Ncam1*) (see Wu et al., 2003). These observations indicate that, whatever process goes awry in *Fgf8* mutant OE, deficits appear very early in development, and the effects of these deficits are long lasting, such that few if any neuronal cells are able to form.

Loss of *Fgf8* results in increased cell death, not decreased cell proliferation

By what mechanism does loss of *Fgf8* cause loss of neuronal stem and progenitor cells in the OE? Potentially, FGF8 could stimulate neurogenesis by promoting proliferation and/or survival of neuronal stem and progenitor cells. Indeed, as described above, recombinant FGF8 – like other FGFs – is able to promote development of OE stem cells and proliferation of INPs in tissue culture assays (see Fig. S3 in the supplementary material) (DeHamer et al., 1994; Shou et al., 2000). To determine the mechanism(s) by which *Fgf8* acts in vivo, we performed in situ assays for both apoptotic cells and proliferating cells in mutant and control animals at early stages of NP invagination and OE development.

To identify apoptotic cells, we performed TUNEL assays on cryosections of OE of mutants and control littermates at E10.5, E12.5 and E14.5. The results are shown in Fig. 6A-C. Large numbers of apoptotic cells were observed throughout the *Fgf8*-expressing domain in mutants (detected in adjacent sections using a probe to *Fgf8 exon 1*, which is expressed, but does not generate functional protein, in *Fgf8* mutants). By contrast, very few apoptotic cells were observed in the *Fgf8*-expressing domain (detected using the *Fgf8 exon2,3* probe) in control littermates. The increase in the number of TUNEL-positive cells was greatest at E10.5, when mutant animals showed a 37-fold increase compared with controls [mutant: 1220±485 (s.e.m.); control: 37±18 (s.e.m.)]. At E12.5, the difference between mutant and control mice was smaller but still significant (Fig. 6B-C), whereas by E14.5 cell death was low, and had decreased to approximately the same level in mutants as in controls (Fig. 6C). Interestingly, *Sox2* expression also appears to be attenuated in the OE regions, showing high levels of apoptosis in mutants, particularly the OE lining the NP in the medial nasal process (Fig. 6A, *Sox2* panel, arrowhead), where FGF8 signaling is strongly reduced in mutants (Fig. 3D).

To determine if absence of functional *Fgf8* results in a change in cell proliferation, we performed immunostaining for the M-phase specific marker, phosphorylated histone H3 (Galli et al., 2004), and for BrdU incorporation (which detects cells in S phase), at E10.5. M-phase cells were observed primarily in the surface (apical) layer of OE in both control and mutant animals, but there was no obvious difference in either the pattern or number of immunopositive cells (Fig. 6D). To confirm this, total numbers of phosphohistone H3-immunopositive cells were counted in serial sections through the entire extent of the nasal pits in mutant and control animals. The results, shown in Fig. 6D,E confirm that there is no significant change in the mitotic index of OE cells in the absence of *Fgf8*. Levels of BrdU immunostaining were high in OE in both mutants and controls, and BrdU+ cells were particularly dense in the basal half of the epithelium in both cases (Fig. 6F). However, quantification of these sections again demonstrated that there was no significant decrease in the number of BrdU-immunopositive cells (i.e. cells in S phase) in the OE of mutant animals, compared with control littermates (Fig. 6G). Thus, the changes in OE neurogenesis seen at E10.5 appear to be the result of increased cell death, not decreased cell proliferation.

Discussion

Although other investigators have noted expression of *Fgf8* in developing olfactory pit (Bachler and Neubuser, 2001; Crossley and Martin, 1995; Mahmood et al., 1995), as well as some effects on OE development in vitro (LaMantia et al., 2000; Shou et al., 2000), the present study is the first to demonstrate that *Fgf8* function is required for development of the OE and maintenance of neurogenesis in this tissue in vivo. *Fgf8* is first expressed within a peripheral domain of ectodermal cells at the rim of the invaginating nasal pit, which we have termed a morphogenetic center. Our data indicate that the cells that initially transcribe *Fgf8* give rise to other cells that contain processed *Fgf8* mRNA and in addition express the neural stem cell marker *Sox2*. From these observations, we conclude that at least some of the *Sox2*-expressing neural stem cells of the OE are derived from the *Fgf8*-expressing ectodermal cells of the morphogenetic center that rims the invaginating nasal pit. In the absence of *Fgf8* function, there is

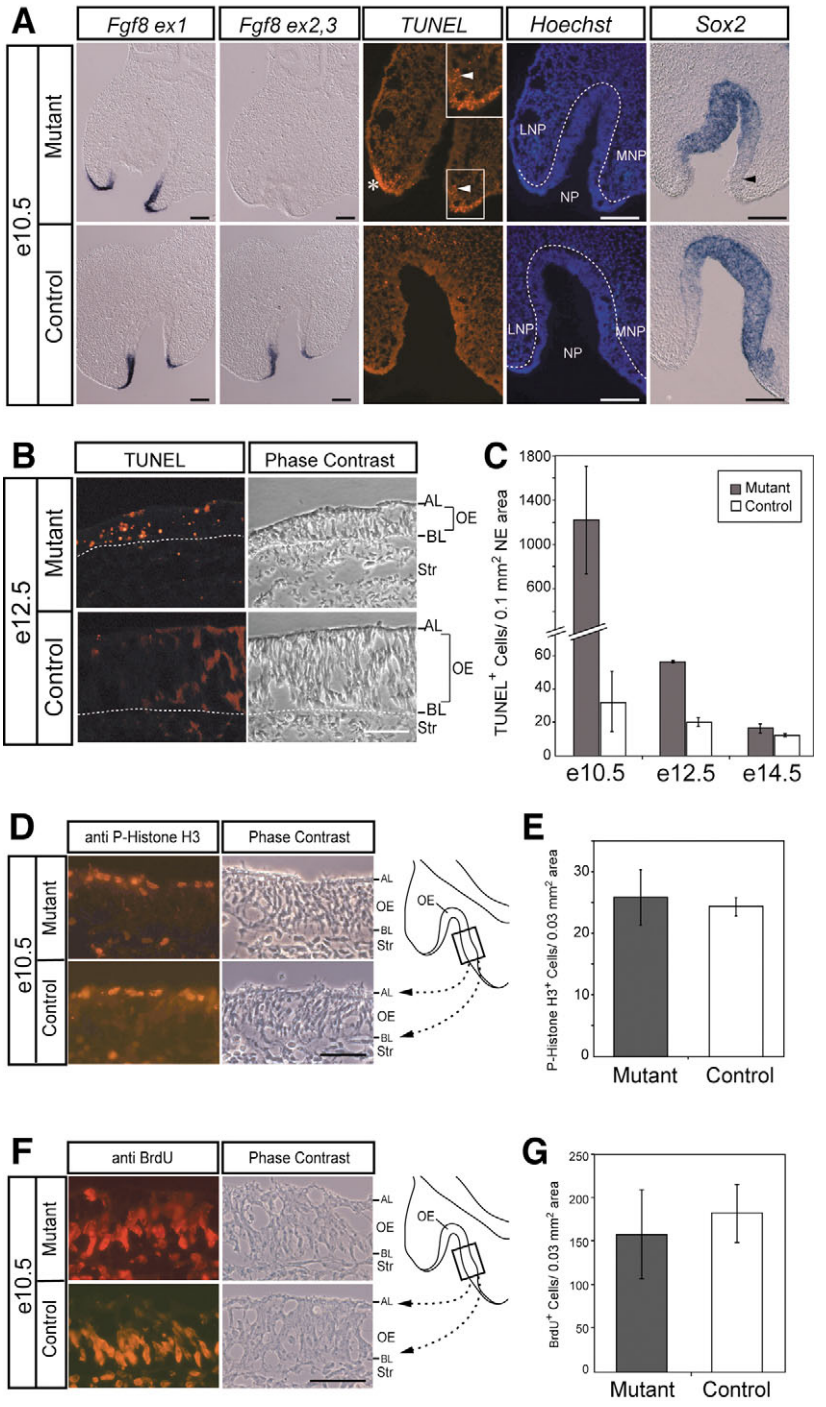

Fig. 6. *Fgf8* is required for cell survival in the neurogenic domain. (A) In situ hybridization for *Fgf8 ex1* probe indicates areas where (nonfunctional) *Fgf8* is expressed in mutants (*Fgf8 ex2,3* probe signal was not detected in mutants). TUNEL panel shows high number of apoptotic cells in mutants in ectoderm (white asterisk) and OE (white arrowhead; magnified in inset) of invaginating nasal pit (NP). Hoechst panel shows extent of invaginating NP. LNP, lateral nasal process; MNP, medial nasal process. *Sox2* expression is reduced in OE of MNP (black arrowhead). Scale bars: 100 μm. (B) High-power micrographs of TUNEL staining in OE of E12.5 Type B mutant and control littermate. Broken white line indicates basal lamina (BL) of OE. AL, apical surface; Str, stroma. Scale bar: 50 μm. (C) Total TUNEL+ cells in identifiable nasal epithelium (NE) were counted, and area of NE measured, in multiple sections at each age indicated. Data for each NP were summed and normalized to 0.1 mm^2, the average total NE area in a section at E12.5. Mean±s.d. for data from individual NPs are shown; NPs from a minimum of 2 animals of each genotype at each age were counted. Differences between mutants and controls were statistically significant at E10.5 (P=0.009, Student's t-test) and E12.5 (P=0.003), but not E14.5. (D) High-power micrographs of anti-phosopho-Histone H3 immunostaining at E10.5. Scale bar: 50 μm. (E) Quantification of data illustrated in D. (F) High-power micrographs of anti-BrdU immunostaining at E10.5. Scale bar: 50 μm. (G) Quantification of data illustrated in F. There are no significant differences between datasets in E and G . Data for each NP were summed and normalized to 0.03 mm^2, the average total OE area in a section at E10.5. Mean±s.d. for data from individual NPs is shown; NPs from a minimum of two animals of each genotype at each age were counted.

a large reduction in FGF-mediated signaling and a wave of apoptosis in cells within the *Fgf8*-expressing domain; this also results in a reduction in the number of *Sox2*-expressing putative neural stem cells. This wave of apoptosis, which begins at the earliest stages of OE development and persists for at least 2 days, severely limits subsequent expansion of the OE and associated neurogenesis. Nasal cavity development is profoundly affected and VNO structures never form. These findings, summarized in Fig. 7, demonstrate that *Fgf8* is a crucial determinant of OE and VNO development, and nasal cavity morphogenesis, and is required for olfactory neurogenesis. In addition, they indicate that cells in the *Fgf8*-expressing domain are crucial for the generation and survival of the stem cells that ultimately generate all cell types in the OE neuronal lineage, and indicate that some neural stem cells must ultimately be derived from *Fgf8*-expresing cells. Thus, when *Fgf8* is absent, neuronal progenitor cell populations cannot be replenished and neurogenesis ultimately ceases.

Fgf8 and the establishment of the OE neuronal lineage

The cells of the OE originate from the olfactory placodes, which first appear as two oval epithelial patches in the anterolateral region of the head around E9 in the mouse (Farbman, 1992). One day later, the placodes invaginate to form the nasal pits (NPs), which continue to deepen and elaborate a stereotyped pattern of folds (nasal turbinates) as development proceeds. The characteristic morphology of the nasal cavity and distribution of different cell types within the OE emerge at about E13-14. At this time, the overall number of mitotic figures decreases, and proliferating cells begin to concentrate in the basal layers of the OE, where neuronal progenitors and stem cells ultimately reside (Cuschieri and Bannister, 1975; Smart, 1971). These changes in cell

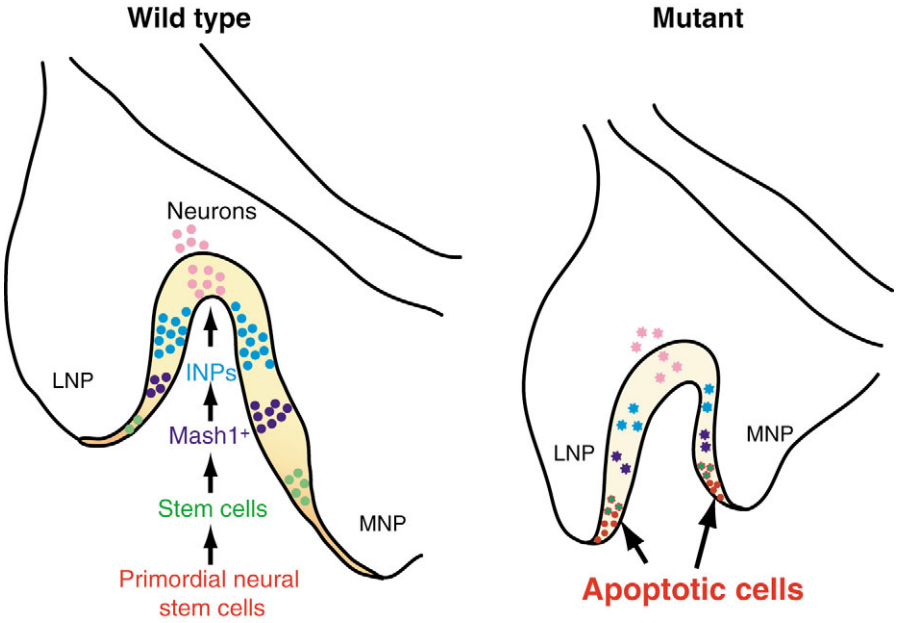

Fig. 7. Role of *Fgf8* in olfactory neurogenesis. Cartoon illustrating the relative positions of the *Fgf8* expression domain (anterior morphogenetic center) and different neuronal cell types during primary neurogenesis at E10.5 in normal OE (wild type) and a model for the role of *Fgf8* in primary olfactory neurogenesis based on the consequences of inactivating *Fgf8* in the anterior morphogenetic center using *Foxg1*-driven Cre (mutant). *Fgf8* expression domain, orange; *Sox2* expression domain (definitive neuroepithelium), yellow; primordial neural stem cells (co-expressing *Sox2* and *Fgf8*), green; *Mash1*-expressing committed neuronal progenitors, dark blue; INPs, light blue; *Ncam1*-expressing neurons, pink. Cells in the *Fgf8* expression domain that undergo apoptosis when *Fgf8* is inactivated are shown in red, and apoptotic primordial neural stem cells in green with a red jagged border. Vestigial populations of other neuronal cell types are shown in their corresponding colors, but with jagged borders. For discussion, see text.

proliferation and location that occur at this time appear to mark a developmental transition from an early morphogenetic form of neurogenesis (primary neurogenesis) to neurogenesis of an established pattern, in which the proportions of different cell types are maintained at a fairly constant level within the OE (established neurogenesis) (see Kawauchi et al., 2004).

What role does *Fgf8* play in establishing the OE neuronal lineage and in primary neurogenesis? Our findings indicate that *Fgf8* is initially transcribed in the most peripheral domain of the invaginating NP, outside of the region where lineage-specific markers such as *Mash1* and *Ngn1* are expressed (Fig. 1). The *Fgf8* mRNA expression domain expands and comes to include neuroepithelial cells that express genes that are definitive markers of olfactory neuroepithelium, including *Sox2*, *Dlx5* and *Pax6* (Fig. 1 and see Fig. S1 in supplementary material). Indeed, recent data suggest that *Sox2* expression characterizes the stem cell population in the OE during established neurogenesis (Kawauchi et al., 2004; Beites, 2005). Our observations therefore suggest that *Fgf8* is expressed in a morphogenetic center, in and around the cells that are responsible for initiating primary olfactory neurogenesis – cells that we now consider to be the primordial neural stem cells of the OE, by analogy to the primordial germ cells that ultimately give rise to gametes. In the absence of *Fgf8* function, severe deficits in primary neurogenesis rapidly take place (Figs 3-5) owing to the high levels of apoptosis that take place in the *Fgf8* expression domain (Fig. 6). This model of *Fgf8* action during primary OE neurogenesis is depicted in Fig. 7.

Fgf8 acts by controlling cell survival

A dramatic phenotype observed in *Fgf8* mutants was the very high level of programmed cell death at E10.5 (Fig. 6). In mutants at this time, cells in the *Fgf8*-expressing domain, as well as surrounding neuroepithelium, are unable to survive and the numbers of all neuronal cell types are subsequently depleted. The few cells already committed to the neuronal lineage appear to be able to continue through the maturation process, as some neuronal cells can be observed in Type B mutants as late as E14.5 (Fig. 5). However, as neuronal stem cells ultimately die in the absence of *Fgf8*, the mature characteristics of the OE and neurogenesis within this epithelium are never established, even in the least severe mutants (Fig. 5). These findings are consistent with observations made in studies of various *Fgf8* mutants in other tissues. For example, when *Fgf8* expression is eliminated from the apical ectodermal ridge (AER) of developing limbs, AER cells and the mesenchyme that underlies them undergo apoptosis (Sun et al., 2002). In addition, Storm and colleagues have reported that telencephalic neural progenitors undergo apoptosis when *Fgf8* is eliminated, the likely cause of the defects in forebrain development observed in our study (Storm et al., 2003) (compare with Fig. 3). Our results, in addition to these and results from a number of labs studying *Fgf8* function, suggest that FGF8 acts as a survival factor for crucial stem cell populations in a wide variety of tissues in which it is expressed (Abu-Issa et al., 2002; Chi et al., 2003; Frank et al., 2002; Storm et al., 2003; Sun et al., 2002; Trumpp et al., 1999).

Although the molecular details of how *Fgf8* prevents cell death are incompletely understood, our analysis of *Pyst1*, which encodes a MAPK-specific phosphatase whose expression is dependent on MAPK signaling (Eblaghie et al., 2003), indicate that the RAS/MAPK pathway activity may be involved. This pathway is known to regulate apoptosis in other systems (Downward, 1998). One possibility, suggested by our data, is that absence of *Fgf8* at this early crucial juncture leads to downregulation of the RAS/MAPK pathway, which ultimately acts as a trigger for death of the primordial neural stem cells that are ultimately responsible for establishing the neurogenic pathway in the OE. Consistent with this notion, in vitro studies have shown a relationship between maintenance of FGF signaling and prevention of apoptosis in P19 cells (Miho et al., 1999).

Are effects of loss of *Fgf8* direct or indirect?

Overall, our observations suggest a model in which FGF8 acts in an autocrine and/or paracrine manner in cells of the

developing OE and surrounding anterior ectoderm. However, the dramatic effects of loss of *Fgf8* function on both craniofacial morphogenesis and forebrain development, observed by us in the present study and by others in this and other *Fgf8* mutants (Abu-Issa et al., 2002; Storm et al., 2003; Trumpp et al., 1999), raise the alternative possibility that effects on OE neurogenesis observed in mutants could be caused indirectly, owing to effects on these other tissues. As we observe a reduction is FGF-mediated signaling in the mesenchyme surrounding the invaginating olfactory neuroepithelium, as well as in the *Fgf8*-expressing neuroepithelium itself (Fig. 3C), the possibility that loss of *Fgf8* disrupts an epithelial-mesenchymal signaling loop that may feed back to promote OE neurogenesis cannot be discounted totally by our data. Because by far the most cell death we observe in mutants is in *Fgf8*-expressing ectoderm and neuroepithelium, and not in underlying mesenchyme (Fig. 6B and data not shown), we do not think that alterations in FGF8-mediated signaling in mesenchyme are responsible for the defects – especially cell death – that we observe in OE. It may still be the case that loss of FGF8 signaling in mesenchyme contributes to the defects in nasal cavity formation we observe, however, possibly by interrupting BMP-mediated signaling in this tissue; we are currently exploring this possibility (S.K., C. E. Crocker and A.L.C., unpublished).

Because *Fgf8* mutants also exhibit strong defects in the developing telencephalon, it might also be argued that proper OE development is dependent on proper formation of the olfactory bulbs (OBs), and thus that effects on OE neurogenesis in mutants might be indirectly mediated via effects on the OB. To test this idea, we examined OE development and OB structure using in situ hybridization in *Fgf8* hypomorphs ($Fgf8^{neo/neo}$), which have been reported to lack most or all OB tissue (Garel et al., 2003; Meyers et al., 1998). Our observations indicate that the normal complement of *Ncam1*-expressing ORNs is present in the OE of *Fgf8* hypomorphs at P0, even though these animals fail to develop any proper OB and have profound reductions in the number of OB neurons and neuronal progenitors (see Fig. S4 in the supplementary material). Similar results have been obtained from studies of other mutant mouse strains in which OBs fail to form during development because of defects in genes other than *Fgf8* [e.g. $extra\text{-}toes^{J}$ (Xt^{J}) mutant mice (Sullivan et al., 1995)]. These observations indicate that *Fgf8* regulates neurogenesis in the OE independently from its regulation of neurogenesis in the OB. Taken together, the data support the conclusion that defects in OE neurogenesis observed in *Fgf8* mutants in the present study are due to direct effects of loss of endogenous *Fgf8* on developing OE, and not to indirect effects resulting from failure of OB formation.

What cell type is dying in *Fgf8* mutants?

Our data from adjacent serial sections indicate that it is *Fgf8*-expressing cells themselves that undergo apoptosis in the absence of FGF8 signaling (Fig. 6). The results of in situ hybridization analysis using probes to intronic versus exonic sequences indicate that ectodermal cells that initially transcribe *Fgf8* RNA give rise to a larger population of neuroepithelial cells, some of which continue to express processed *Fgf8* mRNA (Fig. 1C). Taken together with double-label in situ hybridization results showing overlap between the domains of *Fgf8* and *Sox2* expression (Fig. 1B), these findings strongly suggest that at least some *Fgf8*-expressing cells become what we have termed primordial neural stem cells of the OE (*Fgf8+/Sox2+* cells) [compare Fig. 7 with Kawauchi et al. (Kawauchi et al., 2004)]. Therefore, as apoptosis in mutant OE is most extensive in the *Fgf8+* domain (Fig. 6B), we infer that at least some of the cells that are dying in mutants are primordial neural stem cells, an idea supported by the finding that *Sox2* expression is attenuated in mutant OE in the region where *Fgf8* expression and *Sox2* expression normally overlap (Fig. 6A). However, as the regions of both *Fgf8* expression and apoptosis in the mutant extend beyond the neuroepithelial domain defined by *Sox2* expression, it is also likely to be the case that some of the dying cells in mutants are not committed neural stem cells. Thus, we conclude that apoptotic cells include both *Fgf8*-expressing cells committed to the neural lineage of the OE (i.e. primordial neural stem cells), as well *Fgf8*-expressing cells that may not be destined to undergo this commitment step. Ultimately, the decreased numbers of all neuronal cell types, the failure of the OE and the VNO to develop, and abortive nasal cavity morphogenesis in mutant animals, together demonstrate the profound dependence of both OE neurogenesis and anterior craniofacial development on developmental expression of *Fgf8*.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/23/5211/DC1

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