Developmental Biology 349 (2011) 250–260

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

The canonical Wnt/ß-catenin signaling pathway regulates Fgf signaling for early facial development

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ARTICLE INFO

Article history:
Received for publication 30 May 2010
Revised 1 November 2010
Accepted 2 November 2010
Available online 9 November 2010

Keywords:
Wnt/ß-catenin signaling
Anterior neural ridge (ANR)
Facial ectoderm
Foxg1-Cre
Fgf8
Mice

ABSTRACT

The canonical Wnt/ß-catenin signaling pathway has implications in early facial development; yet, its function and signaling mechanism remain poorly understood. We report here that the frontonasal and upper jaw primordia cannot be formed after conditional ablation of *ß-catenin* with *Foxg1-Cre* mice in the facial ectoderm and the adjacent telencephalic neuroepithelium. Gene expression of several cell-survival and patterning factors, including *Fgf8, Fgf3, and Fgf17*, is dramatically diminished in the anterior neural ridge (ANR, a rostral signaling center) and/or the adjacent frontonasal ectoderm of the ß-catenin conditional mutant mice. In addition, *Shh* expression is diminished in the ventral telencephalon of the mutants, while *Tcfap2a* expression is less affected in the facial primordia. Apoptosis occurs robustly in the rostral head tissues following inactivation of Fgf signaling in the conditional mutants. Consequently, the upper jaw, nasal, ocular and telencephalic structures are absent, but the tongue and mandible are relatively developed in the conditional mutants at birth. Using molecular biological approaches, we demonstrate that the *Fgf8* gene is transcriptionally targeted by Wnt/ß-catenin signaling during early facial and forebrain development. Furthermore, we show that conditional gain-of-function of ß-catenin signaling causes drastic upregulation of *Fgf8* mRNA in the ANR and the entire facial ectoderm, which also arrests facial and forebrain development. Taken together, our results suggest that canonical Wnt/ß-catenin signaling is required for early development of the mammalian face and related head structures, which mainly or partly acts through the initiation and modulation of balanced Fgf signaling activity.

Introduction

The highly complex vertebrate face arises from several facial primordia that consist of an epithelial cover (developed from the ectoderm externally and the endoderm internally), the neural crest-derived mesenchyme (which mainly gives rise to the facial skeleton), and the mesodermal core (which develops facial muscles) (Chai and Maxson, 2006; Creuzet et al., 2005; Minoux and Rijli, 2010; Noden and Francis-West, 2006). Outgrowth and fusion of the paired maxillary prominences (maxp), paired medial and lateral nasal prominences (mnp and lnp), and a single midline frontonasal mass (fnm) establish the mid and upper face, including the upper jaw, upper lip, nose, and forehead (Szabo-Rogers et al., 2010). The maxp is developed from the first pharyngeal arch, while lnp, mnp and fnm originate from a single primordium, the frontonasal process or prominence (fnp). The mandibular prominences (manp) derive from the first pharyngeal arch and form the lower jaw. Several key morphogenetic signaling pathways, such as Wnt, Fgf, Shh, and Bmp, other pathways such as endothelin and Dlx signaling, as well as an increasing array of molecules, are involved in dynamic facial morphogenesis at various developmental sites and stages (Clouthier and Schilling, 2004; Depew et al., 2005; Jiang et al., 2006; Nie et al., 2006a,b; Szabo-Rogers et al., 2010; Wilkie and Morriss-Kay, 2001).

The role of the Wnt/ß-catenin signaling pathway in craniofacial morphogenesis has been revealed mainly through studying the development of neural crest cells and their craniofacial derivatives (Christiansen et al., 2000; Garcia-Castro et al., 2002; Huang and Saint-Jeannet, 2004; Wu et al., 2003). Indeed, conditional deletion of *ß-catenin* in neural crest cells with *Wnt1-Cre* resulted in failure of craniofacial development in mice (Brault et al., 2001). ß-catenin is a key intracellular molecule in the canonical Wnt signaling pathway (Mosimann et al., 2009; Willert and Nusse, 1998). On binding of Wnt ligands to Fzd and Lrp receptors, the intracellular ß-catenin is stabilized by inactivation of a ß-catenin degradation complex (which consists of Gsk3ß, APC, Axin, CK1 and others), and then translocated into the nucleus to activate Tcf/Lef transcription factors binding to the promoter region of the Wnt target genes. The canonical Wnt/ß-catenin signaling pathway plays important roles in development and disease (Logan and Nusse, 2004; MacDonald et al., 2009). Unique activation patterns of several Wnt signaling

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doi:10.1016/j.ydbio.2010.11.004

components and signaling reporters are found in the facial prominences (Brugmann et al., 2007; Lan et al., 2006; Parr et al., 1993; Song et al., 2009; Vendrell et al., 2009). *Lef1* and *Tcf4* double knockout mice exhibit abnormal mid face (Brugmann et al., 2007), and mutations in human *WNT3* and mouse *Wnt9b* genes have been associated with cleft lip/palate (Carroll et al., 2005; Niemann et al., 2004). We have recently demonstrated that Lrp6-mediated Wnt signaling is required for the growth and fusion of facial primordia, and that genetic disruption of Lrp6 signaling in mice leads to cleft lip with cleft palate, which resembles a common birth defect in humans (Song et al., 2009). Yet, the role of the Wnt/ß-catenin signaling pathway in early facial development, particularly in the formation and patterning of facial primordia remains almost unknown.

Other than neural crest cells, both facial ectoderm and forebrain have been suggested as critical for facial morphogenesis in chick models (Hu and Marcucio, 2009; Hu et al., 2003; Marcucio et al., 2005). To address the related mechanisms and the role of the Wnt/ß-catenin signaling pathway in early facial morphogenesis, we employed a widely used loxP-floxed *ß-catenin* conditional gene-targeting mouse line (Brault et al., 2001; Grigoryan et al., 2008) and a well-established *Foxg1*^{kiCre} mouse line (Hebert and McConnell, 2000). We show that ß-catenin in the facial ectoderm and/or telencephalic neuroepithelium is required for formation and patterning of the frontonasal and maxillary processes at the earliest stage of facial development. Combined with molecular biological approaches and gain-of-function analyses, we further demonstrate that the Wnt/ß-catenin signaling pathway controls early facial morphogenesis mainly or partly through regulating Fgf signaling in the anterior neural ridge (ANR) and facial ectoderm in mice.

Materials and methods

Mice

This study used the following genetically modified mice: conditional ß-catenin loss-of-function (LOF) *Catnb1*$^{lox(ex2\text{-}6)}$ (Brault et al., 2001) (Jackson Laboratory stock no. 004152), conditional ß-catenin gain-of-function (GOF) *Catnb1*$^{lox(ex3)}$ (Harada et al., 1999), *Foxg1*^{kiCre} (Hebert and McConnell, 2000) (Jackson Laboratory stock no. 004337), and Cre reporter *Rosa26lacZ* (Soriano, 1999) (Jackson Laboratory stock no. 002292). Mice were housed in the vivarium of the UC Davis School of Medicine (Sacramento, CA, USA). The day of conception was designated embryonic day 0 (E0). All research procedures using mice were approved by the UC Davis Animal Care and Use Committee and conformed to NIH guidelines.

Skeletal and H&E stains

E18.5 embryos were dissected in PBS, fixed in 95% ethanol for one week, and then stained with Alcian Blue and Alizarin Red for bone and cartilage according to a published protocol (McLeod, 1980). For general histological analyses, embryos were fixed in 4% paraformaldehyde (PFA), frozen-embedded in O.C.T. compound (Sakura Finetek USA), sectioned with a Leica cryostat, and then stained with H&E solutions according to standard protocols.

Wholemount in situ hybridization, X-gal staining, immunofluorescence, and TUNEL

These experiments were carried out as described previously (Song et al., 2009). Embryos were fixed in 4% PFA for most experiments. Wholemount *in situ* hybridization was performed according to standard protocols using digoxigenin-labeled antisense RNA probes (Zhou et al., 2008; Zhou et al., 2004). X-gal staining (Wang et al., 2008) was carried out overnight on embryos after brief fixation with 2% PFA. The wholemount embryos after *in situ* hybridization or X-gal staining were sectioned at 50 μm with a vibrotome. Immunofluorescence was carried out on 7- to 10-μm frozen or paraffin-wax embedded tissue sections using appropriate primary antibodies and Alexa fluorescence-conjugated secondary antibodies (Molecular Probes). Rabbit anti-phospho-histone H3 (pHH3, Cell Signaling) and rabbit anti-ß-catenin (Santa Cruz Biotech) were used in this study. TUNEL assays were performed using the Dead End Fluoreometric TUNEL system (Promega), following the manufacturer's instructions.

RNA isolation and real-time quantitative RT-PCR

Total RNA was isolated from tissue dissected from the rostral head region (including both orofacial and forebrain primordia) of E9.5 embryos (wild-type littermate controls and mutants or LiCl/NaCl-treated embryos). Semiquantitative PCR was carried out as described previously (Song et al., 2009). The mRNA levels of *ß-catenin*, *Fgf8*, *Fgf3*, *Fgf17*, *Fgf4*, and *Fgf10* were normalized to the mRNA levels of *Gapdh* (*Glyceraldehyde-3-phosphate dehydrogenase*) to allow comparisons among different experimental groups using the ΔC_t method (Goydos and Gorski, 2003). Primers for these genes are listed in Table 1.

Chromatin immunoprecipitation (ChIP)

In vivo ChIP was carried out as described previously (Song et al., 2009). Briefly, rostral heads including facial and forebrain tissues were dissected from E9.5 embryos in ice-cold PBS. After pipetting and cross-linking with 2% formaldehyde, chromatin extraction and immunoprecipitations were performed with a ChIP assay kit according to the manufacturer's protocols (Upstate Biotechnology). Rabbit anti-ß-catenin antibody (Santa Cruz Biotechnology) was used to pull down the ß-catenin/Tcf/DNA complex; rabbit IgG was used as a negative control. The following PCR primers against the *Fgf8* promoter region were used: Tcf/Lef-binding site 1 (BS1) (Fig. 8A), 5′-TGCTTGCC TCTCTTTAGCC (forward), 5′-ATTTTTGAAGACCA*GGT*GGC (reverse); BS2, 5′-TACCTGTGTCTTGTGACTC (forward), 5′-AGCATATGAGATACT-CAGG (reverse); and BS3, 5′-TGATTGCAGATTCGAGGAAAC (forward), 5′-TGACTGACAGATGCAAATGTG (reverse).

Luciferase reporter assay

The luciferase reporter assay was carried out according to a procedure similar to one we described previously (Song et al., 2009). The 334-bp promoter region of the mouse *Fgf8* gene between −2290bp and −2624bp, which contains a conserved Tcf/Lef-binding site (BS3) (Fig. 8), and the same promoter region without BS3 were amplified and cloned into the luciferase reporter *O-Fluc* upstream of a minimal c-fos promoter. The resulting plasmids were designated *pFgf8-Luc* and *pFgf8-mut-Luc*, respectively, and transfected into L cells with Lipofectamine 2000 reagent following the manufacturer's instructions (Invitrogen). The cells were treated with 0–4 ng Wnt3a or 25 mM LiCl. L cells were also transiently transfected with *pFgf8-Luc*/*pFgf8-mut-Luc* in combination with either a control expression vector (*pcDNA3*), or the expression constructs of Lef1 and constitutively active ß-catenin (aß-catenin) (Lin et al., 2007). Luciferase activity was assayed using the Dual-Luciferase assay kit (Promega) after 24-hour transfection.

Table 1
Primers for real-time PCR.

Gene	Forward	Reverse
ß-catenin	TTAAACTCCTGCACCCACCAT	AGGGCAAGGTTTCGAATCAA
Fgf3	ACCTGGTGCCCAGAGACCTT	GCAGGAAGAGAGAGGACTTTTGTG
Fgf4	GCAGGAAGAGAGAGGACTTTTGTG	CCATTCTGGTAACAAAATTCCAAA
Fgf8	GCTCATTGTGGAGACCGATACTT	TGGCAATTAGCTTCCCCTTCT
Fgf10	CAGTAAGACACGCAAGCATTTACTG	AATCTGATCCAATTCTTCCATGGT
Fgf17	CCAACTCTACAGCAGGAC	CATACAGATGTACTTCTCACTC

Maternal administration of lithium chloride

Pregnant dams were injected with 30 μl of a 600-mM LiCl or NaCl solution each day starting at E7.5 through E9.5. Embryos were fixed and processed by *in situ* hybridization or real-time RT-PCR as described above.

Sample size and statistical analysis

Two to four samples per genotype were used for each experiment (detailed numbers were indicated in the figure legends). A minimum of three samples, or triplicates, were used for quantitative analyses. Student's *t*-test was used for statistical comparisons when appropriate, and differences were considered significant at $P<0.05$.

Results

Conditional gene-targeting of ß-catenin with Foxg1-Cre in the facial ectoderm and telencephalic neuroepithelium

To study the role of ß-catenin signaling in early facial development, we used Foxg1-Cre knockin (*Foxg1*^{kiCre}) mice and the loxP-flanked ß-catenin exons 2–6 *Catnb1*$^{lox(ex2-6)}$ mice for conditional loss-of-function (LOF) analysis of ß-catenin in the Foxg1-expressing tissues. The recombination activity of *Foxg1*^{kiCre} mice was previously reported to be weak in the anterior neural ridge (ANR) and rostral head region at E8.5 and E8.75 in Swiss Webster mice (Hebert and McConnell, 2000). To examine the recombination pattern of *Foxg1*^{kiCre} in the mixed 129 and C57BL mouse strain background used in the current study, we crossed *Foxg1*^{kiCre} with the Cre reporter *Rosa26lacZ* mice in the 129 and C57BL mixed background. We found strong X-gal staining (that represents the recombination activity) in the ANR, facial ectoderm, and adjacent telencephalic neuroepithelium at E8.75 (with 12 to 14 somite pairs) (Fig. 1A, B, and data not shown). This data indicates that *Foxg1*^{kiCre} is a powerful tool for conditional gene-targeting, not only in the forebrain but also in the facial ectoderm. Indeed, we found that ß-catenin-immunoreactivity was drastically lost in the facial ectoderm and telencephalic neuroepithelium, but relatively conserved in the facial mesenchyme (Fig. 1C, D). Real-time RT-PCR for *ß-catenin* mRNA from the rostral head tissue at E9.5 also confirmed the successful deletion of ß-catenin (Fig. 1E).

Facial deformation in the ß-catenin-LOF mutants

The mutant embryos of ß-catenin-LOF mice represented 25% of total embryos recovered, which matches the Mendelian ratio, and they survived until birth. All of the mutants displayed striking facial defects, including the absence of nasal, upper jaw, and ocular structures (Fig. 2). The upper lip and nasal primordia, including the medial nasal prominence, lateral nasal prominence, and maxillary prominence were well-developed in the normal embryos at E10.5 (Song et al., 2009); all of these structures were absent in the mutants (Fig. 2A, B and data not shown). The optic cup and telencephalic vesicle in the mutants were also not developed at this early stage. At E14.5, the facial defects in the mutants were further evident (Fig. 2C, D). In contrast, the tongue and mandible were formed in the mutants. Skeleton preparations for E18.5 heads clearly showed the absence of the premaxillary and maxillary bones and the presence of Meckel's cartilage in the mandible of the mutant embryos (Fig. 2E, F). H&E staining of sagittal head sections at E18.5 revealed that, although the upper jaw and nasal structures in the face and the olfactory bulb and telencephalon in the forebrain were absent, the basal bone of the skull dividing the facial and the forebrain structures was formed in the mutants (Fig. 2G, H). These data suggest that the basal bone-forming mesenchymal cells located between the surface ectoderm and forebrain neuroepithelium are more or less unaffected in ß-catenin-LOF mutants, which is quite consistent with the recombination pattern of Foxg1-Cre in the forehead region.

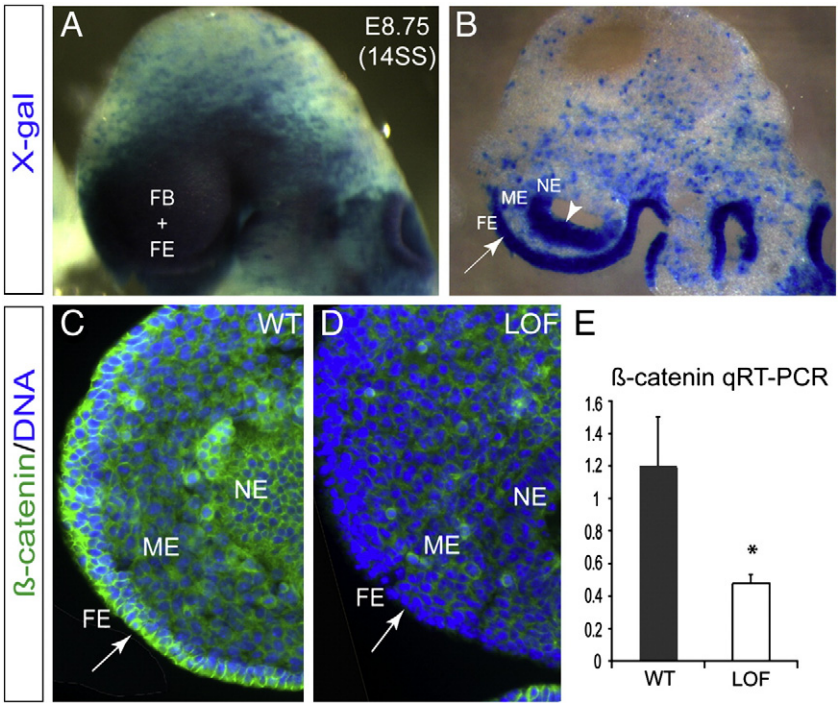

Fig. 1. Recombination activity and conditional inactivation of ß-catenin by Foxg1-Cre in the rostral head. (A, B) A whole head and its representative sagittal section after X-gal staining (*blue*) show the recombination activation sites in the embryo of *Rosa26lacZ;Foxg1*$^{kiCre/+}$ at E8.75 (with 14 somite-pairs) ($n=2$). (C–E) Immunofluorescence and real-time RT-PCR demonstrate the site-specific deletion of ß-catenin by Foxg1-Cre in the rostral head at E9.5 ($n=3$). Arrows indicate the facial ectoderm (FE). FB, forebrain; LOF, loss-of-function (standing for *ß-catenin*$^{lox(Ex2-6)/(Ex2-6)}$*;Foxg1*$^{kiCre/+}$ mice in this study); ME, mesenchyme; NE, neuroepithelium; WT, wild-type littermate control. *$P<0.05$.

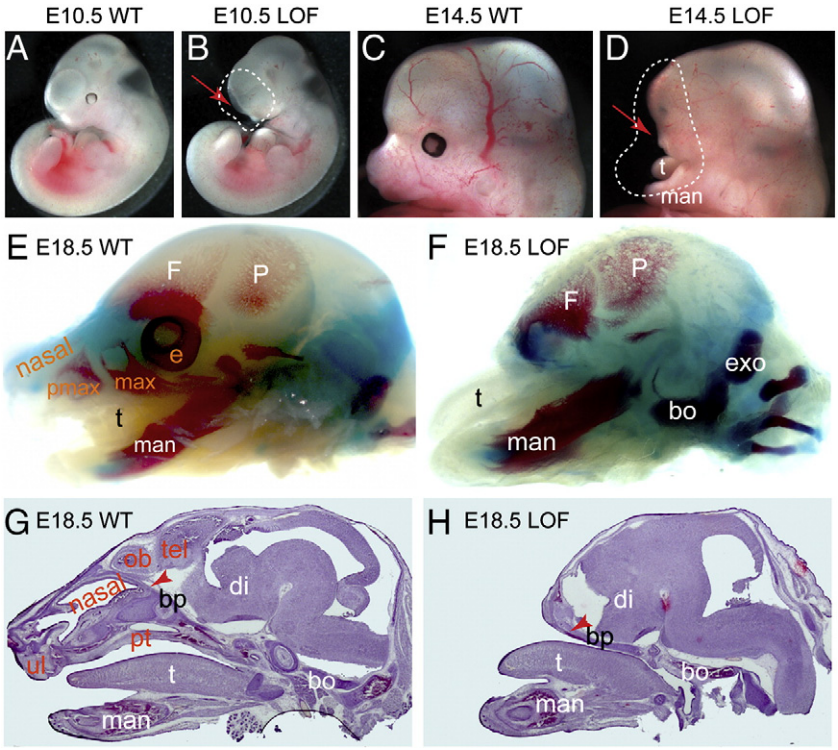

Fig. 2. Dorsal face and upper jaw are absent in the conditional ß-catenin-LOF mutant mice. (A–D) Sagittal views of whole embryos or heads show the defective structures in the rostral head region (*red arrows* and the area circled by the white-dashed line) of the mutants at E10.5 and E14.5. (E, F) Skeletal stain at E18.5 demonstrates the absence of the dorsal facial structures including the upper jaw, nose, and eye (e). The tongue (t) and mandible (man) are apparently formed. (G, H) H&E stain on sagittal head sections at E18.5 more clearly shows the defective facial and forebrain structures in the mutant. Arrowheads indicate the formation of the basisphenoid-like basal bone (bp) of the skull that divides the facial and the forebrain regions in the mutants. bo, basioccipital bone; exo, exoccipital bone; F, frontal bone; max, maxilla; P, parietal bone; pmax, premaxilla; pt, palate; ul, upper lip.

Severe disruption of Fgf signaling in the ANR and facial ectoderm, with less-affected Shh and Tcfap2a signaling in the rostral head primordia of the ß-catenin-LOF mutants

To address the mechanism of facial defects in the ß-catenin-LOF mutants, we first examined several key signaling genes, particularly those involved in Fgf signaling that are critical for both facial and forebrain development. At E9.5, *Fgf8* was expressed in both facial ectoderm and adjacent telencephalic neuroepithelium at the rostral midline-signaling center ANR region, but *Fgf8* was expressed only in the ectoderm around the future nasal pit and pharyngeal arches in the wild-type control embryo (Fig. 3A–F). These *Fgf8* expression domains, particularly in the ANR and adjacent frontonasal ectoderm, were dramatically diminished in the ß-catenin-LOF mutants (Fig. 3G–I). In contrast, expression of *Fgf8* in the mutant brain was relatively conserved in a well-known signaling center, the mid-hindbrain boundary (MHB). At E10.5, the nasal pit was evident by the formation of the medial and lateral nasal prominences in the wild-type embryos. *Fgf8* was expressed in the ectodermal edge of the nasal prominences along the nasal pit in the control embryo, but neither *Fgf8*-expressing nasal prominences nor maxillary prominence formed in the mutants at E10.5 (Fig. 3J, K). In contrast, ectodermal expression of *Fgf8* was relatively conserved in the mandibular prominence of the mutants. These findings indicate that the defective upper jaw and telencephalon in the ß-catenin-LOF mutants might be caused by loss of *Fgf8* mRNA in the ANR and frontonasal ectoderm. Real-time RT-PCR revealed that *Fgf8*, *Fgf3*, and *Fgf17*, but not *Fgf10* or *Fgf4*, were significantly downregulated in the rostral head tissues of the ß-catenin-LOF mutants at E9.5 (Fig. 4A). Additionally, wholemount *in situ* hybridization demonstrated the absence of *Fgf3* and *Fgf17* expression in the mutant ANR at E9.5 (Fig. 4B, C). These findings suggest that ß-catenin is critical for multiple Fgf ligands, particularly Fgf8 expression in the ANR and facial ectoderm.

On the other hand, Shh in the anterior ventral midline tissue and Tcfap2a in neural crest cells both play important roles in facial and/or forebrain development. Wholemount *in situ* hybridization revealed that *Shh* expression in a ventral telencephalic domain, but not in other brain regions, was apparently diminished in the mutants at E9.5 (Fig. 5A–D). In contrast, *Tcfap2a* expression in the facial region was not diminished, or even upregulated in the mutants at E9.5 (Fig. 5E, F). These results suggest that the altered expression of *Shh*, but not *Tcfap2a*, in the developing rostral head may also contribute to the severe defects seen in the mutants.

Drastic apoptosis followed the inactivation of Fgf8 expression in the facial and forebrain primordia of ß-catenin-LOF mutants

Inactivated *Fgf8* expression in the ANR and the facial ectoderm was the most notable change in the gene alterations of the ß-catenin-LOF mutants at E9.5. Because Fgf8 is a critical survival factor for various precursor cells (Chi et al., 2003; Grieshammer et al., 2005; Trumpp et al., 1999), we examined both proliferation and apoptosis in the rostral head of the mutants. We evaluated proliferation by immunolabeling for pHH3 at E9.5 and found no apparent alterations of the rate of the pHH3(+) mitotic cells in the facial ectoderm, adjacent mesenchyme, and telencephalic neuroepithelium of the ß-catenin-LOF mutants compared with wild-type littermate controls (Fig. 6A–C). We then assessed programmed cell death by TUNEL assays and found a marked increase of the apoptotic rate in the mutants (Fig. 6D–F), with particularly high rates of apoptosis in the facial ectoderm and telencephalic neuroepithelium, where Fgf8 expression was lost in the mutants. These results suggest that cell death is a consequence of diminished Fgf8 expression in the mutants. The architecture of the telencephalic neuroepithelium was also dramatically affected, which was shown to be a result of defective cell-cell adhesion in the ß-catenin-LOF mutants (Junghans et al., 2005). The TUNEL(+) facial

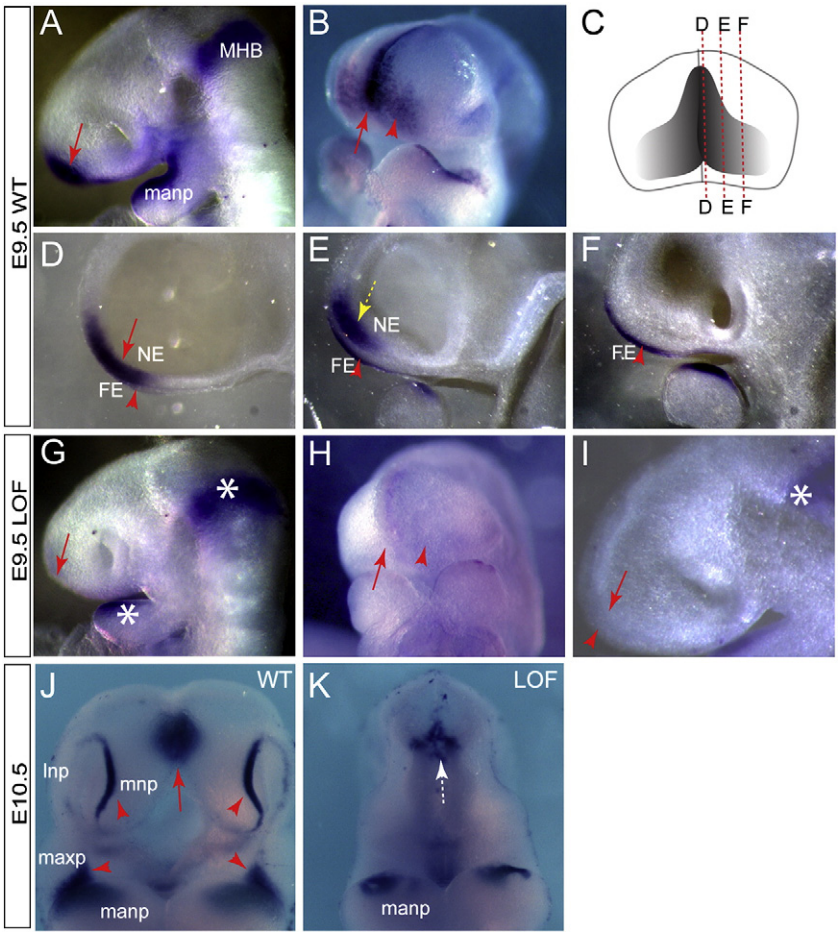

Fig. 3. Loss of *Fgf8* expression in the anterior neural ridge (ANR) and facial ectoderm of the ß-catenin-LOF mutants. (A–F) The normal expression pattern of *Fgf8* in the wild-type head at E9.5. Sagittal (A) and oblique frontal (B) views of a whole head show the *Fgf8* mRNA signals in the ANR (*arrows*) and its adjacent nasal eminence (*arrowhead*), maxillary (maxp) and mandibular (man) processes, and mid-hindbrain boundary (MHB). (C) Illustration of the *Fgf8* expression domain in the frontal face and the approximal positions for the sagittal sections in panels D–F. (D) *Fgf8* mRNA is present in both facial ectoderm (*arrowhead*) and its tightly adjacent neuroepithelium (*arrow*) at the midline region. (E) *Fgf8* mRNA is found in both facial ectoderm (*arrowhead*) and neuroepithelium (dashed arrow), which are divided by a non-Fgf8-expression mesenchymal layer on a proximal lateral section. (F) *Fgf8* mRNA is present only in the facial ectoderm (*arrowhead*) on a distal lateral section. (G–I) *Fgf8* mRNA is absent in both facial ectoderm (*arrowhead* in I) and neuroepithelium (*arrow* in I) of the ANR (*arrows* in G,H) and nasal eminence (*arrowhead* in H) ($n=4$). *Asterisks* indicate the *Fgf8* mRNA signals in the MHB and mandibular process. (J,K) Frontal facial views of the wild-type and mutant embryos with *Fgf8* expression signals at E10.5 ($n=3$). *Red arrow* in J indicates *Fgf8* mRNA in the developed ANR. Arrowheads in J indicate the restricted expression domain of *Fgf8* at the edge of the medial nasal process. *Dashed arrow* in K indicates *Fgf8* expression in a non-ANR brain domain in the mutant. FE, facial ectoderm; lnp, lateral nasal process; manp, mandibular process; maxp, maxillary process; mnp, medial nasal process; NE, neuroepithelium.

mesenchymal cells were also highly increased in the area adjacent to the telencephalic neuroepithelium in the mutants (Fig. 6E), suggesting a secondary effect of the defective Fgf8-expressing neuroepithelium at this developmental stage.

To clarify the causal relationship between the diminished Fgf8 expression and apoptosis in the mutants, we examined Fgf8 expression and TUNEL at E8.75 (Fig. 7). We found a dramatic decrease of the Fgf8 expression domain specifically in the ANR, but not in the MHB or branchial arches in the ß-catenin-LOF mutants at this early stage (Fig. 7A–D). In contrast, no significant changes in TUNEL(+) cells were found in the ANR region between the mutants and littermate controls at E8.75 (Fig. 7E, F). These results suggest that cell death is a consequence of diminished Fgf8 expression in the ß-catenin-LOF mutants during early facial development.

Fgf8 was transcriptionally regulated by Wnt/ß-catenin signaling

To determine the molecular mechanism of the diminished *Fgf8* expression in the ß-catenin-LOF mutants, we examined a 3-kb putative promoter region upstream of the mouse *Fgf8* gene and found three evolutionarily conserved Tcf/Lef binding sites, the Wnt-responsive elements (Fig. 8A). ChIP assays were performed on the extracts from E9.5 rostral head tissue and demonstrated specific binding of ß-catenin/Tcf complex to Tcf/Lef-binding site 3 (BS3) in the putative Fgf8 promoter region (Fig. 8B). Luciferase reporter assays were carried out to investigate the functional significance of BS3 (Fig. 8C, D). The luciferase reporter is driven by the 334-bp promoter region of the mouse *Fgf8* gene between -2290bp and -2624bp, which contains BS3. The reporter was activated by Wnt3a in a dose-dependent manner (Fig. 8C), and by LiCl, a Wnt signaling activator (Fig. 8D). Significantly, the *Fgf8*-promoter reporter was directly activated by the expression constructs of Lef1 and the dominantly active ß-catenin (Fig. 8E). Conversely, these luciferase activities were significantly diminished when BS3 was deleted in the *Fgf8*-promoter construct, demonstrating that *Fgf8*-promoter reporter activation was dependent on the Tcf/Lef consensus site 3. These results strongly suggest that *Fgf8* is a direct target gene of Wnt/ß-catenin signaling during early facial development.

Fgf8 was ectopically upregulated in vivo by gain-of-function of Wnt/ß-catenin signaling

To further demonstrate that *Fgf8* is regulated by Wnt/ß-catenin signaling *in vivo*, we examined the reverse effect of *Fgf8* expression by gain-of-function (GOF) analyses. To activate Wnt/ß-catenin signaling

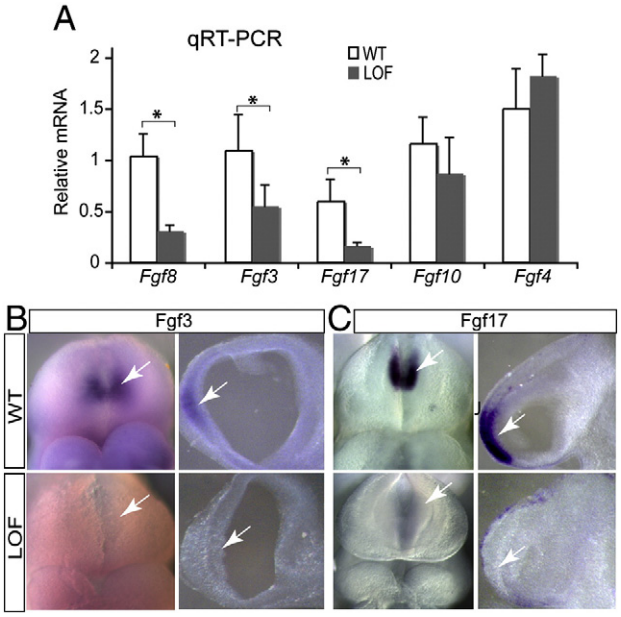

Fig. 4. Alterations of additional Fgf members in the mutant ANR of ß-catenin-LOF at E9.5. (A) Real time RT-PCR results for selective Fgf family members expressed in the rostral head of the normal and mutant embryos. The mRNA level of each gene was normalized against the mRNA level of *Gapdh*. Data were obtained from three independent experiments. $^{*}P<0.05$. (B) Frontal heads or sagittal sections show the defective *Fgf3* expression in the neuroepithelium of the mutant ANR (*arrows*) ($n=3$). (C) Defective expression of *Fgf17* expression in the mutant ANR (*arrows*) ($n=3$).

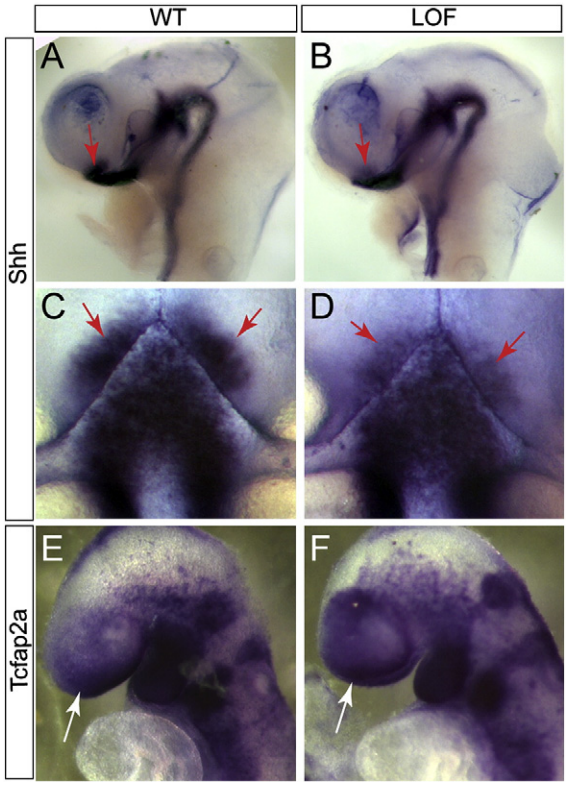

Fig. 5. Alterations of *Shh* and *Tcfap2a* in the mutant head of ß-catenin-LOF at E9.5. Sagittal (A, B) and ventral (C, D) views of the whole heads show the decrease of the ventral telencephalic domain (*arrows*) of *Shh* mRNA in the mutants ($n=3$). (E, F) *Tcfap2a* mRNA was unlikely diminished in the facial region (arrows) but even might be upregulated in the rostral head of the mutants ($n=3$).

in developing tissue, pregnant mice were injected with LiCl, which inhibits Gsk3ß and, in turn, stabilizes ß-catenin for its transcriptional function in the nuclei. After daily maternal administration of LiCl from E7.5, the *Fgf8* mRNA level was upregulated in the ANR and facial region of the normal embryos at E9.5 (Fig. 9A–C). To further verify that *Fgf8* is upregulated *in vivo* by Wnt/ß-catenin signaling, we analyzed Cre/loxP-mediated ß-catenin-GOF by conditionally deleting the ß-catenin exon 3 (encoding for a Gsk3ß-phosphorylation site), which results in constitutive ß-catenin stabilization or activation in Cre-expressing cells (Harada et al., 1999). Strikingly, the *Fgf8* expression domain in the ANR and facial ectoderm was expanded ectopically in the rostral head after conditional activation of ß-catenin with *Foxg1-Cre* at E9.5 (Fig. 9D, F, E, G). At E10.5, the orofacial primordia, including the lateral/medial nasal prominences and maxillary prominence did not form (Fig. 9H vs. Fig. 3J), the *Fgf8* expression domains remained ectopic and clustered horizontally in the upper jaw region and vertically in the head midline of the ß-catenin-GOF mutants (Fig. 9I vs. Fig. 3J). These results indicate that *Fgf8* expression can be efficiently upregulated by GOF of Wnt/ß-catenin signaling *in vivo*, and that the over-activated Fgf8 signaling by ß-catenin may also prevent orofacial development.

Discussion

Conditional gene-targeting with Foxg1^{kiCre} for early facial development

The current study has clearly shown that conditional deletion of *ß-catenin* with *Foxg1^{kiCre}* knockin mice completely prevents the formation of frontonasal (including fnm, mnp, and lnm) and maxillary primordia, which are responsible for failure of mid and upper facial development. The recombination pattern of *Foxg1^{kiCre}* mice was previously reported in the ANR from E8.5 (Hebert and McConnell, 2000). With a similar genetic fate-mapping approach, we have demonstrated the specific and intensive recombination activity of *Foxg1^{kiCre}* in both facial ectoderm and telencephalic neuroepithelium at E8.75 (about 12- to14-somite-pair stage). Another group has also shown *Foxg1^{kiCre}* activity in both cell lineages at E9.5 (Kawauchi et al., 2005). The recombination pattern of *Foxg1^{kiCre}* matches well with the gene expression pattern of endogenous *Foxg1* (formerly *BF1*) that has been previously demonstrated in the presumptive facial ectoderm first, as early as the 4-somite-pair stage, and subsequently in the adjacent neuroectoderm (the future telencephalic neuroepithelium) before the closure of anterior neural folds (Shimamura and Rubenstein, 1997). Also, although numerous studies used *Foxg1* as a classical forebrain developmental marker, their wholemount *in situ* results clearly show expression of *Foxg1* in both facial ectoderm and forebrain primordia at E8.5 or E9.5 in mice (Acampora et al., 2001; Filosa et al., 1997). On the other hand, the original report for *Foxg1^{kiCre}* mice described ectopic recombination patterns in some, but not all, mouse strain backgrounds (Hebert and McConnell, 2000). For instance, BALB/c and CD-1 strains frequently show ectopic recombination patterns of *Foxg1^{kiCre}* in non-*Foxg1*-expressing cell lineages, while the 129SvJ strain shows a recombination pattern that most closely resembles the normal expression pattern of *Foxg1*. In the current study, we used a mixed strain background of 129 and C57BL/6 for both conditional gene-targeting and fate-mapping, and found consistent and reliable results of the *Foxg1^{kiCre}* recombination and the facial phenotypes in the conditional mutants. Taken together, these data indicate that the *Foxg1^{kiCre}* mouse line is a critical tool for conditional gene-targeting in both facial ectoderm and forebrain neuroepithelium in the early developmental stage. However, particular attention needs to be paid to the mouse strain background and use of identical strains in both genetic fate mapping and conditional gene-targeting analyses, not only for *Foxg1^{kiCre}* but perhaps also for all other Cre mouse lines.

Fgfs as the target genes of the Wnt/ß-catenin signaling pathway

The current study provides strong evidence that the expression of several critical morphogenetic signaling molecules, particularly Fgfs,

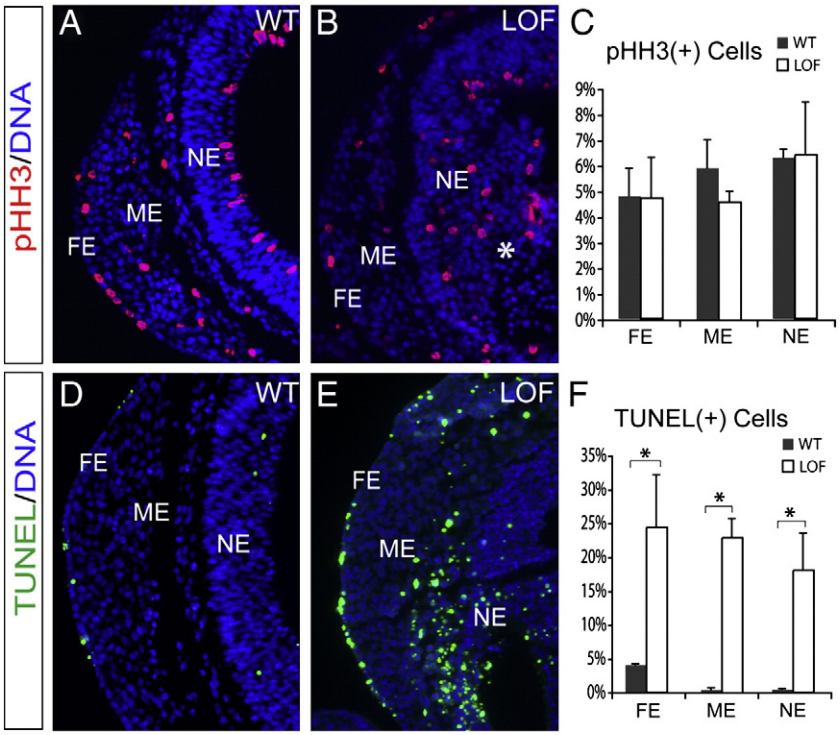

Fig. 6. Proliferation and apoptosis in the ß-catenin-LOF mutants at E9.5. (A-C) Proliferation was assessed by immunofluorescence for pHH3. The proportion of pHH3(+) cells is not significantly changed in the facial ectoderm (FE), facial mesenchyme (ME), and telencephalic neuroepithelium (NE) of the mutants. *Asterisk* in B indicates the disorganized neuroepithelium in the mutants. (D-F) TUNEL(+) apoptotic cells are dramatically increased in the facial ectoderm and telencephalic neuroepithelium as well as in facial mesenchyme.

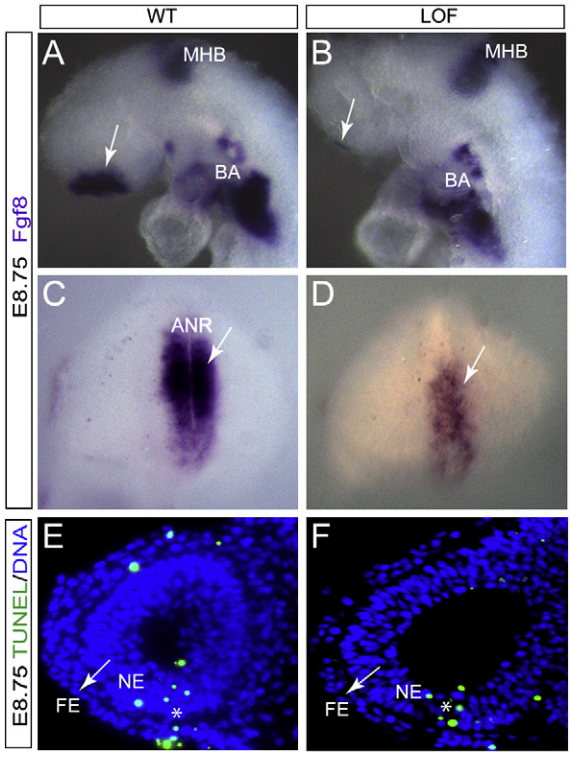

Fig. 7. Loss of Fgf8 precedes programmed cell death in the anterior neural ridge (ANR) of the ß-catenin-LOF mutants at E8.75. Sagittal head (A,B) and frontal views show the dramatic decrease of *Fgf8* mRNA signals in the ANR (*arrows*) but not in mid-hindbrain boundary (MHB) or branchial arches (BA). (E,F) No significant changes of TUNEL(+) cells in the facial ectoderm (FE, *arrows*) and adjacent neuroepithelium (NE) between the controls and mutants at E8.75 ($n=2$ per genotype). *Asterisks* indicate some clustered TUNEL(+) cells in the ventral region of both normal and mutant telencephalon.

is dramatically diminished in the rostral head primordia of ß-catenin-LOF mutants. This may subsequently prevent proper facial development in mutants due to conditional deletion of *ß-catenin* with *Foxg1*^{kiCre} in the facial ectoderm and telencephalic neuroepithelium. Fgfs are evolutionarily conserved polypeptide growth factors playing various important roles in embryonic development (Goldfarb, 1996; Itoh and Ornitz, 2008; Thisse and Thisse, 2005). At E9.5 and E10.5 of mice, genes encoding multiple Fgf ligands (including *Fgf3*, *Fgf8*, *Fgf9*, *Fgf10*, *Fgf15*, *Fgf17*, and *Fgf18*) and two Fgf receptors, *Fgfr1* and *Fgfr2*, are expressed in the facial primordia (Bachler and Neubuser, 2001). Our results suggest that *Fgf8* is a direct target gene regulated by ß-catenin signaling in the ANR and facial ectoderm. This is partially supported by a recent study that a Tcf/Lef binding site located in the intron 3 of the *Fgf8* gene is responsive to Wnt/ß-catenin activation during tooth development (Wang et al., 2009). In contrast, we determined a different Wnt-responsive element in the 5′ promoter region of *Fgf8* in the early developing rostral head, which can be activated by Wnt3a and LiCl, or directly activated by constitutively active ß-catenin and Lef1 constructs. The different Wnt-responsive elements in the *Fgf8* gene in early facial and tooth development suggest the age- and lineage-dependent roles of *Fgf8* activation by the Wnt/ß-catenin signaling pathway. Future transgenic studies may help to determine if these Wnt-responsive elements can actually drive reporter gene activation in respective tissue sites. In addition, we found that *Fgf3* and *Fgf17* are also regulated by Wnt/ß-catenin signaling in the ANR of early mouse embryos. Interestingly, *Fgf4* in the mouse tooth bud (Kratochwil et al., 2002), *Fgf10* in the developing mouse heart (Cohen et al., 2007), and *Fgf20* in cancer cells or *Xenopus* embryos (Chamorro et al., 2005) have previously been determined as the downstream target genes of Wnt/ß-catenin signaling pathway. Taken together, these data suggest that a set of different Fgf ligands is regulated by the canonical Wnt/ß-catenin signaling pathway in a wide range of biological processes.

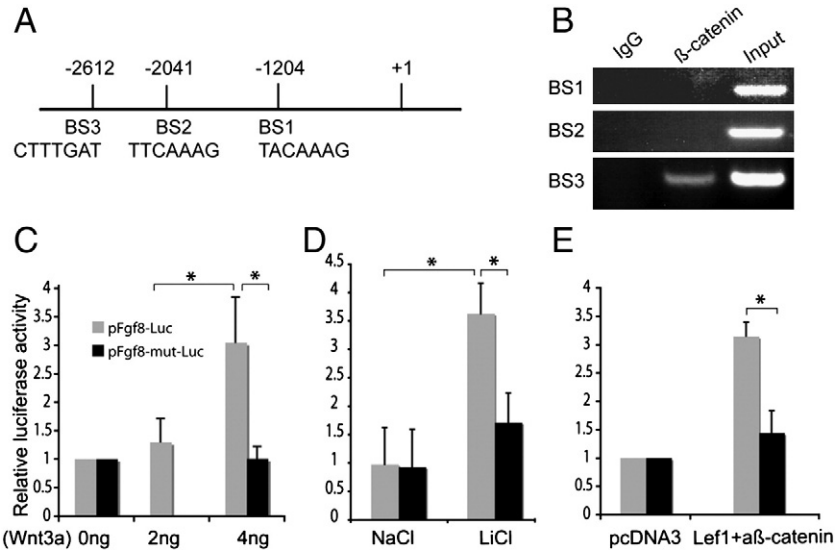

Fig. 8. *Fgf8* is a target gene of Wnt/ß-catenin signaling during facial development. (A) Sequence analysis revealed three Tcf/Lef binding sites (BS1-BS3) within the 3-kb putative promoter region of the mouse *Fgf8*. (B) ChIP assays were performed on the tissue extracts from the rostral head of E9.5 wild-type mice. ß-catenin formed a transcriptional complex with Tcf/Lef at the BS3 in the putative Fgf8 promoter region. (C-E) Luciferase assay results for L cells transfected with *pFgf8-Luc* or *pFgf8-mut-Luc* constructs, stimulated by Wnt3a (C), lithium (D), or co-transfected with pcDNA3 or Lef1 and constitutively active ß-catenin expression constructs (E). The luciferase activity of *pFgf8-Luc* in the control assay was defined as one unit. Luciferase assay results were obtained from three independent experiments, and each was performed in triplicate. $^{*}P<0.05$.

Wnt/ß-catenin signaling activates Fgf signaling for cell survival and facial development

Among the 22 Fgf ligands, Fgf8 is a key molecule in the developing head/brain signaling centers that are required for facial and brain development (Liu and Joyner, 2001; Suzuki-Hirano and Shimogori, 2009; Szabo-Rogers et al., 2010). The current study shows that *Fgf8* expression is dramatically diminished in the ANR and facial ectoderm of the ß-catenin-LOF mice; this may be a major cause of facial defects in these mutants. In support of this, conditional deletion of *Fgf8* with

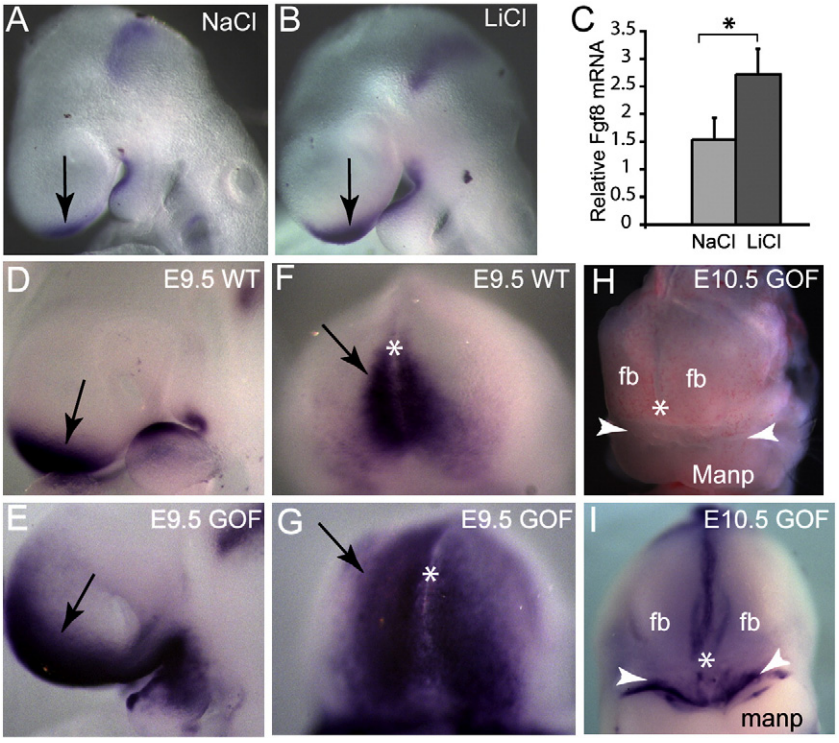

Fig. 9. *Fgf8* is upregulated by gain-of-function (GOF) of Wnt/ß-catenin signaling during facial development. (A–C) Wholemount *in situ* hybridization and real-time RT-PCR demonstrate the increase of *Fgf8* mRNA signals in the anterior neural ridge (arrows) of E9.5 wild-type embryos after maternal administration with the Wnt activator lithium ($n=3$). $^{*}P<0.05$. (D–H) Conditional ß-catenin-GOF with Foxg1-Cre causes dramatic upregulation of *Fgf8* expression in the facial ectoderm (*arrows*) and anterior neural ridge (*asterisks*) at E9.5 ($n=2$), and resulted in defective facial primordia at E10.5 ($n=2$). *Arrowheads* in H (no staining) and I (after *Fgf8 in situ* hybridization) indicate the residual orofacial primordia, including indistinguishable lateral and medial nasal processes and maxillary process, which formed a horizontal line with intensive *Fgf8* expression (I) in the ß-catenin-GOF mutants at E10.5. fb, forebrain; manp, mandibular process.

Nes-Cre1 mice in the ectoderm of the first pharyngeal or branchial arch 1 (BA1) causes extensive cell death in the BA1 mesenchyme and results in severe defects of BA1 derivatives (such as mandible) (Trumpp et al., 1999). The hypomorphic *Fgf8* mutant mice exhibit defective craniofacial development with significantly increased apoptosis in the ANR and other *Fgf8*-expressing regions or migrating neural crest cells (Abu-Issa et al., 2002).

Significantly, conditional deletion of exon 5 in the *Fgf8* gene with *AP2a-Cre* (also called *Tcfap2a-Cre*) in facial ectoderm and neural crest cells leads to the developmental failure of entire facial structures, including both upper jaw and lower jaw, probably due to massive cell death in the mutant pharyngeal arches and other facial primordia (Macatee et al., 2003). This is consistent with the findings in the current study, except that the lower jaw and tongue are formed but the eye is absent in the $Catnb1^{lox(ex2\text{-}6)};Foxg1^{kiCre}$ mutants. The similar facial deformation in these mutants may be due to the fact that the recombination pattern of *Foxg1-Cre* and *AP2a-Cre* are largely overlapped in the facial ectoderm. The different mandibular and ocular phenotypes in these mutants are likely caused by $Foxg1^{kiCre}$ recombination being less active in the pharyngeal arches but highly activated in the telencephalic neuroepithelium, including the optic vesicle, compared to *AP2a-Cre* recombination. Collectively, these results suggest that Fgf8 activated by Wnt/ß-catenin signaling in the facial ectoderm is critical for cell survival in facial ectoderm and facial mesenchyme during the development of the facial primordia. This is supported further by a recent study conducted by Trevor Williams' group. They use their newly developed ectodermal *Cre* transgenic mice with a promoter derived from *Tcfap2a* (or *AP2a*) to conditionally delete *ß-catenin* in the facial ectoderm, which results in similar defects in entire facial primordia associated with diminished expression of Fgf signaling in the facial ectoderm (personal communication from T. Williams). Some minor differences between these studies may be a consequence of the different recombination patterns and timing of the Cre mouse lines used.

Conditional deletion of exons 2 and 3 in the *Fgf8* locus with *Foxg1-Cre* mice results in defective nasal and other craniofacial structures (Kawauchi et al., 2005). However, a small and short snout is formed in the mutant mouse at birth. The less-severe facial defects in the $Fgf8^{lox(exons2,3)};Foxg1^{kiCre}$ mice compared with the extremely severe facial defects in the $Fgf8^{lox(exon5)};AP2a\text{-}Cre$ or the $Catnb1^{lox(ex2\text{-}6)};Foxg1^{kiCre}$ mice indicate that Cre-mediated deletion of $Fgf8^{lox(exons2,3)}$ may create a hypomorphic allele, and that dose-dependent LOF of Fgf8 downstream of ß-catenin signaling may lead to a spectrum of craniofacial disorders with corresponding severities. Related clinical significance is demonstrated by the association of *FGF8* mutations with cleft lip/palate in humans (Riley et al., 2007). We have recently found no dramatic alterations of *Fgf8* expression in the craniofacial primordia of the Wnt coreceptor *Lrp6*-null mice that exhibit full-penetrant cleft lip/palate (Song et al., 2009). However, we cannot exclude that there may be subtle alterations in *Fgf8* expression during lip fusion processes or that Lrp6 and Lrp5 are functionally redundant upstream of ß-catenin in the regulation of Fgf8 signaling. Because the double-null mice of *Lrp6* and *Lrp5* die at gastrulation (Kelly et al., 2004), *Lrp6* conditional gene-targeting mice (Zhou et al., 2010) in combination with the viable/fertile *Lrp5*-null mice (Kato et al., 2002) will allow us to address the dose- and lineage-dependent effects of Wnt signaling on *Fgf8* activation and craniofacial birth defects.

The relationship between facial and forebrain development

We show that a Shh expression domain in the ventral telencephalon is diminished in conditional $Catnb1^{lox(ex2\text{-}6)};Foxg1^{kiCre}$ mutants at E9.5, suggesting that Shh signaling may also be regulated by the Wnt/ß-catenin signaling pathway directly or indirectly in this region. Shh signaling plays important roles in both facial and forebrain development. Mutations in the human *SHH* gene are a cause of holoprosencephaly (HPE), a common midline defect in the forebrain and face (Belloni et al., 1996; Bendavid et al., 2010; Dubourg et al., 2004; Roessler et al., 1996,2009). The completely arrested frontonasal primordia and telencephalic/optic vesicles in the $Catnb1^{lox(ex2\text{-}6)};Foxg1^{kiCre}$ mutants may resemble a severe form of HPE, which also suggests a close relationship between facial and forebrain development by sharing a common signaling mechanism. Wnt signaling may induce both Fgf and Shh signaling pathways in the rostral head signaling centers for facial and forebrain development.

In the chick model, *Shh* and *Fgf8* are expressed in a complementary manner and form a boundary structure in both frontonasal ectoderm and telencephalic neuroepithelium, which is required for both facial and forebrain patterning and growth (Hu et al., 2003; Schneider et al., 2001). Inhibition of Shh signaling in the neuroectoderm of chick embryos not only alters the dorsoventral patterning of the telencephalon, but also disrupts a signaling center in the frontonasal ectoderm, and subsequently affects the facial development (Marcucio et al., 2005). Ectopic Shh signaling in the forebrain splits a single frontonasal ectodermal zone of the chick beak primordium into two separated domains that resemble the paired medial nasal prominences seen in the E10.5 mouse embryo (Hu and Marcucio, 2009). From these studies, the Helms group suggests that the forebrain is not only a structural base supporting facial primordia, but also a source of signals modulating facial development.

However, the signaling-mediating mechanisms from the forebrain to the facial primordia remain unclear. Interestingly, *Fgf8* expression is dramatically diminished in the forebrain neuroepithelium first and then in the nasal pit by the ectopic activation of Shh signaling in the chick forebrain (Hu and Marcucio, 2009), suggesting a stepwise repressive role of Shh on Fgf8 expression in the ANR, and subsequently in the facial ectoderm. In the current study, we have observed a progressive activation pattern of Fgf8 that is expressed specifically in the ANR at E8.75 (after closure of the anterior neural tube), subsequently expands to the facial ectoderm adjacent to ANR at E9.5, and is finally restricted to the nasal pit at E10.5 during normal development. These observations suggest that Fgf8 may first be induced by Wnt/ß-catenin signaling in the ANR, then subsequently Fgf8 regulate its own expression in the facial ectoderm. However, ß-catenin in the facial ectoderm may also modulate Fgf8 activation or expansion through its roles in both Wnt signaling and cell adhesion. Notably, it has been proposed that ß-catenin-mediated cell adhesion, but not the Wnt signaling, is critical for cell survival and organization during forebrain development (Junghans et al., 2005). In disagreement with the conclusion by Junghans et al., the current study has demonstrated that the transcriptional function of ß-catenin signaling is critical for Fgf signaling activation at the ANR, which subsequently modulates cell survival and patterning in forebrain development. As direct evidence, combinatorially deleting Fgf receptors (*Fgfr1*, *Fgfr2*, and *Fgfr3*) with $Foxg1^{kiCre}$ mice demonstrated a dose-dependent effect of Fgf signaling on cell survival and telencephalic development (although no information on facial development was available) (Paek et al., 2009). Interestingly, Paek et al. also observed diminished *Shh* expression in the ventral telencephalon of the triple $Fgfrs1,2,3;Foxg1^{kiCre}$ conditional mutants. Together with our findings of the diminished Shh expression in the same region of $Catnb1^{lox(ex2\text{-}6)};Foxg1^{kiCre}$ mutants, these results suggest that Shh signaling may also be regulated by Fgf signaling downstream of Wnt/ß-catenin signaling for forebrain and facial development. However, the dual functions of ß-catenin in cell adhesion and Wnt signaling may act simultaneously during facial and forebrain development. Our double conditional gene-targeting approaches of Lrp6 and Lrp5 discussed above may clarify these questions in future studies.

In conclusion, the current study demonstrates that conditional inactivation of ß-catenin in the Foxg1-expressing facial ectoderm and neuroectoderm severely impairs the development of the mid/upper

face and the telencephalic/ocular structures in a form that may resemble a severe type of HPE. These defects are mediated by alterations of a set of signaling genes required for cell survival and patterning during early development. Our results provide genetic evidence that the canonical Wnt/ß-catenin signaling pathway is a key upstream factor that initiates and modulates a balanced Fgf signaling activity in the rostral signaling center ANR and the adjacent facial ectoderm required for both early facial and forebrain development.

Acknowledgments

We would like to specially thank M. Taketo for the Catnb1$^{lox(ex3)}$, R. Kemler for the Catnb1$^{lox(ex2-6)}$, and S. McConnell for the Foxg1-Cre mice, and S. Evans for the active ß-catenin and Lef1 expression constructs. We are grateful to T. Williams for sharing unpublished data; M. Hrachova, K. Wang, Y. Li and J. Chan for technical assistance; B. Guo and D. Cortes for critical reading and editing of the manuscript; A. Molotkov for some preliminary studies; Y.Z. Wang, Q. Gan, T. Zhao, and T. Yamagami for general help or discussion. This work was supported by the Shriners Hospitals for Children (research grants 87500 and 86100 to C.Z. and a postdoctoral fellowship to L.S.) and UC Davis School of Medicine (a research award to C.Z.).

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