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The role of Irf6 in tooth epithelial invagination

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ABSTRACT

Thickening and the subsequent invagination of the epithelium are an important initial step in ectodermal organ development. *Ikkα* has been shown to play a critical role in controlling epithelial growth, since *Ikkα* mutant mice show protrusions (evaginations) of incisor tooth, whisker and hair follicle epithelium rather than invagination. We show here that mutation of the Interferon regulatory factor (*Irf*) family, *Irf6* also results in evagination of incisor epithelium. In common with *Ikkα* mutants, *Irf6* mutant evagination occurs in a NF-κB-independent manner and shows the same molecular changes as those in *Ikkα* mutants. *Irf6* thus also plays a critical role in regulating epithelial invagination. In addition, we also found that canonical Wnt signaling is upregulated in evaginated incisor epithelium of both *Ikkα* and *Irf6* mutant embryos.

Introduction

Many organs (e.g. lung, liver, kidney, glands, eyes, hair) develop through reciprocal epithelial–mesenchymal interactions and share similar signaling pathways such as Bmp, Shh, Fgf, Wnt and Tgf at early stages of their development. At the early stages, these organs also show similar morphological features, consisting of a thickening and subsequent invagination of the epithelium. Teeth arise from a series of reciprocal interactions between the oral epithelium and underlying neural crest-derived mesenchyme (for reviews, see Thesleff, 2006; Tucker and Sharpe, 2004). The first morphological sign of tooth development is a narrow band of thickened epithelium on the developing jaw primodia and subsequent localized invagination into underlying mesenchyme to form buds. The bud epithelium progressively takes the form of the cap and bell configurations as differentiation proceeds. Epithelial cells and mesenchymal cells (dental papilla) then differentiate into enamel-producing ameloblasts and dentin-producing odontoblasts, respectively.

The nuclear Factor *kappa* B (NF-κB) pathway plays a major role in many physiological and pathological process including immune response to infection and cancer (for review see Chariot, 2009; Häcker and Karin, 2006; Li and Verma, 2002; Sanz et al., 2010). In mammals, 12 NF-κB homo- or hetero-dimers are formed among the five proteins NF-κB1 (p50, generated from p105), NF-κB2 (p52, generated from p100), RelA (p65), RelB and c-Rel. NF-κB exists in the cytoplasm as an inactive form that is associated with inhibitory proteins termed

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Inhibitor of κB (IκB). Activation of the NF-κB pathway results in nuclear translocation of NF-κB proteins, and can proceed either through classical/canonical, alternative/noncanonical or hybrid pathways. Classical NF-κB activation is usually a rapid and transient response to a wide range of stimuli. In nonstimulated cells, IκB acts to retain NF-κB in the cytoplasm by masking the nuclear localization sequence. Exposure to stimuli results in rapid phosphorylation of IκB that leads to site-specific ubiquitination and degradation. The resulting free NF-κB dimers translocate to the nucleus and regulate target gene transcription. The protein kinase that phosphorylates IκB in response to stimuli is a multiprotein complex, IκB kinase (IKK), composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (also called NEMO). The alternative pathway involves slow activation of NF-κB leading to prolonged activation of NF-κB targets genes. NF-κB inducing kinase (NIK) recruits IKKα that phosphorylates p100, promoting p100 polyubiquitiation and subsequent proteasomal processing to p52, generating RelB/p52 dimers which facilitates full activation of the pathway. In addition, Ikkα is also known to function outside of the IKK complex in an NF-κB-independent manner. *Ikkα* is a critical regulator of keratinocyte differentiation by an NF-κB-independent manner, although the exact mechanism through which Ikkα acts is unclear. Taut undifferentiated epidermis, limb truncation, cleft palate, abnormal molar cusp shape and adhesion of oral epithelium are also observed in *Ikkα* mutant mice (Hu et al., 1999; Li et al., 1999; Takeda et al., 1999). In addition to these phenotypes, incisor tooth epithelium of *Ikkα* mutant mice fails to invaginate into the underlying mesenchyme and instead evaginates into the oral cavity, suggesting that *Ikkα* has a regulatory role in guiding directionality of developing tooth germs. This role of *Ikkα* is independent of the NF-κB (Ohazama et al., 2004).

Interferons (IFNs) play critical roles in many biological processes including the homeostasis and function of immune systems (Bonjardim et al., 2009; Hertzog et al., 2011). Ifn regulatory factor (Irf) genes regulate the transcription of interferons, proteins produced in response to the presence of pathogens, and function as an integral part of the immune system (Platanias, 2005). The Irf family is comprised of nine members (*Irf1*–*Irf9*) that share a highly conserved N-terminal, penta-tryptophan, helix-turn-helix DNA-binding domain and a less well-conserved protein-binding domain (Taniguchi et al., 2001). Many members of Irf family are known to activate the canonical NF-kB pathway (Hiscott, 2007; Taniguchi et al., 2001). Ikkα is also involved in Toll-like receptor (TLR)7- and TLR9-mediated IFNα induction in plasmacytoid dendritic cells via Irf7 phosphorylation as NF-kB independent manners (Hoshino et al., 2006). Mutation in *IRF6* has previously been shown to cause Van der Woude syndrome (VWS) and poplyteal pterigium syndrome (PPS; Kondo et al., 2002). VWS is an autosomal dominant disorder of facial development that is characterized by cleft lip and palate and is the most common form of syndromic orofacial clefting (Van der Woude, 1954). PPS has a similar orofacial phenotype to VWS, but includes popliteal webbing, pterygia, oral synychiae, adhesions between the eyelids, syndactyly and genital anomalies (Bixler et al., 1973; Froster-Iskenius, 1990). *Irf6* mutant mice show cleft palate, adhesion of oral epithelium, limb truncation and taut undifferentiated epidermis, all phenotypes also seen in *Ikkα* mutants (Hu et al., 1999; Ingraham et al., 2006; Li et al., 1999; Richardson et al., 2006; Takeda et al., 1999).

Thickening and the subsequent invagination of the epithelium is an important initial step in tooth development. Although several new insights have recently been revealed, the molecular mechanisms regulating epithelial invagination still remain unclear (Charles et al., 2011; Munne et al., 2009). We show here that null mutation of the *Irf6* results in identical incisor phenotypes (evagination of incisor epithelium) and similar molecular changes to those in *Ikkα* mutants. Despite these shared phenotypes and associated molecular changes, crosses between *Ikkα* and *Irf6* mutants failed to reveal any evidence of a genetic interaction in regulating tooth epithelial invagination.

Materials and methods

Production and analysis of transgenic mice

The production of mice with mutation of *Ikkα*, *Irf6*, *Jagged2* (*Jag2*), *Rip4* and *Stratifin* (*Sfn; Sfn*$^{Er/Er}$) has previously been described (Guenet et al., 1979; Holland et al., 2002; Hu et al., 1999; Jiang et al., 1998; Richardson et al., 2006). Mice overexpressing *Ikkα* under keratin (K) 5 promotor (*K5-Ikkα*) have been described previously (Lomada et al., 2007). Production of the NF-kB reporter [*(Igκ)*$_3$*conalacZ*] and *Axin2* reporter (*Axin2*^{lacZ}) mice has also previously been described (Lustig et al., 2002; Schmidt-Ullrich et al., 1996). The *Irf6* hypomorph allele (*Irf6*^{neo}) was created as an intermediate step toward the construction of an *Irf6* conditional knockout targeting vector. In addition to loxP sites, we inserted the neomycin resistance gene (*neo*), under the control of a constitutive promoter, as a selectable marker for the insertion event. To create this construct, we screened a BAC library derived from 129/SV strain and identified clone RPCI22-516G. From this BAC, we subcloned a 1.8 kb *Kpn*I–*Bam*HI fragment for the 5′-arm and a 3.9 kb *Bam*HI–*Hind*III fragment for the 3′-arm into the *KpnI* and *HindIII* sites of pBluescript II SK(−) vector. The 3 kb *Bam*HI fragment, located between the 5′- and 3′-arms was cloned into the *Sal*I site in ploxP3NeopA vector (kind gift from Dr. Yagi and Dr. Hirabayashi, Osaka University). This vector was cut with *Xho*I, which liberated a 5.8 kb fragment that contained the floxed exons (3 and 4) and the *Pgk-neo* cassette, and was subcloned into the *Bam*HI site located at the junction of the 5′ and 3′ arms. This gene targeting vector was digested with *Not*I and electroporated into ES cells derived from 129/SV strain. We screened 384 ES clones by PCR. After G418 selection, four clones were positive. The positive ES cells were microinjected into C57BL/6 blastocysts, and we obtained nine male chimeras that were mated with C57BL/6 mice. Germline transmission was verified as previously described (Ingraham et al., 2006).

Embryonic day 0 (E0) was taken to be midnight prior to finding a vaginal plug. Embryos were harvested at the appropriate time and genotyped using PCR analysis of genomic DNA extracted from unused embryonic tissue. Embryonic heads were fixed in 4% paraformaldehyde (PFA), wax embedded and serially sectioned at 7 μm. Sections were split over 5–10 slides and prepared for histology and radioactive in situ hybridisation.

In situ hybridisation

Radioactive in situ hybridisation with [^{35}S]UTP-labeled riboprobes was carried out as described previously (Ohazama et al., 2008). Decalcification using 0.5 M EDTA (pH 7.6) was performed after fixation of newborn mice. The radioactive antisense probes were generated from mouse cDNA clones that were gifts from T.A. Mitsiadis (*Notch1*, *Notch2*), A. McMahon (*Shh*) or were obtained from RZPD (*Irf1*, *Irf2*, *Irf3*, *Irf4*, *Irf5*, *Irf7*, *Irf8*, *Irf9*) or Geneservice (*Sfn*).

Immunohistochemistry

After deparaffinization, sections were treated by proteinase K and then incubated with antibody to phosphorylated Smad1, Smad5 and Smad8 (p-Smad1/5/8; Cell signaling Technology) or CD44 (Chemicon). As a negative control, normal rabbit or rat serum was used instead of primary antibody. Tyramide signal amplification system was performed (Perkin Elmer Life Science or DAKO) for detecting primary antibody.