Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 313 (2008) 210–224
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Wnt/β-catenin signaling directs multiple stages of tooth morphogenesis

Fei Liu [a], Emily Y. Chu [a,b], Brenda Watt [a,b], Yuhang Zhang [a], Natalie M. Gallant [a], Thomas Andl [a], Steven H. Yang [c], Min-Min Lu [d], Stefano Piccolo [e], Ruth Schmidt-Ullrich [f], Makoto M. Taketo [g], Edward E. Morrisey [d], Radhika Atit [h], Andrzej A. Dlugosz [c], Sarah E. Millar [a,b,*]

[a] *Departments of Dermatology and Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia PA 19104, USA*
[b] *Cell and Molecular Biology Graduate Group, University of Pennsylvania School of Medicine, Philadelphia PA 19104, USA*
[c] *Department of Dermatology, University of Michigan, Ann Arbor, MI 48109, USA*
[d] *Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia PA 19104, USA*
[e] *Department of Histology, Microbiology and Medical Biotechnologies, Section of Histology and Embryology, University of Padua, 35121 Padua, Italy*
[f] *Max-Delbruck-Center for Molecular Medicine, 13092 Berlin, Germany*
[g] *Department of Pharmacology, Graduate School of Medicine, Kyoto University, Yoshida-Konoé-cho, Sakyo, Kyoto 606-8501, Japan*
[h] *Department of Biology, Case Western Reserve University, Cleveland OH 44106, USA*

Received for publication 22 March 2007; revised 6 October 2007; accepted 16 October 2007
Available online 23 October 2007

Abstract

Wnt/β-catenin signaling plays key roles in tooth development, but how this pathway intersects with the complex interplay of signaling factors regulating dental morphogenesis has been unclear. We demonstrate that Wnt/β-catenin signaling is active at multiple stages of tooth development. Mutation of β-catenin to a constitutively active form in oral epithelium causes formation of large, misshapen tooth buds and ectopic teeth, and expanded expression of signaling molecules important for tooth development. Conversely, expression of key morphogenetic regulators including *Bmp4*, *Msx1*, and *Msx2* is downregulated in embryos expressing the secreted Wnt inhibitor *Dkk1* which blocks signaling in epithelial and underlying mesenchymal cells. Similar phenotypes are observed in embryos lacking epithelial β-catenin, demonstrating a requirement for Wnt signaling within the epithelium. Inducible *Dkk1* expression after the bud stage causes formation of blunted molar cusps, downregulation of the enamel knot marker *p21*, and loss of restricted *ectodin* expression, revealing requirements for Wnt activity in maintaining secondary enamel knots. These data place Wnt/β-catenin signaling upstream of key morphogenetic signaling pathways at multiple stages of tooth development and indicate that tight regulation of this pathway is essential both for patterning tooth development in the dental lamina, and for controlling the shape of individual teeth.

Keywords: Tooth; Mouse; Embryo; Wnt; Molar; Incisor; β-catenin; Dental development

Introduction

Delineating the mechanisms by which oral epithelial cells adopt and maintain dental fates is critical for understanding developmental tooth syndromes and for designing strategies for the regeneration or repair of teeth and enamel. A key question is how the coordinated actions of broadly used signaling pathways result in the formation of a specific organ, in this case the tooth.

An early initiating signal for tooth development arises in the oral ectoderm, causing thickening of the dental lamina and its down-growth into the mesenchyme to form a tooth bud (Lumsden, 1988). The specification of tooth and intervening regions may be regulated by a competition between Fibroblast growth factor (FGF) 8, expressed in pre-tooth epithelium, and Bone morphogenic protein (BMP) 4, expressed in intervening epithelium (Neubuser et al., 1997; St Amand et al., 2000). These factors regulate restricted expression of the homeobox transcription factor *Pitx2* that is required for tooth development beyond the bud stage (Lin et al., 1999). The Sonic hedgehog (Shh) pathway genes *Gli2* and *Gli3*, and the homeobox genes *Msx1* and *Msx2* are important for invagination of the dental

* Corresponding author. Department of Dermatology, University of Pennsylvania School of Medicine, M8D Stellar-Chance Laboratories, 422 Curie Boulevard, Philadelphia PA 19104-6100, USA. Fax: +1 215 573 9102.
E-mail address: millars@mail.med.upenn.edu (S.E. Millar).

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.10.016

lamina to form tooth buds (Bei and Maas, 1998; Hardcastle et al., 1998). However, the mechanisms controlling assignment of tooth fate are incompletely understood.

At the late bud stage, the tooth bud begins to fold at its base in response to mesenchymal signals (Mina and Kollar, 1987). The enamel knot, a non-proliferating, transient epithelial structure, appears at the cap stage and is thought to regulate tooth shape (Vaahtokari et al., 1996). These processes fail to occur in mice lacking the transcription factors LEF1, PITX2, MSX1, and PAX9, which are targets of the intercellular Wnt, BMP and FGF pathways (Bei and Maas, 1998; Kratochwil et al., 1996; Lin et al., 1999; Peters et al., 1998). Subsequent folding morphogenesis (the bell stage) results in the formation of multiple cusps and requires the TNF family member ectodysplasin (EDA), which signals via its receptor EDAR to activate the NF-κB pathway (Courtney et al., 2005; Jernvall et al., 1994; Ohazama et al., 2004; Pispa et al., 1999; Schmidt-Ullrich et al., 2006). Dental papilla cells adjacent to the epithelium differentiate into odontoblasts and begin to secrete dentin (Thesleff and Hurmerinta, 1981), while the epithelial cells differentiate into outer enamel epithelium, stellate reticulum, stratum intermedium, and inner enamel epithelium, lying adjacent to the papilla. The inner enamel epithelium differentiates into preameloblasts and then enamel-secreting ameloblasts (Thesleff and Hurmerinta, 1981).

Wnts form a large family of secreted ligands that activate several receptor-mediated pathways (Logan and Nusse, 2004). In the Wnt/β-catenin pathway, binding of Wnt ligands to Frizzled (FZ) receptors and LDL receptor related protein (LRP) family co-receptors causes β-catenin accumulation, nuclear translocation, and transcriptional activation by complexes of β-catenin and LEF/TCF transcription factor family members (Logan and Nusse, 2004). The Wnt/β-catenin pathway is specifically inactivated by endogenous secreted inhibitors of the Dickkopf (DKK) family, which bind to LRP and to high-affinity receptors of the Kremen family, causing rapid internalization of Kremen–Dkk–LRP complexes and removal of LRP from the plasma membrane (Mao et al., 2002).

Activation of Wnt/β-catenin signaling initiates the de novo formation of ectodermal appendages related to teeth, including hair follicles, feather buds, mammary placodes, and taste papillae (Chu et al., 2004; Gat et al., 1998; Hogan, 1999; Liu et al., 2007; Noramly et al., 1999; Thesleff et al., 1995). Conversely, initiation of hair follicle, mammary, and taste papilla placode development requires Wnt/β-catenin signaling (Andl et al., 2002; Chu et al., 2004; Liu et al., 2007).

Several specific observations indicate that Wnt signaling plays key roles in tooth morphogenesis. Several *Wnt* genes are broadly expressed in oral and dental epithelium, while others are upregulated in developing teeth (Dassule and McMahon, 1998; Kratochwil et al., 2003; Sarkar and Sharpe, 1999). Loss of LEF1 causes arrested tooth development at the late bud stage, loss of expression of a direct LEF1/β-catenin target gene, *Fgf4*, and failure of survival of dental epithelial cells (Kratochwil et al., 2003; Sasaki et al., 2005). Other roles for Wnt signals in developing and postnatal teeth are likely masked in *Lef1*-null mice by functional redundancy of co-expressed *Lef1*, *Tcf1*, and possibly additional *Tcf* family members (Kratochwil et al., 2003; Oosterwegel et al., 1993). Consistent with additional Wnt functions, constitutive ectopic expression of *Dkk1* in the oral epithelia of transgenic mouse embryos causes arrested tooth development at the lamina-early bud stage (Andl et al., 2002), and oral epithelium expressing constitutively active β-catenin develops multiple teeth following transplantation to a kidney capsule (Jarvinen et al., 2006).

Here we investigate the pattern of, and precise requirements for, Wnt pathway activation at multiple stages of tooth development, and use explant culture and in vivo loss and gain of function experiments to determine how Wnt signaling interacts with other factors important for tooth morphogenesis. Our results place Wnt upstream of other key signaling pathways at several stages of tooth development, and suggest the potential use of Wnt activation in strategies for tooth regeneration.

Materials and methods

Generation of mouse lines and genotyping

Wnt activity was monitored using *TOPGAL* (DasGupta and Fuchs, 1999) (Jackson Laboratories, Bar Harbor, ME, USA), *BAT-gal* (Maretto et al., 2003), and *TCF/Lef-LacZ* (Mohamed et al., 2005) Wnt reporter transgenic mice. For epidermal-specific activation of β-catenin signaling, $Ctnnb1^{(Ex3)fl/+}$ mice (Harada et al., 1999) were crossed to *K14-Cre* line 43 transgenic mice (Andl et al., 2004). For epidermal-specific deletion of β-catenin, $Ctnnb1^{fl/fl}$ mice (Brault et al., 2001) (Jackson Laboratories) were crossed to *K14-Cre* line 43 transgenic mice (Andl et al., 2004). To assess the efficiency of *K14-Cre*-mediated recombination in oral and dental epithelia, *K14-Cre* mice were crossed with the *ROSA26R* Cre reporter line (Soriano, 1999) (Jackson Laboratories). For Wnt pathway inhibition, *K5-rtTA tetO-Dkk1* mice were generated and induced by feeding with chow formulated with 1 g/kg doxycycline (BioServ, Laurel, MD, USA) as previously described (Chu et al., 2004). To monitor the efficiency of Wnt pathway activation and inhibition, $Ctnnb1^{(Ex3)fl/+}$ mice and $Ctnnb1^{fl/fl}$ mice were crossed with *TOPGAL* mice and further crossed with *K14-Cre* mice; and *K5-rtTA tetO-Dkk1* mice were crossed with *TOPGAL* mice. To monitor activation of NF-κB signaling, *K5-rtTA tetO-Dkk1* mice were crossed with the NF-κB *LacZ* reporter transgenic mouse line $(Ig)_{3x}cona\text{-}lacZ$ (*NFκB-GAL*) (Schmidt-Ullrich et al., 1996). All experiments were performed with approved animal protocols according to the institutional guidelines established by the University of Pennsylvania IACUC committee.

Analysis of TOPGAL, BAT-gal, and TCF/Lef-LacZ expression

Heads from E11.5-E12.5 embryos were cryosectioned at 10–12 μm followed by X-gal staining. Embryonic mandibles at E12.5–E18.5 were fixed and whole mount stained with X-gal (Furth et al., 1994), photographed and/or paraffin-embedded, sectioned and counterstained with eosin.

Histology, immunofluorescence, BrdU incorporation, TUNEL assays, and in situ hybridization

Tissue preparation, histology, immunofluorescence with anti-β-catenin, BrdU assays, TUNEL staining, and in situ hybridization with digoxygenin-labeled probes were as described previously (Andl et al., 2004, 2002; Chu et al., 2004). Sections were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). *Bmp4*, *Edar*, *Lef1*, *Shh*, *Wnt10b*, *Eda*, *Ctnnb1*, *Msx1*, *Msx2*, *p21*, and *Ectodin* probes were as described previously (Andl et al., 2002; Kassai et al., 2005; Liu et al., 2007; Schmidt-Ullrich et al., 2006). The following PCR products each containing a T7 promoter were used as templates for sense and antisense probe synthesis: *Pitx2*, NM_011098, nt 972–1713; *Pax9*, NM_011041, nt 1431–1643 (Peters et al., 1998); *Ambn*, NM_009664.1, nt 248–957; *Dspp*: MN_010080.2, nt 385–1025 (Wang et al., 2004).