Connective Tissue Research

ISSN: 0300-8207 (Print) 1607-8438 (Online) Journal homepage: https://www.tandfonline.com/loi/icts20

Over- and Ectopic Expression of Wnt3 Causes Progressive Loss of Ameloblasts in Postnatal Mouse Incisor Teeth

S. E. Millar, E. Koyama, S. T. Reddy, T. Andl, T. Gaddapara, R. Piddington & C. W. Gibson

To cite this article: S. E. Millar, E. Koyama, S. T. Reddy, T. Andl, T. Gaddapara, R. Piddington & C. W. Gibson (2003) Over- and Ectopic Expression of Wnt3 Causes Progressive Loss of Ameloblasts in Postnatal Mouse Incisor Teeth, Connective Tissue Research, 44:1, 124-129, DOI: 10.1080/03008200390152205

To link to this article: https://doi.org/10.1080/03008200390152205

Connective Tissue Research, 44(Suppl. 1): 124–129, 2003

0300-8207/03 $12.00 + .00
DOI: 10.1080/03008200390152205

Taylor&Francis
healthsciences

Over- and Ectopic Expression of *Wnt3* Causes Progressive Loss of Ameloblasts in Postnatal Mouse Incisor Teeth

S. E. Millar,[1] E. Koyama,[2] S. T. Reddy,[1] T. Andl,[1] T. Gaddapara,[1] R. Piddington,[2] and C. W. Gibson[2]

[1]*Departments of Dermatology and Cell and Developmental Biology, University of Pennsylvania, Philadelphia, Pennsylvania, USA*

[2]*Department of Anatomy, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA*

Intercellular signaling is essential for the development of teeth during embryogenesis and in maintenance of the continuously growing incisor teeth in postnatal rodents. WNT intercellular signaling molecules have been implicated in the regulation of tooth development, and the *Wnt3* gene shows specific expression in the enamel knot at the cap stage. We demonstrate here that *Wnt3* also is expressed in specific epithelial cell layers in postnatal incisor teeth. To begin to delineate the functions of *Wnt3* in developing and postnatal teeth, we determined the effects of over- and ectopic expression of *Wnt3* in the tooth epithelium of mice carrying a keratin 14-*Wnt3* transgene. Expression of the transgene caused a progressive loss of ameloblasts from postnatal lower incisor teeth. Loss of ameloblasts may be due to defective proliferation or differentiation of ameloblast precursors, progressive apoptosis of ameloblasts, or loss of ameloblast stem cells.

Keywords Ameloblast, Mouse, Tooth, Transgenic, WNT.

INTRODUCTION

The development of teeth during embryogenesis involves a series of reciprocal inductive interactions between cells of the stomodeal or pharyngeal epithelium and underlying mesenchymal cells that arise from the neural crest [1], indicating that intercellular communication is essential for tooth development. Several families of intercellular signaling molecules have been shown to be expressed during tooth morphogenesis and to play key roles in this process. These include bone morphogenetic proteins, fibroblast growth factors (FGFs), Sonic hedgehog (SHH), activin, and WNT [2].

Received 9 November 2001; revised 11 March 2002; accepted 14 March 2002.

Address correspondence to S. E. Millar, M8D Stellar-Chance Laboratories, 422 Curie Boulevard, Philadelphia, PA19104-6100, USA. E-mail: millars@mail.med.upenn.edu

Rodent incisor teeth differ from the molars and from human teeth in that they continue to grow throughout life, providing an accessible model system for investigating proliferation and differentiation in dental tissue [3]. The length of the incisor teeth is normally regulated by the continual grinding of upper and lower incisors, and defects in craniofacial structure that cause malocclusion result in overgrowth of the incisor teeth. Incisors also differ from molar teeth in that enamel is only produced on the labial side of the tooth [3]. Postnatal growth of incisors requires a continuous supply of ameloblast cells that produce enamel and odontoblast cells that produce dentin, suggesting that stem cells for these populations are present in the incisor tooth. Recent studies indicate that putative ameloblast stem cells reside in the cervical loop at the base of the tooth [3]. The proliferation of ameloblast stem cell derivatives is regulated by mesenchymal signals that are likely to include FGF10 [3]. These results indicate that intercellular signaling is necessary for maintenance of growth of the postnatal incisor tooth as well as for tooth development during embryogenesis. In addition to FGF10, expression of SHH and FGF3 has been described in continuously growing postnatal rodent incisor teeth, suggesting that similar networks of regulatory factors may operate to control growth and differentiation in postnatal incisors to those that control tooth development [3, 4].

WNT proteins are secreted paracrine intercellular signaling molecules that play essential roles in many aspects of development and tumorigenesis [5]. In response to binding of WNT proteins to their receptors, a conserved "canonical" signaling pathway is activated that results in inhibition of a complex of proteins that normally acts to degrade cytoplasmic β-catenin [6]. As a result, β-catenin accumulates in the cytoplasm and in the nucleus, where it forms transcriptional complexes with members of the LEF/TCF family of DNA-binding factors, and activates the transcription of WNT target genes [5].

Several lines of evidence suggest that WNT proteins play key roles in tooth development. First, application of an endogenous secreted inhibitor of WNT action, mFRZB1, to molar tooth germs results in retarded tooth development and the formation of smaller teeth [7]. Second, in mice bearing a loss of function mutation in the WNT effector gene *Lef1*, tooth development is arrested at the bud stage [8, 9]. Third, multiple *Wnt* genes are expressed in developing molar teeth [10]. One of these, *Wnt3*, is specifically expressed in the enamel knot at the cap stage [10]. We show here that *Wnt3* also is expressed in the stratum intermedium, stellate reticulum, and outer enamel epithelium of adult incisor teeth. To investigate the functions of *Wnt3* in developing and postnatal teeth, we have determined the effects of over- and ectopic expression of this gene in mice carrying a keratin 14 (K14)-*Wnt3* transgene [11]. The K14 promoter has been shown to direct expression in the basal layer of the epidermis, the outer root sheath of hair follicles, oral epithelium, dental epithelium, enamel epithelium, and ameloblasts [12–14]. Several lines of K14-*Wnt3* transgenic mice have been described previously and show hair fragility due to abnormal differentiation of the hair shaft [11]. Here we describe the tooth phenotypes of these mice and demonstrate that over- and ectopic expression of *Wnt3* in epithelial layers of postnatal lower incisor teeth causes progressive loss of ameloblasts and enamel.

MATERIALS AND METHODS

In Situ Hybridization and Histology

For in situ hybridization and histology of postnatal teeth, mandibles were dissected and fixed overnight in 4% paraformaldehyde. For in situ hybridization, tissues were decalcified in 2% paraformaldehyde, 10% EDTA, dehydrated, paraffin embedded, sectioned at 6 μm, and subjected to in situ hybridization as described previously [15] using antisense probes for *Wnt3* [16] and human growth hormone (*hGH*) [11]. For histological analysis, mandibles were demineralized in 10% EDTA, dehydrated in alcohol, embedded in glycol methacrylate, sectioned at 7μm, and stained with hematoxylin and eosin.

Generation of K14-*Wnt3* Transgenic Mice

The generation of K14-*Wnt3* transgenic mice has been described previously [11]. Briefly, we constructed a K14 expression vector in Bluescript (Stratagene), containing 2kb of human K14 promoter sequences [12] upstream of a crippled version of the *hGH* gene into which a frameshift mutation had been introduced by destroying a *BglII* site in the last exon [17]. The *hGH* sequences provide introns and a polyadenylation site for transgene transcripts. A cDNA containing 508 bp of 5′ human *Wnt3* cDNA and 631 bp of 3′ mouse *Wnt3* cDNA ligated together at a conserved *PstI* site and encoding a hybrid human-mouse WNT3 protein was inserted at a unique *BamHI* site between the K14 promoter and the *hGH* sequences to create transgene K14-*Wnt3*. The transgene was released from plasmid vector sequences by digestion with *EcoRI* and was microinjected into the nuclei of FVB/N or C57BL/6J × FVB/N embryos. Transgenic animals were identified by Southern blot analysis of DNA prepared from tail biopsies, using a ^{32}P-labeled *Wnt3* cDNA fragment or *hGH* sequences as probes. Two lines of transgenic mice, designated TgN(K14-*Wnt3*)7 and TgN(K14-*Wnt3*)11, were maintained on an FVB/N background.

RESULTS

Wnt3 is Endogenously Expressed in Stratum Intermedium, Stellate Reticulum, and Outer Enamel Epithelium

To determine whether *Wnt3* is expressed during postnatal growth of incisor teeth, we hybridized sections of lower incisor teeth from wild type FVB/N mice at 2.5 months and 3.5 months of age with a ^{35}S-labeled probe for *Wnt3*. Expression was detected in cells of the outer enamel epithelium and stellate reticulum and in stratum intermedium cells immediately underlying the ameloblast layer. Expression of *Wnt3* was absent from preameloblasts and ameloblasts (Figure 1).

K14-*Wnt3* Transgene is Expressed in Epithelial Cells

To determine the sites of transgene expression in the oral cavity of K14-*Wnt3* transgenic mice, we used in situ hybridization with a transgene-specific probe and sections of mandibles from 2.5 month-old transgenic mice of a high-expressing K14-*Wnt3* transgenic line, previously designated TgN(K14-*Wnt3*)7 [11]. Transgene expression was detected throughout the epithelium of the cervical loop and in epithelial layers of the incisor teeth including the stratum intermedium, stellate reticulum, outer enamel epithelium, inner enamel epithelium, preameloblasts, and ameloblasts (Figure 2), as well as in surface epithelia of the oral cavity. No expression was detected in the incisor teeth of control, nontransgenic littermates (data not shown). The pattern of K14-*Wnt3* expression in the incisor tooth epithelia of postnatal transgenic mice is broader than that of the endogenous *Wnt3* gene, whose expression is confined to outer enamel epithelium, stellate reticulum, and stratum intermedium (see above). *Wnt3* is therefore overexpressed at endogenous locations in K14-*Wnt3* transgenic incisor teeth and also is ectopically expressed in preameloblasts and ameloblasts.

Lower Incisor Tooth Defects in K14-*Wnt3* Transgenic Mice

To begin to determine the effects of over- and ectopic expression of *Wnt3* in tooth epithelia, we examined the teeth of homozygous transgenic mice of line TgN(K14-*Wnt3*)7 and heterozygous transgenic mice of line TgN(K14-*Wnt3*)11, which express high levels of the K14-*Wnt3* transgene [11]. Examination of the teeth of these mice revealed overgrowth of the upper incisors that was first apparent between 2 and 4 months after birth and was not observed in nontransgenic littermates. Molar teeth appeared grossly normal in these mice. Skeletal preparations did not reveal obvious abnormalities of the jawbones that could have resulted in malocclusion (data not shown), suggesting that incisor overgrowth was due to an intrinsic defect in the incisor teeth. The upper incisor teeth of adult transgenic animals appeared normally enameled and were of normal thickness.

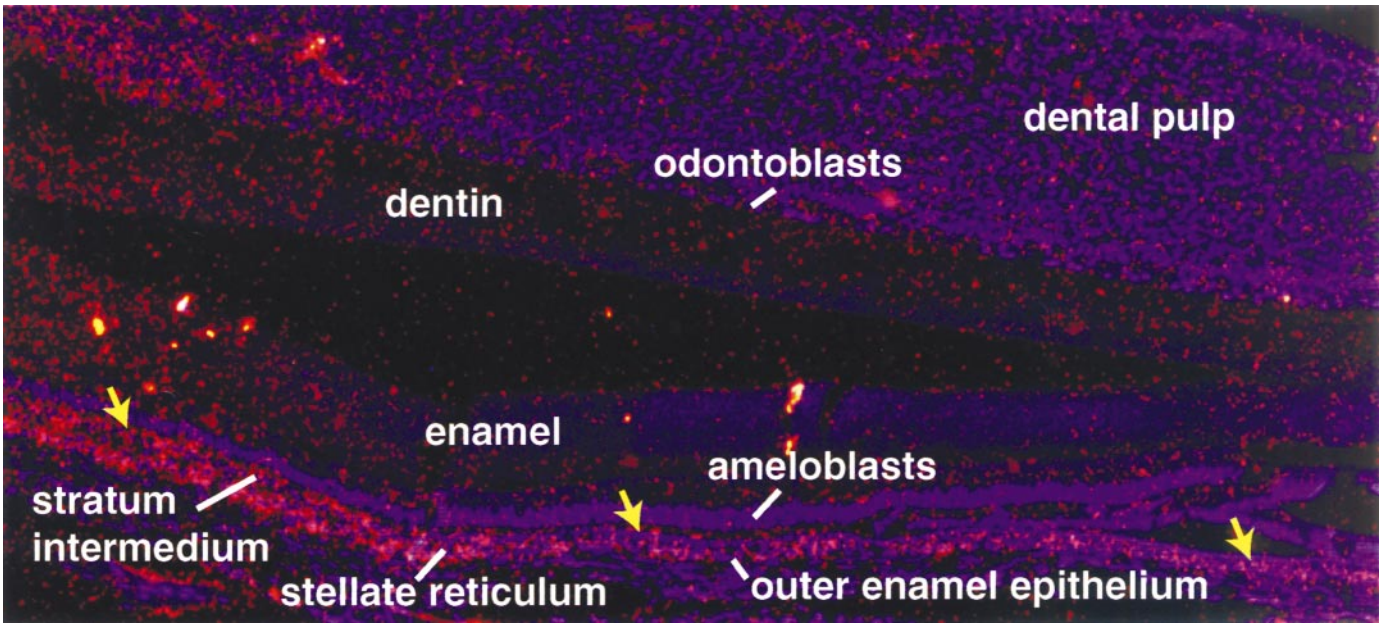

Figure 1. Expression of *Wnt3* in adult mouse incisor teeth. A section of lower left incisor tooth from a 2.5-month-old wild-type mouse was subjected to in situ hybridization with a ^{35}S-labeled probe for *Wnt3*. *Wnt3* is expressed in the stratum intermedium, stellate reticulum, and outer enamel epithelium but not in the ameloblasts. The positive signal appears as red grains. Nuclei are counterstained with Hoechst dye and appear blue. Hybridization signals are indicated by yellow arrows (photographed at 4× magnification).

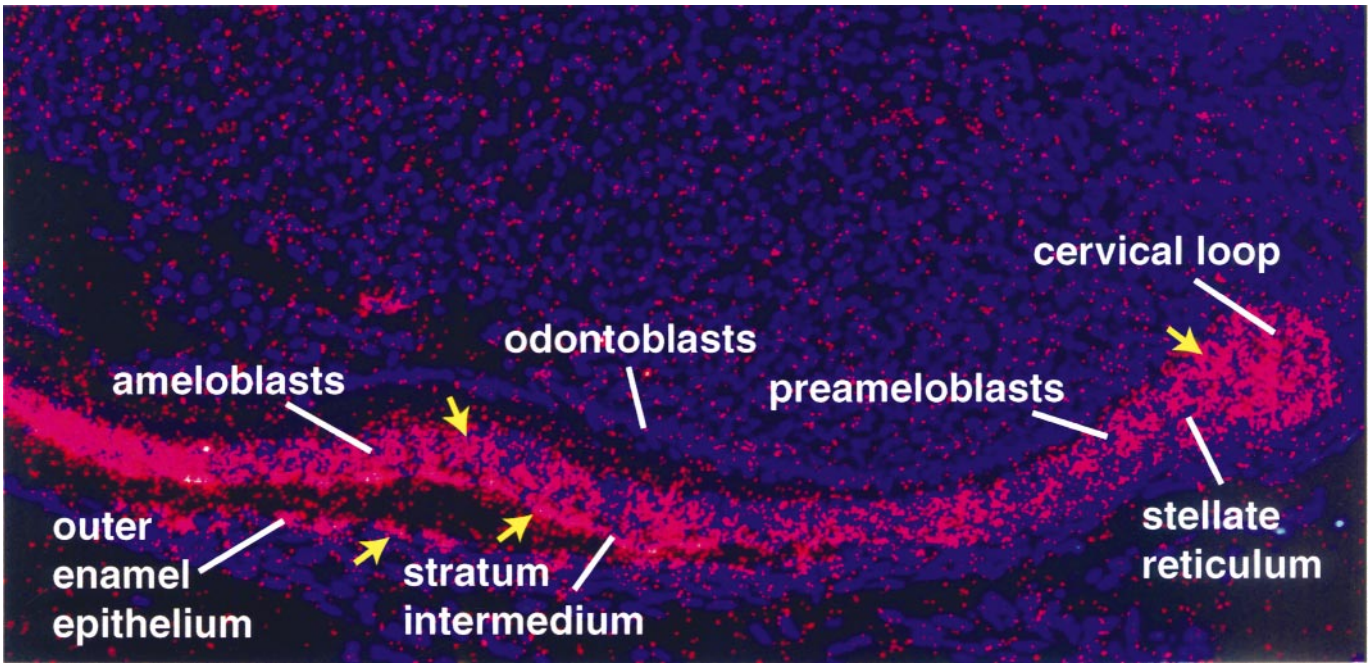

Figure 2. Expression of the K14-*Wnt3* transgene in a postnatal transgenic incisor tooth. A section through the lower left incisor tooth of a homozygous transgenic TgN(K14-*Wnt3*)7 mouse at 2.5 months of age was subjected to in situ hybridization with a probe for *hGH* that hybridizes to transgene-specific transcripts. Transgene expression was detected in the cervical loop, preameloblasts, ameloblasts, stratum intermedium, stellate reticulum, and outer enamel epithelium. Hybridization signals are indicated by yellow arrows (photographed at 10× magnification).

However, examination of the lower incisor teeth revealed that they were misangled, thin, and became progressively shorter with age. These observations suggested that overgrowth of the upper incisor teeth was caused by defects in the lower incisors.

The Incisor Teeth are Histologically Normal at Birth

To determine whether morphogenesis of the incisor or molar teeth was affected by expression of the K14-*Wnt3* transgene, homozygous transgenic mice of line TgN(K14-*Wnt3*)7 and nontransgenic littermates were sacrificed at postnatal day 1 and subjected to histological analysis of the oral cavity. Sectioned incisor and molar teeth were normal in appearance, and well-developed ameloblast and odontoblast cell layers and enamel and dentin were apparent in the incisors of both transgenic and control mice (Figure 3, upper panels). These results suggest that overexpression of *Wnt3* does not have major effects on the morphogenesis of incisor or molar teeth.

Enamel is Significantly Reduced and Ameloblasts are Abnormal or Absent by Seven Weeks of Age

To determine whether histological abnormalities were apparent in adult TgN(K14-*Wnt3*)7 homozygous transgenic incisors, lower jaws were dissected from TgN(K14-*Wnt3*)7 homozygous mice and nontransgenic littermates at 7 weeks and 16 weeks of age and subjected to histological analysis. Molar teeth were normal in appearance in the transgenics. In transgenic lower

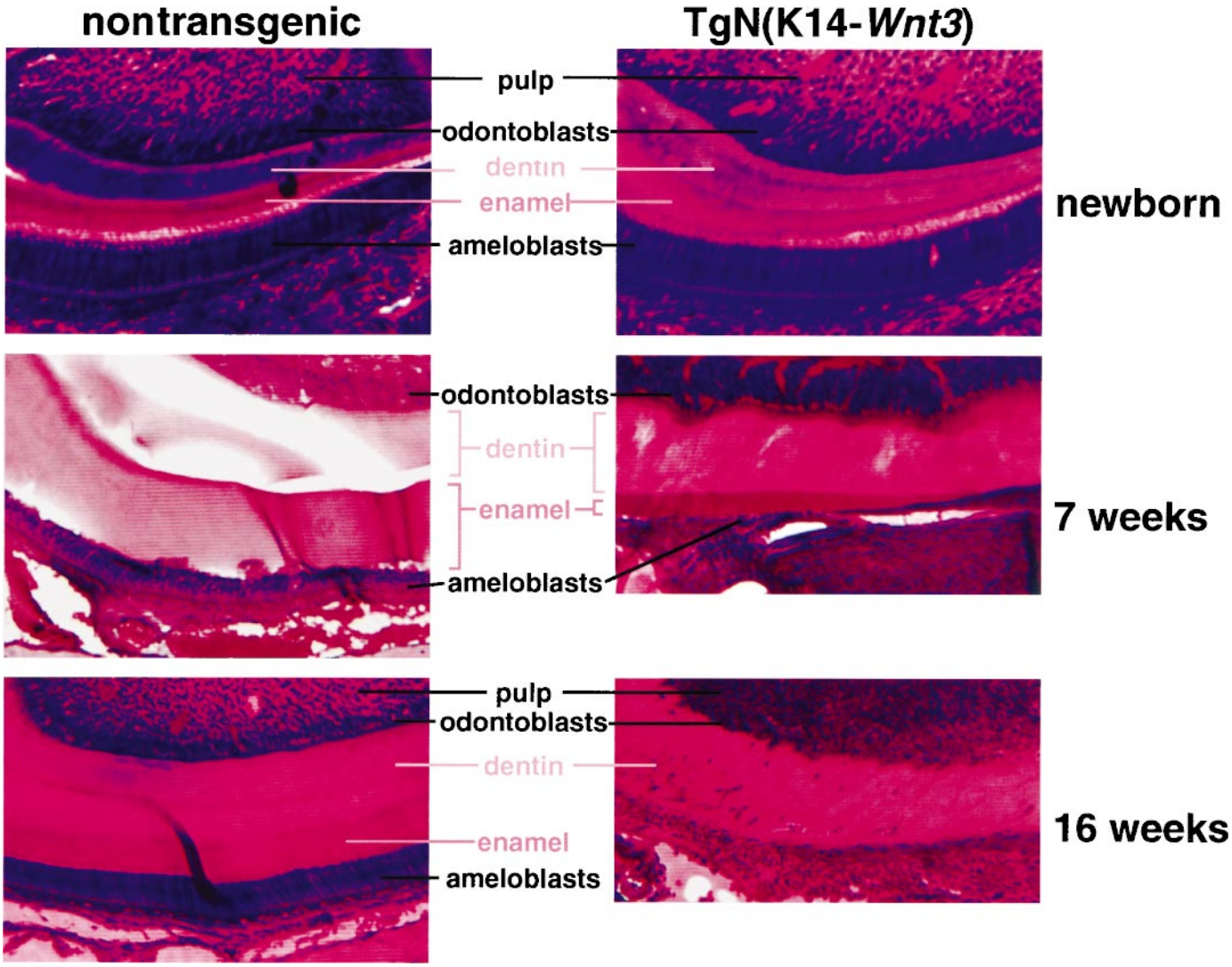

Figure 3. Histology of lower incisor teeth in K14-*Wnt3* transgenic and control mice during postnatal life. Methacrylate embedded sections of lower left incisor teeth from nontransgenic (left panels) and homozygous transgenic TgN(K14-*Wnt3*)7 mice (right panels) at postnatal day 1 (upper panels), 7 weeks of age (middle panels), and 16 weeks (lower panels) were stained with hematoxylin and eosin. Ameloblasts and enamel appeared similar to controls in newborn transgenic mice. At 7 weeks the ameloblast layer was discontinuous and enamel was thin in transgenics, but the dentin appeared normal with the exception of some slight irregularities in the odontoblast-dentin border. In lower incisor teeth from transgenic mice at 16 weeks of age, ameloblasts and enamel were absent, odontoblasts were disorganized, and dentin was present but contained dilated tubules (all panels photographed at 10× magnification).

incisor teeth at 7 weeks of age, the odontoblast and dentin layers appeared identical to controls, with the exception of some slight irregularities in the odontoblast-dentin border on the labial side of the tooth. In contrast, the thickness of the enamel layer was significantly reduced, and the ameloblasts were disorganized and entirely absent in some regions (Figure 3, middle panels). At 4 months of age, well-organized ameloblast and odontoblast cells layers, enamel, and dentin were apparent in control incisors. However, in transgenic incisors the enamel layer was missing, and the ameloblasts and stratum intermedium were completely absent (Figure 3, lower panels). Odontoblasts and dentin appeared normal on the lingual side of the incisor teeth (data not shown). On the labial side, dentin was present, but the odontoblast-dentin junction had become irregular, the dentinal tubules were enlarged and contained cellular material, and in some samples clumps of lymphatic tissue were visible in the pulp and surrounding connective tissue (Figure 3, lower panels). Since these phenotypes appeared later than the defects in the ameloblast layers and enamel, it is likely that they were secondary to the loss of ameloblasts.

DISCUSSION

The development of teeth during morphogenesis is regulated by several different families of intercellular signaling molecules that are re-utilized at successive stages of development [2]. Expression of several of these regulatory factors has been described in continuously growing, postnatal rodent incisor teeth, suggesting that the control of tooth development during embryogenesis and continuous eruption of the incisor in adult animals may be controlled by similar mechanisms [3, 4]. *Wnt3* is one of several *Wnt* genes expressed in developing teeth and shows specific localization to the enamel knot, a signaling center thought to regulate tooth shape [10]. To begin to investigate whether *Wnt* genes also are expressed in postnatal incisor teeth, we carried out in situ hybridization experiments with a probe for *Wnt3* and found that it is expressed in the outer enamel epithelium, the stellate reticulum, and in stratum intermedium cells underlying the ameloblast, enamel-producing layer. We show that in transgenic mice over- and ectopically expressing *Wnt3* in tooth epithelia, the ameloblast layer of the lower incisor teeth progressively deteriorates after birth, resulting in loss of enamel and gross deformities of the tooth. Presumably, these soft lower incisors do not provide an adequate grinding surface for the upper incisors, causing them to overgrow.

Obvious defects in tooth morphogenesis were not observed in transgenic molar or incisor teeth, despite the observation that *Wnt3* is normally expressed in the enamel knot of developing teeth at the cap stage. It is possible that the levels of *Wnt3* expressed in transgenic dental epithelium under the control of the K14 promoter were not sufficient to perturb morphogenesis, or that overexpression of this gene is not capable of altering the fate of developing dental cells. The lack of direct effects of the K14-*Wnt3* transgene on the upper incisor teeth is curious, since these teeth grow continuously and K14 directs expression in the epithelial cells of upper incisor teeth. It is possible that appropriate receptors for *Wnt3* are not expressed in upper incisor teeth, or that maintenance of ameloblasts is achieved through different mechanisms in the upper incisors.

Maintenance of the ameloblast layer of the incisor is thought to require the existence of a pool of ameloblast stem cells, which are capable of self-renewing as well as giving rise to differentiated progeny [3]. Recent studies have identified a rarely dividing population of cells in the cervical loop of the incisor tooth, which give rise to ameloblasts and express *Notch1*, a gene capable of regulating stem cell fate, suggesting these cells are putative ameloblast stem cells [3]. Transient amplifying progeny of the stem cells are thought to proliferate rapidly and to subsequently differentiate into ameloblasts. Since the K14 promoter directs expression throughout the cervical loop as well as in differentiating ameloblasts and other epithelial layers (Figure 2), loss of ameloblasts from transgenic lower incisor teeth might result from failure of activation of stem cells, or from repression of the proliferation or differentiation of transient amplifying ameloblast precursors. Overexpression of *Wnt3* in hair follicles causes altered differentiation of hair shaft precursor cells, consistent with this latter hypothesis [11]. Alternatively, the transgene may cause gradual apoptosis of ameloblast cells. A final possibility is that *Wnt3* causes forced exit of cells from the stem cell compartment, resulting in gradual depletion of the stem cells. This explanation would be consistent with the observed gradual loss of ameloblasts after birth. Interestingly, recent data suggest that WNT signaling causes the activation and proliferation of epithelial stem cells of the hair follicle [18–20], and it is tempting to speculate that similar mechanisms may operate in postnatal incisor teeth. Further investigations will be required to distinguish between these possibilities.

ACKNOWLEDGMENTS

We thank Dr. Elaine Fuchs for the K14 promoter, Dr. David Ornitz for the crippled *hGH* gene, and Drs. Roel Nusse and Henk Roelink for *Wnt3* cDNA and *Wnt3* in situ probe. K14-*Wnt3* transgenic mice were generated in the laboratory of Dr. Gregory Barsh at Stanford University. Dr. Millar is the recipient of a Dermatology Foundation/Dermik Laboratories Career Development Award.

REFERENCES

[1] Thesleff, I., and Sharpe, P. (1997). Signalling networks regulating dental development. *Mech. Dev.* 67(2):111–123.

[2] Jernvall, J., and Thesleff, I. (2000). Reiterative signaling and patterning during mammalian tooth morphogenesis. *Mech. Dev.* 92(1):19–29.

[3] Harada, H., Kettunen, P., Jung, H.S., Mustonen, T., Wang, Y.A., and Thesleff, I. (1999). Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. *J. Cell Biol.* 147(1):105–120.

[4] Koyama, E., Yamaai, T., Iseki, S., Ohuchi, H., Nohno, T., Yoshioka, H., Hayashi, Y., Leatherman, J.L., Golden, E.B., Noji, S., and Pacifici, M. (1996). Polarizing activity, Sonic hedgehog, and tooth development in embryonic and postnatal mouse. *Dev. Dyn.* 206(1):59–72.

[5] Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Ann. Rev. Cell Dev. Biol.* 14:59–88.

[6] Bejsovec, A. (2000). Wnt signaling: An embarrassment of receptors. *Curr. Biol.* 10(24):R919–R922.

[7] Sarkar, L., and Sharpe, P.T. (2000). Inhibition of Wnt signaling by exogenous Mfrzb1 protein affects molar tooth size. *J. Dent. Res.* 79(4):920–925.

[8] Van Genderen, C., Okamura, R.M., Farinas, I., Quo, R.G., Parslow, T.G., Bruhn, L., and Grosschedl, R. (1994). Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev.* 8(22):2691–2703.

[9] Kratochwil, K., Dull, M., Farinas, I., Galceran, J., and Grosschedl, R. (1996). Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. *Genes Dev.* 10(11):1382–1394.

[10] Sarkar, L., and Sharpe, P.T. (1999). Expression of Wnt signalling pathway genes during tooth development. *Mech. Dev.* 85:197–200.

[11] Millar, S.E., Willert, K., Salinas, P.C., Roelink, H., Nusse, R., Sussman, D.J., and Barsh, G.S. (1999). WNT signaling in the control of hair growth and structure. *Dev. Biol.* 207(1):133–149.

[12] Vassar, R., Rosenberg, M., Ross, S., Tyner, A., and Fuchs, E. (1989). Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. *Proc. Natl. Acad. Sci. USA* 86(5):1563–1567.

[13] Dassule, H.R., Lewis, P., Bei, M., Maas, R., and McMahon, A.P. (2000). Sonic hedgehog regulates growth and morphogenesis of the tooth. *Development* 127(22):4775–4785.

[14] Philbrick, W.M., Dreyer, B.E., Nakchbandi, I.A., and Karaplis, A.C. (1998). Parathyroid hormone-related protein is required for tooth eruption. *Proc. Natl. Acad. Sci. USA* 95(20):11846–11851.

[15] Reddy, S., Andl, T., Bagasra, A., Lu, M.M., Epstein, D.J., Morrisey, E.E., and Millar, S.E. (2001). Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a target of Sonic hedgehog in hair follicle morphogenesis. *Mech. Dev.* 107(1–2):69–82.

[16] Roelink, H., and Nusse, R. (1991). Expression of two members of the Wnt family during mouse development—restricted temporal and spatial patterns in the developing neural tube. *Genes Dev.* 5(3):381–388.

[17] Ornitz, D.M., Moreadith, R.W., and Leder, P. (1991). Binary system for regulating transgene expression in mice: Targeting int-2 gene expression with yeast GAL4/UAS control elements. *Proc. Natl. Acad. Sci. USA* 88(3):698–702.

[18] DasGupta, R., and Fuchs, E. (1999). Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* 126(20):4557–4568.

[19] Merrill, B.J., Gat, U., DasGupta, R., and Fuchs, E. (2001). Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin. *Genes Dev.* 15(13):1688–1705.

[20] Huelsken, J., Vogel, R., Erdmann, B., Cotsarelis, G., and Birchmeier, W. (2001). Beta-catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell* 105:533–545.