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ORIGINAL ARTICLE

Immunohistochemical expression of p63 in human prenatal tooth primordia

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Abstract
The aim of this study was to investigate the expression of the p63 gene in normal human tooth buds at different gestational stages. This is the first detailed study of p63 expression in normal human prenatal tooth primordia. The material consisted of sections of the midaxial tissue block from the cranial base of three human fetuses of gestational ages (GA) 11, 15, and 21 weeks. The sections included tooth primordia representing cap stages and bell stages of human tooth morphogenesis. In the present study, immunostaining was carried out using the primary antibody, monoclonal mouse anti human p63 protein, clone 4A4. The sections were counterstained with hematoxylin Mayer. p63 immunoreactivity was identified by microscopy. The study showed a positive reaction of p63 in both the cap stage and the bell stage. In both stages, positivity was observed in the cells of the oral mucosa, the inner and outer enamel epithelium, and in the primary and secondary dental lamina. In the early cap stage, there is a strong positive reaction to p63 in the enamel knot, but not in the late cap stage. We suggest that p63 may have an important regulatory function in the enamel knot.

Key Words: *Development, histochemical, human, malformation, p63 gene, tooth*

Introduction

It is well known from experimental studies that early molecular signaling takes place in the tissues in the developing tooth. Different genes are known to play a regulatory role in the different signaling pathways.

The p63 gene located at 3q27 is important for the intact signaling between ectoderm and mesoderm in a variety of ectodermally derived tissues. This is revealed by absent layering of the skin, malformed extremities, absent teeth, and absent salivary, mammary, and lacrimal glands in p63 knockout mice [1,2].

Mesenchymal expression of *Msx-1* is dependent on ectodermal signaling [3] and it has been shown that *Msx-1* expression is absent in *p63* knockout mice, suggesting that *Msx-1* acts downstream of p63 and that it therefore may play an essential role in the pathogenesis.

It is not yet fully understood what specific function p63 employs in the morphogenesis of the ectodermally derived tissues. However, previous studies indicate that p63 functions as a regulatory gene [1] directing the expression of downstream target genes, which in turn control the signaling between the cells in the ectoderm and the cells in the mesenchyme.

Mutations in p63 have been found in five human malformation syndromes [4]. These syndromes share malformations, which indicates that the mutation disrupts a molecular process specific for ectodermally derived tissues (limb abnormalities, ectodermal dysplasias affecting hair, teeth, nails, and different glands). These are the EEC syndrome (ectrodactyly-ectodermal dysplasia-clefting syndrome), AEC syndrome (ankyloblepharon-ectodermal defects-cleftlip/palate or hay-wells syndrome), LMS syndrome (limb-mammary syndrome), ADULT syndrome (acro-dermato-ungual-lacrimal-tooth syndrome), and non-syndromic split

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Table I. Tooth defects observed in families with p63 mutations

Diagnosis	Tooth defects reported	References
ADULT	Primary hypodontia of permanent teeth. In teenage, permanent teeth are being lost probably due to weak fixation	Propping et al., 2000 [5]
ADULT	Hypodontia, small peg-shaped teeth, mocroretrognathia	O'Brien et al., 2002 [6]
ADULT	Small mouth, multiple oral frenula	Amiel et al., 2001 [7]
EEC	Cone-shaped teeth. Peg-shaped teeth with delayed dentition	Barrow et al., 2002 [8]
EEC	Hypodontia, peg-shaped teeth	Roelfsema & Cobben, 1996 [9]; Buss et al., 1995 [10]
AEC	Hypodontia, maxillary retrognathia	Hay & Wells, 1976 [11]; McGrath et al., 2001 [12]
LMS	Hypodontia	Van Bokhoven et al., 1999 [13]
Rapp-Hodgkin	Partial anodontia, hypodontia, enamel dysplasia, dentine dysplasia, persistence of deciduous teeth	Bougeard et al., 2003 [14]

hand/foot malformation. Tooth defects have been observed in a number of families with p63 mutations suggesting that p63 is important in early human tooth morphogenesis [5–14] (see Table I).

So far, p63 expression has been observed in the oral epithelium of wild-type mice [1]. In p63 knockout mice, tongue and oral epithelium lack the stratification normally seen in the wild type. Furthermore, p63-deficient mice have a truncated maxilla, mandible, and secondary palate, and lack the vibrissae, tooth primordial, and soft-tissue structures [2].

In human tissues, p63 expression has been observed in the nuclei and epithelial cells of stratified epithelia such as skin, esophagus, exocervix, tonsil, and bladder, and in certain subpopulations of basal cells in glandular structures of prostate, breast, and bronchi [15]. The expression is therefore seen primarily in epithelia with a high turnover rate. Furthermore, p63 has been detected in normal human oral mucosa and in benign and malignant oral epithelial lesions.

There are six known different isoforms of p63 which derive from alternative splicing events and encode proteins with two different N-termini (TA and Δ) and three different C-termini (α, β, and γ), and it has been shown that there is a differential expression of the different *P63* isoforms in different layers of stratified epithelia correlating with the normal cellular differentiation [16].

In spite of the fact that patients with p63 mutations may suffer from dental defects, p63 expression was never addressed in human or animal tooth morphogenesis.

The aim of the present study was therefore to map the expression of p63 in normal human tooth buds from different GA in order to correlate it with normal tooth morphogenesis.

Material and methods

Three human fetuses, GA 11, 15, and 21 weeks, spontaneously aborted, were examined at Hvidovre University Hospital, Copenhagen, as part of required autopsy according to legal procedures. Normal development was determined either from a finding of normal chromosomes or, where chromosome analysis was not performed, according to macroscopic and microscopic examination of organs. In this connection, a midaxial tissue block from the cranial base was excised and histologically examined. The sections included the pituitary gland, the nasal septum, the vomeronasal organs, and parts of the maxilla. Tooth primordia were identified in some of these sections.

Immunohistochemistry

The midaxial tissue block from the cranial base of each fetus was decalcified in equal parts of 2% citric acid and 20% sodium citrate from 7 to 15 days, dehydrated, embedded in paraffin, and sectioned sagitally in 4-μm-thick serially cut sections.

Every 5th section was survey stained with toluidine blue (pH 7) in order to characterize the location of the tooth buds morphologically. Paraffin sections were deparaffinized and hydrated in degraded alcohols. Immunohistochemistry was carried out using the EnVision^{TM+}-peroxidase method (K 4065; DAKO, Glostrup, Denmark). The antibody used was monoclonal mouse anti-human p63 protein, clone 4A4. The p63 protein is a member of the p53 family, which includes p73. At least six different transcripts of p63 derive from alternative splicing events and encode proteins with two different N termini (TA and ΔN) and three different C termini (α, β, and γ). The protein isotypes TAap63α, TAp63β, and TAp63γ contain the N-terminal transactivation (TA) domain, whereas the other three isotypes ΔNp63α, ΔNp63β, and ΔNp63γ, lack this domain, and when present in sufficient concentration act in a dominant-negative manner with respect to wild-type p63 and p53 protein. This antibody reacts with all six known different transcripts of p63.

All incubations were carried out at room temperature: Following a 1×5 min wash in TBS, pH 7.6, the sections were encircled with a Pap Pen (DAKO). Sections were then washed 1×5 min in TBS. Endogenous peroxidase was blocked with dual endogenous

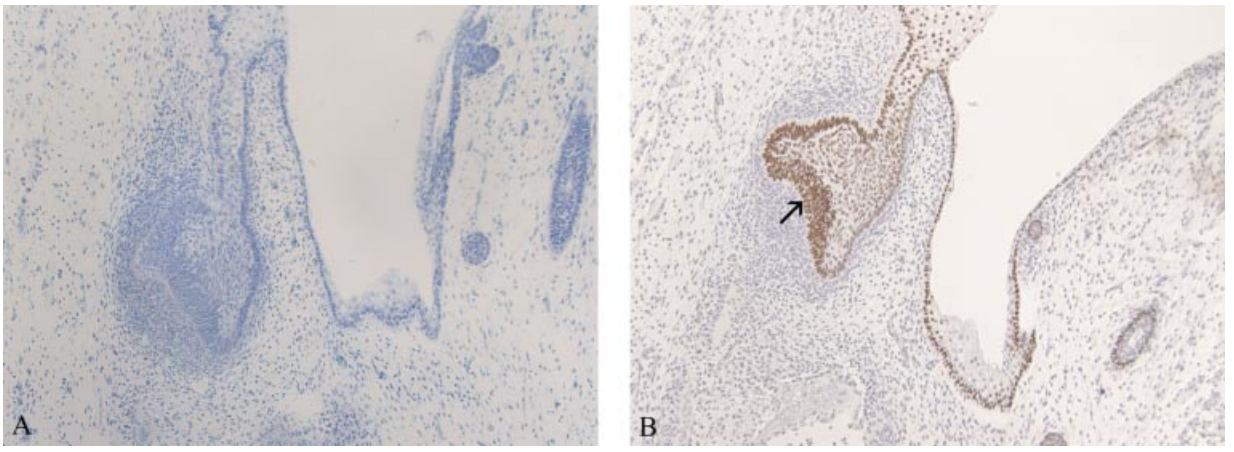

Figure 1. Sagittal sections of a human prenatal incisor tooth bud in the cap stage. Gestational age 11 weeks (×115). A. Toluidine blue staining showing the morphology of the human tooth bud in the cap stage. B. Immunoreactivity for p63 (brown reaction in cell nuclei) shows a positive reaction in the inner and outer enamel epithelium and in the dental lamina. In the oral mucosa, a positive reaction occurs in the basal cell layer and in the proliferating cell layer. A clear nuclear staining of the stellate reticulum and, as indicated by an arrow, a strong staining of the enamel knot can be seen.

enzyme block (K 4065; DAKO) for 10 min wash 1×5 min in TBS.

Incubation in primary antibody, monoclonal mouse anti-human p63 protein, clone 4A4 (M 7247; DAKO) diluted 1 : 50 in antibody diluent (S2022; DAKO) for 60 min. After washes 2×5 min in TBS, the sections were incubated with peroxidase labeled polymer (K 4065; DAKO) for 30 min. Following washes 3×5 min in TBS, sections were incubated in substrate/chromogen DAB (K 4065; DAKO) for 10 min.

The reaction was stopped in water and the tissue sections were counter-stained with hematoxylin Mayer (LAB00254; Bie & Berntsen, Denmark), dehydrated in graded alcohols and coverslipped using Pertex (Histolab, Sweden).

The results were based on the visual identification of p63 immunoreactivity in the tissue sections, using the light microscope (Leica, Germany).

Immunohistochemical controls

Two types of control sections were carried out. In the first control the primary antibody was replaced by an isotype form of the primary antibody (mouse IgG1 antibody, X0931; DAKO); in the second control the primary antibody was deleted and sections were incubated only with antibody diluent. Control sections were processed as described above. Both types of control sections failed to show p63 immunoreactivity.

Results

The tooth primordial from the three fetuses represented the following two stages of tooth morphogenesis.

Cap stage

In the two fetuses of GA 11 and 15 weeks, incisors were identified representing the cap stage (Figures 1A and 2A).

In the fetus of GA 11 weeks, p63 immunostaining was present in the basal cell layer of the outer enamel epithelium, the inner enamel epithelium, the dental lamina, and the overlaying mucosa (Figure 1B). In the overlying mucosa there was also staining in the proliferating cells of the stratified epithelium. Furthermore, a strong nuclear staining in the cells in the stellate reticulum was observed. There was a particularly strong staining of the enamel knot at this stage (Figure 1B).

In the fetus of GA 15 weeks, p63 immunostaining was not observed in the enamel knot. There was nuclear staining in the stellate reticulum comparable to the one seen in the fetus of 11 weeks GA (Figure 2B). There was a marked positive reaction to p63 in the basal cell layer of the oral mucosa and a clear nuclear staining in the proliferating cells in the mucosa. The dental lamina also showed a positive reaction to p63. Furthermore, a positive reaction of p63 was seen in the inner and outer enamel epithelium (Figure 2B).

Bell stage with hard tissue formation

Incisor tooth buds from the fetus of GA 21 weeks represented the dental developmental stage, bell stage, with hard tissue formation (Figure 3A).

p63 immunostaining was localized in the outer and inner enamel epithelium as well as in the oral mucosa (Figure 3B). In the oral mucosa, there was a positive reaction to p63 in the cells of the basal cell layer and in the proliferating cell layer, but not in the cells in the keratinized cell layer. The secondary dental lamina showed a positive reaction to p63 (Figure 3B). A weak nuclear staining in the cells of the stellate reticulum was observed (Figure 3B).

Discussion

The sections of tooth primordia from fetuses of three different gestational ages show a clear expression of

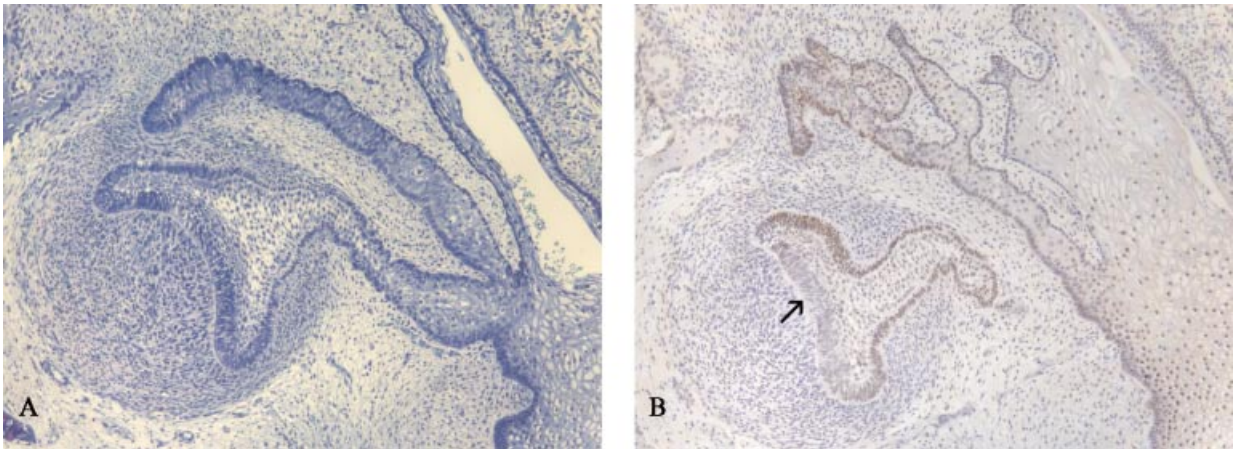

Figure 2. Sagittal sections of a human prenatal incisor tooth bud in the late cap stage. Gestational age 15 weeks (×130). A. Toluidine blue staining showing the morphology of the human tooth bud in the late cap stage. B. Immunoreactivity for p63 (brown reaction in cell nuclei) shows a positive reaction in the inner and outer enamel epithelium and in the dental lamina. In the oral mucosa a positive reaction occurs in the basal cell layer and in the proliferating cell layer. A nuclear staining of the stellate reticulum is observed, but P63 reaction is not observed in the enamel knot (*arrow*).

p63 in different stages of early human tooth development.

At GA 11, 15, and 21 weeks there is expression of p63 in the outer and the inner enamel epithelium as well as in the proliferating cells of the stratified epithelium in the overlying mucosa. Furthermore, there is a strong expression in the enamel knot at GA 11 weeks, which is not present at GA 15 weeks.

The enamel knot is present from the transition of bud stage to cap stage and resides at the transition from cap stage to bell stage [17], when the enamel knot undergoes apoptosis. The enamel knot is proposed to be the prerequisite for the tooth developing into the cap stage [17].

The exact function of the enamel knot is not yet fully clear. It has been suggested, however, that it functions as a signaling center in the tooth morphogenesis and provides positional information for the three-dimensional development of the tooth crown [18]. This is verified by the multiple expressions of genes and signaling molecules in the enamel knot, which are known to have a dominant function in morphogenesis. These are, among others, *Shh*, different bone morphogenetic proteins (*Bmp-2*, *Bmp-4*, and *Bmp-7*), and fibroblast growth factor-4 (*Fgf-4*) [18]. Furthermore, the apoptosis of the enamel knot is shown to be closely linked to the expression of p63 [17].

The formation of the enamel knot and its following disintegration is a natural part of the process and of the complex signaling between the cells in the ectoderm and the mesenchyme. In this signaling, some genes can be regarded as primary regulatory genes, e.g. p21. Others can be regarded as secondary genes, e.g. *Msx-1*, *Msx-2*, *Pax-9*, *Dlx-1*, and *Dlx-2*.

To our knowledge, the expression of p63 has never before been demonstrated in the human tooth primordia. Our results show a spatio-temporal expression of p63 indicating that p63 may play a role in

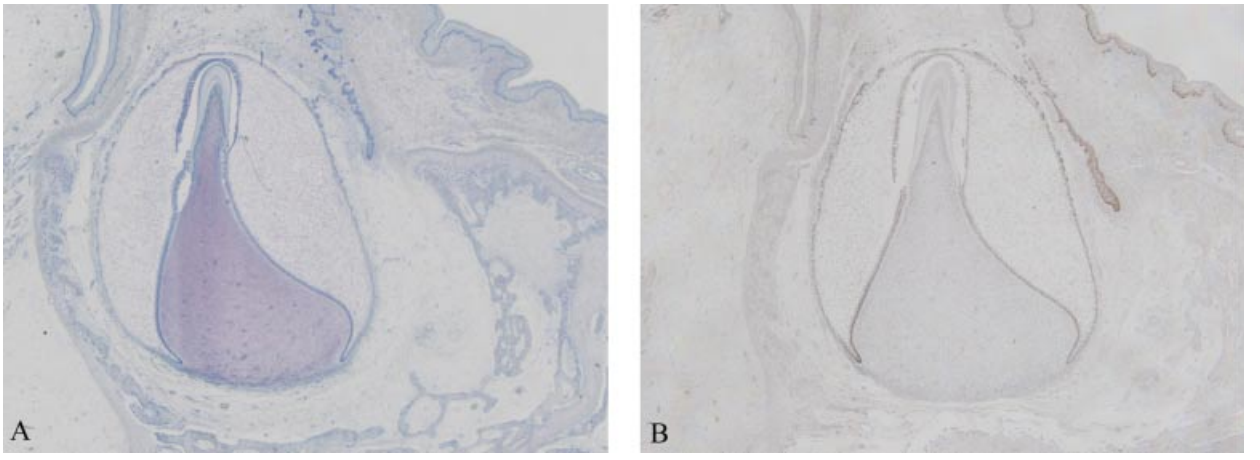

Figure 3. Sagittal section of a human prenatal incisor tooth bud in the bell stage with hard tissue formation. Gestational age 21 weeks (×30). A. Toluidine blue staining showing the morphology of the human tooth bud in the bell stage. B. Immunoreactivity for p63 (brown reaction in cell nuclei) shows a positive reaction in the inner and outer enamel epithelium and in the dental lamina of the permanent successor. In the oral mucosa a positive reaction occurs in the basal cell layer and in the proliferating cell layer. Nuclear staining of the stellate reticulum is weak.

the tooth formation. In particular, the strong reaction in the enamel knot at GA 11 weeks, which is not observed at GA 15 weeks, indicates that p63 may possibly play a role in the signaling in the morphogenesis of the human tooth. Thus, the p63 gene may have a regulatory function in the enamel knot where strong expression is detected until its disintegration towards the end of the cap stage.

In the present study, there is also a marked positive reaction to p63 in both the inner and outer enamel epithelium. The exact meaning of this is unknown, but it can be seen in the cap stage as well as in the bell stage. This finding indicates that p63 could have a regulatory function in the amelogenesis and possibly also in the dentinogenesis, and might explain why the multiple agenesis of teeth seen in individuals with mutations in p63 is often associated with malformations of the present teeth, e.g. conical crowns and enamel hypoplasia [5–14]. The different phenotypes in these syndromes and, in this context, in particular the different dental phenotypes presented in Table I might be related to disturbed expression of specific isotypes of p63, as suggested in the ADULT syndrome [19].

The present study shows that p63 is present in the dental primordia during formation. This is interesting because it is known that different tooth malformations in patients with p63 mutation can be attributed to the expression of p63 in specific domains in the developing human teeth. Future studies are encouraged to clarify the pathogenesis of the different malformations in families with p63 mutations.

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