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Pax9 is required for filiform papilla development and suppresses skin-specific differentiation of the mammalian tongue epithelium

Leon Jonker, Ralf Kist, Andrew Aw, Ilka Wappler, Heiko Peters*

Institute of Human Genetics, International Centre for Life, University of Newcastle upon Tyne, Central Parkway, Newcastle upon Tyne, NE1 3BZ, UK

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Abstract

The epidermis is a derivative of the surface ectoderm. It forms a protective barrier and specific appendages including hair, nails, and different eccrine glands. The surface ectoderm also forms the epithelium of the oral cavity and tongue, which develop a slightly different barrier and form different appendages such as teeth, filiform papillae, taste papillae, and salivary glands. How this region-specific differentiation is genetically controlled is largely unknown. We show here that *Pax9*, which is expressed in the epithelium of the tongue but not in skin, regulates several aspects of tongue-specific epithelial differentiation. In *Pax9*-deficient mice filiform papillae lack the anterior–posterior polarity, a defect that is associated with temporal–spatial changes in *Hoxc13* expression. Barrier formation is disturbed in the mutant tongue and genome-wide expression profiling revealed that the expression of specific keratins (*Krt*), keratin-associated proteins, and members of the epidermal differentiation complex is significantly down-regulated. In situ hybridization demonstrated that several 'hard' keratins, *Krt1-5*, *Krt1-24*, and *Krt2-16*, are not expressed in the absence of *Pax9*. Notably, specific 'soft' keratins, *Krt2-1* and *Krt2-17*, normally weakly expressed in the tongue but present at high levels in skin and in orthokeratinized oral dysplasia are up-regulated in the mutant tongue epithelium. This result indicates a partial trans-differentiation to an epithelium with skin-specific characteristics. Together, our findings show that *Pax9* regulates appendage formation in the mammalian tongue and identify *Pax9* as an important factor for the region-specific differentiation of the surface ectoderm.

Keywords: Pax9; Tongue; Filiform papillae; Keratin; Epithelial differentiation

1. Introduction

Ectodermal appendages, including scales, teeth, feathers, hair, nails, and a variety of eccrine glands such as mammary, sweat, salivary, and lachrymal glands are formed through a series of interactions between epithelial cells derived from the surface ectoderm and the underlying mesenchyme. Despite this diversity and the highly specialized functions of ectodermal appendages the morphological similarities and the utilization of similar signaling pathways during initiation and early phases of morphogenesis suggest that they may have arisen from a common, ancestral ectodermal appendage (reviewed in Pispa and Thesleff, 2003; Sharpe, 2001).

The epithelium of the oral cavity and tongue forms a unique set of appendages (teeth, filiform papillae, taste papillae, and salivary glands) that are all functionally involved in food ingestion. The true tongue, defined as having voluntary muscles and being movable, is a vertebrate-specific organ. The dorsal side of the tongue is covered with filiform papillae (FP), which are small, cone-shaped structures that aid the retention of food (Iwasaki, 2002). Each FP displays a distinct anterior–posterior polarity: the anterior aspect expresses a dense keratohyalin complex in the stratum granulosum and forms a so-called 'soft' orthokeratin, whereas the posterior aspect develops 'hard' orthokeratin (Cameron, 1966; Cane and Spearman, 1969; Hume and Potten, 1976), which is similar, though not identical to that of hair and nails (Dhouailly et al., 1989; Langbein et al., 2001; Tobiasch et al., 1992). One of the few factors known to regulate FP development is *Hoxc13*, a mouse homologue of the *Abdominal-B* gene of *Drosophila*.

Abbreviations: E; EDC; FP; Krt; Krtap; Sprr; Sprrl.

* Corresponding author. Tel.: +44-191-241-8622; fax: +44-191-241-8666.
E-mail address: heiko.peters@ncl.ac.uk (H. Peters).

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In *Hoxc13* deficient mice FP are formed, but have a round appearance and lack the sharply curved talons that normally point posteriorly (Godwin and Capecchi, 1998).

In addition, the epithelium of the skin and upper alimentary tract provides a protective barrier against harmful extra-cellular factors and infectious microorganisms. Barrier formation is achieved by a highly co-ordinated differentiation program of the keratinocytes, which form the so-called cornified envelope (CE). The CE consists of numerous structural proteins, which are cross-linked to form an insoluble macromolecular assembly (Fuchs and Raghavan, 2002; Kalinin et al., 2002). Barrier formation of the mouse skin starts at specific initiation sites during the late phase of gestation and spreads around the body as a moving front (Hardman et al., 1998). In the mouse tongue, barrier formation is initiated around embryonic day 16.5 (E16.5) and is fully established within 48 h (Marshall et al., 2000). Barrier formation in the tongue follows the expression pattern of *Sprr1A*, a member of a family of '*s*mall *p*roline-*r*ich *r*egion proteins' which encode key components of the CE (Steinert et al., 1998). Notably, *Sprr1A* is not expressed in the skin and the analysis of recently identified *Sprr*-like (*Sprrl*) genes in mice and humans (called *l*ate *e*nvelope *p*roteins (*LEP*) genes in humans) within the epidermal differentiation complex (EDC) suggests that these genes are differentially expressed in CEs of the skin and tongue (Marshall et al., 2001; Wang et al., 2001). This results in specific properties of these epithelia and may reflect the need to develop specific barriers in a different (wet or dry) environment. Similarly, specific combinations of keratin genes expressed in the epithelium of skin, oral cavity, and tongue are required to form and maintain a specific epithelial architecture. For example, targeted gene inactivation of *Keratin 6α* and *Keratin 6β* in mice results in severe lesions of the tongue epithelium, whereas the skin is not affected in these mutants (Wong et al., 2000). These region-specific, epithelial differentiation pathways are initiated during embryonic development, however, it is largely unknown how this is genetically controlled.

Pax9 belongs to a group of nine transcription factors characterized by the presence of a DNA-binding 'paired'-domain. In the mouse, *Pax9* is required for the formation of thymus, parathyroids, limbs, secondary palate, teeth, and vertebral column (Neubuser et al., 1995; Peters et al., 1998), whereas mutations in the human *PAX9* gene cause the autosomal dominant disorder oligodontia (Peters and Balling, 1999; Stockton et al., 2000). In the adult mouse, *Pax9* expression is restricted to the tongue, esophagus, salivary glands, and thymus, however, *Pax9* is not expressed in skin (Peters et al., 1997). Analysis of *PAX9* expression in dysplasia and carcinomas of the human esophagus suggested that *PAX9* is involved in the differentiation of epithelial cells within the upper digestive tract (Gerber et al., 2002). We present here a morphological and molecular analysis of the *Pax9* deficient mouse tongue. Our data demonstrate that *Pax9* regulates morphogenesis of FP and modulates various differentiation pathways in the epithelium derived from the anterior surface ectoderm.

2. Results

2.1. Expression of Pax9 in the mouse tongue

To analyze Pax9 protein distribution in the developing and adult tongue epithelium we carried out an immunohistochemical analysis. Pax9 protein is detectable at low levels in the tongue epithelium at E12.5 and E13.5 (data not shown). Subsequently Pax9 is expressed at high levels in the nuclei of epithelial cells of the developing FP throughout embryogenesis (Fig. 1A). The expression is maintained in the adult FP and is also seen in the interpapillary region (Fig. 1B).

2.2. Abnormal morphology and permeability of the Pax9 mutant tongue epithelium

At E18.5 the dorsal surface of the wild-type tongue is covered with FP which are interspersed with taste papillae (Fig. 2A). The surface of a *Pax9*-deficient tongue is smoother and the FP resemble cobblestone-like structures (Fig. 2B). Histological analyses showed that the normal FP exhibit a clear anterior–posterior polarity and have begun to

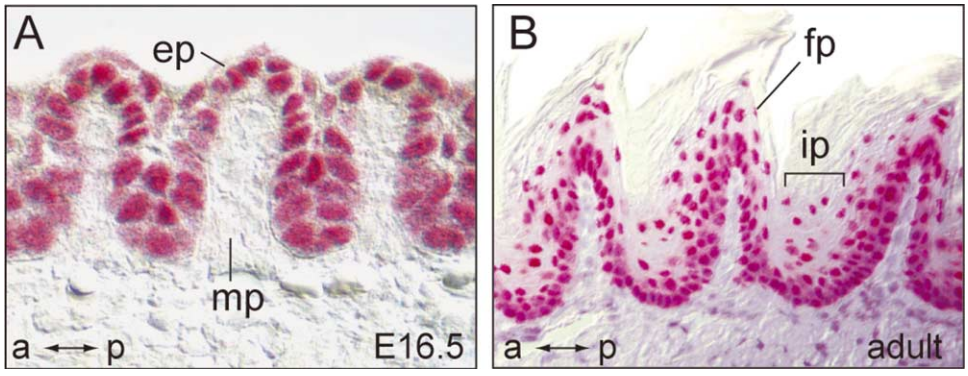

Fig. 1. Pax9 protein expression is restricted to epithelial cells of the mouse tongue. Sagittal sections of E16.5 (A) and adult tongue (B) were stained with an anti-Pax9 antibody. Pax9 is present in developing and adult FP and in epithelial cells between FP, but is absent from mesenchymal cells. a, anterior; ep, epithelium; fp, filiform papilla; ip, interpapillary region; mp, mesenchymal papilla; p, posterior. Magnifications 630× (A) and 400× (B).

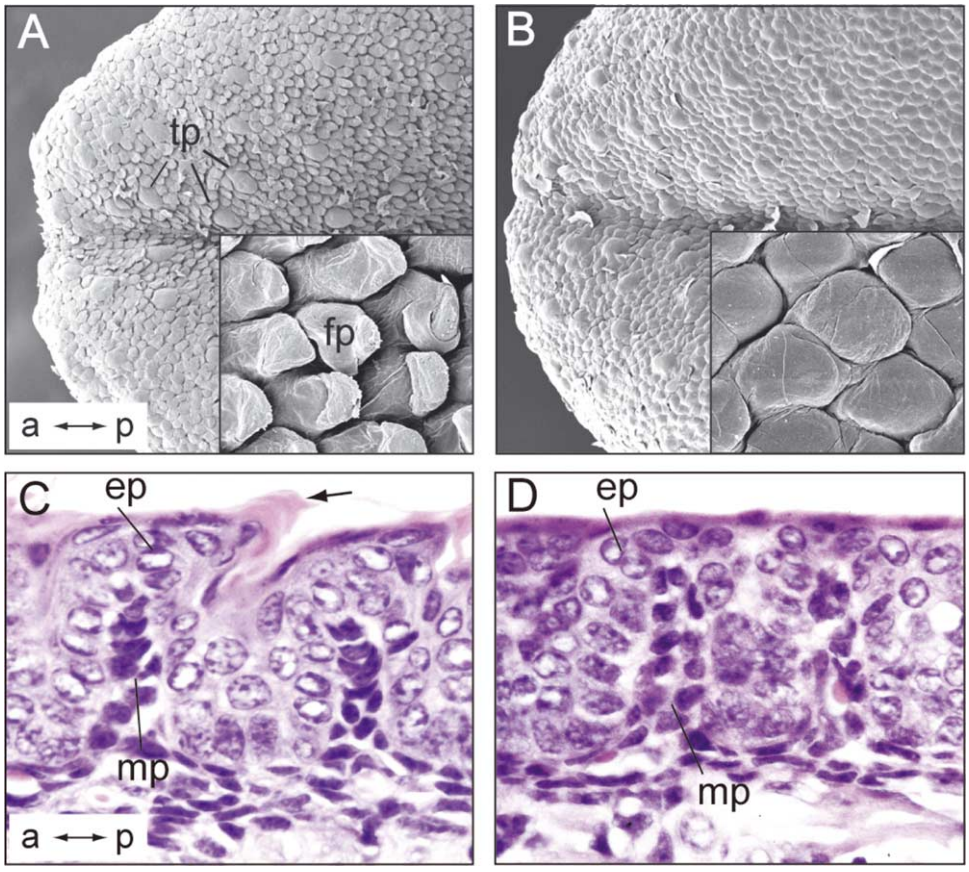

Fig. 2. Morphology of *Pax9*-deficient tongues. (A) SEM of a wild-type tongue at E18.5 showing that the surface of the dorsal tongue is covered with scale-like FP. (B) The surface of the *Pax9* mutant tongue is smoother and scale-like structures are missing (inset). (C) Sagittal section of wild-type FP consisting of epithelial columns separated by mesenchyme. The FP have started to form a cornified layer (arrow). (D) In the absence of *Pax9*, the papilla-like structure lacks an anterior–posterior polarity as well as a cornified layer. a, anterior; ep, epithelium; fp, filiform papilla; mp, mesenchymal papilla; p, posterior; tp, taste papilla. Magnification 630× (C, D).

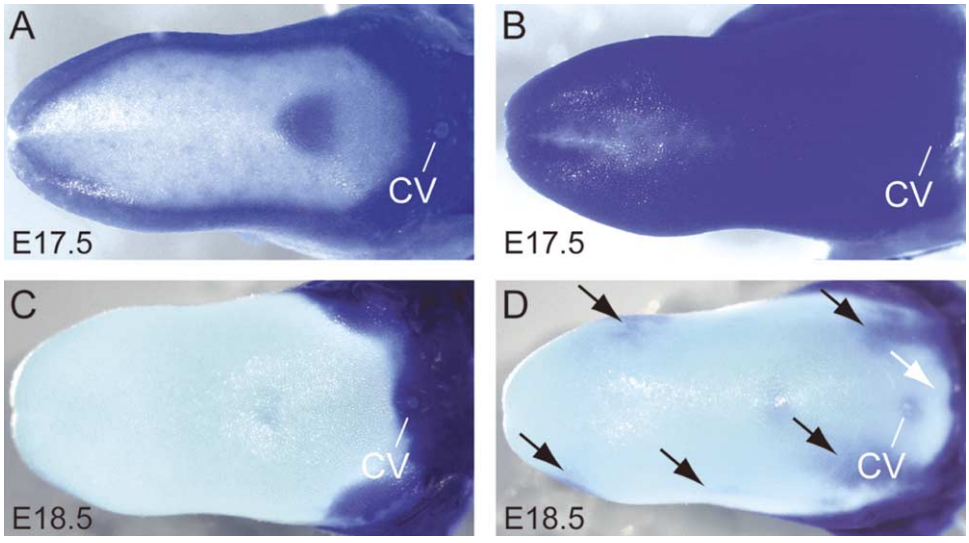

Fig. 3. Abnormal barrier formation in the *Pax9* mutant tongue. (A,C) In wild-type mice, barrier formation is initiated in the majority of the tongue surface epithelium at E17.5 and is completed at E18.5. (B,D) In *Pax9*-deficient mice, formation of the barrier is initiated in a restricted area within the anterior half of the tongue at E17.5. At E18.5 the barrier extends abnormally into the pharyngeal region (white arrow) and areas with incomplete barrier formation are irregularly scattered over the tongue surface (black arrows). cv, circumvallate papilla. Magnification 16-fold (A,B), and 14-fold (C,D).

form a cornified layer (Fig. 2C). In contrast, the mutant FP lack this polarity and a cornified layer is absent (Fig. 2D).

At E17.0, barrier formation has commenced in the wild-type tongue and most of the dorsal side has become impermeable at E17.5 (Fig. 3A). In contrast, the mutant tongue is completely permeable at E17.0 and only a restricted area has completed barrier formation at E17.5 (Fig. 3B). At E18.5, the dorsal epithelium of the wild-type tongue is completely impermeable, except for the posterior region, which is sharply separated from the impermeable area (Fig. 3C). The majority of the *Pax9*-mutant tongue epithelium has also developed a full barrier at E18.5, however, the barrier is abnormally expanded towards the pharyngeal region (Fig. 3D). In addition, a few irregularly distributed areas on the dorsal tongue were consistently found to be still permeable, indicating that barrier formation is delayed and does not follow a co-ordinated pattern.

2.3. De-regulated expression of genes involved in epithelial differentiation

For a genome-wide microarray analysis, RNA was collected from wild-type and *Pax9*-mutant tongues before morphological differences were visible (E15.5), as well as after the phenotype was clearly evident (E18.5). Since *Pax9* is expressed in epithelial cells the analysis of microarray data was focussed on genes involved in epithelial differentiation (Table 1). The microarray analysis indicated that at E15.5 and E18.5, several keratin genes involved in the formation of 'hard' orthokeratin (*Krt1-5*, *Krt1-24*, *Krt1-1*,

Table 1
Summary of epithelial gene expression changes in *Pax9*-deficient tongues identified by microarray analysis

Gene group	Gene	Chr.	E15.5				E18.5			
			Signal +/+	Signal −/−	SLR	Fold Differ.	Signal +/+	Signal −/−	SLR	Fold Differ.
Hard keratins	**Acidic**									
	Krt1-5	11	2464(P)	117(A)	−3.7	13 ↓	589(P)	181(P)	−1.4	2.6 ↓
	Krt1-4	11	398(P)	53(A)	−2.5	6 ↓	64(A)	85(A)		
	Krt1-24	11	425(P)	114(A)	−1.8	3.4 ↓	140(M)	15(A)	−3.2	9.2 ↓
	Krt1-23	11	719(P)	307(P)	−1.1	2.1 ↓	202(P)	334(P)	0.7	1.6 ↑
	Krt1-1	11	272(P)	134(A)	−0.8	1.8 ↓	36(P)	60(P)	−0.6	1.5 ↓
	Basic									
	Krt2-16	15	1171(P)	41(A)	−4.5	23 ↓	436(P)	343(P)	−0.7	1.6 ↓
	Krt2-18	15	350(P)	38(A)	−2.8	7 ↓	147(P)	54(P)	−0.9	1.9 ↓
	Krt2-5	15	1872(P)	619(M)	−1.3	2.5 ↓	1910(P)	2714(P)	<0.6	ns
Soft keratins	**Acidic**									
	Krt1-10	11	1375(P)	828(P)	>−0.6	ns	129(P)	1184(P)	3.2	9.2 ↑
	Basic									
	Krt2-1	15	461(A)	881(P)	1.0	2 ↑	209(P)	2422(P)	3.3	9.8 ↑
	Krt2-17	15	5(A)	40(A)			6(A)	86(P)	3.6	12 ↑
Keratin-associated proteins	*Krtap1-3*	11	749(P)	5(A)	−7.5	181 ↓	na	na		
	Krtap3-3	11	1007(P)	54(A)	−3.9	15 ↓	170(P)	165(P)	>−0.6	ns
	Krtap3-2	11	1427(P)	59(A)	−3.2	9.2 ↓	na	na		
	Krtap13	16	1684(P)	601(A)	−1.6	3 ↓	62(P)	59(P)	>−0.6	ns
EDC	*SprrL1*	3	6726(P)	311(P)	−4.0	16 ↓	410(A)	753(P)	1.3	2.5 ↑
	NICE-1	3	1321(P)	211(P)	−2.8	7 ↓	815(P)	1534(P)	0.8	1.8 ↑
	Sprr1A	3	4436(P)	2107(P)	−1.1	2.1 ↓	374(P)	1391(P)	1.9	3.7 ↑
	SprrL9	3	793(M)	401(A)	−0.7	ns ↓	305(A)	401(A)		
Epithelial differentiation	*suprabasin*	7	4610(P)	2131(P)	−1.1	2.1 ↓	8234(P)	9403(P)	<0.6	ns
	connexin43	10	2303(P)	1139(P)	−1.0	2 ↓	269(P)	267(P)	<0.6	ns
	calmodulin4	13	1453(P)	629(P)	−0.9	1.9 ↓	1152(P)	2053(P)	0.8	1.7 ↑
	sciellin	14	277(P)	160(P)	−0.7	1.6 ↓	228(P)	482(P)	0.8	1.7 ↑

Arbitrary values for signal intensities are given for RNA isolated from wild-type (+/+) and *Pax9*-deficient (−/−) mouse embryos at E15.5 and E18.5. Gene expression was classified as 'present' (P) with *P*-values <0.04, as 'marginal' (M) with *P*-values between 0.04 and 0.06, and as 'absent' with *P*-values >0.06. Signal log ratios (SLR) and 'fold difference' were calculated using the 'Microarray Suite' software and arrows indicate up- or down-regulation, respectively. With exception of *Krt1-23* and *Krt2-5*, all hard keratins were found to be down-regulated at both developmental stages. In contrast, soft keratins were found to be up-regulated. Note that acidic keratins and most of the keratin-associated proteins identified in this screen are clustered on mouse chromosome 11, whereas all basic keratins map to chromosome 15. Members of the 'epidermal differentiation complex' (EDC) on mouse chromosome 3, as well as several other genes involved in epithelial differentiation were found to be down-regulated at E15.5, but were expressed at normal levels or were found to be up-regulated at E18.5. Data on expression of genes not listed in this table are available on request. na, probe sets were not available on the array; ns, not significant.

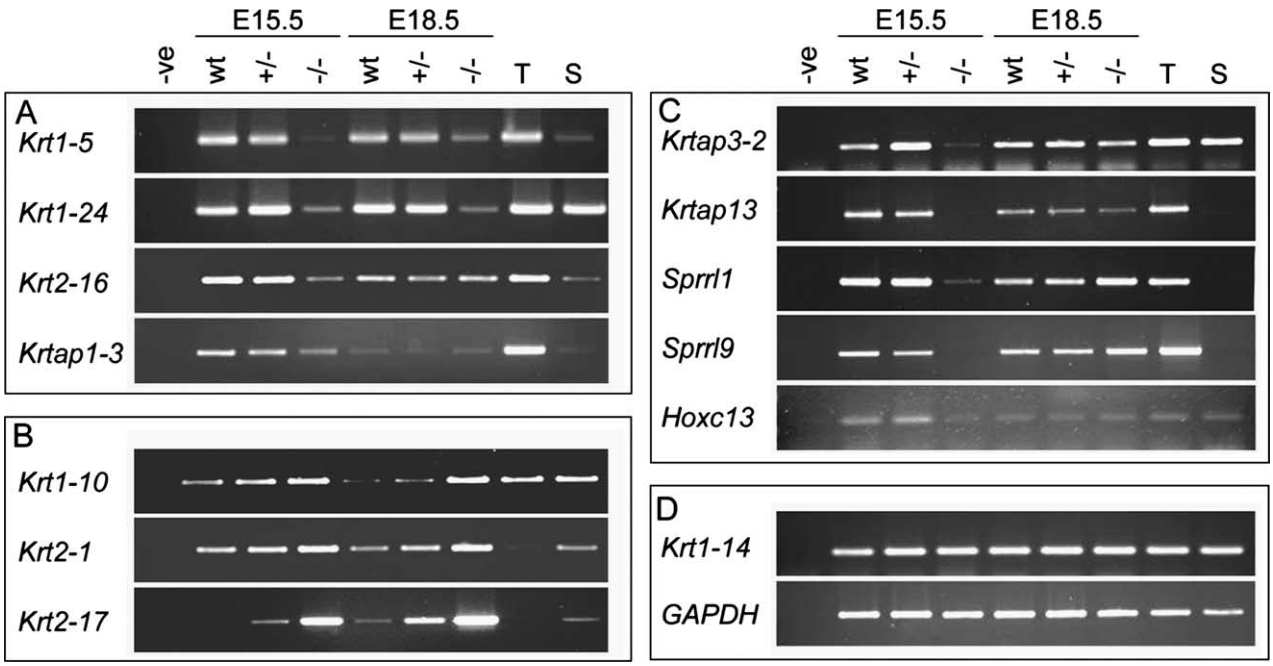

Fig. 4. RT-PCR of genes identified in the microarray analysis showing de-regulated expression. RT-PCR was carried out using RNA from wild-type (wt), *Pax9* heterozygous (+/−), and *Pax9* homozygous (−/−) mutant tongues, as well as from adult tongue (T) and skin (S). (A) Expression of 'hard' keratins and *Krtap1-3* was found to be down-regulated at both stages analyzed. (B) Up-regulation of keratins normally expressed at high levels in 'soft' orthokeratin. (C) Expression of genes showing strong down-regulation in homozygous mutants at E15.5, but similar expression levels at E18.5 in all three genotypes analyzed. (D) RT-PCR of *Krt1-14* and *GADPH* were used as internal controls.

Krt2-16, *Krt2-18*) and several keratin-associated proteins (*Krtap1-3*, *Krtap3-3*, *Krtap3-2*, *Krtap13*) were significantly down-regulated in the *Pax9*-mutant tongue. Decreased expression of genes representing these two groups was confirmed by RT-PCR (Fig. 4A) and in situ hybridization. At E16.5 the expression of *Krt1-5*, *Krt1-24*, *Krt2-16*, and *Krtap1-3* was strongest at the tip of the developing wild-type FP and high expression levels in the posterior compartment of the FP was found at E18.5 (Fig. 5). In agreement with the microarray results none of these genes exhibited significant expression in the *Pax9*-deficient epithelium at both time-points (Fig. 5). In contrast, the microarray data indicated that three keratin genes involved in the formation of 'soft' orthokeratin (*Krt1-10*, *Krt2-1*, *Krt2-17*) were up-regulated. Semi-quantitative RT-PCR revealed that in the adult mouse *Krt2-1* and *Krt2-17* are normally expressed at high levels in the skin but not in the tongue (Fig. 4B), indicating that the *Pax9*-deficient tongue epithelium develops skin-specific characteristics. This was supported by in situ hybridization showing that in the mutant *Krt2-1* and *Krt2-17* are expressed in nearly all cells within the upper epithelial cell layers, whereas in the wild-type mouse the expression is predominantly found in the anterior half of the FP (Fig. 6). A similar expression pattern was detected for *Krt1-10*, a keratin normally expressed at high levels in all suprabasal cells of stratified squamous epithelia which form 'soft' orthokeratin (Fig. 6). Together, these results show that the distorted FP development in *Pax9*-mutant mice is associated with a re-programming of cellular differentiation pathways involved in the organization of the cytoskeleton.

The microarray data also indicated that several genes were initially (E15.5) down-regulated in the *Pax9*-mutant tongue but were expressed at apparently normal or even higher levels at E18.5., including *Krtap1-3*, *Krtap3-3*, *Krtap3-2*, and *Krtap13* (Table 1; Fig. 4C). Some of the genes following the same pattern are located within the EDC on mouse chromosome 3, such as *Sprr1A*, *Sprrl1* (also called *Eig3*), *Sprrl9*, and *NICE-1* (Table 1 and Fig. 4C). Similarly, the expression of *suprabasin* (Park et al., 2002) and *sciellin* (Kvedar et al., 1992), two other factors involved in CE formation was decreased at E15.5 but up-regulated at E18.5. These results are in agreement with the delayed barrier formation of the *Pax9* mutant tongue, which is also indicated by the late onset of *Sprrl1* expression (Fig. 7). Moreover, late onset of epithelial differentiation was indicated by reduced expression of *connexin43*, a member of the connexin family of proteins forming gap junctions (Dahl et al., 1995), and *calmodulin-4*, a member of calmodulins which are known to mediate epithelial differentiation by binding and presenting calcium (Hennings et al., 1980).

Late onset of expression was also detected for *Hoxc13* (Fig. 4C), which was not detectable in the *Pax9* mutant tongue by in situ hybridization at E16.5 (Fig. 7). At E18.5, *Hoxc13* transcripts are predominantly found in the posterior compartment of the wild-type FP, but are irregularly

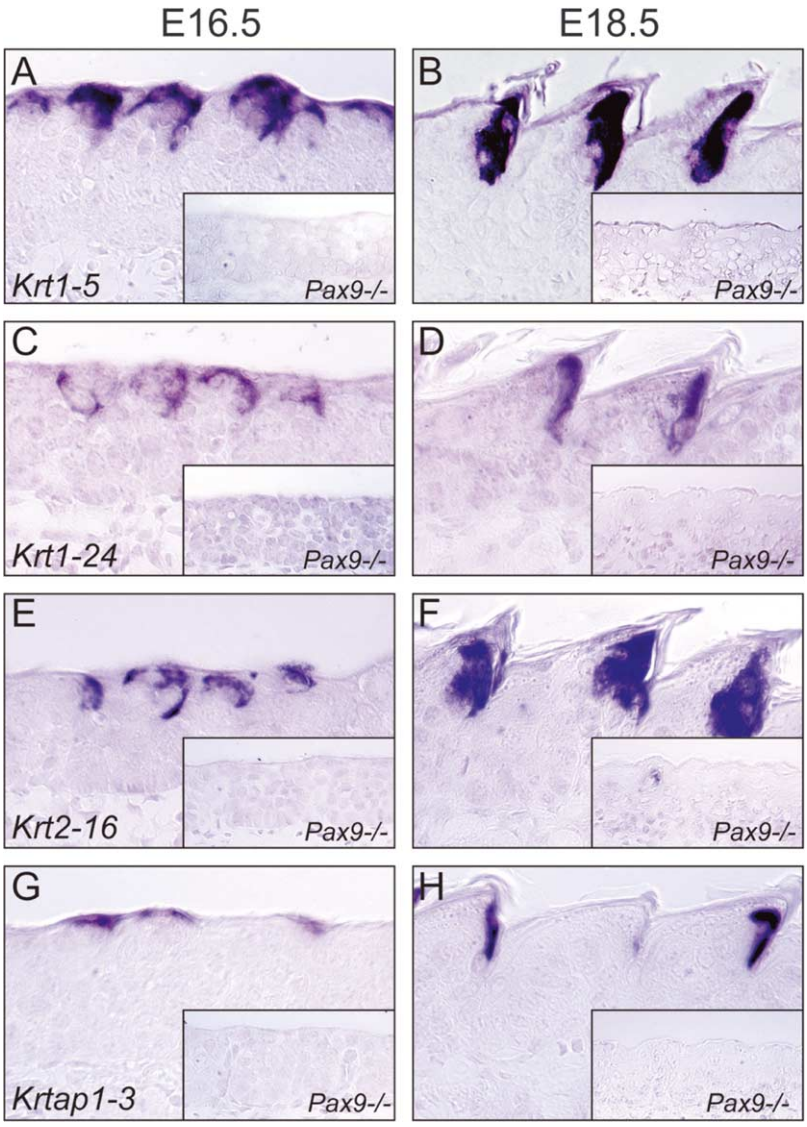

Fig. 5. Gene expression patterns of epithelial markers that require continued *Pax9* activity. Anterior is to the left in all panels and insets in panels A–H demonstrate absence or drastic down-regulation of expression in the *Pax9*-deficient tongue. (A, B) Expression of *Krt1-5* is detectable at E16.5 and is restricted to the posterior half of the FP at E18.5. Similar expression patterns at both developmental stages were observed for *Krt1-24* (C, D) and *Krt2-16* (E, F). (G, H) *Krtap1-3* is expressed in the superficial layer at E16.5 and exhibits a highly restricted expression pattern in the posterior half of the FP at E18.5. Magnification 630×.

scattered within the upper epithelial layers of the *Pax9*-deficient tongue. In contrast, the expression of *Klf4*, encoding a transcription factor that is essential for barrier acquisition throughout the epidermis (Segre et al., 1999), was not altered in homozygous *Pax9*-mutant tongue epithelium at any stage (data not shown).

3. Discussion

Important insight into the transcriptional control regulating the differentiation of stratified squamous epithelia has been provided by gene inactivation of *p63* and *Klf4* in mice. Lack of *p63* results in the absence of stratification and a failure to express epithelial differentiation markers (Mills et al., 1999; Yang et al., 1999). Absence of the Kruppel-like factor four (*Klf4*) affects late differentiation steps, leading to a complete absence of barrier formation (Segre et al., 1999). Whereas *p63* and *Klf4* regulate the differentiation of external (skin) and internal (tongue, oral cavity) stratified squamous epithelia, transcription factors that regulate this differentiation either in external or in internal epithelia have not been identified. Our analyses have identified *Pax9* as an important component of the genetic control that modulates the differentiation of stratified squamous epithelia in a region-specific manner.

In the adult FP three epithelial differentiation programs are executed, resulting in the formation of anterior, posterior, and buttress columns (Hume and Potten, 1976). Our experiments revealed that *Krt1-5*, *Krt1-24*, *Krt2-16*, and *Krtap1-3* were

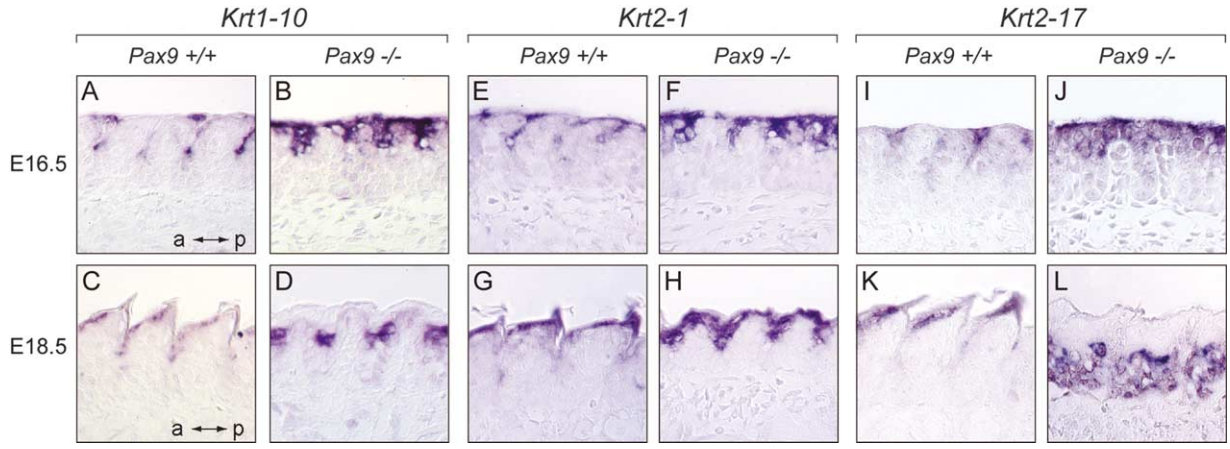

Fig. 6. Ectopic expression of keratin genes normally found in 'soft' orthokeratin. (A, C) *Krt1-10* is weakly expressed in the control epithelium at E16.5 and E18.5. Note that at E18.5, *Krt1-10* is predominantly expressed in the anterior half of the FP. In *Pax9*−/− tongues, *Krt1-10* is strongly expressed in the upper two third of the developing FP at E16.5, and to a lesser degree, at E18.5. Similar observations were made for the expression of *Krt2-1* (E–H) and *Krt2-17* (I–L). Note that in contrast to *Krt1-10* and *Krt2-1*, *Krt2-17* expression is restricted to the lower half of epithelial cells in the *Pax9*-deficient tongue at E18.5. Magnification 630×.

significantly down-regulated in the *Pax9*-deficient mouse tongue epithelium. These genes are normally expressed in the posterior column of the FP and could not be detected in adult back skin (Tobiash et al., 1992). The absence of these genes in the *Pax9*-deficient FP is associated with an apparent loss of characteristics specific for the posterior column, resulting in FP without a visible anterior–posterior polarity. Interestingly, a similar, though less severe defect has been identified in homozygous *Hoxc13*-mutant mice (Godwin and Capecchi, 1998). *Hoxc13* was found to be down-regulated in *Pax9*-deficient FP at E15.5, shortly before the anterior–posterior polarity of the FP is established. At E18.5 *Hoxc13* expression levels are normal in the *Pax9*-deficient tongue epithelium, however, transcripts were irregularly scattered. Similarly, the expression of *Krtap3-3*, *Krtap3-2*, and *Krtap13* was found to be drastically down-regulated at E15.5 and E16.5, but was clearly detectable at E18.5. It is noteworthy that *Krtap13* maps within a cluster of *Krtap* genes on mouse chromosome 16, of which some have previously been shown to be regulated by *Hoxc13* (Tkatchenko et al., 2001).

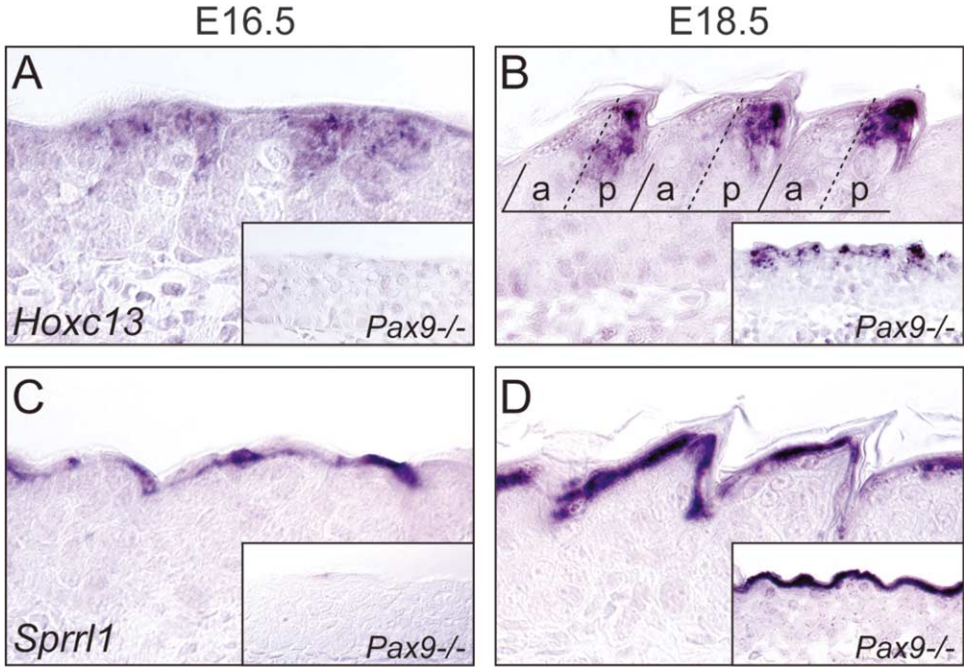

Fig. 7. Defects of FP development and delayed barrier formation are associated with de-regulated expression of *Hoxc13* and *Sprrl1*. (A) Expression of *Hoxc13* is detectable in wild-type mice but is absent in the *Pax9*−/− tongue at E16.5 (inset). (B) At E18.5 *Hoxc13* is predominantly expressed in the posterior half of the developing wild-type FP. In contrast, expression of *Hoxc13* in the *Pax9*-deficient tongue does not show a recognizable pattern (inset). (C) *Sprrl1* expression is initiated in the wild-type tongue epithelium, and is barely detectable in the absence of *Pax9* (inset). (D) At E18.5, both wild-type and mutant epithelium strongly express *Sprrl1* in the outermost epithelial cell layer. a, anterior; p, posterior. Magnification 630×.

In addition, *Hoxc13* was also identified to regulate the expression of specific keratin genes in the hair follicle (Jave-Suarez et al., 2002). Together, this suggests that both patterning and part of the differentiation defects in the *Pax9*-deficient tongue epithelium could result from a delayed onset of *Hoxc13* expression. A delay in the onset of expression was also identified for a subset of genes clustered within the EDC (*Sprr1A*, *Sprrl1*, and *Sprrl9*). Members of this gene family have been implicated in the formation of the CE (Steinert et al., 1998) and the expression of *Sprr1A* was shown to precede barrier formation of the tongue in the same temporal–spatial pattern (Marshall et al., 2000). Hence, the delay in barrier formation observed in the *Pax9*-deficient tongue is likely to be caused by a delayed onset of expression of EDC genes that are required for CE formation.

In addition to missing and delayed onset of gene expression we identified a specific set of keratins to be up-regulated in the *Pax9*-deficient tongue epithelium. The microarray data indicated a 10-fold increase of expression levels of *Krt1-10*, *Krt2-1*, and *Krt2-17* at E18.5, and RT-PCR and in situ hybridization support this result. Notably, a strong up-regulation of the three corresponding human homologous keratin genes (*K10*, *K1*, and *K2e*) has been described in hyperkeratotic lesions of oral dysplasia (Bloor et al., 2003). *K2e* is normally strongly expressed in the skin and only detectable at low levels in the oral mucosa (Collin et al., 1992; Herzog et al., 1994; Smith et al., 1999). Similarly, our results show that *Krt2-17*, and also *Krt2-1* expression is normally weak in the developing mouse tongue but is high in the skin. Severe defects of the tongue epithelium including loss of FP was also seen in transgenic mice over-expressing *K10* under the control of the *K6β* promoter (Santos et al., 2002). Collectively, the ectopic expression of *Krt1-10*, *Krt2-17* and *Krt2-1* in the *Pax9*-mutant tongue epithelium indicates that *Pax9* is involved in the suppression of a skin-specific epithelial differentiation program. Additional studies are required to investigate if the transcriptional activation of these keratins in oral lesions showing skin-like ortho-keratinization depend on a local down-regulation of *Pax9* expression. Conversely, activation of ectopic *Pax9* expression in the skin epithelium using a transgenic approach should help to determine the degree to which *Pax9* can modify a skin-specific epithelial differentiation program.

4. Experimental procedures

4.1. Animals

The *Pax9* mutant allele, $Pax9^{lacZ}$ (Peters et al., 1998), was maintained as a heterozygous mutation on a C57BL/6 genetic background. Heterozygous mutant mice were inter-crossed to obtain wild-type and homozygous mutant tongues, taking the morning of vaginal plug detection as embryonic day 0.5 (E0.5). Due to postnatal lethality of newborn *Pax9*−/− mice (Peters et al., 1998), analyses of time-points later than E18.5 were not possible.

4.2. Barrier formation and histology

Barrier formation was visualized using a 1% toluidine blue solution and was carried out as described previously (Hardman et al., 1998). At least six animals per time-point were analyzed. For histological analysis of the FP, mouse tongues were fixed in 4% paraformaldehyde, dehydrated in ethanol, and embedded in paraffin. Sagittal sections (5 μm) were stained with haematoxilin and eosin using standard procedures.

4.3. RT-PCR and generation of riboprobes

Tissue was homogenized and RNA was isolated from mouse tongue and skin with TRIzol®, according to manufacturer's instructions (Invitrogen). The isolated mRNA was converted into cDNA using Superscript II following manufacturer's instructions (Invitrogen). In three independent experiments, PCR amplifications were performed using gene-specific primers (Table 2; sequences of oligonucleotides available on request), designed to span two or more exons, except for single exon genes. Amplification was carried out for 25–35 cycles and analyzed on an ethidium bromide containing agarose gel. For generating riboprobe templates, cDNA fragments were amplified from mouse mRNA by RT-PCR using gene-specific oligonucleotide primers (Table 2; sequences of oligonucleotides available on request), and cloned into pCRII-Topo (Invitrogen).

Table 2
Gene fragments cloned for RT-PCR and in situ hybridization experiments

Gene	Genebank accession No.	PCR product (bp)	In situ probe (bases)
GAPDH	M32599	522-832 (311)	na
HoxC13	NM_010464	555-815 (261)	725-1225 (501)
Krt1-5	X65506	374-724 (351)	374-724 (351)
Krt1-10	AK014360	973-1312 (340)	1614-1945 (332)
Krt1-14	NM_016958	1384-1588 (205)	na
Krt1-24	NM_016880	118-561 (444)	1106-1710 (605)
Krt2-1	NM_008473	736-1002 (267)	1778-2318 (541)
Krt2-16	AY028607	520-745 (226)	1464-2037 (564)
Krt2-17	X74784	930-1244 (315)	930-1244 (315)
Krtap1-3	AK009730	185-480 (296)	185-727 (543)
Krtap3-2	XM_193648	5-387 (383)	na
Krtap13	NM_010671	23-353 (331)	na
SprrL1	NM_033175	16-411 (396)	16-544 (529)
SprrL9	NM_026335	9-537 (529)	na

Gene specific primers (sequence information available on request) were designed to amplify cDNA fragments. For riboprobe templates the amplified cDNA fragment partly included 3′ untranslated region. The position of the bases covered by the primers within the cDNA are followed by the size of the PCR product in brackets. na, not applicable.

4.4. In situ hybridization and immunohistochemistry

Non-radioactive in situ hybridization was performed as described in the 'In situ Hybridization Manual' supplied by Roche, using slight modifications. Digoxigenin (DIG) labeled probes were generated using a DIG-labeling kit (Roche) and sections were hybridized overnight in a humidified chamber. RNA hybrids were detected with an alkaline phosphatase-conjugated anti-DIG antibody and visualized using NBT/BCIP as a substrate. Tongues from at least two different embryos for each genotype were used for in situ hybridization. Expression of Pax9 protein on paraffin sections was visualized using a monoclonal anti-Pax9 antibody as described (Gerber et al., 2002). The expression was analyzed in tongues from wild-type embryos at E12.5, E13.5, E14.5, E16.5, E18.5, and from adult mice ($N>3$ for each stage).

4.5. Microarray analysis

The anterior two-third of the tongue (E15.5 or E18.5) was isolated from wild-type and homozygous *Pax9* mutant (*Pax9*−/−) mouse embryos, respectively. For each microarray experiment a minimum of three wild-type or *Pax9*−/− tongues were collected and pooled together, and RNA was isolated. The mRNA was converted to labeled cRNA and hybridized to MG-U74 (E15.5) or Mouse430 (E18.5) microarrays as recommended by the manufacturer (Affymetrix). For data analysis, the software 'Affymetrix Microarray Suite' was used, applying default parameters for detection and comparison of gene expression.

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