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Sox9 Is Essential for Outer Root Sheath Differentiation and the Formation of the Hair Stem Cell Compartment

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Summary

Background: The mammalian hair represents an unparalleled model system to understand both developmental processes and stem cell biology. The hair follicle consists of several concentric epithelial sheaths with the outer root sheath (ORS) forming the outermost layer. Functionally, the ORS has been implicated in the migration of hair stem cells from the stem cell niche toward the hair bulb. However, factors required for the differentiation of this critical cell lineage remain to be identified. Here, we describe an unexpected role of the HMG-box-containing gene *Sox9* in hair development.

Results: *Sox9* expression can be first detected in the epithelial component of the hair placode but then becomes restricted to the outer root sheath (ORS) and the hair stem cell compartment (bulge). Using tissue-specific inactivation of *Sox9*, we demonstrate that this gene serves a crucial role in hair differentiation and that skin deleted for *Sox9* lacks external hair. Strikingly, the ORS acquires epidermal characteristics with ectopic expression of GATA3. Moreover, Sox9 knock hair show severe proliferative defects and the stem cell niche never forms. Finally, we show that *Sox9* expression depends on sonic hedgehog (Shh) signaling and demonstrate overexpression in skin tumors in mouse and man.

Conclusions: We conclude that although Sox9 is dispensable for hair induction, it directs differentiation of the ORS and is required for the formation of the hair stem cell compartment. Our genetic analysis places *Sox9* in a molecular cascade downstream of sonic hedgehog and suggests that this gene is involved in basal cell carcinoma.

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Introduction

The mammalian hair develops through intricate signaling between the epithelial ectoderm and the underlying mesenchyme (for review see [1]). The generation and analysis of transgenic and knockout mice have identified numerous molecular players involved in this finely tuned cross talk (for review see [2]). In the mouse, development of hair pelage commences at E14.5 in response to a mesenchymal signal, which induces a thickening of the ectodermal epithelium (hair placode). Epithelial cells continue to proliferate, invade, and then engulf the underlying mesenchymal cells, the latter of which will constitute the permanent dermal papilla. The epithelial cells in close contact to the papilla make up the highly proliferative matrix of the hair bulb. In response to cues coming from the dermal papilla, matrix cells rapidly divide and, while moving upwards, differentiate into the three cell layers of the hair shaft (medulla, cortex, and cuticle) as well as the different concentric cylinders of epithelial cells surrounding the hair shaft. The inner root sheath (IRS) represents the most inner epithelial lineage and is composed of three distinct cell layers: the IRS cuticle, the Huxley's layer and the Henle's layer. The IRS is surrounded by the outer root sheath (ORS), an epithelial compartment that is directly connected with the basal layer of the epidermis.

In contrast to all other mammalian organs, the hair undergoes continuous cycling throughout adult life [3, 4]. In the mouse, a complete hair cycle lasts approximately 25 days and can be divided into three phases: a phase of growth (anagen) characterized by significant proliferation in the matrix of the hair, leading to an extension of the hair into the deeper layers of the dermis and the growth of the hair shaft; regression (catagen), which is mediated through apoptosis in the lower part of the hair and which causes retraction of the hair follicle; and, finally, a phase of inactivity (telogen). Regrowth of a new hair involves the stimulation of stem cells residing in a specific compartment of the hair contiguous to the ORS and located near the arrector pili muscle, the bulge [5–7]. This growth-induction process seems to be mediated at least in part by signaling through Shh and Wnt/β-catenin [8–12]. Stem cells leave the bulge and migrate toward the matrix, where they begin to proliferate and subsequently differentiate into a new hair shaft [13]. How stem cells find their way to the matrix is so far poorly understood, but it has been shown that they migrate along the ORS, a finding that may suggest that this cell layer provides important cues to guide cells toward the hair bulb.

Sox9 is a member of the *Sox* gene family, which is

characterized by the presence of an HMG box with more than 50% homology to the sex determining gene *Sry* [14]. The HMG box can bind and bend DNA, and it has been proposed that *Sox* genes encode architectural DNA binding proteins. In addition to the HMG box, Sox9 possesses a transactivation domain at its C terminus [15], and it has been shown that it can activate the genes *Mis* in testis [16, 17] and *Col2a1* [18] during chondrogenesis in vitro and in vivo. Moreover, in vitro studies suggest that *Sox* genes may also have a role in RNA splicing [19], but in vivo evidence for such a function is still missing.

Human patients carrying mutations/rearrangements in *SOX9* locus suffer from campomelic dysplasia, a skeletal malformation syndrome characterized by bowing of the long bones, and high frequency of XY sex reversal [20–22]. Studies in mice have confirmed the pivotal role of Sox9 during chondrogenesis [23–25] but also demonstrated an important function for this gene in the formation of many organs including the testis [26], the heart [27], the neural tube [28], and the neural crest [29].

In this report, we describe a so-far-unknown role for *Sox9* in the development and maintenance of the mammalian hair. We show that *Sox9* is activated in hair placodes at the time of induction and is continuously expressed in the bulge and ORS of the hair follicle. We further provide data that while *Sox9* seems to be dispensable for hair induction, it is essential for normal hair formation and cycling. Indeed absence of *Sox9* leads to a complete loss of CD34-positive stem cells, suggesting that it is required for the maintenance of this important cell lineage.

Results

Analysis of *Sox9* Expression during Hair Formation and Cycling

Sox9 expression was analyzed by in situ hybridization during hair follicle development. Activation was found at E12.5 in whisker pads (data not shown) and as early as E14.5 in hair placodes (Figure 1). Sectioning of in situ hybridized tissues or immunofluorescent analysis with Sox9 antibodies demonstrated strong staining in the thickening epithelial component and absence from the underlying mesenchyme. Sox9 remained to be expressed during the downgrowth of the placode but at E18.5, became restricted to the presumptive outer root sheath (ORS) layer. Sox9 could be detected in the nuclei of K14-positive cells, a specific marker for the ORS. Sox9 expression was maintained at all stages of hair cycling. During anagen (P8) Sox9-positive cells were abundant; however, at the lower part of the hair follicle, ORS cells lacked Sox9 expression (Figures 1D and 1E). Similarly, ORS cells at the infundibulum appeared to be negative for Sox9. Sox9 remained to be expressed at catagen (P17) (Figure 1F). At telogen stage (P21), a small number of cells positive for Sox9 were located at the base of the follicle epithelium, presumably representing bulge cells (Figure 1G).

The bulge compartment contains slowly cycling stem cells that are important for replenishing the pool of follicle cells during hair cycling. Stem cells in the bulge have been demonstrated to express high levels of CD34 [6, 30–32]. Double labeling with CD34 demonstrated that Sox9 is expressed in most, if not all, stem cells (Figure 1H). To clearly identify whether all CD34 positive cells also express Sox9, we performed confocal-microscope-aided 3D reconstruction. Careful analysis demonstrated that all CD34-positive cells also showed Sox9 staining (see Movie S1). Apart from the hair follicle, Sox9-positive cells were also present in the sebaceous gland (data not shown).

Skin-Specific Inactivation of *Sox9* Leads to Alopecia

To analyze the function of *Sox9* in hair formation, we made use of a Cre transgenic line (*Y10:Cre*) that was recently generated in our lab and showed strong ectopic expression of the Cre recombinase in the developing epithelial layer. Developmental analysis of Cre activity with a PGK-driven indicator mouse strain (*pgklxneoLacZ*) [33] showed activation of the Cre recombinase in the epithelial component of developing skin as early as E14.5 (Figure 2 and data not shown).

To investigate the role of *Sox9* during hair development, we crossed *Sox9*$^{flox/flox}$ mice with *Y10:Cre* transgenic animals. Immunofluorescent analysis was performed to determine the efficiency of Cre-mediated excision of *Sox9* at various stages of development. At E15.5, deletion of *Sox9* was incomplete in skin epithelium, and most of the hair placodes still expressed the protein (data not shown). At E18.5, Sox9 protein was dramatically reduced and at P2 virtually absent from caudal skin of *Y10:Cre; Sox9*$^{flox/flox}$ mice (Figures 2D and 2E). Careful immunofluorescent analysis at various stages after birth showed that only very few hair follicles (<1%) were able to escape Cre-mediated excision of Sox9 (data not shown).

Heterozygous deletion of Sox9 (*Y10:Cre; Sox9*$^{flox/+}$) in skin had no obvious effect on development and animals showed normal coat formation. In contrast, *Y10:Cre; Sox9*$^{flox/flox}$ animals developed severe hair abnormalities and appeared hairless in the caudal part of the body (Figure 2C). Despite the nude appearance of *Sox9* knockout mice, careful observation demonstrated the presence of small, atrophic hair. The mouse has three main hair types (Guard, zig-zag, and awl), which can be clearly distinguished by their size and shape (Figure 2F) [34]. Macroscopic analysis of plucked hair from P21 *Y10:Cre; Sox9*$^{flox/flox}$ mice demonstrated dramatically smaller hair, and the different types were virtually impossible to distinguish (Figure 2G). Moreover, during the plucking, we noticed that hair from knockout skin broke off easily, pointing to a fragility of the hair shaft. Scanning electron microscopy confirmed these findings on an ultrastructural level (Figures 2H and 2I).

Histological Analysis of Hair Development in *Sox9* Knockout Skin

We next performed histological analysis to study the knockout phenotype during hair follicle development and cycling. From E15.5 to E18.5, no obvious histological abnormalities could be found and the number of hair placodes in *Y10:Cre; Sox9*$^{flox/flox}$ animals was similar to that in control littermates (compare Figure 3B

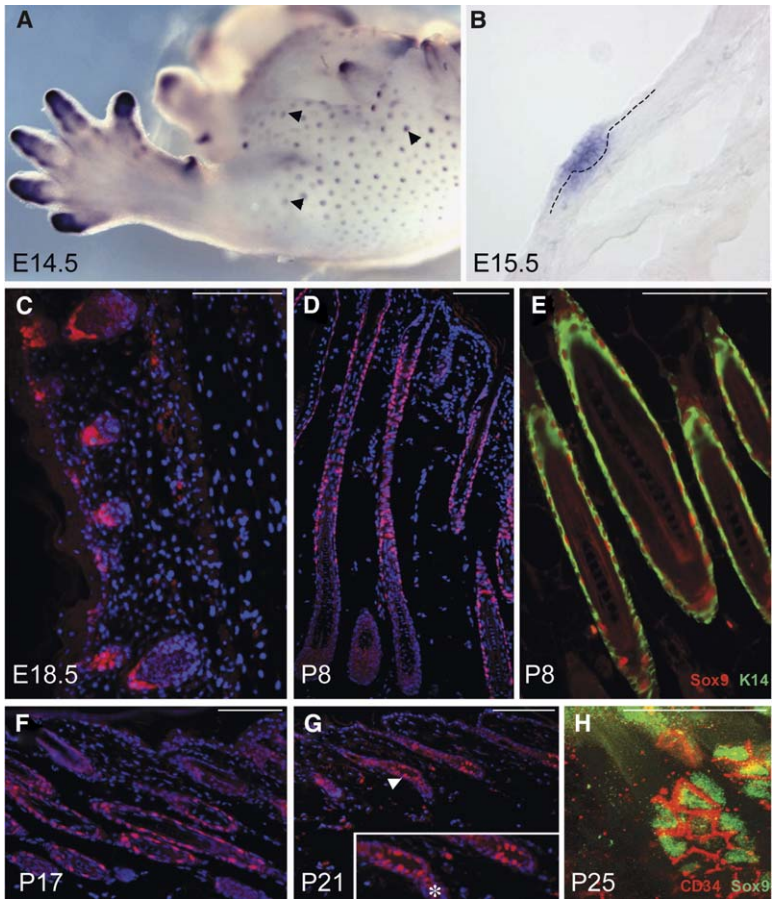

Figure 1. *Sox9* Expression Analysis during Hair Development and Cycling

(A) In situ hybridization analysis at E14.5 demonstrates *Sox9* expression in the developing hair placode (arrowheads).

(B) Cross-section of in situ hybridized tissue at E15.5 reveals strong staining in the epithelial ectoderm of the developing hair placode. A dashed line demarcates the border between the epithelial and the mesenchymal component.

(C–G) Sox9 protein (red) localization during hair follicle development and cycling (blue, DAPI). (C) At E18.5 Sox9 protein can be detected in the downgrowing hair germ. The presumptive matrix cells are negative. (D) P8 (first anagen). With exception of the lower and upper (infundibulum) part of the follicle, Sox9 activation can be seen along the entire length of the ORS. (E) Costaining with the ORS marker K14. (F) P17 (Catagen). (G) P21 (Telogen). Sox9 is restricted to few cells at the lower portion of the hair follicle. Note the absence of signal in the secondary hair germ (asterisk).

(H) At P25, Sox9 (green) is located in the nuclei of the hair stem cells, which express the molecular marker CD34 (red). Scale bars represent 100 μm.

with Figure 3A). Between P6 and P14, knockout animals showed a marked change in the follicular epithelium. By P8, there appeared to be increased cellularity in the dermis, and the number of bulb matrix cells appeared to be decreased (Figure 3D).

The normal hair cycle in the mouse lasts approxi-

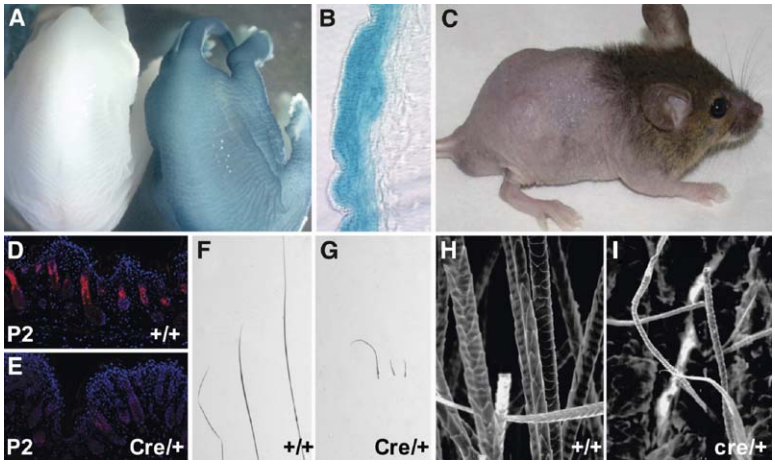

Figure 2. Tissue-Specific Knockout of Sox9 in the Epidermis

(A and B) The Cre-mediated deletion of the *Y10:Cre* line was analyzed by crossing this strain with *pgklxneolacZ* mice. Blue staining was restricted to the epithelial component of the developing skin.

(C) *Y10:Cre* crossed onto the *Sox9*$^{flox/flox}$ background leads to alopecia in the caudal region of the body. Note: the presence of hair in the head/shoulder region is due to an inefficient Cre activity in the *Y10:Cre* line (data not shown).

(D and E) Comparison of Sox9 antibody staining between wild-type and *Y10:Cre; Sox9*^{flox}/*Sox9*^{flox} dorsal skin at P2. Note the complete absence of Sox9 in knockout skin (E).

(F and G) In contrast to the clearly distinguishable main hair types (zig-zag, awl, guard) in wild-type animals, hair in knockout mice is atrophic and morphologically indistinguishable.

(H and I) Scanning electron microscopic analysis confirming the short and highly abnormal hair in KO tissue (I) compared to wild-type hair (H).

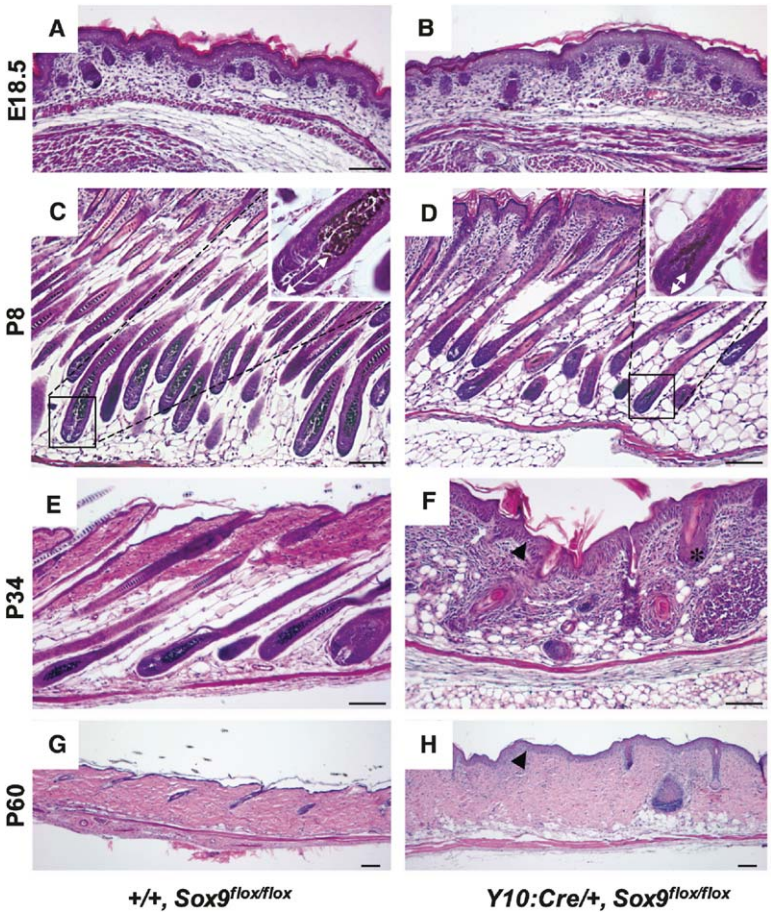

Figure 3. Histological Analysis throughout Development and Hair Cycling

(A and B) At E18.5, animals depleted for *Sox9* in the skin develop hair placodes (B) similar to their control littermates (A).

(C and D) At P8, a marked reduction of the length of the hair bulb is detected in the absence of Sox9 (D), when compared to control littermates (double-headed white arrows in the insets) (C).

(E–H) After the first hair cycle, the number of hair follicles decreases. Dramatic degeneration of the hair follicle is observed (asterisk in [F]). Note the thickening of the epidermis (arrowheads in [F] and [H]). Scale bars represent 100 μm.

mately 25 days with the onset of catagen at approximately P16 and telogen at P21. The second phase of hair growth commences at P25 by a process that requires Wnt/β-catenin and Shh signaling [11]. Hair follicles in *Y10:Cre; Sox9*$^{flox/flox}$ animals remained after the first hair cycle (P25), although their number appeared to be reduced with a marked decrease at P60 (compare Figure 3H with Figure 3G). Beside the severely abnormal hair follicle, we also noticed a significant thickening of the epidermis with scarring at the later time points (P60).

Ectopic Expression of Epidermal Markers in the Mutant ORS and Absence of the Bulge

To detect differences in differentiation, we analyzed the distribution of a variety of molecular markers in wild-type and mutant animals. Cytokeratin expression correlates with differentiation and shows specific pattern of expression in developing and adult skin. K17, an ORS-specific marker, remained to be expressed in mutant animals (Figures 4A and 4B and data not shown). Similarly, K5 and K14, two genes normally present in the ORS and basal cell layer of the epidermis were expressed in the expected pattern (Figures 4C, 4D, 4F, and 4G and data not shown). Surprisingly, markers K1 and K10, which in wild-type skin are restricted to the suprabasal cell layer of the epidermis (Figures 4C and 4F), were now also detected within the hair follicles in *Y10:Cre; Sox9*$^{flox/flox}$ animals (Figures 4D, 4E, 4G, and 4H). K1 and K10 were, however, absent from the most basal layer of the epidermal sheath of the mutant hair, as demonstrated by double labeling with K14 (Figures 4E and 4H).

Recent studies have identified several transcription factors with important functions during hair follicle development. Foxe1, a member of the winged helix family, is also expressed in the ORS but, in contrast to Sox9, is switched on at a later stage during development (E17.5) and then restricted to the lower part of the hair follicle ([35] and Figure 4I). Expression analysis at P4, P8, and P17 detected strong Foxe1 expression in knockout tissue (Figure 4J and data not shown), demonstrating that this factor is initially not affected by the absence of *Sox9*. By P35, however, Foxe1 protein remained in only a few cells in the highly abnormal hair follicles (data not shown).

GATA3 belongs to the GATA transcription factor family and has recently been shown to have an important function in determining the IRS cell lineage [36]. GATA3 expression can be found in the IRS at the lower part of the hair follicle and in the basal cell layer of the epidermis (Figure 4K). In *Sox9* knockout mice, GATA3 remained to be expressed in the proximal part of the IRS. Surprisingly, additional GATA3-positive cells were now

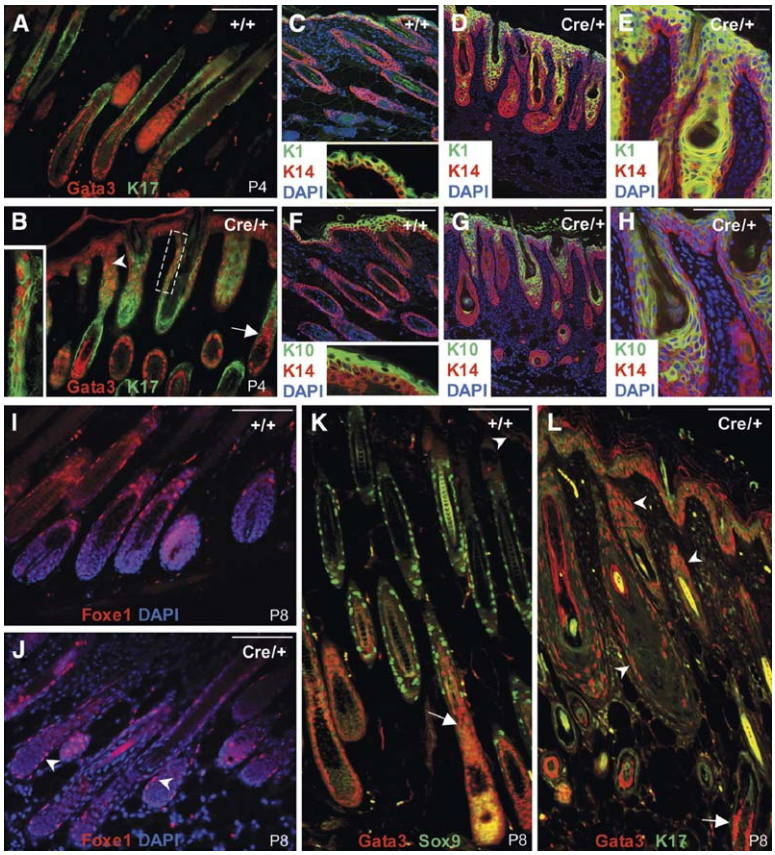

Figure 4. Aberrant Expression of Skin-Specific Marker Genes

(A and B) The ORS marker K17 remains to be expressed in the mutant epidermal sheath. In contrast, a dramatic expansion of GATA3 (red) expression domain from the basal layer to K17-positive cells (green) of the ORS (arrowhead) can be detected (compare also with [K] and [L]).

(C–H) Expression of K1 (green) and the ORS marker K14 (red) and (F–H) K10 (green) and K14 (red). K1 and K10 expression in wild-type tissue (C and F) are restricted to the suprabasal cell layer of the epidermis, whereas knockout skin shows strong staining within the hair follicle at P17.

(I and J) Expression of the transcription factor Foxe1 (red) in the lower ORS is not affected by the knockout of *Sox9* (arrowhead).

(K) GATA3 (red) in wild-type tissue is restricted to the lower part of the IRS (arrow) and the basal layer of the epidermis (arrowhead). No Sox9 (green)/GATA3 (red) double-positive cells could be detected.

(L) In contrast, in *Sox9* knockout skin, GATA3 can be seen along the length of the hair shaft (arrowheads) as well as in the IRS (arrow). Scale bars represent 100 μm.

also detected in the most outer layer of the abnormal hair follicle (Figure 4L). Double staining with the ORS marker K17 demonstrated that the GATA3-positive cells still have characteristics of the ORS (Figure 4B). Interestingly, GATA3 expression in knockout ORS cells was already detectable at P4, a time point when histological abnormalities are still relatively minor. We conclude that lack of *Sox9* leads to the acquirement of epidermal characteristics in the ORS with a multicellular appearance and the expression of GATA3 and, in the more apical cells, the expression of K1 and K10.

The bulge is part of the ORS and constitutes a reservoir of stem cells for the skin and hair. Stem cells in the bulge express the surface marker CD34, keratin15, and S100-A6, among others [32]. Sox9 is expressed in most, if not all, CD34-positive cells (Figures 1H and 5A and Movie S1). To test whether Sox9 affects the formation of the bulge, we analyzed tissues at different stages after the first hair cycle. In contrast to wild-type skin, CD34-expressing cells never appeared in *Y10:Cre; Sox9$^{flox/flox}$* (Figure 5B). Similarly, keratin 15 and S100-A6 signal was dramatically reduced in Sox9 knockout animals (compare Figures 5C–5F), indicating that Sox9 has an important function for the formation of the bulge.

Proliferation Defects in the Hair Matrix Cells

Histological analysis of *Sox9* mutant skin suggested a reduction of cell number in the highly proliferating matrix cells of the hair bulb (Figures 3C and 3D). To address whether this decrease was caused by reduced cell proliferation or by an increase in apoptosis, we performed PCNA/BrdU and TUNEL analyses, respectively. Proliferating cells in normal mouse skin are mostly found in the basal cell layer of the epidermis, the ORS and the actively dividing matrix cells of the hair bulb (Figures 6A and 6B). Analysis of knockout mice showed no significant difference of proliferation at E18.5, P2, or P4, suggesting that the initial proliferative capacity within the hair follicle is not disturbed (data not shown). From P8, a marked reduction of cell proliferation in the matrix region could be observed with BrdU/PCNA labeling (Figures 6C and 6D).

The growing hair (anagen) normally shows very little apoptosis and at P8. TUNEL staining in control animals was restricted to a small proportion of cells within the hair shaft (Figure 5E). In the hair bulb, no apoptotic cells were detectable (Figure 5F). In contrast, *Sox9* knockout skin demonstrated a significant increase of apoptosis in the lower part of the ORS and in particular the precortex of the hair bulb (Figures 5G and 5H).

Sox9 Is Dispensable for Early Hair Follicle Development

The early time point of *Sox9* expression at E14.5 within the hair placode raised the possibility that this gene serves an important function during hair induction. Although the analysis with the *Y10:Cre* line demonstrated an important function of *Sox9* within the outer root sheath, the Cre activity was insufficient to completely

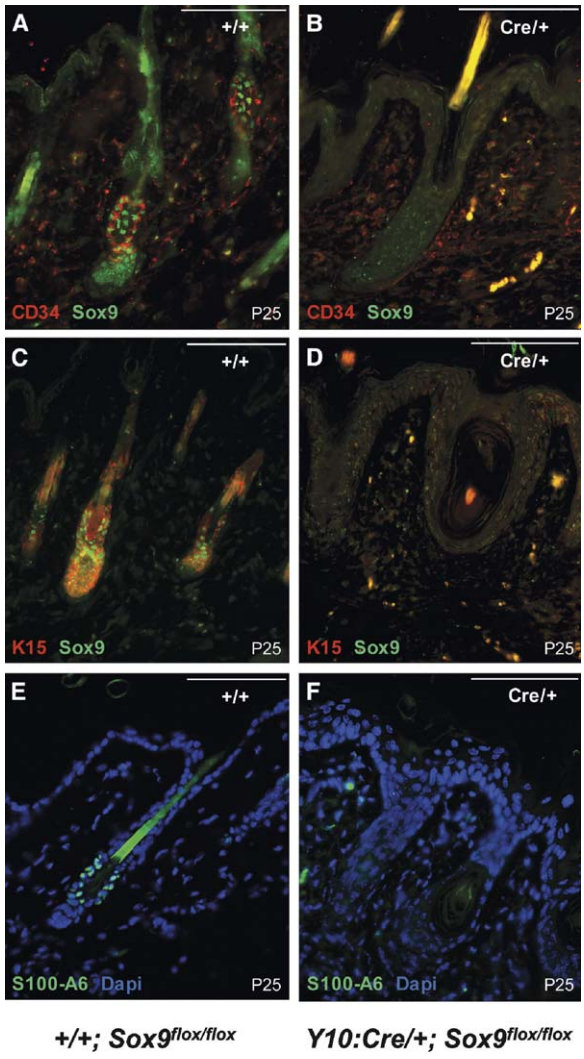

Figure 5. Lack of Sox9 Results in the Absence of Hair Stem Cells as Detected by Immunofluorescent Staining for CD34, K15, and S100-A6

(A and B) Double staining for CD34 (red) and Sox9 (green).
(C and D) Double staining for K15 (red) and Sox9 (green).
(E and F) Detection of S100-A6 (green). Scale bars represent 100 μm.

delete *Sox9* at E14.5 during hair formation, and a proportion of cells continued to express Sox9 protein until P2. To test the early function of *Sox9* during hair formation, we made use of homozygous knockout animals that were created with germline-specific Cre lines (XX *ZP3:Cre*; $Sox9^{flox/flox}$ × XY *Prm:Cre*; $Sox9^{flox/flox}$) [26]. Homozygous knockout animals die at E11.5 because of heart failure, which precludes a straightforward analysis of hair formation in these animals [27]. *Sox9* expression in whisker formation can be detected as early as E12.5 (data not shown), and we tested whether transplantation experiments may allow us to address *Sox9* function in early hair formation. The upper lips of wild-type control and homozygous *Sox9* knockout animals were transplanted under the kidney capsule of nude mice and development allowed to proceed in vivo (Figure 7A). 10 days after transplantation, clear hair formation with strong Sox9 staining in the ORS could be detected in wild-type animals (Figures 7C and 7E). *Sox9* mutant tissues also showed the formation of hair development (Figures 7B and 7D). Immunohistological analysis confirmed the absence of Sox9 protein in knockout tissue, whereas K14 remained to be expressed in the newly formed ORS (Figure 7F). We conclude that *Sox9* does not appear to be essential for early development of the hair follicle.

Sox9 Expression Depends on Shh Signaling and Is Activated in Basal Cell Carcinomas

The early expression pattern of *Sox9* in the epithelial placode is reminiscent of *Shh* expression. To test whether *Sox9* acts upstream or downstream of this signaling protein, we analyzed tissue samples from *Shh* knockout animals [37]. At E18.5 Sox9-positive signal was virtually absent from $Shh^{-/-}$ tissues suggesting that its expression depends on the presence of this signaling molecule (Figure 8B). In the skin, Shh appears to act mainly through the transcriptional activator Gli2. Similarly to our findings in $Shh^{-/-}$ tissues, Sox9 antibody staining on $Gli2^{-/-}$ sections showed a dramatic reduction of Sox9 signal (Figure 8C).

Ectopic expression of *Shh*, *Gli2*, or an activated version of *Gli2* missing its N-terminal repression domain (Δ*NGli2*) has been shown to cause skin tumors [38–40]. To test whether Shh signaling is not only required but also sufficient to induce *Sox9* expression, we tested samples from mice ectopically expressing Δ*NGli2* under control of the basal-cell-layer-specific K5 promoter [41]. Indeed, nuclear Sox9 protein could be readily detected in tumor tissues from *ΔNGli2* transgenic mice (Figure 8D). Strikingly, also the epidermal basal cell layer that did not show signs of tumor formation exhibited Sox9-positive cells, suggesting that the activation of *Sox9* by Gli2 is an early, if not direct, event (Figure 8D, inset).

The expression of Sox9 in mouse Gli2 tumor samples raised the possibility that SOX9 is also upregulated in human skin tumors. SOX9 antibody staining on human control skin showed strong nuclear staining in the ORS, sebaceous glands, and sweat glands (Figure 8E and data not shown). Additional cytoplasmic staining was present in the epidermis, which, however, seemed to be unspecific because no expression in this tissue could be detected by in situ hybridization (Figure 8F). We next investigated biopsies from patients suffering from pilomatricoma, squamous cell carcinoma (SCC), or basal cell carcinoma (BCC). Antibody staining in samples from pilomatricoma and SCC were found to be negative for SOX9 expression. In contrast, SOX9 was highly expressed in four out of four BCC tumors analyzed (Figures 8G and 8H).

Discussion

The ORS forms the outer-most epithelial layer of the hair follicle and as such represents a direct continuation of the basal layer of the epidermis. Aside from the demonstration that stem cells migrate along the ORS to reach the hair bulb, little is known about the role of this cell lineage during hair formation [13]. Our present

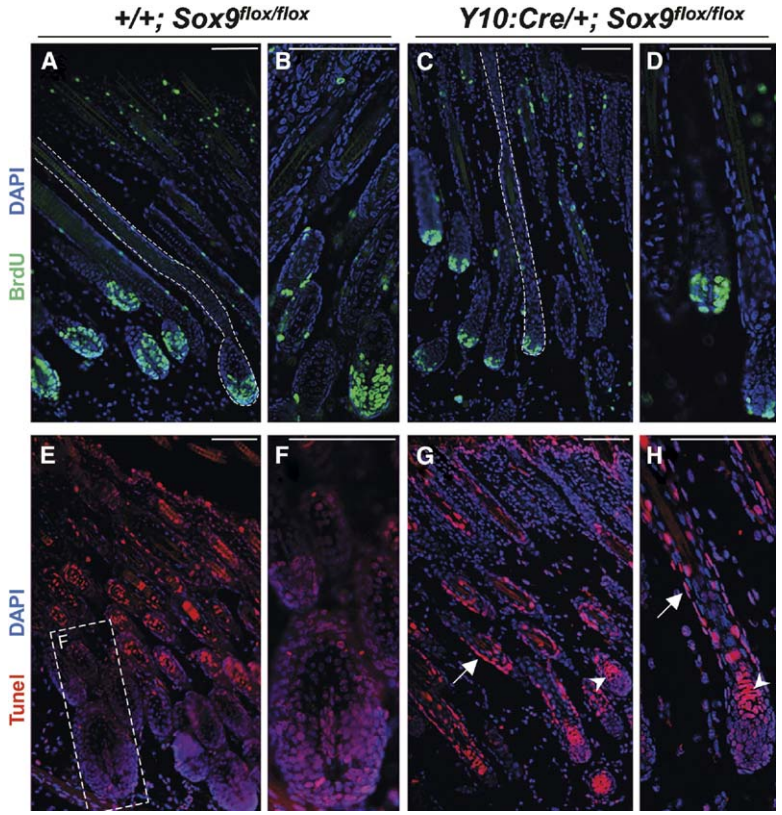

Figure 6. *Sox9* Null Hair Follicles Show a Dramatic Decrease in Proliferation and an Increase in Apoptosis

(A–D) BrdU proliferation analysis at P8 demonstrates a substantial reduction of proliferating cells in the matrix of the *Y10:Cre; Sox9 flox/flox* hair bulb (C–D) compared to wild-type littermates (A–B). Dotted lines outline hair follicles.

(E–H) In contrast, apoptotic activity (TUNEL assay) is markedly increased in the precortex (arrowheads) and the ORS (arrows) of knockout tissue (G–H). (F) and (H) are higher magnifications (40×) of (E) and (G) (20×), respectively. Scale bars represent 100 μm.

study has now uncovered a crucial role for the transcription factor Sox9 in ORS formation and the maintenance of the hair throughout life.

Sox9 Is Dispensable for Early Hair Follicle Formation during Embryogenesis

Sox9 activation during hair development occurs as early as E14.5 during mouse embryogenesis in the epithelial hair placode. As such, *Sox9* is one of the first genes known to be expressed during hair follicle induction. Despite this early expression, tissue-specific deletion of *Sox9* with our Cre line resulted in the same number of hair follicles, although one has to take into account that *Sox9* deletion at this early time point was not complete. However, taken together with our results that upper lips from *Sox9*-null embryos developed hair follicles when transplanted under kidney capsules, these data suggest that *Sox9* is not essential for early follicle formation. Alternatively, there could be another member of the *Sox* gene family that may complement for the loss of *Sox9*. Partial functional redundancy between Sox9 and other genes of the same *Sox* subtype has already been observed before in other tissues [26, 28]. To our knowledge, the only other three *Sox* genes described to be expressed during hair development are the mesenchyme-specific gene *Sox18* and the melanocyte regulators *Sox10* and *Sox13* [42–45]. The formation of hair follicles in *Sox9*-null embryos may not be too surprising in the light of the Shh-dependent *Sox9* activation. *Shh* knockout animals also form hair follicles, which, however, arrest at an early time of development [46].

Sox9 Is Required for the Maintenance of the ORS

At birth, the follicle has reached into deeper areas of the underlying mesenchyme (dermis), and the first hair shafts develop. At this time point, expression of Sox9 in wild-type tissue becomes restricted to the ORS of the developing hair. Hair bulb, matrix, and precortex in skin lacking Sox9 appear to develop at least initially normally, as demonstrated by histological analysis, normal proliferation figures at P2, and the expression of cell-type-specific markers such as GATA3 (IRS) and Foxe1 (lower part of the ORS). Morphological abnormalities in knockout skin first become apparent at P4/P6, with dramatic changes occurring by P8. Because Sox9 expression at this time point is restricted to the ORS, we can conclude that this cell layer has an essential function for the development and maintenance of the hair follicle and the growth of the hair shaft.

Our molecular analysis points to a function of Sox9 in the maintenance of ORS cell identity, and lack of Sox9 leads to the activation of several markers that are normally restricted to the epidermis. These include expression of the transcription factor GATA3, which, apart from IRS, can also be found in the basal cell layer of the epidermis [36]. Moreover, we observed that the normally single-cell layer of the ORS developed into a multicellular layer in the absence of Sox9. Suprabasal layers of this abnormal sheath expressed the markers

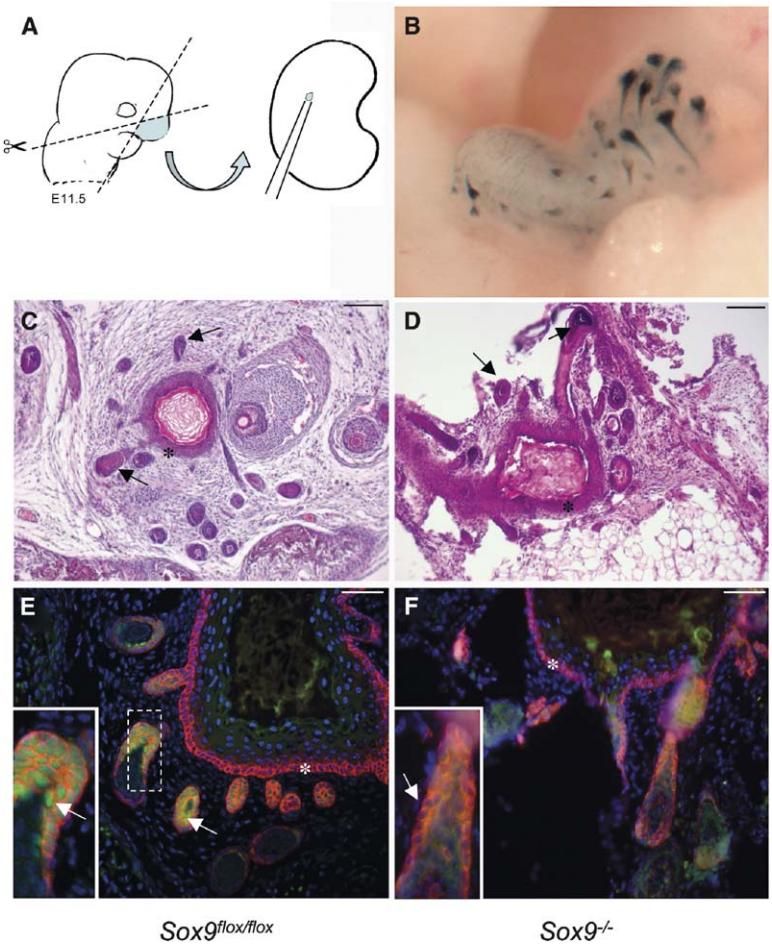

Figure 7. *Sox9* Function Is Dispensable for Hair Follicle Formation

(A) The upper lip of E11.5 wild-type or homozygous *Sox9* knockout embryos (see text) were transplanted under the kidney capsule of nude mice.

(B) Macroscopic view of *Zp3:Cre; Prm3:Cre; Sox9 flox/flox* tissue 10 days after transplantation. Note the presence of highly pigmented hair follicles.

(C and D) Hematoxylin- and eosin-stained sections of wild-type (C) and mutant transplants (D). Black asterisk, epidermis; arrow, hair follicle.

(E and F) Immunofluorescent analysis of transplanted material with antibodies against Sox9 (green) and K14 (red). Note the presence of Sox9 nuclear stain in wild-type (E) and a complete absence of Sox9 staining in mutant (F) hair bulbs (white arrow). White asterisk, K14-positive basal layer of the epidermis. Scale bars represent 100 μm.

K1 and K10, which are normally restricted to the suprabasal layers of the epidermis and infundibulum. Because of the absence of infundibulum-specific markers, we cannot completely exclude the possibility that ORS cells in Sox9 mutants are replaced by infundibular cells. However, in the epithelial sheath of mutant hair, the

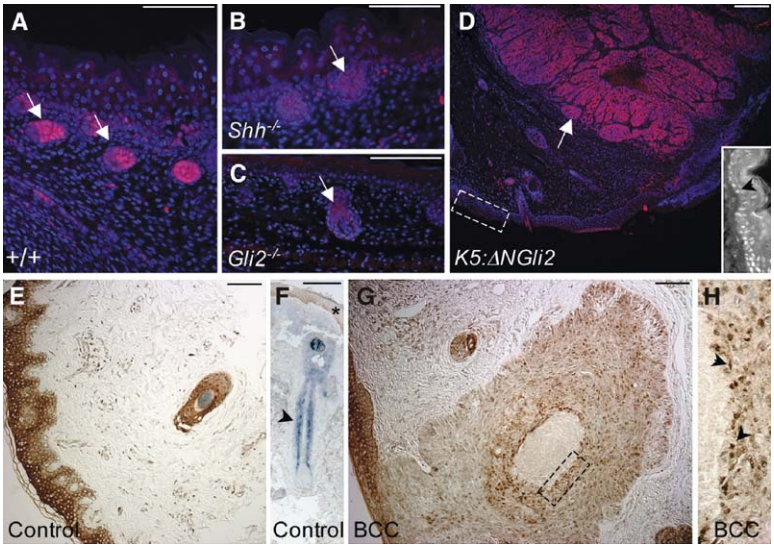

Figure 8. Sox9 Acts Downstream of Shh and Gli2 and Is Upregulated in Basal Cell Carcinoma

(A–C) Wild-type E18.5 tissue samples with strong expression of Sox9 in the downgrowing hair germ (white arrows). In contrast, *Shh*$^{-/-}$ (B) or *Gli2*$^{-/-}$ (C) skin show a dramatic reduction of Sox9 expression in hair germs.

(D) Dramatic upregulation of Sox9 in a basal cell carcinoma (arrow) in mice expressing a constitutively active version of Gli2 (ΔNGli2) under control of the K5 promoter. Inset: high-power view of an epidermal part of (D). Note that Sox9 is already expressed in the basal layer of *K5:ΔNGli2* transgenic mice before the development of a tumor (black arrowhead).

(E) Human tissue samples of control skin show strong nuclear staining with a Sox9 antibody in the ORS of hair follicles. Cytoplasmic staining in the epidermis is nonspecific background as demonstrated by in situ hybridization with a Sox9 antisense probe (F).

(G and H) Basal cell carcinoma shows upregulation of SOX9 protein with strong nuclear staining (arrowheads in [H]). Scale bars represent 100 μm.

strong staining of GATA3, which at least in our hand, was absent from normal infundibulum, would argue against this explanation and support the hypothesis of a differentiation of the ORS into an epidermal cell type. This *trans*-differentiation is, however, incomplete because the ORS-specific marker K17 remains to be expressed in the hair shaft. We conclude that Sox9 functions to maintain ORS identity and that loss of this transcription factor leads to the acquirement of epidermal characteristics.

Sox9 and the Bulge: A Role in Stem Cell Maturation/Cell Migration?

Compelling evidence from recent publications demonstrate that hair follicle stem cells reside in the bulge, which becomes apparent at about 3 weeks after birth. It is clear that stem cells must already exist at an earlier time point to supply the growing hair continuously with cells but possibly also to contribute to wound healing. Although these cells have not been identified so far, they are likely to reside within the ORS. Interestingly, hair follicles of our *Sox9* knockout mice showed a marked decrease of proliferation in the matrix as early as P8. The fact that Sox9 is not expressed in the matrix suggests that this proliferative defect is secondary. Since decreased proliferation occurs very early after *Sox9* deletion, we may speculate that *Sox9* is either required for the survival of these stem cells or, alternatively, for guiding stem cells to the hair bulb.

After completion of the first hair cycle, the bulge is readily detectable by the presence of CD34-positive cells. Our results indicate that *Sox9* is expressed in bulge stem cells. Although several genes have been identified by microarray and quantitative PCR analysis on enriched hair follicle stem cells [6, 30, 32], to our knowledge, Sox9 represents the first transcription factor that has been confirmed to be expressed on a cellular level in this cell type. Interestingly, hair-stem-cell-specific markers were absent from the follicles lacking Sox9. Moreover, the reduced proliferation rate in matrix cells, together with the fact that hair follicles get lost over time, may suggest that *Sox9* is not only expressed in stem cells but has also an essential function for their survival.

Sox9 as a Downstream Target of Shh Signaling: A Connection to Basal Cell Carcinoma

Shh is a signaling molecule involved in many epithelial mesenchymal interactions. *Shh* knockout animals develop hair placodes but fail to form mature hair follicles, suggesting that *Shh* is required for proliferation and downgrowth of the hair placode [46, 47]. Proliferative defects in *Shh*-null animals are at least partly caused by a failure of cyclinD1 and cyclinD2 activation. Expression of Shh at early stages of development resembled that of Sox9, which prompted us to test a possible interrelation of these two molecules. The complete absence of Sox9 signal in $Shh^{-/-}$ skin demonstrates a direct dependence of *Sox9* expression in skin. A similar activation of *Sox9* by Shh signaling has been reported previously during somatic chondrogenesis [48, 49]. Although Sox9 expression completely depended on Shh signaling during hair induction (Figure 7), Sox9 protein was expressed in the absence of *Shh* in cartilaginous and other tissues (data not shown). This is in agreement with the phenotype observed in *Shh* knockout mice, which does not completely abolish bone formation [37]. This may at least in part be due to the presence of other hedgehog molecules such as Indian hedgehog [50]. However in the skin *IHH* expression is restricted to sebaceous glands [51].

Shh acts by binding to its receptor Patched, which triggers the activation of members of the Gli family proteins. Recent data demonstrate that Gli2 serves a key role in Shh signaling during embryonic hair follicle development [41, 52]. The results in the present study support this hypothesis and mice lacking Gli2 show a dramatic reduction of Sox9 in the hair follicle, whereas mice expressing a constitutively active version of Gli2 in the basal cell layer of the epidermis (K5:ΔNGli2) now express Sox9 ectopically in this cell layer. Taken together, these data point to a very early, possibly direct, activation of *Sox9* by Gli2.

Genetically modified mice ectopically expressing *Shh*, *Gli2*, or *ΔNGli2* in the basal layer develop basal cell carcinoma [39, 40, 53]. Our results demonstrate that tumor samples from K5:ΔNGli2 mice express high amounts of Sox9 in the malignant tissue. Consistent with this finding, four out of four human BCC samples analyzed in this study showed strong SOX9 expression in the tumor tissue. At present, we cannot distinguish between a mere activation of *Sox9* by Gli2 and a causative role of Sox9 in tumor formation. It is, however, striking that Sox9 has recently been demonstrated to be expressed in colon cancer, in which it seems to be under control of the Wnt signaling pathway [54]. Indeed, there is now increasing evidence for a role of *Sox* genes in cancer [55]. Taken together with the expression of Sox9 in hair stem cells and the proliferative defects seen in Sox9 knockout mice, a more general function of Sox9 in tumorigenesis is an attractive hypothesis that remains to be tested.

Conclusions

The data represented in this study show that Sox9 is a crucial factor to maintain the ORS identity and the development or survival of the stem cell lineage. The activation of *Sox9* by the Shh pathway and its upregulation in skin tumors suggests a role for this gene in tumorigenesis.

Experimental Procedures

Mouse Strains

Transgenic mice expressing the *Y10:Cre* transgene were generated by microinjection of the Cre recombinase under control of a human WT1 YAC construct [56]. Details on the construction of this transgene are available on request. The *Y10:Cre* construct was isolated according to [57] and injected into fertilized oocytes collected from C57Bl6/CBA F1 matings. Mice depleted for *Sox9* in the skin were generated by crossing animals carrying the *Y10:Cre* transgene with mice homozygous for the $Sox9^{flox}$ allele [58]. Animals ($Sox9^{flox/flox}$; *Y10:Cre/+*) were obtained by cross breeding and kept on a mixed genetic background 129/C57Bl6. Mice homozygous for *Sox9* deletion were generated by crossing males (*Prm1:Cre*; $Sox9^{flox/flox}$) with females (*Zp3:Cre*; $Sox9^{flox/flox}$) as described [26]. 8-week-old Swiss nude female mice were purchased from Charles River and used for the upper lips transplantations. The indicator Cre mouse line [33]

was used to monitor the expression of the *Y10:Cre* transgene in mutant animals.

Genotyping of Mice

The presence of the *Y10:Cre* transgene was detected with primers specific for the *Cre* recombinase gene [26].Wild-type, *Sox9^{flox}*, and *Sox9* knockout alleles were detected with primers as described [26].

Histological and Immunological Analyses

Skin samples from exactly aged knockout and control litter mates were collected, fixed with 4% paraformadehyde overnight at 4°C, and then embedded in paraffin. Microtome sections of 7 μm thickness were stained with hematoxylin and eosin. For imunohistochemistry, antigenes were retrieved in 10 mM Na citrate (pH 6) and then incubated for 45 min in blocking solution (3% BSA, 10% donkey serum, 0.1% Triton) at room temperature. Blocking solution was replaced by the primary antibodies prepared in diluent (3% BSA, 3% donkey serum, 0.1% Triton) at the following concentrations: K1 (rabbit, 1:100, Covance, PRB-165P), K5 (rabbit, 1:100, Covance, PRB-160P), K6 (rabbit, 1:100, Covance, PRB-169P), K10 (rabbit, 1:100, Covance, PRB-159P9), CD34 (rat, PharMingen, 1:100, Clone RAM34), Gata-3 (mouse, 1:100, Santa Cruz, HCG3-31), Cytokeratin 14 (mouse, 1:100, BioTrend, clone LL002), Foxe1 (rabbit, 1:600 [35]), Sox9 (1:1000 [28]), K17 (rabbit, 1:1000, gift of P. Coulombe), K15 (chicken, 1:300, Covance, PCK-153P), S100-A6 (rabbit, 1:100, Lab Vision, RB-1805). When staining with mouse antibodies, we used the reagents from the MOM Basic Kit according to the manufacturer's protocol (Vector Labs). Relevant Cy3- or Cy2-conjugated anti-rabbit or anti-rat antibodies (1:150, Jackson Laboratories) were used for detection of primary antibodies. Slides were mounted by using vectashield with DAPI as mounting reagent (Vector Labs). For light microscopy analysis, biotinylated anti-rabbit IgG (1:150, Vector Labs) were used. They were detected with extravidin peroxidase (1:150, Sigma-Aldrich), and staining was developed with DAB (Sigma-Aldrich). For BrdU labeling, pups were weighed and injected intraperitoneally with BrdU (50 μg/g of body weight, Sigma-Aldrich). Pups were sacrificed 2 hr after injection, and skin samples were fixed overnight in 4% paraformaldehyde and then embedded in paraffin. Sections were cut and dewaxed, and antigen retrieved as indicated above. BrdU incorporation was monitored with 5-Bromo-2′-deoxy-uridine labeling and detection kit (Roche Applied Science) following the manufacturer's protocol. Apoptosis assay was carried out with in situ cell-death detection kit (Roche Applied Science) according to the manufacturer's indications. Confocal analyses were carried out on 37-μm-thick sections with a Zeiss confocal microscope LMD510. The three-dimensional reconstructions of images were performed with Volocity program. Light and fluorescent studies were performed with a Leica microscope DMLB, and pictures were taken with a spot RT-slider camera (Diagnostic instruments) and processed with Adobe Photoshop.

Upper Lips Transplantation

Embryos homozygous for the *Sox9* knockout allele were collected in utero at E11.5 just before they died. Upper lips were dissected out and transplanted under the capsule of anaesthetized athymic mice. 10 days after transplantation, mice were sacrificed by cervical dislocation. Kidneys were fixed in 4% PFA overnight, and transplants analyzed by histological techniques (see below).

In Situ Hybridization Analysis

Embryos were fixed with 4% paraformaldehyde in PBS overnight at 4°C. Further processing of the embryos and in situ hybridization was carried out as described [59]. Sox9 riboprobes were synthesized as described [60]. In situ analysis on human sections was performed according to [61].

X-Gal Staining

Embryos were fixed in 0.2% glutaraldehyde in 0.1 M phosphate buffer, 2 mM $MgCl_2$, and 5 mM EGTA for 35 min and processed as described [62].

Morphological Analyses

Hairs were picked by pulling them with forceps from the back of transgenic and control litter mice sacrificed 21 days after birth. For scanning electron microscopy, back skin was fixed in 2% glutaraldehyde at 4°C overnight, dehydrated, and then critical-point dried and mounted on metal stubs and sputter coated with gold palladium. Samples were visualized with a JEOL JSM T300 scanning electron microscope.

Supplemental Data

Supplemental Data include a movie and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/15/1340/DC1/.

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