Molecular Pathogenesis of Genetic and Inherited Diseases

Hedgehog Signaling, Keratin 6 Induction, and Sebaceous Gland Morphogenesis

Implications for Pachyonychia Congenita and Related Conditions

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Keratins 6a and b (K6a, K6b) belong to a subset of keratin genes with constitutive expression in epithelial appendages, and inducible expression in additional epithelia, when subjected to environmental challenges or disease. Mutations in K6a or K6b cause a broad spectrum of epithelial lesions that differentially affect nail, hair, and glands in humans. Some lesions reflect a loss of the structural support function shared by K6, other keratins, and intermediate filament proteins. The formation of sebaceous gland-derived epithelial cysts does not fit this paradigm, raising the question of the unique functions of different K6 isoforms in this setting. Here, we exploit a mouse model of constitutively expressed Gli2, a Hedgehog (Hh) signal effector, to show that K6a expression correlates with duct fate in sebaceous glands (SGs). Whether in the setting of Gli2 transgenic mice skin, which develops a prominent SG duct and additional pairs of highly branched SGs, or in wild-type mouse skin, K6a expression consistently coincides with Hh signaling in ductal tissue. Gli2 expression modestly transactivates a K6a promoter-driven reporter in heterologous systems. Our findings thus identify K6 as a marker of duct fate in SGs, partly in response to Hh signaling, with implications for the pathological expansion of SGs that arises in the context of certain keratin-based diseases and related disorders. (*Am J Pathol* 2008, 173:752–761; DOI: 10.2353/ajpath.2008.071089)

Genetically determined mutations in individual intermediate filament protein-encoding genes account for, or are associated with, more than 70 distinct human disorders.¹

These disorders tend to be individually rare but collectively affect a broad range of tissues, reflecting the tissue- and cell type-specific transcriptional regulation of intermediate filament genes.^{1–3} For several of these disorders, at least part of the underlying pathophysiology involves the expression of cellular fragility following exposure to mechanical trauma, brought about by a partial loss of the structural support function provided by intermediate filament polymers. There are, however, many examples of lesions that do not fit the classical paradigm of trauma-induced cell lysis; whether the newly emerging, non-mechanical functions of intermediate filament proteins^{4,5} are compromised in such settings is unknown.

A group of four keratin genes, the type II keratin paralogues 6a and 6b (*K6a*, *K6b*), and type I keratins 16 and 17 (*K16* and *K17*), show an intriguing regulation comprising a constitutive component in all major types of epithelial appendages (eg, hair, nail, glands) and an inducible component that either follows a “challenge” (eg, injury, infection) or mirrors an ongoing pathology (eg, psoriasis, carcinoma).^{6,7} Unlike several other keratin pairings, the expression of *K6a*, *K6b*, *K16*, and *K17* does not correlate with execution of a specific program of terminal differentiation.^{6,8,9} Several functions have been associated with these keratins from the study of various types of genetically modified mice, including structural support,^{10,11} modulation of keratinocyte migration,^{12–14} of tumor necrosis factor- α -induced apoptosis (for *K17*)¹⁵ and of protein synthesis.¹⁶ Conversely, small mutations in these

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keratin genes act dominantly to produce an unusually broad spectrum of epithelial lesions resembling ectodermal dysplasias,¹⁷ and which predominantly affect one or many epithelial appendages. These disorders (and the affected target proteins) include type 1 (K6a, K16) and type 2 (K6b, K17) pachyonychia congenita, steatocystoma multiplex (K17), and two palmoplantar keratoderma variants (K16) (see Human Intermediate Filament Database, <http://www.interfil.org/>)^{1,3} There is pronounced phenotypic heterogeneity among these conditions, and many aspects of their pathophysiology cannot be readily explained through a loss of structural support in the relevant epithelial cell population(s).^{3,7,18}

A puzzling element that is frequently (>50%) associated with mutations in K6a, K6b and K17 is the development of epithelial cysts, generally, at the time of puberty.^{19,20} Other than steatocystoma multiplex, a "pure" glandular disorder, epidermal inclusion cysts are seen in type 1 and type 2 pachyonychia congenita, while vellus hair cysts and steatocystomas are additionally seen in type 2 pachyonychia congenita.²⁰ The content of these cysts bear a resemblance to sebaceous glands (SGs); in the case of steatocystoma multiplex they are believed, in fact, to originate from SG ducts.^{20–22} There is no molecular rationale, at present, for the development and proliferation of cysts in the skin of individuals bearing mutations in those keratin genes.

Here, we report that K6a gene expression preferentially marks ductal tissue in SGs. We discovered this phenomenon while studying *Gli2* transgenic mice (*Gli2*^{TG}), which as reported offer the distinct advantage of showing markedly enlarged SGs.²³ We show that this anomaly begins with an enlargement and elongation of the ductal portion in SG tissue, and is closely paralleled by local enhancement of Hedgehog (Hh) signaling, and by *K6a* expression at the mRNA and protein levels. Thereafter, additional SGs appear according to a well defined spatiotemporal pattern in *Gli2*^{TG} mice, and Hh signaling along with *K6a* expression consistently marks SG ductal tissue during this process. Hh signaling and *K6a* expression also marks ductal tissue in the normal setting of wild-type mouse skin. Finally, we present molecular evidence that suggests a causal link, likely indirect or partial, between Hh signaling and *K6a* expression. These original findings have implications for how mutations affecting *K6a* and its type I keratin partner genes *K16* and *K17* cause appendageal defects that frequently include an abnormal expansion of SG tissue.^{20,24–28}

Materials and Methods

Animal Models and Whole Mount Epidermal Sheets Preparation

The following transgenic lines were used: *hK6a-lacZ* mice (23-1p line),²⁹ harboring a transgene consisting of 5.2-kb of 5'upstream sequence from the *hK6a* gene fused to the *lacZ* coding sequence modified with a nuclear localization signal; *Ptch-lacZ* mice (C57Bl/6 strain),³⁰ in which the *LacZ* sequence has been knocked-in the *Patched* locus;

Gli2^{TG} mice (C57Bl/6 strain),²³ in which the mouse *Gli2* sequence is downstream from the bovine *K5* promoter. *Gli2*^{TG}/*K6a-lacZ* and *Gli2*^{TG}/*Ptch-lacZ* double-transgenic mice were generated through selective crosses. Genotyping for the *K5-Gli2* transgene were performed via PCR. Genotyping for *hK6a-lacZ* and *Ptch-lacZ* were performed using β -galactosidase assay *in situ*.³¹ All whole mount epidermal sheets were prepared from the upper one-third region on the dorsal side of male mouse tail skin, as described.^{31,32} All protocols involving mice were reviewed and approved by the Johns Hopkins University Animal Care Use Committee.

Antibodies, Probes, Plasmids, and Other Reagents

The following antibodies were used: rabbit polyclonal antisera directed against K6 ("K6gen"),^{33,34} K17³³; a mouse monoclonal antibody directed against K14 (LLOO1).³⁵ Rhodamine- and fluorescein isothiocyanate-conjugated goat anti-mouse and anti-rabbit secondary antibodies were obtained from Kirkegaard and Perry Labs (Gaithersburg, MD).

In situ hybridization for the *mK6a* mRNA was done as described elsewhere.³¹ The *hK6a* promoter-luciferase construct was generated by subcloning 3.3 kb of 5'upstream sequence from the *hK6a* gene (obtained by NcoI digestion)³⁶ into pGL3-firefly (Promega, Madison, WI). Rat FoxE1 was a gift from Dr. Caterina Missero (Ceinge Biotechnologie, Napoli, Italy); mouse *Gli2* was a gift from Dr. Philip Beachy. Nuclear factor kappa-B/p65³⁷ and IgK-interferon promoter-luciferase constructs³⁸ were provided by Dr. Mollie Meffert (Johns Hopkins University, Baltimore, MD). Transcription factor coding sequences were subcloned into mammalian expression vectors (nuclear factor kappa-B /p65 in pEGFP; *Gli2* in pRK5; FoxE1 in pCMV6). Empty vectors served as controls.

Morphological Analyses

Indirect immunofluorescence and β -galactosidase histochemistry on whole mount epidermal sheets were performed as described.³¹ For β -galactosidase histochemistry, the incubation were performed 1 hour at 37°C for *hK6a-lacZ* samples, and overnight for *Ptch-lacZ* samples. For Oil Red O histochemistry, whole mount epidermal sheets were fixed with 10% formalin for 5 minutes and rinsed in three changes of distilled water. Samples were transferred to Oil Red O working solution (60% of 0.5% Oil Red O/isopropanol in distilled water). After 1 hour, samples were rinsed in 60% isopropanol for 5 minutes and in three changes of distilled water. Samples were mounted with Crystal mounting media (Biomed, Foster City, CA) and analyzed by microscopy.

Mouse skin tissues (ear, tail, paw, tongue, and eyelid) were obtained from male mice at various ages. Samples were fresh frozen and embedded in optimal cutting temperature compound (Sakura Finetec, Torrance, CA), or were fixed in Bouin's solution and embedded in paraffin. Normal adult human skin samples, also paraffin-embedded, were

used. Five to twenty- μm thick sections (frozen or paraffin) were subjected to H&E staining, indirect immunofluorescence, Oil Red O histochemistry, or β -galactosidase histochemistry as described above. Final preparations were visualized using either a Zeiss Axioplan-2 microscope equipped for fluorescence imaging or a PerkinElmer Ultra-View confocal microscope.

Transient Transfection and Luciferase Assays

Monkey kidney Cos-1 epithelial cells or alternatively, mouse 308 skin keratinocytes,³⁹ were seeded in 24-well plates at a density of 2×10^4 cells/ml and incubated at 37°C for one day before transfection. Transient transfection of mouse *K6a* (cf. above) and mouse *K17* gene promoters⁴⁰ and transcription factors (or corresponding empty vectors, used as controls; cf. above) was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in serum-free medium as described.⁴¹ Cells were harvested 48 hours later for analysis. Cells were cotransfected with plasmid featuring a given promoter (see description below) subcloned in a pGL3-basic vector (Firefly luciferase, Promega), the promoter-less pGL3-basic plasmid (included as a negative control), and the pRL-TK plasmid (Renilla luciferase, Promega; included to normalize data for transfection efficiency). The assays were controlled, and normalized, by parallel transfection of the relevant empty vectors. Optimal transfection conditions consisted of 1.2 μg total plasmid DNA per well, and a ratio of pGL3 to pRL-TK of 10:1. To assay for luciferase activity, cells were lysed with 100 μl /well of lysis buffer provided with the Dual Luciferase Reporter Assay kit (Promega); lysates were stored at -20°C until analyzed. Assays for firefly luciferase and *Renilla* luciferase activity were sequentially performed in one reaction well in 96-well plates using 20 μl aliquots of cell lysates. Luciferase activity was measured using a microplate luminometer (Fluoroskan Ascent FL, Labsystems, Helsinki, Finland) according to the manufacturer's instructions. Three independent experiments were performed to yield a given reporter activity, which was calculated as "x-fold expression" relative to the control luciferase activity.

Additional Studies on *Gli2*^{TG} Mouse Skin

For analysis of the hair follicle cycle, skin tissue was always obtained from the mid-back region of age- and gender-matched mouse littermates. Skin pigmentation provides a reliable index of progression through the hair follicle cycle, reflecting the strict coupling of the latter with follicular melanogenesis.⁴² Shaved mouse back skin was photographed, at predetermined ages, before histological study. For the latter, skin tissue was Bouin's-fixed, paraffin-embedded, and 5 to 10 μm sections were counterstained with H&E. Hair cycle stage was determined based on mouse age along with well established morphological criteria.⁴²

Morphological analyses of tumor tissue was performed as described in the main text of this article. To determine the levels of *K6* and *K17* mRNA transcripts by semiquan-

titative RT-PCR, total RNA was isolated from ear tumor of *Gli2*^{TG} mice and ear tissue of normal littermate mice. Reverse transcription and semiquantitative PCR were performed¹⁵ using β -tubulin as an internal control. The oligonucleotide primers used for PCR were used as follows: *K17*, Forward: 5'-GATGGAGCAGCAGAACCGAGTA-3', Reverse: 5'-GGTCTCAAGCATAGGAATGCTGGGG-3'; *K6a*, Forward: 5'-GAGCTGGCTTTGGTGGTG-3', Reverse: 5'-GTCCTCCACTGTGTCCTG-3'; β -tubulin, Forward: 5'-CAACGTCAAGACGCCGTGTG-3', Reverse: 5'-GACAGAGGCAAACTGAGCACC-3'.

Results

Dysmorphology and Increased Number of SGs in *Gli2*^{TG} Mice

We noted a tremendous expansion of SG tissue in the course of studying whole mount preparations of tail skin epithelia from *Gli2*^{TG} mice. Normally, a single pair of SGs occurs above the hair bulge/arrector pili muscle unit in hair follicles from tail skin (Figure 1A). This is so for the lifetime of wild-type mice (unpublished data). Owing to its shortness, SG duct tissue is not readily visible on whole-mount preparations (Figure 1A) or tissue sections (data not shown). In *Gli2*^{TG} mice, however, SG become aberrantly shaped and show markedly elongated and enlarged ducts starting at $\sim\text{p25}$ (Figure 1B). At $\sim\text{P45}$, a second pair of SGs appears above the existing one (Figure 1C). At ~ 2 months of age, third and fourth pairs of SGs develop, again in an upward direction along the follicle axis (Figure 1D). Still later on, additional SGs develop at infundibulum-epidermal junctions (Figure 1E) and in the proximal interfollicular epidermis (Figure 1F). In parallel to SG duplication, the original SG and oldest supernumerary ones adopt a complex, branched morphology (Figure 1, G and H). Ectopic SG units can also be seen in *Gli2*^{TG} skin sections stained with H&E (Figure 1, I-L). While especially prominent in tail tissue, this SG phenotype is also seen in the skin of other body sites in *Gli2*^{TG} mice (data not shown).

Enhanced Hedgehog Signaling Correlate with Enlargement of SG Ducts in *Gli2*^{TG} Mice

Ptch-lacZ mice³⁰ provide a read-out of sites of Hh-dependent transcription in mouse skin epithelia.⁴³ We crossed *Ptch-lacZ* and *Gli2*^{TG} mice and analyzed the double-transgenic progeny by whole-mount X-Gal staining. As illustrated in Figure 2A, *Ptch-lacZ* reporter signal is strongest to the elongated ducts in SGs from *Gli2*^{TG}/*Ptch-lacZ* mice (Figure 2B). Preparations dual-stained for LacZ activity and nuclear DNA (via Hoechst) suggest that the *Patched* reporter is excluded from the outermost cell layer (Figure 2C). X-Gal staining intensity in SG ducts was comparable to that seen in newly formed hair bulb at the onset of anagen in *Ptch-lacZ* hair follicles (data not shown). These observations establish that the onset of alterations in SG morphology correlate, early on, with

Figure 1. Comparing sebaceous gland (sg) development in wild-type and *Gli2*^{TG} mice. **A–H:** Whole mount tail epidermal preparations, stained with Oil Red O, from wild-type (Wt) (**A**) and *Gli2*^{TG} mice (**B–H**). In (**A–F**), a summary schematic of the pilosebaceous unit and associated epidermis is shown at left (red: SG; green: hair bulge). **A:** SG morphology in 25-day-old (P25, left) and 5-month-old (right) wild-type mice. **B–H:** *Gli2*^{TG} mice develop a spectacular SG phenotype over time. **B:** Expansion and elongation of SG ducts (see arrows) in *Gli2*^{TG} mice between P25 and P45. **C:** Appearance of an additional pair of SGs in *Gli2*^{TG} mice, typically seen between P45 and P56. **D:** Formation of a third, and additional pairs of SGs, in *Gli2*^{TG} mice after 2 months of age. The original SG is numbered 1 in **B–D**. **E–F:** Oil Red O staining reveals the present of ectopic SGs in the infundibulum region of hair follicles (**E**), and in interfollicular epidermis (**F**), in a 10-month-old *Gli2*^{TG} mouse. **G–H:** Complex arborization of SGs, at lower magnification, in Oil Red O-stained whole mounts from a 10-month-old *Gli2*^{TG} mouse. **I–L:** H&E-stained sections prepared from paraffin-embedded *Gli2*^{TG} tail tissue. Between two and five pairs of SGs can be distinguished. The arrow in frame (**L**) shows an example of SG seemingly originating from interfollicular epidermis. Epi, epidermis; hf, hair follicle; hs, hair shaft. Scale bars = 50 μm.

local activation of Hh-dependent transcription. As a side note, the perplexing lack of *Ptch-lacZ* reporter activity at sites where the *Gli2*^{TG} transgene is knowingly expressed (eg, epidermis), for at least 4 weeks after birth, has recently been explained by powerful mechanisms mediating Gli degradation.^{43,44}

Abnormal SG Ducts Show Enhanced Keratin 6a Expression in *Gli2*^{TG} Mice

Mutations altering the coding sequence of K6a, K6b, K16, or K17 cause pachyonychia congenita, which often

features marked anomalies in SGs. We crossed the *Gli2*^{TG} mice with *hK6a-lacZ* reporter mice to gain further insight into the SG lesions elicited by local Hh signaling. In addition to being up-regulated after skin injury,²⁹ the *hK6a-lacZ* reporter is regulated in a hair cycle-dependent fashion in the companion layer of hair follicles.³¹ LacZ reporter activity is readily detectable in the SG ducts of *Gli2*^{TG}/*hK6a-lacZ* mice (Figure 2D). X-Gal staining covers the entire duct (Figure 2D), and in some cases, extends distally to glandular cells (Figure 2E). Endogenous *mK6a* is also expressed in ductal tissue, as shown by *in situ* hybridization (Figure 2F) and antibody staining of whole

Figure 2. Elongation of SG ducts is accompanied by enhanced Hh signaling and *K6a* expression in *Gli2*^{TG} mice. **A:** Summary of *Ptch-lacZ* and *hK6a-lacZ* expression, which is very similar, during the development of the SG phenotype between P25 and P56 in *Gli2*^{TG} mice. **B:** Activity of the *Ptch-lacZ* transgene, reflecting Hh signaling, is conspicuously restricted to the elongated ducts of SGs in *Gli2*^{TG} skin. Several examples are shown (see arrows). **C, C':** Dual-staining of a phenotypic SG in *Gli2*^{TG} skin for lacZ activity (**C**) and Hoechst (**C'**), to reveal nuclei, confirms that the outermost layer of SG ducts is devoid of *Ptch-lacZ* reporter activity (compare the white arrowheads, depicting the outermost layer, with the black arrow in **C**: marking in inner location of X-Gal staining). **D:** The *hK6a-lacZ* transgene is also active in the elongated segment of SG ducts in *Gli2*^{TG} mice. Several examples are shown (see arrows). **E:** Occasionally, *hK6a-lacZ* transgene activity is detected in the distal portion of SG, as shown here; in many cases, interestingly, the duct phenotype is not as pronounced. **F:** Detection, by *in situ* hybridization, of the endogenous *K6a* mRNA in the elongated segment of SG ducts in *Gli2*^{TG} mice. Signal also occurs in the hair follicle companion layer (cl), as expected.³¹ **G–I:** Indirect immunofluorescence for specific antigens on whole mount skin tail preparations (**G–H**) and tissue sections (**I**) from *Gli2*^{TG} mice. **F:** Whole mount preparation showing K6 immunoreactivity in SG ducts (see arrow) and the lower segment of hair follicles, as described.³¹ **G:** Immunoreactivity for K17 in more broadly distributed, although is also includes SG ducts (see arrows). **I:** K6 immunoreactivity in a tail tissue section from a *Gli2*^{TG} mouse. Signal is preferentially localized to the inner layer(s) of ductal epithelium (see arrows). hs, hair shaft; sg, sebaceous gland. Scale bars = 50 μ m.

mount preparations (Figure 2G), suggesting that the *hK6a-lacZ* transgene faithfully reports on its activity. By comparison, K17 shows a broader distribution in the pilosebaceous unit, that also includes the ductal epithelium of SG (Figure 2H). The restriction of K6 immunoreactivity to the innermost portion of SG ducts can be readily appreciated in tissue sections (Figure 2I).

Hedgehog Signaling and K6 Expression Also Coincide in the SG Ducts of Wild-Type Skin

We analyzed the skin of *Ptch-lacZ* and *hK6a-lacZ* mice for reporter transgene expression, with a particular emphasis on the P15–P35 age window. In *Ptch-lacZ* skin, X-Gal staining was present at the base of growing (anagen) hair follicles, as expected (Figure 3A"; also see⁴³), as well as in SG ducts (Figure 3A'). In *hK6a-lacZ* skin, X-Gal staining also occurred in SG ducts, in addition to a narrow ring located below the SG (Figure 3B; see³¹). Of note, no X-Gal staining is detectable in wild-type mouse skin (data

not shown), confirming the specificity of these findings. *In situ* hybridization performed on whole-mount epidermal sheets of wild-type mouse skin using a probe specific for the *mK6a* mRNA also gave rise to a signal in, or near, SG ducts (data not shown). The shortness of the duct, and/or its masking by the gland, precluded us from identifying the precise location of the signal. Unlike *Ptch-lacZ* (Figure 3A), however, neither *hK6a-lacZ* nor endogenous *mK6a* is expressed in the newly formed hair tissue at anagen onset (Figure 3B; see³¹). These findings indicate that Hh-dependent transcription and *K6a* expression temporally and spatially coincide in SG ducts of normal mouse skin tissue.

In sections prepared from hair follicle-bearing human skin, K6 immunoreactivity occurs in the ductal portion of SG (Figure 3, C and D). K6 antigens show a more restricted distribution than K14 (Figure 3, C' and C"), as expected. As is the case in mouse skin (Figure 2I), K6 antigens are largely excluded from the outermost layer of epithelial cells encasing SG ducts (Figure 3D).

Figure 3. Hh signaling and *K6a* expression occurs at the base of sebaceous glands in wild-type mice. **A:** Summary of *Ptch-lacZ* transgene activity in otherwise wild-type mouse skin after onset of anagen at P25-P28. **A':** Detection of *Ptch-lacZ* activity in whole mount epidermal sheets prepared from mouse tails, showing *lacZ* activity in the secondary hair germ (see double arrows) as expected,⁴³ and at the base of SGs near their point of attachment to hair follicles (see arrows). Follicles shown are late telogen (left image) and early anagen stages (right image). **B:** Detection of *hK6a-lacZ* activity at the base of SGs (see arrow), and in a ring-like pattern (see "ring") as described.³¹ **C-C'':** Dual immunostaining for K6 (C; green) and K14 (C'; red) in normal human hair-bearing skin. (C'') show the merged signals. Unlike K14, K6 does not occur in epidermis (epi) of normal skin, and its distribution in the pilosebaceous unit is more restricted, and includes the innermost portion of the SG ducts (see arrows). Frame (D) shows a region adjacent to (C); K6 immunoreactivity is strong in the innermost layers of the outer root sheath (ors) in addition to innermost aspect of the SG ducts (arrowheads). hs, hair shaft; sg, sebaceous gland. Scale bars = 50 μ m.

The Human *K6a* Promoter Is Modestly Responsive to *Gli2* Co-expression in Heterologous Systems

The parallel occurrence of hedgehog signaling and *K6a* expression in SG ducts raises the prospect that *Gli2* stimulates *K6a* gene transcription. This possibility is strengthened by the notion that *K17*, which encodes a type I partner keratin for *K6a/K6b* in several epithelial settings,³³ is a *bona fide* *Gli2* target gene.^{40,45} To test this notion, we performed standard, internally controlled transient transfection assays with luciferase reporter constructs in Cos-1 cells and in mouse skin keratinocytes. Expression of nuclear factor kappa-B (used as a positive control; see⁴⁶) enhances the intrinsic activity of the *K6b* promoter by ~16-fold in Cos-1 cells (Figure 4A), as previously reported,^{46,47} thereby establishing the validity of our experimental setting. Expression of *Gli2* also enhances *K6a* promoter activity, by ~4.4-fold in Cos-1 cells (Figure 4A), and ~3.2-fold in skin keratinocytes (Figure 4B). The transcription factor FoxE1, a *Gli2* target gene whose activity is increased during hair follicle morphogenesis and in basal cell carcinoma,^{48,49} fails to stimulate *K6a* promoter activity (eg, Figure 4A), showing the specificity of *Gli2*'s effect on the *K6a* promoter. On the other hand, and consistent with previous findings,^{45,40} we find that *Gli2* has a considerably stronger impact on the *K17* promoter compared to the *K6a* promoter activity in both Cos-1 cells and skin keratinocytes (Figure 4, A and B).

Additional Observations in *Gli2*^{TG} Mouse Skin

Hh signaling is essential for the morphogenesis and post-natal cycling of hair follicles.^{43,50–53} Mouse skin tissue becomes sensitive to constitutive *Gli1* and *Gli2* overexpression only at the onset of the first postnatal anagen

during the fourth week postbirth.^{43,44} This time frame also corresponds to the onset of SG anomalies in *Gli2*^{TG}, prompting us to examine the postnatal hair cycle in the back skin of these mice. Entry into the first iteration of the catagen and telogen stages occurs at similar times in *Gli2*^{TG} and wild-type littermates (data not shown). However, anagen re-entry is delayed and occurs at ~P30/P31 in *Gli2*^{TG} mice, instead of ~P25-P28 as seen in wild-type controls (Supplementary Figure S1 at <http://ajp.amjpathol.org>).⁴² The initial alterations affecting SGs thus coincide with a delay in anagen re-entry in *Gli2*^{TG} hair follicles, and these events occur significantly ahead of

Figure 4. Overexpression of *Gli2* activates human *K6a* promoter activity in Cos-1 epithelial cells (A) and 308 mouse skin keratinocytes (B). The *hK6a*-Luc, *mK17*-Luc constructs, or control empty vectors, were transiently transfected with or without the identified transcription factor (TF) in Cos-1 cells. Luciferase activity was measured 48 hours later, and normalized, as described in Materials and Methods. Cotransfection of *Gli2* increases *K6a* promoter activity in both Cos-1 cells (4.4-fold; see A) and mouse skin keratinocytes (3.2-fold; see B), though markedly less so than *mK17* promoter activity (>50-fold in both instances). Results are representative of three experiments (mean + SD). See text for details.

tumor formation in this mouse model (unpublished observations).²³

Previous reports indicate that several Hedgehog target genes, including *Patched* and *K17*, are robustly expressed in skin tumors arising in older *Gli2*^{TG} mice, and in tumor-proximal hyperplastic epithelium.^{23,43,44} If *K6a* expression is under the (direct or indirect) control of *Gli2*, then it should be induced in *Gli2*^{TG} tumor tissue. K6 immunoreactivity can indeed be detected in pretumoral epithelial downgrowths (Supplementary Figure S1C, C' at <http://ajp.amjpathol.org>) and in tumor tissue as well (Supplementary Figure S1D at <http://ajp.amjpathol.org>). In the latter, however, K6 immunoreactivity is only patchy, and is strongest in donut-shaped structures (Supplementary Figure S1D at <http://ajp.amjpathol.org>) which, on H&E staining, are reminiscent of glandular ducts (Supplementary Figure S1E at <http://ajp.amjpathol.org>). This pattern is distinct from the uniform, pan-tumor staining seen when staining similar sections for K17 (Supplementary Figure S1F at <http://ajp.amjpathol.org>), and extends previous reports of spotty K6 expression in human BCC tumors.^{54,55} Away from such skin lesions, K6 immunoreactivity is restricted to epithelial appendages as is the case for wild-type skin (Supplementary Figure S1G, G' at <http://ajp.amjpathol.org>). Again, the *hK6a-lacZ* reporter shows an identical activity profile when examined in the skin of *Gli2*^{TG}/*hK6a-lacZ* double-transgenic mice (data not shown). Confirming these findings, RT-PCR analyses show that the endogenous *K6a* and *K17* mRNAs are elevated in ear tumor tissue harvested from adult *Gli2*^{TG} mice, as compared with wild-type (Supplementary Figure S1H at <http://ajp.amjpathol.org>).

Discussion

The morphogenesis of sebaceous glands, and their homeostasis, is not well understood. Sebocytes arise from a pool of mitotically active, transit-amplifying "basal cells" located in the outermost aspect of the gland per se, and undergo holocrine secretion in the hair canal, calling for their replenishment on a continuous basis.^{56–58} The ultimate source for the sebocyte lineage, in adult mouse skin, is a small pool of committed progenitor cells tucked within the outer root sheath at or near the point of SG branching from hair follicles. There is evidence that a transcriptional repressor, Blimp-1, helps maintain these cells in a relatively quiescent state.⁵⁸ The ductal epithelium, which comprises three layers (basal, intermediate, granular layers), lies between these quiescent stem cells and the transit-amplifying "basal" cells that encapsulate the distal part of the SG.⁵⁷ Whether the various cell types in the ductal epithelium and gland per se represent different steps along a single path to differentiation, or are the product of distinct differentiation programs from a common progenitor, is unknown, as is the pattern of cell movement within SG.

Molecularly, gain- and loss-of-function studies have shown that activation of Hedgehog signaling^{59,60} and c-Myc^{61,62} stimulates SG morphogenesis and, eventually, cause SG tumors in mouse skin.⁶³ Likewise, interference

with Lef1-dependent Wnt signaling, resulting from either a dominant-negative strategy in mice (via Δ NLef1)⁶⁰ or somatic mutations in humans,⁶⁴ leads to *de novo* formation of SGs and their tumorigenesis as well. Intriguingly, overexpressed Δ NLef1 causes an up-regulation of Indian hedgehog in mature sebocytes, which on its secretion and limited diffusion would activate the proliferation of committed SG progenitors, causing SG enlargement.⁶⁰ The idea of an interplay between Wnt and Hh signaling, and likely other pathways, in determining lineage choices followed by organ morphogenesis in skin epithelia has gained a lot of support in recent years.^{60,65,66}

Our results establish that Hh signal reception is strongest within the duct segment of SG. This is so not only in the skin of wild-type mice but also in *Gli2*-overexpressing mice starting at 4 weeks postbirth. Previous work showed that Hh signaling plays a key role during ductal and branching morphogenesis in other glandular organs including prostate,⁶⁷ mammary gland,⁶⁸ salivary gland,⁶⁹ lung,⁷⁰ and pancreas.⁷¹ Our findings of "duct-preferred" Hh signaling, and of an aberrant branching phenotype in *Gli2*^{TG} skin, suggest that Hh signaling plays a similar role in SG as well. Hh signaling is also key to initiation of a new anagen phase in the resting follicles of adult skin^{51,53,72} and, interestingly, the initial enlargement and elongation of SG ducts in *Gli2*^{TG} mice temporally coincides with a delay in the onset of the first postnatal anagen (growth) phase in hair follicles. Therefore, the "broad" ectopic expression of *Gli2* in this setting, and at ~3.5 weeks postbirth, could stimulate early progenitor cells to divide, and contribute to re-direct their fate from hair follicle to SG. Bulge stem cells, which are proximal to SG ducts but normally give rise to hair follicle lineages, can indeed be directed toward SG and epidermal fates under special circumstances.^{58,73–75} Such a mechanism has been proposed to underlie the continued formation of sebum-filled dermal cysts in *Hairless* mice, which fail to re-initiate anagen.^{76,77} It may prove relevant to investigate the status of signaling effectors known to promote SG morphogenesis, such as Gli, c-Myc, and others, in steatocystoma lesions from pachyonychia congenita patients.

Another observation having potential implications for stem cell location, and regulation, is the highly ordered pattern of *de novo* SG morphogenesis observed along the hair follicle axis, and in the interfollicular epidermis, of adult *Gli2*^{TG} mice. The specific spatial pattern seen may reflect the location of early progenitor, bipotent cells in the upper segment of skin epithelia, as recently suggested.⁷⁸

We report here that *K6a* is primarily expressed in the suprabasal layers of SG ducts in both wild-type and *Gli2*^{TG} mice, and in human. These findings extend previous reports of K6 expression in the ductal epithelium of sweat glands⁷⁹ and developing mammary glands,^{80–82} and of K6b isoform expression, specifically, in sweat gland ducts of human skin.⁸³ The role of K6 isoforms in glandular epithelia remains an enigma (eg, ref. 82) despite the creation and study of several relevant mouse models, in particular, *K6a/K6b* double null mice.^{10,84} Clearly, SGs can form in the absence of these keratins (unpublished data),^{14,84} but the significance of this ob-

servation is unclear as K5 and possibly K75 (formerly K6hf) may well compensate for the loss of K6.^{34,84} The early postnatal death of K6a/K6b null mice¹⁰ also makes it impossible to examine SG homeostasis in the adult setting. What about functions revealed in non-glandular epithelia? Studies in K6a/K6b null mice evidenced the essential structural support role of these two keratins in the oral mucosa,¹⁰ and during wound repair after acute injury to skin.¹⁴ Constitutive K6 expression often occurs in contexts where an epithelial sheet is moving alongside a stationary epithelial phase.⁴ In addition to the wound edge, this is so at the interface between the outer root sheath and companion layer in hair follicles, and between the nail bed and nail plate.^{4,31,85,86} Both mechanical support and regulation of cell migration could account for the presence of K6 in sweat gland ducts. In addition to better mouse models, however, resolution of this issue awaits a better understanding of cellular homeostasis in mature SGs. As a side note, the frequent occurrence of steatocystoma lesions and, though in a rare manner, of premature tooth eruption^{19,20} lends further credence to the notion that K6 protein(s) (and their partners) may fulfill roles other than structural support in epithelia. In this vein, the positive role of K17¹⁶ and other skin keratins⁸⁷ toward the regulation of protein synthesis could be enhanced by mutations in K6 isoforms, and/or in K17.

Finally, there is the issue of whether *K6a* is a direct target for Gli2 transcription factors *in vivo*. Relative to the *K17* proximal promoter, which is exquisitely sensitive to Gli2 (>70-fold response), the *K6a* promoter shows a very modest response (3- to 4-fold range; see Figure 4). Unlike K17,⁸⁸ the epidermis of newborn and young adult *Gli2*^{TG} mice is negative for K6 antigens, though later on, spotty K6 expression can be observed in tumors and tumor-proximal hyperplastic epidermis (Supplementary Figure S1 at <http://ajp.amjpathol.org>). Such arguments favor the possibility that K6 is an indirect target of Gli2, or alternatively, that the latter works cooperatively with another factor(s) to transactivate the *K6a* promoter. While the 5' upstream sequence of both *hK6a* and *mK6a* do not feature the strong Gli2-responsive element identified in the *K17* proximal promoter,⁴⁰ another Gli-binding site, GGACACCCA, is present (–1925 bp to –1917 bp from the translational start site of *hK6a*; data not shown). This particular site confers a similar responsiveness to the *FoxE1* gene promoter.⁴⁸ Although our findings definitely establish that *K17* and *K6a* represent Gli2 targets of high and low sensitivity, respectively, further studies are required to establish the mechanism of Hh signal-associated *K6a* gene expression *in vivo*.

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