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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.
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 provided by intermediate filament polymers. 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. 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. 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 are compromised in such settings is unknown.

A group of four keratin genes, the type II keratin paralogs 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). 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. Several functions have been found to be associated with these keratins from the study of various types of genetically modified mice, including structural support, modulation of keratinocyte migration, and of tumor necrosis factor-α-induced apoptosis (for K17) and of protein synthesis. 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, and which predominantly affect one or many epithelial appendages. These disorders (and the affected target proteins) include type I (K6α, K16) and type 2 (K6b, K17) pachyonychia congenita, steato-cystoma multiplex (K17), and two palmoplantar keratoderma variants (K16) (see Human Intermediate Filament Database, http://www.interfil.org/). 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). A puzzling element that is frequently (>50%) associated with mutations in K6α, K6b and K17 is the development of epithelial cysts, generally, at the time of puberty. Other than steato-cystoma multiplex, a “pure” glandular disorder, epidermal inclusion cysts are seen in type 1 and type 2 pachyonychia congenita, while vellus hair cysts and steato-cystomas are additionally seen in type 2 pachyonychia congenita. The content of these cysts bear a resemblance to sebaceous glands (SGs); in the case of steato-cystomas they are believed, in fact, to originate from SG ducts. 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 K6α gene expression preferentially marks ductal tissue in SGs. We discovered this phenomenon while studying Gli2 transgenic mice (Gli2TG), which as reported offer the distinct advantage of showing markedly enlarged SGs. 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 K6α expression at the mRNA and protein levels. Thereafter, additional SGs appear according to a well defined spatiotemporal pattern in Gli2TG mice, and Hh signaling along with K6α expression consistently marks SG ductal tissue during this process. Hh signaling and K6α 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 involving a loss of structural support in the relevant epithelial cell population(s).

Materials and Methods

Animal Models and Whole Mount Epidermal Sheets Preparation

The following transgenic lines were used: hK6α-lacZ mice (23-1p line), harboring a transgene consisting of 5.2-kb of 5′ upstream sequence from the hK6α gene fused to the lacZ coding sequence modified with a nuclear localization signal; Ptch-lacZ mice (C57Bl/6 strain), in which the LacZ sequence has been knocked-in the Patched locus; Gli2TG mice (C57Bl/6 strain), in which the mouse Gli2 sequence is downstream from the bovine K5 promoter. Gli2TG/K6α-lacZ and Gli2TG/Ptch-lacZ double-transgenic mice were generated through selective crosses. Genotyping for the K5-Gli2 transgene were performed via PCR. Genotyping for hK6α-lacZ and Ptch-lacZ were performed using β-galactosidase assay in situ. All whole mount epidermal sheets were prepared from the upper one-third region on the dorsal side of male mouse tail skin, as described. 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”), K17; a mouse monoclonal antibody directed against K14 (LLO01); 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 mK6α mRNA was done as described elsewhere. The hK6α promoter-luciferase construct was generated by subcloning 3.3 kb of 5′ upstream sequence from the hK6α gene (obtained by NcoI digestion) 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/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. For β-galactosidase histochemistry, the incubation were performed 1 hour at 37°C for hK6α-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 (Biomedia, 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

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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 UltraView confocal microscope.

**Transient Transfection and Luciferase Assays**

Monkey kidney Cos-1 epithelial cells or alternatively, mouse 308 skin keratinocytes were seeded in 24-well plates at a density of 2 x 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 promoters 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.

Cells were harvested 48 hours later for analysis. Cells were cotransfected with plasmid featuring a given promoter (see description below) subcloned in a pGL3-vector (Firefly luciferase, Promega); the promoter-less pGL3-basic 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. 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.

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 semiquantitative 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 performed using β-tubulin as an internal control. The oligonucleotide primers used for PCR were used as follows: K17, Forward: 5′-GATGGAGCCAGCAGAACCAGGAGTA-3′; Reverse: 5′-GGTCTCAAGCATAGAATGCTGGG-3′; K6a, Forward: 5′-GAGCTGCGTTGTTGTTG-3′. Reverse: 5′-GTCCCTCTAGTGTCCGT-3′, β-tubulin, Forward: 5′-CAACGTCAAGACCGCTGTTG-3′, Reverse: 5′-GACAGAGGCAAACCTGAGCACC-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/rector pil 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 ~p25 (Figure 1B). At ~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**

_Ptc-lacZ_ mice provide a read-out of sites of Hh-dependent transcription in mouse skin epithelia. We crossed _Ptc-lacZ_ and Gli2^Tg_ mice and analyzed the double-transgenic progeny by whole-mount _X-Gal_ staining. As illustrated in Figure 2A, _Ptc-lacZ_ reporter signal is strongest to the elongated ducts in SGs from Gli2^Tg/Ptc-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 _Ptc-lacZ_ hair follicles (data not shown). These observations establish that the onset of alterations in SG morphology correlate, early on, with
local activation of Hh-dependent transcription. As a side note, the perplexing lack of Ptch-lacZ reporter activity at sites where the Gli2TG transgene is knowingly expressed (e.g., 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 Gli2TG 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 Gli2TG 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 Gli2TG/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 mkK6a is also expressed in ductal tissue, as shown by in situ hybridization (Figure 2F) and antibody staining of whole

Figure 1. Comparing sebaceous gland (sg) development in wild-type and Gli2TG mice. A–H: Whole mount tail epidermal preparations, stained with Oil Red O, from wild-type (Wt) (A) and Gli2TG 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: Gli2TG mice develop a spectacular SG phenotype over time. B: Expansion and elongation of SG ducts (see arrows) in Gli2TG mice between P25 and P45. C: Appearance of an additional pair of SGs in Gli2TG mice, typically seen between P45 and P56. D: Formation of a third, and additional pairs of SGs, in Gli2TG 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 Gli2TG mouse. G–H: Complex arborization of SGs, at lower magnification, in Oil Red O-stained whole mounts from a 10-month-old Gli2TG mouse. I–L: H&E-stained sections prepared from paraffin-embedded Gli2TG 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 in interfollicular epidermis. Epi, epidermis; hf, hair follicle; hs, hair shaft. Scale bars = 50 μm.
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 see31), 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 hK6α-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 K6α 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 outmost layer of epithelial cells encasing SG ducts (Figure 3D).
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, is a bona fide Gli2 target gene. 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; see Figure 4A) enhances the intrinsic activity of the K6b promoter by ~16-fold in Cos-1 cells (Figure 4A), as previously reported, 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, 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, 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 Gli2TG Mouse Skin

Hh signaling is essential for the morphogenesis and postnatal cycling of hair follicles. 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. This time frame also corresponds to the onset of SG anomalies in Gli2TG, 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 Gli2TG and wild-type littersmates (data not shown). However, anagen re-entry is delayed and occurs at ~P30/P31 in Gli2TG mice, instead of ~P25-P28 as seen in wild-type controls (Supplementary Figure S1 at http://ajp.amipathol.org). The initial alterations affecting SGs thus coincide with a delay in anagen re-entry in Gli2TG hair follicles, and these events occur significantly ahead of...
tumor formation in this mouse model (unpublished observations).\textsuperscript{23,25}

Previous reports indicate that several Hedgehog target genes, including Patched and K17, are robustly expressed in skin tumors arising in older Gli2\textsuperscript{TG} mice, and in tumor-proximal hyperplastic epithelium.\textsuperscript{23,43,44} If K6a expression is under (the direct or indirect) control of Gli2, then it should be induced in Gli2\textsuperscript{TG} tumor tissue. K6 immunoreactivity can indeed be detected in premalignant epithelial downgrowths (Supplementary Figure S1C, C’ at http://aja.amjpathol.org) and in tumor tissue as well (Supplementary Figure S1D at http://aja.amjpathol.org). In the latter, however, K6 immunoreactivity is only patchy, and is strongest in donut-shaped structures (Supplementary Figure S1D at http://aja.amjpathol.org) which, on H&E staining, are reminiscent of glandular ducts (Supplementary Figure S1E at http://aja.amjpathol.org). This pattern is distinct from the uniform, pan-tumor staining seen when staining similar sections for K17 (Supplementary Figure S1F at http://aja.amjpathol.org), and extends previous reports of spotty K6 expression in human BCC tumors.\textsuperscript{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://aja.amjpathol.org). Again, the hK6a-lacZ reporter shows an identical activity profile when examined in the skin of Gli2\textsuperscript{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\textsuperscript{TG} mice, as compared with wild-type (Supplementary Figure S1H at http://aja.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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsuperscript{59,60} and c-Myc\textsuperscript{61,62} stimulates SG morphogenesis and, eventually, cause SG tumors in mouse skin.\textsuperscript{63} Likewise, interference with Lef1-dependent Wnt signaling, resulting from either a dominant-negative strategy in mice (via ΔNLeF1)\textsuperscript{60} or somatic mutations in humans,\textsuperscript{54} leads to de novo formation of SGs and their tumorigenesis as well. Intriguingly, overexpressed ΔNLeF1 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.\textsuperscript{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.\textsuperscript{63,65–66}

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,\textsuperscript{67} mammary gland,\textsuperscript{68} salivary gland,\textsuperscript{69} lung,\textsuperscript{70} and pancreas.\textsuperscript{71} Our findings of "duct-preferred" Hh signaling, and of an aberrant branching phenotype in Gli2\textsuperscript{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\textsuperscript{64,65} and, interestingly, the initial enlargement and elongation of SG ducts in Gli2\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsuperscript{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.\textsuperscript{78}

We report here that K6a is primarily expressed in the suprabasal layers of SG ducts in both wild-type and Gli2\textsuperscript{TG} mice, and in human. These findings extend previous reports of K6 expression in the ductal epithelium of sweat glands\textsuperscript{79} and developing mammary glands,\textsuperscript{80–82} and of K6b isoform expression, specifically, in sweat gland ducts of human skin.\textsuperscript{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.\textsuperscript{10,84} Clearly, SGs can form in the absence of these keratins (unpublished data),\textsuperscript{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 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 eruption18,20 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 keratins85 toward the regulation of protein synthesis could be enhanced by mutations in K6 isoforms, and/or 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 exclusively 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−/− mice is negative for K6 antigens, though later on, spotty K6 expression can be observed in tumors and epithelium. 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.40 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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