Short Communication

Hedgehog Signaling Regulates Sebaceous Gland Development

Mary Allen,* Marina Grachtchouk,* Hong Sheng,* Vladimir Grachtchouk,* Anna Wang,* Lebing Wei,* Jianhong Liu,* Angel Ramirez,† Daniel Metzger,‡ Pierre Chambon,‡ Jose Jorcano,† and Andrzej A. Dlugosz*

From the Department of Dermatology and Comprehensive Cancer Center,* University of Michigan, Ann Arbor, Michigan; Project on Cell and Molecular Biology and Gene Therapy,† Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas, Madrid, Spain; and Institut de Genetique et de Biologie Moleculaire et Cellulaire,‡ College de France, Communaute Urbaine de Strasbourg, Strasbourg, France

Epithelial progenitor cells in skin give rise to multiple lineages, comprising the hair follicle, an associated sebaceous gland, and overlying epidermis; however, the signals that regulate sebocyte development are poorly understood. We tested the potential involvement of the Hedgehog pathway in sebaceous gland development using transgenes designed to either block or stimulate Hedgehog signaling in cutaneous keratinocytes in vivo. Whereas inhibition of the Hedgehog pathway selectively suppressed sebocyte development, Hedgehog pathway activation led to a striking increase both in size and number of sebaceous glands. Remarkably, ectopic Hedgehog signaling also triggered the formation of sebaceous glands from footpad epidermis, in regions normally devoid of hair follicles and associated structures. These ectopic sebaceous glands expressed molecular markers of sebocyte differentiation and were functional, secreting their contents directly onto the skin's surface instead of into a hair canal. The Hedgehog pathway thus plays a key role in sebocyte cell fate decisions and is a potential target for treatment of skin disorders linked to abnormal sebaceous gland function, such as acne. (Am J Pathol 2003, 163:2173–2178)

Epithelial progenitor cells in skin give rise to epidermis as well as the epithelial component of skin appendages, including hair follicles and associated sebaceous glands.1,2,3 Early-stage follicles consist of an epithelial thickening, called a placode, and an adjacent mesenchymal condensate in the developing dermis.4 The follicle epithelium grows downward through the dermis and into the subcutaneous fat, where it surrounds the condensate-derived hair follicle papilla to form the hair bulb. Rapidly proliferating matrix cells in the hair bulb give rise to the hair shaft and inner root sheath lineages, which are driven upward toward the skin's surface during hair maturation. Surrounding these cell layers is the follicle outer root sheath, which is continuous with the interfollicular epidermis and contains epithelial stem cells in a region called the bulge.5,6 The first differentiated cell type to appear in the developing follicle is the oil-rich sebocyte, which arises from cells within the superficial hair follicle.7 Over the course of several days, the expanding pool of sebocytes forms a gland located outside of the hair follicle, with sebocytes releasing their contents into the hair canal not far below the skin's surface.

Proper hair follicle development is dependent on a series of inductive signals traveling between epithelial and mesenchymal follicle progenitors.8 Recent studies have begun to identify some of the molecules regulating follicle morphogenesis and cell fate, and similar to many other organ systems, hair follicles use both the Wnt and Hedgehog pathways to guide their assembly. Wnt signaling regulates follicle initiation and reactivation of follicle growth during postnatal hair cycling.9–12 At later stages of follicle maturation, the Wnt pathway also plays an important role in terminal differentiation of hair lineages.13,14 Hedgehog signaling plays a complementary role, as it is essential for the proliferative expansion of hair follicle epithelium but is not required during follicle initiation or hair lineage differentiation.15–17

Given the involvement of Hedgehog proteins in regulating cell lineage specification in several other organs,18 we tested the potential involvement of this pathway in cell fate decisions in skin using both loss-of-function and

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Address reprint requests to Andrzej A. Dlugosz, M.D., U-M Cancer Center/Dermatology, 3310 CCGC, Box 0932, 1500E Medical Center Drive, Ann Arbor, MI 48109-0932. E-mail: dlugosza@umich.edu.

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gain-of-function transgenic mouse models. We find that inhibition of Hedgehog signaling in cutaneous keratinocytes selectively blocks the formation of sebocytes. In contrast, ectopic activation of the Hedgehog pathway promotes sebocyte development, even in regions that normally do not contain hair follicles or associated sebaceous glands. Our findings strongly implicate Hedgehog signaling in sebocyte cell fate decisions, and suggest that modulation of the Hedgehog pathway may provide a novel means of regulating sebaceous gland function.

Materials and Methods

Construction and Breeding of Transgenic Mice

Because Hedgehog signaling modulates gene expression via Gli proteins, we generated transgenic mice expressing a skin-targeted dominant-negative Gli mutant designed to block Hedgehog responsiveness in cutaneous epithelium. We produced a pcDNA3.1Flag-Gli2 (kindly provided by Drs. Hiroshi Sasaki and Chi-chung Hui) with the following elements: rabbit NotI, cDNA with bovine growth hormone polyA signal, flanked by NheI sites in reverse orientation, was inserted into the K5 transgenic cassette. M2SMO cDNA was subcloned into the Nhel site, yielding a construct with the following elements: bovine K5 promoter, rabbit β-globin intron, loxp, EGFP, bovine growth hormone polyA, loxP, M2SMO, and 2xSV40 polyA, which we designated K5-flxGFP-M2SMO. Eight transgenic founders were produced and several mouse lines established, and these will be characterized in a subsequent publication. To activate M2SMO expression, K5-flxGFP-M2SMO mice were crossed with either K5-Cre or K5-CreERT2 mice to generate double-transgenic progeny. Whereas recombination in mice harboring the K5-CreERT2 transgene was previously shown to be dependent on treatment with 4-hydroxytamoxifen, low-level recombination took place in untreated double-transgenic mice described in this report (see Figure 2I). This most likely reflects the variable sensitivity of different floxed alleles to Cre-mediated recombination. All mice were housed and maintained according to University of Michigan institutional guidelines.

Results

For hematoxylin and eosin (H&E) staining, we fixed skin overnight in neutral-buffered formalin, transferred to 70% EtOH, processed, and embedded in paraffin. Skin was also embedded in Optimum Cutting Temperature compound for frozen sections. Oil Red O staining was performed using a modification of a protocol kindly provided by Dr. Karin Müller-Decker (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Frozen sections were fixed in 1% neutral-buffered formalin for 5 minutes, washed in deionized water, and incubated in 60% isopropanol for 5 minutes. Sections were stained with filtered Oil Red O working solution, prepared immediately before use by making a 6:4 mixture of stock (0.5% Oil Red O in 99% isopropanol) and deionized water. Sections were transferred to 60% isopropanol, washed in deionized water, counterstained using hematoxylin, and mounted using 50% glycerol in PBS. A similar protocol was used for Oil Red O staining of whole-mounds, except samples were subsequently washed and stored in deionized water.

Semiquantitative RT-PCR

We isolated RNA from skin lysates prepared using Trizol (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Semiquantitative reverse-transcription-polymerase chain reaction (RT-PCR) was performed using 1-µg samples of total RNA for first-strand DNA synthesis (Superscript II RT kit, Invitrogen). Primers were selected to span an intron whenever possible, or PCR was performed in the absence of reverse transcriptase to rule out the possibility that amplification products were derived from contaminating DNA. PCR conditions are available on request; the following primers were used: actin (421 bp) forward 5′-TACCACAGGCATTTGATGAGGA-3′, reverse 5′-CAACGTCACTTCTGTAGTGG-3′; c-Myc (548 bp) forward 5′-AGTCATGATCCCTAGTGTCCTTCCCTACT-3′, reverse 5′-CGAGCTGCCTTCCCTAC-3′; Gli1 (364 bp) forward 5′-TCTGGGAAGCTCTATTCAGGC-3′, reverse 5′-CATGCTCTTCTTCTCAGGC-3′; M2SMO (435 bp) forward 5′-AAACCGGATCAAGAGAGGA-3′, reverse 5′-GGAGAGCTCGAGGAATGTTGTA-3′; M2SMO (364 bp) forward 5′-AAATCCGATGCGAGAGAGGA-3′, reverse 5′-GGTAGCGCAAGGCGATTGTA-3′; Mc5r (490 bp) forward 5′-AAATCCGATGCGAGAGAGGA-3′, reverse 5′-GGAGAGCTCGAGGAATGTTGTA-3′; Scd3 (809 bp) forward 5′-CTTGGATAACCCCTGGGTG-3′, reverse 5′-CTTCCTGGAAACATCCACGCTTC-3′; Shh (241 bp) forward 5′-TCTGGATGAACCGATGTCGCC-3′, reverse 5′-GCCACCGGATTCTGCTT-3′.
tion in a dominant-negative manner. To inhibit Hedgehog signaling selectively in cutaneous epithelium, we used the bovine K5 promoter to generate K5-Gli2ΔC4 transgenic mice. The K5 promoter is active in the epidermal basal layer, embryonic hair follicle progenitor cells, and follicle outer root sheath, including the bulge region which harbors multipotent stem cells.

Hair follicles in K5-Gli2ΔC4 mice were shorter than in control littermates but expressed multiple markers for epidermal and hair follicle cell lineages (H. Sheng et al., manuscript in preparation). However, while 7-day-old control dorsal skin contained numerous hair follicle-associated sebaceous glands (Figure 1A), these structures were rarely seen in dorsal skin from K5-Gli2ΔC4 littermates (Figure 1B). Sebaceous glands were also deficient in transgenic mice at 13 days of age (not shown), but analysis at later times was not possible because of impaired viability of these mice. Staining with Oil Red O to identify lipids revealed subcutaneous adipose tissue in both control and K5-Gli2ΔC4 skin sections (Figure 1A). Extensive sebaceous gland development normally occurs during the perinatal period, reflected by the accumulation of transcripts encoding the sebocyte markers Scd3 and Mc5r between day 1 and 9 in control skin (Figure 1E). In skin from 9-day-old K5-Gli2ΔC4 mice, however, Scd3 mRNA was nearly undetectable and Mc5r transcripts were present at levels comparable to day 1. In retrospect, there was also a selective deficiency of sebaceous glands in Shh mutant skin, which harbors multipotent stem cells.

Since multiple markers for epidermal and hair follicle lineages are detected both in Shh mutant and K5-Gli2ΔC4 skin, Shh/Gli signaling appears to be specifically required for sebocyte development.

Shh acts on target cells by inhibiting the function of its receptor Ptc, which normally represses the signal transducer Smo. To expand the pool of keratinocytes in which the Shh pathway is active, we generated K5 promoter-driven mice using a gain-of-function SMO mutant designated M2SMO, which is largely resistant to inhibition by Ptc. We bypassed the perinatal lethality previously reported in K5-M2SMO mice by modifying the K5 transgenic cassette to enable Cre-dependent induction of M2SMO, as described in the Materials and Methods. K5 promoter-driven M2SMO expression resulted in an increase in both the number and size of sebaceous glands in haired skin (Figure 2, A and B). The appearance of ectopic sebocytes within the epidermis (Figure 2B, inset) suggested that Hedgehog signaling could act as a switch directing competent keratinocytes to enter the sebocyte lineage. To further explore this possibility, we examined regions of volar skin that are normally devoid of hair follicles or sebaceous glands. In striking contrast to controls, volar skin from M2SMO-expressing mice was thicker and contained numerous, well-formed ectopic sebocytes (Figure 2, C and D). Much of the adjacent epidermis contained typical keratinocytes, suggesting that only a subset of epidermal cells was capable of forming sebocytes in response to M2SMO. Intriguingly,
during early stages of development, ectopic sebocytes arose from cells at the base of epidermal invaginations (Figure 2D, inset), one of the sites where epidermal stem cells have been proposed to reside in non-hairy skin.35 The ectopic sebocytes in $M2SMO$-expressing volar skin stained with Oil Red O and were frequently organized into glands (Figure 2, E and F). In the absence of hair follicles, many of these glands released their contents into ducts leading directly to the skin’s surface (Figure 2F, inset), instead of into hair canals. Thus, the ectopic sebocytes exhibited a remarkable degree of autonomy with regard to assembly into glandular structures with rudimentary ducts. To better assess the extent of ectopic sebaceous gland development, footpads were removed from control and $M2SMO$-expressing mice, stained with Oil Red O, and examined as whole mounts. Large numbers of ectopic, Oil Red O-positive sebaceous glands were detected in footpads from $M2SMO$-expressing mice but not controls (Figure 2I). As expected, the transgenic samples contained $M2SMO$ mRNA and elevated levels of the Shh target gene Gli1, confirming enhanced Hedgehog pathway activity. $M2SMO$ samples did not contain detectable levels of Shh mRNA, arguing that the appearance of sebocytes in transgenic skin was not the result of up-regulated Shh expression. In light of recent studies documenting enhanced sebocyte development in mice expressing MYC in skin,36,37 it is noteworthy that c-Myc expression appeared to be up-regulated in volar skin of $M2SMO$ mice, compared to controls.

Discussion

The results of our studies strongly implicate Hedgehog signaling in the development of sebocytes from competent progenitor cells. Although sebaceous glands are normally derived from hair follicles, ectopic Hedgehog signaling leads to sebocyte formation within the epidermis of hairy skin, as well as hairless volar skin. Previous work using hairless skin38,39 or even corneal epithelium40 has demonstrated the presence of multipotent progenitor cells capable of giving rise to hair follicle and sebocyte cell lineages. However, these studies entailed the use of tissue or cell-suspension recombinants, and did not iden-
Hedgehog Signaling and Sebaceous Glands

While this manuscript was under review, a report was published implicating Indian hedgehog in the proliferation of sebocyte progenitors (Niemann C, Unden AB, Lyle S, Zouboulis CC, Toftgard R, Watt FM: Indian hedgehog and b-catenin signaling: role in the sebaceous lineage of normal and neoplastic mammalian epidermis. Proc Natl Acad Sci USA 2003, 100(Suppl 1):11873–11880).

Note Added in Proof

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References

12. Van Mater D, Kolligs FT, Dlugosz AA, Fearon ER: Transient activation of β-catenin signaling in cutaneous keratinocytes is sufficient to trigger the active growth phase of the hair cycle in mice. Genes Dev 2003, 17:1219–1224


33. Roop DR: Deregulated expression of DeltaNLef1 in mouse epidermis results in differentiation of hair follicles from epidermal cells in skin grafts that might be important for hair follicle development. J Invest Dermatol 1995, 104:21S–22S.


