Correction

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The authors note that Jessica Svärd and Stephan Teglund should be added to the author list between Anja Schwäger and Nick Barker. Jessica Svärd should be credited with contributing new reagents/analytical tools. Stephan Teglund should be credited with contributing new reagents/analytical tools. The corrected author and affiliation lines, and author contributions appear below. The online version has been corrected.

Maria Kaspera,1, Viljar Jaksa,b,1, Alexandra Area, Åsa Bergströma, Anja Schwägera, Jessica Svärda, Stephan Teglunda, Nick Barkerc,2, and Rune Toftgårda,3

aCenter for Biosciences and Department of Biosciences and Nutrition, Karolinska Institutet, Novum, 141 83 Huddinge, Sweden; bInstitute of Molecular and Cell Biology and Estonian Biocentre, University of Tartu, 51010 Tartu, Estonia; and cHubrecht Institute, Koninklijke Nederlandse Akademie van Wetenschappen and University Medical Center Utrecht, 3584CT Utrecht, The Netherlands


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Wounding enhances epidermal tumorigenesis by recruiting hair follicle keratinocytes

Maria Kasper1,2, Viljar Jaks3,4, Alexandra Are2, Åsa Bergström5, Anja Schwäger3, Jessica Svärd3, Stephan teglund3, Nick Barker2,6, and Rune Toftgard2,7

1Center for Biosciences and Department of Biosciences and Nutrition, Karolinska Institutet, Novum, 141 85 Huddinge, Sweden; 2Institute of Molecular and Cell Biology and Estonian Biocentre, University of Tartu, 51010 Tartu, Estonia; and 3Hubrecht Institute, Koninklijke Nederlandse Akademie van Wetenschappen and University Medical Center Utrecht, 3584CT Utrecht, The Netherlands

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Chronic wounds and acute trauma constitute well-established risk factors for development of epithelial-derived skin tumors, although the underlying mechanisms are largely unknown. Basal cell carcinomas (BCCs) are the most common skin cancers displaying a number of features reminiscent of hair follicle (HF)-derived cells and are dependent on deregulated Hedgehog (Hh)/GLI signaling. Here we show, in a mouse model conditionally expressing GLI1 and in a model with homozygous inactivation of Ptch1, mimicking the situation in human BCCs, that the wound environment accelerates the initiation frequency and growth of BCC-like lesions. Lineage tracing reveals that both oncogene activation and wounding induce emigration of keratinocytes residing in the lower bulge and the nonpermanent part of the HFs toward the interfollicular epidermis (IFE). However, only oncogene activation in combination with a wound environment enables the participation of such cells in the initiation of BCC-like lesions at the HF openings and in the IFE. We conclude that, in addition to the direct enhancement of BCC growth, the tumor-promoting effect of the wound environment is due to recruitment of tumor-initiating cells originating from the neighboring HFs, establishing a link between epidermal wounds and skin cancer risk.

Lgr5 | stem cells | wound healing | carcinogenesis

In the skin, the hair follicle (HF) is a reservoir for epithelial stem and progenitor cells, which reside in different niches (1). One population marked by expression of Keratin 15 (K15) and CD34 is present in the bulge, a structure below the opening of the sebaceous gland at the attachment site of the musculus arrector pili (2). Other stem cell (SC) populations marked by expression of Lgr6 and Lrig1 are found in the junctional zone above the bulge, in close proximity to the sebaceous gland (isthmus) and the neck of the HF (infundibulum) (3, 4). In addition, a SC population marked by expression of Lgr5 is present in the bulge and secondary hair germ (SHG) in the resting telogen hair follicle but relocates to the outer root sheath (ORS) of the growing part in actively cycling anagen HFs (5). Genetic lineage tracing has revealed that bulge and Lgr5-marked SCs maintain all parts of the hair follicle below the sebaceous gland opening (5). Under normal circumstances, HFs and the interfollicular epidermis (IFE) are maintained by separate cell populations (6); however, during cutaneous wound repair, the cells originating from the HF, including the bulge SCs, leave the HF and contribute to wound reepithelialization (7, 8). Though both chronic and acute wounds have been recognized as promoters of the formation of epithelial tumors, the underlying cellular mechanisms have not been studied in detail (9, 10). By using two different genetic mouse models of human BCC, we show that the wound environment enhances the tumor initiation and progression. Lineage tracing of Lgr5+ cells revealed that keratinocytes originating from the lower bulge and the nonpermanent part of the HF contribute to wound reepithelialization and the formation of basaloid proliferations in the IFE of the wound area. Furthermore, Lgr5+ cells with an activated Hh pathway can initiate new BCC-like lesions in the wound IFE, suggesting that the enhancement of the BCC formation caused by wounding can be at least in part attributed to cellular contribution from neighboring HFs.

Results

Postnatal Activation of Hh Signaling via Overexpression of GLI1 or Deletion of Ptch1 Induces BCC-Like Lesions in Transgenic Mice. In humans, sporadic BCCs occur predominantly during the later phase of life due to the deregulation of the Hedgehog (Hh) pathway by inactivation of its negative regulator, PTCH, and subsequent activation of the Hh pathway effectors, the GLI transcription factors (11). Hh activation in mouse skin can give rise to a spectrum of tumors with hair follicle-like differentiation ranging from trichoblastoma over hamartoma, trichofolliculoma, trichoeithelioma to tumors having characteristics of nodular and superficial BCC; however, the tumor phenotype is largely dependent on the level of Hh signaling activity (12, 13). To mimic such a situation, we activated the Hh pathway in the basal layer of the mouse epidermis after completion of the HF morphogenesis in two different genetically engineered mouse models.

First, we generated K5tTA/TREGLI1 mice harboring the tetracycline-regulated transcriptional activator (Tta) expressed under the control of the bovine keratin 5 (K5) promoter and a tretynose element (TRE) controlling the expression of human GLI1 (13–16). To induce GLI1 expression, the tetracycline analog doxycycline was removed from the drinking water at postnatal day 16 (P16; Fig. 1A). The GLI1 protein could first be detected 2 wk after doxycycline removal (Fig. SLA). The K5tTA/TREGLI1 mice developed a characteristic phenotype including standing hair, hyperkeratosis, and hyperpigmentation (Fig. 1B).

To confirm the identity of the early proliferations as precursors for BCC-like lesions, we obtained skin samples at different stages of tumor progression (Fig. 1A), which we defined by the size and character of the basaloid proliferations in the IFE. To distinguish the early proliferations from the normal IFE, we stained the sections with the following markers: K5, which labels the basal cells and basaloid proliferations; Sox9, a BCC and an ORS marker not found in the normal IFE (17, 18); P-cadherin, a secondary hair germ marker also expressed in BCC-like lesions (19); cytoke
6 (K6), which marks the inner root sheath (IRS) and hyperproliferative areas during wound healing and in other dermal pathologies (20); and K67, which labels proliferative cells.

In the first stage of tumor formation, small buds consisting of 2–3 layers of K5-expressing basal cells appeared, which could be distinguished from the surrounding IFE by positive staining for Sox9 and P-cadherin and were negative for K6 (Fig. 1A and Fig. S1B), indicating a characteristic ORS-like differentiation pattern (21). When the lesions progressed to stage 2, they became clearly distinguishable from the IFE while retaining the characteristic marker expression pattern as seen in stage 1 (Fig. 1D and G). At this stage the upper cell layer of the IFE starts to express K6 due to overall epidermal hyperproliferation induced by GLI1; nevertheless, the tips of the lesions remain K6 negative. The absence of K6 expression in these areas may facilitate proliferation and migration of tumor cells, as it has been shown that K6-negative mouse keratinocytes are less rigid (22). Stage 3 lesions penetrated deeper into the dermis and began to exhibit a branched phenotype (Fig. 1 E and H). More advanced lesions, which we classified as stage 4, appeared to originate both from the IFE and from the HF and consisted of keratinocyte strands invading the epidermis and occasionally anastomosing with each other (Fig. 1F and Fig. S1C). In rare cases the lesions resembled human fibroepithelioma and nodular BCC (Fig. S1D).

Second, we generated K5Cre*PR1/Ptch1fl/fl mice, which express the Cre recombinase/progesterone receptor fusion protein under the control of the K5 promoter to allow homozygous inactivation of Ptch1 (23). In K5Cre*PR1/Ptch1fl/fl mice the recombination and subsequent activation of the Hh pathway was induced by RU486 administration (Fig. 1I and SI Materials and Methods). It has been shown previously that biallelic deletion of Ptch1 in mouse epidermis results in formation of lesions closely resembling human BCCs (24, 25). Deletion of Ptch1 in the basal compartment resulted in the formation of K5-expressing basaloid proliferations in dorsal skin originating both from HFs and IFE following a largely similar timing of appearance as in K5tTA/TREGLI1 mice, although with a different preference of cellular location during the initial stages. In stage 1, basaloid proliferations were only observed in association with HFs (Fig. 1J and M). In stage 2, early lesions in addition appeared in the IFE, and stage 3 and 4 lesions (described in ref. 25) were observed on the ears (Fig. 1L) but not in the dorsal skin at the time of sacrifice. The lesions present in the K5Cre*PR1/Ptch1fl/fl epidermis also expressed Sox9 and P-cadherin, and were negative for K6, similarly to the BCC-like lesions arising in the GLI1-overexpressing epidermis (Fig. 1M).

Wounding Promotes the Formation of BCC-Like Lesions. To study the effect of wounding on the formation of BCC-like lesions, full-thickness excisional wounds (3 mm in diameter) were introduced to the dorsal skin of K5tTA/TREGLI1 and K5Cre*PR1/Ptch1fl/fl mice (SI Materials and Methods). In K5tTA/TREGLI1 mice the induction of GLI1 expression was initiated at P16. The wounds were created at the time when the hGLI1 transgene expression was first detected at the protein level (Fig. S1A, and the mice were killed when small IFE-associated lesions corresponding to stage 1 and 2 were present in the unwounded dorsal skin (Fig. 2A). In contrast to unwounded skin, the wound area displayed branching stage 3 lesions, which were associated with the HF openings and IFE (Fig. 2B). In the absence of GLI1 transgene expression, no lesions developed in the wound and neighboring areas (Fig. 2C), and wounding before Hh pathway activation had no significant effect on tumor formation (Fig. S2). We found a significant threefold increase in the size of the lesions in the wound area compared with unwounded epidermis; however, no increase in the overall number of proliferations was seen (Fig. 2D). The proliferations that emerged in the wound area exhibited the typical marker gene expression pattern of BCC-like lesions (Fig. 2E) and were distinguished from normal wound epithelium by Sox9 expression (Fig. S3).

The K5Cre*PR1/Ptch1fl/fl and K5Cre*PR1/Ptch1fl/+ mice were injected four times with RU486, and full-thickness excisional wounds were created (SI Materials and Methods). The mice were killed at late stage 1, when HF- and the first small IFE-associated basaloid lesions emerged in the unwounded epidermis of mice with homozygous deletion of the Ptch1 gene (Fig. 2F). The wound area of the K5Cre*PR1/Ptch1fl/+ epidermis contained an increased number of basaloid proliferations corresponding to stage 2, which exhibited the characteristic pattern of K5, Sox9, P-cadherin, K6, and K67 expression (Fig. 2G and J). Analysis of the lesions revealed a 2.4-fold increase in number and a 2.8-fold
increase in the average size of the lesions in the wound area compared with the unwounded epidermis. This observation suggests that, in addition to promoting tumor growth, the wound environment also increases the initiation frequency of BCC-like lesions in this model (Fig. 2I). No proliferations developed in the wound areas of the K5Cre*PR1/Ptc1flfl mice (Fig. 2H).

**Keratinocytes from the Bulge and the Nonpermanent Part of the HF Contribute to Tumor Formation in the IFE only in the Wound Area.**

Because cells originating from the HF actively participate in the wound healing process, we hypothesized that HF keratinocytes might contribute to the enhancement of tumor growth caused by the wound environment. To verify this we used the previously described lineage tracing approach based on Lgr5-EGFP-IRES-creER<sup>T2</sup>/R26R mice (5, 26). Upon activation of Cre by tamoxifen, which induces recombination in the R26R locus, the Lgr5+ cells and their progeny residing in the bulge and the nonpermanent part of the HF, are marked by permanent expression of LacZ.

Lgr5+ cells were labeled at P14, full-thickness wounds were introduced in the dorsal skin at the age of 5 wk and mice were killed 2 wk later (Fig. 3A). In unwounded skin the HF below the sebaceous gland opening was fully labeled (Fig. 3B). In the HFs situated close to the wound edges, labeled cells migrated out of the HFs and repopulated the infundibular area as expected (Fig. 3C). Labeled keratinocytes also integrated into the basal layer of the newly formed wound epidermis (Fig. 3D). We did not detect activity of the Lgr5 promoter during the wound-healing process in any cellular location where it is not normally expressed (Fig. S3 C and F), supporting the HF origin of labeled keratinocytes.

To evaluate the participation of Lgr5+ progeny in the formation of the BCC-like lesions, we generated quadruple transgenic mice by crossing Lgr5-EGFP-IRES-creER<sup>T2</sup>/R26R and K5tat/ TREGLI1 mice. To ensure that the Lgr5+ cells were labeled before the GLI1 transgene expression started, tamoxifen was injected at P14 and the doxycycline was removed at P16 (Fig. 3E). Activation of Hh signaling led to formation of BCC-like lesions as in the K5tat/TREGLI1 mice (Fig. 3H). At 5 wk of age, when early lesions corresponding to stage 1 were detected in the IFE, Lgr5+ progeny was present below the sebaceous gland opening in the normal unwounded skin (Fig. 3F). By 6 wk of age, the Lgr5+ progeny was present above the sebaceous gland opening, extending into the infundibulum of the HF, resembling the tracing pattern of HFs close to a normal wound (Fig. 3G, compare with Fig. 3C). At 7 wk of age, when stage 2 and stage 3 basaloid proliferations had developed in the epidermis, the Lgr5+ progeny was present in the infundibulum and HF opening, and moreover found to be incorporated into the dermis. However, LacZ-positive traced cells were not detected in the IFE-associated BCC-like lesions, illustrating the inability of HF-derived keratinocytes, originating from the bulge and the nonpermanent part, to integrate into the IFE-associated proliferations (Fig. 3H).

Note that the HF-associated lesions, which also develop in the K5tat/TREGLI1 mice, originate from Lgr5+ cells (Fig. S4).

Next, we introduced wounds in the dorsal skin of 5-wk-old K5tat/TREGLI1/Lgr5-EGFP-IRES-creER<sup>T2</sup>/R26R mice, treated with doxycycline and tamoxifen as described (Fig. 3F). As in normal skin, the Lgr5+ progeny was able to integrate into the basal layer of the newly formed wound epidermis (Fig. 3I). Remarkably, LacZ-labeled cells representing progeny of Lgr5+ cells also contributed to the IFE-associated Sox9- and GLI1-expressing basaloid proliferations developing in the wound epidermis (Fig. 3K and L and Fig. S4F), showing that the wound microenvironment allows recruitment of cells of follicular origin into the forming IFE-associated tumors.

**HF Keratinocytes with Activated Hh Signaling Initiate Tumor Formation in the Infundibulum and IFE upon Wounding.** The K5 promoter used in the previous experiments to direct Hh pathway activation is active throughout the basal compartment of the epidermis, including the newly formed wound epithelium. Therefore, it is possible that the cells originating from the bulge and the nonpermanent part of HF were attracted by the wound environment contributing to the forming tumors but have no autonomous tumor initiation ability. To test this possibility, we generated Lgr5-EGFP-IRES-creER<sup>T2</sup>/Ptc1flfi mice in which the Hh pathway was activated only in the Lgr5-expressing cells and their progeny. These mice were treated with tamoxifen so that high recombination efficiency could be achieved before the onset of the first anagen (similarly to the K5cre*PR1/Ptc1flfi; SI Materials and Methods), and excisional full-thickness wounds were created at 5 wk (the same timing as for the K5tat/TREGLI1/Lgr5-EGFP-
In unwounded skin, conditional inactivation of Pch1 in the Lgr5-expressing cells resulted in formation of HF-associated BCC-like lesions, whereas the skin of Lgr5-EGFP-ires-creERT2/+/Pch1fl/fl mice showed a normal histological appearance (Fig. 4A, B, and D). The morphology of the lesions ranged from prevalent simple nodular basaloid proliferations surrounded by a layer of palisading cells in the dorsal skin (Fig. 4B and C), to less frequent, more advanced multinodular and branched BCC-like lesions in the ventral skin, confirming that Lgr5+ cells can act as cells of origin for BCC-like lesions (Fig. S5). Subsequent wounding experiments were performed on the dorsal skin, where no IFE-associate lesions were observed in unwounded skin (Fig. 4B and C). Intriguingly, in the wound areas, multiple lesions developed, which were associated with infundibula of the neighboring HFs and wound IFE (Fig. 4E and F). The lesions were morphologically similar to the basaloid proliferations seen in the IFE of K5tTA/TREGLI1 and K5Cre*PR1/Pch1fl/fl mice, because they showed the characteristic expression pattern of K6, Sox9, and P-cadherin, and contained relatively high numbers of Ki67-positive cells (Fig. 4G). This shows that tumor-initiating cells can migrate from the bulge and the nonpermanent part of the HF into the permanent part of the HF and to the IFE during wound healing and contribute to tumor formation also at this location.

**Discussion**

Though chronic wounds are a well-recognized risk factor for cancer formation, little is known about the molecular and cellular mechanisms underlying this effect. Although several case reports depicting the association between BCC and skin injury have been published, to our knowledge there exist only single studies addressing this association (10, 27). The authors point out that both chronic and acute trauma should be considered as etiologic factors for BCC. The possibility that an engagement of cells with high proliferative capacity or tumorigenic potential originating from neighboring areas of the tissue might be an important component of the mechanism by which a wound environment promotes tumor formation is an interesting open question addressed in this study. Using three different transgenic mouse models for human BCC, we show that a wound environment can enhance the development of BCC via two distinct mechanisms: promotion of tumor growth and enhancement of tumor initiation frequency. In the first model, the K5tTA/TREGLI1 mice, wounding does not increase the tumor initiation frequency, whereas in the K5Cre*PR1/Pch1fl/fl mice, the number of early proliferations in unwounded areas is considerably lower, the wound increases not only the size of the tumors but also their number. Our hypothesis is that in the K5tTA/TREGLI1 epidermis, more...
cells, if not all K5+ basal cells in the IFE, which are competent to accumulate GL11, do express GL11. Wounding in this setting promotes tumor growth but not an increase in the number of lesions. In the case of the K5Cre×PR1/K5Cre×PR1 mice, not all cells in the K5+ compartment are subjected to Pch1 deletion due to the relative inefficiency of Cre activation, which results in a smaller number of cells having an active Hh pathway. Hence, in smaller numbers of cells carrying a homozygous deletion of Pch1 and thereby significantly increases the number of lesions in the wound area, and as shown in Lgr5-EGFP-ires-CreERT2/Pch1fl/fl control mice are devoid of basaloid lesions. (E and F) The wound areas of mice shown in Fig. 4 B and C contain lesions associated with the infundibulum of the HFs as well as IFE-associated lesions. (G) Immunostaining of marker genes identifies the infundibular- and IFE-associated lesions as basaloid lesions. Arrowheads show early infundibular and IFE-associated BCC-like lesions. (A–F) H&E staining. (C Right and G) Hematoxylin counterstain. (Scale bars: 100 μm.)

cells is not yet known. An intriguing possibility is that the tumor-initiating cells in the IFE may correspond to the Lgr6+ SCs, which give rise to the SG and the IFE during skin homeostasis (3).

Cancer development and wound healing share several common features, which has led to the view that “tumors are wounds that do not heal” (31). Indeed, the lineage-tracing experiments in K5/TREGL11 mice show that oncogenic signaling in the epidermis also induces emigration of cells from the bulge and the nonpermanent part of the HF analogously to wound healing; however, these cells were unable to integrate into the HF infundibula or IFE and instead underwent terminal differentiation. This indicates that, although Hh pathway activation induces cell migration, it does not provide a receptive microenvironment allowing homing of cells to the IFE, unlike the situation in a healing wound. Evidence that the homing ability of a cell is largely dependent on the host environment comes from our experiments using Lgr5-EGFP-ires-CreERT2/Pch1fl/fl mice where keratinocytes with an activated Hh pathway were able to migrate out of the nonpermanent part of HFs and resettle at HF infundibula and in the wound IFE, thereby initiating new BCC-like lesions. Our finding that wounding causes enhanced formation of BCC-like lesions by redirecting the fate of hair follicle progeny in the context of activated Hh signaling provides an explanation for the

Fig. 4. Lgr5+ keratinocytes represent tumor-initiating cells for HF-associated lesions and for IFE-associated lesions after wounding. (A) Tamoxifen-injected Lgr5-EGFP-IRES-creER<sup>ERT2</sup>/Pch1<sup>fl/fl</sup> control mice show no phenotype in unwounded skin. (B) Basaloid proliferations in unwounded dorsal skin of 2-mo-old Lgr5-EGFP-IRES-creER<sup>ERT2</sup>/Pch1<sup>fl/fl</sup> mouse. (Left) H&E staining. (Right) K6 immunostaining. (D) Dorsal wound areas of tamoxifen-injected Lgr5-EGFP-IRES-creER<sup>ERT2</sup>/Pch1<sup>fl/fl</sup> control mice are devoid of basaloid lesions. (E and F) The wound areas of mice shown in Fig. 4 B and C contain lesions associated with the infundibulum of the HFs as well as IFE-associated lesions. (G) Immunostaining of marker genes identifies the infundibular- and IFE-associated lesions as basaloid lesions. Arrowheads show early infundibular and IFE-associated BCC-like lesions. (A–F) H&E staining. (C Right and G) Hematoxylin counterstain. (Scale bars: 100 μm.)

Fig. 5. Full-thickness cutaneous wounding is required to induce HF keratinocyte migration to the IFE. (A) Lgr5+ lineage tracing in untreated Lgr5-EGFP-ires-CreERT2/R26R mice. (B–F) Tamoxifen was administered at P14; trauma was introduced on dorsal telogen skin. The samples were collected 7 d after trauma (B–E) and 14 d after repeated TPA treatment (F). (B) Superficial incisions induced K6 expression in the regenerating IFE but did not induce keratinocyte migration from the HF. (C) Hair plucking induced anagen in the affected HFs without inducing keratinocyte migration into the IFE. (D and E) Full-thickness incisions and excisional wounds induced integration of cells originating from the bulge and the nonpermanent part of the HF into the IFE. The incisional wound areas were identified by K6 staining in the IFE and the damaged muscle layer (arrows). (F) Repeated TPA treatment twice a week for 2 wk induced a hyperproliferative state in the IFE and emigration of Lgr5+ progeny into the IFE. (Inset) Non–TPA-treated skin of the same animal. (A–F) LacZ staining, K6 immunostaining, hematoxylin counterstain. (Scale bars: 100 μm.)
association between tissue injury from excessive UV radiation exposure (32) or chronic ulceration (33) with BCC development. In these situations an increased mutational load is the result of inflammation generating reactive oxygen species or infliction of direct physical DNA damage (9, 34, 35). In line with this scenario we found that TPA treatment can stimulate migration of Lgr5+ progeny to the IFE. TPA treatment induces a strong inflammatory and hyperproliferative response in mouse skin and is typically used for epidermal tumor promotion, and TPA has been shown to be as effective as deep skin wounding in promoting papilloma formation in mice (36). Although treatment with TPA can provide the important signals responsible for the migration of the potential tumor initiating cells, the key signals for HF cell migration into the wound are as of yet unclear. Our findings suggest that molecular events during the initial phase of acute wound healing are crucial, because wounding preceding Hh pathway activation fails to promote tumor development. Moreover, only full-thickness wounds induce the migration of cells from neighboring HFs to the place of injury. The molecular mechanism underlying both the attraction and homing of potential tumor-initiating cells or cells contributing to tumor formation remains an important topic for future studies and is likely to depend on the specific cytokine and growth factor milieu and involve alteration of epigenetic patterns (37).

Materials and Methods

Mice. Lgr5-EGFP-IRES-creER

(22)(R26R, K5tTA/TREGLI1, K5tTA/TREGLI1/Lgr5-EGFP-IRES-creER

(22)R26R, K5Cre*PR1/Ptch1

(19), and Lgr5-EGFP-IRES-creER

(22)Ptch1

(19) mice were generated by interbreeding mice carrying the following alleles: Lgr5-EGFP-IRES-creER

(22) (26), R26R (mice were obtained from Jackson Laboratory), K5T(A14), TREGLI1 (18), K5Cre*PR1 (23), and Ptch1

(19)Ptch1

(19) (a kind gift from S. Teglund, Karolinska Institutet).

Hedgehog Pathway Activation. The GLI1 transgene expression in mice carrying a K5T and a TREGLI1 allele was induced by doxycycline removal from the drinking water (2 mg mL–1 doxycycline, 5% sucrose) at P16. The deletion of the Ptch1 gene of mice carrying K5Cre*PR1 or Lgr5-EGFP-IRES-creER

(22) and Ptch1

(19) alleles was induced by i.p. injection of either 4 mg RU486 or 4 mg of tamoxifen (SI Materials and Methods).

Lineage Tracing. Mice aged 14 d (P14) were injected i.p. with 2 mg of tamoxifen (20 mg/mL in sunflower oil).

Wounding and TPA Treatment. Full-thickness wounds, 3 mm in diameter, were created on the back skin of mice. Superficial incisions, hair plucking, and TPA treatment were performed on telogen mouse skin as described in SI Materials and Methods. All animal experiments were performed according to the regulations of Sweden.

La2C Analysis. To determine the pattern of recombination at the Rosa26-LacZ(R26R) reporter locus, fresh dorsal (if not stated otherwise) skin tissue fixation of GLI1 in the mammary gland disrupts pregnancy-induced maturation and causes lactation failure. J Biol Chem 277:5187-5192.

Immunohistochemistry. Freshly obtained skin samples were fixed in 4% formaldehyde and embedded in paraffin. Staining with antibodies recognizing K5, K6, GLI1, K67, Sox9, P-cadherin, CD34, or EGFP was performed as described in SI Materials and Methods.

Quantification of Tumor Formation. Tumors were identified by Sox9 expression, and wound areas were defined by the absence of underlying panniculus carnosus and by increased expression of K6. Partly overlapping images spanning 1-cm-wide skin samples were merged into a panorama, and the number and area of the identified tumors were measured using ImageJ software (National Institutes of Health, USA) as described in SI Materials and Methods.

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(19) mice. D. Roop for providing K5Cre*PR1 mice and unpublished data concerning K5Cre*PR1/R26-LacZ mice, and B. Rozell and H. Clevers for constructive comments on the manuscript. Support for this work was provided by the Swedish Research Council (R.T.), a Marie-Curie Intra-European Fellowship (to V.J.C.), and a fellowship from the Wenner-Gren Foundation (to M.K.).

4. Jensen KB, et al. (2009) Lrig1 expression de...
Supporting Information

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SI Materials and Methods

Mice. K5tTA/TREGLI1 mice. GLI1 expression was suppressed throughout the embryonic development and postnatally until P16 by adding doxycycline (2 g/L in 5% sucrose) to the drinking water. Skin samples were collected at time points according to the predefined stages (see main text). Mice were wounded in the first anagen (P27–P33), when transgene expression at the protein level could be detected (Fig. S1A), and killed when stage 2 proliferations were detected in unwounded skin (n = 3). TREGLI1 control mice were treated identically.

K5Cre mice. Lgr5-EGFP-IREs-creERT2/fl mice. To induce Cre activity, 1 mg of a progesterone analog RU486 (10 mg/mL in sunflower oil) was injected i.p. four times during the first (P18–P24) telogen. Wounds were made simultaneously with the last injection. To achieve a similar timing with respect to Hh pathway activation, wounds were created at an earlier time point compared with K5tTA/TREGLI1 mice, because the K5tTA/TREGLI1 mice have a delay in GLI1 expression at the protein level compared with K5Cre/fl mice (Fig. S1B). Mice were killed when stage 2 proliferations were detected in unwounded skin (n = 3). K5Cre*PPI/Pch1+/- control mice were treated identically.

Lgr5-EGFP-IREs-creERT2/fl mice. To induce Cre activity mice were injected with 1 mg of the estrogen analog tamoxifen (20 mg/mL in sunflower oil) every second day between P14 and P20 (four injections). Wounds were introduced at 5 wk (P35), and mice were killed at 8 wk (n = 3; Fig. 4A, B, D, and E) when the overall health condition of the mice declined. The wounding time point was chosen to coincide with the wounding in quadruple-transgenic mice (Fig. 3I–L). Injection with a low dose of tamoxifen (1 mg, once at P13) resulted in longer survival (4 mo) of the experimental animal and appearance of advanced BCC-like lesions in the skin (n = 1) (Fig. 4C, F, and G).

For the K5Cre*PPI and Lgr5-EGFP-IREs-creERT2 models, the RU486 or tamoxifen administration schemes were based on previous experience to achieve efficient recombination and broad pathway activation.

The mice were fed ad libitum and housed at the animal facility of Karolinska Institutet. All animal experiments were performed according to the regulations of Sweden.

Animal Treatments. All procedures were performed on dorsal skin. Excisional wounding was performed by removing a full-thickness skin piece, 3 mm in diameter, with sharp scissors. Incisional wounding consisted of a single 3-mm-long full-thickness incision performed with scissors (n = 3). Superficial incisions were performed on shaved skin using a scalpel (n = 3). TPA was dissolved in acetone at 10 μg/mL, and 100 μL of TPA solution was administered topically onto shaved skin twice a week (day 1, day 4, day 8, and day 11). The skin samples were collected 7 d after wounding or 14 d after the start of the TPA treatment (n = 3). All experiments were performed during telogen except for full-thickness wounding, for which the specific time points are given in the figures or in the previous paragraph.

LacZ (β-Galactosidase) Staining Protocol. Freshly obtained skin tissue was fixed (2% paraformaldehyde, 0.2% glutaraldehyde in PBS) for 30 min at room temperature (RT). Tissues were washed three times for 15 min with rinse buffer (2 mM MgCl2, 0.01% Nonidet P-40 in PBS). Subsequently, the β-galactosidase substrate solution [1 mg mL−1 X-Gal, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 0.3H2O in rinse buffer] was added and the tissues were incubated for 36–40 h at 37 °C in the dark. The substrate was removed, and the tissues were washed twice in PBS for 10 min and kept in 70% ethanol until embedding (maximum 48 h). The stained tissues were processed into paraffin blocks according to standard procedures. Tissue sections (4 μm) were prepared and counterstained with eosin or H&E, or subjected to immunohistochemistry.

Immunohistochemistry. The skin tissue samples were fixed overnight in 4% FA (4% formaldehyde in PBS) at RT. The FA solution was removed and the tissues were washed twice in PBS for 10 min, then embedded in paraffin using standard methods and sectioned (4 μm). The sections were dewaxed by immersion in xylene (2 × 15 min), 100% EtOH (2 × 5 min), 95% EtOH (1 × 5 min), 70% EtOH (1 × 5 min), and distilled water (1 × 5 min) followed by antigen retrieval in 10 mM citrate buffer or Diva Decloaker (Biocare Medical; 20 min, 97 °C) as detailed below. The slides were allowed to cool in the antigen retrieval solution for 20 min at RT and washed in TBS (3 × 5 min). Sections were blocked in 4% normal serum of rabbit or goat, depending on the origin of the secondary antibody, for 30 min at RT and then incubated with the primary antibody (diluted in the respective blocking solution) as detailed below. Subsequently, sections were washed in TBS (3 × 5 min) and incubated with biotinylated secondary antibody (Vector Laboratories; diluted 1:200 in the corresponding blocking solution for 30 min at RT followed by wash in TBS (3 × 5 min)). The sections were incubated with the HRP-streptavidin conjugate (Zymed) and the signal was detected using the DAB Plus Substrate Kit (Zymed) according to the manufacturer’s instructions. Counterstaining was performed with Mayer’s hematoxylin for 30 s followed by rinsing with tap water and incubation for 1 min in PBS. Sections were dehydrated by serial immersion for 1 min each in 70% EtOH, 95% EtOH, 2 × 100% EtOH, and xylene (2 × 5 min). The slides were mounted in Pertex mounting medium.

Primary antibodies (provider/ordering number, dilution, incubation time, antigen retrieval): rabbit anti–β-catenin (Epitomics; 1:2477-1, 1:3,000, 1 h RT, 10 mM citrate), rabbit anti-EGFP (Cell Signaling; D5:1, 1:100, overnight at 4 °C, Diva Decloaker), rabbit anti-CDCP (Santa Cruz; sc-13024, 1:100, 1 h RT, Diva Decloaker), rabbit anti-K5 (Covance; PRB-160P, 1:1,000, overnight at 4 °C, 10 mM citrate), rabbit anti-K6 (Covance; PRB-169P, 1:1,000, overnight at 4 °C, 10 mM citrate), rabbit anti-Ki67 (Novocasta; NCL-Ki67p, 1:2,000, overnight at 4 °C, Diva Decloaker), rabbit anti-Sox9 (Millipore; ab5535, 1:200, overnight at 4 °C, Diva Decloaker); rat anti-CD34 (eBioscience; 51-0531, 1:100, overnight at 4 °C, Diva Decloaker); goat anti-human GLI1 (RnD Systems; AF3324, 1:100, 1 h RT, 10 mM citrate).

Quantification of Tumor Formation. Panorama images spanning the entire width of the sample were obtained from Sox9- and K6-stained consecutive sections of wounded and unwounded skin. The wound areas were defined by the absence of the muscle layer and/or by intense K6 staining. Next, the Sox9-expressing lesions were identified in the defined regions of interest, and their number was counted and tumor areas measured using ImageJ software. Finally, the average area per lesion was calculated by dividing the number of individual lesions by the length of the region of interest. For each genotype, the results of three independent experiments were averaged and a two-tailed t test performed.

Fig. S1. Different stages of tumor progression in K5tTA/TREGLI1 mice. (A) The doxycycline-off system gives a delay in GLI1 protein accumulation. At 3 d after doxycycline withdrawal, no GLI1 protein expression was detected. The DAB developing time was prolonged to be able to pick up low GLI1 expression, resulting in overall background staining (asterisks). At 4 wk (11 d after doxycycline removal), the GLI1 protein was detected in a few cells in the IFE. Stage 1 proliferations consist of cells with variable levels of GLI1 expression. (B and C) Expression pattern of K5, Sox9, P-cadherin, K6, and Ki67 in early (stage 1) and advanced (stage 4) lesions. Basaloid proliferations retain their positive staining for K5 and Sox9 and heterogeneous staining for the proliferation marker Ki67. P-cadherin is initially expressed in the cells having direct contact with the dermis. In advanced stage 4 lesions, P-cadherin expression is retained at the tips of the proliferations. The overlaying IFE of early proliferations does not contain K6-expressing cells. During later stages, K6 is expressed in the overlaying IFE and in the areas of the lesions adjacent to the IFE. The K6-positive areas generally show no or little P-cadherin expression and vice versa. (D) Examples of advanced basaoid proliferations at stage 4 obtained from K5tTA/TREGLI1 mice. (A–C) Hematoxylin counterstain. (D) H&E staining. (Scale bars: 100 μm.)

Fig. S2. Wounding before Hh pathway activation does not significantly accelerate BCC development. (A) Experimental timeline for K5tTA/TREGLI1 mice. (B–D) Wounds were made at two different time points on the dorsal skin of the same animal. (B) Unwounded skin of the same animal. (C) A 3-mm wound was made at P20 and analyzed at 6 wk. (D) A 3-mm wound was made at 4.5 wk and analyzed at 6 wk. Only the latter wound, made after Hh pathway activation (Fig. S1A), resulted in enhancement of tumor formation compared with unwounded skin. (B–D) H&E staining. (Scale bars: 100 μm.)
Fig. S3. Expression of K6, Sox9, and Lgr5 (EGFP) in wound epithelium and HFs. A 10-d-old wound in an Lgr5-EGFP-IRES-creERT2 mouse (A–C) compared with unwounded skin (D–F). (A and D) The wound area is strongly K6-positive, whereas in unwounded skin, K6 stains only the HF. (B and E) Sox9 is expressed in the HFs but not in the wound epidermis. (C and F) The Lgr5 promoter is not active in cells residing in the wound areas as shown by the absence of Lgr5 promoter-driven EGFP expression, but is detected in the bulge and SHG of the HF only. (A–F) Hematoxylin counterstain. Asterisks indicated unspecific staining in the keratinized upper layer of the epidermis. (Scale bars: 100 μm.)

Fig. S4. Lgr5+ progeny contribute to HF-associated lesions in unwounded skin but contribute to IFE-associated lesions only upon wounding. (A) Experimental scheme of tumor induction and lineage tracing in K5tTA/TREGLI1/Lgr5-EGFP-IRES-creERT2/R26R mice. (B–D) K5tTA/TREGLI1/Lgr5-EGFP-IRES-creERT2/R26R mouse skin develops HF- and IFE-associated lesions. In unwounded skin only, HF-associated lesions have Lgr5+ cells as a cell of origin. (E) Experimental timeline for wounding of K5tTA/TREGLI1/Lgr5-EGFP-IRES-creERT2/R26R mice. (F) Lgr5+ progeny constituting early basaloid lesions include GLI1-positive cells, demonstrating the presence of an active Hh signaling pathway. (B) H&E staining, dorsal skin. (C and D) LacZ and eosin staining of dorsal (C) and ventral (D) skin. (F) Hematoxylin counterstain. Arrowhead indicates HF opening. (Scale bars: B–D, 100 μm; F, 50 μm.)

Fig. S5. Advanced BCC-like lesions derived from Lgr5+ cells. Examples of BCC-like lesions on the ventral skin of a 4-mo-old Lgr5-EGFP-IRES-creERT2/Ptchfl/fl mouse given 1 mg of tamoxifen at P13. (Scale bars: 100 μm.)
Fig. S6. Homozygous deletion of Ptch1 induces basaloid proliferations with SHG identity. (A–C) Consecutive sections of unwounded Lgr5-EGFP-IRES-creER<sup>T2</sup>/Ptch<sup>fl/fl</sup> mouse skin stained with antibodies recognizing EGFP (Lgr5+) and the bulge SC marker CD34. The cells present in the HF-associated proliferations have characteristics similar to Lgr5+ cells in the SHG (Lgr5+/CD34−); expression of the bulge area SC marker CD34 is unaffected. Arrows indicate CD34+ bulge cells; in C, bulge of a neighboring HF. (A and B) Telogen skin. (C) Late catagen skin. (A–C) Hematoxylin counterstain. (Scale bars: 100 μm.)