**Lgr6 is a stem cell marker in mouse skin squamous cell carcinoma**

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The G-protein-coupled receptors LGR4, LGR5 and LGR6 are Wnt signaling mediators, but their functions in squamous cell carcinoma (SCC) are unclear. Using lineage tracing in Lgr5-EGFP-CreERT2/Rosa26-Tomato and Lgr6-EGFP-CreERT2/Rosa26-Tomato reporter mice, we demonstrate that Lgr6, but not Lgr5, acts as an epithelial stem cell marker in SCCs *in vivo*. We identify, by single-molecule *in situ* hybridization and cell sorting, rare cells positive for Lgr6 expression in immortalized keratinocytes and show that their frequency increases in advanced SCCs. Lgr6 expression is enriched in cells with stem cell characteristics, and Lgr6 downregulation *in vivo* causes increased epidermal proliferation with expanded lineage tracing from epidermal stem cells positive for Lgr6 expression. Surprisingly, mice with germine knockout of Lgr6 are predisposed to SCC development, through a mechanism that includes compensatory upregulation of Lgr5. These data provide a model for human patients with germline loss-of-function mutations in Wnt pathway genes, including RSP01 or LGR4, who show increased susceptibility to squamous tumor development.

The mouse skin hair follicle comprises several distinct stem cell populations. Expression of *Lrig1* (refs 3, 4) defines a cell population in the hair follicle functional zone that serves to maintain the hair follicle infundibulum, while quiescent stem cells with *Krt15* expression (Krt15+) the bulge region act as a reserve stem cell pool that becomes activated in response to stress. *Lgr4*, *Lgr5* and *Lgr6* are expressed in stem cells in many tissues, including the small intestine, breast, ovary and hematopoietic system. In the skin, *Lgr5* has been genetically linked to a network of genes that are expressed specifically in the hair follicle, while *Lgr6* is expressed in the isthmus region, sebaceous gland and interfollicular epidermis. The LGR receptors bind R-spondins (RSPO) to enhance Wnt/β-catenin signaling. Wnt signaling is known to be activated in the hair germ and hair follicle bulge during the transition from telogen to anagen phase and is critical for the formation of new follicles. However, several reports have also indicated a negative role for Lgr5 in Wnt signaling, including in human colorectal cancers. Moreover, loss-of-function mutations in human RSP01 (ref 28) and LGR4 (ref 29) increase susceptibility to skin squamous carcinoma development and LGR6 has been implicated both as a potential tumor-suppressor gene and a breast cancer germline susceptibility gene. Thus, the exact roles of LGR receptors in Wnt signaling and tumorigenesis appear to vary depending on the specific LGR and RSPO family members that are expressed and interact in a given cellular context.

The relationships between normal tissue stem cells and ‘cancer stem cells’ (CSCs; also known as ‘tumor-initiating cells’) are controversial and unresolved. Lgr5 has been reported to be a marker of both normal stem cells and CSCs in intestinal adenoma and in gastric cancer, but it remains unclear whether members of this gene family are expressed and, in particular, have a functional role in CSCs in other tumor types including cutaneous SCC.

Here we identify a specific role for Lgr6 as a cutaneous CSC marker. Expression of Lgr6, but not Lgr5, increases during tumor progression and metastasis, while lineage tracing in established tumors shows that only the progeny of Lgr6+ cells form distinct epithelial colonies within SCCs. Nevertheless, germine deletion of Lgr6 predisposes mice to development of SCCs. These data underline the parallels between this mouse model and human patients with germline loss of genes in this pathway, including RSP01 and LGR4, who also have an increased propensity for SCC development.

**RESULTS**

*Lgr6 is a stem cell marker in squamous carcinoma*

Mouse skin SCCs can be initiated with a single dose of dimethylbenzanthracene (DMBA) and promoted by repeated treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA). Tumors from this model have shown increased susceptibility to squamous tumor development.

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and matched metastatic tumors from an interspecific FVBBX backcross population. While Lgr6 expression continued to increase during progression, Lgr5 expression showed a decrease, suggesting that Lgr5 may not be required for tumor maintenance (Fig. 1a).

We investigated the localized expression of Lgr5 and Lgr6 in SCCs from mice carrying an EGFP reporter gene under the control of the Lgr5 or Lgr6 promoter (Lgr5-EGFP-CreERT2 and Lgr6-EGFP-CreERT2 mice). Staining of tumors using antibodies against GFP (expression from the Lgr5 or Lgr6 promoter; green) or keratin 14 (KRT14; red) (Fig. 1b–e and Supplementary Fig. 1) showed that Lgr6 (Fig. 1c) but not Lgr5 (Fig. 1c) was expressed in distinct colonies of cells distributed throughout the SCCs. Patterns of co-staining for GFP and KRT14 suggest that, while the vast majority of Lgr6+ cells are in the epithelial compartment, the average expression levels of KRT14 in these cells are relatively low as compared to those in adjacent, more differentiated cells (Supplementary Fig. 1, inset). These data are thus compatible with the hypothesis that Lgr6 may act as a stem cell marker in squamous carcinomas, whereas Lgr5 does not.

Lgr5+ cells have been reported to be capable of acting as the cells of origin of intestinal and gastric adenomas, cutaneous basal cell carcinomas (BCCs) and SCCs. Schepers et al. exploited the Lgr5-EGFP-CreERT2/+ targeted knock-in mouse to activate Wnt signaling in Lgr5+ crypt stem cells and showed that the resulting adenomas continued to express Lgr5. We asked whether induction of Ras gene mutations within Lgr5+ stem cells in the hair follicle gives rise to SCCs that continue to express Lgr5 or if it is dispensable for tumor maintenance. We crossed Lgr5-EGFP-CreERT2/+ or Lgr6-EGFP-CreERT2+/- mice with Kras<sup>SL+G12D</sup> mice carrying a floxed (loxP-flanked) mutant Kras allele. Activation of oncopgenic Kras with topical 4-hydroxytamoxifen (4OHT) in Lgr5+ stem cells resulted in some limited sebaceous gland hyperplasia (Supplementary Fig. 2a), but, when the mice were further challenged with a back wound stimulus, papillomas readily arose within 4 weeks (Supplementary Fig. 2b). Similar observations were made after activation of Kras in the Lgr6+ stem cell population (data not shown). These results are in agreement with the observations of Lapouge et al. but contrast with the findings of another study, possibly owing to differences in the genetic background of the mice used in the different laboratories.

Surgical removal of papillomas in Lgr5-EGFP-CreERT2+/-/Kras<sup>SL+G12D</sup> mice led to progression to aggressive spindle carcinomas (Supplementary Fig. 2c). Lgr5 expression could be detected in the outer root sheath (ORS) of hair follicles in normal skin from Lgr5-EGFP-CreERT2+/-/Kras<sup>SL+G12D</sup> mice (Supplementary Fig. 2d) and in hair follicles close to the carcinomas, but not in the epithelial carcinoma cells (Supplementary Fig. 2e). TaqMan and immunofluorescence analyses of three cell lines independently derived from these spindle carcinomas showed an absence of Lgr5 expression (Supplementary Fig. 2f). We conclude that even tumors arising from Lgr5+ cells in the hair follicle fail to express Lgr5 during tumor progression and that Lgr5 is not a positive driver of SCC growth.

**Lineage tracing of Lgr6+ stem cells in squamous tumors**

CSCs can give rise to progeny within tumors that continue to fuel cancer development and progression. We carried out lineage tracing within chemically induced skin tumors from Lgr6-EGFP-CreERT2 or Lgr5-EGFP-CreERT2 mice crossed with Rosa26-LSL-Tomato reporter mice (hereafter Lgr6 and Lgr5 reporter mice, respectively). Early-stage papillomas were induced in both double-transgenic lines by standard DMBA treatment followed by 8 weeks of tumor promotion using TPA (Supplementary Fig. 3). At this stage, mice were treated

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**Figure 1**: Lgr6 expression increases with squamous tumor progression and Lgr6-GFP<sup>+</sup> cells, not Lgr5-GFP<sup>+</sup> cells, are localized within tumor epithelium. (a) Levels of Lgr5 and Lgr6 expression during tumor progression were analyzed in samples of normal skin, papillomas, primary carcinomas and matched metastatic tumors from an interspecific FVBBX backcross population. Lgr6 expression continues to increase through the benign, malignant carcinoma and metastasis stages, whereas Lgr5 expression shows a progressive decrease. Each box plot shows the median and 25th and 75th quartiles (interquartile range, IQR). Whiskers extend to the smallest and largest values, except for values more than 1.5 times the IQR, which are marked as outliers. Normal skin, n = 15; papillomas, n = 27; carcinomas, n = 25; metastases, n = 45; where n is the number of independent biological samples. (b–e) Localized expression of Lgr5-GFP and Lgr6-GFP was investigated within primary squamous carcinomas (at 25 weeks after initial TPA treatment) by immunostaining against GFP (green) and KRT14 (red) to identify cell populations specifically expressing stem cell and basal cell markers, respectively. Representative sections from squamous tumors demonstrate that Lgr6-GFP (arrows) (e), but not Lgr5-GFP (c), is clearly expressed in distinct colonies of cells distributed throughout the SCCs. (b, d) Hematoxylin and eosin staining of serial sections of the immunostaining depicted in c and e. The white dashed lines indicate the epithelial border indicated by KRT14 (red) expression. DAPI (blue) localizes cell nuclei. Scale bars, 50 µm.
Lgr6 reporter mice but not in Lgr5 reporter mice (Fig. 2a,b). The number of Tomato+ traced cells increased at 3 weeks and 6 weeks, with these cells giving rise to distinct clones that showed evidence of migration from the initial Lgr6+ cells (Fig. 2d,f). Some Tomato+ traced cells in Lgr6 reporter mice were also observed in the dermis/stromal compartment (Supplementary Fig. 4b,d), but these were only a minor population, the significance of which is unknown. In contrast, papillomas from Lgr5 reporter mice displayed very few Tomato+ traced cells, even after 6 weeks, and the majority of these were found in the dermal compartment (Fig. 2e,g and Supplementary Fig. 4a,c).

We also carried out lineage tracing from Lgr6+ cells in primary carcinomas that arose from 24–30 weeks after tumor initiation. At 2 d (Fig. 3a,b), 2 weeks (Fig. 3c,d) and 4 weeks (Fig. 3e,f) after tamoxifen treatment, there was a significant increase in Tomato+ cell numbers in comparison to papillomas. Lgr6-traced Tomato+ clones grew rapidly and occupied a major proportion of the carcinoma 4 weeks after Cre-mediated activation (Fig. 4e,f). Again, no significant level of tracing progeny cells was seen in the equivalent study of Lgr5 reporter mice (Supplementary Fig. 5a–d). Interestingly, Driessens et al. demonstrated that clonal populations arising in skin tumors from Krt14-CreER/Rosa26 reporter mice treated with tamoxifen showed a strong decrease in clone numbers over several weeks, but no such decrease was seen in clone number in our studies of serial biopsies of carcinomas from Lgr6-CreER/RosaYFP reporter mice (Fig. 3). This suggests that Krt14 initially labels many transit-amplifying cells with poor self-renewal capacity, whereas the Lgr6+ population is already enriched in stem cells that are capable of long-term self-renewal.

The transcription factor SOX2 is expressed in papillomas and squamous carcinomas and was reported to mark a stem cell population that is enriched in tumor-initiating cells. We investigated possible overlap between Sox2+ cells and Lgr6+ cells in SCCs from the DMBA and TPA model by staining for SOX2 in tumors from Lgr6-EGFP-CreERT2 mice. The data in Supplementary Figure 6 show that SOX2 is more widely expressed in a larger population of cells than Lgr6, with minimal overlap between the cells expressing these two distinct markers.

**Figure 2** Clonal expansion derived from Lgr6+ cells is observed in benign squamous tumors. Lgr5- and Lgr6-derived lineage tracing was performed within chemically induced skin tumors from Lgr6-EGFP-CreERT2 or Lgr5-EGFP-CreERT2 mice crossed with Rosa26-LSL-Tomato reporter mice by standard DMBA treatment followed by 8 weeks of tumor promotion using TPA. Mice were then treated with tamoxifen (TAM) and papillomas were collected at 2 d, 3 weeks and 6 weeks thereafter, to measure the extent of lineage tracing from either Lgr5-GFP+ or Lgr6-GFP+ stem cells. (a,b) At 2 d after tamoxifen addition, no Lgr5-derived Tomato+ cells (red) were readily observed in papilloma epithelium (a); however, in Lgr6 reporter mice, rare Lgr6-GFP+Tomato+ cells were localized in the epithelial compartment (b). (c–g) Over time, rare Lgr5-derived, Tomato+ cells (arrows) were observed in papilloma epithelium (c,e,g); in contrast, Lgr6-derived, Tomato+ traced cell numbers increased at 3 weeks and 6 weeks, giving rise to distinct epithelial clones (d,f,g). The yellow dashed boxes demarcate the magnified regions in the figure insets. The white dashed lines indicate the epithelial–dermal border. DAPI (blue) localizes cell nuclei. Scale bars, 50 μm.

with tamoxifen and papillomas were collected at different times thereafter (2 d, 3 weeks and 6 weeks), to measure the extent of lineage tracing from either Lgr5+ (Fig. 2a,c,e,g) or Lgr6+ (Fig. 2b,d,f,g) stem cells. Imaging of fluorescent cell populations from papillomas 2 d after tamoxifen treatment identified rare GFP and Tomato double-positive cells in the epithelial compartment in Lgr6 reporter mice but not in Lgr5 reporter mice (Fig. 2a,b). The number of Tomato+ traced cells increased at 3 weeks and 6 weeks, with these cells giving rise to distinct clones that showed evidence of migration from the initial Lgr6+ cells (Fig. 2d,f). Some Tomato+ traced cells in Lgr6 reporter mice were also observed in the dermis/stromal compartment (Supplementary Fig. 4b,d), but these were only a minor population, the significance of which is unknown. In contrast, papillomas from Lgr5 reporter mice displayed very few Tomato+ traced cells, even after 6 weeks, and the majority of these were found in the dermal compartment (Fig. 2e,g and Supplementary Fig. 4a,c).

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**Single-cell analysis of Lgr5 and Lgr6 coexpression in normal keratinocytes and tumor cell lines**

Analysis of gene expression at the single-cell level is important in the context of CSCs, of which only a small and variable number may be present in solid tumors or tumor cell lines. We carried out gene expression microarray analysis of Lgr4, Lgr5 and Lgr6 levels in mouse cell lines representing various stages of chemically induced tumors. Lgr5 was not expressed above background levels in any of these cell lines, while Lgr4 and Lgr6 were expressed at varying levels (Supplementary Fig. 7). TaqMan analysis (Fig. 4a) confirmed that Lgr5 was not expressed above the limits of detection except in positive-control back skin samples.

To address the possibility that Lgr5 may be expressed in rare single cells in keratinocyte cultures, we performed Lgr4, Lgr5 and Lgr6 multiplex in situ hybridization. We also analyzed a BCC cell line (Asz001), as Lgr5 has been reported to be expressed in BCC cells. Expression of Lgr5 was detected in the BCC cells by TaqMan and qPCR analyses, albeit at very low levels (Fig. 4b, arrow). In situ hybridization analysis showed that, while Lgr4 was broadly expressed in Asz001 cells, Lgr5 and Lgr6 were more restricted in expression. Interestingly, all three family members were coexpressed in rare single cells (Fig. 4c and inset) at a frequency of about 0.75% (4 in 533 cells). This observation raises the intriguing possibility that these rare Lgr4, Lgr5 and Lgr6 triple-positive cells are CSCs in
the BCC cell line, although further work would be needed to test this hypothesis.

In contrast to the BCC cell line, none of the SCC and keratinocyte cell lines showed substantial Lgr5 expression (Fig. 4d,e and Supplementary Fig. 8). The SCC and keratinocyte cell lines showed different patterns of Lgr6 expression depending on the degree of malignant transformation. In immortalized keratinocytes (C5N cells; Fig. 4d) and NK cells (Supplementary Fig. 8), Lgr6 was expressed in single cells, together with Lgr4. A much larger population of cells expressed Lgr4 but not Lgr6, as also seen in all of the other cell lines tested (Fig. 4 and Supplementary Fig. 8). Cell lines derived from tumors at more advanced stages (E4 cells, derived from a squamous lung metastasis; Fig. 4e) showed a high proportion of Lgr6+ cells, in agreement with the observation of high expression levels in these cells by microarray analysis (Supplementary Fig. 7). These data therefore demonstrate that, at the single-cell level, there appears to be a hierarchy in expression patterns of the LGR gene family. BCCs have distinct Lgr5+ cells, some of which are triple-positive cells that express all three family members. Immortalized keratinocytes lack Lgr5 expression but have rare Lgr6 and Lgr4 double-positive cells, and these appear to increase in number in more advanced tumors.

Multiplex analysis of LGR family gene expression using PCR-activated cell sorting

To determine the levels of coexpression of Lgr4, Lgr5 and Lgr6 at a more quantitative level, we used a novel PCR-activated cell sorting (PACS) technology55–57 (Online Methods). With this method, we were able to identify and sort single cells by performing single-cell TaqMan RT–PCR reactions in >100,000 cells targeting Lgr4, Lgr5 and Lgr6 mRNA sequences. We validated this approach using a subset of the cell lines that were analyzed for LGR gene expression by in situ hybridization and microarrays. Figure 4f shows that almost 100% of the single NK (immortalized keratinocyte), E4 (metastatic squamous carcinoma cell) and ASZ001 (BCC) cells expressed Lgr4, in agreement with the results of in situ hybridization. In contrast, about 1% of the BCC cells expressed Lgr5, however, there was no significant expression in NK cells (Fig. 4f). Interestingly, this extremely sensitive approach also identified a small population of E4 cells (around 0.4%) that expressed some Lgr5 (Fig. 4f), despite the fact that expression analysis by TaqMan (Fig. 4a), microarray (Supplementary Fig. 7) or in situ hybridization (Fig. 4e) of the whole cell population did not detect any significant Lgr5 transcripts. Lgr6 was expressed in over 90% of the E4 cells and in about 10% of BCC cells, but only in about 1–2% of the NK cells, again in line with the in situ data (Supplementary Fig. 8). We also analyzed expression of the same genes in keratinocytes from normal mouse skin. Lgr4 was quite widely expressed (20–30% of keratinocytes), in agreement with previous data on expression in mouse and human skin54,58, while Lgr6, known to be expressed in the upper isthmus as well as the interfollicular epidermis16, was found in around 18% and Lgr5 was found in around 2% of single keratinocytes (Fig. 4f).

Further analysis of these data (Fig. 4g) confirmed the presence of Lgr4, Lgr5 and Lgr6 triple-positive cells in vivo in normal keratinocytes. Around 1.5–2% of normal dorsal telogen skin keratinocytes expressed Lgr5 (Fig. 4f), and of these about 20% (0.3% of the total population) were triple positive (Fig. 4g). Comparable results were seen for the BCC cell line, where of the 1% of the total cell population that expressed Lgr5 (Fig. 4f) about 18% (0.18% of the total) were triple positive for expression of all three genes (Fig. 4g). The concordance between the in situ hybridization and PACS sorting data for the cell lines indicates that in vivo there are specific subpopulations of Lgr5+ cells that also coexpress the other family members. Whether these coexpression patterns are involved in cell fate decisions in normal skin homeostasis in vivo remains to be addressed. Interestingly, PACS analysis of primary squamous carcinoma also identified the presence of a small population of triple-positive cells (Supplementary Fig. 8d,e). These cells, however, do not appear to be clonogenic in tumors, as the lineage-tracing studies using the Lgr5-EGFP-CreERT2 allele failed to identify a major population of traced epithelial tumor cells.

Lgr6 is enriched in keratinocyte cells with stem cell properties

To test the possibility that Lgr6+ cells represent a stem cell subpopulation, we used the Aldefluor assay59–62 to isolate Aldefluor+ and Aldefluor− subpopulations from C5N and D3 cultures. Lgr6 expression was elevated in the Aldefluor+ fraction in comparison to the Aldefluor− fraction (Fig. 5a,b), and Aldefluor+ cells sorted from C5N cultures were capable of forming more and larger colonies in vitro than Aldefluor− cells (Fig. 5c). We conclude that Lgr6, and

Figure 3 | Clonal expansion derived from Lgr6+ cells in primary SCCs. Lgr6-derived lineage tracing was performed within chemically induced primary (1°) SCCs from Lgr6-EGFP-CreERT2 mice crossed with Rosa26-LSL-Tomato reporter mice by standard DMBA treatment followed by 20 weeks of tumor promotion using TPA. (a,b) Once SCCs were readily visible, mice were treated with tamoxifen and a biopsy was taken 2 d later, showing abundant Lgr6-GFP+Tomato+ cells (red) localized in the epithelial compartment as well as some Lgr6-GFP+ cells (green; asterisks) lacking recombination (b). The hematoxylin and eosin sections in a provide tumor histology for the images in b. (c,d) In the same tumor 2 weeks after tamoxifen treatment, distinct Lgr6-GFP+Tomato+ clones were observed (d, inset). (e,f) At 4 weeks after labeling, substantial Tomato+ cell labeling was observed within the epithelial tumor tissue. The yellow dashed boxes demarcate the magnified regions in the figure insets. The white lines indicate the epithelial–dermal border. DAPI (blue) was used to visualize cell nuclei. Arrows indicate GFP and Tomato double-positive cells. Scale bars, 50 μm.

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not Lgr5, acts as a marker of a stem-like cell population in squamous epidermal cells.

Lgr6 suppresses epidermal cell growth in vitro and in vivo

We next overexpressed Lgr6 in immortalized keratinocytes (NK cells), which have low endogenous levels of Lgr6 mRNA (Supplementary Figs. 7 and 8). Transfection of NK cells with an inducible Lgr6 expression construct (Fig. 5d) led to growth retardation after addition of doxycycline (Fig. 5e). To validate this growth-suppressive effect of Lgr6 in epidermal cells, we knocked down Lgr6 in an SCC cell line (E4), which expresses relatively high endogenous levels of Lgr6 (Fig. 4e and Supplementary Fig. 7). We transduced E4 cells with an inducible Lgr6 shRNA construct, which, upon doxycycline administration, elicits efficient knockdown of Lgr6 without altering the levels of either Lgr4 or Lgr5 (Fig. 5f). Consistent with a growth-suppressive role for Lgr6 in epidermal cells, doxycycline treatment of transduced E4 cells led to increased growth but had no effect on unmodified E4 cells (Fig. 5g).

Lgr6 knockout in vivo expands lineage tracing in the skin

Lgr6 is a marker of infundibulum stem cells, but a possible functional role in control of homeostasis in vivo has not been investigated. Although the Lgr6<sup>−/−</sup> mouse is viable<sup>63</sup>, developmental adaptation to loss of expression could lead to compensatory mechanisms for controlling skin homeostasis in the adult mouse. We therefore generated Lgr6-knockdown mice, which possess an Lgr6 shRNA construct knocked into the Rosa26 locus that can be induced by addition of doxycycline.

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Lgr6 activates Wnt signaling but is a germline tumor-suppressor gene

Lgr5 has been reported to be overexpressed in hepatocellular carcinoma, colon and ovarian cancers, and has also been demonstrated to promote the tumorigenicity of BCC cells. Paradoxically, however, Lgr5 has also been shown to reduce the growth of colorectal cancer cells, and tumor-suppressive roles have been suggested for Lgr4 in mice treated with 4-OHT. Germline loss of RSPO1, the upstream ligand for the LGR family of receptors, or of LGR4 also causes predisposition to the development of squamous carcinoma in humans. We therefore investigated the potential role of Lgr6 as an inducer of Wnt signaling in squamous cells and as a germline tumor-suppressor gene.

We assessed the effects of Lgr6 deregulation on Wnt signaling in keratinocyte cell lines using the well-characterized TOPFlash assay. Overexpression of Lgr6 in multiple epidermal cell lines consistently led to activation of this pathway (Fig. 6h). Finally, analysis of gene expression data for human head and neck SCCs from The Cancer Genome Atlas (TCGA) (Supplementary Tables 1 and 2) showed a strong connection to the Wnt pathway only for LGR6 and not to any significant extent with LGR5 or LGR4. This supports the notion that LGR6 is linked to Wnt signaling and may have a unique role in tumors of squamous origin.

To assess the effect of germline inactivation of Lgr6 on tumor susceptibility, we bred Lgr6−/− mice through multiple generations onto a pure FVB/N genetic background and exposed them to the DMBA/TPA chemical carcinogenesis protocol. The knockout mice showed a highly significant increase in susceptibility to...
Doxycycline, the Tet repressor sits on the H1-tetO promoter and prevents transcription of the Lgr6 shRNA. In the presence of doxycycline, the Tet repressor is bound by the drug and prevents association with the H1-tetO promoter; this in turn allows for transcription of the Lgr6 shRNA.

(b) Lgr4, Lgr5 and Lgr6 mRNA levels in back skin samples were determined through TaqMan analysis. In the absence of doxycycline, the Lgr6 mRNA expression is low levels of phosphorylated β-catenin and high levels of Lgr6, in spite of its activity as a negative stimulator of Wnt signaling, acts as a germline suppressor of SCC development, thus mimicking the functions of human Wnt activators RSPO1 and LGR4 as SCC-suppressor genes. One possible explanation for these apparently contradictory results is that papilloma development (Fig. 6i), as well as shortened carcinoma-free survival (Fig. 6j). We conclude that Lgr6, in spite of its activity as a positive stimulator of Wnt signaling, acts as a germline suppressor of SCC development, thus mimicking the functions of human Wnt activators RSPO1 and LGR4 as SCC-suppressor genes. One possible explanation for these apparently contradictory results is that...
germline loss of Lgr6 could cause developmental upregulation of other pathway components that may compensate for loss of Lgr6 activity. Stem cells in normal skin or intestine display considerable plasticity, such that ablating of Lgr5+ cells can stimulate their regeneration from alternative stem cell populations. To investigate possible mechanisms of compensation, we derived a series of cell lines from Lgr6−/− skin SCCs and analyzed expression levels of the other LGR family members. The wild-type mouse carcinomas showed variable activation of the Wnt pathway, as demonstrated by differences in levels of phosphorylated β-catenin. Of the three carcinomas tested, the highest Wnt activity was seen in the cell line (168 cells) that also had the highest level of Lgr6 expression (Fig. 6f, left). Variable levels of Wnt activity were also seen in the carcinomas from Lgr6−/− knockout mice (Fig. 6f, right, and Supplementary Fig. 10), but in three of four cases with high activity the level of Lgr5 expression was strongly upregulated. This is particularly noteworthy as all of our expression data show that, in SCCs from wild-type mice, Lgr5 is switched off even at the single-cell level and has no major role as an epithelial cancer stem cell marker. These data therefore identify a mechanism by which cancer stem cells in Lgr6−/− mice can be regenerated through a feedback mechanism resulting in upregulation of Lgr5.

**DISCUSSION**

Using a combination of gene expression analysis, lineage tracing, single-molecule in situ hybridization and PACS analysis of single tumor cells, we have shown that Lgr6 satisfies many of the criteria of an SCC stem cell marker. Lineage tracing identified a subpopulation of Lgr6+ tumor cells that give rise to progressively growing epithelial clones. Lgr6 was enriched in Aldehyde dehydrogenase+ cells with stem cell properties, and its inhibition resulted in increased proliferation and lineage tracing in the epidermis. Although Lgr5 was not significantly associated with squamous tumor development or progression, it was expressed in rare single cells in a BCC cell line, some of which were triple positive for all three LGR family members. The detection of these triple-positive cells in vivo in basal keratinocyte cells, as well as in primary carcinomas, suggests that they may have a distinct role in stem cell homeostasis, but this question will require further investigation.

The model that we believe best explains our results is shown in Supplementary Figure 11. In this model, an early stem cell population may express Lgr5 and Lgr6 as well as Lgr4, with the initial cell fate choice being determined by competitive interactions of these GPCRs with their R-spondin ligands and other potential effector molecules such as Rnf43 (ref. 70) and Znrf3 (ref. 71). Lgr5 expression is maintained in some cells that can give rise to BCCs but lost in cells that commit to the epidermal cell fate. In this stem cell compartment, Lgr6 functions as a brake toward further epidermal commitment and differentiation, such that loss of Lgr6 leads to increased epidermal lineage differentiation and proliferation. It is possible that loss of Lgr6 expression (but continued expression of Lgr4) may be linked to commitment to a more differentiated keratinocyte state. Indeed, the highest levels of Lgr4 expression are seen in cell lines with the most ‘epithelial’ characteristics such as the immortalized keratinocytes C5N and NK or the differentiated papilloma cell lines MscP1 and MscP5 (Supplementary Fig. 7).

Germline deletion of Lgr6 in the mouse caused predisposition to SCC development, as also seen in humans with germline loss of LGR4 (ref. 29) or RSPO1 (ref. 28). Tumors that arose in Lgr6−/− mice in fact showed upregulation of Lgr5, particularly in those tumors with the highest levels of Wnt signaling. These data highlight the plasticity and complex control of Wnt/β-catenin signaling in the skin, where activation of the pathway can maintain hair follicle and epidermal stem cell growth, but impairment in hair follicle stem cells stimulates commitment to the epidermal lineage. Elevated expression of β-catenin is negatively associated with normal epidermal proliferation, but its localization in the nucleus is positively associated with growth of Hras-transformed cells. Successful targeting of the Wnt pathway for SCC therapy will require resolution of the complex mechanisms that distinguish signaling in these different biological contexts.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

P.Y.H., E.K. and A.B. designed experiments. J. Seibler and V.R. generated and provided ES cells for creation of mouse strains. P.Y.H., E.K., J. Sjolund, A.K., R.D., H.C.K., A.J., M.P. and S.E.W. performed experiments. P.Y.H., E.K. and A.B. analyzed data. K.H. and M.M. carried out statistical analyses. P.Y.H., E.K. and A.B. wrote the manuscript, with contributions from the other authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Mouse treatments, chemical carcinogenesis and lineage tracing in skin. Lgr6-knockdown mice were generated in a similar fashion to previously reported doxycycline-inducible knockdown mice80, using the Lgr6 shRNA sequence 5′-GGATGACACTGGAAGACAAT-3′. Recombinant embryonic stem (ES) cells were injected into blastocysts, and chimeric animals were obtained at the UCSF Cancer Center Transgenic and Targeted Mutagenesis Core Facility. Doxycycline-containing water was prepared by dissolving 2 g of doxycycline (RPI) in 1 L of water containing 50 g of sucrose (Affymetrix). For BrdU studies, mice were injected intraperitoneally with BrdU labeling reagent (Life Technologies) at a concentration of 10 µg/ml body weight and killed 3 h after injection. For chemical carcinogenesis experiments, 8- to 12-week-old male and female mice were randomly assigned to control and treatment groups. Initiation was carried out using a single dose of DMAB (25 µg per mouse in acetone) applied to back skin. Starting 1 week later, the mice were administered topical treatments of TPA (200 µl of a 10−4 M solution in acetone) twice a week for 20 weeks. The animals were then monitored for papilloma number and progression to carcinoma. Mice were killed when their first carcinoma reached 1–1.5 cm in diameter. For fluorescent Lgr5-EGFP-Tomato and Lgr6-EGFP-Tomato lineage-tracing experiments, papillomas were established following the chemical carcinogenesis protocol. After 8 weeks of TPA treatment, one topical dose of tamoxifen (200 µl of 25 mg/ml tamoxifen in 100% ethanol) was administered to shaved back skin and papillomas were then surgically removed 2, 3 weeks and 6 weeks after the initial tamoxifen treatment for analysis. Similar lineage tracing was carried out with mice bearing carcinomas that arose 24–30 weeks after initiation with DMAB. To preserve Lgr5- or Lgr6-driven GFP and Tomato reporter expression, before embedding, excised tumors were briefly fixed in 4% paraformaldehyde for 2 h and then placed in 30% sucrose overnight at 4 °C before freezing in OCT for subsequent cryosectioning. For Lgr6-knockdown lineage-tracing experiments, 8- to 10-week-old Lgr6 KD+/−/Lgr6-EGFP-CreERT2+/−/R26RloxZ′/+ mice were administered either doxycycline- or vehicle-containing water for 10 d and then given one topical dose of 4OHT (1 mg in 200 µl of 100% ethanol) (Sigma-Aldrich) on back skin to induce Cre expression. Mice were killed in triplicates 7 d after induction. Animals were housed in standard conditions, allowed to feed ad libitum and treated in accordance with the rules and protocols stipulated by the UCSC Institutional Animal Care and Use Committee (IACUC). All mouse experiments were approved by the University of California at San Francisco Laboratory Animal Resource Center. Age-matched animals of the required genotypes were randomly assigned to treatment groups (for instance, wounded or unwounded (Supplementary Fig. 2) and vehicle or doxycycline treated (Fig. 6c–g)). When assessing experimental outcomes in animal samples (for instance, BrdU staining in Fig. 6c and X-gal staining in Fig. 6d–g), the investigator was blinded through the use of unique animal IDs for the samples that do not indicate treatment conditions.

X-gal staining. Back skin samples were surgically removed from 4OHT- or vehicle-treated mice and cut into small pieces. These were added to 4% formaldehyde diluted in PBS and placed on a shaker at room temperature for 10 min. After three washes with PBS, the skin samples were incubated in X-gal staining solution (1 mg/ml X-gal, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, and 2 mM MgCl2 in PBS) at 37 °C in the dark. The next day, the samples were washed with PBS, transferred to 70% ethanol and made into paraffin blocks. 6-µm sections were cut on a microtome and counterstained with nuclear fast red (Vector Laboratories).

Multiplex in situ hybridization. ViewRNAISH Cell Assay probes for mouse Lgr4, Lgr5 and Lgr6 were obtained from Panomics, and in situ hybridization was performed according to the manufacturer’s protocol on cells grown in polylysine-coated, glass-bottom 96-well plates (In Vitro Scientific). Images were acquired on the 6D high-throughput microscope in the UCSF Nikon Imaging Center and analyzed with the NIS Elements and ImageJ software packages.

Single-cell RT–PCR analysis. Single-cell analysis of Lgr4, Lgr5 and Lgr6 expression was performed using the Mission Bio PACS platform55–57. Briefly, single-keratinocyte suspensions of disaggregated back skin, NK cells, E4 SCC cells, BCC cells or primary SCC cells were stained with a calcein violet viability dye and encapsulated in microfluidic droplets. Droplets containing individually lysed cells were further processed with the PACS microfluidic workflow to enable droplet-based TaqMan RT–PCR detection of target transcripts. The multiplex RT–PCR reactions consisted of FAM-labeled Lgr5, HEX-labeled Lgr6 and Cy5-labeled Lgr4 TaqMan probes. Thermocycling conditions were as follows: 50 °C for 15 min, 93 °C for 2 min, and 35–40 cycles of 92 °C for 15 s and 60 °C for 1 min. Following thermocycling, the droplets were rehydrated into a microfluidic device for fluorescence analysis using four coincident lasers (405 nm, 473 nm, 532 nm and 640 nm; CNI lasers) to excite the cell viability stain and TaqMan probes. The resulting fluorescence channels (centered at 440 nm, 510 nm, 572 nm and 680 nm) were separated from the lasers and each other with dichroic filters (Semrock) before being detected by four PMTs (Thorlabs). Detection was processed in real time using LabVIEW, and droplet scatterplots were generated using MATLAB.

Single cells were analyzed with the multiplexed TaqMan assay (7,334 back skin cells, 14,454 NK cells, 21,596 E4 SCC cells, 16,914 BCC cells and 2,617 primary SCC cells). Three independent replicates were performed for each cell or sample type.

Immunofluorescence. The first, deparaffinization and antigen retrieval were performed on 6-µm back skin sections using Trilogy pretreatment solution (Cell Marque). Then, the sections were incubated in 10% donkey serum (Abcam) diluted in PBS containing 0.3% Triton X-100, at room temperature for 1 h. Subsequently, the sections were incubated with primary antibody against BrdU (rat; Abcam) or GFP (rabbit; Santa Cruz) at 4 °C overnight. After washing three times with PBS containing 0.1% Tween-20, the sections were incubated with the appropriate Alexa Fluor 488/555–conjugated secondary antibody (Molecular Probes) for 1 h at room temperature. After washing three times with PBS containing 0.1% Tween-20, the samples were finally mounted in Vectorshard hardset mounting medium with DAPI (Vector Laboratories). Lgr5-EGFP, Lgr6-EGFP and KRT14 expression in primary SCC cyroslices was visualized by incubation with antibodies to GFP (chicken; Abcam) and KRT14 (rabbit; Covance).

Western blotting and RT–qPCR analysis. Lgr6-wild-type and Lgr6-knockout cell lines were derived from SCCs generated by DMAB and TPA treatment of wild-type or Lgr6−/−FVB mice87. SCCs were dissected and digested in 4 mg/ml collagenase A (Sigma-Aldrich) at 37 °C for 60 min with agitation, followed by 30 min of incubation in 0.25% trypsin-EDTA solution. Dissociated cells were then passed through a 40-µm filter (Greiner Bio-One) and seeded into a 10-cm dish with regular DMEM. For western blot analysis, cells were prepared with lysis buffer (Cell Signaling Technology) supplemented with protease inhibitors, 100 µM NaF and 100 µM Na3VO4. Protein lysates were run by SDS–PAGE and transferred onto PVDF membranes (Bio-Rad). After blocking, membranes were incubated overnight at 4 °C with antibodies to phosphorylated β-catenin (8814; Cell Signaling Technology) or β-actin (4967; Cell Signaling Technology). HRP-linked anti-rabbit secondary antibody was used (7074; Cell Signaling Technology), and ECL was used for detection. The membrane used for detection of phosphorylated β-catenin was stripped for 30 min at room temperature in Restore Plus Stripping buffer (Thermo Fisher), reblocked and incubated with antibody to β-catenin (1247-1, Epitomics). For LGR gene expression analysis, RNA was extracted from Lgr6-wild-type and Lgr6-knockout cells with TRIzol reagent (Thermo Fisher) and reverse transcribed using the QuantiTect RT kit (Qiagen). qPCR expression analyses were performed with the indicated primer sets (Supplementary Table 3) by using Power-Up SYBR Green Mastermix (Thermo Scientific, A25742) and run on an AB7900HT instrument (Applied Biosystems). Relative gene expression was calculated using RPL19 as a housekeeping gene to generate ΔCt values. ΔCt values from 1425-L1 Lgr6-wild-type cells were used as the reference to generate ΔΔCt values, and relative gene expression was calculated as 2−ΔΔCt or is presented as copy number/1,000 copies of L19 for gene expression. All reactions were run in duplicate. For primer sequences, see Supplementary Table 3.

Cell culture treatments and MTT and TOPFlash assays. Doxycycline was administered, where appropriate, at a concentration of 1 µg/ml in culture medium. The shRNA sequence used to target Lgr6 in E4 cells was identical to the one used to generate the Lgr6-knockdown mice (see above), and
this was cloned into the doxycycline-inducible F1HUTG lentiviral vector. For MTT assays, 2,000 (Lgr6 ox and null NK) or 4,000 (Lgr6 shRNA and null E4) cells were seeded in each well of a 96-well plate and the culture medium was replaced every third day. The Vybrant MTT Cell Proliferation Assay (Life Technologies) was performed according to the manufacturer’s protocol at the indicated time points after initial seeding. For TOPFlash assays, cells grown in six-well plates were first transfected with 2 μg of the M50 Super 8× TOPFlash plasmid and 0.1 μg of the pRL-TK Renilla luciferase plasmid per well, using Lipofectamine 2000 (Invitrogen). After 6 h, the medium was replaced and doxycycline was added, where appropriate. Two days later, luciferase activity was determined for each well using the Dual-Luciferase Reporter Assay System (Promega) with the GloMax Luminometer, according to the manufacturer’s instructions.

Cell lines and microarray analysis. The cell lines shown in Supplementary Figure 7 were described previously (refs. 55, 51, 82, 83 and references therein). NK cells and C5 cells are immortalized keratinocytes derived from wild-type (C5) and Tprp53-null (NK) mice. Hras-null cells were derived from a carcinoma induced by DMBA and TPA in Hras-knockout mice. All of the other lines carry initiating mutations in the Hras gene. Cells were grown in serum-supplemented medium in the log phase. RNA extraction with TRIzol reagent and reverse transcription was performed using the High-Capacity Reverse Tran

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