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Epidermal expression of Lgr6 is dependent on nerve endings and Schwann cells

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Abstract

Lgr5/6 proteins are stem cell markers in various tissues. However, what determines their restricted expression pattern in these tissues remains unknown. We found that in skin, Lgr6 is not only expressed in the central isthmus, directly above the hair follicle bulge cells as reported previously, but also in the interfollicular epidermis. Lgr6 expression in skin is highly correlated with the innervation sites of cutaneous nerves. In the hair follicle, Lgr6 closely localizes with the surrounding nerve endings and their corresponding Schwann cells throughout the entire hair cycle. Furthermore, ablation of cutaneous nerves leads to degeneration of Schwann cells and diminished expression of Lgr6. Our results demonstrate that the nerve endings/Schwann cells control Lgr6 expression in skin, implying that they play a role in regulation of skin epithelial cells.

Keywords

Lgr6; nerve endings; intercellular junction; Schwann cells; epidermis

Introduction

Lgr4, 5, and 6 constitute the type B family of Lgr (leucine-rich repeat-containing G-protein-coupled receptor) proteins [1, 2]. They are specifically expressed in some populations of adult stem/progenitor cells and play important roles in organogenesis and adult cell homeostasis in various tissues, including the intestine, hair follicle, cornea, distal stomach, mammary gland, tongue, kidney, prostate, testes and uterus [3–14]. Recent studies have revealed that Lgr4/5/6 proteins are facultative components of the Lrp/Fzd Wnt receptor complex and are able to potentiate canonical Wnt signaling through binding to their common ligands, R-spondins [15–18].

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Author contributions

XL and HN designed the experiments, analyzed the data, and wrote the manuscript. XL conducted the experiments.

Conflict of interests

The authors declare no conflict of interest.

Lgr4, 5, and 6 exhibit different expression patterns in the adult hair follicle: Lgr4 is expressed in the entire bulge below the sebaceous gland, Lgr5 is expressed in the lower bulge while Lgr6 is expressed in the central isthmus, directly above the bulge [11, 12]. Although Lgr proteins have recently been intensively studied as markers and regulators of stem cells, the specific factors that establish their expression patterns in various tissues have yet to be elucidated.

Nerve fibers innervate skin in the interfollicular epidermis (IFE) and in the upper region of hair follicle [19–22]. In the upper region, nerve endings dock to epidermal cells together with terminal Schwann cells, forming a specialized structure similar to the neuromuscular junction, enabling the hair follicle to sense the environment [23, 24]. The crosstalk between nerves and epidermal cells is important for morphogenesis and homeostasis [25–30].

In contrast with previous reports [11], we found in this study that Lgr6 is not only expressed in the central isthmus, but also in a portion of the interfollicular epidermis. Surprisingly, Lgr6 protein closely localizes with nerve endings/Schwann cells and forms a fiber-like structure at the isthmus region. When back skin was denervated, Schwann cells degenerated and Lgr6 expression was lost. Our results demonstrate that Lgr6 expression is dependent on the presence of intact nerve endings/Schwann cells, suggesting that nerve endings/Schwann cells might directly regulate stem cell compartment in the skin.

Materials and Methods

Animals

Lgr6-EGFP-CreERT2 [11], *S100B-EGFP-CreERT2* and *ROSA26-tdTomato-EGFP* [31] mice were imported from Jackson Laboratory. *Lgr6-EGFP-CreERT2* and *S100B-EGFP-CreERT2* mice were both crossed with the reporter line *ROSA26-tdTomato-EGFP*. Mice were injected intradermally with tamoxifen to test LGR6 expression activity and to trace *S100B*⁺ Schwann cells in skin. All mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility at Baylor College of Medicine (BCM). This study was approved by the BCM Institutional Animal Care and Use Committee (IACUC).

Skin embedding and immunostaining

For routine immunostaining, back skins were embedded and frozen on dry ice in OCT compound (Sakura Finetek). For detection of GFP in tissue sections, back skins were pre-fixed with 4% PFA at room temperature for 2 hrs, washed with PBS, soaked in 10 % sucrose at room temperature for 2 hrs and then in 30 % sucrose at 4°C overnight; skins were then equilibrated with 30 % sucrose/OCT (1:1) at room temperature for 6 hrs and embedded and frozen on dry ice in OCT compound.

Skin sections (6–9 µm) were fixed in 4% PFA and stained with the following antibodies: rabbit anti-neurofilament (AB5539; Fisher Scientific) 1:500, rabbit anti-S100B (Z0311; Daco) 1:500, rabbit anti-Lgr6 (IMGEX; IMG-71579) 1:100, rabbit anti-GFP (Molecular Probes; G10362) 1:300, rat anti-cd104 (β4 integrin) (BD Pharmingen; 553745) 1:200; rat anti-cd34 (BD Pharmingen; 553731) 1:300.

Denervation surgery

Denervation of dorsal skin in mice was performed under sterile conditions as described in previous reports [32, 33]. Mice at least 20 days old were anesthetized and shaved and a 25–30 mm dorsal midline incision was made through the full thickness of the skin. The skin was then elevated to allow visualization of the dorsal cutaneous nerves (DCNs) under the dissecting microscope, and left-side DCNs of T3–12 were divided close to their exit point the muscle and close to their entry into the skin. The right-side DCNs were left intact as a control. Care was taken to avoid cutting the larger blood vessels in the skin. After denervation, the skin incision was closed with nylon suture material placed at 5-mm intervals. Buprenorphine was administered for post-surgical analgesia.

Results

Lgr6 is expressed in the interfollicular epidermis

The GFP reporter signal in *Lgr6-EGFP-CreERT2* knock-in back skin [11] is detectable but dim when directly visualized by fluorescent microscopy. We amplified this signal by immunostaining with anti-GFP antibody, and clearly observed the GFP reporter in the central isthmus, directly above the hair follicle bulge, as previously described, as well as in the interfollicular epidermis (IFE) (Fig. 1a). Surprisingly, the interfollicular basal layer contained distinct *Lgr6*^{GFP}-positive and -negative populations; ~25% $\pm$ 17.3% (mean $\pm$ SD, n=6) of total interfollicular basal cells were *Lgr6*^{GFP} positive during the second postnatal telogen.

As described previously [11], *Lgr6*^{GFP} was highly enriched in the central isthmus, directly adjacent to the sebaceous gland (Fig. 1b). However, *Lgr6*^{GFP} was also found occasionally in the sebaceous gland itself (Fig. 1c) and in the CD34⁺ hair follicle stem cells (Fig. 1d). A similar pattern of expression was previously reported in an independent study [34].

Lgr6 closely localizes with nerve endings/Schwann cells

Skin is innervated by sensory nerves in the IFE, as well as in the isthmus ([20] and Fig. 2a), both sites of *Lgr6* expression. This correlation led us to closely examine the spatial relationship between *Lgr6*-expressing cells and nerve endings. To stain for axonal projections and Schwann cells, we used the axonal marker neurofilament (NF) and S100B, respectively. Indeed, we found nerve fibers surrounding *Lgr6*^{GFP} positive cells in the isthmus (Fig. 2b). We also noticed that Schwann cells, which wrap around axons, were highly concentrated around the basal membrane where *Lgr6*^{GFP} positive cells are located (Fig. 2c).

Since the cytoplasmic reporter GFP does not reflect the subcellular localization of endogenous *Lgr6*, we used a commercial *Lgr6* antibody to co-stain for *Lgr6* and the axonal marker NF. This confirmed the enrichment of *Lgr6* expression in the central isthmus, in close approximation with neuronal axons in a fiber-like architecture. This relationship was especially marked in guard hair follicles (Fig. 2d). By immunostaining, we also detected *Lgr6* in the IFE (Fig. 2d), albeit at a much lower expression level than in the isthmus

Nerve endings/Schwann cells surround Lgr6⁺ cells throughout the hair cycle

During the growth phase of the hair cycle, Lgr6 is still expressed in the IFE and the central isthmus, including the remnant (club hair) follicle from the previous cycle (Fig. 2e), and we observed that nerve fibers/Schwann cells surrounded the central isthmus of both new and old hair follicles (Fig. 2e and 2f). A transverse view of hair follicle demonstrated that nerve endings/Schwann cells tightly wrap around the entire hair follicle unit containing several hair shafts (Fig. 2g). All of these data demonstrate a close correlation between Lgr6 expression and the presence of nerve endings/Schwann cells, strongly indicating a possible interplay between epidermal cells, nerve fibers, and Schwann cells.

Cutaneous denervation leads to degeneration of Schwann cells

To directly address whether nerve endings interact with epidermal cells, we surgically ablated the dorsal cutaneous nerves (DCNs). One month following denervation, we observed a significant reduction in staining intensity of the Schwann cell marker S100B in the isthmus area (Fig. 3a). After 3.5 months, S100B was completely lost in most of the hair follicles (Fig. 3b).

Loss expression of S100B suggests loss of Schwann cells, but it is possible that Schwann cells remain but only the expression of S100B disappears following the loss of trophic effects from the ablated nerves. To distinguish these two possibilities, we utilized *S100B-EGFP-CreERT2*, *ROSA26-tdTomato-EGFP* mice to trace the fate of S100B-lineage cells. After turning on the reporter GFP with several consecutive doses of tamoxifen, we ablated the DCNs in the left-side back skin, while leaving right side intact as a control. After 3 months, we observed GFP⁺ Schwann cells surrounding the hair follicles on the control side, but no GFP⁺ cells on the denervated side (Fig. 3c). This result indicates that S100B⁺ Schwann cells degenerated after loss of trophic support from nerves.

Cutaneous denervation leads to loss of Lgr6 expression

Together with loss of S100B expression, endogenous Lgr6 expression was also diminished in the isthmus region following denervation (Fig. 3d.). We also found a loss of Lgr6^{GFP} reporter activity in both the isthmus and in the IFE in *Lgr6-EGFP-CreERT2* mice (Fig. 3e). Alternatively, we used lineage tracing in *Lgr6-EGFP-CreERT2*, *ROSA26-tdTomato-EGFP* mice to further test *Lgr6* promoter activity after denervation. We again ablated the DCNs in the left-side back skin, leaving the right side intact as a control. One month later, we administered tamoxifen to the mice for 5 consecutive days and harvested skin after a further 5 days. Only the bright GFP under the *ACTB* promoter in *ROSAS26-tdTomato-EGFP* could be visualized by direct fluorescent microscopy. Consistently, in comparison with the control side, fewer GFP⁺ cells were detected on the denervated side, indicating a loss of *Lgr6* promoter activity (Fig. 3f).

Discussion

Our study demonstrates that Lgr6 activity is not restricted to the central isthmus, but is also present in the IFE and occasionally in the sebaceous gland and the lower bulge region. These data are consistent with recent results published by others [34]. In the original

lineage-tracing study using *Lgr6* knock-in mice, the presence of *Lgr6*-lineage clones in both the hair follicle and IFE was interpreted to mean that *Lgr6*⁺ cells in the isthmus possess the capacity to generate the IFE, the sebaceous gland, and the hair follicle [35–37]. However, our observations here suggest that IFE, sebaceous gland and hair follicle are more likely to be generated from *Lgr6*⁺ cells in their own compartments. This is consistent with reports demonstrating the segregation of these lineages [34, 38, 39]. Our finding that *Lgr6*-lineage clones can arise within the IFE resolves an apparent inconsistency between the *Lgr6* lineage-tracing results and those previously obtained using *Shh-Cre* and *Lrig1-CreER*, in which the *Lgr6*-expressing domain within the HF was labeled but was never seen to incorporate into the IFE during normal skin homeostasis [34, 38].

In addition, we found that only about 25% of interfollicular basal cells express *Lgr6*. This heterogeneous expression of *Lgr6* in the interfollicular basal cells raises the questions whether *Lgr6*⁺ and *Lgr6*[−] basal cells are functionally different and whether they have differential stem cell potential. However, it is important to exercise caution in interpreting these results. GFP expression in *Lgr5-EGFP-CreERT2* is variegated, as noted by the mice depositor (from website of Jackson Laboratory). Although GFP should faithfully represent the *Lgr6* expression pattern in the knock-in mice, the possibility should not be excluded that *Lgr6* reporter expression may be subject to silencing or other alterations due to the introduction of foreign DNA elements in these mice.

Lgr6 expression both in IFE and the central isthmus correlates well with the presence of dense nerve endings and Schwann cells in the skin. When *LGR6*^{GFP} skin was co-stained with specific markers, we observed that endogenous *Lgr6* and nerve ending fibers were closely associated in the isthmus. This strongly suggests that *Lgr6* expression is related to nerve endings and/or Schwann cell activity. Indeed, when nerves were surgically ablated, *Lgr6* expression was diminished as well, both in the IFE and in the isthmus. Since both nerve fibers and Schwann cells degenerated after denervation, we could not conclude whether *Lgr6* expression is determined by nerve fibers, Schwann cells or both.

Lgr5 itself is a downstream target protein of Wnt pathway [13, 40]. In the intestine, *Lgr5*⁺ stem cells are physically neighbored by Wnt3-expressing Paneth cells [41]. However, in a separate study, Paneth cells were found to be neither essential for *Lgr5* expression nor for stem cells maintenance [42]. Interestingly, Schwann cells are of neural crest origin and express the neural crest marker Wnt1 [43]. It would be interesting to investigate whether Wnt1 from Schwann cells mediates the expression of *Lgr6* in skin.

Lgr6 is not the first epithelial marker whose expression is affected by nerve endings/Schwann cells. *Gli1* expression in the upper bulge cells has been shown to depend on Sonic hedgehog signals produced by neighboring neurons [32]. Nerve endings and terminal Schwann cells have also been shown to secrete numerous trophic factors to maintain normal architecture at the neuromuscular junction [44, 45]. Since nerve endings/Schwann cells dock directly adjacent to the hair follicle stem cell niche, and are concentrated around interfollicular basal cells, it will be very interesting to closely examine the relationship between nerve endings/Schwann cell activity and epidermal stem/progenitor cell function.

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Figure 1. *Lgr6* is expressed in the IFE, central isthmus, and occasionally sebaceous gland and hair follicle bulge

(a–d) Immunofluorescence images of adult (P69) *Lgr6-EGFP-CreERT2* knock-in mouse backs using antibodies against GFP, $\beta 4$ -integrin, and CD34. *Lgr6* is expressed in a subpopulation of basal cells in the IFE (a), the central isthmus (b), the sebaceous gland (c) and the CD34⁺ hair follicle bulge stem cells (d). Arrows point to *Lgr6* signals in the sebaceous gland (c) and the bulge stem cells (d). Scale bar, 20 μ m.

Figure 2. *Lgr6* localizes closely with nerve endings/Schwann Cells throughout the hair cycle Immunofluorescence images of backskins of *Lgr6-EGFP-CreERT2* knock-in mice. (a) Nerve endings and Schwann cells, marked by neurofilament (NF) and S100B, densely surround the isthmus, directly above the bulge in telogen. (b) *Lgr6* and nerve endings localize closely at the isthmus, shown by immunostaining using antibodies against GFP and NF. (c) S100B⁺ Schwann cells are concentrated around basal membrane, the interface where *Lgr6*⁺ basal cells localize in the IFE. (d) Immunostaining using antibodies against *Lgr6* and NF shows that endogenous *Lgr6* and neurofilament localize closely in a fiber-like structure in the isthmus. (e–g) Nerve endings/Schwann cells surround both new and remnant (club) hair follicles throughout the hair cycle. At anagen phase, *Lgr6* and nerve endings also localize closely, as shown by immunostaining using antibodies against GFP and NF (e). Nerve endings (NF⁺) and Schwann cells (S100B⁺) tightly surround the isthmus of both new and old hair follicles, represented by longitudinal (f) and transversal views (g). Scale bar, 20 μ m. SG, sebaceous gland; HS, hair shaft. Arrows point to club (old) hair follicles.

Figure 3. Denervation leads to degeneration of Schwann cells and diminished expression of *Lgr6*
 Immunofluorescence images of control and denervated skins. Dorsal cutaneous nerves of mice were unilaterally ablated at the left side, while nerves at the right side were left intact as control. (a) Short-term denervation (1 month) leads to decline of S100B expression in Schwann cells. (b) Long-Term denervation (3.5 months) leads to depletion of S100B in Schwann cells. (c) GFP reporter was induced by tamoxifen application in *S100B*-*CreERT2*, *ROSA26-tdTomato-EGFP* mice. After denervation for 3 months, GFP labeled Schwann cells were not detected. (d–e) After denervation for 1 month, endogenous *Lgr6* (d) and GFP in *Lgr6-EGFP-CreERT2* knock-in mice (e) were diminished. (f) *Lgr6-EGFP-CreERT2*, *ROSA26-tdTomato-EGFP* mice were denervated for a month and then administered with tamoxifen to induce GFP expression. Microscope was set to only detect bright GFP under *ACTB* promoter in *ROSA26* locus and not the dim GFP under *Lgr6* promoter. In comparison with control side, induction of GFP in denervated skin is largely abolished. Scale bar, 20 μm.