GPR39 marks specific cells within the sebaceous gland and contributes to skin wound healing

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G protein-coupled receptors (GPCRs) mediate multiple key biological processes in the body. As one of the most important groups of drug targets, GPCRs have been intensively studied in recent decades. In recent years, the roles of GPCRs as novel stem cell markers and in stem cell regulation have received attention. GPR39 is an orphan GPCR that is conserved across vertebrates1. It is expressed in a range of tissues and functions in a variety of physiological processes, such as insulin secretion2,3, synaptic signalling4 and gastric emptying5. The metabolic roles of GPR39 are well recognised, in strong contrast to the lack of knowledge about its functions in stem cell biology. However, several reports have implied its involvement in stem cell biology. GPR39 is upregulated in both normal human foetal kidney and stem-like Wilms’ tumour xenografts6,7. It has also been reported to be a signature gene in human synovium-derived mesenchymal stem cells (MSCs)8.

The mammalian skin epidermis comprises the stratified epithelium and its appendages including the hair follicle (HF), the sweat gland and the sebaceous gland (SG). Various populations of stem cells have been identified in different regions of the epithelium; they maintain homeostasis and the renewal of the entire skin epithelium, as well as play critical roles in skin repair after injuries. SG is an epidermal appendage that is important in skin barrier function and is involved in common skin diseases, such as acne vulgaris and androgenic alopecia9. A SG is usually intimately attached to an HF, forming a pilosebaceous unit10. Recent investigations have elucidated SG cellular dynamics during morphogenesis and homeostasis11. Importantly, stem cells of the SG have been identified, with the specific expression of the transcription factor Blimp112. However, SG stem cells have been investigated less thoroughly than other stem cell populations in the HF, largely because of a lack of specific cell surface markers.

Using a reporter mouse model in which GPR39 expression was monitored by LacZ staining, we serendipitously discovered that in murine skin, GPR39 was uniquely expressed in SGs and colocalised with SG stem cells. Furthermore, using a skin wound model, we revealed intriguing spatiotemporal expression dynamics of GPR39 at the wound site and the positive involvement of GPR39 in the wound healing process.
Results

Unique promoter activity and mRNA expression of Gpr39 in SGs. Using a previously described mouse model in which targeted insertion of the lacZ reporter gene disrupted the Gpr39 locus, we monitored the expression of Gpr39 by staining with X-gal. As shown in Fig. 1a, LacZ staining of the tail skin in 20-day-old Gpr39<sup>-/-</sup>lacZ mice indicated specific Gpr39 promoter activity at the openings of SGs (Fig. 1a, top). Gpr39<sup>-/-</sup> skin from negative-control littermate mice showed no such staining (Fig. 1a, bottom). In a separated hair follicle, lacZ<sup>+</sup> cell clusters were surrounded by sebocytes positive for Oil-Red-O (ORO) (Fig. 1b, left panel). After these sebocytes had been removed, the lacZ<sup>+</sup> cell cluster was clearly observed attached to the isthmus of the HF (Fig. 1b, right panel). We performed RT-PCR to examine the mRNA expression of Gpr39 in various epidermal compartments. The separation of the epidermal compartments was verified by the expression of different markers in their corresponding compartments (Fig. S1). As indicated in Fig. 1c, Gpr39 expression was detected in the pilosebaceous unit rather than in the interfollicular epidermis (IFE) (Fig. 1c). RNA in situ hybridisation also confirmed the existence of Gpr39 mRNA within the SG opening (Fig. 1d). lacZ signals were also detected in the dorsal skin and exhibited a temporal upregulation around postnatal Day 24, when hair follicles entered a new anagen phase (Fig. 1e), indicating the cellular dynamics of Gpr39 expression during the hair cycle.

GPR39<sup>+</sup> cells colocalise with Blimp1<sup>+</sup> SG stem cells. GPR39 protein expression in wild-type mice was examined by immunofluorescence staining. As shown in Fig. 2a, the membrane location of GPR39 was clear in the cells at the SG opening, where unipotent Blimp1<sup>+</sup> SG stem cells have been reported to reside<sup>9</sup>. To analyse the relationship between the GPR39<sup>+</sup> cells and the Blimp1<sup>+</sup> cells, we stained with these markers and found that GPR39 and Blimp1 largely marked the same cell population within the SG (Fig. 2b). GPR39 expression was also compared to the expression of the isthmus stem cell marker Lrig1. Whereas Lrig1 staining exhibited a typical isthmus distribution, GPR39<sup>+</sup> cells were observed adjacent to Lrig1<sup>+</sup> cells, with partial overlapping (Fig. 2c). After dispase digestion, we stained GPR39 and Blimp1 in detached SGs (Fig. 2d). These results also verified that GPR39 was expressed in small cells at the SG opening (Fig. 2e) and colocalised with Blimp1 (Fig. 2f). A flow cytometric analysis indicated that the GPR39<sup>+</sup> cells accounted for approximately 0.05% of all epidermal cells (Fig. 2g). In human skin, GPR39 expression was also restricted to the opening of the SG (Fig. 2h). In summary, the cell-specific expression of GPR39 and its colocalisation with Blimp1 suggested that GPR39 may mark a population of putative SG stem cells.

GPR39<sup>+</sup> cells differentiate and generate ORO<sup>+</sup> sebocytes in vitro. We also characterised GPR39<sup>+</sup> SG cells in vitro. Tail SGs from 7- to 8-week-old mice were cultured in vitro. On D0 of culture, the SGs were positive for ORO (Fig. S2b), whereas only the small cells at the SG opening were positive for GPR39 (Fig. S2c). On D2 of culture, the GPR39<sup>+</sup> cells proliferated and migrated outwards (Fig. S2f), whereas the ORO<sup>+</sup> sebocytes remained as clusters (Fig. S2e). After transient trypsinisation, the ORO<sup>+</sup> sebocytes detached from the culture dish, whereas the GPR39<sup>+</sup> cells remained attached (Fig. S2g, h and i). On D5, ORO<sup>+</sup> cells reappeared in the culture, whereas the GPR39<sup>+</sup> cell number had decreased sharply (Fig. S2j, k and l), suggesting that the GPR39<sup>+</sup> cells had differentiated into ORO<sup>+</sup> cells. This in vitro assay implied that GPR39<sup>+</sup> cells are progenitor-like cells that can differentiate into ORO<sup>+</sup> sebocytes.

GPR39 shows dynamic spatiotemporal expression during skin wound healing. The expression pattern of GPR39 suggested that it might play a role in the skin. However, Gpr39<sup>-/-</sup> mice showed no

Figure 1 | Promoter activity and mRNA expression of Gpr39 in the skin. (a) Microscopic observation of LacZ staining in tail skin of Gpr39<sup>-/-</sup>lacZ (top) and Gpr39<sup>-/-</sup> (bottom) mice. Arrows indicate lacZ signals at the openings of SGs. (b) Whole-mount LacZ staining of an isolated intact hair follicle (HF) (left) and an HF with the sebocytes removed (right). The tissues were counter-stained with ORO. (c) RT-PCR examination of Gpr39 and Actb (as an internal control) expression in separate epidermal compartments. NEG, negative control; POS, positive control. (d) RNA in situ hybridisation of Gpr39 in the SG. (e) LacZ staining in the dorsal skin of Gpr39<sup>-/-</sup> mice on different postnatal days. HF, hair follicle; SG, sebaceous gland; Epi, epidermis; HS, hair shaft; P20, postnatal Day 20. Bar (a) = 100 μm, (b) = 20 μm, (d) = 25 μm, (e) = 20 μm.
obvious defects in skin development or homeostasis. Therefore, we created a skin wound environment to further explore the function of GPR39. After making 1/8-inch, circular, full-thickness cutaneous wounds in the backs of \(Gpr39^{-/-}\) mice (7- to 8-week-old), we noted that GPR39 had intriguing expression dynamics in the wound area. On D2 after wounding, \(lacZ\) signals were barely detected in the epidermis (Fig. 3 a, b; first column). However, by D4, \(lacZ\) signals became evident in the epidermis at the edge of the wound (Fig. 3 a, b; second column). Along with the healing, the epidermal \(lacZ\) signals became restricted to the area within the wound on D6 (Fig. 3 a, b; third column). Upon wound closure, LacZ staining in the epidermis had decreased (Fig. 3 a, b; last column). The expression of the GPR39 protein in the wounded epidermis from D2 to D8 was also confirmed by immunostaining (Fig. 3c).

GPR39-deficient mice show a delay in skin wound repair during the intermediate stage. The intriguing expression pattern of GPR39 at the wound site implied a function of GPR39 in wound repair. To assess whether GPR39 was involved in skin wound repair, we made uniform skin wounds (1/8-inch, circular, full-thickness cutaneous wounds) in 7- to 8-week-old \(Gpr39^{-/-}\) mice and in \(Gpr39^{+/-}\) mice in which both \(Gpr39\) alleles had been replaced by \(lacZ\) sequences. We monitored and measured the wound size from D0 to D12 post wounding. Wound closure was slower in the \(Gpr39^{-/-}\) mice than in their wild-type siblings (Fig. 4a). A statistical analysis of wound size change also indicated a significant delay in the wound closure rate in the \(Gpr39^{-/-}\) mice on D6 and D8 (Fig. 4b), indicating that GPR39 contributes to skin wound healing.

Discussion

Although the \(Gpr39\) gene was cloned in 1997, research on its biological function has only emerged over the last 10 years. The vast majority of studies on this newly discovered orphan receptor have focused on its functions in metabolism. Our data on GPR39 expression in mouse skin suggest that GPR39 is a novel surface marker of putative SG stem cells. We also demonstrated that GPR39 is involved in skin wound repair. To our knowledge, the present study is the first report to examine GPR39 in skin tissue.

Several studies had reported the expression of GPR39 in stem/progenitor cell populations. Microarray data have indicated the upregulation of \(Gpr39\) expression in stem-like Wilms’ tumour xenografts, which are highly rich in progenitor cells. In chondrogenic human synovium-derived MSCs, the CpG-rich region of the \(Gpr39\) gene promoter was shown to be hypomethylated, and \(Gpr39\) expression was found to be downregulated upon differentiation towards chondrocytes. Here, using different methods, we clearly demonstrated the expression of GPR39 at the openings of SGs, at the exact position where SG stem cells reside. The colocalisation of GPR39 and the SG stem cell marker Blimp1 in the same cell population and the presence of GPR39– cells adjacent to Lrig1

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Figure 2 | Localisation of GPR39 protein expression in HFs. (a) Immunostaining of GPR39 (green) in the HF and SG. PI was used for counterstaining (red). (b) Costaining of GPR39 (red) and Blimp1 (green) in the HF. Hoechst was used for counterstaining (blue). Inserted in the panel are separate colour channels and an enlarged picture of the SG section. (c) Costaining of GPR39 (red) and Lrig1 (green) in the HF. (d) SGs detached from the tail skin after dispase digestion. (e) Immunostaining of GPR39 in detached SGs. PI was used for counter staining (red). (f) Costaining of GPR39 (red) and Blimp1 (green) in a detached SG. The cells were counterstained with Hoechst (blue). (g) Flow cytometric analysis of epidermal cells using a PE-conjugated GPR39 antibody. The upper panel shows cells stained with the GPR39 antibody, and the lower panel shows the negative control without antibody staining. Inserted in the upper panel is an image of a sorted cell with PE fluorescence. (h) Expression of GPR39 in a human hair follicle. Arrows indicate positive signals. HF, hair follicle; SG, sebaceous gland; Epi, epidermis. Bar (a) = 25 \(\mu m\), (b, c) = 20 \(\mu m\), (d) = 5 \(\mu m\), (e, f, g, h) = 20 \(\mu m\).
Isthmus stem cells further supported GPR39 as a putative signature gene for SG stem cells. Unfortunately, the limited number of GPR39 SG cells and our inability to culture them in vitro made it difficult to fully characterise these cells. However, our preliminary in vitro data support the notion that GPR39 SG cells can differentiate into sebocytes. Interestingly, the expression of GPR39 in SGs in dorsal skin was phase dependent, whereas it was robust in most of the tail SGs. The possible explanation is that tail SGs are more well-developed than dorsal SGs and tail skin has less drastic morphological change during the hair cycle progression compared with dorsal skin. Despite its abundant expression in putative SG stem cells, GPR39 seemed to function only as a marker, rather than as an important regulator: Grp39−/− SGs or epidermis showed no obvious differences from their wild-type counterparts. Intriguingly, several other GPCRs have recently been identified as definitive markers for different stem/progenitor cell populations within the HF, and for epithelial stem cells in other tissues. These GPCRs include the highly concerned leucine-rich repeat-containing GPCRs, LGR4/5/614–16.

GPR39 was previously found to be involved in cell migration and epithelial repair in vitro in HaCaT cells, an immortalised human keratinocyte cell line17. Here, we discovered the role of GPR39 in skin wound healing in vivo. Skin wound healing is a complex biological process involving changes in the epidermis, dermis and vasculature; it is characterised by three continuous, overlapping phases: an inflammatory phase, a proliferative phase and a contraction and remodelling phase18. The proliferative phase further contains two interdependent stages: an initial keratinocyte-mediated stage, in which epidermal cells proliferate and migrate to seal the epithelial barrier, followed by a fibroblast-mediated phase, in which the dermis is remodelled19. Given that GPR39 expression was found only in SGs and wounded epidermis, we consider that the function of GPR39 is also restricted to the epidermis. Interestingly, GPR39 expression in the wounded epidermis was the highest during D4–6 after wounding, when the wounded epidermis exhibited a drastic increase in thickness. These data suggest that GPR39 might be associated with the proliferation and/or migration of epidermal cells during D4–6; as a consequence, GPR39-mutant mice showed significantly impaired wound closure later during D6–8. However because GPR39 was not involved in other events, such as dermal remodelling and contraction, its loss was not sufficient to devastate the entire wound healing process. It is why GPR39-mutant wounds finally closed during D13–14, which is also the case for many other defects18,20.

Figure 3 | Expression dynamics of GPR39 after wounding. (a) Whole-mount LacZ staining in wounded skin of Gpr39lacZ mice on different days after wounding. Inserted in the D4 image is a negative control showing no LacZ staining in Gpr39−/− mice. (b) Microscopic observation of lacZ expression in wounded skin of Gpr39lacZ mice on different days after wounding. The upper panels are magnifications of the framed areas in the lower panels. (c) GPR39 immunostaining on D2, 4, 6 and 8 after wounding. The upper panels are magnifications of the framed areas in the lower panels. Black arrows indicate lacZ+ cells. Asterisks indicate the wound site. Epi, epidermis; HF, hair follicle. Bar = 50 μm.

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Epidermal regeneration after wounding involves the contributions of various stem/progenitor cell populations, depending on the circumstances, although normal epidermal homeostasis is dependent on distinct stem cell populations replacing local dead cells21. The progeny of various stem/progenitor cell populations, depending on the circumstances, is involved in multiple stages of skin morphogenesis and activated GPR391,27,28. In HaCaT cells, GPR39-mediated epithelial repair was also triggered by Zn2+ released from injured cells29. Recent research revealed that GPR39 is a regulator of the Hedgehog pathway29. The Hedgehog pathway, mainly the Sonic Hedgehog (Shh) pathway, is involved in multiple stages of skin morphogenesis and may have the ability to modulate several aspects of wound healing30,31. The Hedgehog pathway is also known to play a key role in regulating SG development32 and the proliferation of SG progenitors33. It is possible that the roles of GPR39 in SGs and epidermal wound repair are associated with the Hedgehog pathway. Nevertheless, to fully dissect the mechanism of GPR39 function in the skin requires exploration of its ligand and intracellular signal transduction.

In summary, our pioneering work exposed a novel role for GPR39 in SGs and epidermal wound repair. Given the importance of GPCRs as cellular drug targets, further comprehensive investigations into GPR39 under skin wounding and pathological conditions might shed light on treatments for cutaneous wound repair and skin disorders such as acne vulgaris.

**Methods**

*Mice.* Mouse husbandry, breeding and use were performed according to the "Guidelines for the Care and Use of Animals in Research" of the Institute of Zoology, Chinese Academy of Sciences. All of the mice were maintained under specific-pathogen-free (SPF) conditions with a constant photoperiod (12L:12D) and free access to water and food. All of the experiments involving the use of laboratory mice were approved by the ethics committee of the Institute of Zoology, Chinese Academy of Sciences.

Heterozygous mice (*Gpr39*+/−) were generated as previously described by Lexicon Genetics, Inc. (Woodlands, TX)16. In these mice, the targeted insertion of the lacZ reporter gene disrupted the Gpr39 locus, leading to the replacement of endogenous Gpr39 expression by lacZ expression. Thus, homozygous mutants (*Gpr39*−/−) were GPR39 knockouts (*Gpr39*−/−). For mouse genotyping, the primers 5′-ACCTCATCCTGTTGACCTC-3′ and 5′-ATGAGGCTCAAACGTGAG-3′ were used to amplify the wild-type-specific band, whereas the primers 5′-GGAACCTCICACTCGAGTGCG-3′ and 5′-GCAAACGATCGCTTCTAT-3′ were used to amplify the mutation-specific band.

β-galactosidase (lacZ) staining and Oil-Red-O (ORO) counterstaining. Epidermal sheets and skin tissues were isolated at the indicated time points from *Gpr39*+/− and *Gpr39*−/− mice. Whole tissues were fixed in a 4% paraformaldehyde (PFA) solution (containing 0.1 mol/L PIPES buffer [pH 6.9], 2 mM MgCl2, and 5 mM EGTA) (Sigma) for 30 minutes at 4 °C. The fixed tissues were rinsed in phosphate-buffered saline (PBS, containing 2 mM MgCl2, 0.1% sodium deoxycholate and 0.02% NP-40, 5 mM potassium ferrocyanide and 1 mg/mL X-Gal) (Sigma) overnight at 37 °C. Alcohol (70%) was used to stop the reaction and to store the tissues.

To accurately localise lacZ expression in the SGs, some epidermal sheets were counterstained with ORO (Sigma). After being pre-incubated in 60% isopropanol for 5 minutes, tissue samples were counterstained in an ORO staining solution containing 0.5% ORO in a mixture of propanol and distilled water at a volume ratio of 3:2. The SGs were clearly observed within 5 minutes because of ORO absorption. The tissue samples were washed with 60% isopropanol to remove any remaining staining solution and were stored in PBS.

For more detailed histological observations, whole-mount-stained tissues were dehydrated with gradient alcohol and were embedded in paraffin. Sections were cut at a thickness of 20 μm. After being deparaffinised and rehydrated, the tissue sections were counterstained with eosin.

**RNA extraction and RT-PCR.** Different regions of the mouse tail epidermis were dissected for RNA extraction and RT-PCR, as indicated in Fig. 1c. The entire epidermal sheet (with hair follicles) was peeled off from the dermis after an overnight incubation with dispase (1 mg/mL, Gibco) at 4 °C. The hair follicles were subsequently dissected from the epidermal sheet with 0.1 mm forceps, with the remaining tissue being the IFE. The RNasy Micro Kit (Qiagen) was used to extract total RNA from the separate epidermal regions described above. cDNA was generated with M-MuLV reverse transcriptase (New England Biolabs). PCR was performed using Promega PCR Master Mix. The PCR cycle for *Actb* (β-actin) or other genes was as follows: 95 °C for 4 minutes; 25 or 30 cycles of 95 °C for 30 seconds, 57 °C or 60 °C for 30 seconds and 72 °C for 30 seconds; and 72 °C for 5 minutes. The primers used to amplify different genes were listed in Table S1. Actb was used as the internal control.

**RNA in situ hybridisation (ISH).** In *in situ* hybridization with digoxigenin-labeled antisense RNA probes was performed on frozen sections as previously described34. The primers used to generate the antisense probes were 5′-GTGGTCTCGGGCGCTC-3′ and 5′-GGCCAGCTGGGCTTCTTCT-3′. The sense probes were used as the negative control.

**Immunofluorescence staining.** Frozen sections (20 μm thick) were fixed in 4% PFA/PBS for 10 minutes at room temperature. After being penetrated with 0.5% Triton for 10 minutes at room temperature, the sections were blocked in PBS containing 5% bovine serum albumin (BSA) or 5% rabbit serum for 1 hour in 37 °C, followed by incubation with the corresponding primary antibodies diluted in 1% BSA/PBS or 1% rabbit serum for 4 hours in a humid chamber. After being washed with PBS three times, the sections were incubated with secondary antibodies diluted in 1% BSA/PBS or 1% rabbit serum for 1 hour in 37 °C. Propidium iodide (PI, Sigma) or Hoechst 33342 (Sigma) was used to counterstain for *in situ* hybridisation experiments.
SG isolation and in vitro culture. After overnight dispase digestion (1 mg/mL in PBS) at 4 °C, Sgs from 7- to 8-week-old mice were easily detached from the tail epidermis. Intact Sgs were subsequently fixed with 4% PFA/PBS for staining or were cultured in DMEM (Gibco) with 20% fetal bovine serum (FBS, Hyclone). Sgs and SG growth were transiently digested with 0.05% trypsin on D2 of culture to remove sebocytes. The cultures were fixed for ORO staining and GPR39 immunostaining on D0, D2 and D5.

Flow cytometry. The entire epidermal sheets (with hair follicles) from the backs of 7- to 8-week-old mice were peeled off from the dermis after dispase digestion and were further trypanosed into single cells and incubated with a PE-conjugated rabbit anti-GPR39 antibody (bs-5789R-PE, Bios) diluted with 5% FBS in PBS. After serial washes, epidermal cells were analysed on a MoFlo flow cytometer.

In vivo wound healing assay and statistical analysis. Gpr39<sup>−/−</sup> mice (n = 5) and their wild-type littermates (n = 5) at 7–8 weeks were used in the skin wound healing assay. Two 1/8-inch, circular, full-thickness cutaneous wounds were made with a punch (FISKARS) on the back of each mouse. The wounds were photographed on Days 0, 2, 4, 6, 8, 10, and 12 post-wounding. The wound area was calculated using NIH ImageJ software and was indicated as the percentage of the area on D0.

A statistical comparison of the wound areas between Gpr39<sup>−/−</sup> and Gpr39<sup>+/+</sup> mice was performed using Student’s t-test (two-tailed), and all the data are presented as the means ± SEM. Differences were considered significant when P < 0.05.

The same procedure was used to generate wounds on the backs of Gpr3<sup>−/−</sup> and Gpr3<sup>+/+</sup> mice. Wounded tissue and the surrounding healthy skin was collected for histological analysis on different days after injury. The collected skin samples were stored at -80 °C for cryosectioning or were fixed in 4% PFA solution for LaC2 staining.

Flow cytometry.

In vivo wound healing assay and statistical analysis.

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