Lgr4 is crucial for skin carcinogenesis by regulating MEK/ERK and Wnt/β-catenin signaling pathways

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Introduction

Lgr4, also known as Gpr48, belongs to the leucine-rich, G protein–coupled receptor family of proteins, and has recently been shown to augment Wnt/β-catenin signaling via binding to Wnt agonists R-spondins. It plays an important role in skin development, but its involvement in skin tumorigenesis is unclear. Here, we report that mice deficient for Lgr4 are resistant to 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced keratinocyte proliferation and papilloma formation. We show that TPA treatment activates MEK1, ERK1/2 and downstream effector AP-1 in wild-type (WT) epidermal cells and mice, but not in cells or mice where Lgr4 is depleted. Wnt/β-catenin signaling is also dramatically activated by TPA treatment, and this activation is abolished when Lgr4 is deleted. We provide evidences that blocking both MEK1/ERK1/2 and Wnt/β-catenin pathways prevents TPA-induced increase in the expression of Ccnd1 (cyclin D1), a known Wnt/β-catenin target gene, and that the activation of MEK1/ERK1/2 pathway lies upstream of Wnt/β-catenin signal pathway. Collectively, our findings identify Lgr4 as a critical positive factor for skin tumorigenesis by mediating the activation of MEK1/ERK1/2 and Wnt/β-catenin pathways.

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All animal experiments were performed with 8-week-old WT and Lgr4 KO mice. Each experimental group consists of 12 female and 12 male mice. Mice were shaved, double-stranded oligonucleotides encoding shRNA were provided with food and water ad libitum, and were housed under standard conditions, with a constant temperature and a 12-h light/dark cycle. All experiments were approved by the Animal Care and Use Committee of East China Normal University.

Skin chemical carcinogenesis

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For fractionation, cells were treated with TPA (200 ng/ml) or the vehicle control for 4 h prior to harvesting. 10^-5 cells were lysed for 10 min on ice in the cytoplasmic lysis buffer (10 mM Hepes at pH 8.0, 15 mM MgCl2, 10 mM KCl, 0.5 mM EDTA, 300 mM sucrose, 0.1% NP-40, 10 mM NaF, 20 mM β-glycerophosphate, 10 mM Na3VO4, 1 × protease inhibitors, 0.5 mM PMSF) for 10 min on ice and then quick-spun for 15 s to collect cytosolic lysate. In addition, 4 × 10^-5 cells were lysed with nuclear lysis buffer (50 mM Hepes at pH 7.9, 250 mM KCl, 0.1 mM EDTA, 0.1% NP-40, 0.1% glycerol, 10 mM NaF, 10 mM Na3VO4, 1 mM DTT, 1 × protease inhibitors, 0.5 mM PMSF) for 30 min on ice. The lysates were spun for 20 min at 15,000 rpm at 4°C to collect nuclear lysates. Lysates were then run in SDS-PAGE for Western blot analysis.

Statistical analysis

All data are presented as mean ± SD. Student’s t-test was performed to determine the statistical significance of differences between the groups. p < 0.05 was considered statistically significant.

Results

Lgr4 is crucial for DMBA/TPA-induced skin tumorigenesis in mice

To explore the function of Lgr4 in skin tumorigenesis, we followed DMBA-initiated and TPA-promoted two-stage chemical carcinogenesis protocol and compared tumor incidence and tumor multiplicity of WT and Lgr4-deficient mice. WT mice developed papillomas as early as 8 weeks after TPA treatment. By 12 weeks post-DMBA/TPA administration, 90% of the WT mice had developed papillomas. In contrast, of a total of 12 KO-mice examined, only 1 developed a tumor-like structure at 15 weeks after TPA treatment (Fig. 1a and b). On average, Lgr4^-/- mice developed 5.02 tumors per mouse with 20 weeks of TPA treatment, whereas the average number of tumor-like structures formed per Lgr4^-/- mouse is only 0.08 (Fig. 1c). The tumor-like structures in Lgr4^-/- mice were also considerably smaller than the tumors in Lgr4^+/+ mice (Fig. 1d). All skin tumors derived from Lgr4^-/- mice were well-differentiated squamous cell papillomas (Fig. 1e), albeit with different histopathological grades (Supplemental Fig. S1). In contrast, the tumor-like growths produced by the KO mice contained only a hyperproliferative epidermis (Fig. 1e). Thus, loss of Lgr4 renders the mice near-completely resistant to chemically induced skin tumorigenesis.

Lgr4 deficiency reduces TPA-dependent epidermal hyperplasia and keratinocyte proliferation

To understand the mechanism underlying the resistance to carcinogenesis in Lgr4 KO mice, we examined the effects of short-term TPA treatment on epidermal cell proliferation. As shown in Fig. 2a, Lgr4^-/- mouse skin treated with TPA for one week or two weeks exhibited a marked increase in epidermal thickness compared with Lgr4 WT mouse skin. The numbers of cells positive for proliferating cell nuclear antigen (PCNA) or for Ki67 was significantly higher in Lgr4 KO skin than that in the WT (Fig. 2b and c). Thus, the resistance to tumor formation in Lgr4 KO mouse skin correlates with a decrease in epidermal cell proliferation.

To further determine the effects of Lgr4 on epidermal cell proliferation, we used a shRNA knockdown approach to deplete endogenous Lgr4 protein in A431 cells, a human epidermoid carcinoma cell line (Supplemental Fig. 2a and 2b). As shown in Fig. 3a, Lgr4 knockdown resulted in a dramatic reduction in cell number. Moreover, flow cytometric analysis revealed a significant reduction in the number of cells progressing through G1 (Fig. 3b). In addition, Lgr4 knockdown showed an approximate 50% reduction in cloning efficiency in soft agar when compared with control cells (Fig. 3c and d). Importantly, primary keratinocytes derived from Lgr4 KO mice also showed decreased proliferation compared with their WT counterparts (Fig. 3e).

Resistance to tumorigenesis in Lgr4-deficient mice correlates with reduced MEK/ERK signaling

It is known that mice lacking epidermal Mek1 protein develop fewer papillomas than WT mice following DMBA/TPA treatment.
To address how Lgr4 affects skin keratinocyte proliferation, we measured the activity/level of MAPK pathway components. As shown in Fig. 4a, marked elevation of p-ERK1/2, p-c-fos and p-c-jun levels was observed in negative control shRNA (shNC) A431 cells after TPA treatment, whereas shLgr4 cells were less responsive. Consistently, the extent of activation of MEK1/ERK/AP-1 in primary keratinocytes from Lgr4−/− was significantly lower than that in those from control littermates (Fig. 4b).

We next compared MEK/ERK pathway status in WT and Lgr4 KO mice. The levels of phosphorylated MEK1 and ERK1/2 were significantly increased in WT mice following TPA treatment (Fig. 4c). The levels of AP-1 proteins (c-fos and c-jun) were also increased. In Lgr4 KO mice, however, TPA treatment induced only a slight alteration in the levels of p-MEK1 and p-ERK1/2 (Fig. 4c). Together, our results indicate that Lgr4 deficiency compromises the TPA-induced activation of MEK1/ERK1/2 signaling both in vitro and in vivo.
Lgr4 is required for maximal Wnt/β-catenin signaling in skin cells

It has been shown that the β-catenin/TCF pathway is constitutively activated in non-melanocytic skin tumors in the two-stage chemical carcinogenesis model [13]. We thus investigated the relationship between Lgr4 and Wnt/β-catenin signaling in our model. As shown in Fig. 5a, primary keratinocytes derived from WT mice showed an obvious increase in the level of β-catenin protein after TPA treatment, whereas cells from Lgr4 KO mice were insensitive to TPA in terms of β-catenin elevation. GSK3β is known to phosphorylate β-catenin and cause its degradation [22]. Phosphorylation of GSK3β on Ser9 blocks the interaction between GSK3β and β-catenin, thereby increasing β-catenin stability [23]. We therefore asked whether Lgr4 deletion affects β-catenin protein level by regulating Ser9 phosphorylation of GSK3β. Indeed, p-GSK3β (Ser9) level was greatly increased in keratinocytes from WT mice after TPA treatment, whereas the extent of increase was significantly reduced in Lgr4 KO keratinocytes (Fig. 5a). Consistently, TPA treatment markedly enhanced β-catenin and p-GSK3β (Ser9) levels in WT skin, but not in skin of Lgr4 KO mice (Fig. 5b). Interestingly, even under physiological (untreated) conditions, the levels of β-catenin and p-GSK3β (Ser9) were slightly higher in WT skin than in Lgr4 KO skin (Fig. 5b). Moreover, in A431 cells, TPA elicited an increase in β-catenin and p-GSK3β (Ser9) levels in a time-dependent manner, but this increase was not obvious when Lgr4 was depleted (Fig. 5c).

To further determine the effects of Lgr4 on β-catenin activity, we performed indirect immunofluorescence to examine β-catenin accumulation in primary keratinocytes. As expected, TPA treatment induced a more prominent nuclear accumulation of β-catenin in keratinocytes from WT mice than keratinocytes from the Lgr4 KO mice (Fig. 5d and e). Similarly, TPA induced more obvious nuclear accumulation of β-catenin in shNC A431 cells than shLgr4 cells (Fig. 5f and g). These results demonstrate a cell-autonomous inhibition of Lgr4 deficiency on TPA-induced β-catenin accumulation in skin epidermal cells. Results of cell fractionation experiments corroborated this notion, as after TPA treatment, a higher increase in the level of nuclear β-catenin was detected in WT than Lgr4 KO primary keratinocytes (Fig. 5h).

In addition, we examined the expression of β-catenin target genes by RT-qPCR. The levels of Ccnd1 and c-Myc transcripts were considerably lower in Lgr4 KO skin compared with WT skin, especially after TPA treatment (Fig. 5i). Consistently, the expression of CCND1 and c-MYC was markedly increased in shNC A431 cells after TPA treatment compared with shLgr4 cells (Fig. 5j). Together, these in vivo and in vitro findings show a dependence of Wnt/β-catenin signaling output on Lgr4.

Both MEK/ERK and Wnt/β-catenin signaling contributes to skin cancer formation

Given that both ERK1/2 and β-catenin pathways were activated by TPA in WT skin, we wondered about their possible relationship. In WT mouse primary keratinocytes, ERK activation occurred at 4 h and peaked at 6 h after TPA treatment (Fig. 6a). The accumulation of β-catenin followed the same time course. In human SCC samples, higher expression of both p-ERK and β-catenin was observed compared with normal skin. Moreover, Lgr4 showed a positive correlation with both p-ERK and β-catenin in human SCC samples (Fig. 6b).

Next, we examined the interdependence of the two signaling pathways by using specific pathway inhibitors. As shown in Fig. 6c, MEK/ERK inhibitor U0126 significantly blocked TPA-induced ERK1/2 activation in primary keratinocytes derived from both WT and Lgr4 KO mice. Interestingly, p-GSK3β and β-catenin were also markedly reduced in level by U0126. Moreover, TPA-induced increase in cyclin D1 protein was greatly inhibited by U0126 (Fig. 6d). Similar results were obtained in A431 cells upon Lgr4 knockdown (Fig. 6d). On the other hand, although β-Catenin/TCF inhibitor FH535 effectively blocked the increase in cyclin D1 protein level, p-ERK level was not altered (Fig. 6c).

The above findings suggest a dependence of β-Catenin/TCF pathway activity on MEK/ERK signaling in epidermal cells. To further test this hypothesis, we next attempted to reduce the
expression of ERK1/2 by using shRNA-expressing lentiviruses. Both shERK1 and shERK2 effectively reduced the levels of p-ERK1/2 in primary keratinocytes (Fig. 6e). Consistent with data described above, TPA treatment significantly increased p-ERK and β-catenin levels in primary keratinocytes. Depletion of ERK1/2 resulted in a marked reduction of β-catenin levels in these cells (Fig. 6e). Furthermore, both U0126 and FH535 inhibited the expression of cyclin D1 as well as TPA-induced proliferation of A431 cells (Fig. 6f). Taken together, these results show that MEK/ERK activation lies upstream of β-catenin signal pathway in normal and cancerous epidermal cells.

Discussion

Our work presents the detailed functional study on the involvement of Lgr4 in skin tumorigenesis. Specifically, we show that Lgr4 is required for papilloma formation in a two-stage skin carcinogenesis mouse model. Moreover, we provide both in vitro and in vivo evidences suggesting that Lgr4 likely does so by promoting TPA-induced keratinocyte proliferation and by regulating MAPK/ERK and Wnt/β-catenin signaling pathways.

The mitogen-induced ERK/MAPKs are linked to cell proliferation and survival [24]. ERK1 and ERK2 are activated by mitogens and are upregulated in several types of human tumors. Mutant BRAFV600E acts as a homodimer to drive consecutive ERK pathway activation, leading to the formation of melanoma, thyroid and colon cancer [25]. TPA can substitute for diacylglycerol (DAG) for activating protein kinase C (PKC) and is used as a classical skin tumor promoter. PKC is well known as an activator of the ERK pathway [26]. In WT mice, TPA treatment markedly increased the activation of MEK1 and ERK1/2, while it failed to do so in Lgr4 KO mice. These results suggest that Lgr4 is required for TPA-induced MEK/ERK activation during skin tumor formation. Consistently, Wang et al. also found Lgr4 to mediate keratinocyte proliferation by increasing the phosphorylation of EGFR, ERK and STAT3 [27]. A mechanistic understanding of exactly how Lgr4 regulates the ERK pathway is still lacking and awaits future work that lies outside the scope of this study.

Several previous studies showed that Lgr4 plays an important role in multiple organ development processes and in promoting colon cancer cell proliferation/metastasis by activating Wnt signaling [18,20,28]. In the present study, we found that Lgr4 deletion negatively impacts TPA-induced nuclear accumulation of β-catenin and expression of β-catenin target genes, raising the possibility that activation of β-catenin at least in part mediates the role of Lgr4 in accelerating skin tumor formation. A possible sequence of events in WT mice is that TPA stimulation upregulates ERK activity, which in turn leads to the phosphorylation of GSK3β and consequently increased Wnt/β-catenin pathway activity. Our data suggest that Lgr4 impinges on the early step(s) of this

![Fig. 5](image.png)

**Fig. 5.** Lgr4 is required for TPA-induced changes in Wnt/β-catenin pathway components. (a) Lgr4 deletion inhibited TPA-induced upregulation of β-catenin expression. Primary keratinocytes were treated with or without TPA for 4 h, and cell lysates were subjected to immunoblotting analysis. (b) Immunoblot analysis of β-catenin and p-GSK3β (Ser9) in the skin treated with or without TPA from WT and Lgr4 KO mice. (c) Knockdown of Lgr4 by shRNA inhibited the TPA-induced activation of Wnt/β-catenin signaling. A431 cells were treated with or without TPA for specified time points, and cell lysates were subjected to immunoblotting analysis. (d) Immunofluorescence for β-catenin in primary keratinocytes treated with or without TPA for 4 h. Cells were counterstained with DAPI (blue). Scale bars, 100 μm. (e) Primary keratinocytes with β-catenin’ nuclei per group were quantified. Data are mean ± SD from three independent experiments; *p < 0.05, **p < 0.01. (f) Immunofluorescence for β-catenin in A431 cells treated with or without TPA for 4 h. Cells are counterstained with DAPI (blue). Scale bars, 100 μm. (g) A431 cells with β-catenin’ nuclei per group were quantified. The data were expressed as numbers of nuclear β-catenin per 100 cells from three independent experiments; *p < 0.05, **p < 0.01. (h) Primary keratinocytes treated with or without TPA for 4 h were fractionated into nuclear and cytoplasmic fractions, and 50 μg of protein for each fraction was analyzed by Western blotting for β-catenin, actin, and histon H3. Actin was used as a loading control, and histon H3 for purity of the nuclear fraction. (i) Quantitative RT-PCR analysis of c-Myc and Ccnd1 in the skin treated with or without TPA from WT and Lgr4 KO mice. (j) A431 cells were treated with or without TPA for 4 h, and cells were subjected to real-time PCR analysis for c-Myc and Ccnd1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 5. (continued).
sequence. The function of Lgr4 in regulating both ERK and β-catenin signaling is reminiscent of that of its homolog, Lgr5, which has been shown to regulate pro-survival MEK/ERK signaling and proliferative Wnt/β-catenin signaling in neuroblastoma [29].

We found that ERK inhibitor and siRNA effectively suppressed TPA-induced increase in β-catenin, whereas a β-catenin/TCF inhibitor did not alter p-ERK level. These data suggest that activation of MEK/ERK signaling lies upstream of activation of Wnt/β-catenin signaling during chemically induced skin tumorigenesis. In the absence of Lgr4, ERK is not activated by TPA treatment and β-catenin activation is also compromised. These findings are consistent with a model where Lgr4 positively regulates Wnt/β-catenin signaling pathway during skin tumorigenesis in a manner that requires the activation of MEK/ERK signaling.

It is well known that the phosphorylation of β-catenin by GSK3β triggers its degradation. In our study, the levels of p-GSK3β (ser9) positively correlated with β-catenin accumulation after TPA treatment in vitro and in vivo. It has been reported that the ERK1/2
cascade is associated with phosphorylation of GSK3β at Ser9, resulting in inactivation of GSK3β and upregulation of β-catenin [30,31]. Thus, it seems plausible that TPA-induced activation of ERK in the skin of WT mice contributes to the phosphorylation of GSK3β and consequently β-catenin accumulation. AKT also has the potential to promote the phosphorylation of GSK3β at Ser9 [32]. However, we did not find any obvious change in p-AKT level in keratinocyte after TPA treatment.

Together, our study identifies Lgr4 as a critical promoting factor of skin carcinogenesis and a potential therapeutic target that may be explored in the future for skin cancer therapy. Heterozygous loss of LGR4 in human is reported to be associated with increased risk of skin SCC [33], a finding that is in apparent conflict with our results. Towards verifying the role Lgr4 in human skin cancer, we examined its expression in human normal skin, SCC, basal cell carcinoma (BCC), and melanoma samples by immunohistochemical staining. Lgr4 protein was seen in the basal layer of normal skin, as well as in the basal layer-derived regions of early-stage SCC and BCC samples [Supplemental Fig. 3]. However, Lgr4 expression was barely detectable in the advanced cancer tissues of SCC and BCC. In human melanoma tissues, Lgr4 showed more positive staining than advanced SCC and BCC [Supplemental Fig. 3]. It is important to note that exposure to ultraviolet (UV) radiation is a major risk factor for most human skin cancers, and as such human skin carcinogenesis presents some differences from DMBA/TPA-induced skin carcinogenesis in the mouse model. Future studies are necessary to elucidate the detailed molecular mechanism of Lgr4 action in human SCC.

In conclusion, our data support a model in which Lgr4 promotes skin tumor formation in a DMBA/TPA animal model by activating MEK/ERK and Wnt/β-catenin signaling pathways. In the skin of Lgr4 WT mice, TPA stimulation upregulates ERK activity, leading to phosphorylation of GSK3β and increased activity of Wnt/β-catenin signaling.

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Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2016.09.005.

References


Fig. 7. A proposed model for the role of Lgr4 in skin carcinogenesis. Lgr4 promotes skin tumor formation in a DMBA/TPA animal model by activating MEK/ERK and Wnt/β-catenin signaling pathways. In the skin of Lgr4 WT mice, TPA stimulation upregulates ERK activity, leading to phosphorylation of GSK3β and increased activity of Wnt/β-catenin signaling.