Aberrant Wnt signalling induces comedo-like changes in the upper hair follicle

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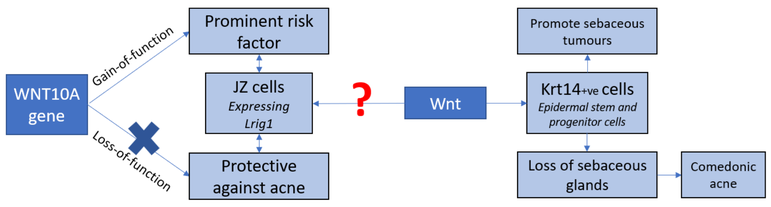
Introduction

Acne is a significant public health problem with current treatments having significant downsides. Development of new treatments for acne has been inhibited by little insight into the pathogenesis of acne. Acne comedones are formed from abnormal differentiation and proliferation of junctional zone (JZ) cells, causing atrophy of sebaceous glands (SGs) and comedones formation. The JZ contains progenitor cells for SGs. Currently, it is hypothesised that abnormal proliferation of the cells of the junctional zone predisposes comedo formation.

A genome-wide association study in humans suggested that loss-of-function of WNT10A, an important signal for stem cells, was protective against acne. These support the involvement of JZ stem cells in the pathogenesis of acne. However, while Wnt is known to be important in acne formation, the exact role of Wnt in the upper follicle pathway is not fully understood. This is possibly due to the overly broad targeting of Wnt signalling on multiple epidermal stem cells.

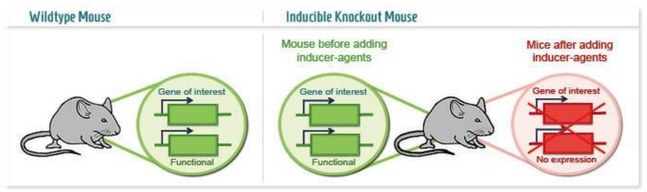
This paper aims to test the hypothesis that Wnt signalling controls the fate of JZ stem cells. This is done through modulating cells in the junctional zone through gain and loss-of-function of Wnt signalling. Wnt activation will be done through deletion of *Apc*. Wnt inhibition will be done through deletion of β-catenin.



Materials and methods

Phase 1: Modulating Wnt signalling in Lrig1-CreERT2 mice via topical applications on the dorsal

- Lrig1 is a gene expressed by progenitor cells in the junctional zone of hair follicles
- In Lrig1-CreERT2 mice, the Lrig1 promoter drives expression of the Cre recombinase specifically in junctional zone progenitor cells, when tamoxifen is given. The Cre recombinase subsequently excises LoxP sites that have been inserted into the target gene, constituting a conditional knockout.
  - Lrig1-APC mice: Gain of function in Wnt signalling
  - Lrig1-βcat mice: Loss of function in Wnt signalling



Phase 2A: Harvesting of dorsal skins to obtain tissue sections for histology, immunofluorescence and RNA in situ hybridization

- External inspection of mice
  - Autopsy performed for APC cKO mice, which died within 11 days of induction due to stem cell disruption in the intestine
- Microscopic imaging to study morphology of hair follicle (HF), comprising junctional zone, infundibulum and sebaceous glands
- Pattern of distribution of immunostained cell proliferation markers (Ki67) within the epidermis

Phase 2B: Quantification of sebaceous gland volumes and infundibulum dimensions

- Statistical analysis of quantitative data

Results and Analysis

Modulation of *Wnt* signalling results in contrasting upper hair follicle and sebaceous gland phenotypes

Lrig-Apc mutants:

- <u>*Upper hair follicle*</u>: the JZ began to dilate on Day 7 (with upregulated Axin2 expression, a reliable indicator of *Wnt* activity). This progressed to massive cysts, mainly confined in the JZ while infundibulum thickness did not differ significantly from controls. (Figure 1,3)
- <u>*Sebaceous gland*</u>: significantly smaller than that of controls ($0.62*10^5$ μm^3 compared to $0.89*10^5$ μm^3) (Figure 1,3)

Lrig-βcat mutants:

- <u>*Upper hair follicle*</u>: elongated and hyperplastic infundibulum and JZ in 4 weeks of induction (Figure 2,4)
- <u>*Sebaceous gland*</u>: twice the size of that in controls (mean volume of $1.6*10^5$ μm^3 versus $0.8*10^5$ μm^3) (Figure 2,4)

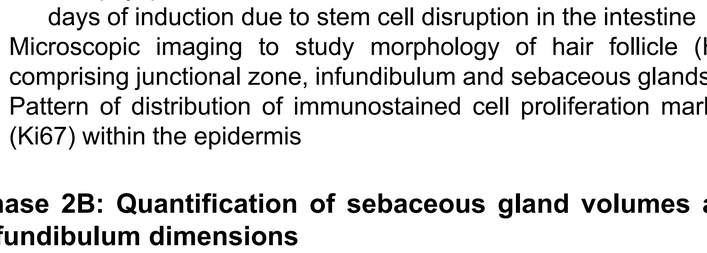


Figure 1: H&E stained sections of dorsal skin from Lrig1-Apc mutant and control mice (APC control) 7 days and 11 days after 4-OHT treatment.

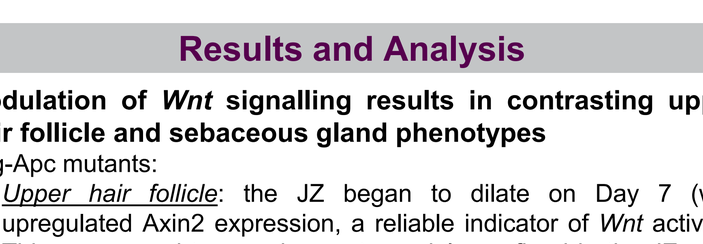


Figure 2: H&E stained sections of dorsal skin from Lrig1-βcat mutant and control mice (βcat Ctrl) 2 weeks and 4 weeks after 4-OHT treatment.

Results and Analysis

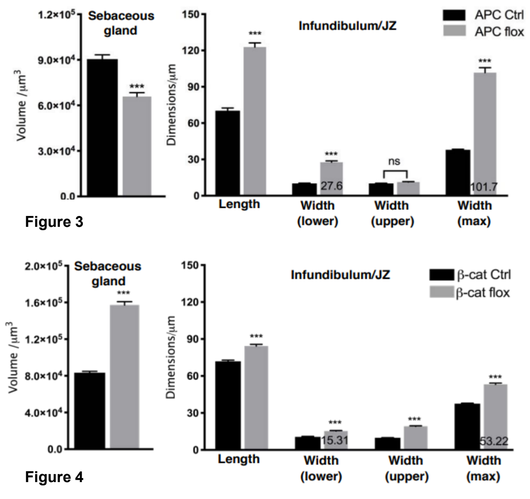


Figure 3

Figure 4

Figure 3 and 4: Quantifications of infundibulum/junctional zone dimensions and the sebaceous glands volumes. Length: measurement from IFE to the point of SG duct connecting to the HFs; Width (lower): thickness of cell layers in the junctional zone; Width (upper): thickness of the cell layers in the infundibulum; Width (max) maximum diameters of the JZ cysts. N=60-180 imaged hair follicles from 2-5 mice in each group, respectively. N=110-290 sebaceous glands from 2-5 mice. Data are mean±SEM. p<0.001.

Wnt-activated junctional zone cysts contain large number of cells expressing stem cell marker *Lgr6* and *Lgr5* (Figure 5)

Lgr6, a *Wnt* signalling pathway mediator and stem cell marker, is expressed in hair follicle isthmus stem cells that give rise to HFs, SGs and IFE.

- Lrig1-Apc skin: *Lgr6* was strongly upregulated in the basal cells of the junctional zone cysts
- Lrig1-βcat mutant skin: *Lgr6* expression did not change appreciably

Lgr5, which marks stem cells in the hair bulge and has likewise been reported as a *Wnt* target gene, is not typically expressed in the JZ.

- Lrig1-Apc skin: *Lgr5* expression was increased
- Lrig1-βcat mutant skin: *Lgr5* expression was unchanged

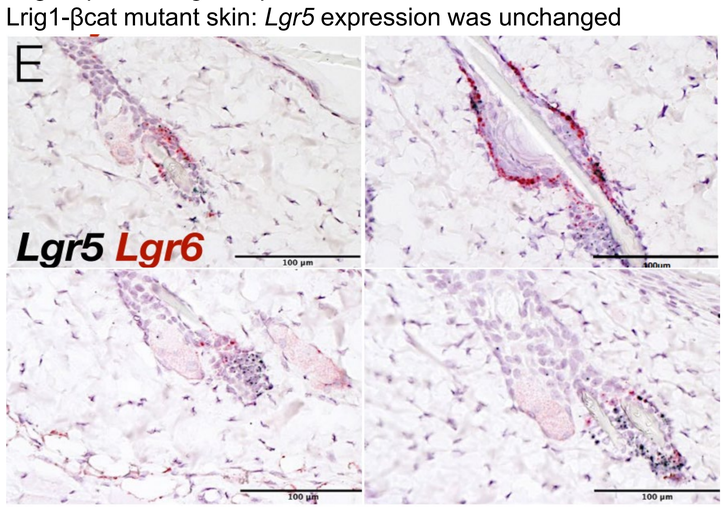


Figure 5: Representative images of RNA in situ hybridization for *Wnt* targeted genes: Lgr6 and Lgr5 as performed on Lrig1-Apc and Lrig1- βcat mutants and controls.

Contributions of *Lrig1*$^{+ve}$ stem cells upon deletion of *Apc* or *Ctnnb1* gene

To determine if the modulation of *Wnt* signalling indeed affects the movement and contribution of *Lrig1*$^{+ve}$ stem cells to the upper pilosebaceous unit (PSU) lineages, lineage tracing was done. Lineage tracing, whereby a cell is identified by expression of a reporter gene, was conducted by tracing mGFP^{+ve} (GFP: green fluorescent protein) cell distribution (supported by red fluorescence of membrane tdTomato protein). (Figure 6)

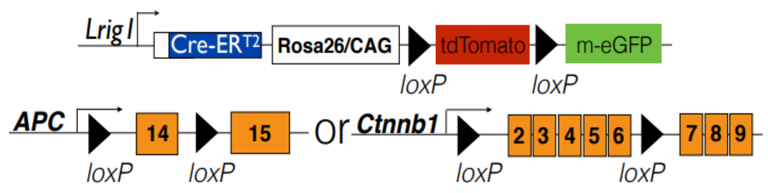


Figure 6: Schematic representation of the genetic elements for lineage tracing

<u>Lineage tracing results on day 11 (Figure 7)</u>

- *Lrig1-Apc-mTmG*: mGFP^{+ve} cells remained mostly restricted to the JZ cyst (with high *Axin2* expression in basal cells of JZ cysts and low Axin2 expression in differentiated sebocytes) and contributed less to SGs than was the case in controls
- *Lrig1-βcat-mTmG*: mGFP^{+ve} stem cells that had lost β-catenin contributed extensively to the entire upper PSU, including the elongated INF, JZ and enlarged SG

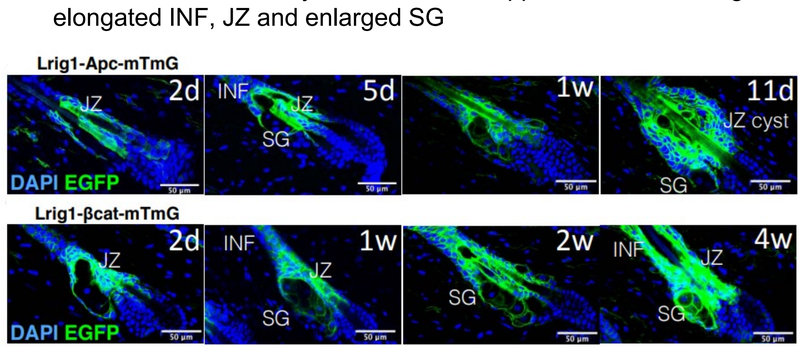


Figure 7: Dorsal skins collected at serial time points as indicated after 4-OHT treatment and stained with DAPI, a blue-fluorescent DNA stain.

Hence, *Wnt* signalling maintains JZ cells in a proliferative stem cell state, and loss of *Wnt* signalling drives fate commitment and differentiation toward the specified cell lineages in the entire upper PSU, including the elongated INF, JZ and enlarged SG.

Sonic hedgehog (*Shh*) signalling drives *Lrig1-Apc* junctional zone cyst formation

To investigate which other signalling pathways might be implicated in Wnt-induced cyst formation, expressions of several genes were tracked. Sonic hedgehog (*Shh*) is a well-known target of canonical Wnt signalling in the skin.[1] Indian hedgehog (*Ihh*) and Desert hedgehog (*Dhh*) are other signalling molecules in the Hedgehog (Hh) family. *Wnt* signalling is known to upregulate *Smo* expression[2] (which in turn encodes for hedgehog signalling transducer). *Gli1* and *Ptch1* are Hedgehog target genes.

Result and Analysis

	Hedgehog ligand and pathway target genes					
	Shh	Ihh	Dhh	Smo	Ptch1	Gli1
Lrig-Apc mice	++	-	-	+	++	++
Lrig-βcat mice	-	-	-	-	-	-

++: Markedly increased expression relative to control
+: Increased expression relative to control
-: Little or no change in expression relative to control

The data suggests that upregulated Wnt signalling activates *Shh* ligand production and the Hedgehog signalling cascade, which may lead to JZ cyst formation. *Smo* antagonist TAK-441 treatment is used to inhibit *Smo* activity during the treatment period. Since *Smo* encodes the Hedgehog signalling transducer, an inhibition of *Smo*'s activity tests for the effects of the absence of the Hedgehog signalling cascade on cyst formation.

	Cell layers (number and thickness)	Maximum cyst diameter	Length of upper PSU	Axin2	Shh	Smo	Ptch1	Gli1	Lgr6
Lrig-Apc mice (TAK-441 treatment)	---	---	-	-	-	--	---	---	---

Control mice (no TAK-441 treatment) demonstrated minimal changes in terms of length of upper HF, width of JZ and maximum diameter.

++: Markedly increased relative to control
+: Increased relative to control
-: Little or no change relative to control
--: Decreased relative to control
---: Markedly decreased relative to control

The data suggests that Wnt-induced cyst formation is mediated at least in part by Shh signalling.

All-trans retinoic acid (atRA) rescues the Lrig1-Apc mice phenotype

atRA, a metabolite of vitamin A_1, is a component of established treatment of comedonic and inflammatory acne[3] and is known to antagonise *Wnt* and Hedgehog signalling.[4]

	Axin2	Lgr6	Shh	Ptch1	Gli1	βcat	Dkk4
Lrig-Apc mice (atRA treatment)	--	--	--	--	--	--	+

+: Increased expression relative to control
-: Little or no change in expression relative to control
--: Decreased expression relative to control

The data reflects the inhibitory effect of atRA on key Hedgehog ligand and pathway genes. Qualitatively, Lrig1-Apc mice treated with atRA also demonstrated reduced dilation in upper HFs and smaller cysts.

Discussion

The study indicates that loss of Wnt signalling causes enlargement of JZ, infundibulum and SGs. Gain of function of Wnt signalling, on the other hand, leads to cyst formation in JZs and shrinkage of its corresponding SGs. The lower part of the hair follicle is unaffected. The relationship between Wnt signalling and JZ, SGs can be explained by the following:

(1) Wnt signalling upregulates *Lgr6* in JZ basal cells, which further enhances Wnt/*β*-catenin signalling.
(2) Other transcription factors such as *c-Myc* work alongside *β*-catenin in determining JZ identity. *c-Myc* was investigated due to its similar involvement in Wnt/*β*-catenin signalling in colorectal cancer. *c-Myc* was upregulated in both gain- and loss of function conditions. This can be attributed to the bidirectional nature of *c-Myc* response to Wnt signalling. The data also suggests that other cofactors and multiple regulatory pathways are present.
(3) Shh signaling contributed partly to driving Lrig1-*Apc* junctional zone cyst formation, with the Smoothened inhibitor effectively downregulating phenotype development. This can be seen in the shrunken SGs upon Hedgehog activation induced by *Ptch1* ablation in Lrig+ve cells.

Limitations in the study

(1) *Lrig1* was expressed in basal layer of SGs and hair bulge, making it difficult to exclude the influence of Lrig1 bulge stem cells on JZ cyst formation.
(2) Deletion of *Apc* was used instead of β-catenin so as to exclude the latter's role in cell adhesion. This, however, may be confounded by involvement of *Apc* deficiency in β-catenin independent processes, such as YAP activation in promoting cell proliferation.

Overall, our data supports the hypothesis that comedo formation occurs due to aberrant JZ differentiation. Acne interventions can target Wnt and Shh signalling for better therapeutic outcome. Pre-existing acne therapies such as atRA have proven so by ameliorating comedo-like Lrig-Apc JZ cysts in APC cKO mice.

Acknowledgements

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