

# Scientific Studies

## TrichoTech™



# ***In Vitro* Effects of the Phytocomplex TrichoTech™ on Human Fibroblasts: Proliferative Potential and Effects on Gene Expression of FGF-7 and FGF-10**

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## **Abstract**

The human hair follicle, a mini-organ formed with neuroectodermal-mesodermal interaction, is a complex structure, in the active steady state (anagen) the dermal papilla can be considered as a ball of extracellular matrix, surrounding specialized fibroblasts. The cross-talk of dermal papilla with neighbouring matrix cells results in the maintenance of hair fibre production. This study aimed to investigate the proliferative potential of the compound Trichotech™, a phytocomplex obtained from a mixture of essential oils, on cultured human fibroblasts and its ability to modulate the gene expression of FGF-7 and FGF-10. Trichotech™ was shown to enhance fibroblasts proliferation in concentrations of 0.5% to 2.0%, and also increase the percentage of cells in the S/G2/M phases of the cell cycle. Trichotech™ at both 1.0% and 2.0% induced a statistically significant effect on wound healing assay compared to the untreated control. We examined the interaction between cell survival (PI3K/Akt) and mitogenic (Ras/MAPK) signal transduction pathways after Trichotech™ treatment (1.0% and 2.0%) on the fibroblast cell line. Trichotech™ caused phosphorylation of ERK1/2, as well as greater phosphorylation of MEK in comparison with both the untreated control and ERK1/2. PI3K and AKT, however, were not shown to be significantly more phosphorylated following Trichotech™ exposure. To verify the relative expression of

mRNA for FGF-7 and FGF-10 genes, a real-time polymerase chain reaction (qPCR) protocol was used. Results show the increase in mRNA expression by fibroblasts after treatment with Trichotech™. In both concentrations tested, Trichotech™ was found to increase the expression of FGF-7 and FGF-10. Sirius red staining allows for rapid assessment of collagen content, it showed a significant increase in collagen content in treated fibroblasts. Further investigation concerning Trichotech™ could be helpful towards the development of new bioactive phytocomplexes for dermatological and trichological use.

## Keywords

Trichotech™, Fibroblasts, Proliferation, FGF-7, FGF-10

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## 1. Introduction

The human hair follicle, a mini-organ formed with neuroectodermal-mesodermal interaction [1], is a complex structure consisting of an outer root sheath, an inner root sheath, the hair shaft, the bulge and the sebaceous gland [2].

The follicle undergoes successive steps of fibre production, regression and rest, which in humans last for an average of 3 years, 3 weeks and a few months, respectively. An additional phase involving the active release of the club fibre has also been described, and is thought to be independent from the rest of the hair cycle [3], while bearing no direct consequence on fibre production initiation [4].

Human hair follicle dynamics are regulated through a bi-stable equilibrium state, including an active steady state (the anagen stage) and a resting steady state (the telogen stage); the transition between these two steady states involves either a degradation phase (the catagen phase) or a neo-morphogenesis phase (the neogen phase). It is now believed that mesenchymal and epithelial oscillators control the stochastic autonomous switching between these two steady states [5].

In the active steady state (anagen), the dermal papilla can be considered as a ball of extracellular matrix, surrounding specialized fibroblasts. The cross-talk of dermal papilla with neighbouring matrix cells results in the maintenance of hair fibre production [6].

The dermal papilla maintains bulge stem cells and secondary hair germ cells quiescent during telogen through production of bone morphogenetic protein 4 (BMP4) and fibroblast growth factor 18 (FGF-18). Cell proliferation during anagen is triggered via production of BMP inhibitors (e.g. *Sosrdc1* and *Bmbi*), as well as secretion of FGF-7 and FGF-10 [7] [8]. Thus, a combination of factors secreted by dermal papilla fibroblasts generates a signaling environment that dictates whether hair follicles will remain dormant or enter the anagen stage.

In a recent study, [9] found that topical application of FGF-10 was able to induce significant hair growth in C57BL/6 mice, linked to induction of anagen phase and increase in the number of hair follicles due, at least in part, to upre-

gulation of  $\beta$ -catenin and Shh signaling.

The use of certain phytochemicals as stimulants of hair growth has been considered an effective secondary measure for the treatment of hair loss, especially when common first-line treatments such as minoxidil application or finasteride administration yield poor results or cause adverse reactions. Many plant extracts and fractions thereof have been shown to elicit hair growth in mice [10] [11] [12], thus the prospecting of plant extracts as a source of hair growth-promoting compounds is a promising strategy.

The present study aimed to investigate the proliferative potential of the compound Trichotech™, a phytocomplex obtained from a mixture of essential oils, on cultured human fibroblasts and its ability to modulate the gene expression of FGF-7 and FGF-10, as well as to propose further applications on hair growth.

## 2. Materials and Methods

### 2.1. Chemicals

Propidium iodide, Direct Red 80 (Sirius Red) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The AnnexinV/FITC Apoptosis Detection Kit was obtained from BD Pharmingen (CA, USA). Iscove's Modified Dulbecco's Medium (IMDM) and all cell culture reagents were purchased from Life Technologies (Thermo Fisher Scientific, USA). MEK, ERK, PI3K and AKT primary antibodies and Alexa Fluor 488-conjugate monoclonal antibodies were acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

### 2.2. Cell Culture

CCD-1072Sk (ATCC® CRL2088™) fibroblasts were cultured in ISCOVE'S medium with 10% fetal bovine serum, 0.292 g/l L-glutamine, 1.0 g/l D-glucose, 2.2 g/l NaHCO<sub>3</sub>, 10.000 UI penicillin, and 0.060 g/l streptomycin. Cells were kept in 25 cm<sup>2</sup> flasks (1 × 10<sup>5</sup> cells/ml) in a humidified incubator at 37°C with an atmosphere of 5% CO<sub>2</sub> for a maximum of 30 population doublings. In all experiments, the fibroblast cultures were subjected to cell viability assays using Trypan blue dye, and readings were performed in a hemocytometric chamber under a light microscope. All experiments described were performed when cell viability was equal or above 95%.

### 2.3. MTT Reduction Cell Viability Assay

The MTT reduction assay ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) is employed with great success for estimating the number of viable cells in initial drug screenings. Its interpretation serves as an indicative of cellular metabolic activity, and the redox reactions occur in both the mitochondria and the cytosol. The reduction of the MTT salt to formazan happens mainly because of the succinate dehydrogenase enzyme, and results in purple insoluble formazan crystals. The intensity of the color is used to measure mitochondrial activity and, therefore, cell viability [13]. Cells were seeded at a density of 5 × 10<sup>4</sup>

cells/well and treated with different concentrations of Trichotech™ (0.5, 1.0, 1.5 and 2.0%) for 24 hours. Next, 10 µl of a 5 mg/ml MTT solution (Sigma-Aldrich) were added to each well. After 4 hours the samples were reincubated with 100 µl of Sodium dodecyl sulfate (SDS) solution [10%] for 12 hours, and then optical density was measured in a FlexStation® 3 multimode Benchtop Reader (Molecular Devices, CA, USA) at 540 nm.

#### **2.4. Propidium Iodide (PI) Incorporation Assay**

Propidium iodide incorporation assays were performed using flow cytometry to assess the cellular fraction in the S/G2/M phase of the cell cycle (*i.e.* proliferating cells). To summarize, cells were seeded in 24-well plates at an initial density of  $2 \times 10^5$  cells/well, to which was added a hypotonic fluorochrome solution (HFS—0.1% w/v sodium citrate, 0.5% w/v Triton X-100 and 50 µg/ml propidium iodide). After an incubation period of 4 h at 4°C and shielded from light, the cells and supernatant were collected and analyzed. A FACS can flow cytometer and the CellQuest software were employed, and the data obtained were analyzed with WinMDI 2.8, considering 20,000 events per analysis for each assay.

#### **2.5. Wound Healing**

For this assay, fibroblasts were seeded in 6-well microplates and cultured as described above until observation of a confluent monolayer. The cell monolayers were carefully “scratched” with a sterile pipette tip, and washed with saline and PBS to remove loose cells and debris. Next, the cells were incubated at 37°C with culture medium without fetal bovine serum (nutrient deprivation) and with 0.2% low molecular weight HA. Reference points near the “wound” were demarcated to ensure the same area of image acquisition. Images were obtained at different times using a digital camera attached to the microscope, and the percentage of wound closure was calculated using IMAGEJ (NIH, USA).

#### **2.6. Measurement of MEK/ERK and PI3K/AKT Signaling Activity in Fibroblasts**

Protein phosphorylation is a dynamic process controlled by the enzymatic activities of kinases and phosphatases. In order to inhibit these processes rapidly, fixation was done by adding BD FACS™ Lysing Solution. Following fixation, the cells were pelleted by centrifugation, and cell permeabilization was carried out either by use of a saponin solution, for analysis of cytoplasmatic proteins, or Triton X-100 solution for nuclear proteins. Following fixation and permeabilization, the samples were washed once with PBS and processed for antibody labeling. Antibody dilutions were prepared using PBS with BSA and sodium azide, with primary antibody concentrations according to the manufacturer’s instructions. Samples were incubated for 2 hours at room temperature for labeling. Then the samples were washed and incubated with the secondary antibody for 45 minutes also at room temperature. After labeling with the primary and secondary antibodies, samples were read at 100,000 events in a BD Accuri™ C6 flow cyto-

meter.

## 2.7. Real-Time PCR (qPCR)

Total RNA extracted from fibroblast samples was converted to cDNA using a SuperScript® III RT kit (Invitrogen, Carlsbad, CA). A qPCR analysis was performed in 10 µL reactions with the SYBR GREEN PCR Master Mix and analyzed on a StepOnePlus™ Real-Time PCR instrument (Invitrogen, Carlsbad, CA). Relative standard curves were generated by serial dilutions and all samples were run in triplicates. Primers used are: FGF-7 forward (5'-ATCAGGACAGTGGCAGT TGG A-3'); FGF-7 reverse (5'-AACATTTCCCCTCCGTTGTGT-3') and FGF-10 forward (5'-CACATTGTGCCTCAGCCTTTC-3'); FGF-10 reverse (5'-AGGTGA TTGTAGCTCCGCACA-3'). The PCR reaction was performed under the following conditions: 50°C (2 min), 95°C (10 min), and 40 cycles of 95°C (15 s) and 55°C (1 min). GAPDH was used as a control gene.

## 2.8. Sirius Red Collagen Quantification

After cells were cultured, the medium was removed and the wells were washed three times with 0.1 M PBS. Next, 100 µL of Bouin's solution (picric acid 0.9%, formaldehyde 9.0% and glacial acetic acid 5.0%) were added for fixation for 1 h. Samples were washed with PBS, then the Sirius Red dye was added. After 1 h, the maximum possible amount of dye was removed, followed by washing with 150 µL of a 0.01 M hydrochloric acid solution for 30 seconds to remove the dye that did not bind to collagen. Next, the dye was removed from cell layers by the addition of 0.1 M NaOH for 30 min. 100 µL aliquots of the solution contained in the wells were transferred to a new plate. Absorbance was measured with an Elx-800-UV (Bio-Tek Instruments, USA) microplate reader at 570 nm.

## 2.9. Statistical Analysis

Results were given as mean ± SEM (standard error of the mean). The results obtained were statistically analyzed using a one-way analysis of variance (ANOVA), followed by Tukey's test a posteriori. Semi-quantifications were analyzed using Student's t-test. P-values < 0.05 were considered significantly different. Analyses were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., CA, USA).

# 3. Results and Discussion

## 3.1. MTT to Formazan Reduction Assay

The MTT colorimetric assay is an established method of determining viable cell number in proliferation and cytotoxicity studies. This colorimetric assay provides accurate and reliable quantification of viable cell number and is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial enzymes, and the amount of formazan produced is directly proportional to the number of living, not dead cells, present during

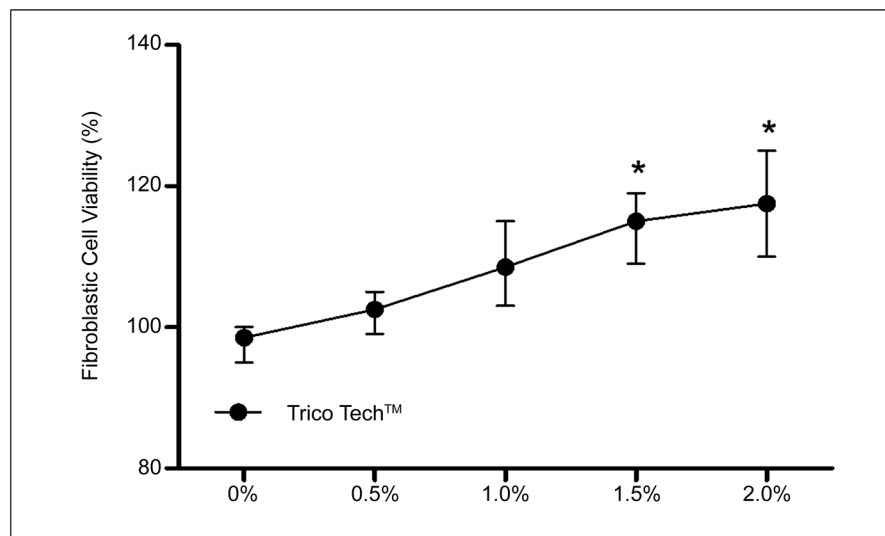
MTT exposure [14]. Trichotech™ was shown to enhance fibroblasts proliferation (Figure 1). This effect of Trichotech™ in concentrations of 0.5% to 2.0% was visible by MTT assay after 24 hours of treatment ( $P < 0.05$ ). Trichotech™ at concentrations of 1.5% and 2.0% significantly enhanced the proliferation of fibroblasts compared to the untreated group. Notice a dose-dependent increase in fibroblast proliferation.

### 3.2. Propidium Iodide (PI) Incorporation Assay

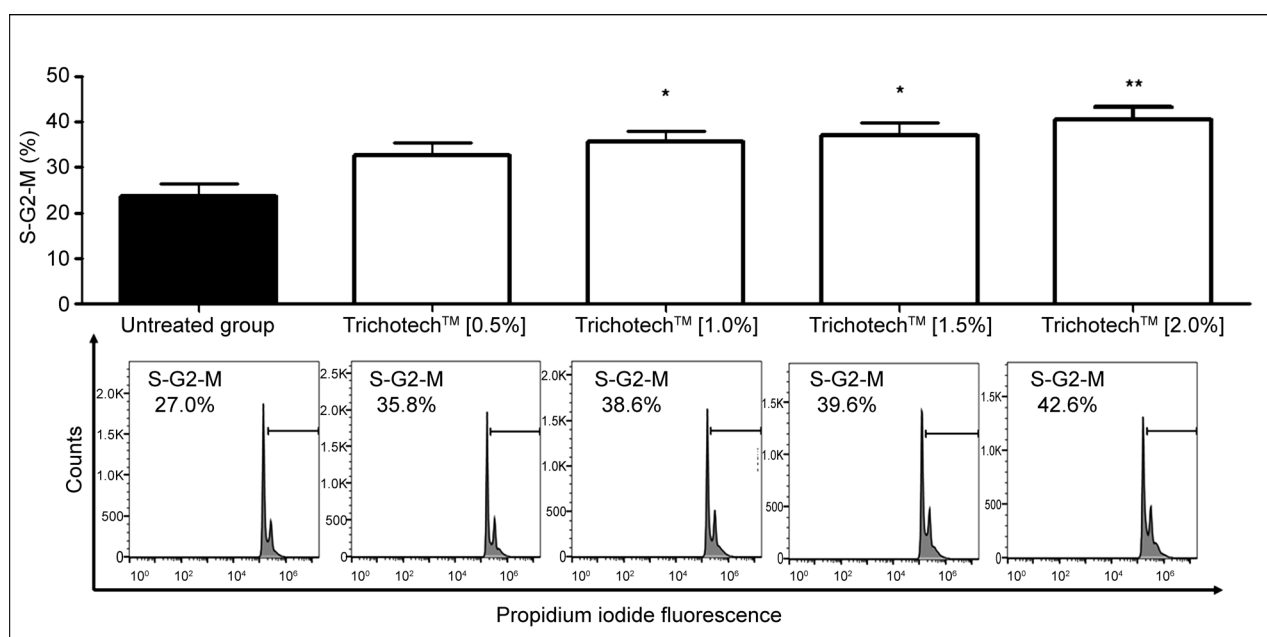
Progression through the cell cycle is one of the most fundamental features of cells and can be measured by staining cells with propidium iodide (PI). The level of PI fluorescence in a cell is, directly proportional to the DNA content of that cell, the quantification of which indicates the percentage of cells in each phase of the cell cycle in a sample [15]. As seen in Figure 2, the percentage of cells in the S/G2/M phases of the cell cycle increased in a dose-dependent fashion upon Trichotech™ treatment. This shows that Trichotech™ acts in accordance with several lines of evidence which support a molecular mechanism in the response to stimulation by natural compounds, increasing G2/M phase in fibroblasts [16] [17].

### 3.3. Wound Healing

Cell migration and proliferation coupled with controlled cell cycle are beneficial for the repair of sagged and wrinkled skin, dermal, and gastrointestinal wound healing. The *in vitro* scratch assay is a well-developed method to measure cell migration and its steps involve creating a “scratch” in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the scratch, and comparing the images to quantify the migration rate of the



**Figure 1.** Results of cell proliferation from MTT reduction assay after 24-hour exposure to different concentrations of Tricotech™. Before starting the tests, cells were deprived of fetal bovine serum. (\*)  $P < 0.05$ —significant in relation to control. ANOVA, Tukey. Assays performed in triplicate. GraphPad Prism v5.0.

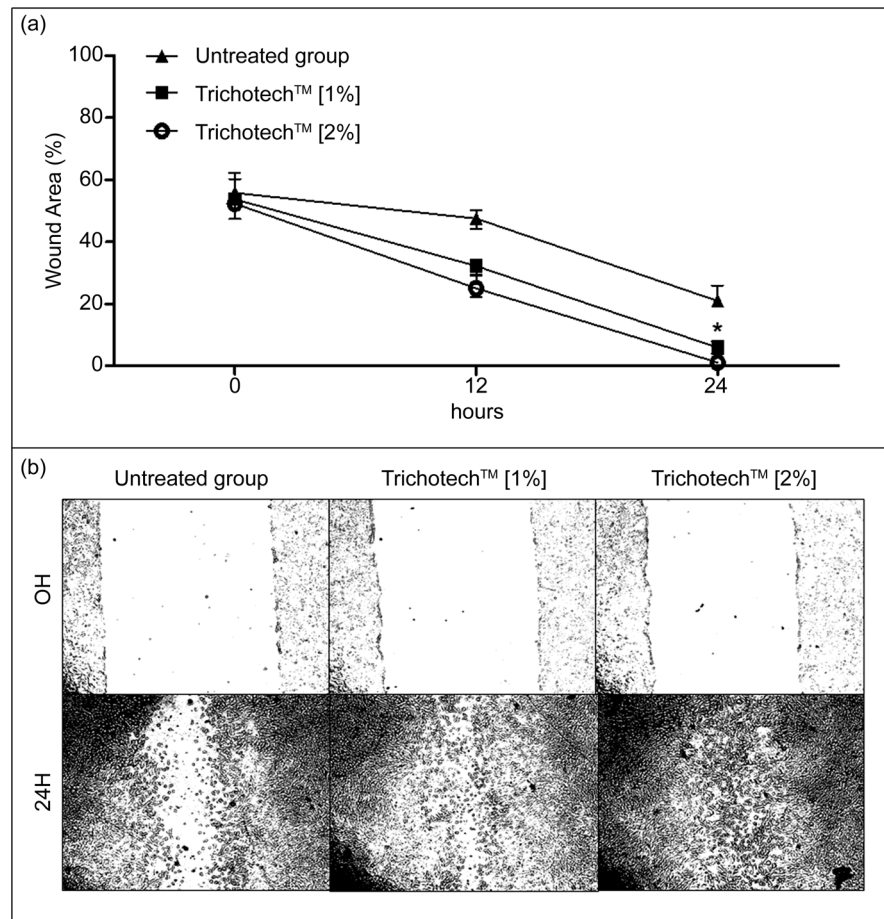


**Figure 2.** (a) Percentage of cells in S/G2/M phase obtained after 24-hour exposure of CCD-1072Sk cells to different concentrations of Trichotech™. Before starting the tests, cells were deprived of fetal bovine serum. (\*)  $P < 0.05$ —significant in relation to control. ANOVA, Tukey. GraphPad Prism v5.0. (b) Histogram representing statistically significant concentration (10%). FlowJo v10.0.

cells [18]. We evaluated Trichotech™ wound healing stimulating activity on fibroblast cells using the scratch assay. Scratches were made on confluent fibroblast monolayers, which were then exposed to Trichotech™ for 24 h at two concentrations (1.0% and 2.0%). Trichotech™ at both 1.0% and 2.0% induced a statistically significant effect on wound closure compared to the untreated control (Figure 3(b)). In Figure 3(a) and Figure 3(b), respectively, we can see images obtained at different times and the graphic representation of the distance between the edges of the scratch.

### 3.4. Measurement of MEK/ERK and PI3K/AKT Signaling Activity in Fibroblasts

An understanding of the mechanisms that regulate the cell migration and proliferation of dermal fibroblast cells by a natural compound could be beneficial in devising novel therapies to regulate fibrosis and wound contraction to ultimately improve the wound healing process [19]. The most highly studied intracellular signaling cascades in the context of cancer are the mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT pathways [20]. We examined the interaction between cell survival (PI3K/Akt) and mitogenic (Ras/MAPK) signal transduction pathways after Trichotech™ treatment on the fibroblast cell line. The cells were stimulated with 1.0% and 2.0% Trichotech™. The treatment with Trichotech™ caused phosphorylation of ERK1/2, as well as greater phosphorylation of MEK in comparison with both the untreated control and ERK1/2 (Figure 4). PI3K and AKT, however, were not shown to be significantly more phosphorylated following Trichotech™ exposure. Similar results

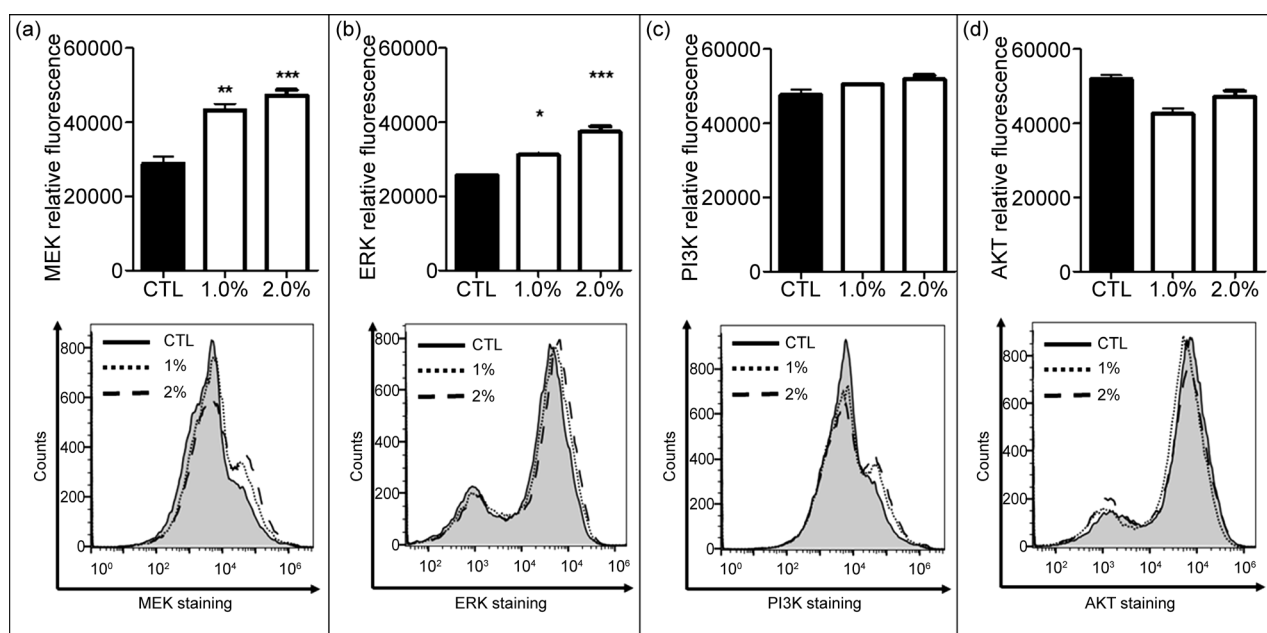


**Figure 3.** (a) Photographic representation of *in vitro* samples subjected to a simulated wound and exposed for 24 hours to different concentrations of Trichotech™ (1% and 2%). (b) Representative graphic of the percentage of wounded area at 0 h and 24 h after the same treatment. Before starting the tests, cells were deprived of fetal bovine serum. (\*)  $P < 0.05$ —significant in relation to group 0 h treated with Trichotech™, Student's t-test, GraphPad Prism v5.0.

were obtained [21], whereby the proliferative effects of camphor were shown to be mediated by the PI3K/AKT/mTOR and MAP kinase pathways—the key signaling pathways involved in the control of cell proliferation. In this same study, camphor-induced phosphorylation of ERK, but not PI3K and AKT, was also reported. Taken together, this evidence indicates that Trichotech™ induced fibroblast proliferation possibly through upregulation of MAP kinase signaling pathways.

### 3.5. Real-Time PCR (qPCR)

Several growth factors (e.g., FGF-1, FGF-2, FGF-7 and FGF-10) can promote cell cycle and proliferation and have the potential to rescue hair loss and facilitate hair cell regeneration *in vivo* and *in vitro* [9]. Among these FGF genes, FGF-7 was found to be expressed in the hair follicle. FGF-7 RNA is localized to the dermal papilla during anagen, but expression is down-regulated by the late-anagen VI stage [22]. Besides, no FGF-7 RNA was detected in follicles during

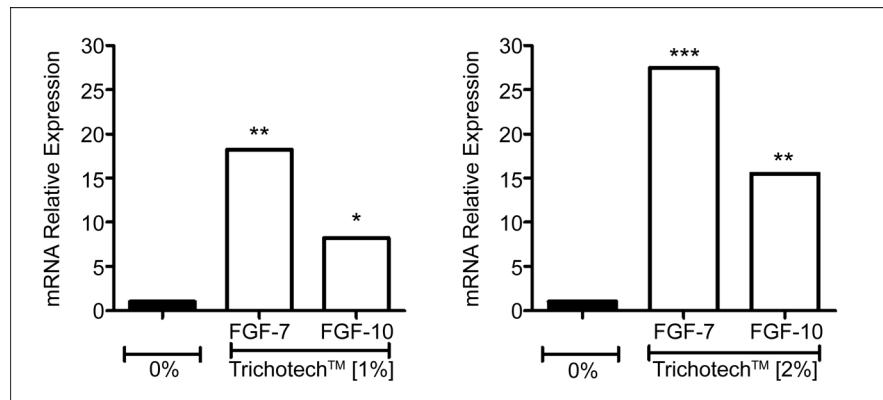


**Figure 4.** Graphic and corresponding representative histogram of MEK (a), ERK (b), PI3K (c) and AKT (d) phosphorylated proteins signaling after exposure to Trichotech™ [1%] and [2%] for 1 h. Results are expressed by the MFI (median fluorescence intensity) and compared with the untreated group (CTL). (\*)  $P < 0.05$ —significant in relation to CTL group. ANOVA, Tukey, GraphPad Prism v5.0.

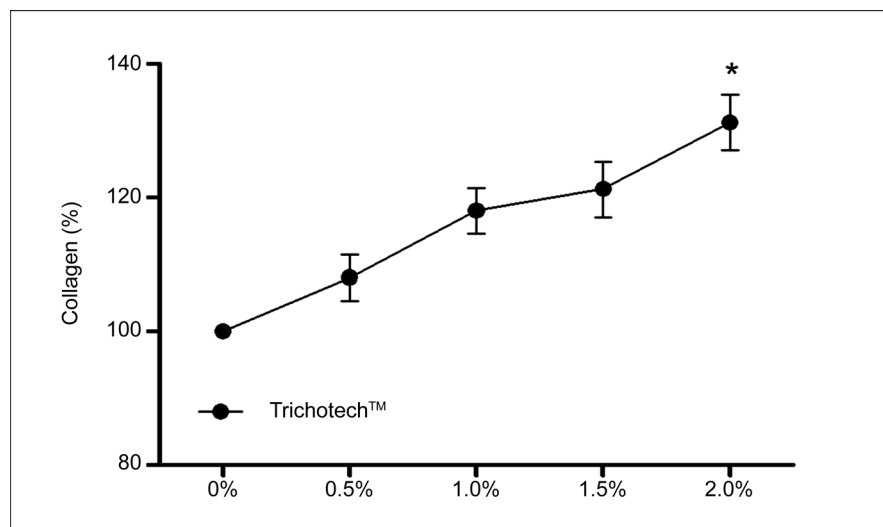
catagen or telogen [23]. FGF-10 is found in the dermal papilla fibroblasts and its receptor FGFR2IIIb is found in the neighboring outer root sheath of the keratinocytes [24], suggesting that FGF-10 is a mesenchymally derived stimulator of hair follicle cells, which contribute to the hair-promoting activity. To verify the relative expression of mRNA for fibroblast growth factor-7 (FGF-7) and fibroblast growth factor-10 (FGF-10) genes, a real-time polymerase chain reaction (qPCR) protocol was used. **Figure 5** shows the increase in mRNA expression by fibroblasts after treatment with Trichotech™. In both concentrations tested, Trichotech™ was found to increase the expression of FGF-7 and FGF-10 by several fold, thereby constituting a dermal papilla signal instructing hair germ cells to proliferate and initiate a new hair cycle.

### 3.6. Sirius Red Collagen Quantification

Sirius red staining of collagen has been used for many years. The present colorimetric plate assay allows for rapid assessment of collagen content [25]. The sirius red assay showed a significant increase in collagen content in treated fibroblasts (**Figure 6**). The magnitude of the increase in collagen between control and treated samples was markedly increased following treatment with 2.0% Trichotech™. Previous reports have demonstrated similar increases in collagen production following exposure to natural compounds, akin to the data obtained in the present work. Jung [26] showed that *Camellia japonica* oil was capable of inducing type I collagen synthesis in fibroblasts. Studies also observed increased type I collagen content in human dermal fibroblasts following exposure to ginseng and cinnamon preparations, respectively [27] [28].



**Figure 5.** Relative expression levels of mRNA for FGF-7 and FGF-10 in human fibroblasts assessed by quantitative RT-PCR. Bars represent the range of relative expression. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.0001$  compared to internal control GAPDH. Graphs were plotted with GraphPad Prism v5.0.



**Figure 6.** Total collagen content in fibroblasts as measured by incorporation of Sirius Red dye after 24-hour exposure to different concentrations of Trichotech™. Before starting the tests, cells were deprived of fetal bovine serum. The exposure did not cause a significant increase in relation to the control group (untreated cells). (\*)  $P < 0.05$ —significant in relation to control. Student's t-test. GraphPad Prism v5.0.

#### 4. Conclusion

Fibroblasts are found in the dermal papilla of hair follicles, and as such are heavily implicated in hair growth regulation. Taken together, our data show a stimulating effect of Trichotech™ on cultured fibroblasts. Indeed, a pronounced increase in cell growth was observed after exposure to Trichotech™ at concentrations of 0.5% - 2.0%. Our results suggest that Trichotech™ induced fibroblast proliferation by activating ERK signaling pathways. In addition, FGF-7 and FGF-10 mRNA levels were shown to be increased compared with untreated controls. Further investigation concerning Trichotech™ could be helpful towards the development of new bioactive phytocomplexes for dermatological and trichological use.

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## The human hair follicle, a bistable organ?

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**Abstract:** The hair cycle and its control remain today an object of debate. A number of factors, which can modulate this process, have been identified but its choreography remains elusive. For years, the hunt for the conductor has been on, but nobody ever caught him. Intuitively, the process being considered as cyclic, an automaton controlling this cycle should be looked for, by analogy with a clock. However, the putative hair follicle oscillator that would control hair cycle failed to be identified and characterized. In fact, we have revealed that human hair follicle has an autonomous behaviour and that the transitions from one phase to

the next occur independently for each follicle, after time intervals given stochastically by a lognormal distribution characterized by a mean and a variance. From this analysis, one can conclude that instead of a cyclical behaviour with an intrinsic automaton, a bistable steady state controls human hair follicle behaviour, which under a stochastic way jumps from the dormant to the active steady state and vice versa.

**Key words:** bistable steady state – hair cycle – hair follicle – neogen

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### Introduction

For decades, the hair cycle and its control have been an object of debate. Although several systems, like endometrium, cycle in the mammalian body, the hair follicle is clearly one of the only organs in mammals, together with the mammary gland for example (1), which 'cyclically' degenerates and regenerates from stem cells (2). The understanding of such a unique behaviour would certainly give clues to tissue homeostasis and regeneration. Interestingly, a number of factors that can modulate, trigger, stimulate or repress this process have been identified (3). Furthermore, the stem cells have been identified, localized and even molecularly characterized (4–6), although recent data suggest an impressive diversity in hair follicle stem cell populations (7). Although the list of actors keeps steadily increasing, the choreography remains elusive. For years, the hunt for the chief of orchestra has been on, but nobody ever caught him. Intuitively, the process being considered as cyclic, an oscillator controlling this cycle should be looked for, by analogy with a clock (8). Even though circadian clock genes were recently identified as possible contributors to the regulation of hair follicle cycling (9), a famous paper evidenced the failure in finding the regulators of the hair cycle (3) and the question, Why is it so difficult to identify and characterize this oscillator? remains. My answer is simple: it simply does not exist.

### Neogen – a new phase taking into consideration the morphogenesis process

In fact, by carrying out monthly phototrichograms during 14 years on a group of ten male, alopecic and non-alopecic volunteers (10), we studied the behaviour of 930 individual follicles and recorded about 9000 hair cycles. We then discovered that the duration of each phase of the so-called hair cycle was highly variable, from a few weeks to several years, generating an apparently chaotic behaviour shared by all follicles, whatever the alopecia grade. We had indeed revealed that each follicle had an autonomous stochastic behaviour, the probability of duration of each phase fitting with a lognormal equation (11,12). Of note, even though a deterministic model would predict the average durations

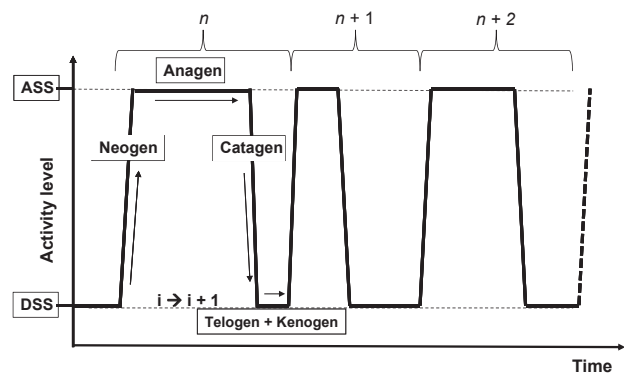
of anagen, telogen and kenogen phases around which fluctuations are observed, it would not be capable of accounting for these fluctuations of phase durations (12). Considering this peculiar dynamics, characterized by an absence of synchronized oscillations, one should reconsider the entire process of degeneration–regeneration of the hair follicle. Classically, the follicle undergoes successive steps of fibre production (anagen), regression (catagen) and rest (telogen), which in humans last for an average of 3 years, 3 weeks and a few months, respectively. A side phase, termed 'exogen', has been described, independent from the rest of the hair cycle, during which the club fibre is actively released (13) without direct consequence on anagen initiation (14). After hair loss, a latency period is observed in 80% of hair cycles (10), between elimination of a hair in exogen (14) and the appearance of the replacement hair in anagen. The duration of this period, called kenogen (15), varies from 2 to 5 months on average (10). Interestingly enough, if catagen designates the shift from anagen to telogen, no name characterizes the shift from telogen to anagen, only anagen stages being given (16). Indeed, to date, anagen phase includes a very quick and active morphogenetic process followed by a long-lasting steady fibre production state. It is nevertheless striking that the hair follicle undergoes steady periods (telogen and anagen) that are interrupted by short and intensively active periods of remodelling, regression and regeneration. If regression phase is termed catagen, I propose to call the regeneration phase 'neogen' in order to highlight, in a symmetric way to catagen, the dynamic and short-lasting character of this crucial process. The entire process of resting, regeneration, fibre production and regression would thus include four main successive phases, namely telogen, neogen, anagen and catagen. Two of those are very short, neogen and catagen, and two are quite long, telogen and anagen.

### The hair follicle, a bistable organ

Instead of a cycle, the human hair follicle behaviour would rather be described as a stochastic process operating on a bistability. The hair follicle would exist in two steady states, active and dormant. From time to time, under a stochastic way, the follicle would

jump from one state to another one. Thus, the follicle would not have a cyclic behaviour, but would undergo a succession of steady states (Fig. 1). Considering that many of the individual cell-fate decisions which control organism and organ development are binary in nature (life or death, proliferation or quiescence), that stochasticity of gene expression could lead to bimodal output (17) and bistable gene expression (18) and that binary choices are typically made by bistable switches (19,20), one might define the hair follicle – at a higher order – as a bistable organ.

Under this model, the bistable steady state is controlled by a combination of numerous factors with stochastic incremental variations, and the jump from one steady state to the other one would be triggered when given thresholds are reached. To capture this behaviour, an integrative multiparametric equation remains to be elaborated, which would include as variables all the factors so far identified in hair growth control, like growth factors, hormones, nuclear receptors, transcription factors and circadian molecular clock genes (21). Owing to the existence of thresholds, one prediction of this model is that both steady states would be endowed with refractory and competent phases, as recently shown for telogen (22–24). Indeed, refractory telogen is characterized by high bone morphogenetic proteins (BMPs) while competent telogen is characterized by low BMPs (22), a condition required for neogen to take place. Depending on the follicle considered, neogen would thus start after a variable time in the competent telogen phase (22) and last until full anagen development. Similarly, one could predict that anagen would be characterized by competent and refractory phases; the former involving IGF-1, HGF, GDNF and VEGF signalling, and the latter involving FGF5, TGF $\beta$  and BDNF in the onset of catagen (3). By analogy with the bistable calcium-/calmodulin-dependent protein kinase II switch that could control long-term memory upon stable persistent activation (25), a second prediction is that follicle could be blocked on either steady states that is an active state or a dormant state. In fact, several examples of unlimited hair growth have been recently reported in China, with anagen duration over 25 years (26). On the contrary, eyelashes are an example of follicles mainly blocked in dormant state, since 70–90% of them are in telogen (27). Chronic telogen effluvium and androgenetic alopecia would in fact translate subtle

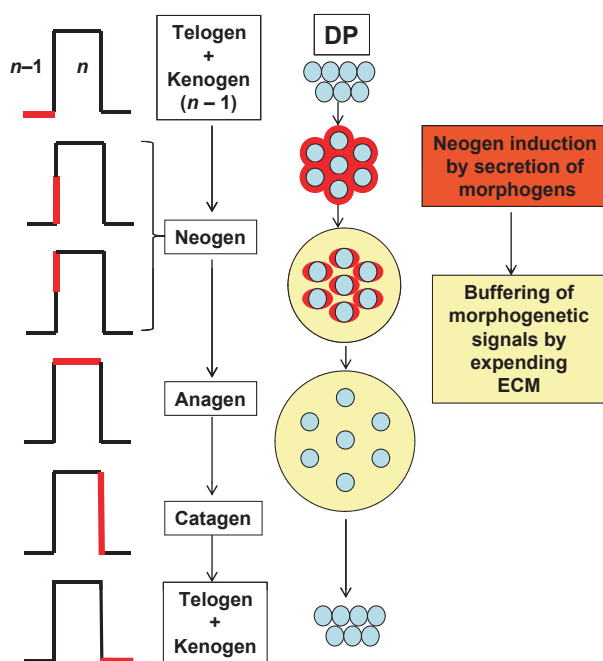


**Figure 1.** A new representation of the hair follicle behaviour, with an active steady state (ASS) of fibre production (anagen) and a dormant steady state (DSS) (telogen/kenogen), interspaced by short-lasting phases of neomorphogenesis (neogen) and regression (catagen). Three successive periods ( $n$ ,  $n + 1$ ,  $n + 2$ ) are represented.

changes in the general multiparametric equation controlling the stochasticity of hair follicle behaviour (28) and more specifically the transition thresholds of the bistable steady states.

### The dermal papilla, a key controller

Finally, if it is conceivable that catagen translates the jump from active to dormant state and stops when the follicle regression is completed, how can we explain that the neogen phase also stops, when it reaches the active steady state? Probably, this is partially controlled by the balance between extracellular matrix (ECM) and morphogens production by the dermal papilla (DP), linked to its dynamics. Indeed, DP is voluminous with the cells far apart in anagen, and flattened with the cells compacted in telogen (29). In anagen phase, the DP can be considered as a ball of ECM, surrounding specialized fibroblasts. The cross-talk of DP with neighbour matrix cells results in the maintenance of hair fibre production. An alteration in this cross-talk, induced by modifications of some variables of the multiparametric equation controlling the steady states, such as the transient FGF5 expression (30), would result in the onset of catagen and initiate the jump to the dormant steady state. During this phase, DP is left behind the regressing follicle, while its ECM starts degrading. When the DP ultimately reaches the telogen follicle, it is a simple cell aggregate, with no ECM. A new cross-talk can take place, and morphogenetic signals can be exchanged which, after having reached a given threshold, trigger the neogen phase, that is the jump from dormant to active steady state. Simultaneously, the synthesis of DP ECM is reinitiated. This ECM is rich in components like GAGs



**Figure 2.** Dynamics of dermal papilla (DP) and consequences. On the left column are represented the successive phases of human hair follicle behaviour (see Fig 1). ( $n - 1$ ) and ( $n$ ) correspond to two successive periods. The red fragment denotes the phase corresponding to associated DP drawings (on the right column). In this model, the competition between morphogens and extracellular matrix (ECM) secretion is postulated to regulate the duration and extent of neogen phase. Morphogens secretion corresponds to the red area surrounding DP cells (depicted in blue) while DP ECM is depicted in pale yellow.

(31), which can progressively trap morphogenetic factors and buffer matrix cells activating factors (Fig. 2). A new steady state is established, the neogen phase is finished, and the anagen phase starts.

### Conclusion

Although seasonal changes and periodicity in the growth and shedding of human hair have been reported (32,33), large-amplitude oscillations resulting from follicular synchronization are not observed in human scalp, likely because each follicle behaves independently of each other and hair regeneration/degeneration only depends on intrinsic activation/inhibition mechanisms. Of note, desynchronized hair follicle behaviour looks specific for human being, as a different type of dynamic behaviour with periodic moulting is observed in a number of mammalian species (34–37). We have previously demonstrated that moultings would correspond to oscillations of follicular cycles resulting from synchronization by periodic external and/or hormonal signal(s) (34–37), which would trigger the transition from anagen to telogen phase

(12). Moreover, regenerative wave patterns in adult mouse hair follicle populations have been linked to macroenvironmental regulation of stem cell activity instead of – or superimposed to – hair follicle intrinsic microenvironment (22,23).

To summarize, the human hair follicle appears as a prototypic systems biology model (38) and as such, the first example of an organ under the control of bistable steady state, which under a stochastic way jumps from dormant to active state and vice versa. This implies that minute variations of some key variables might trigger these jumps to neomorphogenesis or degeneration. If this concept holds true, it might be of value to consider tissue homeostasis as the result of a bistable steady state and to identify key variables involved in the control of normal and pathological epithelial–mesenchymal interactions, the hair follicle being a true paradigm of this type of interactions.

### Conflict of interests

Bruno A. Bernard is an employee of L'Oréal Company.

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# Regenerating the skin: a task for the heterogeneous stem cell pool and surrounding niche

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**Abstract** | In the past years, our view of the molecular and cellular mechanisms that ensure the self-renewal of the skin has dramatically changed. Several populations of stem cells have been identified that differ in their spatio-temporal contribution to their compartment in steady-state and damaged conditions, suggesting that epidermal stem cell heterogeneity is far greater than previously anticipated. There is also increasing evidence that these different stem cells require a tightly controlled spatial and temporal communication between other skin residents to carry out their function.

## Cornified cells

Epidermal keratinocytes that have undergone a process of terminal differentiation whereby they form a mesh of crosslinked proteins that confers impermeability and barrier protection.

## Lineage-tracing techniques

Genetic tagging of a certain cell type that allows following its fate and that of its progeny during a particular process, such as development, homeostasis or carcinogenesis.

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The skin is a multilayered organ that protects the organism against external aggressions. The outermost layer of the skin is the epidermis, which has a high turnover rate owing to the continuous shedding of the uppermost cornified cells. It is also perhaps the tissue that withstands the highest number of injuries because of this inherent function. As in every tissue with a high degree of turnover, the role of its resident stem cells is crucial for maintaining the equilibrium between cell loss and cell division (homeostasis), as well as for repairing damaged areas.

Scattered in the epidermis are the hair follicles. These highly dynamic appendages are responsible for generating the pelage, a protective and social feature of many mammals. The hair follicles cycle between growth (anagen), destructive (catagen) and resting (telogen) phases<sup>1</sup>. The first label-retaining experiments described a population of slow-cycling cells residing at a protrusion beneath the sebaceous glands named the bulge<sup>2</sup>. These bulge label-retaining cells were later shown to be able to regenerate the cycling portion of the hair follicle after each growth cycle, confirming that they act as hair follicle stem cells<sup>3–6</sup>. However, bulge stem cells do not contribute to the interfollicular epidermis unless it has been damaged<sup>4,7–9</sup>, and later reports showed that different stem cell populations are responsible for the maintenance of the parts of the hair follicle above the bulge<sup>7,10</sup>. These were the first indications that different epidermal stem cell pools with specific properties and lineage preferences cohabit within hair follicles.

Since stem cells were first identified in the epidermis<sup>11</sup>, a continuous search for new epidermal stem cell markers has resulted in more efficient means to isolating

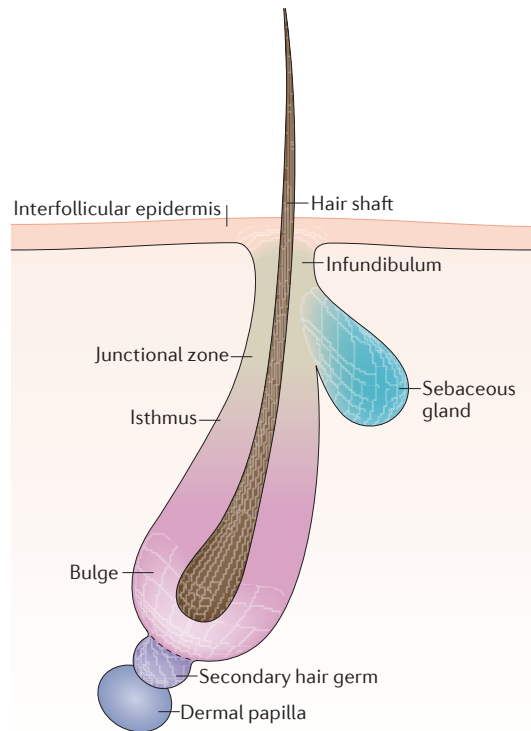
them and studying their properties. The use of the promoter regions to render expression of a given reporter gene in different subsets of epidermal stem cells has been helpful for their isolation, molecular study and lineage-tracing techniques<sup>5,6,12,13</sup>. All these studies are constantly revising and correcting the hypotheses and dogmas pertaining to the biology of the skin.

In this Review, we discuss the latest advances in our knowledge of epidermal stem cells, focusing on the recent discoveries of a growing number of stem cell pools with distinct features. We also compile the information on newly described niche cells that ensure skin homeostasis and discuss whether these niche inhabitants help to generate and maintain epidermal stem cell heterogeneity throughout development and adult life.

## Skin stem cells

The epidermis comprises the interfollicular epidermis, hair follicles, sebaceous glands and sweat glands (FIG. 1). Each of these tissues has its resident stem cells, which are discussed below.

**Interfollicular epidermis.** The interfollicular epidermis is defined as the region of stratified epidermis flanked by hair follicles. The classic hypothesis of interfollicular stem cell behaviour, based on early label-retaining studies, stated that stem cells located at the basal layer give rise to short-lived progenitors that undergo several rounds of division, known as transit-amplifying cells<sup>14</sup>. These cells would amplify the keratinocyte population and migrate upwards as they differentiate, constituting one epidermal proliferative unit<sup>14,15</sup>.



**Figure 1 | The epidermis.** Generalized structure of the epidermis and the hair follicle. The interfollicular epidermis generates a protective barrier against dehydration and external aggressions. Hair follicles are scattered in the interfollicular epidermis and give rise to the hair shafts. The permanent portion of the hair follicle consists of the infundibulum, the junctional zone, isthmus and bulge. The schematic shows the hair follicle in resting phase (telogen), during which the dermal papilla lies right below the secondary hair germ. Attached to the side of the hair follicle is the sebaceous gland, which contributes to the generation of the isolating barrier of the epidermis with its secretions.

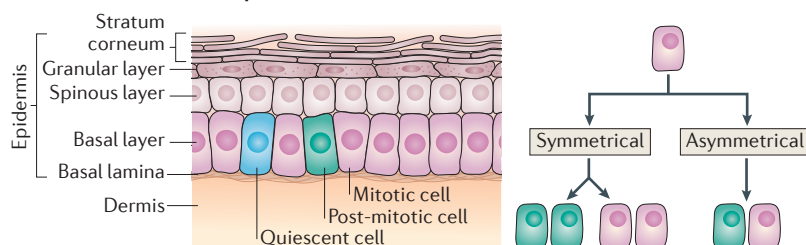
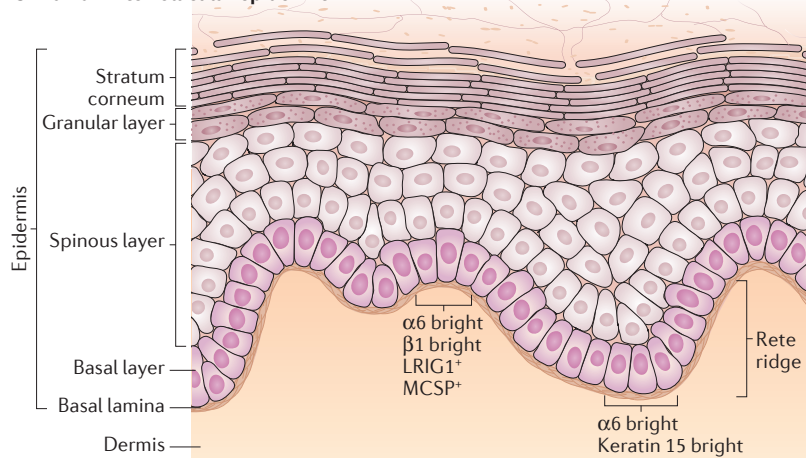
However, long-term lineage-tracing experiments in the mouse epidermis have indicated that basal clones are not organized into these discrete functional units<sup>4,7,16–18</sup>. In these experiments, some clones were observed to increase their size parallel to the underlying basement membrane (lateral expansion), and their growth at the basal layer of the epidermis remained linear with time, constituting a unipotent population of cells, later termed committed progenitors<sup>17,18</sup>. Interestingly, the fate of the progeny of committed progenitors is stochastic; they can become either two mitotic cells, two post-mitotic cells or one cell of each type, with a 10:10:80 fixed probability in healthy tissue<sup>17</sup> (FIG. 2a). Epidermal homeostasis is then maintained by a neutral drift mechanism (based on the expansion in the size of the clones) and a reduction in the number of clones. This neutral competition among the stem cell progeny is not unique to the epidermis and is also used by other tissues, such as the endoderm-derived squamous oesophagus<sup>19</sup> and the intestinal epithelium<sup>20,21</sup>. Interestingly, cancer stem cells in experimental mouse cutaneous squamous cell carcinomas no longer follow this mode of action and favour symmetric cell division,

generating two daughter cancer stem cells<sup>22</sup>. This indicates that the process of neutral competition is essential for maintaining homeostasis.

Interestingly, although the fate of dividing basal interfollicular committed progenitor cells is stochastic, the timing of their proliferation and differentiation occurs in a controlled manner through circadian rhythms<sup>23–26</sup>. These ensure that tissues and organs coordinately carry out their required function in a time-of-the-day-dependent way<sup>27</sup> and are essential for tissue homeostasis. This mechanism also ensures that proliferation of basal interfollicular progenitors is uncoupled from oxidative stress and ultraviolet (UV) radiation-induced damage in the morning, by predominantly occurring in the late afternoon and evening. Several lines of evidence underscore the importance of this control mechanism of basal interfollicular progenitors. For instance, in mice in which the circadian rhythm is perturbed specifically in this tissue, the interfollicular epidermis is prematurely aged and is differently predisposed towards oncogene-induced tumorigenesis<sup>23</sup> (BOX 1). In addition, mice chronically exposed to UV radiation at night develop twice the number of epidermal tumours than those exposed to UV light in the morning<sup>24</sup>.

Recent studies suggest, however, that the basal layer of the mouse interfollicular epidermis is heterogeneous and also contains basal cells that do not behave like committed progenitors. By labelling two different promoters in these cells — one targeting committed progenitors and the other targeting a more quiescent label-retaining population — a recent study showed that whereas the progeny of committed progenitor clones transiently contributes to the initial stages of wound healing, the clones derived from the more quiescent population last longer in the repaired epidermis<sup>28</sup>. These results suggest that the interfollicular epidermis contains a reservoir of quiescent basal cells, predominantly for regenerative purposes, that is possibly compartmentalized around the hair follicles<sup>28</sup> (FIG. 2a). A molecular analysis of these two populations revealed that committed progenitors express lower levels of stem cell markers, such as  $\alpha 6$  and  $\beta 1$  integrin, and higher levels of proteins controlling the epidermal and keratinocyte differentiation programme, like Notch 3, GRHL3 and some members of the SPPR family. In addition, committed progenitors show higher rates of proliferation than the quiescent stem cell population from which they originate<sup>28</sup>.

Interestingly, the higher expression of  $\beta 1$  integrin by quiescent mouse interfollicular stem cells has also been observed for human epidermal stem cells, with quiescent and proliferative pools being distinguished by several markers<sup>28</sup>. For instance, quiescent human interfollicular stem cells not only express higher levels of  $\alpha 6$  and  $\beta 1$  integrins<sup>29,30</sup> but also lower levels of the transferrin receptor CD71 (REF. 31). Quiescence of human interfollicular stem cells also relies on the activity of Delta 1 (REF. 32), MCSP (melanoma-associated chondroitin sulphate proteoglycan)<sup>33</sup>, LRIG1 (Leu-rich repeats and immunoglobulin-like domains 1)<sup>34</sup>, the polycomb protein CBX4 (chromobox protein homologue 4)<sup>35</sup> or on low levels of DSG3 (desmoglein 3)<sup>36</sup> and EGFR (epidermal growth factor receptor)<sup>34,37</sup>.

**a Mouse interfollicular epidermis****b Human interfollicular epidermis**

**Figure 2 | Architecture of the interfollicular epidermis.** **a** | The basal layer of the mouse interfollicular epidermis contains a unipotent population of cells termed committed progenitors. These divide in a stochastic manner with fixed probabilities, giving rise to either two mitotic or two post-mitotic cells (symmetrical cell division) or one cell of each (asymmetrical cell division). In addition, the basal layer contains a quiescent population reserved for regeneration. **b** | Quiescent human interfollicular epidermis stem cells express high levels of  $\alpha 6$  and  $\beta 1$  integrins, as well as LRIG1 (Leu-rich repeats and immunoglobulin-like domains 1) and MCSP (melanoma-associated chondroitin sulphate proteoglycan). Whether this population, or the one expressing high levels of  $\alpha 6$  integrin and keratin 15 at the bottom of rete ridges, is the most quiescent remains controversial.

However, the location of human quiescent epidermal stem cells within the basal layer is still under debate, and three different hypotheses have been proposed. One of these suggests that cells with the highest expression of  $\alpha 6$  integrin and keratin 15 located at the bottom of the rete ridges (structures on the basal layer of the human interfollicular epidermis) are relatively quiescent and display a higher clonogenic potential than the more proliferative and keratin 15 dimmer upper-rete ridge basal cells<sup>31,38,39</sup> (FIG. 2b). However, there is also strong evidence indicating that the brightest  $\beta 1$  integrin, MCSP and LRIG1<sup>+</sup> quiescent cells clustered at the top of the rete ridges harbour the highest stemness of all basal cells<sup>30,33,34,40</sup>. Interestingly, the basal layer of the epidermis of infants shows homogeneous expression of keratin 15 throughout, whereas in the adult human epidermis basal cells at the tip of the ridge express higher levels of this protein than those at the bottom<sup>31</sup>. Considering that the studies regarding  $\alpha 6$  integrin and keratin 15 used adult keratinocytes, whereas those characterizing  $\beta 1$  integrin<sup>+</sup> and LRIG1<sup>+</sup> cells were carried out in keratinocytes obtained from young donors,

this apparent controversy might simply reflect the different age of the epidermis used (see BOX 1 for a detailed discussion on the age-associated molecular and morphological changes of the human epidermis).

Nevertheless, and contradicting both views, a third hypothesis based on lineage-tracing experiments in human grafts suggests that all epidermal basal cells, irrespective of their location, contribute equally to the maintenance of the epidermis<sup>41</sup>. New approaches will be required to solve this conundrum, but regardless of whether quiescent stem cells reside at the tip or at the top of the ridges, the human epidermis does contain proliferative and quiescent pools of basal cells. This raises the possibility that the mechanisms ensuring its daily renewal might not differ greatly from those identified in the mouse interfollicular epidermis. The fact that the quiescent pool harbours the highest potential to sustain long-term regeneration suggests that this is the case<sup>38</sup>. That said, we do not know whether the human interfollicular epidermis relies on the neutral competition of the progeny of its stem cells to sustain homeostasis, as occurs in mice.

**Giving rise to the hair follicle.** The first step of hair follicle generation is the aggregation of a condensate of dermal fibroblasts around an area of the developing epidermis that secretes signals essential for hair follicle formation. The signals that control hair follicle development have been reviewed extensively elsewhere<sup>1,42</sup>. Embryonic hair follicle progenitors are characterized by the expression of different factors that, in some cases, are later required to maintain hair follicle stem cells throughout adulthood. These include the transcription factors SOX9 (REFS 43,44), LHX2 (LIM homeobox 2)<sup>45,46</sup>, NFATC1 (nuclear factor of activated T cells cytoplasmic 1)<sup>47</sup>, TCF3 (T cell factor 3) or TCF4 (REFS 48,49) and TBX1 (T-box transcription factor 1)<sup>50</sup>, which are normally involved in developmental processes. Once morphogenesis is completed, approximately 18 days after birth, the first adult hair follicle cycle starts. Degeneration (catagen), resting (telogen) and regeneration (anagen) cycles will sequentially follow during the whole life of the mouse<sup>1,51</sup>.

The resting stage of the hair cycle is much more dynamic than previously suggested. During approximately the first 28 days of telogen, hair follicles remain unresponsive to activating signals owing to inhibitory signals from the dermis (mainly bone morphogenetic protein (BMP)), leading to a telogen state that is refractory to anagen<sup>52</sup>. Once the inhibitory signals are counteracted by the expression of negative regulators of BMP<sup>50,53</sup>, hair follicles become prone to activating cues, a state known as competent telogen<sup>54</sup>. Notably, cells of the hair germ, an epithelial aggregate that is found below the bulge and that appears after the first postnatal catagen<sup>55,56</sup>, is the first region in the hair follicle to respond to activating signals at the onset of anagen<sup>53,57</sup> (see below).

Interestingly, the oscillating expression of BMP in dermal cells is out of phase with that of WNT activating signals, leading to a combined regulation between the hair follicle epithelium and the surrounding dermis controlling hair follicle cycling<sup>54</sup>. Once a threshold of

# Box 1 | Effects of ageing on epidermal stem cells

Skin ageing is likely to be a combination of the natural process of cellular decay and the result of the exposure to continuous external aggressions such as ultraviolet light. In aged human skin, the undulating pattern that forms the rete ridges of the basal layer is flattened, and the expression of stem cell markers is lower<sup>29,33,120</sup>, which results in a reduced potential to self-renew *in vitro*<sup>121</sup>. In aged mouse epidermis, although the morphology of the hair follicles and sebaceous glands is altered<sup>122</sup>, the total number of bulge stem cells remains constant<sup>122–124</sup>.

The reasons underlying skin stem cell ageing are not entirely clear. Bulge stem cells do not prevent their DNA from accumulating mutations through asymmetrical chromosome segregation during mitosis<sup>63,125</sup>, but they inherently repair DNA damage faster than other hair follicle cells by expressing more BCL-2 (B cell lymphoma 2) and transiently stabilizing p53, as well as through BRCA1 (breast cancer 1)-dependent survival mechanisms<sup>126,127</sup>. They also have the longer telomeres and lower rates of telomere shortening upon division, which might prevent replication-induced genomic instability<sup>123,128</sup>.

Nevertheless, aged bulge stem cells show reduced clonogenic potential<sup>123,124</sup>. One possible cause might rely on the progressive alteration of the pathways that control their self-renewal or the transition from quiescence to activation, both resulting in bulge stem cell ageing<sup>50,129</sup>. Recently, a reduction in the activity of the transcription factor TBX1 (T-box transcription factor 1) has been shown to lower the self-renewal potential of bulge stem cells during ageing<sup>50</sup>. In addition, aberrant activation of the JAK–STAT (Janus kinase–signal transducer and activator of transcription) pathway in the aged bulge generates an altered cytokine environment that affects bulge stem cell self-renewal in an autocrine manner<sup>124</sup>.

Microenvironmental and systemic changes might also affect bulge and interfollicular stem cell ageing. In aged skin, dermal cellularity and thickness decrease, the subcutaneous fat layer thickens and the presence of immune cells is reduced<sup>122</sup>. Changes in the expression of extracellular matrix proteins and collagen deposition also correlate with ageing<sup>81</sup>. Last, although macroenvironmental cues can markedly affect the self-renewal and regenerative capabilities of stem cells in different tissues, this remains to be proven in the skin<sup>130</sup>.

BMP inhibitory signals is reached<sup>54,58</sup> and nuclear stabilization of the WNT effector  $\beta$ -catenin has occurred<sup>59</sup>, the hair follicle is primed to enter anagen. During this phase, the follicle grows downwards into the dermal layer by expansion of a rapidly dividing compartment at the invading tip of the hair bulb known as the matrix.

But, is a unique set of hair follicle stem cells responsible for hair cycling, or do different spatially distinct populations contribute to different phases of the hair cycle? If so, which population kick-starts anagen? Where do the other structures of the hair follicle originate from and how and when are they renewed? New reports are uncovering an unexpected heterogeneity in stem cells of the hair follicle. Different pools of stem cells are distributed along the longitudinal and vertical axes of the follicle, which are defined by the expression of genetic markers (FIG. 3; TABLE 1). These different pools have been shown to contribute to different temporal phases of the hair cycle and to different regions of the follicles. However, although this heterogeneity is complex, some of the hair follicle stem cell pools can substitute for each other under stress conditions, which indicates that the expression of different markers does not necessarily imply a different function or lineage preference (discussed below).

**Bulge stem cells.** Bulge stem cells give rise to the cycling portion of the hair follicle in every anagen phase<sup>4–6,53,56</sup> and regenerate the entire hair follicle and interfollicular epidermis when transplanted<sup>3,5–8,10,13,60,61</sup>. Among the

bulge stem cell population, the first level of heterogeneity stems from the fact that only a portion of bulge stem cells is activated during anagen<sup>5</sup> and that different bulge stem cells show distinct propensity to proliferate. For instance, the basal and suprabasal layers of the bulge have been shown to contain cells that express different levels of  $\alpha 6$  integrin, with the highest levels in the cell layer attached to the basal lamina<sup>13</sup>. The clonogenic potential or regenerative capabilities upon transplantation of both populations is the same; however, basal bulge stem cells are more proliferative than suprabasal bulge stem cells *in vivo* during anagen. Although the specific contribution of suprabasal bulge stem cells to hair cycling has not been determined, these findings suggest that the distance between bulge stem cells and the basal lamina modulates their proliferative capacity<sup>13</sup>.

Circadian rhythms also have a role in determining the differential response of bulge stem cells to activating stimuli<sup>23</sup>. It has been shown recently that the expression of specific signals controlling the activation, quiescence and differentiation of bulge stem cells, including elements of the WNT, transforming growth factor- $\beta$  (TGF $\beta$ ) and Notch signalling pathways, are under circadian control<sup>23</sup>. Interestingly, two populations of clock-antiphasic stem cells coexist in telogen hair follicles, one that is prone to activation (WNT responsive<sup>high</sup>) and one that is predisposed to remain quiescent (WNT responsive<sup>low</sup>). The circadian expression of proteins involved in WNT signalling thus dictates which bulge stem cells will become active and which will remain dormant during anagen<sup>23</sup>. Although epidermal-specific arrhythmia only causes progressive delays in hair follicle cycling as mice age, whole body circadian arrhythmic mice already show a delay in the onset of the second postnatal anagen (at postnatal days 20–34). This suggests that the circadian clock of niche cells, and/or circadian systemic cues, is also important for timing hair follicle stem cell function<sup>62</sup> (see below).

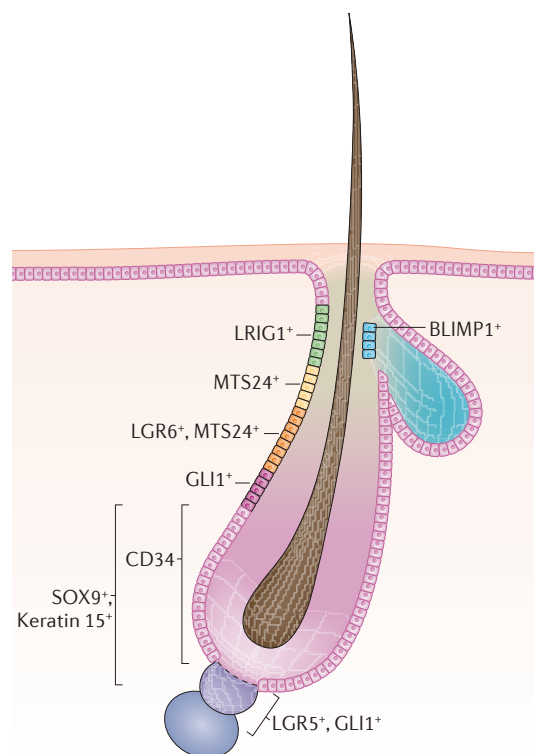
Apart from the distance to the basal lamina and circadian rhythms, the vertical axis in the bulge also distinguishes between stem cells located in the mid- and upper bulge regions (which show lower levels of proliferation) and rapidly cycling stem cells found in the lower bulge (all of which differ in their spatio-temporal contribution to hair follicle cycling)<sup>63–65</sup>. The more proliferative cells in the lower portion of the bulge are in close contact with the secondary hair germ and are enriched in the expression of the previously identified intestinal stem cell marker LGR5 (Leu-rich repeat-containing G protein-coupled receptor 5)<sup>66</sup>. These LGR5<sup>+</sup> cells also express the Hedgehog-activated transcription factor GLI1 (FIG. 3), which is absent in mid-bulge cells but, interestingly, present again in the upper-bulge region<sup>67</sup>. During telogen, the high expression of LGR5 and GLI1 also extends down to the secondary hair germ, overlapping only partially with other bulge markers such as CD34 and keratin 15. However, in anagen hair follicles, the localization of LGR5<sup>+</sup> and GLI1<sup>+</sup> cells shifts to the lower part of the outer root sheath (ORS) of the forming hair follicle, where they lose the expression of bulge stem cell markers. This change in localization is suggestive

of a possible trafficking of cells from the bulge to the growing portion of the hair follicle, as confirmed later by histone H2B (H2B)–GFP label-retaining assays<sup>56</sup>.

A common question in the field of stem cell research is when the progeny of stem cells reach a point of no return regarding the multipotent state. Loss of cell cycle quiescence is not enough, as actively proliferating stem cells are still fully capable of regenerating hair follicles in reconstitution assays<sup>1</sup>. Likewise, egressing from the niche is not a determining factor. For instance, some of the upper cells of the ORS that have exited the niche and divided a few times during anagen are later recycled to the new bulge after completion of the hair cycle<sup>56</sup>. This population of ORS cells retains the expression of some stem cell markers such as SOX9, TCF3 and LGR5 while outside the bulge and reacquires the expression of other bulge markers such as CD34 as it returns to the bulge niche<sup>56</sup>. Interestingly, the progeny of bulge stem cells that reach lower parts of the ORS during anagen adopt different fates. For example, some will establish the secondary hair germ, which, as mentioned above, is the first region in the hair follicle to respond to activating signals. Secondary hair germ cells are molecularly more similar to bulge stem cells than the highly proliferative lower matrix cells, the progeny of which is destined to differentiation into the hair shaft, and they even retain the expression of some bulge stem cell markers such as keratin 15, SOX9 and LGR5 (REF. 53). They also display a multipotent capacity, as their progeny can mark whole hair follicles in lineage-tracing experiments<sup>53</sup> and dedifferentiate into bulge stem cells when this compartment is depleted<sup>68</sup>. That said, hair germ and bulge cells show different sensitivities to the signals emanating from the dermal papilla (see below), which might depend on their different distance from these specialized fibroblasts<sup>53</sup>, or might be due to the fact that the hair germ is formed by a group of recycled ORS cells that have used more of their dividing 'credit' than those that will form the new bulge<sup>56</sup>. This might explain why hair germ cells are clonogenic for fewer passages than bulge stem cells *in vitro*<sup>53</sup>. The molecular mechanisms that control the function of hair follicle stem cells and their progeny has been extensively reviewed elsewhere<sup>1,69,70</sup>.

**Neighbours above the bulge.** Hair follicles connect with the epidermis through the junctional zone, which lies immediately above the bulge, and the isthmus, which extends from the junctional zone up to the interfollicular epidermis (FIG. 1). These two areas have recently attracted much attention, as they seem to contain several subsets of specialized stem cells (FIG. 3).

The first pool of stem cells is located at the upper fringe of the bulge, although they can still be considered as part of it on the basis of their location<sup>67</sup>. They differ from lower bulge populations in that they do not express CD34 and keratin 15 and have high levels of GLI1 (REF. 67). Intriguingly, although hair germ and lower bulge cells are the first to contribute to hair cycling, upper bulge stem cells are as efficient as their lower counterparts in contributing to all the hair follicle lineages during anagen. However, lower keratin



**Figure 3 | Hair follicle stem cell pools.** Hair follicles contain several pools of spatially distributed stem cells that are defined by unique molecular signatures and differentially contribute to hair follicle cycling. The vertical axis of the hair follicle determines different proliferative and regenerative capabilities. Apart from that, attachment to the basal lamina also dictates the proliferative behaviour of bulge stem cells. TABLE 1 summarizes the different follicular stem cell pools and their contribution to tissue homeostasis in normal, repair and regenerating conditions. The more proliferative cells in the lower portion of the bulge are in close contact with the secondary hair germ and express LGR5 (Leu-rich repeat-containing G protein-coupled receptor 5) and the Hedgehog transcription factor GLI1. A separate population at the lower bulge cells expresses high levels of SOX9, keratin 15 and CD34. At the upper fringe of the bulge is a population that is rich in expression of GLI1 and might act as a reserve population, with roles in wound repair. Above this is a population of LGR6<sup>+</sup> and MTS24<sup>+</sup> stem cells that predominantly contributes to the maintenance of the junctional zone and sebaceous glands. An MTS24<sup>+</sup> stem cell population that does not express LGR6 has also been identified. Further up resides a quiescent population of stem cells that expresses LRIG1 (Leu-rich repeats and immunoglobulin-like domains 1) and contributes to follicular, sebaceous and interfollicular lineages. BLIMP1<sup>+</sup> (B lymphocyte-induced maturation protein 1) cells have also been identified and act as sebaceous gland progenitors.

15<sup>+</sup> bulge cells contribute to the healing of interfollicular epidermis wounds in a transient way<sup>8</sup>, whereas the progeny of keratin 15<sup>+</sup> and GLI1<sup>+</sup> upper bulge cells stably remain at the wounded area long after it has been repaired<sup>67</sup>. This suggests that upper bulge cells might

Table 1 | **Differential contributions of hair follicle stem cells to epidermal structures in basal and challenging conditions**

Stem cell or progenitor pool	Lineage contribution in steady state			Regeneration (in transplants)			Wound and/or epilation repair		
	Hair follicle	Sebaceous gland	Interfollicular epidermis	Hair follicle	Sebaceous gland	Interfollicular epidermis	Hair follicle	Sebaceous gland	Interfollicular epidermis
LRIG1 <sup>+</sup>	–	+	+	+	+	+	NA	NA	NA
BLIMP1 <sup>+</sup>	–	+	–	NA	NA	NA	–	+	–
LGR6 <sup>+</sup>	Sporadic	+	+	+	+	+	+	+	Permanent*
Upper bulge GLI1 <sup>+</sup>	+	–	–	NA	NA	NA	+	–	Permanent*
Keratin 15 <sup>+</sup> and CD34 <sup>+</sup>	+	+	–	+	+	+	+	+	Transient†
Keratin 15 <sup>+</sup> and LGR5 <sup>+</sup> or keratin 15 <sup>+</sup> and GLI1 <sup>+</sup>	+	–	–	+	+	+	+	–	NA
Secondary hair germ	+	–	–	NA	NA	NA	+	NA	NA

BLIMP1, B lymphocyte-induced maturation protein 1; LGR6, Leu-rich repeat-containing G protein-coupled receptor 6; LRIG1, Leu-rich repeats and immunoglobulin-like domains 1; NA, not assessed. \*Stem cells are recruited for the repair and their progeny remain there long after the wound has been repaired. †Stem cells are recruited to the wound site, but after the wound is healed their progeny remain there only transiently.

constitute a long-term reservoir of stem cells poised to contribute to the repair of the interfollicular epidermis in a stable manner, similar to the stem cell heterogeneity observed in the mouse interfollicular epidermis (see above).

Moving upwards from GLI1<sup>+</sup> bulge cells, a second population of cycling stem cells is located at the junctional zone and is defined by the expression of LGR6 (REF. 71) (FIG. 3). These cells appear early during embryonic development in the hair follicle placodes and are later restricted to the lower junctional area in postnatal hair follicles throughout adulthood. LGR6<sup>+</sup> cells show enriched expression of the epithelial stem cell marker MTS24 (REFS 71–73) but do not express bulge stem cell markers such as CD34 or keratin 15. LGR6<sup>+</sup> hair follicle cells predominantly contribute to the maintenance of the junctional zone and sebaceous glands<sup>71</sup>, although they show some sporadic contribution to the entire hair follicle and some areas of the interfollicular epidermis. Whether this unequal contribution to different compartments is due to the heterogeneity of the LGR6<sup>+</sup> pool is still debated. It is worth noting that the expression of MTS24 is broader than that of LGR6 and extends upwards into the junctional zone. This suggests that two distinct stem cell types coexist in the junctional area, one in the lower territory (LGR6<sup>+</sup>) and the second in the upper region (MTS24<sup>+</sup>)<sup>71–73</sup>. However, the specific function of upper MTS24<sup>+</sup> cells has not yet been described.

Further climbing up along the vertical axis of the hair follicle junctional zone, we find a quiescent pool of cells that expresses LRIG1<sup>+</sup> (REF. 74) (FIG. 3). When transplanted into a wounded area of recipient mice, LRIG1<sup>+</sup> cells act as multipotent stem cells that stably contribute to all the follicular, sebaceous and interfollicular lineages<sup>74</sup>. However, during normal homeostasis, they are lineage-biased and predominantly contribute to the interfollicular epidermis and sebaceous glands but not to the hair follicles. LRIG1 is not only a marker defining

stem cells at the junctional zone, but also regulates the intensity of EGF signalling (a factor that triggers stem cell proliferation) by generating a negative feedback loop that maintains the quiescent state of these stem cells<sup>34,74</sup>. It exerts a similar role in the intestinal epithelium and human interfollicular stem cells, suggesting that LRIG1 is a general rheostat of adult stem cell proliferation<sup>34,75,76</sup>.

An intriguing picture emerges from all these studies. First, the maintenance of the junctional zone requires two types of stem cell (LGR6<sup>+</sup> and LRIG1<sup>+</sup>). Second, the progeny of both LGR6<sup>+</sup> and LRIG1<sup>+</sup> stem cells feeds into the interfollicular epidermis, although it is not known whether this contribution is random or restricted to specific areas, and if so, for what reason. With regard to the sebaceous glands, things are even more complex, as at least three types of stem cells, LRIG1<sup>+</sup>, LGR6<sup>+</sup> and bulge cells, contribute to their maintenance<sup>3,6,10,13</sup>. In addition to these, a fourth population of cells that expresses BLIMP1 (B lymphocyte-induced maturation protein 1) (FIG. 3) and that arises from bulge stem cells acts as unipotent sebaceous gland progenitors<sup>77</sup>. Whether all these stem cells equally contribute to sebaceous gland maintenance in healthy epidermis or do so differently under specific circumstances is not currently known.

**Sweat glands.** Sweat glands are not part of the pilosebaceous unit comprising hair follicles and sebaceous glands, but they regulate the temperature of the skin and whole body by secreting an aqueous solution. Sweat buds emerge during late embryonic development from keratin 14<sup>+</sup> progenitors and share some developmental traits with other glands such as those of the mammary tissue. However, in adulthood they are distinguished from these in that they do not require branching but instead a coiling process that generates the ductal and glandular parts of the gland. In addition, they undergo little renewal during adult life compared with other glandular structures that show extensive morphogenesis. In

## Placodes

Condensate of embryonic epidermal cells that invaginate into the dermis to form the embryonic hair follicle structures.

any case, sweat glands do undergo self-renewal, but until recently nothing was known about the identity of their progenitors in adulthood<sup>78</sup>.

Sweat glands contain ductal, myoepithelial and luminal progenitors (the latter two in the glandular part)<sup>78</sup>. Similar to the mammary gland, each of these progenitors is unipotent (that is, only contributing to its own lineage). Although ductal and epidermal progenitors can help to repair the gland when wounded, the myoepithelial and luminal glandular components cannot. Interestingly, however, when removed from their natural environment, both ductal and myoepithelial progenitors, but not luminal progenitors, can become multipotent in a manner dependent on the microenvironment. For instance, when transplanted into the shoulder fat or mammary fat pads of non-lactating recipients, both types of progenitors reconstituted functional sweat glands, but intriguingly, when transplanted into the mammary fat pad of lactating mice, 20% displayed features of mammary glands. However, when transplanted into the dorsal skin they only generated a stratified epidermis<sup>76</sup>.

It should be noted that in the human skin there are two types of sweat glands: apocrine and eccrine. The latter (which is the most abundant in the human skin) are absent in most laboratory animal models. Recent work has unveiled a role in wound repair for keratinocyte outgrowths emerging from eccrine sweat glands, which suggests that these glandular structures might represent another reservoir of cells for repairing the interfollicular epidermis<sup>79</sup>.

### Microenvironmental influence on hair follicles

New evidence indicates that hair follicle stem cells rely on both cell-autonomous mechanisms and their microenvironment for their correct functioning. Bulge stem cells retain their identity and potential to regenerate hair follicles upon transplantation even when having been passaged in culture, suggesting that their identity can be maintained, at least in the short-term, without any surrounding niche cues<sup>13,50</sup>. However, transplanted bulge stem cells recruit and regenerate a new niche<sup>5,13,80</sup>. This might depend on the secretion of specific factors and extracellular or transmembrane proteins, both of which are enriched in the transcriptome signature of bulge stem cells<sup>5</sup>. This specialized environment subsequently dictates the behaviour of bulge stem cells<sup>81</sup>. For instance, the distance of bulge stem cells to the basal lamina might be one important factor regulating their proliferation<sup>13</sup> (see above), and pathways that transmit the information from the basal extracellular matrix to the cell, such as  $\alpha 6$  integrin and the small GTPase RAC1, are essential for the maintenance and quiescence of bulge stem cells<sup>82–84</sup>. In addition, the basement membrane provides polarization cues for the orientation of stem cells during mitosis<sup>85</sup>.

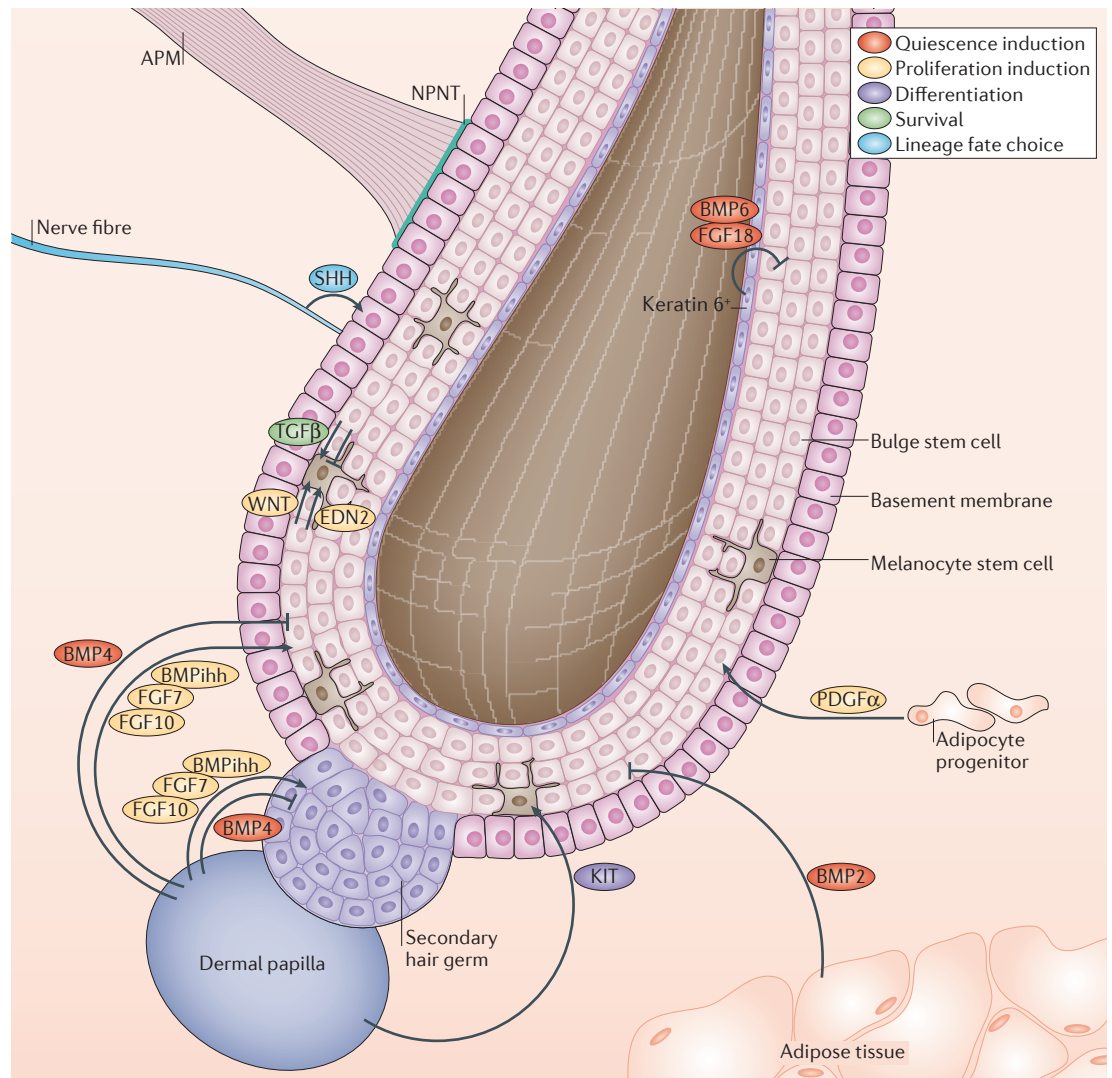
Further underscoring the importance of the niche, new studies indicate that bulge stem cells communicate with various cell types, including specialized dermal fibroblasts, muscle cells, adipocytes and neurons (FIG. 4). Interestingly, bulge stem cells even interact with their own progeny that exit the niche at the onset of anagen,

and that later return to the bulge to generate a pro-quiescence environment in telogen<sup>56</sup>. During anagen, these cells egress the bulge and progressively lose their clonogenic and regenerative capabilities as they trail along the ORS. Upon completion of the hair cycle, they are spared from apoptosis and climb back into the inner layer of the bulge, where they retain the expression of basal bulge cell markers but are distinguished from basal bulge stem cells in that they express keratin 6 (FIG. 4). Although they have lost all their stem cell potential, keratin 6<sup>+</sup> bulge stem cells are essential to maintain bulge stem cell quiescence by secreting negative regulators of proliferation such as fibroblast growth factor 18 (FGF18) and BMP6 (REFS 13,53). Recent studies suggest that the returning properties of these keratin 6<sup>+</sup> non-clonogenic bulge stem cells depend on the activity of the transcription factor HOPX (HOP homeobox)<sup>86</sup>.

**Dermal papilla fibroblasts.** The dermal papilla is composed of a condensate of fibroblasts at the tip of telogen hair, which is then embedded in the hair matrix during anagen. The importance of the hair inductive properties of dermal papilla cells was initially shown by transplantation assays<sup>87</sup>, and recent reports revealed that although ablation of dermal papilla condensates does not affect the survival of hair follicle keratinocytes, it completely stalls hair follicles in telogen<sup>88,89</sup>. Interestingly, dermal papilla cells progressively lose their hair inductive properties when serially passaged in culture, which indicates that either their organization into a three-dimensional structure or their close interaction with keratinocytes *in vivo* are necessary to maintain their hair-inductive properties during folliculogenesis<sup>90</sup>.

The size of the dermal papilla also has an important role in hair follicle cycling and specification. Dermal papilla size increases during the first stages of anagen to accommodate the increase in the area of the bulb of the growing hair follicle<sup>91</sup>. This concomitant increase in the size of the growing tip of anagen hair follicles and their associated dermal papillae is likely to be essential to maintain the communication between both cell types during the hair cycle. Supporting this is the fact that BMP signals arising from hair follicle keratinocytes are essential to maintain the hair-inductive properties of dermal papilla cells during anagen<sup>92</sup>. The increase in the size of the dermal papilla and lower hair follicle area does not depend uniquely on proliferation. In the case of the hair follicle, proliferation coupled to the coordinated movement of keratinocytes contributes to its increase in size<sup>89</sup>. By contrast, increased dermal papilla size during anagen might originate from the enlargement of the dermal papilla fibroblasts themselves, but also either from the migration into the dermal papilla of other dermal fibroblasts surrounding the hair follicles or of those lining the ORS, which form the dermal sheath<sup>91,93,94</sup>.

Interestingly, in mice the size of the dermal papilla might not only be important for hair follicle morphogenesis but also for hair follicle specification. Mice contain four types of hair follicles that vary in size and shape: guard, awl, auchene and zigzag. Guard and awl hairs have a straight pattern, but the former



**Figure 4 | Microenvironment surrounding hair follicle stem cells.** The diagram shows the interactions between hair follicle stem cells with neighbouring cell populations and the surrounding stroma. Signals emanating from structures adjacent to the bulge (that is, the basal membrane) or from specialized cell types (that is, the dermal papilla, the keratin 6<sup>+</sup> bulge layer or mature and progenitor adipocytes) control the behaviour of bulge stem cells and consequently affect hair follicle cycling. The dermal papilla maintains bulge stem cells and secondary hair germ cells quiescent during telogen by producing bone morphogenetic protein 4 (BMP4) and triggers their proliferation during anagen through BMP inhibitors (BMPi), fibroblast growth factor 7 (FGF7) and FGF10. Adipocytes maintain bulge stem cell quiescence through BMP2 secretion, whereas adipocyte progenitors promote their proliferation through platelet-derived growth factor-α (PDGFα) secretion. Keratin 6<sup>+</sup> cells promote bulge stem cell quiescence through the secretion of BMP6 and FGF18. Hair follicle stem cell lineage determination is also dictated by microenvironmental cues, such as nerve-derived sonic hedgehog (SHH) signalling. The crosstalk is mutual, as signals generated by hair follicle stem cells affect other cell types, including muscle cells and melanocyte stem cells. Positional cues for the correct attachment of the arrector pili muscle (APM) to the bulge depend on nephronectin (NPNT) deposition by bulge stem cells. The survival (transforming growth factor-β (TGFβ)), quiescence, proliferation (WNT and endothelin 2 (END2)) or differentiation (KIT) of melanocyte stem cells relies on signals from hair follicle stem cells and dermal papilla fibroblasts, which ensure their coordinated behaviour with the rest of the hair follicle cells during each stage of the hair cycle.

are longer and the latter are blunt and thicker. By contrast, auchene hairs are thinner and bent in the middle, and zigzag hairs are the thinnest and alternate oblique angle bends<sup>95</sup>. Recently, it was shown that hair follicles can switch between these hair follicle types after each round of anagen, depending on the size of the dermal papilla<sup>88</sup>. Thus, it is plausible that changes in the size of

the dermal papilla might be one of the factors underlying human alopecia, which is associated with a reduction in the size of hair follicles<sup>96</sup>.

The communication between dermal papilla cells and hair follicle cells regulates different aspects of the hair cycle<sup>97</sup>. For instance, dermal papilla cells undergo substantial molecular changes preceding the activation

of hair follicles by expressing increased levels of factors that stimulate hair follicle growth, such as BMP inhibitors, FGF7 and FGF10. These cues, together with WNT ligands also secreted by dermal papilla cells, ultimately result in the activation of secondary hair germ cells that fuel the initial stages of anagen<sup>53,54,98,99</sup>. However, dermal papilla cells also maintain the growth-refractory phase of bulge and secondary hair germ cells during telogen by secreting BMP4 (REF. 54).

Dermal papilla cells specifically express Corin, TBX18, SOX2 and CD133 during different stages of epidermal development and in adult skin<sup>100–103</sup>. However, similar to hair follicle cells, dermal papilla cells are heterogeneous, as SOX2<sup>+</sup> and CD133<sup>+</sup> dermal papilla cells are associated with guard, awl and auchene hair follicles whereas dermal papilla cells from zigzag hair follicles are SOX2<sup>−</sup> and CD133<sup>+</sup> (REF. 101). One of the determining factors in hair follicle size control might in fact be SOX2, as its expression by dermal papilla cells influences the migration speed of hair shaft progenitors and hence the length of the hair shaft<sup>103</sup>. Considering that hair follicles can switch between hair types<sup>88</sup>, it will be interesting to determine what molecular mechanisms dictate the transition from one type of dermal papilla to another, and whether they are dermal papilla autonomous or paracrine. The fact that SOX2<sup>−</sup> dermal papilla cells cannot be converted into SOX2<sup>+</sup> cells in three-dimensional cultures suggests that a paracrine mechanism is required<sup>90</sup>.

**Melanocytes.** Melanocytes are neural crest-derived cells that provide pigment to different structures such as eyes, skin and hair. Although in human skin melanocytes are found interspersed between interfollicular epidermis keratinocytes and hair follicles, in mouse skin they can be found only within follicular structures. In hair follicles, melanocyte stem cells are scattered throughout bulge stem cells<sup>104</sup>, where they share signalling pathways essential for their survival and function<sup>105,106</sup>. In telogen hair follicles, the interaction with bulge stem cells not only serves as a scaffold niche for melanocyte stem cells, but TGFβ signals secreted by bulge stem cells also help to maintain melanocyte stem cells<sup>106,107</sup>. Later on, at the onset of anagen, activation of melanocyte stem cells and bulge stem cells is synchronized in a manner dependent on WNT signals secreted by bulge stem cells<sup>105</sup>. Interestingly, the activation of the WNT pathway in bulge stem cells not only signals to melanocyte stem cells but also drives the surrounding dermal fibroblasts to proliferate and generate a dermal matrix competent for hair growth<sup>108</sup> (FIG. 4).

In addition to WNT, the expression of endothelin 2, a vasoconstrictive secreted peptide that can also be mitogenic, by bulge stem cells downstream of the transcription factor nuclear factor 1 B-type (NFIB) is essential for the coordinated behaviour of both stem cell types<sup>109</sup> (FIG. 4). Once melanocyte stem cells have been activated, their differentiating progeny migrate to the hair bulb, where they transfer pigment to matrix cells and the new hair shaft. Interestingly, this differentiation depends on the secretion of KIT ligand by dermal papilla cells (FIG. 4), which indicates that melanocyte behaviour during the

entire hair cycle is spatio-temporally coordinated by several cell types. In fact, perturbing the concerted communication of bulge and dermal papilla cells with melanocyte stem cells reduces melanocyte survival<sup>106</sup>. This loss of communication between melanocyte stem cells and melanocytes might underlie the progressive apoptotic cell death of melanocyte stem cells observed during physiological ageing, which results in hair greying<sup>106,110</sup>.

**Adipose tissue and adipocyte progenitors.** Hair follicles also communicate with the subcutaneous adipose tissue during all stages of their cycling. During telogen, the subcutaneous fat expresses BMP2, rendering hair follicle stem cells insensitive to anagen-inducing signals, thereby contributing to the telogen period refractory to anagen<sup>54</sup> (FIG. 4). Secretion of BMP2 then progressively decreases (through an unknown mechanism), allowing anagen-promoting signals to propagate and to generate waves of anagen-competent areas that organize hair follicle regeneration as single entities<sup>111</sup>. In turn, during anagen, the thickness of the subcutaneous adipose tissue increases owing to the enhanced proliferation of adipocytes<sup>54,112</sup>, but the mechanism underlying this has not been determined.

Interestingly, the relationship between hair follicles and dermal adipose tissue is not restricted to mature adipocytes, as adipocyte progenitors also promote hair follicle cells to enter anagen by secreting the mitogenic molecule platelet-derived growth factor-α (PDGFα)<sup>112</sup>. Thus, altogether a combination of factors secreted by dermal papilla fibroblasts, adipocytes and adipocyte progenitors generates a signalling environment that dictates whether hair follicles will remain dormant or enter anagen.

**Muscle, nerve and systemic signals.** Hair follicles regenerated by co-transplanted bulge and dermal cells reconstruct a functional niche, including *de novo* neural fibre generation and piloerection<sup>80</sup>. Piloerection is mediated by the sympathetic stimulation of the arrector pili muscle (APM), raising the hair shaft to prevent temperature loss. A recent study has shown that bulge cells secrete nephronectin (NPNT) into their basement membrane, which anchors to α8 integrin expressed by the APM fibres, and this determines where the muscle attaches to hair follicles<sup>113</sup> (FIG. 4).

Besides the APM, sensory nerves surrounding the bulge compose another niche structure. Bulge-associated nerve cells release sonic hedgehog (SHH), generating a population of upper bulge stem cells that express GLI1 but not other bulge markers such as keratin 15 and CD34 (FIG. 4). These upper GLI1<sup>+</sup> bulge cells contribute to all cell lineages of the hair follicle, but unlike the progeny of lower bulge counterparts (which remain in the repaired area only transiently), their progeny is maintained in a repaired area of wounded interfollicular epidermis. Interestingly, this SHH signal is essential for the contribution of upper bulge stem cells to wound repair of the interfollicular epidermis, but not for hair follicle cycling. This underscores the relevance of the niche towards the function of different populations of hair follicle stem cell lineages<sup>67</sup>.

# Concluding remarks

In the past years, our view of the mechanisms that govern skin homeostasis and regeneration have markedly changed. New populations of stem cells have been identified that behave spatio-temporally differently in healthy tissues and in situations of damage, indicating that a great level of stem cell heterogeneity is present in the skin.

However, these studies have raised many new questions. For instance, it is not known whether the different stem cells that spread along the vertical axis of the hair follicle contribute to hair follicle cycling at different times, and if so, what the significance of this temporal difference is. In addition, we do not know whether the function of all the dermal lineages known to communicate with hair follicle cells is coordinated, or whether they act in a cell-autonomous manner. All these signals must also be coupled to cell-autonomous mechanisms and physical signals that modulate stem cell function. For instance, distinct populations of human basal interfollicular epidermis cells with different propensities to differentiate are generated in culture in a cell-autonomous manner<sup>114</sup>, but also the shape that these cells adopt according to their underlying extracellular matrix is a potent modulator of their differentiation<sup>115</sup>.

The role of the microenvironment in the behaviour of interfollicular epidermal stem cells has not been addressed in depth. However, given the richness

of structures such as blood vessels, subcutaneous fat, innervation and the accumulation of fibroblasts under the upper parts of the rete ridges (in the case of human skin), it is reasonable to speculate that the microenvironment might be essential for interfollicular epidermal homeostasis. Moreover, the fact that keratinocytes partially differentiated *in vitro* can reconstitute an epidermis in graft assays demonstrates that the microenvironment surrounding a wound can modify the behaviour of post-mitotic keratinocytes and force them to re-enter the cell cycle and dedifferentiate to contribute to healing<sup>116</sup>.

The bloodstream is probably the main source of long-range signals reaching the skin, and cues provided by the vascular niche might be essential for skin homeostasis. Vascular endothelial growth factor (VEGF) signals promote the self-renewal of cancer stem cells in squamous tumours<sup>117</sup>, and a population of nestin-expressing bulge cells generates new blood vessels around transplanted hair follicles, suggesting that there is a communication between hair follicle stem cells and the vessels surrounding them<sup>118</sup>. Inter-organ communication has been shown between other tissues<sup>54</sup>, and a gut–brain–skin axis has been proposed recently<sup>119</sup>. Future work will undoubtedly provide exciting and intriguing insights into how all these mechanisms are coordinated.

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### Review

# Mesenchymal–epithelial interactions during hair follicle morphogenesis and cycling

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### ABSTRACT

Embryonic hair follicle induction and formation are regulated by mesenchymal–epithelial interactions between specialized dermal cells and epidermal stem cells that switch to a hair fate. Similarly, during postnatal hair growth, communication between mesenchymal dermal papilla cells and surrounding epithelial matrix cells coordinates hair shaft production. Adult hair follicle regeneration in the hair cycle again is thought to be controlled by activating signals originating from the mesenchymal compartment and acting on hair follicle stem cells. Although many signaling pathways are implicated in hair follicle formation and growth, the precise nature, timing, and intersection of these inductive and regulatory signals remains elusive. The goal of this review is to summarize our current understanding and to discuss recent new insights into mesenchymal–epithelial interactions during hair follicle morphogenesis and cycling.

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### 1. Introduction

A hair follicle is the primary unit that produces a single out-growing visible hair shaft. In mice, multiple hairs are induced all over the body and patterned to form rows of eyelashes, discrete whiskers, or densely clustered pelage hairs. All fulfill a wide range

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of functions, including control of body temperature, providing physical protection, relaying sensory and tactile input, and serving decorative purposes for social interactions. At least eight different major hair types can be distinguished in mice [1], and the hair coat alone contains four separate hair subtypes [2].

All hair follicles have the same basic arrangement, with epithelial progenitor cells at the base giving rise to multiple intermediary cell lineages that form the hair shaft and its guiding channel. Epithelial progenitors themselves surround a core cluster of mesenchymal cells, the dermal papilla (DP), which is thought to provide signals to coordinate hair growth [3]. The exchange of molecular cues between epithelial and mesenchymal compartments begins during embryogenesis, when hair follicles are first formed [4]. Remarkably, many of the fundamental signaling programs required for hair morphogenesis are evolutionarily conserved across species with different types of skin appendages, such as feathers and scales [5]. Furthermore, parallels exist between the mechanisms driving hair, tooth and mammary gland formation, all of which require mesenchymal–epithelial interactions [6]. After initial hair follicle formation and a prolonged period of growth, follicles undergo cycles of destruction and regeneration throughout life [7]. For new hair re-growth, signal exchange between DP cells and stem/progenitor cells is thought to occur in a process that is reminiscent of embryonic hair follicle formation [8]. Many diverse developmental programs require coordinated mesenchymal–epithelial interactions for completion, and studies of hair growth provide an exquisite system in which to study the complexities of this universally important process.

Numerous methods have been used to characterize the interplay of signals exchanged between the mesenchymal and epithelial components during embryonic follicle initiation, postnatal growth and adult regeneration. An early approach involved tissue recombination experiments, which determined that dermal signals initiate follicle formation [9]. Subsequent microdissection and transplantation experiments revealed the inductive and nurturing role of specialized DP cells [10] and localized multipotent epithelial stem cells to the follicle bulge [11]. The identification of putative ligands and receptors involved in mesenchymal–epithelial interactions came from tissue stainings performed since the 1990s, and more recently from studies systematically assessing gene expression with the help of genetic fluorescent reporter tools [12–15]. The functional relevance of many ligands has been explored by bead implantation experiments, complete gene knockout mice and spontaneous mouse mutants [16]. Most recently, compartment-specific gene ablation [17] and transgenic overexpression in the epidermis [18] and bulge stem cells [19] of candidate ligands and receptors yielded many insights into the requirement and timing of several signaling pathways for hair morphogenesis. In this review, we will highlight the basic concepts of hair follicle development, discuss our current understanding of the signal exchange during this process, and review recent new insights into the mesenchymal–epithelial interactions driving follicle induction, growth and regeneration.

## 2. Overview of hair follicle development, growth and regeneration

### 2.1. Hair follicle formation

Classically, the initiation of hair follicle morphogenesis is described in terms of an ordered series of mesenchymal–epithelial interactions: a “first signal” emanating from the dermis acts on an unspecified epidermis, and the formation of morphologically recognizable hair placodes follows next [4,8]. Several studies have proposed that mechanisms of lateral inhibition, mediated by diffusible signals that act within the epidermal compartment,

coordinate the even spacing of these placodes [20–22]. As development progresses, stabilized placodes signal to underlying dermal cells, prompting the formation of dermal condensates or clusters of DP precursor cells. Finally, these condensates are believed to signal back to the epithelial compartment to stimulate proliferation and downgrowth of hair germs [4]. Hair follicle stem cells arise from epidermal progenitors early on [23] but remain located in the upper portion of the follicle while supplying rapidly dividing cells at the tip that allow further downgrowth of the hair peg. As the epithelial component of the nascent follicle extends deep into the skin, DP precursor cells remain at the leading edge and are eventually engulfed. The dermal component of the mature hair follicle consists of these DP cells, which remain in the bulb region, and an adjoining connective tissue sheath that encircles the follicle in its entirety [4].

The first epithelial placodes appear at embryonic day E14.5, and eventually develop into primary guard hair follicles. These unique hairs comprise only 1–5% of the adult mouse coat, and are distinguished by their large follicle size and longer shaft length. Primary placodes have already progressed to form prominent downgrowths by E16.5, when a second wave of placode formation initiates. Secondary placodes appear in an even distribution between established guard follicles and give rise to awl and auchene hairs. These contribute to twenty percent of the final adult coat, with smaller follicles and shorter shaft lengths compared to primary guard hairs. A third and final wave of placode formation begins at E18.5, giving rise to zig-zag hairs that represent the vast majority of the adult coat [2,24].

### 2.2. Hair growth phase

After initial hair follicle downgrowth, the DP is completely encased by the lowest part of the hair bulb, although it remains separated from the epithelial compartment by an enveloping basement membrane. From this position, the DP lies adjacent to a population of transit-amplifying matrix cells and is thought to emit signals crucial for regulating their proliferation and differentiation into the hair shaft and its channel, the inner root sheath [3,16,25]. The hair shaft is in the center and consists of a medulla, cortex and a cuticle layer. The inner root sheath surrounds the hair shaft and consists of cuticle, Henley and Huxley layers. It is bordered by the outer root sheath layer that contains proliferating cells derived from stem cells in the bulge that feed into the matrix compartment of the bulb. Melanocytes reside above the DP within the epithelial compartment and provide pigmentation to the hair shaft [26]. Morphogenesis initiated during all three waves continues well into postnatal development, when hair shafts eventually erupt from the skin around postnatal day P5 and follicles reach the most advanced stage of postnatal hair growth by days P13–15 [27].

### 2.3. Regeneration in the hair growth cycle

Once morphogenesis is complete, follicles are prompted to enter the first hair cycle by an unknown stimulus, either presumed to emanate from the DP, or by the absence of continuous growth stimuli from the DP [7]. Fully formed follicles transition into catagen, a destructive phase characterized by profound apoptosis in the epithelial compartment of the lower follicle including the matrix cells and all differentiating layers. The DP remains intact and moves upwards toward the permanent portion of the hair follicle, which contains epithelial and melanocyte stem cells in the bulge [28,29]. Most outer root sheath cells survive as well and move upwards to give rise to a second bulge containing new stem cells and the hair germ of transit-amplifying cells [30]. Whether this movement of DP and outer root sheath is due to active migration or a passive external tug is unknown; regardless, this shift brings the DP into

close contact with the newly formed bulge and hair germ around P19 in the first hair cycle. After a short period of rest until P21, the DP emits signals that induce stem cell activation and proliferation of hair germ cells that grow down together with the DP to generate a new complete follicle, resembling the activation of epidermal stem cells during embryonic hair follicle induction [27,31].

### 3. Mesenchymal–epithelial interactions during embryonic hair follicle formation

#### 3.1. Integrative overview of inductive signals and events

The early stages of hair follicle formation involve the tight temporal and spatial regulation of inductive signals in what is thought to be a sequential process of secreted molecules alternating from epidermis and dermis [4]. However, efforts to definitively place the major players such as Wnt, *Eda*, *Fgf*, and *Bmp* in such a cascade are complicated by the multifactorial nature of these interactions and the limited time frame in which these exchanges occur (Fig. 1). Nevertheless, widespread Wnt ligand expression in the epidermis seems to be most upstream event (Fig. 1A) [32]. Secreted Wnts from the epidermis are thought to incite similarly broad Wnt signaling activity within the dermis [32,33], which could in turn drive expression of the elusive first dermal signal(s) necessary to bring about hair follicle induction (Fig. 1B) [4,8,16]. Given that the concept of an inductive dermis was first described many years ago [34,35], it is remarkable that the underlying molecular mechanisms remain obscure. However, a singular epithelial signal promoting dermal cell condensation has not been definitively described either; rather, a number of molecules are thought to promote condensate formation and maintenance (discussed below) [4]. Therefore, it is possible that multiple dermal factors are involved to initiate induction as well.

Multiple molecular markers such as Wnt10b, *Edar*, *Dkk4* and *K17* pattern the epidermis before any visible signs of hair placodes [36–39]. Similarly, beneath these epidermal “pre-placodes”, new markers such as *Sox2* and *Sdc1* identify groups of specialized dermal cells [40–42]. At this point in development, parsing out the precise timing and function of each signaling molecule or other genes within the greater scheme of mesenchymal–epithelial interactions becomes difficult because they appear virtually simultaneously. As a result, a comprehensive understanding of how all pathways interact remains incomplete. In the following chapter we provide a detailed discussion of individual signaling pathways implicated in morphogenesis, while noting confirmed upstream and downstream effectors, in an attempt to piece together a model of how these molecules cooperate during hair induction. These relationships are further depicted in Fig. 1C.

The factors that specifically promote follicle growth after induction are slightly more well-defined, since several mutants exist in which hair follicles are induced, but do not mature. In this regard, epithelial *Shh* and *Pdgfra*, in addition to *Fgf* and *Tgfb2* ligands emitted from the dermis, are central to promoting hair germ formation (Fig. 1D). A balance of dermal *Inhba* (activin- $\beta$ A) secretion and epidermal follistatin expression is similarly important for early progression of hair peg growth (Fig. 1E). In the future, advances in molecular analysis and tools to genetically and/or inducibly target specific compartments at precise time points during hair development will be invaluable to define the subtext underlying epidermal–dermal conversations.

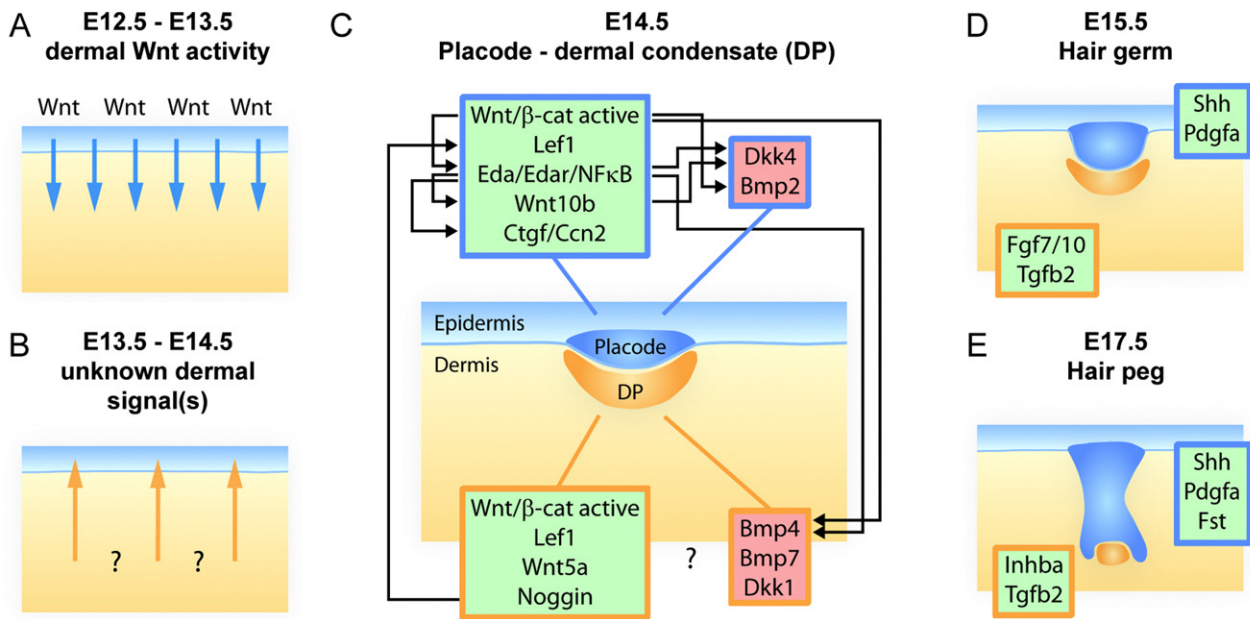
#### 3.2. Inductive signals in embryonic skin

The foundations of modern skin and hair development research were established many years ago by a “cut-and-paste” approach

(reviewed in [8,9]). These classic experiments employed tissue recombination techniques to explore the functional basis of mesenchymal–epithelial interactions in skin appendage formation. Epidermal and dermal layers were separated from early mouse embryos, and recombined such that dermis from the hairy back was paired with epidermis from a glabrous region (e.g. hairless foot pad) – or vice versa – before further culture and assessment of hair growth [34,35]. The results of these grafts revealed that only dermis from hairy mouse backskin induced appendage formation, but dermis from hairless regions did not, regardless of the origin of the epidermal tissue. Therefore the inductive potential lies within the dermis, since the origin of dermal tissue dictated whether skin appendages developed.

Morphologically recognizable hair placodes in backskin first appear around E14.5, along with concomitant expression of signaling genes [4,16], and many studies have looked into the roles of these factors in orchestrating follicle induction and subsequent hair formation. The functions of canonical Wnt/ $\beta$ -catenin signaling [43] in epidermis and dermis are especially well-characterized, and it is clear that this pathway is necessary for hair induction [44]. Mutant mice lacking the transcription factor *Lef1*, a  $\beta$ -catenin binding partner, formed only rudimentary mammary gland, tooth and hair structures providing early evidence of the central role of Wnt signaling in skin appendage development [45]. Subsequent studies confirmed *Lef1* activation modulates hair growth: transgenic *Lef1* overexpression in epidermis resulted in pelage follicle crowding and ectopic hair growth within other epithelial tissues [46]. Further recombination experiments using wild-type and knockout skin demonstrated a selective requirement for dermal *Lef1* expression in mediating normal hair growth [47]. In direct studies of Wnt signaling, transgenic expression of stabilized  $\beta$ -catenin in the epidermis led to de novo hair follicle formation [48], an effect confirmed later with inducible expression of stable  $\beta$ -catenin or epidermal deletion of the intracellular  $\beta$ -catenin inhibitor APC [49–51]. Moreover, early and sustained Wnt activation by epidermal expression of constitutively active  $\beta$ -catenin resulted in increased dermal fibroblast proliferation, precocious placode formation and later switched the entire epidermis to a hair fate or induced excessive, ectopic follicles [52–54]. Correspondingly, selective  $\beta$ -catenin ablation in the epidermis entirely prevented epithelial placode formation [33,55]. Forced expression of constitutively activated  $\beta$ -catenin within the dermis led to major skin phenotypes as well: overproliferation of mesenchymal fibroblasts and excessive follicle morphogenesis following precocious dermal condensate establishment [32]. Thus, a role for Wnt signaling in hair induction is well-established.

Wnt signaling reporter mouse lines have been particularly helpful for defining dynamic patterns of Wnt signaling activity during skin development [33,56–58]. Broad dermal activity driven by widespread epidermal Wnt ligand secretion (Fig. 1A) [32] precedes Wnt signaling in epidermal placodes [33]. Ablation of dermal  $\beta$ -catenin prior to hair induction precludes the expression of any placode markers by the epidermis and results in the failure of first wave hair formation. This suggests that widespread Wnt signaling in dermal cells regulates the first signal(s) to directly or indirectly promote hair fate specification in the epidermis (Fig. 1B) [32,59,60]. Concomitant with Wnt signaling activity in pre-placodes, dermal Wnt activity becomes intensified in underlying dermal condensates. Interestingly, ablating  $\beta$ -catenin in placodes abrogated this focused Wnt activity and resulted in a failure of dermal condensate formation [33,55]. The mechanisms that specifically support dermal condensate formation are not yet clear; *Shh* and *Pdgfra* signaling have been proposed in the past, but epidermal Wnt ligands themselves might also play a central role [4,61]. Wnt10a and Wnt10b are upregulated in the placode as morphogenesis begins and might perpetuate focused Wnt signaling activity within both placode and condensate [36]. Both Wnt5a, produced by dermal condensates,



**Fig. 1.** Mesenchymal–epithelial signal exchange during hair follicle induction. Developmental stages (A–E) are represented schematically. (A) Epidermal Wnts activate dermal Wnt/β-catenin signaling. (B) Unknown dermal signal(s) induce an epidermal response leading to placode formation. (C) Activating (green) and inhibitory (red) signals from placodes and dermal condensates (DP precursors) consolidate pattern formation through reinforcing placode/DP fate and lateral inhibition on neighboring epidermis. The network diagram depicts known hierarchies and regulatory connections between signaling pathways (as described in text). (D and E) Signals regulating hair downgrowth at hair germ and peg stages.

and Wnt10a, turned on in dermis during downgrowth, may contribute as well.

Patterns of Wnt inhibitors in the developing skin are similarly dynamic and compartment specific, in that Dkk1 is expressed in the mesenchyme surrounding follicles during the first stages of downgrowth but is conspicuously absent from the follicle itself [20,36,62]. When this secreted Wnt inhibitor was misexpressed in transgenic epidermis, effectively blocking Wnt signaling in both adjoining epithelial and dermal compartments, the appearance of physical dermal condensates and downgrowths was completely abolished [63]. In contrast, Dkk4 is expressed in the placode of primary wave follicles [38]. It has been proposed to act in a lateral fashion along with BMP ligands to affect placode spacing (to be discussed further below). Intriguingly, overexpression of this factor affects only secondary wave hair morphogenesis while primary guard hairs form normally [64]. The role that these inhibitors play in compartmental crosstalk remains to be clarified.

In addition to Wnt, Ectodysplasin (Eda) signaling is similarly essential for hair follicle induction [6,65]. Eda is a Tnf family ligand [66] that signals through downstream NFκB transcriptional activation after binding to the corresponding Ectodysplasin receptor (Edar) [67,68]. The central role Eda signaling plays in skin appendage morphogenesis was first recognized because mutations in pathway components lead to human disorders of hair, tooth, and mammary bud formation [69]. Mouse models of mutated Edar (*downless*) or ligand Eda (*tabby*) have similar phenotypes [70,71], and are characterized by a sparse coat and absent guard hair formation [65]. During embryonic stages, Eda is widely detectable throughout the epidermis while Edar expression becomes confined to early placode structures. As development continues, Eda expression is progressively confined to the interfollicular epidermis [72]. Because both ligand and receptor are expressed only by the epidermis, Edar signaling appears to act as a purely intraepithelial method of communication, and indeed a number of studies suggest that this pathway is important for placode stabilization and patterning, but not necessarily for initial placode induction [33,73].

Recently the timing and hierarchy of Eda signaling with respect to Wnt/β-catenin signaling was clarified. Using reporter mice for both β-catenin and NFκB activity revealed that Wnt signaling precedes Edar activation, and crossing reporters with knockouts confirmed that Wnt signaling could be activated in the absence of Eda [33]. Conversely, inhibiting Wnt precludes Edar expression and NFκB activation, definitively placing Edar signaling downstream of Wnt pathway components during early hair induction (Fig. 1C). Nevertheless, placodal Wnt10b itself is a direct target of NFκB signaling likely reinforcing placode fate stabilization (Fig. 1C) [33]. Additionally, multiple studies found that the expression of Wnt inhibitor Dkk4 appears downstream of Edar signaling [38,64,74]. In terms of facilitating mesenchymal–epithelial interactions, Eda overexpression in Eda null skin explants identified both dermal Bmp4/7 and epidermal Bmp inhibitors to be downstream targets of Edar signaling [75]. This allows a model in which Dkk4 and Bmp4/7 diffuse laterally to act on surrounding interfollicular epidermis to suppress placode induction. In this reaction-diffusion model, the central placode remains unperturbed, thanks to the expression of Bmp inhibitors Ccn2 and Ctgf also downstream of Edar activation [20,22,63,75–77]. Finally, Shh has been identified as a downstream target of Edar signaling [77] which promotes initial follicle growth following induction.

Apart from Wnt and Eda signaling as promoters of hair induction, BMP signaling activity in embryonic skin has an inhibitory role. During early follicle formation, the BMP receptor Bmpr1a is expressed in the epidermal compartment along with BMP2. BMP4 expression is selectively upregulated in dermal condensates [78]. Noggin, a BMP inhibitor, is also expressed from this compartment; a balance of these contradictory signals is thought to fine-tune the dermal messages sent to an epidermal target at this stage of development (Fig. 1C) [79]. Neutralization of BMPs by noggin overexpression stimulated robust formation of excess placodes [80], while constitutive deletion of noggin impaired the induction phase of follicle generation [79,81]. Secondary follicle induction was specifically inhibited in noggin null embryos, and although primary follicles did form, they arrested at an early downgrowth stage

lacking Lef1 and Shh expression. Interestingly, impaired epidermal BMP signaling in receptor-null mice promoted accelerated placode development, but was not sufficient to drive excessive follicle formation [82]. To add further complexity, when BMP signaling was abnormally sustained in noggin null skin, it could act back on the epidermal compartment to downregulate Lef1 and Wnt/ $\beta$ -catenin activity [83]. Such observations highlight the complex, overlapping nature of the signals involved in this process, and the intricate balance that needs to be maintained for successful morphogenesis.

Besides Wnt, Eda, and BMP pathways as major mediators of follicle induction, Fgf signaling has been implicated as well, although its role is less clearly defined. Multiple receptor and ligand isoforms are present during the early stages of hair development [84–88]. Transgenic mice expressing a soluble, dominant-negative Fgfr2IIIb isoform failed to develop hair [89], and Fgfr2IIIb knockout mice displayed delayed induction suggesting that Fgf ligands work to promote placode establishment [90,91]. However, more recent investigations conclude that Fgf signaling actually deters induction. Immunostaining for Fgfr2IIIb reveals widespread expression throughout E13.5 epidermis, and then subsequent downregulation in placodes [42]. The role of Fgf signaling in normal hair follicle induction thus requires further study and clarification.

### 3.3. Initial growth after induction

After induction, placode cells start to proliferate and generate morphologically recognizable downgrowths under the direction of two central signaling pathways: Shh and Pdgf. Shh is first expressed in the developed placode and then localized to the tip of the downgrowing bulb in contact with the DP as development proceeds [78,92]. The Shh receptor Patched is expressed by both epidermal and dermal compartments from an early stage [61]. Shh knockout mice revealed an important role for this signaling pathway in mediating early hair formation [93,94], since hair germs arrested at the early downgrowth stage. Both epidermal and dermal components of these early follicles were already recognizable suggesting that Shh signaling, while dispensable for induction, is crucial for these slightly later stages. To place this pathway in the context of mesenchymal–epithelial interactions, studies used epithelial or dermal-specific ablation of primary cilia components to effectively abrogate Shh signaling separately within each compartment [95,96]. Only dermal-specific knockout mice had a similar hair phenotype as Shh mutants, suggesting that secreted Shh activates effector pathways in a responsive dermis that directly or indirectly supports placode proliferation (Fig. 1D). Very recently, studies in which Smoothed was knocked out in early embryonic dermis have conclusively proven that Shh signaling within dermal condensate cells is crucial for DP development and subsequent hair follicle maturation [97]. Earlier studies of Shh pathway knockouts found normal Wnt10b, Lef1, and Bmp2/4 expression in arrested follicles, indicating that hedgehog signaling either lies downstream or functions independently of these inductive molecules [93,94]. Complementary analyses have confirmed abrogated Shh expression in mice lacking epithelial Wnt or Eda, thus implicating it as a target [55,73–75,77]. However, other dermal factors such as Wnt5a and Pdgf receptor Pdgfra were found to be dysregulated in Shh null follicles [36,61]. Wnt5a expression was completely missing from stalled follicles in Shh mutants, while Pdgfra expressing dermal cells were still present but abnormally dispersed [61]. Since these mice displayed normal Pdgfa ligand expression, the study concluded that downstream targets of Shh signaling within the dermis mediate Pdgf responsiveness and the effects of these two pathways are jointly important for maintenance of the DP.

The role of Pdgf signaling in hair morphogenesis was recognized because Pdgfa knockout mice have sparse coats that degenerate with age. This system provides a clear example of

mesenchymal–epithelial interactions, as the ligand is secreted solely by epidermis and the Pdgfra receptor is uniquely expressed in the dermis (Fig. 1D) [61]. Pdgfa expression is initially robust and widespread in E13.5 epidermis before becoming concentrated in early stage placodes [61]. On the dermal side, Pdgfra expression is broadly present throughout the upper dermis early on, but becomes progressively restricted to cells within the DP and along the dermal sheath. A significant percentage of Pdgfa knockout mice die during embryogenesis, but those that survive display abnormally sparse hair and thin skin phenotypes due to diminished white adipose tissue stores. The hair follicles that do appear form normally, suggesting that the signaling pathway is not essential for induction, but the primary coat cannot be maintained and the secondary coat, which usually appears at the first postnatal anagen starting after day P21, is never generated [61]. Pdgfra knockouts die during embryogenesis, but analysis of early skin reveals that follicles form normally, confirming that this signaling axis is not necessarily involved in induction.

Tgfb signaling also promotes hair germ growth; in particular, mesenchymally-expressed Tgfb2 acts on epithelial receptors (Fig. 1D) [98–100]. Full Tgfb2 knockout mice displayed delayed and/or arrested follicle growth at E18.5 reminiscent of Shh null mutants. Furthermore, culturing skin explants in vitro with exogenous Tgfb2 promoted excessive follicle growth [101]. Finally, a role of Tgfb/Activin signaling in hair morphogenesis was recognized because Inhba (activin- $\beta$ A) ligand knockout mice lack vibrissae at birth [102,103]. Moreover, epidermal-specific receptor knockout mice produced fewer and misshapen follicles that degenerate over time, suggesting dermally-generated ligands are needed to direct both early and late stages of differentiation within the epidermal compartment [104]. The related molecule follistatin, which inhibits activin and Bmp ligands, is expressed from the epithelial compartment and has been investigated in the context of hair growth as well. Surprisingly, full knockouts resemble Inhba knockouts, with fewer, stunted follicles at birth. These findings suggest follistatin works to fine-tune inputs from separate Tgf signaling avenues before morphogenesis can move forward [105,106].

From several of the above-mentioned studies the idea emerges that varying input from multiple signaling cascades leads to the specification of unique hair types. For example, mouse models with compromised Edar signaling lack only guard hairs, indicating that this cascade is uniquely necessary for first wave follicle induction [37]. Conversely, only guard hairs can form in the absence of noggin [81], suggesting that inhibition of Bmp signaling is distinctly required for second and third wave induction. When Shh is overexpressed in the epidermis, both first and second wave follicles are missing, and only third wave zigzag follicles are induced to form [107]. Unique gene expression profiles within the mesenchymal component of the HF specify hair type as well [40]. A differential requirement for Wnt signaling in either compartment has not yet been described, except that epidermal overexpression of Dkk4 appears to affect only second wave morphogenesis [64]. Taken together, evidence from these mutants suggests that the correct balance of morphogens is necessary for the development of discrete hair types, adding yet another layer of complexity for defining a hierarchy of the central signaling pathways implicated in hair formation.

## 4. Postnatal hair follicle induction capacity

### 4.1. Inductive capacity of mature DP

Dermal condensates in embryonic hair follicles are precursor cells of the DP in fully formed hair follicles. Although it is believed

that dermal condensates require stimuli from the placode to form, mature DP cells retain hair inducing activity independent of placodal signals. Early studies demonstrated that microdissected DPs could induce new hair growth after transplantation into glabrous skin of the foot pad [108]. Similarly, adult rat DPs from pelage follicles were microdissected, cultured as single cells and then implanted as cell clumps below foot pad epidermis to induce hair follicle formation from overlying afollicular epidermis [109]. Subsequent refinement of hair induction protocols by growing hairs at the skin surface in chamber grafts [110] or deep in the subcutaneous skin tissue [111] now allows hair induction to be assessed for hundreds of hairs simultaneously. Using such methods, pure DP cells isolated based on fluorescent markers from postnatal backskin retained hair induction capacity when transplanted together with postnatal epidermal cells [14,40].

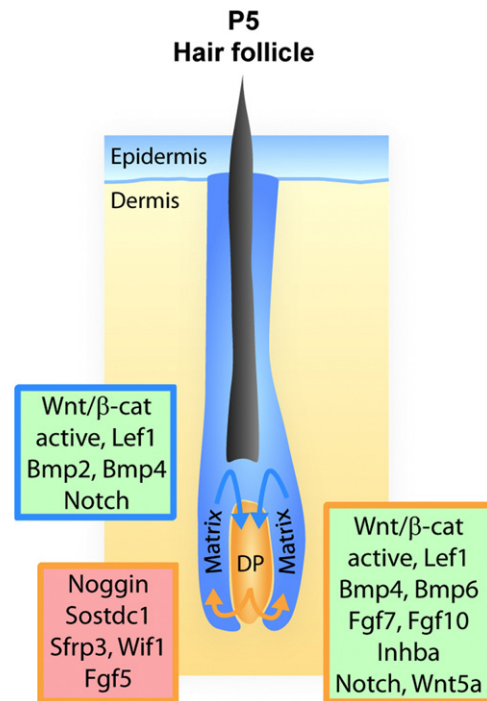
Interestingly, the hair type origin of DP cells also determines the type of experimentally induced hair follicles; for example, whisker DP cells induce whisker-like follicles on mouse ears [109]. Recent transcriptional profiling of DP cells from pelage follicles generated a DP gene signature [14] and DPs from pelage hair cell types retain a core signature but also exhibit distinct gene expression profiles [40]. Sox2, for example, is robustly expressed in guard and awl/auchene DP, but not in zigzag DP. The functional importance of this difference was recently illustrated by isolating pelage DP based on Sox2 expression prior to using these cells in separate hair-reconstitution assays. Isolated Sox2-negative DPs, when combined with keratinocytes in chamber graft assays, produced only zigzag type hairs. These experiments highlight the importance of mesenchymal–epithelial interactions in hair formation and provide powerful evidence that such interactions help drive hair type specification during morphogenesis [40].

#### 4.2. Adult follicle neogenesis after wounding

According to common knowledge, *de novo* hair follicle morphogenesis is a one-time affair that is limited to embryogenesis and early postnatal development. However, over half a century ago observations in adult rabbits, mice and even humans suggested the potential of new hair follicle formation in the context of a wound response [112–115]. Recently wounding-induced hair follicle formation was confirmed with elegant experiments in mice, in which definitive genetic fate mapping demonstrated the origin of new follicles, including their stem cells, from neighboring epidermal cells during reepithelialization [116]. Ablation of Wnt signaling in the healing wound completely abrogated new hair formation. The potential role of an inductive mesenchyme and the origin of the newly formed DPs has yet to be examined in this context.

### 5. Compartmental crosstalk during postnatal hair growth

After the early stages of downgrowth are complete, the DP is thought to direct neighboring epithelial matrix cells to proliferate and differentiate into the multiple cell types that form the hair shaft and its channel [3]. Several signaling programs central to induction are involved in these later stages of follicle maturation as well (Fig. 2); for example, Wnt signaling activity and nuclear Lef1 and  $\beta$ -catenin expression in maturing hair shaft precursors point to an important role of this pathway [56,117]. Hair shaft keratins are regulated by Wnt signaling activity [117], and forced activation of Wnt signaling drove matrix cells into differentiating hair masses resembling human benign hair tumors [48,118]. Inducible  $\beta$ -catenin ablation to block Wnt signaling activity in the matrix cells specifically during the hair growth phase has not yet been performed. However, active signaling in the dermal compartment is important at this stage; cultured DP cells grown in the presence



**Fig. 2.** Signaling between matrix and DP during hair follicle growth. Multiple positive and negative regulators are in both compartments that may also signal in an autocrine fashion.

of Wnt ligands retained hair inductive capabilities [119], and postnatal ablation of  $\beta$ -catenin in the DP compromised hair growth [120].

The importance of Bmp signaling is also reiterated during postnatal hair growth. Follicles formed when the Bmp receptor was selectively deleted within the epithelial compartment, but matrix cells were unable to undergo the proper program of maturation and differentiation [82,121,122]. Ultimately, highly abnormal follicles were generated because of an inherent inability of epithelial progenitors to stabilize Lef1 and activate Wnt signaling. In other investigations of Bmp signaling in postnatal growth, ligand overexpression inhibited proliferation within the outer root sheath resulting in small and misshapen follicles that were unable to regenerate [123]. Overexpression of the Bmp inhibitor noggin leads to excessive matrix cell proliferation and prevented hair shaft maturation [124]. An important role for Bmp activity within DP cells exists as well [125]. Ablation of Bmp signaling in isolated DP cells abolished their ability to organize hair growth in a chamber graft assay, suggesting that Bmp activity within the DP is required for instructive capabilities.

Another pathway important for organizing hair growth during postnatal morphogenesis is Fgf signaling through Fgf7/Fgf10 ligands [86]. Neonatal Fgfr2IIIb null skin, insensitive to both ligands, displayed cystic or misaligned follicle growth when cultured in grafting experiments [91]. Finally, Notch signaling also appears to participate in hair maturation, since mice with disrupted dermal Notch signaling developed intrinsic hair shaft defects [126]. Decreased Wnt5a in DP and reduced Foxn1 in matrix cells were part of the mechanism behind this phenotype. Notch signaling within matrix progenitors is also necessary to maintain proper terminal hair differentiation [127,128].

Several intrinsic transcriptional regulators such as Cutl1, Gata3, Hoxc13, Foxn1 and Msx2 directly affect hair shaft differentiation, structure and shape (reviewed in [2]). Whether mesenchymal–epithelial interactions are involved or these factors function in a compartment-autonomous manner remains to be

determined. Egf, Igf and Tgfa signaling pathway activation can also affect hair shape [2].

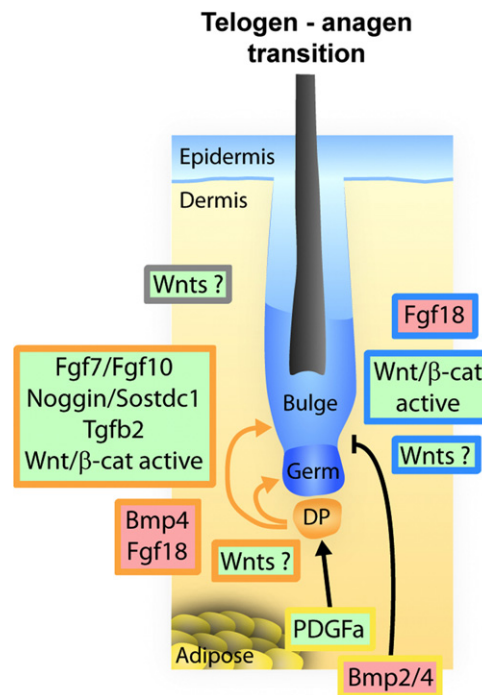
After the anagen growth period, follicles enter the catagen destruction phase, which also seems to be regulated by mesenchymal–epithelial interactions and influences from the macroenvironment [16]. Knockout mice lacking Fgf5, which is expressed in DP, are characterized by abnormally long hair due to a prolonged anagen phase, indicating that signaling through this ligand promotes catagen entry [129]. Other examples of factors that advance the anagen/catagen transition include Bdnf, IL1b, Ntf3, Tgfb1 and Tnf, while Hgf, Igf1 and Vegf promote anagen maintenance (reviewed in [130,131]). The direct source of origin and the potential involvement of mesenchymal–epithelial interactions for many of these molecules remain to be clarified.

## 6. Signals during hair regeneration

### 6.1. Signals from the dermal papilla

During the anagen growth phase DP cells in the bulb are far removed from bulge epithelial stem cells in the upper part of the follicle, and most likely do not contribute to regulation of stem cell quiescence [15,132,133]. Other cell types in the immediate stem cell microenvironment or niche, such as endothelial cells, Schwann cells and nerve endings, and dermal sheath cells are considered to provide signals keeping the stem cells in a quiescent state [31,134]. Although tantalizing gene expression analyses in the stem cells suggest such a model [12,13,15], direct evidence is lacking. The same analyses proposed secreted factors generated by stem cells may regulate their own behavior in an autocrine fashion. In addition, bulge epithelial stem cells affect neighboring melanocyte stem cells [135,136] and muscle progenitor cells just outside the bulge that give rise to the arrector pili muscle [137], and in return these cells may influence epithelial stem cell behavior as well. On the other hand, many stem cell intrinsic factors, such as transcription factors Lhx2, Nfatc1, Runx1, Sox9, Stat3, Tcf3/Tcf4 were shown in loss of function studies to directly affect stem cell quiescence and activation, and subsequent hair regrowth during the hair cycle [23,138–144]. Again, direct regulation of these factors by interactions of the epithelial stem cells with the neighboring mesenchyme has not been established yet, leaving the possibility that these essential genes are regulated cell-autonomously and not necessarily influenced by mesenchymal–epithelial interactions.

As the hair cycle ensues, DP cells move upwards toward the skin surface during the catagen destruction phase and come to rest next to the bulge stem cells and hair germ progenitor cells during the telogen resting phase. It is not clear whether DP cells join the niche efforts to regulate stem cell quiescence, but historically the presence of DP cells next to the stem cell compartment is considered essential for activating stem/germ cells to regenerate the follicle in a new anagen growth (Fig. 3) [3]. While conceptually appealing, this model lacked substantiating evidence until very recently because of the absence of DP-specific inducible gene targeting tools to directly interrogate the role of genes in the DP for stem cell activation in the bulge. Nevertheless, without such tools, the activating role of the DP was confirmed by using laser ablation to selectively target DP cells in vivo during hair cycling [145]. After DP cells were physically disrupted corresponding follicles became quiescent while neighboring unaffected follicles continued to cycle. Other examples supporting the instructive role of DP cells during hair re-growth came from hairless (Hr) and vitamin D receptor (Vdr) mutant mice, in which DP cells fail to move upwards toward the bulge during the catagen destruction phase, leaving DP cells stranded deep in the dermis [146,147]. New hair follicle regeneration at the end of telogen is absent, suggesting that the presence of DP cells next to bulge stem cells is important for inducing new hair re-growth. More



**Fig. 3.** Signals regulating stem cell quiescence and activation during the hair cycle. Bmp2/4 from DP/adipose tissue and Fgf18 from bulge/DP inhibit stem cell activation. Activation of Wnt signaling in the bulge and secreted Fgf7/10 and Bmp inhibitors from the DP activate stem cells to re-grow a new follicle during hair regeneration.

recent, albeit indirect evidence comes from work demonstrating that DP-derived Fgf7 and Fgf10 are involved in promoting hair follicle regeneration during the anagen to telogen transition [148]. Exogenously supplied Fgf7, normally expressed in DP cells [14], induced bulge/hair germ proliferation, suggesting that DP-derived Fgf7 could be a stem cell activating signal (Fig. 3). Another cytokine that could act on the stem cells both in an autocrine fashion and through mesenchymal–epithelial interactions is Fgf18, which was found to be expressed in bulge cells and to inhibit bulge cell proliferation in vitro [13]. More recently, Fgf18 expression was described as high in both DP and bulge cells during mid-telogen, and ablation of the factor in the stem cell compartment prompted rapid progression into active hair growth (Fig. 3). Additionally, Fgf18 could suppress hair growth in studies involving the injection of recombinant Fgf18 protein [149]. Genetic tools to selectively target genes of interest in the DP will be necessary to understand the molecular mechanisms behind DP-induced stem cell activation during hair cycling.

Many recent studies have also demonstrated a critical role for Wnt and Bmp signaling during hair regeneration in terms of controlling stem cell quiescence and activation [28,150,151]. Forced activation of Wnt signaling through expression of stabilized  $\beta$ -catenin led to precocious stem cell activation in the bulge [50,152,153]. Conditional and inducible ablation of  $\beta$ -catenin in the bulge during telogen showed a loss of quiescence and depletion of stem cells [152]. Therefore inhibition of Wnt signaling by Tcf3 within the stem cells [142] and by secreted Wnt inhibitors from the stem cells [15] and the niche [148] appear to be crucial for maintaining stem cell quiescence, while activation of Wnt signaling is required for the transition to a new hair growth phase (Fig. 3). In a reversed role to Wnt signaling, active Bmp signaling is required for stem cell quiescence, since ablation of Bmp receptors in stem cells leads to aberrant stem cell activation [154,155]. It appears that for stem cell activation and new hair follicle regrowth to occur, upregulation of Bmp inhibitors in the DP [148,156] and downregulation

of long-range Bmp signals from deep in the dermis (see below) have to coincide with activation of Wnt signaling in the bulge (Fig. 3).

Most recent evidence also implicated an essential role of Tgfb signaling in the stem cell compartment. By selectively ablating the Tgfb2 receptor expressed in stem cells, these studies demonstrated that Tgfb2 ligands generated in the DP act on the epithelial compartment to promote a switch from quiescence to active regeneration [157]. Downstream of activated Tgfb2 signaling, target genes suppress propagation of Bmp signaling and allow onset of a new round of follicle cycling. This is consistent with earlier studies, in which authors were able to provoke premature anagen by injecting recombinant Tgfb into skin [101].

## 6.2. Role of the macroenvironment

Besides influences from the local stem cell microenvironment, fat tissue deeper in the dermis was recently described as a heretofore unrecognized niche cell population, capable of secreting factors to influence hair cycling from a distance. Fat-derived Pdgf in particular was proposed to act on DP cells which in turn regulate induction of follicle regeneration in the hair cycle (Fig. 3) [158]. Mutant mice with defects in skin adipocyte precursor cells, which normally express high levels of Pdgfa ligand, lacked Pdgfra receptor activation in DP cells. Hair re-growth failed during the cycle, but could be recovered by injecting beads soaked in Pdgfa, suggesting that fat-stimulated activation of this signaling pathway in the DP niche elicits downstream events to trigger follicle regeneration.

Influences from fat may regulate the behavior of cohorts of hair follicles at once, providing macroenvironmental cues that can affect larger domains of the hair coat in which all follicles cycle together in a dynamic fashion. Such a model is supported by recent findings of cyclical Bmp expression in the fat domain [159]. High Bmp levels reach the bulge area and help to keep Wnt-repressed stem cells quiescent, thereby promoting a refractory telogen phase. Together with activation of Wnt/ $\beta$ -catenin signaling, widespread downregulation of long-range Bmp signals then promotes stem cell activation and new hair re-growth during an “induction competent” phase [160].

## 7. Concluding remarks

Hair follicle morphogenesis is an excellent model system in which to explore universal developmental themes, and studies of mesenchymal–epithelial interactions in this context have been particularly robust. As described in this review, numerous aspects of the communication between epidermis and dermis during hair induction, growth and regeneration have been uncovered. Nevertheless, despite decades of increasingly meticulous investigation, many details of the complex mechanisms driving hair follicle morphogenesis and cycling remain obscure. Studies have been hindered by multiple signaling isoforms that impart redundancy, as well as intricate pathway intersections and feedback loops that are difficult to untangle using mouse models. Two central mysteries that remain to be explored are the nature of the first dermal signal(s) during embryonic hair follicle induction and the activating signal(s) from DP cells during hair regeneration in the cycle. Clarification of timing, origins, and targets of important signaling pathway components will be necessary as well. Additionally, advances have been hampered by the absence of tools to specifically manipulate gene expression in inductive DP precursors during early formation stages and adult DP cells during regeneration. Compartment-specific genetic drivers to target the placode and lineages in the mature hair follicle will be useful as well. As our tools continue to be refined, so too will our understanding of how epithelial and mesenchymal tissues cooperate to create such

elaborate and patterned structures as the hair follicle, imparting a greater understanding of developmental paradigms and potentially information about hair growth that will be useful in clinical applications.

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