Niche–induced cell death and epithelial phagocytosis regulate hair follicle stem cell pool

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Tissue homeostasis is achieved through a balance of cell production (growth) and elimination (regression)1–3. In contrast to tissue growth, the cells and molecular signals required for tissue regression remain unknown. To investigate physiological tissue regression, we use the mouse hair follicle, which cycles stereotypically between phases of growth and regression while maintaining a pool of stem cells to perpetuate tissue regeneration4. Here we show by intravital microscopy in live mice4–6 that the regression phase eliminates the majority of the epithelial cells by two distinct mechanisms: terminal differentiation of suprabasal cells and a spatial gradient of apoptosis of basal cells. Furthermore, we demonstrate that basal epithelial cells collectively act as phagocytes to clear dying epithelial neighbours. Through cellular and genetic ablation we show that epithelial cell death is extrinsically induced through transforming growth factor (TGF)-β activation and mesenchymal crosstalk. Strikingly, our data show that regression acts to reduce the stem cell pool, as inhibition of regression results in excess basal epithelial cells with regenerative abilities. This study identifies the cellular behaviours and molecular mechanisms of regression that counterbalance growth to maintain tissue homeostasis.

‘Tissue regression in the hair follicle is thought to be mediated through programmed cell death’. However, it is unclear which cells within the follicle are removed and whether this process is a result of intrinsic cellular exhaustion or active elimination by extrinsic factors. We used our established intravital microscopy technique4 to visualize cell behaviours non-invasively in live mice during hair follicle regression (Fig. 1a, Extended Data Fig. 1 and Supplementary Video 1). Unexpectedly, time-lapse recordings of epithelial nuclei (made visible using H2B–green fluorescent protein (GFP) driven by the keratin 14 promoter (K14-H2BGFP)) revealed a lack of cell death by nuclear fragmentation in the suprabasal (inner) layers. Furthermore, time-lapse recordings and genetic lineage-tracing approaches showed that inner layers were eliminated through upward terminal differentiation4 (Fig. 1b, c, Extended Data Fig. 2 and Supplementary Video 2).

In contrast, using live imaging we captured cell death in the basal epithelial layer. Furthermore, we found that apoptotic debris was retained within the basal epithelium and relocated around neighbouring nuclei, suggesting that basal epithelial cells may act as phagocytes to remove epithelial cellular debris during hair follicle regression (Fig. 1d and Supplementary Video 3). To test this, we induced mosaic expression of a cytoplasmic tdTomato fluorescent reporter in the basal layer. This showed internalization of tdTomato+ epithelial debris into neighbouring tdTomato+ basal epithelial cells (Fig. 1e). Ultrastructure analysis confirmed phagocytosis of apoptotic bodies by basal epithelial cells (Fig. 1f and Extended Data Fig. 3). Tracking this process in real time with cytoskeletal and nuclear labelling demonstrated that apoptotic debris from a single cell was dispersed within the surrounding epithelium and collectively internalized by neighbouring basal epithelial cells (Fig. 1g and Supplementary Videos 4–6). Consistent with these findings, professional phagocytes were neither present inside the regressing hair follicles nor did they colocalize with epithelial cell debris (Extended Data Fig. 4 and Supplementary Videos 7, 8). Taken together, these data reveal two modes of epithelial cell elimination during hair follicle regression. While suprabasal cells undergo terminal differentiation, basal epithelial cells undergo apoptosis and are collectively removed by their basal epithelial neighbours. These findings, along with work done on the mammary gland9–13, support a new paradigm of physiological epithelial self-clearance.

Thus far, we have demonstrated that the basal epithelium adopts new cellular behaviours from growth to regression14,2. During growth, highly mitotic cells fuel downwards extension of the basal epithelium. These basal cells, located in the lower follicle, are also more likely to be eliminated during regression, suggesting a model in which mitotic exhaustion primes cells for death2. An alternative model could be that cell death is driven by extrinsic cues based on spatial location in the basal epithelium. To test these models, we promoted survival intrinsically in the basal epithelium using the Wnt/β-catenin signalling pathway, which is expressed in the suprabasal layers and has been implicated in survival of these cells13 (Fig. 2a and Extended Data Fig. 5). We used a Cre-inducible genetic model to activate β-catenin signalling ectopically in single cells of the basal epithelium and track survival during regression in vivo (Fig. 2b). Control experiments confirmed a spatial bias of cell survival in the upper basal layer, as suggested by previous work12. Although β-catenin activation was observed to enhance cell survival throughout the follicle, the spatial bias of cell survival seen in controls was retained in the β-catenin-activated follicles (Fig. 2c, d). These data suggest that cell intrinsic factors such as Wnt/β-catenin signalling alone do not explain the pattern of cell survival observed and implicate extrinsic factors to induce cell death in the basal epithelium.

These results prompted us to ask whether the observed pattern of basal cell survival was the result of spatially regulated induction of cell death. Quantifications of cell death events in time-lapse recordings of various stages of regression revealed an initial localized induction of cell death at the bottom of the follicle, which is in direct contact with the hair follicle mesenchymal dermal papilla niche (Fig. 3a and Supplementary Video 9). Therefore, we hypothesized that interaction with the dermal papilla promotes cell death along the basal epithelium of the hair follicle. To test this, we used two-photon laser ablation4 specifically to remove the dermal papilla at the onset of regression and revisited the same hair follicles over time (Fig. 3b). Dermal papilla ablation resulted in significantly reduced death of basal epithelial cells as measured by hair follicle length when compared to neighbouring unablated hair follicles (Fig. 3c and Extended Data Fig. 6). Significant differences in ablated and unablated hair follicle lengths are seen as early as 2 days after ablation, suggesting that the dermal papilla directly promotes regression (Fig. 3d). The difference in length of ablated and unablated hair follicles could be

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Figure 1 | Basal epithelial cells collectively act as phagocytes to clear neighbouring epithelial cell debris. a, Schematic of hair follicle in regression, indicating the basal and suprabasal (inner) epithelial layers, using K14-H2BGFP mice. b, Single optical sections showing upward collective movement of inner layers in relation to surrounding basal epithelial cells at successive time points, 2.5 h apart (compare position of yellow and white dashed lines). c, Single-cell lineage tracing of inner layer cells during regression (n = 30 cells, in 4 mice). Labelled cells were revisited daily. Asterisk indicates mesenchymal dermal papilla. d, Single optical sections showing cell death (nuclear fragmentation) at successive time points. Note that fragments (green) relocate (white arrow) around neighbouring epithelial nuclei (yellow, red and blue). e, Whole-mount staining showing engulfment of neighbouring basal epithelial cellular content by phalloidin staining (blue) in with mosaic Cre induction in basal layer. Nucleus is indicated in green and cytoplasm in red. f, Electron micrograph illustrating multiple apoptotic bodies (red arrowhead) present in basal epithelial cells. Basal, basal epithelial cell; Der, dermis. Inset shows high-magnification electron micrograph depicting desmosomal junctions (arrowhead) of phagocytic epithelial cells. Scale bar, 500 nm. g, Single optical sections of both coronal and transverse planes (x-y and x-z) at successive time points 4 min apart showing internalization of an apoptotic body (yellow border) by a neighbouring basal epithelial cell. Nucleus is indicated in red and cell cortex in green. h, Scheme of the two modes of elimination of epithelial cells and collective phagocytic uptake of basal epithelial apoptotic bodies by neighbouring basal epithelial cells during regression. Scale bars, 20 μm unless otherwise indicated.

was significantly reduced compared to control follicles at this initial time point. The debris generated from these follicles by day 2 was cleared by day 4, similar to control follicles, suggesting that cell clearance is relatively unaffected by dermal papilla removal (Fig. 3e). Collectively, this establishes a functional role for the mesenchymal niche to promote basal epithelial cell death.

To understand the molecular signalling that facilitates basal epithelial cell death, we investigated the TGF-β signalling pathway, as exogenous administration of TGF-β1 ligand has been shown to induce precocious hair follicle regression. We found that TGF-β ligands are expressed by the mesenchymal dermal papilla, whereas TGF-β signalling is active in the basal epithelium during the regression phase (Fig. 3f–h and Extended Data Figs 7, 8a). To test the functional role of TGF-β signalling in basal epithelial cell death during regression, we conditionally eliminated TGF-β receptor 1 (TGF-βR1) in the basal layer (Extended Data Fig. 8b, c). Removal of TGF-βR1 at the onset of regression resulted in aberrant accumulation of basal cells by the end of regression when compared to control littermates (Fig. 3i–k). Together, these data demonstrate that extrinsic regulation through TGF-β signalling and epithelial–mesenchymal crosstalk induces cell death along the basal epithelium while sparing a restricted pool of stem cells.

This work raises the question of whether hair follicle regression serves to eliminate either exhausted basal cells or functional cells from an expanded stem cell pool. To address this question, we used an approach to remove the dermal papilla transiently during regression (Fig. 4a). Strikingly, as neighbouring unablated follicles began a new round of growth, dermal-papilla-ablated follicles that had failed to complete
regression also initiated hair growth from the bottom of their aberrantly long basal epithelium. Furthermore, ablated hair follicles appeared grossly normal, with proper generation of differentiated suprabasal layers, similar to neighbouring unablated hair follicles (Fig. 4b). These findings demonstrate that basal epithelial cells of the hair follicle are not intrinsically committed for cell death, but rather retain a capacity to regenerate tissue. This suggests that regression functions to reduce an expanded stem cell pool following tissue growth.

We show that physiological regression is an extrinsically regulated process that reduces the size of the hair follicle stem cell compartment (postnatal day (P)22–P35). White arrowhead indicates differentiated inner layers. Observations shown represent n = 3 mice. Scale bars, 25 μm.

Figure 4 | Basal epithelial cells targeted for cell death retain regenerative potential. a, Scheme of laser ablation experiment. b, Sequential revisits of hair follicles after dermal papilla (DP) ablation during the next round of growth (postnatal day (P)22–P35). White arrowhead indicates differentiated inner layers. Observations shown represent n = 3 mice. Scale bars, 25 μm.
while leaving terminal differentiation programs unaffected. Regression is regulated through TGF-\(\beta\) signalling initiated by the mesenchymal niche to induce spatially restricted cell death in the basal epithelium. Clearance of apoptotic cells is a self-contained process driven by epithelial phagocytosis within the regressing basal epithelium. Finally, inhibition of regression through transient loss of the mesenchymal niche demonstrates that cells throughout the hair follicle basal epithelium maintain regenerative competency when in proximity to the mesenchymal niche (Extended Data Fig. 9). All together, we demonstrate that tissue regression relies on spatially coordinated cellular behaviours, and establish a new understanding of the extrinsic regulation that counterbalances tissue growth over the lifespan of an organism.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** K.R.M. and V.G. designed experiments and wrote the manuscript; K.R.M. performed the experiments and analysed the data; P.R. generated the K14-H2BmCherry mouse line and assisted with two-photon time-lapse imaging; G.Z. and P.M. performed immunofluorescence. S.B. performed skin whole-mount staining. T.Y.S. assisted with technical aspects. K.R.M., D.Q.G. and A.M.H. performed three-dimensional imaging analysis. K.B.B. helped with data analysis.

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METHODS

Mice. K14-H2BmCherry, Lef1-RFP<sup>+</sup> and K14-GFP<Actin><sup>+</sup> were obtained from the Fuchs Laboratory, Tgβgf<sup>F</sup> mice were obtained from V. Kaartinen<sup>17</sup>. Cnna<sub>1</sub>-Pol<sup>+</sup>/<sup>-</sup> mice were obtained from M. Taketo<sup>20</sup>. Lgr5-CreER (Cleveres Laboratory), Shh-CreER (Tahin Laboratory), LysM-Cre (Foerster Laboratory), Cxcr<sub>3</sub>-GFP (Littman Laboratory) and Rosa-stop-tdTomato (Zeng Laboratory) were obtained from Jackson Laboratory (JAX)<sup>21–25</sup>. The Yale Transgenic Facility generated the same optics as used for acquisition. An 800 nm laser beam was used to scan tail mix (15 mg ml<sup>-1</sup>) of tissue. We revisited the same 4% osmium tetroxide for 1 h. The samples were rinsed and en bloc stained in 1% tannic acid/0.1 M HEPES for 1 h, then rinsed in 50 mM Tris/50 mM maleate and placed in 2% uric acid/50 mM Tris/50 mM maleate for 1 h. After rinsing, they were dehydrated through a graded series 50% to 95% of ethanol at 4 °C, then infiltrated with 50:50 ethanol/LR White (EMS) for 1 h followed by several changes of pure 100% LR White overnight on a rotator at 4 °C. Samples were polymerized at 60 °C for 18 h. Fifty-nanometre resin sections were cut on a Leica UC7 ultra-microtome and collected on nickel formvar/carbon grids, and immunolabelled using a primary chicken anti-GFP (Abcam) diluted to 1:50 for 1 h, rinsed and placed on protein A gold secondary 1:50 (University of Utrecht). The sections were counterstained with 2% uranyl acetate and lead citrate. Grids were viewed with FEI Tecnai BiowTEM at 80 kV. Images were taken using Morada CCD and iTEM (Olympus) software. Immunostaining on paraffin sections and whole-mount skin. Skin was fixed in 4% PFA for whole mount or in 10% formalin for paraffin embedding and used for histological analysis as previously described<sup>26</sup>. Immunohistochemistry was performed by incubating sections at 4 °C overnight with primary antibodies as follows: mouse anti-β-catenin (1:100, BD #610153; 14/Beta-Catenin), rat anti-CD11b (1:250, ebioscience #14-0112; M1/70), goat anti-P-cadherin (1:100, R&D #AF671), rabbit anti-pSmad2 (Ser465/467) (1:100, Cell Signaling #3108; 13BD4), and rabbit anti-Lef1 (1:100, Cell Signaling #2286; C18A7). pSmad2 was immunostaining required TSA Plus kit (PerkinElmer). For bright-field immunohistochemistry, biotinylated species-specific secondary antibodies, followed by detection using the ABC kit (Vector Labs) and DAB kit (Vector Labs), were used according to the manufacturer’s instructions. M.O. kit was used for mouse antibodies (Vector Laboratories). Secondary antibodies conjugated with FITC, RRX and Cy5 (Jackson Immunoresearch Laboratories) were used at a concentration of 1:100 for 1 h at room temperature. Alexafluor 350 phallolidin (Life Technologies) was used according to the manufacturer’s instructions. FACs. Back skins of K14-H2BGBP, Lef1-RFP and Lgr5-CreER, Tgβgf<sup>F</sup> or Tgβgf<sup>R</sup>; tdTomato, K14-H2BmCherry mice were harvested at P12, P16 or P20 and were placed derm on 0.2% collagenase (Sigma) at 37 °C for 20 min, and then placed on 0.25% trypsin (Gibco) at 37 °C for 10 min to obtain epithelial cells as previously described<sup>27</sup>. Cells were stained for 10 min with biotinylated rat anti-CD34 (1:50, ebioscience #14-0341; RAM34), biotinylated rat anti-CD45 (1:50, BD #553077; 30-F11), biotinylated rat anti-CD117 (1:50, BD #553335; 2B8) and goat anti-integrin-<a href="#" name="a9" class="footnote">9</a> (1:90, R&D #AF3827). Cells were washed for 5 min and then incubated with streptavidin-Pacific blue (1:200, Invitrogen) and Alexafluor 647 donkey anti-goat IgG (Jackson Immunoresearch Laboratories). Cells were isolated on DAPI exclusion and by the following criteria: dermal papilla <span class="MathJaxMml">CD11b<sup>−</sup></span>, <span class="MathJaxMml">CD34<sup>−</sup></span>, <span class="MathJaxMml">GFPHigh</span> using a FACs 4 (Becton, Dickinson & Company). Cells were sorted into RNA lysis buffer for RNA isolation (RNeasy Mini Kit, Qiagen). FACs profiles were analysed through Flowjo software.

RT-qPCR. cDNA was made using Superscript III First-Strand Synthesis kit (Invitrogen). RT–qPCR was performed in triplicate with SYBER Green I reagents (Invitrogen) using 5.0 ng cDNA per reaction on the ViiATM 7 Real-Time PCR system (Life Technologies). Gene-specific primers were designed and are listed in Supplementary Table 1.

Statistical analysis. Data are expressed as percentages, box and whisker plots (error bars represent maximum and minimum), or mean ± s.d. An unpaired Student’s <a href="#" name="a10" class="footnote">t</a>-test was used to analyse data sets with two groups and <a href="#" name="a11" class="footnote">P</a> < 0.05 to **<a href="#" name="a12" class="footnote">P</a> < 0.001 indicated a significant difference. Two-tailed Student’s <a href="#" name="a13" class="footnote">t</a>-test was used to compare two means. Data were analysed using GraphPad (GraphPad). No statistical method was used to predetermine sample size.

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Extended Data Figure 1 | Hair follicle regeneration cycle. Hair follicle growth (Anagen) is characterized by downward expansion and generation of several epithelial layers. The most external layer, the outer root sheath (ORS), consists of relatively undifferentiated basal epithelial cells. Inner layers are generated by a committed progenitor pool, the matrix, which gives rise to several differentiated layers including companion layer, inner root sheath (IRS) and hair shaft. After growth, the majority of the newly formed layers are lost during the regression phase (Catagen), leaving a small surviving fraction of cells that reconstitute a new stem cell/progeny (Bulge/Hair Germ) compartment at the rest phase (Telogen).
Extended Data Figure 2 | Hair follicle inner layers resist apoptosis and continue upward terminal differentiation. a, Upward movement of hair follicle inner layers during growth and regression. Single optical sections show upward collective movement of inner layers relative to surrounding basal cells at two time points 130 min apart. Compare the position of labelled cells and dashed line of basal (red) to inner layers (yellow). b, Upward movement of hair shaft during regression. Optical sections of top view (epidermis) and side view (hair follicle) at two time points, 1 day apart. Note the extrusion of hair shafts from regressing hair follicles. Observations shown represent n = 3 mice. c, Companion layer lineage tracing during regression. Representative example of matrix progenitors of the companion layer traced during regression in Lgr5-CreER;tdTomato;K14-H2BGFP mice (n = 20 or 7 lineages, in 4 mice). d, Terminal differentiation of inner layer progenitor cells. Representative example of single-cell lineages (n = 35 or 9 lineages, in 3 mice) traced during the initial transition of hair follicle growth to regression in Shh-CreER;tdTomato;K14-H2BGFP mice. Scale bars, 25 μm.
Extended Data Figure 3 | Apoptotic bodies are cleared by neighbouring basal epithelial cells. a, Toluidine-blue-stained section of regressing hair follicles used for ultrastructure analysis. b, Electron micrograph illustrating multiple apoptotic bodies (red arrowheads) present in hair follicle basal epithelium, but absent in inner layers. c, Electron micrograph showing a hair follicle in regression (white dashed line). d, Electron micrograph showing the restriction of apoptotic bodies (red arrowheads) and phagocytic activity to the basal epithelium. e, High-magnification electron micrograph with immune-gold labelling for GFP protein expressed by K14-H2BGFP cells. Positive GFP labelling is present in both apoptotic bodies (Ap) and phagocytic basal epithelial nuclei (n). Observations shown represent n = 2 mice. Scale bars, 25 μm unless otherwise indicated.
Extended Data Figure 4 | Professional phagocytes are not present in regressing hair follicles. a, Professional phagocytes do not enter regressing hair follicles. Single optical sections showing absence of myeloid populations inside the hair follicle 2.5 h after epithelial cell death (arrowhead) in LysM-Cre;tdTomato;K14-H2BGFP mice. b, Immunofluorescent staining of myeloid populations in skin during hair follicle regression. DAPI, blue; CD11b, red; P-cadherin, green. Observations shown represent n = 4 mice. Scale bar, 25 μm.
Extended Data Figure 5 | Wnt/β-catenin activity is restricted to the inner layers during regression.  

(a, b) Immunohistochemistry (a) and immunofluorescent (b) staining highlighting active (nuclear) β-catenin of hair follicle inner layers (dashed line) at the onset of regression.  

(c) Immunofluorescent staining of the Wnt/β-catenin target gene, Lef1, during hair follicle regression. DAPI, blue; Lef1, red; P-cadherin, green. Asterisk indicates mesenchymal dermal papilla. Observations shown represent n = 2 mice. Scale bars, 50 μm.
Extended Data Figure 6 | Late and partial mesenchymal dermal papilla removal does not affect hair follicle regression. a, Sequential revisits of hair follicles after dermal papilla (DP) ablation during late regression. b, Box plot quantification of hair follicle length immediately after ablation, 4 days and 11 days after dermal papilla ablation (n = 20 follicles, in 4 mice; error bars represent maximum and minimum). c, Sequential revisits of hair follicles after partial dermal papilla ablation during early regression (n = 12 follicles, in 3 mice). d, Schematic illustration of the results from mesenchymal dermal papilla ablation experiments. Dermal papilla ablation during early regression results in failed elimination of the basal epithelium, while the inner layers continue upward in terminal differentiation, yet dermal papilla ablation during late regression does not impair hair follicle regression. Asterisk indicates auto-fluorescence from the two-photon laser. NS, not significant; P < 0.05, mean ± s.d. Scale bars, 25 μm.
Extended Data Figure 7 | Characterization of TGF-β pathway in mesenchymal dermal papilla and basal epithelial cell populations during regression. a, Schematic of skin digestion and cell isolation with representative images before and after tissue digestion in K14-H2BGFP;Lef1-RFP mice. b, Representative fluorescent-activated cell sorting (FACS) scheme for isolating mesenchymal dermal papilla (DP; RFP<sup>1</sup>, CD34<sup>+</sup>, CD45<sup>+</sup>, CD117<sup>+</sup>, integrin-α9<sup>+</sup>) and enriched hair follicle basal epithelium (RFP<sup>2</sup>, GFP<sup>High</sup>) cells. c, Validation of mesenchymal dermal-papilla-sorted population enrichment by Sox2 expression. d, Validation of basal-epithelial-sorted population enrichment by keratin 14 (K14) expression. e, TGF-β ligand 2 and 3 expression in the mesenchymal dermal papilla throughout the hair cycle. TGF-β1 expression in basal epithelium, mesenchymal dermal papilla, and all sorted cells during regression. f, Differential expression of TGF-β target genes: Smad7, Tmeff1, p15INK4B (also known as Cdkn2b) and in the hair follicle basal epithelium throughout the hair cycle (mean ± s.d.; n = 3 technical replicates). Scale bars, 100 μm. *P < 0.05, **P < 0.01, ***P < 0.001.
Extended Data Figure 8 | Local TGF-β activation during regression and validation of Cre-induced loss of TGF-βR1 expression. a, TGF-β activation shown by immunofluorescent staining of pSmad2 during the transition from hair follicle growth to regression. DAPI, blue; pSmad2, red; P-cadherin, green. Observations shown represent $n = 4$ mice. b, Representative FACS scheme for isolating tdTomato-Cre-reporter-positive basal epithelial cells (tdTomato⁺, GFP⁺) from Tgfbr1⁻/⁻β and Tgfbr1⁺/⁺ mice. c, Expression of TGF-βR1 and the TGF-β target gene, Smad7, in Cre-recombined basal epithelial cells from Tgfbr1⁻/⁻β and Tgfbr1⁺/⁺ mice ($P < 0.01$, mean ± s.d.; $n = 3$ technical replicates). Scale bar, 50 μm.
Extended Data Figure 9 | Extrinsic induction of hair follicle regression dictates the regenerative (stem cell) pool. Crosstalk with the mesenchymal niche during regression results in localized TGF-β activation, promoting a spatially restricted gradient of cell death in the basal epithelium. Clearance of apoptotic cells by neighbouring basal epithelial cells results in a limited pool of surviving stem cells. Inhibition of this regression process results in excessive amounts of basal epithelial cells capable of fuelling a new round of growth when in contact with the mesenchymal dermal papilla.