Reprogramming adult dermis to a neonatal state through epidermal activation of β-catenin

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SUMMARY

Hair follicle formation depends on reciprocal epidermal-dermal interactions and occurs during skin development, but not in adult life. This suggests that the properties of dermal fibroblasts change during postnatal development. To examine this, we used a PdgfraEGFP mouse line to isolate GFP-positive fibroblasts from neonatal skin, adult telogen and anagen skin and adult skin in which ectopic hair follicles had been induced by transgenic epidermal activation of β-catenin (EF skin). We also isolated epidermal cells from each mouse. The gene expression profile of EF epidermis was most similar to that of anagen epidermis, consistent with activation of β-catenin signalling. By contrast, adult dermis with ectopic hair follicles more closely resembled neonatal dermis than adult telogen or anagen dermis. In particular, genes associated with mitosis were upregulated and extracellular matrix-associated genes were downregulated in neonatal and EF fibroblasts. We confirmed that sustained epidermal β-catenin activation stimulated fibroblasts to proliferate to reach the high cell density of neonatal skin. In addition, the extracellular matrix was comprehensively remodelled, with mature collagen being replaced by collagen subtypes normally present only in developing skin. The changes in proliferation and extracellular matrix composition originated from a specific subpopulation of fibroblasts located beneath the sebaceous gland. Our results show that adult dermis is an unexpectedly plastic tissue that can be reprogrammed to acquire the molecular, cellular and structural characteristics of neonatal dermis in response to cues from the overlying epidermis.

KEY WORDS: Wnt, Dermis, Epidermis, Fibroblast, Niche, Stem cell, Mouse

INTRODUCTION

Stem cell behaviour is determined by the interplay between intrinsic transcriptional programmes and external signals from the local environment, or niche (Schofield, 1978; Fuchs et al., 2004; Watt and Driskell, 2010). How and when most mammalian stem cell niches form is little understood (Fuchs et al., 2004), yet this is crucial to defining the mechanisms by which stem cell activity is regulated during tissue regeneration and in disease.

One of the most prominent niche cell types is the fibroblast, a ubiquitous mesenchymal cell that plays a key role in constructing and maintaining the extracellular matrix (ECM) through the production of matrix proteins, metalloproteinases and inhibitors (Kalluri and Zeisburg, 2006). Fibroblasts are constituents of almost every tissue in the body, and are also active in the stroma of tumours (Kalluri and Zeisburg, 2006; Vermeulen et al., 2010). Their properties are known to change in different contexts. For example, there are differences between fibroblasts in developing and adult skin and between undamaged and wounded skin, including differences in cell migration and contractility and in growth factor and cytokine expression (Sandulache et al., 2007; Werner et al., 2007).

In skin, the formation of hair follicles from developing epidermis requires signals from fibroblasts in the underlying dermis (Millar, 2002; Fuchs, 2008; Yang and Cotsonis, 2010). Hair follicle morphogenesis takes place during the late embryonic and early neonatal period (Millar, 2002; Fuchs, 2008), and adult skin does not normally give rise to new follicles. Nevertheless, hair follicle neogenesis can be induced in adult mouse skin in response to transgenic or wound-induced epidermal activation of Wnt/β-catenin (Lo Celso et al., 2004; Silva-Vargas et al., 2005; Ito et al., 2007). The new hair follicles have associated dermal papillae (DP) (Lo Celso et al., 2004; Silva-Vargas et al., 2005; Ito et al., 2007), implying crosstalk between Wnt-activated epidermis and dermis. The signalling events involved have barely been explored, partly owing to a lack of fibroblast-specific reagents for direct isolation of purified fibroblast populations.

We have now used PdgfraEGFP reporter mice to directly isolate pure populations of dermal fibroblasts, allowing their transcriptional profiling and functional characterisation. Our results demonstrate that the dermal niche that supports epidermal differentiation is not a fixed entity, but is capable of comprehensive remodelling in response to extrinsic cues.

MATERIALS AND METHODS

Mouse strains

Experiments were performed in accordance with the UK Government Animals (Scientific Procedures) Act 1986. PdgfraEGFP (PdgfraH2B-eGFP) mice (Hamilton et al., 2003) were obtained from Jackson Laboratories and maintained on a C57BL/6 CBA mixed background. The D2 line of K14ANβ-cateninER (K14β-catER) mice was used (Lo Celso et al., 2004).

K14β-catER mice were treated at 6–8 weeks of age, corresponding to the telogen (resting) phase of the hair cycle. To activate expression of the transgene, the back skin of mice was treated with 1.5 mg 4-OHT (Sigma) dissolved in 0.1 ml acetone. For transient activation of β-catenin mice were treated once, and for sustained activation mice were treated three times per week for 2 weeks. In those experiments all mice were analysed 2 weeks following the first treatment. In the timecourse experiment, mice were treated three times per week for 1, 2 or 3 weeks and then analysed 3 days following the final treatment. In some experiments, mice were injected with BrdU in PBS (50 mg/kg) 2 hours before sacrifice.
Gene expression profiling

Total RNA was purified from sorted cell populations using the Preplink Micro-to-Midi Total RNA Purification System Kit (Invitrogen), including an on-column DNase treatment step. RNA was isolated from itg6-positive epidermal keratinocytes and PdgfraEGFP-positive dermal fibroblasts from four different experimental groups.

Genome-wide expression profiling was carried out by the Paterson Institute Microarray Core Facility. The data are deposited in the NIH GEO repository under accession number GSE32966. RNA quality was assessed using an Agilent Bioanalyzer and assays were performed in triplicate using samples with RNA integrity number (RIN) values of >8. A NuGEN Pico Kit was used to generate cDNA from 5 ng input RNA samples and cDNA was hybridised to Affymetrix MG430.2.A arrays. Array images produced by the Affymetrix PICR 3000 scanner were imported as CEL files into Genespring GX11 (Agilent) for analysis. RMA (robust multi-chip average) normalisation (baseline to median of all samples) was used.

Analyses were performed on genes selected for expression above the bottom twentieth percentile in all three samples within at least one of four experimental groups. Datasets derived from keratinocytes and from fibroblasts were treated as two separate experiments. To identify differentially expressed genes, we compared the four groups in each experiment using ANOVA followed by the Tukey HSD post-hoc test, with a P-value cut-off of 0.05. For each experiment, we constructed six lists of significantly regulated entities (ANOVA P<0.05) with a fold change of greater than 2 (supplementary material Tables S1, S2). These lists formed the basis of further analyses.

Quantitative (q) PCR

Total RNA was purified from sorted cell populations as described above and cDNA prepared using the Superscript III First-Strand Synthesis Supermix for qRT-PCR Kit (Invitrogen). qRT-PCR was carried out using the TaqMan Fast Universal PCR Master Mix and TaqMan probes and an ABI Prism HT7900 Sequence Detection System (Applied Biosystems). The following probes were used: Gapdh endogenous control (4352932E), Itg6 (Mm00434375_m1), Crabp1 (Mm00442776_m1), Krt14 (Mm00516876_m1), Krt10 (Mm03099211_m1), Lvl (Mm00515219_s1), Pdgfra (Mm01211694_m1), Ivl (Mm01333430_m1), Cola2 (Mm01165187_m1), Des (Mm00802455_m1) and Alx4 (Mm00431780_m1). Values were normalised to Gapdh expression using the ACT method (Jensen et al., 2009). Each assay was performed in triplicate on samples from four mice.

Histology and immunohistochemistry

Tissues were fixed in formal saline and embedded in paraffin. For frozen sections, tissues were placed in OCT compound (Tissue-Tek) and then frozen on dry ice. Neonatal dermis was minced and enzymatically dissociated using 0.4 mg/ml collagenase type I (Sigma) for 30 minutes at 37°C, with DNase I (20 U/ml) added for the final 5 minutes. Adult dermis was minced and dissociated for 20 minutes at 37°C using a mixture of 1.25 mg/ml collagenase type I (Invitrogen), 0.5 mg/ml collagenase type II ( Worthington), 0.5 mg/ml collagenase type IV (Sigma), 0.1 mg/ml hyaluronidase IVS (Sigma) and 50 U/ml DNase I. Enzyme activity was neutralised by the addition of serum-containing medium and preparations were passed through a 70-μm cell sieve to remove debris. The resultant single-cell suspensions contained a mixture of fibroblasts, hair follicle keratinocytes and other cells.

Flow cytometry

Cells were labelled with the following primary antibodies: rat anti-Itg6 (CD49f; Serotec), rat anti-CD45-PE (eBioscience), rat anti-CD31-PE (eBioscience) and rat anti-CD117-PE (c-Kit) (eBioscience). Non-conjugated primary antibodies were detected using chicken anti-rat Alexa Fluor 647 (Invitrogen). DAPI staining was used to exclude dead cells. Non-labelled cells and cells labelled with secondary antibody only were used as controls to set gates. An APC BrdU Flow Kit (BD Pharmingen) was used to perform BrdU staining. Flow cytometry analyses were carried out using a CyAn ADP Analyzer (Beckman Coulter). Sorting was performed using a MoFlo cell sorter (DakoCytomation). Re-analysis of sorted cells in each experiment showed that PdgfraEGFP-positive populations were of >97-100% purity and Itg6-positive populations of >97-98% purity.

Image acquisition and processing

Microscopy was carried out using a Leica SP5 confocal microscope utilising 405, 488 and 561 nm lasers and Leica Application Suite version 8.2.2. Images were obtained using a 20× HCX PL APO CS dry objective with an L1 405/UV correction optic and a 63× HCX PL APO water-immersion objective with L8 405 and L10 UV correction optics (Leica). Images were optimised globally for brightness and contrast using Photoshop CS4 and assembled into figures with Illustrator CS4 (Adobe).

RESULTS

Direct isolation of dermal fibroblasts using PdgfraEGFP

Platelet-derived growth factor receptor α (Pdgfrα) was previously reported to be a marker of the pre-DP dermal condensate in developing mouse skin (Karlsson et al., 1999). This led us to examine the skin of PdgfraEGFP mice, in which histone H2B-eGFP is expressed from the Pdgfra locus (Hamilton et al., 2003). In wholemounts of neonatal PdgfraEGFP back skin, GFP was expressed widely throughout the dermis (Fig. 1E,F), although the endogenous Pdgfrα protein (Fig. 1E,F), although the endogenous Pdgfrα protein was expressed throughout the dermis and within the DP at the base of developing follicle (Fig. 1A). Examination of neonatal mice with fluorescence illumination showed that GFP was expressed in the skin of all body regions (Fig. 1B). Immunohistochemistry of sections from developing and adult skin showed that GFP was absent from keratin 14 (Krt14)-positive epidermal keratinocytes but was expressed widely throughout the dermis (Fig. 1C–D). The same was true of endogenous Pdgfrα protein (Fig. 1E,F), although the endogenous Pdgfrα protein was expressed at the cell surface and in the
cytoplasm, whereas GFP was expressed in the nucleus (Fig. 1G). Double labelling for GFP and Pdgfrα confirmed extensive coexpression (Fig. 1H).

Flow cytometry of PdgfraEGFP+ cells isolated from adult back skin showed that GFP+ cells were negative for CD31 (Pecam1), CD117 (c-Kit) and CD45 (Ptprc) (Fig. 1I-L). PdgfraEGFP+ cells did not therefore express markers of endothelial cells, melanocytes and haemopoietic cells, respectively.

To confirm that PdgfraEGFP+ cells were indeed fibroblasts, we isolated three distinct populations from dissociated adult telogen back skin: GFP+ cells; Itga6+ GFP– keratinocytes; and viable cells negative for both markers (Fig. 2A). qPCR analysis showed that the GFP-positive population was highly enriched for Pdgfra mRNA and also for mRNA of the skin fibroblast marker genes Col1a2 and vimentin (Vim) (Fig. 2B-D) (Kalluri and Zeisberg, 2006). GFP+ cells were enriched for the DP markers Alx4 and Crabp1 (Fig. 2E,F) and contained very low levels of mRNA corresponding to markers (Itga6 and Krt14) of undifferentiated keratinocytes (Fig. 2G,H). Immunohistochemistry of neonatal back skin confirmed that PdgfraEGFP+ cells coexpressed Vim and Alx4 (Fig. 2I-L).

Col1a2 mRNA is a well-established marker of skin fibroblasts (Kalluri and Zeisberg, 2006). Since the GFP-positive population accounted for all of the detectable Col1a2 mRNA in the three cell populations, we conclude that PdgfraEGFP identifies the entire dermal fibroblast population. Furthermore, the lack of Krt14 mRNA establishes that the GFP-positive population does not contain keratinocytes.

In order to determine whether PdgfraEGFP+ fibroblasts could support hair follicle formation, we carried out skin reconstitution assays (Lichti et al., 2008) (Fig. 2M-P). Grafts of 3 × 10^6 neonatal keratinocytes alone (n=3) did not result in hair growth (Fig. 2O). However, grafts of keratinocytes combined with 3 × 10^6 flow-sorted PdgfraEGFP+ neonatal fibroblasts gave rise to hair in 3/3 grafts (Fig. 2N,P). GFP+ cells gave rise to all of the mesenchymal components of the hair follicle, including the DP, dermal cup, DP, dermal papilla, DS, dermal sheath. Scale bars: 150 µm in A; 50 µm in C; 200 µm in D,E,G,H; 40 µm in F.

Gene expression profiling of purified fibroblasts and keratinocytes during hair follicle neogenesis

We hypothesized that fibroblasts from developing and adult skin would have distinct characteristics relating to the presence or absence of hair-inductive capacity. To explore this, we carried out gene expression profiling of keratinocytes and fibroblasts isolated...
from neonatal and adult skin. In addition, we profiled cells from adult K14Δβ-cateninER (K14Δβ-catER) skin, in which the formation of ectopic follicles is induced by sustained epidermal activation of β-catenin using a 4-hydroxytamoxifen (4-OHT)-inducible transgene (Lo Celso et al., 2004). Applying 4-OHT six times over a 2-week period induces both ectopic follicles and anagen of existing follicles, whereas application of a single dose of 4-OHT results in anagen re-entry without ectopic follicle formation (Lo Celso et al., 2004; Van Mater et al., 2003) (supplementary material Fig. S1). Thus, by varying the number of 4-OHT treatments, we were able to distinguish gene regulation specific to anagen from gene regulation specifically associated with ectopic follicle formation.

We isolated RNA from epidermal keratinocytes (Itg6+) and dermal fibroblasts (PdgfraEGFP) purified from adult telogen skin, adult anagen skin (K14Δβ-catER, transient activation), adult anagen skin with ectopic follicles (EF) (K14Δβ-catER, sustained activation) and neonatal skin (Fig. 3A-H). Examination of histological sections showed that ectopic follicles were encapsulated by GFP-expressing cells and that all ectopic DP were GFP+ (Fig. 3C,D).

Gene expression profiling was performed on triplicate samples and datasets generated from epidermal keratinocytes and from dermal fibroblasts were treated as two separate experiments. For each experiment, we compared each pair of groups (a total of six pairs derived from four groups) to generate six lists of significantly
regulated entities (ANOVA $P<0.05$, fold change greater than 2) (supplementary material Tables S1, S2). These lists formed the basis of further analysis.

In the keratinocyte array, a total of 3057 entities were significantly regulated (ANOVA $P<0.05$) by more than 2-fold within one or more of six possible pairs of groups (Fig. 3I). In the fibroblast array, the total was 5536 entities (Fig. 3J). Pairwise comparison of the ‘Telogen’ (adult wild-type) group with the ‘Ectopic follicles’ (K14β-catER, sustained activation) group revealed significant regulation of 1068 entities in keratinocytes and 3002 entities in dermal fibroblasts (supplementary material Tables S1, S2). Therefore, both keratinocytes and fibroblasts are subject to dynamic transcriptional regulation in different developmental states. Epidermal activation of β-catenin results in substantial transcriptional regulation of niche fibroblasts.

**Reprogramming of the adult dermal fibroblast population to a neonatal state**

We carried out hierarchical clustering of all significantly regulated entities ($P<0.05$, fold change greater than 2) based on both entities and samples (Fig. 3I, J). In the keratinocyte experiment, the most closely related experimental groups were ‘Anagen’ (K14β-catER, transient activation) and ‘Ectopic follicles’ (K14β-catER, sustained activation), and these two groups were more closely related to the ‘Telogen’ (adult wild-type) group than to the ‘Neonatal’ group, which formed a distinct cluster (Fig. 3I). Therefore, in epidermal keratinocytes the most important determinant of variance in transcriptional regulation was the age (neonatal or adult) of the mouse. The level of upregulation of a selection of genes previously reported to be positively regulated by the Wnt pathway was similar when ‘Anagen’, ‘Neonatal’ and ‘Ectopic follicles’ epidermal arrays were compared with ‘Telogen’ (supplementary material Table S3).
The limited overlap in gene expression between the ‘Ectopic follicles’ and ‘Neonatal’ epidermal datasets might reflect, at least in part, the presence of pre-existing adult anagen follicles in the ‘Ectopic follicles’ sample.

In the fibroblast experiment, the adult ‘Telogen’ and ‘Anagen’ experimental groups were closely related. However, the adult ‘Ectopic follicles’ group was more closely related to the ‘Neonatal’ group than to either of the other adult groups (Fig. 3J). This result demonstrates that ectopic follicle formation in K14β-catER mice involves reprogramming of adult dermal fibroblasts to a state resembling neonatal fibroblasts.

**Dynamic expression of DP signature genes**

In the dermal fibroblast array, certain ‘core’ fibroblast genes, including *Pdgfra, Colla2* and *Vim*, were expressed at similar levels in all four experimental groups, showing that fibroblasts maintain essential characteristics that are independent of the developmental state (Fig. 4A). By contrast, expression of DP genes varied between experimental groups. Two hundred and twenty out of two hundred and twenty-five (98%) genes in a previously published DP signature list (Rendl et al., 2005) were detected above background levels in 3/3 samples in at least one of four experimental groups (not shown), consistent with the presence of DP cells within our cell isolates. Using a Venn diagram, the DP signature was overlaid onto the list of total regulated entities (5536). This showed that 113/225 (50%) of DP signature genes were significantly regulated (Fig. 4B).

Hierarchical clustering of regulated DP signature genes revealed several distinct clusters (Fig. 4C). Some DP genes, such as *Crabp1* (Collins and Watt, 2008), were highly expressed in all adult groups relative to the ‘Neonatal’ group. Consistent with a previous report (Enshell-Seijffers et al., 2008), the DP-specific serine protease 5194

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**Fig. 4. Sustained epidermal activation of β-catenin reprograms adult dermal fibroblasts toward a neonatal state.** (A) Heatmap representations of three ‘core’ fibroblast genes expressed at similar levels in all four experimental groups. (B) Venn diagram constructed from two lists comprising (1) all significantly regulated entities (*P*<0.05, fold change greater than 2) in the dermal fibroblast array and (2) 225 DP signature genes (Rendl et al., 2005). (C) Hierarchical clustering (based on entities) of 113 regulated DP signature genes derived from the region of overlap in B. (D-G) GO analysis of significantly regulated genes in the dermal fibroblast array experiment. Hierarchical clustering was performed using genes grouped under three selected GO terms (from a total of 275 significantly enriched terms, *P*<0.05), as indicated. Some genes are represented by multiple entities. Scale bar represents fold regulation (baseline to median of all samples).
Corin was highly upregulated in both the ‘Anagen’ group and ‘Ectopic follicles’ group (in which anagen follicles are also present) relative to the ‘Telogen’ group. Some DP genes were upregulated in both the ‘Ectopic follicles’ and ‘Neonatal’ groups, relative to the other two adult groups, implying a specific association with hair follicle neogenesis. These included Sox18 and Pth1r. Pth1r encodes the parathyroid hormone receptor, which activates β-catenin signalling through recruitment of Dishevelled (Romero et al., 2010) (Fig. 4C; supplementary material Table S2). We conclude that DP genes are expressed in all dermal preparations, but that the precise DP signature varies between different developmental states. The formation of ectopic follicles in adult skin involves the regulation of certain DP genes that are normally associated with hair follicle neogenesis during development.

**Gene Ontology analysis of dermal fibroblast array datasets**

Since DP genes represented only a small subset of the total genes regulated during dermal reprogramming (113/5536; 2%), we used Gene Ontology (GO) analysis to explore the list of total regulated entities (5536) in the dermal fibroblast array experiment. Two hundred and seventy-five GO terms satisfied the corrected P-value cut-off of 0.05. Of 5002 entities encompassed within at least one GO term, 31% were classified under ‘cellular component’, 31% under ‘biological process’ and 38% under ‘molecular function’.

Lists of genes associated with selected GO terms of interest were used to carry out hierarchical clustering (based on entities) (Fig. 4D-G). Many regulated genes fell within GO terms associated with developmental processes, such as ‘anatomical structure morphogenesis’ (Fig. 4D). In addition, numerous significant GO terms were associated with the ECM and secreted proteins, of which ‘extracellular space’ is represented as an example [see also the list of matrix metalloproteinase (MMP) genes in supplementary material Table S4]. There was a general downregulation of matrix and secreted proteins in both the ‘Ectopic follicles’ and ‘Neonatal’ groups (Fig. 4E). However, in both these groups a small number of genes encoding secreted proteins were strongly upregulated. These included the Wnt inhibitor Dkk1, which regulates hair follicle spacing (Sick et al., 2006), and Frzb, which promotes diffusion of Wnts through extracellular interactions (Mii and Taira, 2009). Also downregulated in the ‘Ectopic follicles’ and ‘Neonatal’ groups were many genes associated with the GO term ‘positive regulation of cell migration’ (Fig. 4F). Some of the most prominent groups of genes upregulated in both the ‘Ectopic follicles’ and ‘Neonatal’ groups were those associated with cell division, as exemplified by the GO term ‘mitosis’ (Fig. 4G). This implies an association of fibroblast proliferation with the formation of new hair follicles.

In summary, GO analysis indicates that key elements of dermal reprogramming during ectopic follicle formation are the general downregulation of genes associated with the ECM and cell migration and the strong upregulation of genes regulating mitosis.

**Sustained epidermal activation of β-catenin results in comprehensive remodelling of the dermis**

Gene expression profiling predicted that sustained epidermal activation of β-catenin stimulates dermal fibroblast proliferation and remodelling of the ECM. Labelling skin sections with antibodies to Vim or GFP confirmed that the number of dermal fibroblasts increased (Fig. 5A-H). The increase in the number of PdgfraEGFP+ cells was, as predicted, confined to the dermis (Fig. 5E-H).
To visualise the dermal ECM we performed classic histochemical staining using Herovici’s method (Fitzgerald et al., 1996), which stains mature collagen bright pink and immature collagen blue. Collagen in adult telogen and adult anagen skin was predominantly stained pink, consistent with the presence of a mature ECM undergoing relatively slow turnover during the hair cycle (Fig. 5I, J). Neonatal sections stained only weakly and the small amount of collagen present formed fine blue fibrils, consistent with an immature matrix (Fig. 5L). Sections from the ‘Ectopic follicles’ group also stained blue, and contained only sparse patches of mature pink collagen (Fig. 5K). Therefore, the mature matrix had been replaced with new, immature collagen (Fig. 5I-L).

**Sustained epidermal activation of β-catenin stimulates dermal fibroblast proliferation**

In light of the upregulation of genes associated with cell division (Fig. 4G; supplementary material Table S2) and the increased number of Vim+ and PdgfraEGFP+ fibroblasts (Fig. 5A-H) associated with the sustained epidermal activation of β-catenin, we predicted that fibroblast proliferation would be stimulated. When mice were injected with BrdU to label S-phase cells, there was a marked increase in GFP+ BrdU+ cells in the dermis following sustained activation of β-catenin, to a level that was comparable with neonatal dermis (Fig. 5A-F).

We used flow cytometry to quantify the absolute numbers of GFP+ fibroblasts and proliferating GFP+ BrdU+ fibroblasts per unit area of back skin (Fig. 5G). In comparison to telogen skin, sustained epidermal activation of β-catenin (six 4-OHT treatments) led to a 27-fold increase in the total number of PdgfraEGFP+ fibroblasts/cm², to a density approaching that of neonatal skin. Induction of anagen (one 4-OHT treatment) resulted in only a 1.5-fold increase. Similarly, the number of GFP+ BrdU+ fibroblasts/cm² increased only modestly in response to anagen but 35-fold in response to sustained β-catenin activation, to about half the density in neonatal skin (Fig. 5A-I).

Cell cycle analysis of PdgfraEGFP+ cells showed an increased percentage of cells in the mitotic (G2+M) phases of the cell cycle for both the ‘Ectopic follicles’ and ‘Neonatal’ groups. The high ratio of G2+M to S-phase (BrdU+) cells implies an increased rate of cell division (Fig. 6J-M). Thus, the formation of ectopic follicles in adult skin involves dramatic proliferative expansion of dermal fibroblasts, resulting in a densely populated dermis.

We conclude that sustained epidermal activation of Wnt/β-catenin signalling stimulates fibroblast proliferation and leads to structural remodelling of the entire dermis, not only the regions closely associated with ectopic follicles.
Adult dermal reprogramming originates from a subpopulation of fibroblasts associated with the hair follicle junctional zone

In order to establish the timing and dynamics of dermal reprogramming, K14\beta-catER/PdgfraEGFP mice were analysed after 4-OHT treatment for 0 days (no 4-OHT), 7 days (three doses), 14 days (six doses) or 21 days (nine doses) (Fig. 7). Consistent with earlier work (Lo Celso et al., 2004), in 7 day-treated back skin the original hair follicles had initiated anagen re-entry but no ectopic follicles had formed. Ectopic follicles were present in 14 day- and 21 day-treated skin.

Remodelling of ECM was first evident in the dermis adjacent to the epidermal junctional zone. The junctional zone is the region between the sebaceous glands, hair follicle infundibulum and bulge, and is the location of Lrig1\(^+\) epidermal stem cells (Jensen et al., 2009). Herovici staining showed that at 7 days the dermis adjacent to the junctional zone already comprised fine blue collagen fibrils, consistent with ECM remodelling. At this stage, the rest of the deep dermis consisted of a mixture of mature pink and immature fine blue fibrils collagen. (E-H) Frozen sections labelled with anti-Col11\alpha1 (red) and showing GFP fluorescence (green). Nuclei were stained with DAPI (blue). (I-L) Paraffin sections stained for Krt14 (green) and BrdU (red). (M,N) Frozen section of ectopic follicle-forming K14\beta-catER back skin labelled with anti-Lrig1 (red) and showing GFP fluorescence (green). The boxed regions in I and M are enlarged in J and N, respectively. Arrows point to: (E) a small population of PdgfraEGFP\(^+\) fibroblasts associated with the telogen junctional zone; (B,F) regions of recently regenerated collagen at the junctional zone; (C,D,G,N) ectopic follicles; (J,K) BrdU-labelled junctional zone fibroblasts; and (L) the DP of an original hair follicle, which is not labelled with BrdU. (O) The progressive remodelling of adult dermis originating from fibroblasts associated with the hair follicle junctional zone. Scale bars: 200 \(\mu\)m.
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During the timecourse we observed a progressive accumulation of PdgfraEGFP+ fibroblasts in regions associated with ectopic follicle formation and in the junctional zone at the top of hair follicles. This was consistent with earlier studies (Enshel-Seijffers et al., 2010; Chi et al., 2010; Horne and Jahoda, 1992). Our experiments established that the origin of new DP cells. The existence of the junctional zone as a common site of dermal reorganisation and ectopic hair follicle formation leads us to speculate that the altered mesenchymal niche formed there by epidermal β-catenin activation endows the adjacent epidermal cells with the ability to form ectopic hair follicles. Analysis of the keratinocyte microarrays identified a number of candidate fibroblast mitogens, including Fgf5, Fgf2, Tgfβ2 and Shh, that were upregulated in response to β-catenin activation (supplementary material Tables S1, S3, S5).

There is growing evidence for skin fibroblast heterogeneity (e.g. Driskell et al., 2009), reflecting, at least in part, different embryonic origins (Fliniaux et al., 2004) and body sites (Rinn et al., 2006). However, whether dermal fibroblasts are organised within a differentiation hierarchy, in which some cells have greater intrinsic proliferative and differentiation potential than others (Biernaskie et al., 2009) or whether proliferative heterogeneity is primarily determined through signals from the local microenvironment, remains to be investigated. It will also be interesting to compare, quantitatively, the ability of neonatal and reprogrammed adult fibroblasts to support hair growth in skin reconstitution assays.

The impact of epidermal Wnt/β-catenin on the dermal niche is important in light of the well-established role of Wnt signalling in epithelial cancers (Clevers, 2006). Epidermal activation of β-catenin led to the upregulation of a small subset of matrix genes including Col11a1, a prognostic marker of malignant stomach lesions (Zhao et al., 2009) and of colorectal cancers (Suzeveau et al., 2009). We speculate that the stroma of human sebaceous gland tumours bearing LEFI mutations that inhibit Wnt signalling (Takeda et al., 2006) will have different characteristics to the stroma of pilomatrixomas with activating β-catenin mutations (Chan et al., 1999). Given the normal role of Wnt signalling in many other adult epithelia, including intestine and lung, it will be interesting to determine whether the connective tissue stroma in other tissues can also be reprogrammed to an earlier developmental stage and, if so, whether this is of significance for tumour progression.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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