BJD
British Journal of Dermatology

Deciphering the molecular morphology of the human hair cycle: Wnt signalling during the telogen–anagen transformation

N.J. Hawkshaw,[1] J.A. Hardman,[1] M. Alam,[2,3,4] F. Jimenez[2,4] and R. Paus[1,3,5]

[1]Centre for Dermatology Research, The University of Manchester and NIHR Biomedical Research Centre, Manchester, U.K.
[2]Mediteknia Skin and Hair Lab, Las Palmas de Gran Canaria, Spain
[3]Monasterium Laboratory Skin and Hair Research Solutions GmbH, Münster, Germany
[4]Universidad Fernando Pessoa-Canarias, Las Palmas de Gran Canaria, Spain
[5]Dr Phillip Frost Department of Dermatology and Cutaneous Surgery, University of Miami Miller School of Medicine, Miami, FL, U.S.A.

Summary

Background The signals that induce anagen (growth) in 'quiescent' human telogen hair follicles (HFs) are as yet unknown. Their identification promises better targeted therapeutic hair growth interventions.
Objectives Recognizing the central role of Wnt signalling in hair biology, the aim was to delineate the differential expression of key agonists, antagonists and target genes of this pathway during the telogen-to-anagen transformation of human scalp HFs.
Methods This differential expression was studied by *in situ* hybridization in human telogen and early-anagen scalp HF sections.
Results On anagen induction, gene expression of the Wnt ligands *WNT3*, *WNT4* and *WNT10B*, the Wnt ligand secretion regulator *WLS*, and the Wnt target genes *AXIN2* and *LEF1*, is significantly increased within the secondary hair germ and the dermal papilla. Conversely, expression of the secreted Wnt inhibitor *SFRP1* (secreted frizzled-related protein 1) is reduced. Human epithelial HF stem cells upregulate *WNT4* and *WNT10A* expression, suggesting that these Wnt agonists are important for stem cell activation.
Conclusions We provide the first evidence that key changes in Wnt signalling that drive murine anagen induction also occur in human scalp HFs, yet with notable differences. This provides a rational basis for Wnt-targeting therapeutic interventions to manipulate human hair growth disorders.

Correspondence
Nathan Hawkshaw and Ralf Paus.
E-mail: Nathan.hawkshaw@manchester.ac.uk; rxp803@med.miami.edu; Ralf.Paus@manchester.ac.uk

Accepted for publication
12 July 2019

Funding sources
Supported in part by a PhD fellowship to N.H. from Giuliani (Milan), as well as by a basic research grants from Monasterium Laboratory (Muenster), the NIHR Manchester Biomedical Research Centre, Inflammatory Hair Diseases Programme, and start-up funds from University of Miami Miller School of Medicine to R.P.

Conflicts of interest
R.P. serves as a consultant for Giuliani, and is founder and managing owner of Monasterium Laboratory.

DOI 10.1111/bjd.18356

What's already known about this topic?

- Upregulation of Wnt agonists and downregulation of Wnt antagonists in the secondary hair germ and/or dermal papilla drives hair growth (anagen) induction in mice.
- Autocrine Wnt signalling in murine epithelial hair follicle stem cells is required to maintain their stem cell function.
- Reduction of Wnt ligands or increased expression of Wnt antagonists induces dysregulation of the murine hair follicle cycle and causes alopecia.

What does this study add?

- This study demonstrates for the first time that key Wnt pathway regulatory agonists, antagonists and target genes, are expressed in the human telogen-to-early-anagen transformation.

- On human anagen induction the Wnt ligands *WNT3*, *WNT4* and *WNT10B* are increased in the regenerating epithelium, whereas the Wnt antagonist, *SFRP1* (secreted frizzled-related protein 1), is reduced.
- Human anagen induction has fundamental differences in the expression of Wnt ligands compared with the murine system.

What is the translational message?

- Regulation of these Wnt ligands permits targeted therapeutic interventions in human hair growth disorders and informs development of new drugs that promote or suppress anagen induction.

Abnormalities in hair follicle (HF) cycling, that is the premature entry of growing (anagen) HFs into the regression stage of the hair cycle (catagen) and/or the retarded anagen induction in 'quiescent' (telogen) HFs, lie at the basis of most clinically important hair growth disorders.[1–4] While the molecular signals that drive HF cycling are well understood in the murine system,[5,6] these controls are as yet unexplored in human HFs. Although the organ culture of microdissected human anagen scalp HFs pioneered by Philpott and colleagues[7,8] has permitted major insights into key controls that regulate the anagen-to-catagen transformation,[9] such as changes in transforming growth factor-β and insulin-like growth factor-1 signalling,[10,11] the main molecular signals that induce anagen in human telogen HFs are unknown.[12] This has greatly hindered the rational development of potent new hair growth-modulatory drugs that target key anagen induction signals.

It is widely assumed that these signals mimic those identified in mice.[6] However, the substantial signalling differences between murine and human hair growth control[5] caution against the development of novel agents for the treatment of human hair growth disorders based on speculative assumptions rather than experimental evidence. Yet, generating the latter has been greatly hampered by the difficulty of microdissecting and organ-culturing human telogen and early-anagen HFs from human skin,[8] unless one has access to fully cycling human scalp HF xenotransplants on immunocompromised mice *in vivo*.[13] Therefore, the signalling mechanisms that underlie the telogen-to-anagen transition have been almost exclusively investigated in the murine HF.[12] As there are fundamental differences between murine and human HF biology, such as the expression of transcription factors[14] and bulge epithelial HF stem cell (eHFSC) markers[15] or the response to important hair growth-regulatory hormones like 17-β-oestradiol and prolactin,[16–19] it is unknown to what extent the mechanistic concepts identified in mice[4] apply to anagen induction in human HFs.

The main hurdle for clinical research in the study of the telogen-to-anagen transition in humans is the difficulty of obtaining '*in vivo*' fresh telogen samples. However, our group has recently reported a method of obtaining human telogen and early-anagen HFs by stereomicroscopic selection during the follicular unit (FU) hair transplantation process. When harvested, FU grafts are being stereomicroscopically visualized in order to classify them as one to four or more hair FU grafts, the suspected telogen or early-anagen follicles can be selected according to rigorous morphological criteria[13] and delineated using methylene blue staining.[1,20] In these freshly harvested human telogen and early-anagen scalp HFs, selected gene and protein expression changes can then be characterized by *in situ* hybridization (ISH) or immunofluorescence microscopy.

In the current study, we have undertaken the first step towards charting the 'molecular morphology' of human anagen induction in healthy human telogen HFs, using ISH to characterize gene expression changes for key molecular players of the Wnt pathway. This is because Wnt signalling is one of the most fundamental pathways for regulating the HF cycle in mice.[21–24] Specifically, Wnt activity is responsible for anagen induction by inducing proliferation within the secondary hair germ (SHG) and eHFSCs leading to regeneration of the HF.[21–23,25–27] While it has been doubted whether the SHG exists in human HFs,[12] morphological evidence clearly suggests that it does (Fig. 1).[13] This charting activity is a critical prerequisite for the rational, hypothesis-driven development of anagen-inducing or anagen-suppressing hair drugs that target Wnt signalling for the more effective management of hair growth disorders. These are based, at least in part, on insufficient/retarded anagen induction leading to telogen effluvium or alopecia such as androgenetic alopecia,[3] or on excessive anagen induction in telogen HFs leading to hirsutism/hypertrichosis.[28]

Materials and methods

Human hair follicle collection

Male occipital scalp HFs were obtained from five patients undergoing hair transplant surgery using the FU excision technique at Mediteknia Skin and Hair Lab, Gran Canaria, Spain. Tissue samples were harvested after obtaining written informed patient consent and ethical institutional approval (University of Las Palmas de Gran Canaria, CEIH-2014-06). Depending on the probe, three to 15 HFs were examined for quantification of either telogen or early-anagen HFs and derived from three to five donors.

Human hair follicle isolation

FUs were placed in physiological saline solution after extraction, with HFs microdissected and separated based on macroscopic morphology.[1] HFs were immediately embedded in optimal cutting temperature compound (OCT) and frozen in liquid nitrogen. Samples were stored at −80 °C prior to subsequent use.

Tissue processing

Using an OTF5000 cryostat (Bright Instruments, London, U.K.), 6-μm frozen sections from microdissected fresh telogen and early-anagen HFs were made. Haematoxylin stain was used to ensure that HFs were in the correct hair cycle stage, based on distinct morphological criteria.[13]

In situ hybridization

OCT-embedded HFs were sectioned at 6 μm, dried at −20 °C for 1 h, then stored at −80 °C for subsequent use. Sections were then processed for RNA *in situ* detection using the RNAscope 2·5 HD Assay-Red kit (Advanced Cell Diagnostics, Newark, CA, U.S.A.) following the manufacturer's instructions. The following probes were used: SFRP1 (NM_003012·4, target 401–1971), DKK1 (NM_012242·2, target 229–1523), AXIN2 (NM_004655·3, target 502–1674), LEF1 (NM_001166119·1, target 793–1919), WLS (NM_001193334·1, target 355–1325), WNT1 (NM_005430·3_target 390–1863), WNT2 (NR_024047·1, target 1324–2598), WNT3 (NM_030753·4, target 1014–1945), WNT3A (NM_033131·3, target 1212–2328), WNT4 (NM_030761·4, target 132–1770), WNT10A (NM_025216·2, target 658–1949), WNT10B (NM_003394·3, target 865–2282), PPIB as a positive control (NM_000942·4, region 139–989), and DapB as a negative control (EF191515, target 414–862).

Secreted frizzled-related protein 1/keratin 15 immunofluorescence

For eHFSC visualization and SFRP1 protein immunostaining, frozen HF sections of 6 μm were fixed in 4% paraformaldehyde for 20 min at 4 °C, then washed with Tris-buffered saline (TBS) (all wash steps used TBS). Tissue was then permeabilized with 0·5% Triton X-100 (in TBS) for 10 min. Sections were washed and incubated with 10% normal goat serum (NGS) (in TBS) for 30 min. Next, sections were incubated with secreted frizzled-related protein 1 (SFRP1) (1 : 200 in 10% NGS; cat. no. ab4193; Abcam, Cambridge, U.K.) and keratin 15 (K15) (1 : 500 in 10% NGS primary antibodies; cat. no. 80522; Abcam) primary antibodies overnight at 4 °C. The following day, sections were washed and incubated with Alexa Fluor 488 secondary antibody (1 : 200 in TBS; cat. no. A11008; Thermo Fisher Scientific, Waltham, MA, U.S.A.) and Alexa Fluor 594 secondary antibody (1 : 200 in TBS; cat. no. A11032; Thermo Fisher Scientific) for 1 h at room temperature. Sections were then washed and nuclei counterstained by using DAPI (4′,6-diamidino-2-phenylindole) 1 μg mL^{-1} in phosphate-buffered saline for 1 min. For negative controls, primary antibodies raised against SFRP1 and K15 were omitted. Congruence of the observed SFRP1 and K15 immunostaining patterns with the ones reported for human scalp HFs[14,32] was used as positive control.

Quantitative and qualitative morphometry of mRNA expression patterns

ISH and immunofluorescence were imaged using an Olympus BX53 upright microscope (Olympus, Tokyo, Japan). For representative immunofluorescence images, the contrast was changed globally within Microsoft PowerPoint (Microsoft Corporation, Redmond, WA, U.S.A.), and matching settings were applied to both telogen and early-anagen HFs. ImageJ software (https://imagej.nih.gov) was used to quantify the number of mRNA transcripts in the mesenchymal [connective tissue sheath and dermal papilla (DP)] and epithelial cells (SHG and regenerating hair-matrix) of telogen and early-anagen HFs. For qualitative analysis of eHFSCs two patient samples were used to confirm staining pattern.

Statistical analysis

The average mRNA signal for each donor sample was used for statistical analysis ($n = 3–5$ male donors for each mRNA probe target). To compare the anatomical regions of epithelial and mesenchymal cells but also between telogen and early-anagen human HFs, a two-way ANOVA with Šidák's multiple comparisons test was used to determine statistically significant changes. A P-value $< 0·05$ was considered statistically significant. Analysis was performed using GraphPad Prism version 7 software (GraphPad Software, La Jolla, CA, U.S.A.).

Results

Follicular units obtained during hair transplantation surgery permit one to interrogate gene expression changes during the human telogen-anagen transformation

FUs obtained during hair transplantation surgery permit interrogation of gene expression changes during the human telogen-to-anagen transformation. To selectively compare Wnt signalling read-out parameters between human telogen and early-anagen HFs (Fig. 1a), we turned to freshly isolated FU punch grafts, as they are routinely prepared and used during single-unit hair transplantation surgery.[29–31] During the hair transplant surgery process, on average 1000–1500 FUs were extracted from the patient's donor scalp and individually inspected under the stereomicroscope for FUs that contained macroscopically suspected telogen HFs, and then further microdissected into individual HFs (Fig. 1b). These HFs were histologically verified to be in either telogen or early-anagen by haematoxylin histochemistry (Fig. 1c) to ascertain their exact hair cycle stage by applying the standardized classification criteria.[13]

To investigate Wnt signalling activity and its regulatory components in the telogen-to-anagen transition we focused on

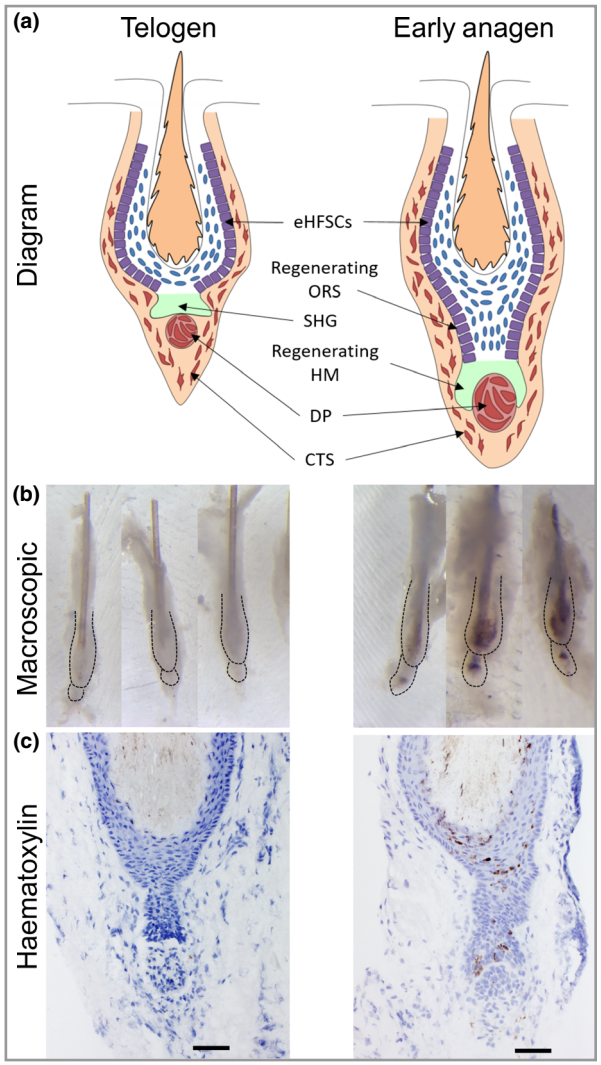

Fig 1. Isolation of human telogen and early-anagen hair follicles (HFs). (a) Diagram of the anatomical regions of human scalp HFs. (b) Human scalp telogen and early-anagen HFs were identified macroscopically from follicular units during hair transplantation. (c) These HFs were validated further by haematoxylin staining and morphology. Dashed lines depict anatomical regions in macroscopic images. CTS, connective tissue sheath; DP, dermal papilla; eHFSCs, epithelial hair follicle stem cells; HM, hair matrix; ORS, outer root sheath; SHG, secondary hair germ. Scale bars = 50 μm.

two compartments: (i) the SHG and (ii) the bulge region. This is because, in mice, anagen induction occurs in a two-step process: initially the SHG proliferates and forms the hair matrix, and next eHFSCs in the bulge region give rise to the outer root sheath (ORS) of the regenerating HF.[4,25]

Wnt signalling is elevated in both the regenerating epithelium and mesenchyme of human early-anagen HFs

To examine whether intrafollicular Wnt signalling occurs in the human system, these freshly isolated human telogen and early-anagen scalp HFs were subjected to single-molecule ISH,[32] and the canonical Wnt target genes *AXIN2* (Fig. 2c, d) and *LEF1*[22] (Fig. 2e, f) were analysed. Through this, low basal levels of *AXIN2* and *LEF1* mRNA in the SHG and DP of telogen HFs could be detected and were significantly higher in both selected compartments of early-anagen HFs (Fig. 2d, f), suggesting that Wnt signalling is increased on human anagen induction.

Wnt ligand expression is increased within the regenerating epithelium on anagen induction

Next, we wanted to determine which cells and Wnt ligands could be responsible for this increased Wnt signalling on human anagen induction. To do this, we analysed the key regulator of Wnt secretion, *WLS*,[23] and a panel of Wnt ligands (*WNT1*, *WNT2*, *WNT3*, *WNT3A*, *WNT4*, *WNT10A* and *WNT10B*). This revealed that both the SHG and DP of human telogen HFs express low levels of *WLS* and that this is elevated within the regenerating epithelium and mesenchyme of early-anagen HFs (Fig. 3a, b), suggesting that both HF compartments have the capacity to secrete Wnt ligands. However, of the seven Wnt ligands we analysed, *WNT3*, *WNT4* and *WNT10B* could be detected and were specifically elevated in epithelial cells and not fibroblasts (Fig. 3d, e, g), suggesting that the SHG is the main source of Wnt ligands in the human early-anagen HF. Notably, *WNT3* is the most abundant Wnt ligand on anagen induction (Fig. 3c, d) signifying that *WNT3* is particularly vital for the regeneration of the human HF.

The Wnt antagonist, secreted frizzled-related protein 1, is downregulated in epithelial cells and fibroblasts of human early-anagen hair follicles

As the balance of Wnt ligands and Wnt inhibitors determines activation of Wnt signalling,[33] we next wanted to ascertain if this increased Wnt signalling (Fig. 2) is also due to a reduction of Wnt inhibitors. To investigate the production of Wnt antagonists we analysed two secreted Wnt ligand inhibitors, *DKK1* and *SFRP1* (Fig. 4). This revealed that negligible levels of *DKK1* mRNA could be detected in telogen or early-anagen HFs (Fig. 4e), which is in agreement with previous murine data.[34] Conversely, *SFRP1* mRNA is not restricted to eHFSCs[22] and is expressed in the telogen SHG and fibroblasts (Fig. 4a, d). *SFRP1* is then reduced in both epithelial and mesenchymal cells within early-anagen HFs (Fig. 4a, d). To determine if the changes we detected by ISH at the mRNA level translated to protein, we analysed SFRP1 protein by immunofluorescence (Fig. 4b, c). Through this we detected prominent SFPR1 protein expression in the telogen HF, and on anagen induction SFRP1 immunoreactivity was reduced in both epithelial and mesenchymal cells (Fig. 4b, c). Collectively, this suggests that *SFRP1* is a more prominent Wnt inhibitor than *DKK1* in the human telogen HF and therefore may contribute to regulating Wnt ligand activity in the telogen-to-anagen transition.

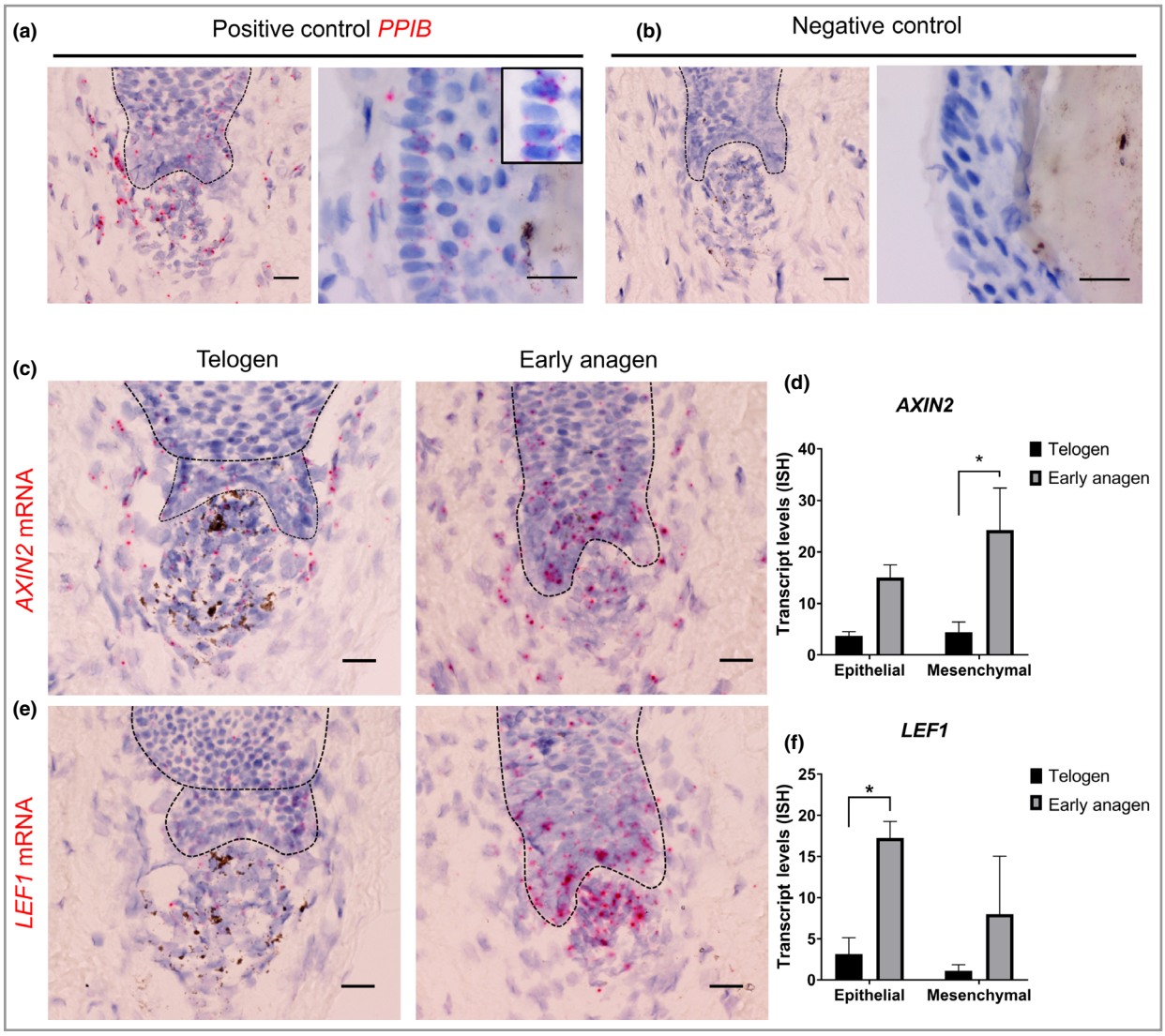

Fig 2. Wnt signalling (*AXIN2* and *LEF1* mRNA) is increased in human early-anagen hair follicles (HFs). (a) Positive and (b) negative controls for *in situ* hybridization (ISH) in the anatomical regions, dermal papilla, secondary hair germ (SHG) and bulge epithelial HF stem cells. (c) Representative images of *AXIN2* mRNA in telogen and early-anagen HFs. (d) Quantification by ISH of *AXIN2* mRNA signal. (e) Representative images of *LEF1* mRNA in telogen and early-anagen HFs. (f) Quantification of *LEF1* mRNA signal by ISH. Dashed lines depict the boundary between the SHG or regenerating hair matrix and the base of the bulge region. Inserts depicts positive mRNA signal. Scale bars = 20 μm. n = 3–5 male patient samples used for quantification of mRNA signal. Data are expressed as mean ± SEM. A two-way ANOVA with Šidák's multiple comparisons test was used to determine statistically significant changes. *$P < 0{\cdot}05$.

Human bulge epithelial hair follicle stem cells self-regulate Wnt activity

Finally, we qualitatively analysed the expression of Wnt signalling molecules within the human bulge region of telogen and early-anagen HFs (Fig. 5). Recently it was identified that murine bulge eHFSCs self-regulate Wnt activity;[22] therefore, we next wanted to determine if these specific Wnt signalling molecules identified in mice are also expressed in human telogen eHFSCs. We detected *AXIN2* mRNA in telogen basal eHFSCs (Fig. 5a) and these cells continued to be *AXIN2*$^+$ in early-anagen HFs (Fig. 5b), suggesting that Wnt signalling is constantly active in these cells. This further corroborates that telogen is not a 'resting' phase and that the 'quiescence' of the HF in this hair cycle window is, at best, a relative one, not only in mice,[4] but also in human scalp HFs. Next, we wanted to define the cells that are responsible for this Wnt activity by analysing *WLS* and Wnt ligand mRNA. *WLS* mRNA was detected in both the outer and the inner bulge layer of human telogen and early-anagen HFs (Fig. 5a, b), demonstrating that both layers have the capacity to secrete Wnt ligands.

Of the seven Wnt ligands we examined, none could be detected in the telogen bulge (Fig. 5a), despite the use of positive (Fig. 2a) and negative controls (Fig. 2b). Therefore, different Wnt ligands other than the ones we have examined here are responsible for regulating Wnt signalling during the telogen

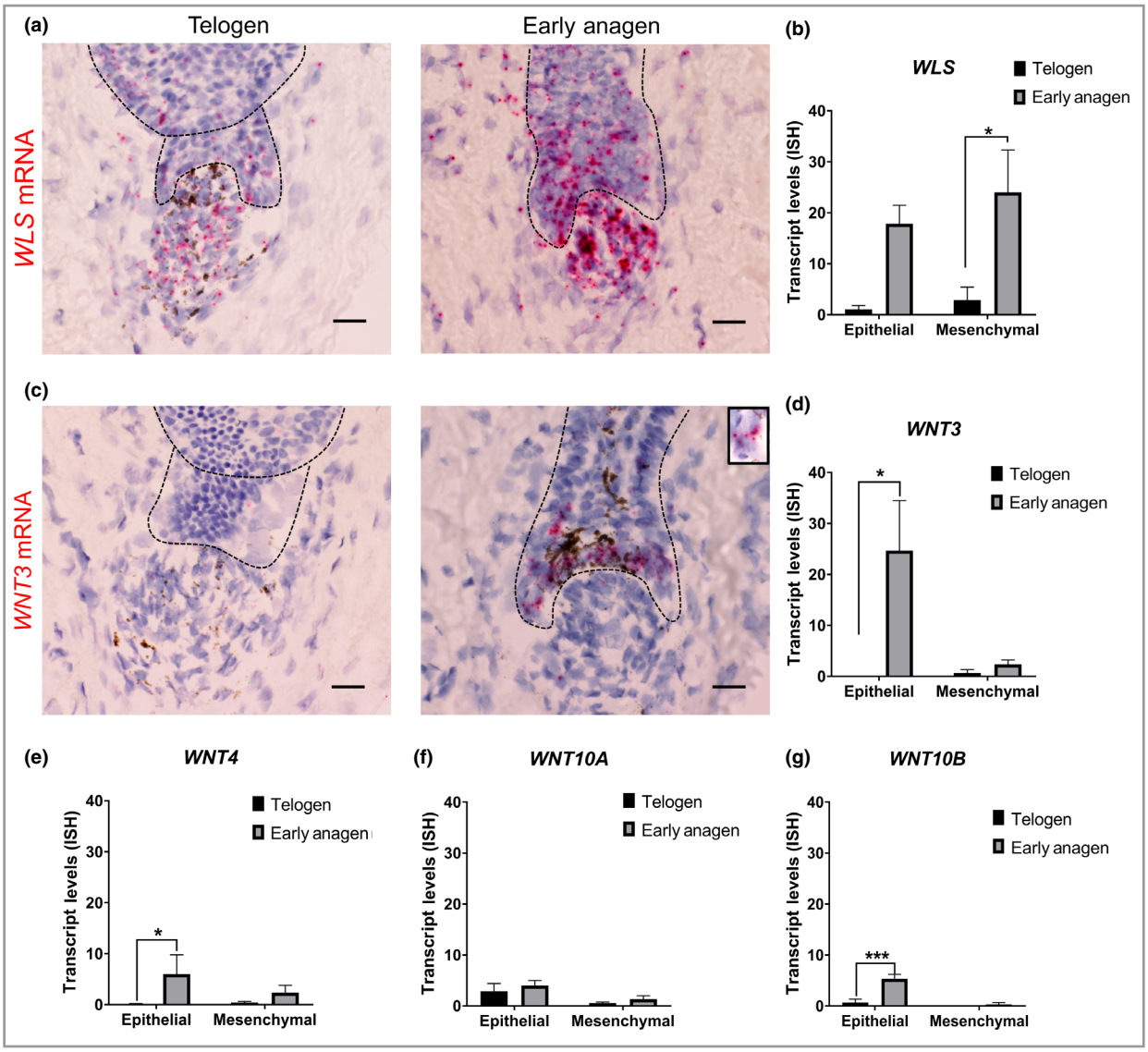

Fig 3. The Wnt ligand mediator, *WLS*, and *WNT3* mRNA is increased in the regenerating epithelium of early-anagen hair follicles (HFs). (a) Representative images of *WLS* mRNA in telogen and early-anagen HFs. (b) Quantification of *WLS* mRNA signal by *in situ* hybridization (ISH). (c) Representative images of *WNT3* mRNA in telogen and early-anagen HFs. (d) Quantification of *WNT3* mRNA signal by ISH. (e–g) Quantification of *WNT4*, *WNT10A* and *WNT10B* mRNA signal by ISH. Dashed lines depict the boundary between secondary hair germ or regenerating hair matrix and base of the bulge region. Insert depicts positive mRNA signal. Scale bars = 20 μm. n = 3–5 male patient samples used for quantification of mRNA signal. Data are expressed as mean ± SEM. A two-way ANOVA with Šidák's multiple comparisons test was used to determine statistically significant changes. *$P < 0{\cdot}05$, ***$P < 0{\cdot}001$.

phase. Conversely, in early-anagen, bulge eHFSCs are positive for *WNT4* and *WNT10A* (Fig. 5b), suggesting that these Wnt ligands are responsible for Wnt signalling during early-anagen.

Finally, we wanted to determine if these cells also express Wnt antagonists to moderate Wnt activity. This revealed *DKK1* mRNA is negative in the bulge region, whereas *SFRP1* mRNA transcripts and protein are detectable in both the inner and outer bulge region of human telogen (Fig. 5c–e) and early-anagen HFs (Fig. 5f–h). Collectively, this demonstrates that human eHFSCs are Wnt active, Wnt secreting and Wnt inhibitory, and therefore capable of self-regulating Wnt activity.

Discussion

Even though Wnt signalling is regarded as one of the most fundamental pathways for anagen induction,[4,35–37] there is no evidence yet that this also applies to human HFs.[12] In this detailed study, utilizing fresh human telogen and early-anagen HFs, we were able to demonstrate for the first time which components of the Wnt pathway can be detected in the telogen–anagen transition of the human HF cycle (Fig. 6). Moreover, with high sensitivity, we have identified which cells are Wnt active, Wnt secreting and Wnt inhibitory. Through this, we confirm that the core components of the Wnt pathway in

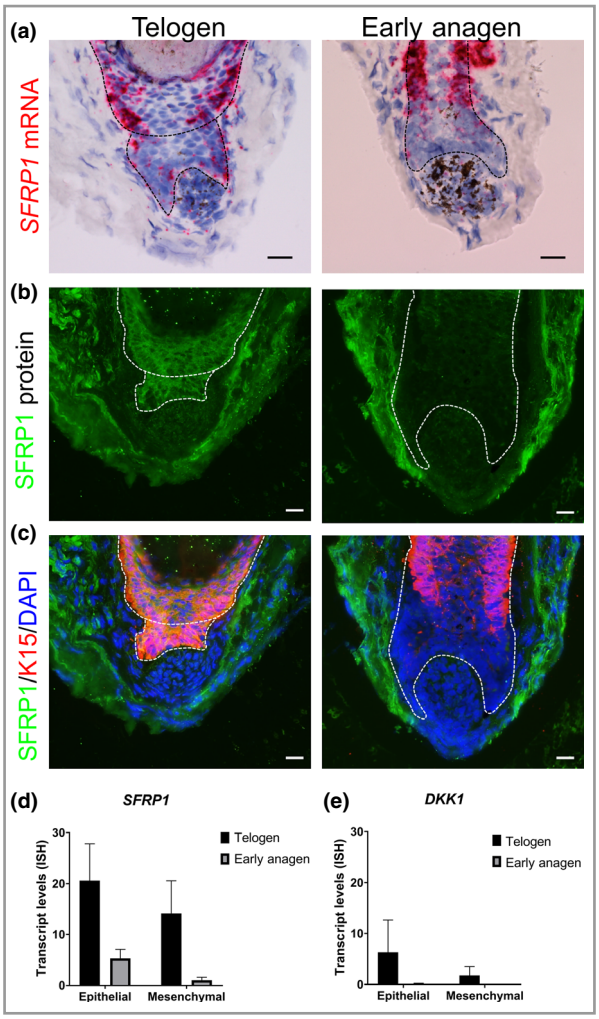

Fig 4. The Wnt antagonist, secreted frizzled-related protein 1 (SFRP1), is prominently expressed in human telogen hair follicles (HFs) and is reduced on anagen induction. (a) Representative images of *SFRP1* mRNA in telogen and early-anagen HFs. (b–c) Representative images of SFRP1 protein with the stem cell marker, keratin 15 (K15), by immunofluorescence microscopy in telogen and early-anagen HFs. (d) Quantification of *SFRP1* mRNA signal by *in situ* hybridization (ISH). (e) Quantification of *DKK1* mRNA signal by ISH. Dashed lines depict the boundary the between secondary hair germ or regenerating hair matrix and the base of the bulge region. Scale bars = 20 μm. n = 3–5 male patient samples used for quantification of mRNA signal. A two-way ANOVA with Šidák's multiple comparisons test was used to determine statistically significant changes. Data are expressed as mean ± SEM.

the human HF correspond with those of mice. That is: Wnt signalling is elevated on anagen induction in the SHG and DP; the mediator of Wnt ligand secretion, *WLS*, is elevated in both selected epithelial and mesenchymal compartments; and Wnt inhibitory signals are reduced on anagen induction.

While we demonstrate the differential mRNA expression of key Wnt signalling molecules and SFRP1 protein upon human anagen induction, further systematic follow-up and confirmation at the protein level is required (e.g. WNT3, WNT4, WNT10A/B, DKK1). In addition, the expression of cognate receptors (frizzled) remain to be systematically characterized, which is required to next ascertain signalling of autocrine and/or paracrine interactions.

While these core components of the Wnt pathway in humans resemble those of the murine HF, there are fundamental discrepancies in the regulatory modulators of the Wnt pathway. These discrepancies relate to the specific Wnt ligands and Wnt antagonists that are expressed, and whether or not their mRNA levels alter upon anagen induction.

More specifically, in the regenerating human epithelium we identify that *WNT3* is the most prominent Wnt ligand, whereas in mice *Wnt10b* is one of the most abundant ligands in this region.[38] In addition, murine *Wnt10a* mRNA is known to be highly expressed within the DP of early-anagen HFs,[38] whereas in humans we could detect only extremely low levels of *WNT10A* in the DP, which does not alter on anagen induction. Therefore, *WNT10A* cannot account for Wnt signalling in the human DP. Conversely, in mice eHFSCs, autocrine Wnt ligands are required to maintain β-catenin activity during both telogen and early-anagen.[22] While we could also detect Wnt signalling (*AXIN2* mRNA), the ligands in mice reported to sustain this (*Wnt1* and *Wnt4*)[22] are negative in humans, suggesting that Wnt ligands other than those we analysed are responsible for telogen eHFSC Wnt activation. In addition, the Wnt inhibitor *Dkk1* is regarded as a key Wnt antagonist capable of negatively regulating the telogen–anagen transition in mice.[39,40] However, we could only detect low *DKK1* mRNA in normal human telogen and early-anagen HFs. Instead, we verify that in the human telogen bulge, *SFRP1* is expressed not only in eHFSCs, just as in mouse HFs,[22] but also in the SHG and DP, and that SFRP1 expression declines during early-anagen development. Thus, in contrast to human anagen VI HFs,[32] in human telogen HFs, the *SFRP1* gene is also transcribed in distinct regions of the HF epithelium (Fig. 6). This suggests that, in the human telogen HF, DKK1 is not the predominant Wnt antagonist to modulate Wnt ligand activity on anagen induction, but rather that SFRP1 modulates Wnt for HF regeneration. Collectively, this underscores the clinical importance of directly using human tissue to ensure that therapeutic targets identified in the murine system are applicable to human skin.

From a clinical translational perspective, considering that aberrations in Wnt pathways result in changes in HF telogen–anagen status, modulating Wnt signalling through specific Wnt agonists and antagonists could have therapeutic consequences. In particular, activation of the most highly expressed Wnt ligand in anagen induction (*WNT3*) and/or inhibition of SFRP1, the most prominent Wnt-inhibitor that we found in this study, could be of significant therapeutic value for specific alopecias. The manipulation of hair growth by means of acting on these specific Wnt agonists and antagonists involved in human HF cycling, as demonstrated here, could render more valuable drug targets than using other Wnt proteins that may not have a significant role in human follicle formation[41] or whose hair growth effects are based on murine models.[42] Moreover, our demonstration that *WNT10A* is specifically expressed and switched on during early-anagen in bulge eHFSCs suggests that *WNT10A* is required for regenerating the ORS during the human hair cycle. This adds direct morphological

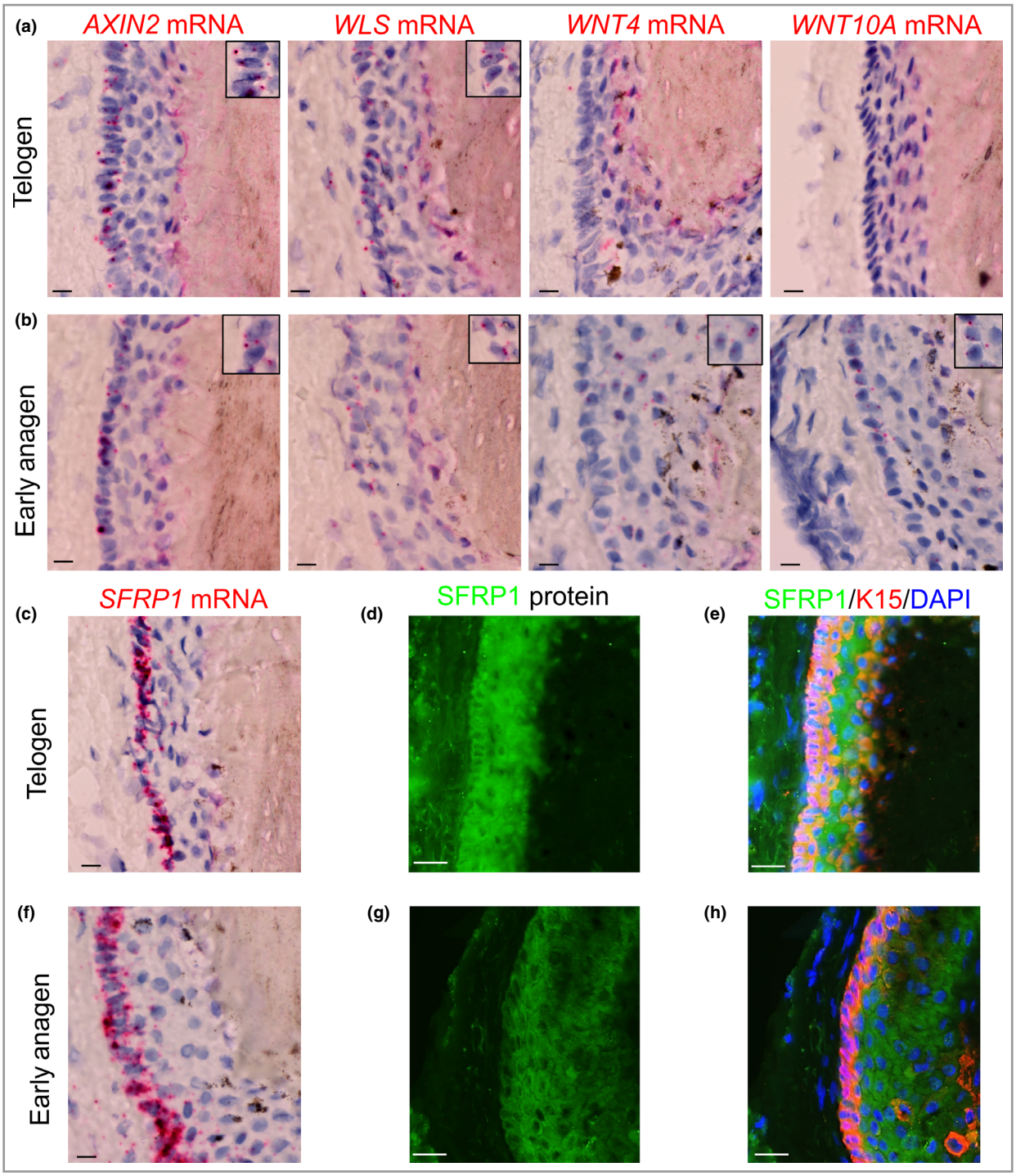

Fig 5. Detection of Wnt signalling molecules in human telogen and early-anagen bulge epithelial hair follicle stem cells (eHFSCs). (a) Representative images of the Wnt pathway in human bulge eHFSCs of telogen HFs. (b) Representative images of the Wnt pathway in human bulge eHFSCs of early-anagen HFs. (c) Representative image of secreted frizzled-related protein 1 (SFRP1) mRNA in telogen bulge eHFSCs. (d, e) Representative images of SFRP1 protein with the stem cell marker, keratin 15 (K15), by immunofluorescence microscopy in telogen HFs. (f) Representative image of SFRP1 mRNA in early-anagen bulge eHFSCs. (g, h) Representative images of SFRP1 protein with the stem cell marker K15 by immunofluorescence microscopy in early-anagen HFs. Inserts depict positive mRNA signal. Scale bars: a–c, f = 10 μm; d, e, g, h = 20 μm. DAPI, 4′,6-diamidino-2-phenylindole.

and cellular context to genome-wide association studies of androgenetic alopecia that identify a reduction in *WNT10A*,[43] and also helps to explain why patients diagnosed with ectodermal dysplasia[44] and Schöpf–Schulz–Passarge syndrome[45] who have a loss-of-function mutation in *WNT10A* suffer from alopecia or hypotrichosis.

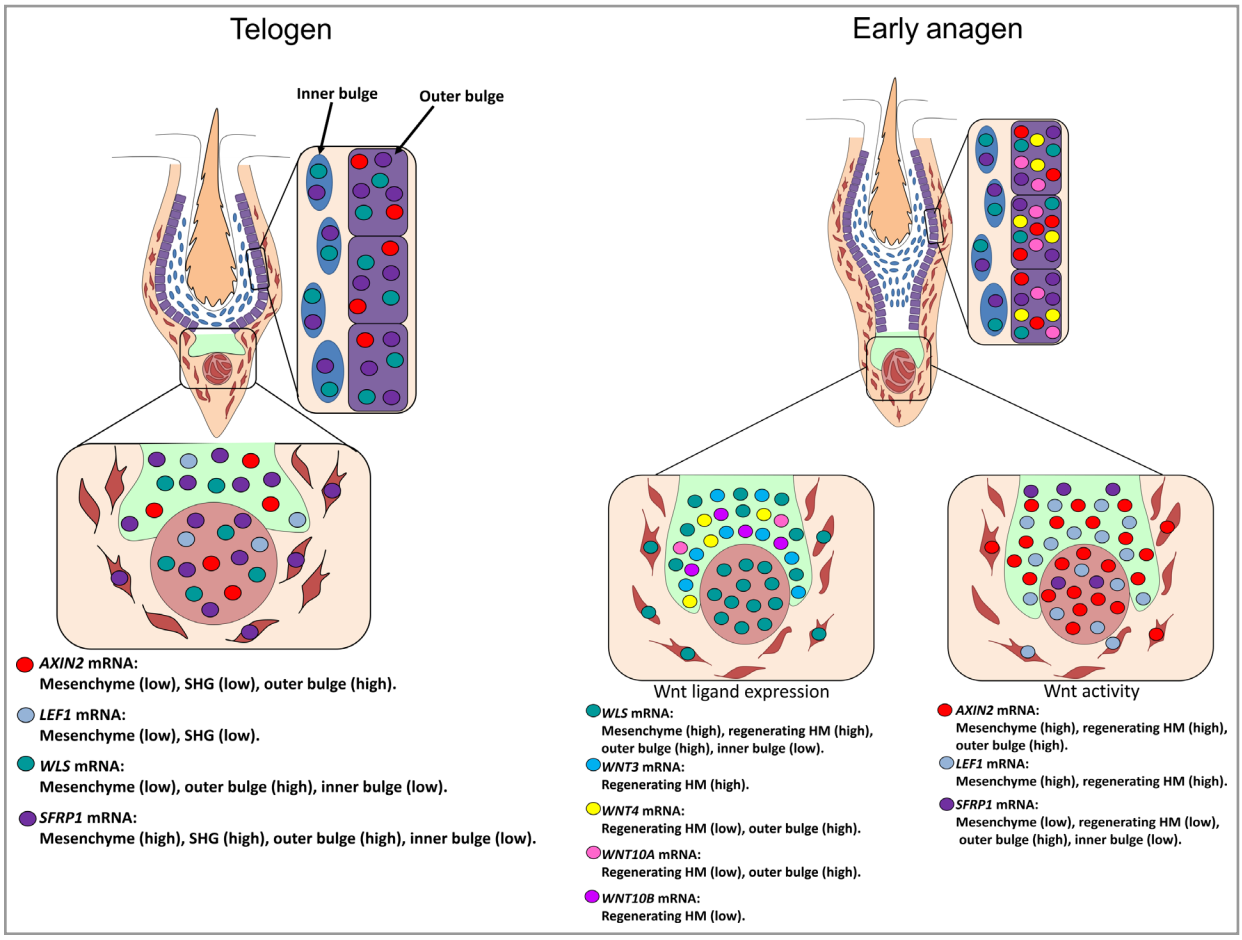

Fig 6. Illustration depicting the mRNA expression of key Wnt signalling molecules in human telogen and early-anagen hair follicles (HFs). In human telogen HFs the secondary hair germ (SHG) and mesenchyme express low levels of Wnt target genes (*AXIN*2 and *LEF1*), the Wnt ligand secretion mediator *WLS*, and Wnt ligands, whereas the secreted Wnt inhibitor, secreted frizzled-related protein 1 (*SFRP1*), is highly expressed within these anatomical regions. Conversely, bulge epithelial hair follicle stem cells express high levels of *AXIN*2, *WLS* and *SFRP1*, whereas the Wnt ligands we analysed in this study are negative (*WNT1*, *WNT2*, *WNT3*, *WNT3A*, *WNT4*, *WNT10A*, *WNT10B*). On anagen induction, the regenerating epithelium increases expression of *WLS* and Wnt ligands (*WNT3*, *WNT4* and *WNT10B*), whereas the mesenchyme increases expression of *WLS* and only negligible levels of Wnt ligands could be detected in this region. This also corresponded with high levels of Wnt target genes (*AXIN*2 and *LEF1*) in both the regenerating epithelium and mesenchyme and a reduction in *SFRP1*. The bulge region of early-anagen HFs continues to express *AXIN*2, *WLS* and *SFRP1*, and induces expression of *WNT4* and *WNT10A*. SHG, secondary hair germ; HM, hair matrix.

In addition to hair-loss disorders, the SHG and bulge are of particular clinical relevance because they may give rise to at least some basal cell carcinoma (BCC) in human skin,[46,47] and dysregulated Wnt signalling has also been implicated in BCC pathobiology.[48–50] Clinically, this renders a systematic examination of Wnt signalling in these key epithelial progenitor cell compartments of human HFs[15] important also from an oncological perspective.

Acknowledgments

The authors thank the industry sponsor of this PhD project, Giuliani, Milan, Italy, and in particular Giammaria Giuliani. We would also like to thank Dr Irene Hernandez and Derek Pye for their technical support.

References

1 Hernandez I, Alam M, Platt C *et al.* A technique for more precise distinction between catagen and telogen human hair follicles ex vivo. *J Am Acad Dermatol* 2018; **79**:558–9.
2 Paus R, Cotsarelis G. The biology of hair follicles. *N Engl J Med* 1999; **341**:491–7.
3 Qi J, Garza LA. An overview of alopecias. *Cold Spring Harb Perspect Med* 2014; **4**:pii: a013615.
4 Geyfman M, Plikus MV, Treffeisen E *et al.* Resting no more: re-defining telogen, the maintenance stage of the hair growth cycle. *Biol Rev Camb Philos Soc* 2015; **90**:1179–96.
5 Schneider MR, Schmidt-Ullrich R, Paus R. The hair follicle as a dynamic miniorgan. *Curr Biol* 2009; **19**:R132–42.
6 Rezza A, Wang Z, Sennett R *et al.* Signaling networks among stem cell precursors, transit-amplifying progenitors, and their niche in developing hair follicles. *Cell Rep* 2016; **14**:3001–18.

7 Philpott MP, Green MR, Kealey T. Human hair growth in vitro. *J Cell Sci* 1990; **97** (Pt 3):463–71.
8 Langan EA, Philpott MP, Kloepper JE, Paus R. Human hair follicle organ culture: theory, application and perspectives. *Exp Dermatol* 2015; **24**:903–11.
9 Kloepper JE, Sugawara K, Al-Nuaimi Y *et al.* Methods in hair research: how to objectively distinguish between anagen and catagen in human hair follicle organ culture. *Exp Dermatol* 2010; **19**:305–12.
10 Philpott MP, Sanders DA, Kealey T. Effects of insulin and insulin-like growth factors on cultured human hair follicles: IGF-I at physiologic concentrations is an important regulator of hair follicle growth *in vitro*. *J Invest Dermatol* 1994; **102**:857–61.
11 Soma T, Tsuji Y, Hibino T. Involvement of transforming growth factor-β2 in catagen induction during the human hair cycle. *J Invest Dermatol* 2002; **118**:993–7.
12 Panteleyev AA. Functional anatomy of the hair follicle: the secondary hair germ. *Exp Dermatol* 2018; **27**:701–20.
13 Oh JW, Kloepper J, Langan EA *et al.* A guide to studying human hair follicle cycling in vivo. *J Invest Dermatol* 2016; **136**:34–44.
14 Hawkshaw NJ, Haslam IS, Ansell DM *et al.* Re-evaluating cyclosporine A as a hair growth-promoting agent in human scalp hair follicles. *J Invest Dermatol* 2015; **135**:2129–32.
15 Purba TS, Haslam IS, Poblet E *et al.* Human epithelial hair follicle stem cells and their progeny: current state of knowledge, the widening gap in translational research and future challenges. *Bioessays* 2014; **36**:513–25.
16 Langan EA, Ramot Y, Goffin V *et al.* Mind the (gender) gap: does prolactin exert gender and/or site-specific effects on the human hair follicle? *J Invest Dermatol* 2010; **130**:886–91.
17 Langan EA, Griffiths CEM, Paus R. Utilizing the hair follicle to dissect the regulation and autocrine/paracrine activities of prolactin in humans. *Am J Physiol Endocrinol Metab* 2012; **302**:E1311–12.
18 Paus R, Langan EA, Vidali S *et al.* Neuroendocrinology of the hair follicle: principles and clinical perspectives. *Trends Mol Med* 2014; **20**:559–70.
19 Ohnemus U, Uenalan M, Inzunza J *et al.* The hair follicle as an estrogen target and source. *Endocr Rev* 2006; **27**:677–706.
20 Alam M, Hernandez I, Paus R, Jimenez F. Image Gallery: Intravital visualization of the dynamic changes in human hair follicle cycling. *Br J Dermatol* 2018; **178**:e396.
21 Lien WH, Polak L, Lin M *et al.* *In vivo* transcriptional governance of hair follicle stem cells by canonical Wnt regulators. *Nat Cell Biol* 2014; **16**:179–190.
22 Lim X, Tan SH, Yu KL *et al.* *Axin2* marks quiescent hair follicle bulge stem cells that are maintained by autocrine Wnt/β-catenin signaling. *Proc Natl Acad Sci U S A* 2016; **113**:E1498–505.
23 Myung PS, Takeo M, Ito M, Atit RP. Epithelial Wnt ligand secretion is required for adult hair follicle growth and regeneration. *J Invest Dermatol* 2013; **133**:31–41.
24 Enshell-Seijffers D, Lindon C, Kashiwagi M, Morgan BA. β-catenin activity in the dermal papilla regulates morphogenesis and regeneration of hair. *Dev Cell* 2010; **18**:633–42.
25 Greco V, Chen T, Rendl M *et al.* A two-step mechanism for stem cell activation during hair regeneration. *Cell Stem Cell* 2009; **4**:155–69.
26 Rompolas P, Deschene ER, Zito G *et al.* Live imaging of stem cell and progeny behaviour in physiological hair-follicle regeneration. *Nature* 2012; **487**:496–99.
27 Hsu YC, Pasolli HA, Fuchs E. Dynamics between stem cells, niche, and progeny in the hair follicle. *Cell* 2011; **144**:92–105.
28 Blume-Peytavi U. How to diagnose and treat medically women with excessive hair. *Dermatol Clin* 2013; **31**:57–65.
29 Jimenez F, Shiell RC. The Okuda papers: an extraordinary – but unfortunately unrecognized – piece of work that could have changed the history of hair transplantation. *Exp Dermatol* 2015; **24**:185–6.
30 Vogel JE, Jimenez F, Cole J *et al.* Hair restoration surgery: the state of the art. *Aesthetic Surg J* 2013; **33**:128–51.
31 Martínez ML, Escario E, Poblet E *et al.* Hair follicle-containing punch grafts accelerate chronic ulcer healing: a randomized controlled trial. *J Am Acad Dermatol* 2016; **75**:1007–14.
32 Hawkshaw NJ, Hardman JA, Haslam IS *et al.* Identifying novel strategies for treating human hair loss disorders: cyclosporine A suppresses the Wnt inhibitor, SFRP1, in the dermal papilla of human scalp hair follicles. *PLOS Biol* 2018; **16**:e2003705.
33 Clevers H, Nusse R. Wnt/β-catenin signaling and disease. *Cell* 2012; **149**:1192–205.
34 Kwack MH, Kim MK, Kim JC, Sung YK. Dickkopf 1 promotes regression of hair follicles. *J Invest Dermatol* 2012; **132**:1554–60.
35 Rompolas P, Greco V. Stem cell dynamics in the hair follicle niche. *Semin Cell Dev Biol* 2014; **25–26**:34–42.
36 Sennett R, Rendl M. Mesenchymal–epithelial interactions during hair follicle morphogenesis and cycling. *Semin Cell Dev Biol* 2012; **23**:917–27.
37 Lim X, Nusse R. Wnt signaling in skin development, homeostasis, and disease. *Cold Spring Harb Perspect Biol* 2013; **5**:pii: a008029.
38 Reddy S, Andl T, Bagasra A *et al.* Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a target of Sonic hedgehog in hair follicle morphogenesis. *Mech Dev* 2001; **107**:69–82.
39 Choi YS, Zhang Y, Xu M *et al.* Distinct functions for Wnt/β-catenin in hair follicle stem cell proliferation and survival and interfollicular epidermal homeostasis. *Cell Stem Cell* 2013; **13**:720–33.
40 Qiu W, Lei M, Zhou L *et al.* Hair follicle stem cell proliferation, Akt and Wnt signaling activation in TPA-induced hair regeneration. *Histochem Cell Biol* 2017; **147**:749–58.
41 Zimber MP, Ziering C, Zeigler F *et al.* Hair regrowth following a Wnt- and follistatin containing treatment: safety and efficacy in a first-in-man phase 1 clinical trial. *J Drugs Dermatol* 2011; **10**:1308–12.
42 Lee S-H, Yoon J, Shin SH *et al.* Valproic acid induces hair regeneration in murine model and activates alkaline phosphatase activity in human dermal papilla cells. *PLOS ONE* 2012; **7**:e34152.
43 Heilmann S, Kiefer AK, Fricker N *et al.* Androgenetic alopecia: identification of four genetic risk loci and evidence for the contribution of WNT signaling to its etiology. *J Invest Dermatol* 2013; **133**:1489–96.
44 Xu M, Horrell J, Snitow M *et al.* *WNT10A* mutation causes ectodermal dysplasia by impairing progenitor cell proliferation and KLF4-mediated differentiation. *Nat Commun* 2017; **8**:15397.
45 Tziotzios C, Petrof G, Liu L *et al.* Clinical features and *WNT10A* mutations in seven unrelated cases of Schöpf–Schulz–Passarge syndrome. *Br J Dermatol* 2014; **171**:1211–14.
46 Sellheyer K, Krahl D. Basal cell (trichoblastic) carcinoma common expression pattern for epithelial cell adhesion molecule links basal cell carcinoma to early follicular embryogenesis, secondary hair germ, and outer root sheath of the vellus hair follicle: a clue to the adnexal nature of basal cell carcinoma? *J Am Acad Dermatol* 2008; **58**:158–67.
47 Quist SR, Eckardt M, Kriesche A, Gollnick HP. Expression of epidermal stem cell markers in skin and adnexal malignancies. *Br J Dermatol* 2016; **175**:520–30.
48 Mohan SV, Chang ALS. Advanced basal cell carcinoma: epidemiology and therapeutic innovations. *Curr Dermatol Rep* 2014; **3**:40–5.
49 Do Carmo NG, Sakamoto LHT, Pogue R *et al.* Altered expression of *PRKX*, *WNT3* and *WNT16* in human nodular basal cell carcinoma. *Anticancer Res* 2016; **36**:4545–51.
50 Brinkhuizen T, van den Hurk K, Winnepenninckx VJL *et al.* Epigenetic changes in basal cell carcinoma affect SHH and WNT signaling components. *PLOS ONE* 2012; **7**:e51710.