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An Updated Classification of Hair Follicle Morphogenesis

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Abstract

Hair follicle formation in developing embryonic skin requires stepwise signaling between the epithelial epidermis and mesenchymal dermis, and their specialized derivatives, the placode/germ/peg and dermal condensate/papilla, respectively. Classically, distinct stages of hair follicle morphogenesis have been defined, in the mouse model, based on 1) changes in cell morphology and aggregation, 2) expression of few known molecular markers, 3) the extent of follicle downgrowth, and 4) the presence of differentiating cell types. Refined genetic strategies and recent emerging technologies, such as live imaging and transcriptome analyses of isolated cell populations or single cells, have enabled a closer dissection of the signaling requirements at different stages of hair follicle formation, particularly early on. They have also led to the discovery of precursor cells for placode, dermal condensate and future bulge stem cells that, combined with molecular insights into their fate specification and subsequent formation, serve as novel landmarks for early hair follicle morphogenetic events and studies of the signaling networks mediating these processes. In this review, we integrate the emergence of hair follicle precursor cell states and novel molecular markers of fate and formation to update the widely used 20-year old seminal classification guide of hair follicle morphogenetic stages by Paus and colleagues. We then temporally describe the latest insights into the early cellular and molecular events and signaling requirements for hair follicle morphogenesis in relation to one another in a holistic manner.

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AUTHOR CONTRIBUTION

N.S., K.W.M. and M.R. have conceived and written the manuscript and designed the figures. All the authors have read the manuscript and have approved this submission.

CONFLICT OF INTEREST

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1 | INTRODUCTION

The mature hair follicle (HF) is structurally complex, belying its small size. It is predominantly comprised of concentric rings of epithelial cells that form the hair shaft and inner root sheath (1), with reserve stem cells in the bulge region (2–7) and their progenitors, transit-amplifying matrix cells, at the bulbar base. Surrounded by the matrix is a central cluster of mesenchymal cells, the dermal papilla (DP), which acts as an instructive signaling niche (8–10) for these transit-amplifying progenitors to proliferate, migrate upwards and differentiate into the several layers of shaft and inner root sheath cell lineages during the hair growth phase (10–12). Adding to the complexity is the presence of other HF resident cell types: sebocytes that make up the mature sebaceous gland (13–15), and melanocytes that pigment the hair (16). The specification of the epithelial cell types of the HF and of the mesenchymal DP, from the embryonic placode (Pc) and dermal condensate (DC), respectively, and later emergence of other HF resident cell types, is a tightly controlled process during embryogenesis, both temporally and spatially (17).

Hair inductive capacity lies within the dermis, which has been demonstrated in “cut-and-paste” tissue recombination experiments; recombined hair-forming dermis and non-hairy glabrous epidermis, both derived from murine embryonic skin before HF morphogenesis, are able to form hair, while dermis from glabrous regions is unable to induce follicles even in hairy epidermis (18,19). Remarkably, xenograft experiments recombining feather-forming dermis from chicken with scale-forming epidermis from lizards or hair forming dermis from mouse with chicken epidermis results in the production of scale or feather structures, respectively, suggesting that the induction of epidermal appendages is controlled by the mesenchyme (19,20). While the “first dermal signal” for patterned initiation of HF morphogenesis in the epidermis remains elusive (21–23), it is known that widespread Wnt signaling activity in the upper dermis by embryonic day (E) 12.5 precedes HF formation (24) and is required for HF induction (25). This signal acts on the epidermis to induce Pc formation at ~E14.0 (17,26), which produce the “first epithelial signal” that acts on the underlying fibroblasts. One or more such signals, including FGF20 (27), lead to the formation of the DC, local condensations of upper dermal fibroblasts, at E14.5 (17). The DC then secretes still unknown “second dermal signals” to catalyze proliferation of Pc progenitors and downgrowth of the HF (17). Xenograft experiments have failed to completely form feathers, scales or HFs suggesting that species-specific signaling crosstalk between the epithelial and mesenchymal compartments initiated by the “second dermal signal” is necessary for proper downgrowth and differentiation (28,29). Complete understanding of these crucial reciprocal signals in early HF morphogenesis remains elusive, owing to the rapidity with which epithelial-mesenchymal crosstalk and subsequent morphological changes occur, although many individual components have been parsed.

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The evaluation of HF morphogenetic stages has historically relied upon progressive changes in cellular shape and morphology, dynamic aggregation of cells, emergence of follicle resident cell types, extent of HF downgrowth (17,30), and the usage of few known molecular markers, IL-1RI (30), TGF-βRII (31), and Alkaline Phosphatase (AP) (30,32). In a seminal classification guide from 20 years ago, Paus and colleagues summarized these key characteristics of HF morphogenesis to provide a well-defined classification system for greater spatiotemporal clarification of the major HF morphogenesis stages (30). Since its establishment, advanced mouse genetic methods have enabled numerous functional studies that uncovered the essential roles of major signaling pathways, such as Wnt, Eda/Edar, Fgf, Bmp, Shh and TGFβ signaling (21–23,26). Furthermore, many emerging technologies such as live imaging, multicolor fluorescent labeling and isolation of distinct cell types from specific stages, as well as high sensitivity transcriptomics at both the population and single-cell level, have enabled a more fine-toothed dissection of the cellular and molecular dynamics of HF morphogenesis. Such advances have permitted the definition of molecular signatures of Pc and DC, and neighboring cell types (33–35), as well as identified migration as the main cellular mechanism of Pc and DC formation (36–38). They have also allowed for the discovery of precursors to the Pc (pre-PC) (24,39–41), multipotent fibroblasts that give rise to the DC (42) and the fated precursors of DC (pre-DC) (43), by their molecular properties and prior to identifiable changes in cell morphologies and arrangement. Finally, they have enabled identification of suprabasal $SOX9^{+}$ precursors to HF stem cells after placode formation (44,45).

In this review, we update the well-established classification guide of HF morphogenesis stages by incorporating the recently discovered early precursor cell states and the many new cellular and molecular insights into early HF fate specification and formation. We then describe the current knowledge of reciprocal mesenchymal-epithelial interaction to provide a comprehensive overview of the dynamism of HF morphogenesis, focusing on early cellular, molecular and signaling events during the first wave of embryonic hair follicle formation.

2 | UPDATED STAGING OF HF MORPHOGENESIS

2.1 - Early Morphogenesis

To account for the recently discovered precursor cell states and new molecular events during the earliest phase of HF formation (“molecular placode” pre-Pc before morphological Pc; fated pre-DC before DC formation; HFSC precursors before bulge formation), we subdivided the previous stage 0 from the original classification (30) into 2 new stages, stages 1 and 2 (Figure 1). These are prefaced by a new stage 0 during which HF induction from the dermis is set up before any patterned molecular or cellular events. The previously classified advanced Pc/DC and germ stages then succeed the new precursor stages (Figure 1). All molecular markers related to early morphogenesis described in this review are featured in a comprehensive Figure 5 that is, for easy reference, color-coded by cell type and stage, and lists the corresponding cited publications.

For all stages, we describe the updated classification in the context of the first wave of primary guard hair formation, for which most new cellular and molecular insights have been discovered in recent years, likely due to the ability to study first wave hair formation in

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isolation. In the experimental mouse model, the touch sensitive (tylotrich) guard hairs are induced starting at approximately embryonic day (E)13.5, before the formation of secondary, non-tylotrich coat hair types (2^{nd} wave: awl, auchene, initiated at ~E15.5; 3^{rd} wave: zigzag, initiated at ~E17.5-E18.5) that make up the majority of adult hairs (27,46,47). Finally, while we provide the approximate gestational ages for each HF stage as they first appear, it is important to note that, due to variability of developmental timing, the early stages of first wave hair formation co-exist in parallel (e.g. at E15.0) and can be identified and distinguished by the stage-specific criteria defined below.

Stage 0 ––Before HF morphogenesis begins at ~E13.5, basal epidermal cells are a uniform layer without any morphological signs or patterned molecular distinctions of HF formation (Figure 1, Figure 2, stage 0). At stage 0, widespread Wnt signaling activity in the upper dermis (24) is important for setting up HF inductivity by supplying the critical, but still unknown “first dermal signal”: Epidermal ablation of Wntless (Wls), a mediator of broad epidermal Wnt ligand secretion, at E13.5 and broad dermal ablation of β-catenin both result in a loss of Wnt signaling activity in the dermis and subsequent absence of Pre-Pc induction (25), demonstrating the requirement of Wnt signaling upstream of still unknown target genes that act as inductive signals towards the epidermis. Knockout of the epidermal transcription factor *ΔNp63* prevents expression of Wnt target genes in early epidermal progenitors and subsequent HF formation (48), further confirming the important role of epidermis-derived Wnts and broad dermal Wnt signaling activity in pre-Pc fate specification and HF induction. Interestingly, recent single-cell RNA sequencing analyses suggest that upper dermal fibroblasts are transitioning toward DC fate specification at this early induction stage prior to morphogenesis (42).

Before pre-Pc specification, neural crest-derived melanoblasts, precursors of HF-resident melanocytes, are already present in the dermis. During Stage 0, melanoblasts begin migrating upward into the epidermis (49). At this stage of skin development, melanoblasts express Sox10 (50), Mitf (51), Pax3 (52), Dct (53), Kit (54) and Tyrp1 (53).

Stage 1 ––Still unknown signals from Wnt-responsive upper dermal cells act on the uniform epidermis to induce Pc formation, termed the “first dermal signal”. At stage 1 around E13.5 – 13.75, the molecular Pc precursor (pre-Pc) cell fate is focally induced in epidermal progenitors at sites of future HF morphogenesis, prior to any morphological signs (Figure 1, Figure 2 stage 1). Several markers for the pre-Pc state (“molecular placode”) have been identified, such as active Wnt signaling (24,55,56), Edar (57–59) and Fgf20 (27). Other genes are expressed in molecular placodes, but the precise timing of their expression with relation to DC fate acquisition in Stage 2 follicles is unclear. These include Wnt10b (60), its downstream target Dkk4 (40,61), and Cxcr4 (62) (Figure 2, Figure 5). The timing of signaling pathways and other inter- and intracellular molecular machinery that regulate expression of these genes has yet to be fully dissected. At this earliest pre-Pc stage, Wnt signaling remains widely active in the upper dermis.

Stage 2 ––The establishment of the pre-Pc then sparks the induction of the DC cell fate in the closest neighboring fibroblasts at ~E14.0 that precedes stereotypic aggregation of the mesenchymal DC cluster (Figure 2, stage 2) (43). These DC precursors (pre-DC) are at a

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transitional state from fibroblasts towards the DC fate. Pre-DC specification requires the pre-placodal production of Fgf20, an important component of the “first epithelial signal” (43) that is also required for DC aggregation and maintenance at the following stages (36,37). At this stage, the pre-Pc shows active Wnt signaling and expression of Edar, as well as Cxcr4 (62), Dkk4 (40,61) and Wnt10b (60). Whether Shh (24,55), Pcad (34) and Lhx2 (33,34), three bona-fide stage 3 Pc markers, are already expressed at stage 1 or 2 is currently unclear.

In contrast to the other fibroblast-type cells in the mesenchyme, including fibroblasts that will transition into pre-DC, pre-DC cells are already post-mitotic, indicating that acquisition of DC fate is concomitant with the shutdown of the cell cycle machinery (37,42,43). Like the pre-Pc, there are high levels of Wnt signaling activity in pre-DC (24), when compared to other dermal fibroblasts; in fact, Wnt activation is necessary for acquisition of DC fate (42) and DC formation (46). Pre-DC cells, ranging from 1 to ~15–20 cells, are randomly dispersed amongst dermal fibroblasts and located right below pre-Pc cells (separated by the basement membrane), which by contrast form a contiguous unit (Figure 2). Pre-DC can be discerned by *de novo* expression of Foxd1 and Sox2, as well as by upregulation of Tbx18 and highest expression of pandermal fibroblast marker Twist2 (43), which begins ramping up expression prior to acquisition of DC fate amongst upper dermal fibroblasts (42) (Figure 2). Foxd1 (35), Sox2 (8,63), Tbx18 (64), Bmp4 (25,65,66), Bmp7 (25), Hhip (62), and Fgf10 (41) have been previously identified as highly expressed signature genes in the aggregated DC (Figure 5).

Stage 3 ––Stage 3 closely resembles Stage 1 from Paus *et al* (30), and both the Pc and DC are morphologically identifiable in addition to expression of several signature genes (Figure 1, Figure 3, stage 3). The Pc, starting at ~E14.5, can now be distinguished from the rest of the epidermis because it appears thicker and is comprised of larger, tightly packed vertically oriented keratinocytes that slightly invaginate into the underlying dermis with basement membrane at the leading edge (Figure 3, stage 3). Most pre-Pc genes remain expressed joining an expanded Pc signature (Figure 3) (35) (http://hair-gel.net). Intriguingly, Cxcr4 expression wanes as the Pc matures, and is completely lost by Stage 4 HF morphogenesis, although the gene, itself, appears dispensable for HF morphogenesis (62). Shh (24,55),Pcad (34) and Lhx2 (33,34), a downstream target of Edar, are now expressed within these basal matrix progenitors. At this stage, $Sox9^{+}$ suprabasal HFSC precursors are specified during perpendicular asymmetric cell divisions of Shh-expressing basal Pc progenitors with high Wnt signaling activity (45,67) (Figure 1, Figure 3, stage 3), and, once suprabasal, have very low levels of Wnt signaling. Shh and Sox9 expression in basal Pc and suprabasal HFSC precursors, respectively, is largely non-overlapping (45,67). These HFSC precursors will, through the course of morphogenesis give rise to the all epithelial cells of the hair follicle, including the sebaceous gland (44,68) and Merkel cells (69) (Figure 4). Basal Pc progenitors themselves are precursors of the progenitors at the leading edge of downgrowing HFs that become replaced by HFSC progeny by the end of morphogenesis (Figure 4) (44).

The DC can now be recognized as an early cluster of aggregating cells. At this stage, the DC contains a greater number of cells (~35+) than pre-DC (Figure 3, stage 3). It begins to become polarized along the anterior-posterior (A-P) axis; this polarization becomes more prominent at stage 4, described below. They are also molecularly distinct from pre-DC:

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Sox2, Foxd1, Tbx18 expression is further upregulated and Twist2 expression is lost (43) (Figure 3). Earlier molecular studies of aggregated DC at E14.5 showed upregulated expression of genes associated with cell migration, axon guidance, canonical Wnt signaling, and Notch signaling (35), as well as additional molecular signature genes that can be queried at the accompanying web database (http://hair-gel.net) (Figure 3, Figure 5): Cxcr4 (62), Enpp2 (70), Nrp2 (71), Prdm1 (72,73), Sdc1 (74), Prom1 (75), and Trps1 (76). At this stage, DC weakly express AP, which is readily used as a marker for mature DP in postnatal skin (30).

Stage 4 ––By Stage 4, the Pc has elongated into the hair germ at ~E15.0, which has a more pronounced invagination into the dermis (Figure 1, Figure 3, stage 4). During Pc downgrowth, the germ is now prominently polarized as, through PCP-dependent cell rearrangements, the $Sox9^+$ HFSC precursors for the entire pilosebaceous unit migrate toward the posterior side of the developing HF with Pcad, Shh, Lhx2 expressing matrix precursors at the leading anterior edge (77). Bipotent HFSC precursors, for both the future bulge stem cells and sebaceous gland, additionally express Lrig1 (68,78). DC at this stage are positioned on the anterior cap of the developing HF, which is crucial for maintaining appropriate spatial organization of HFSC precursors (77). DC at Stage 4 are further clustered than DC at Stage 3, and are comprised of more aggregating non-proliferative cells (up to 90+ cells). Though, they are also molecularly distinct, although many markers are shared (35,43) (Figure 3, Figure 5). Finally, they express AP more highly, indicating differentiation toward the mature DP (30).

2.2 - Late Morphogenesis

Late morphogenetic events of Stage 5, beginning at ~E15.5, through Stage 10 closely resemble Stage 3 to Stage 8 in the original classification guide (30) (Figure 1). As such, we will reinforce the previously established staging, but will integrate the previously undescribed $Sox9^+$ HFSC precursors colonizing the bulge region. (Figure 4).

Stage 5 ––This stage is marked by more pronounced downgrowth of the HF, from the hair germ stage to the hair peg stage (Figure 1, Figure 4, stage 5). This is associated with an elongated epithelial cell morphology and concentric orientation around the axis of the future HF. Basal Pc cells continue to express Shh and Pcad, while the entire developing outer root sheath (ORS) expresses K5 (79). The upper portion of the stage 5 HF, where the eventual bulge is formed, co-expresses Sox9 and Lrig1 (44,78). The DC remains on the leading edge of the downgrowing hair peg and assumes a more rounded morphology, preceding its eventual engulfment by the HF matrix and transition into the mature DP.

Stage 6 ––By Stage 6, the hair peg begins to resemble a mature HF more closely. The lower portion of the hair peg, in closest contact with the DC, begins to form a bulb-like shape (Figure 1, Figure 4, stage 6). The HF lineages are also becoming specified; at this stage the inner root sheath (IRS) begins to develop, expressing Blimp1 in the Henle layer (72) and Gata3 in its Huxley and cuticle layers (80,81). Sox9 and Lrig1-expressing HFSC bipotent precursors remain in the upper ORS (78). The DC is in the process of engulfment; by Stage 6 the DC is more than 50% engulfed by the surrounding matrix cells at the base of

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the hair peg. The morphology of the DC is also becoming more akin to the mature DP; the DC is elongating and is longer than it is wide. $Sox10^+$ melanoblasts begin migrating into the developing HF from the epidermis (50,82) while melanoblasts at the epidermis gradually disappear (49).

Stage 7 ––—During Stage 7, the IRS is elongating up through the developing follicle, and the hair canal begins developing (Figure 1, Figure 4, stage 7). The hair shaft begins to form and expresses nuclear Lef1 and has active Wnt signaling in its pre-cortex (83). In pigmented mice, melanin granules are also detectable in the differentiating precortex region, above the DC. $Sox9^+$ HFSC precursors reside in the location of the future bulge of the mature HF in the upper ORS. The sebaceous gland lineage is delineated at this stage; Oil Red O^+ $Lrig1^+$ sebocytes are first visible in the upper part of the hair follicle, at the site of the future sebaceous gland (pre-SG) (30,78,84). The DC is almost entirely engulfed by the surrounding matrix.

Stage 8 ––—The growing HF has elongated past the boundary of the lower dermis, and the hair canal is morphologically visible (Figure 1, Figure 4, stage 8). The IRS has reached the level of the hair canal and starts to express trichohyalin, detected by AE15 antibodies (85), also expressed in the medulla of the hair shaft (80)(81). The maturing hair shaft can be recognized by AE13-stained hair keratins and marking of the cortex by Foxn1 (86). Other markers including K71 (K6irs1/Krt2–6g) (87), Cutl1 (88), and Hoxc13 (89) are expressed by the IRS at later stages, but it is unclear at which stage they first appear. The companion layer, which expresses K6 (81), can be found between the IRS and the K5+ ORS. Sebocytes form the sebaceous gland (SG), just above the bulge on the posterior side of the HF. The DP is now fully engulfed by matrix cells, and appears morphologically thinner than in Stage 7 DC. Melanoblasts are separated into two populations at two distinct locations: $Sox10^-$ precursors to melanocyte stem cells localize at the bulge region and $Sox10^+$ melanocyte precursors localize next to the DP in the hair matrix compartment (49,50,82).

Stage 9 ––—By Stage 9 of HF development, the tip of the hair shaft leaves the IRS and enters the hair canal (Figure 1, Figure 4, stage 9). The DP is even more fully narrowed.

Stage 10 ––—The HF has reached maximal length by Stage 10; it extends to the subcutaneous level (Figure 1, Figure 4, stage 10). The hair shaft emerges through the epidermis.

3 - ESSENTIAL SIGNALING PATHWAYS FOR HAIR FOLLICLE MORPHOGENESIS

3.1 - Initial HF induction

Broad dermal Wnt signaling activity is the upstream initiating event for HF morphogenesis. Subsequent Wnt signaling activation, alternating in epidermal Pc and dermal DC, leads to downstream signaling events to control HF formation. Several additional pathways, such as Eda, Fgf, Bmp and Shh signaling, have been identified over the past two decades to be

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essential in either compartment and their stage-specific roles will be described in the following.

3.1.1 - Sequential Wnt activity in dermis and epidermis—The importance of Wnt/β-catenin signaling in HF formation has been well demonstrated over the years (21–23,26,90–92). Mutant mice with eliminated expression of the transcription factor Lef1, a β-catenin binding partner, lack HF as well as other skin appendages such as teeth and mammary gland (93). Conversely, transgenic epidermal overexpression of Lef1 leads to abnormal HF clustering and ectopic HF formation in hairless epithelium (94), while mice expressing stabilized β-catenin in the epidermis exhibited de novo HF morphogenesis (95). Wnt10b expression (60) and Wnt signaling is localized in the nascent stage 1 and 2 pre-Pc (83), and it is essential for Pc induction as epidermal β-catenin ablation (24,55) prevented Pc formation. Likewise, forced broad epidermal misexpression of Wnt inhibitors Dkk1, normally found in the dermal fibroblasts surrounding early HFs, and Dkk2, a surrogate for pre-Pc marker Dkk4, both blocked Pc formation (40,56). In fact, the balance between Wnt signaling activators and inhibitors is thought to limit the number of HFs by setting up a pre-pattern through lateral inhibition that follows the reaction-diffusion model (40,96), famously proposed by Alan Turing nearly 70 years ago (97,98). In this model, short-range activation signals are counteracted by long-range inhibition signals, consistent with a wider diffusion range of smaller Dkks compared to larger, hydrophobic Wnts (96,99,100), thereby limiting the HF induction field. Besides driving Pc initiation, localized epidermal Wnt activity is also required for Pc formation during the transition from pre-Pc (stages 1 and 2) to Pc (stage 3) by coordinating cell migration (36): live imaging and tracking cell divisions during early Pc morphogenesis demonstrated that Wnt and Eda signaling mediate Pc formation through directed migration and cytoskeletal rearrangements, rather than cell proliferation.

Preceding focal Wnt signaling and Pc formation, broad uniform Wnt signaling activity in the upper dermis (24) is essential for pre-Pc induction at stage 0: dermal specific *β-catenin* ablation blocked localized Pc Wnt signaling and subsequent Pc formation (25). While broad dermal Wnt signaling is an absolute requirement for Pc fate specification, the Wnt target gene(s) that serve as key Pc inductive signal(s), i.e. the first dermal signal(s) is/are still unknown. Dermal Wnt activity itself requires production of epidermal Wnt ligands, as blocking Wnt ligand secretion in Wntless (Wls) mutants results in a failure of dermal Wnt signaling (25). Epidermal Wnt ligand expression is controlled by the transcription factor ΔNp63 (48), a key regulator of epidermal fate specification (101,102).

Finally, after HF induction and following localized Wnt signaling in the pre-Pc and Pc, intensified Wnt activity was also found in the early clustered DC of stage 3 HFs, which is required for HF progression (24,46). Also at stage 3, localized high Wnt signaling in basal Pc progenitors together with active Shh signaling (45) and a suprabasal Wnt^{low} signaling environment (67) are essential for suprabasal Sox9+ HFSC fate acquisition before HF downgrowth in the following stages. For this process, asymmetric cell division in the basal layer of stage 3 Pcs, perpendicular to the basal-suprabasal plane, is required for the emergence of suprabasal $SOX9^{+}$ HFSC precursors (45).

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3.1.2 - Eda signaling—Upon binding of TNF family member ligand Ectodysplasin (Eda) to its receptor Edar, downstream NFκB activation triggers transcriptional regulation essential for placode development (103,104). Mutations in Eda (*tabby*) and Edar (*downless*) in both humans and mouse models fail to form skin appendages such as HFs and teeth (105,106). During HF initiation, Eda is uniformly expressed throughout the epidermis, while Edar expression is confined to the stage 1 and 2 pre-Pc and later to the stage 3 Pc (39). Eda signaling is downstream of Wnt signaling; abolishing Wnt activity in the epidermis eliminates Edar expression and NFκB activation, while Wnt activity persists even after genetic abrogation of Eda signaling (24). Although Edar is one the earliest markers for pre-Pc, Eda/Edar signaling appears to be dispensable for pre-Pc induction of first wave HF. In the absence of Eda pre-Pcs remain stuck at the stage 1–2 stage, and is therefore required for further development to stage 3 Pc (24). Second and third wave HFs did form, but third wave HFs lost the characteristic zigzag shape, suggesting that Eda signaling also plays a role in establishing the molecular mechanism for hair shaft bending (107). In addition, this also indicates that molecular controls of HF morphogenesis can have intrinsic differences between first, second and third wave HFs.

3.1.3 - Fgf20 signaling—After pre-Pc fate initiation at stage 1, a “first epithelial signal” leads to specification and formation of the underlying DC. So far, Fgf20 has been the only identified epithelial signal that is directly required for DC formation; upon gene ablation of this Pc-derived factor, formation of aggregated DC and subsequent HF morphogenesis was abolished in all first and most second wave HFs (27). The same group very recently demonstrated that formation of the clustered DC is achieved through Fgf20-dependent cell migration and aggregation, and not proliferation (37). The intercellular machinery driving migration to form the condensed DC remains unknown, but recent profiling of DC suggested that actin remodeling and, intriguingly, axonal guidance genes may play a role (35). Preceding its role in DC cluster formation (stage 3), very recent work demonstrated that Fgf20 is already required for DC fate specification from fibroblasts before aggregation takes place (43). DC precursors, or pre-DC, are unclustered cells at stage 2 underneath pre-Pcs that transition from a fibroblast fate to acquire the DC gene expression program. In the absence of Fgf20, pre-DC fail to become specified.

Besides failure of DC specification and formation, Pc morphology was also severely altered in Fgf20 mutants, with Pcs forming stripe-like pattern instead of rounded shapes (27). Pc expansion beyond normal size may be due to impaired lateral inhibition from the KO pre-Pcs and the absent DCs that normally produce Dkk4 and Bmp2, and Bmp4, respectively. While Fgf20 is a downstream target of Wnt and Eda signaling, Edar expression levels were also severely reduced in Fgf20 knockout Pcs (27). Both cases raise the question of whether Fgf20 acts cell-autonomously on the pre-Pc, or whether perturbed placode development could be a secondary consequence of altered signaling inputs from the pre-DC (stage 2) or DC (stage 3). Taken together, as the first known epithelial signal from the pre-Pc (stage 1) towards the dermis, Fgf20 is responsible for promoting the transition of fibroblasts to the DC fate (43). Then Fgf20 instructs pre-DC cells to migrate and aggregate to form the clustered DC (37).

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3.1.4 - Bmp signaling—While Wnt, Eda and Fgf signaling promote HF morphogenesis, BMP signaling acts in an inhibitory fashion, likely to fine-tune and reinforce the lateral inhibition already set up by Wnt/Dkk diffusion gradients for proper spacing of HFs in the reaction-diffusion model (97). During HF morphogenesis, the ligands Bmp2 and Bmp4 are enriched in Pc and DC (25), respectively, and thought to inhibit a HF fate in neighboring epidermis where the receptor Bmpr1a is expressed (65). Conversely, the BMP inhibitor Noggin is enriched in the DC and thought to activate and promote Pc formation as short-range Bmp inhibitory signal: Overexpression of Noggin results in formation of excessive Pcs (108), while secondary HF fail to form in Noggin null mice (109,110).

3.2 - Hair follicle downgrowth

After initial HF induction, Pc and DC are formed after sequential first dermal and epithelial signals, respectively. The DC then produces the secondary dermal signal, which triggers proliferation of Pc progenitors for HF downgrowth. Continued signal crosstalk between the epidermal and dermal compartments is thought to be crucial for HF formation. To date, none of the second dermal signals have been identified yet, but epithelial Shh, Pdgfa, and Tgfβ signals have been shown to act on the dermal compartment during subsequent signal interplay for HF downgrowth (111–114). Shh signaling was also shown to be key for regulating specification of $Sox9^+$ suprabasal future bulge SCs (45), as well as for maintaining the proper cellular movement of developing Pc cells (77).

3.2.1 - Shh signaling—Shh is expressed in the pre-Pc and Pc at all stages, while the receptor Patched (Ptch) can be found in both epidermal and dermal compartments (113). Shh null mice display an arrest of HF development at the stage 4 germ, despite normal induction of both Pc and DC (111,112). Dermal specific ablation of Shh pathway component Smo abolished dermal Shh signaling and was shown to be important for Noggin expression in the DC and for maintaining the DC (115). Conversely, Pcs in Noggin null skins failed to express Shh mRNA and protein (110), suggesting that Shh signaling in DCs and Noggin-mediated BMP signaling inhibition in Pcs establish a positive feedback loop in developing HFs.

In addition to its role in maintaining the DC, two recent studies have shown that Shh is also indispensable for the development of the HF epithelial fate and formation. Ouspenskaia and colleagues demonstrated that Shh signaling is essential for the expansion of suprabasal $Sox9^+$ HFSC precursors (45). In Shh-null mice, there was a significant decrease in both the number of $Sox9^+$ suprabasal cells and levels of Sox9 expression. Moreover, high Wnt activity in basal Pc progenitors, the precursors of future matrix cells, is required for SHH expression, which then triggers symmetric divisions of overlying $Sox9^+$ suprabasal cells. Besides establishing HFSC, Shh signaling is also essential for placode invagination and counter-rotational placode cell movements that set up polarization during the stage 3 to stage 4 transition of early HF morphogenesis (Figure 4) (77). $Sox9^+$ suprabasal cells, which first appear around the Shh-expressing basal cells, migrate to the posterior position, while Shh-expressing cells move anteriorly. This cellular rearrangement is also DC dependent, as DC laser ablation resulted in aberrant Sox9 expression in anterior placode cells (77).

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3.2.2 - PDGFA signaling—During HF morphogenesis, the ligand Pdgfa is broadly expressed in the epidermis while the receptor, Pdgfra, is uniformly expressed in the dermis (113). Pdgfa ablation revealed a requirement of Pdgfa signaling for HF downgrowth; despite normal HF induction, the mice had a sparse hair coat due to retarded HF development (113). However, whether this a specific HF development defect has been called into question with a more recent study: Rezza and colleagues reported unperturbed HF induction and development following dermal specific Pdgfra ablation (116). As arrested HF phenotypes were only found in Pdgfa mutants with a severe systemic phenotype (113), and given that Pdgfa signaling is essential for many developmental aspects (117), the retarded HF development could stem from secondary effects of abrograted systemic Pdgfa signaling.

3.2.3 - TGFβ signaling—Besides Shh signaling, Tgfβ signaling has also been implicated as crucial for HF downgrowth. The ligand TGFβ2 is secreted by the dermis and acts on the receptor expressed by the epithelium (31,118,119). Studies in Tgfβ2 null mice revealed a requirement of Tgfβ signaling for HF downgrowth as HF development was arrested early (114). A second study of Tgfb receptor ablation demonstrated fewer and growth-retarded HFs (120). Interestingly, a recent study placed Tgfβ signaling downstream of Eda signaling in a NF-κB/Lhx2/Tgfβ signaling axis (33). Both mice with suppressed NF-κB activity and knockouts of NF-κB target Lhx2 have impaired TGFβ signalling, demonstrating that Eda signaling is not only important for Pc maintenance during stage 1 and 2 of HF formation, but is also essential for HF downgrowth by activating TGFβ signaling.

In summary, after the first dermal signals kick-start the initiation of HF morphogenesis, activation of Wnt and Eda signaling promotes pre-Pc formation and Pc stabilization. The nascent pre-Pc in turn provides the first epithelial signals, such as Fgf20, for inducing DC fate in unclustered precursors, and then aggregation of the maturing DC. At the same time, inhibitory signals including Dkk4 and Bmp4 from the Pc and DC, respectively, suppress HF formation in the interfollicular area to maintain even spacing between established HF. Second dermal signals from the DC then promote the proliferation of Pc progenitors for HF downgrowth. Continued signal interplay through Shh and Tgfb signaling further promotes HF development towards the formation of the mature HF.

4 – CONCLUDING REMARKS

The HF morphogenesis staging guide described by Paus and colleagues in 1999 has been widely used for 20 years; it has served as an important standard of HF development for classifying and comparing HF morphogenetic defects across numerous HF morphogenesis studies. In light of the recent discoveries of specific early precursor cell states in both the epithelial and mesenchymal HF compartments, as well as of the myriad novel molecular insights driving the classification of new stages, we propose this update here to the classical staging guide. To account for Pc induction at a “molecular placode” pre-Pc stage, for DC cell fate acquisition and pre-DC specification, and for the emergence of precursors to HFSC, we subdivided the previously defined stage 0 into 2 new stages that are prefaced by a new stage 0 with no specific patterned molecular or cellular events, while at the same time preserving previous established advanced stages that succeed the new precursor stages.

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Continued and concerted efforts over the past 20 years to parse out essential signals and controls of HF initiation, in both the epidermal and dermal compartments, have provided many important insights that we summarized here. Nevertheless, many details regarding the initiating events of the “first dermal signal” and relevant crosstalk between the Pc and DC in the “first epithelial signal” and “second dermal signal” remain elusive and the full spatiotemporal account of all HF morphogenetic signaling is complete. The near simultaneous activity of many known signaling pathways, in both the epidermis and dermis, and interplay of positive and negative regulation between them further complicates the story. A more granular perspective of early HF morphogenetic events, in combination with integrated *in vivo*, *in vitro*, and *in silico* approaches may aid in deconvoluting the roles of many of these signaling pathways to paint a more complete picture.

Technological advances have permitted the identification of stage-specific marker genes in embryonic epidermis and dermis for identification and isolation of relevant cell types, as well generation of more specific genetic drivers for *in* vivo study. Critically, single-cell RNA-sequencing has allowed for a more dynamic understanding of developmentally associated transcriptional dynamics, through the use of computational modeling, as well as identification of cellular heterogeneity. Since pseudotemporal ordering of cell fates in the dermis allowed for revealing a putative differentiation trajectory from upper dermal fibroblasts to pre-DC to Stage 3 and 4 DC (42,43), it is conceivable that cognate analyses of epidermal differentiation will add in the future increased temporal resolution and, in combination with dermal differentiation analyses, will shed light on critical signaling crosstalk. Through observations of *in vivo* phenomena in genetic abrogation studies, substantial insights into the staging of early HF morphogenesis have been made, to drive understanding of signaling necessity. And in combination with the modeling ability of *in silico* approaches, we predict that we will continue to move steadily toward gaining a clearer picture of the identity of crucial dermal and epithelial signals.

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et al 1999</th><th>Saxena
et al 2019</th><th>After Embryonic Day (E)</th><th colspan="3">Epithelium</th><th>Mesenchyme</th></tr>
<tr><td></td><td>Stage 0</td><td>~E12.5</td><td colspan="3">Unspecified Epidermis</td><td>Unspecified Upper Dermis
WNT^{hi}</td></tr>
<tr><td rowspan="2">Stage 0</td><td>Stage 1</td><td>~E13.5</td><td colspan="3">pre-Pc
Single Layer of Molecular Placode</td><td>Unspecified Upper Dermis
WNT^{hi}</td></tr>
<tr><td>Stage 2</td><td>~E14.0</td><td colspan="3">pre-Pc
Single Layer of Molecular Placode</td><td>pre-DC
Unclustered Precursors</td></tr>
<tr><td>Stage 1</td><td>Stage 3</td><td>~E14.5</td><td>Pc
pre-Mx Basal</td><td colspan="2">Pc
pre-HFSC Suprabasal,
Bipotent for SG & HF</td><td>DC
Clustered</td></tr>
<tr><td>Stage 2</td><td>Stage 4</td><td>~E15.0</td><td>Germ
pre-Mx at Leading Edge,
Anterior Polarized</td><td colspan="2">Germ
pre-HFSC Suprabasal
Posterior Polarized, Bipotent for SG & HF</td><td>DC
Clustered,
Polarized</td></tr>
<tr><td>Stage 3</td><td>Stage 5</td><td>~E15.5</td><td>Peg
pre-Mx at Leading Edge</td><td colspan="2">Peg
pre-HFSC in Upper ORS
Bipotent for SG & HF</td><td>DC
<50% Engulfed</td></tr>
<tr><td>Stages 4-5</td><td>Stages 6-7</td><td>~E16.0
onwards</td><td>Bulbous Peg
Mx in Bulb</td><td>Bulbous Peg
HFSC in Upper ORS
Unipotent for HF</td><td>Bulbous Peg
SG in Upper ORS
Unipotent for SG</td><td>DC
>50% Engulfed</td></tr>
<tr><td>Stages 6-8</td><td>Stages 8-10</td><td>~E17.5
onwards</td><td>Follicle
Mx in Bulb</td><td>Follicle
HFSC in Bulge
Unipotent for HF</td><td>Follicle
SG
Unipotent for SG</td><td>DP
100% Engulfed</td></tr>
</table>

Figure 1. Updated Classification of Hair Follicle Morphogenesis Stages

Updated hair follicle morphogenetic classification including new stages for emergence and spatial localization of recently discovered precursor cell states in the epithelial and mesenchymal compartments, including pre-Pc, pre-DC and HFSC precursors. Differentiation of the pre-Pc into the Pc, then into Mx (matrix), HFSC, and SG (sebaceous gland), as well as of the pre-DC into the DP serve as hallmarks for stage identification. The previous classification system by Paus *et al*, 1999 is outlined. The approximate gestational ages are provided for HF stages as they first appear during the first wave of primary guard hair formation.

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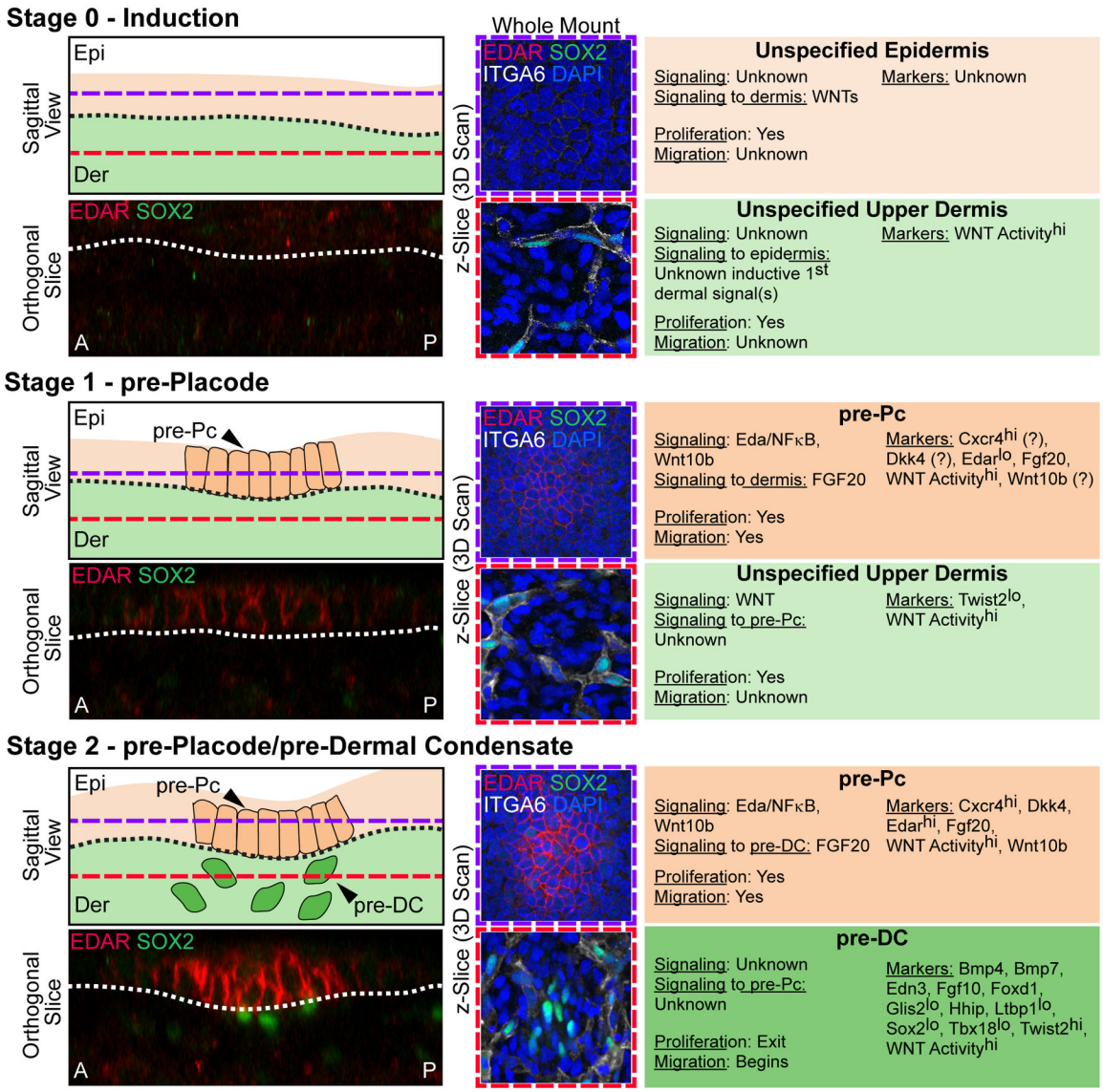

Figure 2. Stages 0–2 of Hair Follicle Morphogenesis

Left top: Sagittal view schematic of HF morphogenesis stages. Purple dashed lines mark the epidermal Z-plane, and red dashed lines mark the dermal Z-plane of 3D-imaged confocal scans of whole mount immunofluorescence of E15.0 back skin. A and P denote anterior and posterior orientation of embryonic skin (head is left). Left bottom: Orthogonal slice from a 3D reconstruction of whole mount immunofluorescence for EDAR and SOX2. Middle: Whole mount immunofluorescence for EDAR, SOX2, and ITGA6 in the epithelial plane (top, purple frame), and dermal plane (bottom, red frame). DAPI marks all nuclei. Right: Description of autocrine and paracrine signaling, proliferation and migration status, and markers of the relevant epithelial and mesenchymal populations at each stage.

Stage 0 – Induction. The uniform unspecified multipotent epidermis resides over an unspecified dermis. EDAR is not expressed in the epidermal compartment and only $SOX2^{+}$/$ITGA6^{+}$ Schwann cells are present in the dermis. Widespread Wnt signaling activity in the upper dermis sets up HF induction by the critical, but still unknown “first dermal signal(s)”.

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Stage 1 – pre-Placode. Emergence of placode precursors (pre-Pc), the fated "molecular placode", in the epidermis over an unspecified dermis. The pre-Pc in the epidermal plane expresses EDAR. Only $SOX2^{+}$/ $ITGA6^{+}$ Schwann cells are present in the dermal compartment. Markers with question marks refer to known expression by *in situ* hybridization and/or protein staining at E13.5, in which the presence of recently discovered pre-DC (stage 2) cannot be ruled out.

Stage 2 – pre-Placode/pre-Dermal Condensate. pre-Pc in the epidermis over dermal condensate precursors (pre-DC) in the dermis. The pre-Pc in the epidermal plane expresses EDAR more strongly. Pre-DC emerge as low-level $SOX2^{+}$/$ITGA6^{-}$ unclustered cells underneath pre-Pc.

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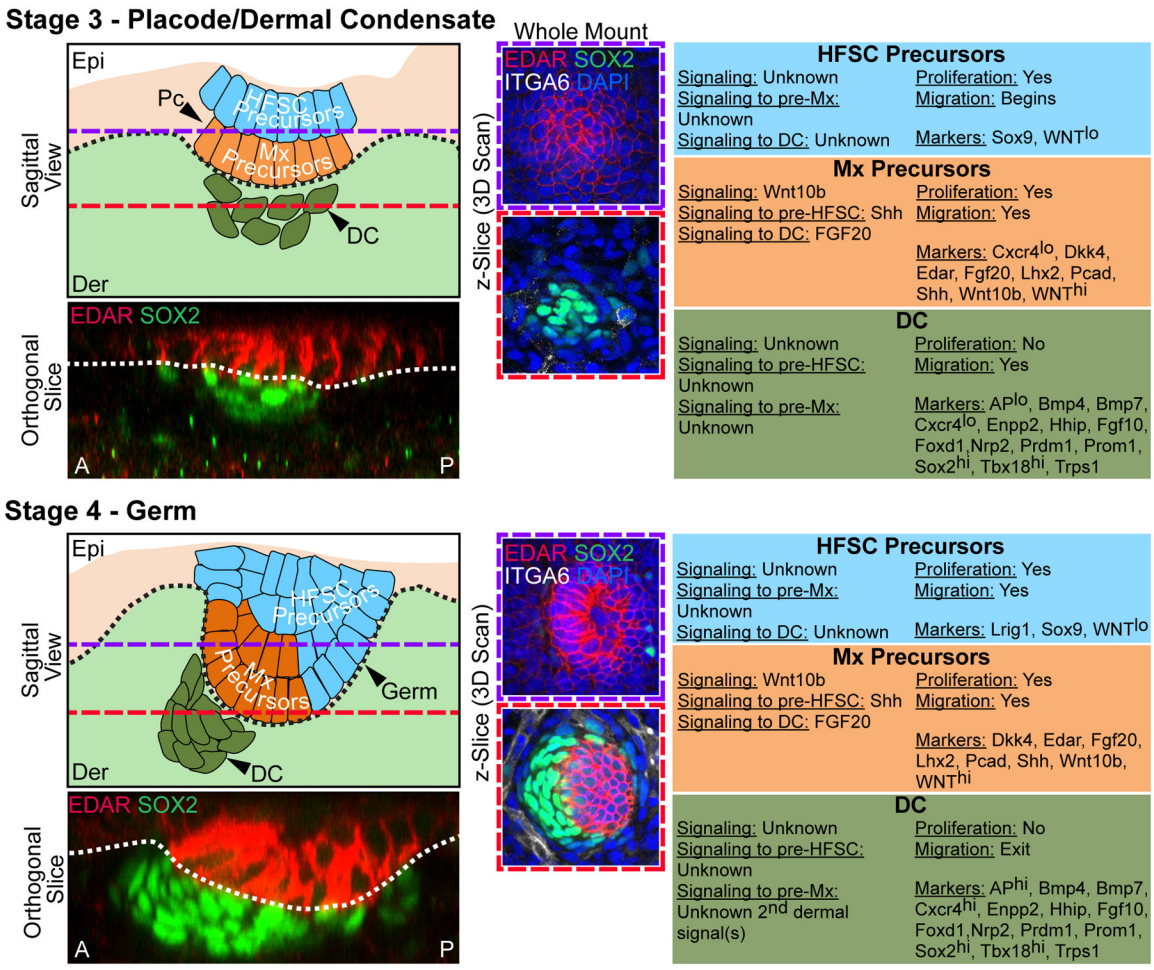

Figure 3. Stages 3–4 of Hair Follicle Morphogenesis

Left top: Sagittal view schematic of HF morphogenesis stages. Purple dashed lines mark the epidermal Z-plane, and red dashed lines mark the dermal Z-plane of 3D-imaged confocal scans of whole mount immunofluorescence of E15.0 back skin. A and P denote anterior and posterior orientation of embryonic skin (head is left). Left bottom: Orthogonal slice from a 3D reconstruction of whole mount immunofluorescence for EDAR and SOX2. Middle: Whole mount immunofluorescence for EDAR, SOX2, and ITGA6 in the epithelial plane (top, purple frame), and dermal plane (bottom, red frame). DAPI marks all nuclei. Right: Description of autocrine and paracrine signaling, proliferation and migration status, and markers of the relevant epithelial and mesenchymal populations at each stage.

Stage 3 – Placode/Dermal Condensate. Pc in the epithelium, with matrix (Mx) precursors at the basal layer and HFSC precursors in the suprabasal layer, above an aggregating DC in the dermis. Pronounced EDAR expression is detectable in maturing placodes. Pronounced SOX2 expression is present in the maturing DC.

Stage 4 – Germ. Downgrown germ in the epithelium, with matrix (Mx) precursors at the basal layer and HFSC precursors in the suprabasal layers, above an aggregating and polarized DC in the dermis. At this stage, the DC has more cells and is further condensed. Note anterior polarization (towards the head) of the downgrowing germ with the DC at the

leading edge. EDAR continues to be highly expressed at the germ stage. SOX2 is highly expressed in the DC.

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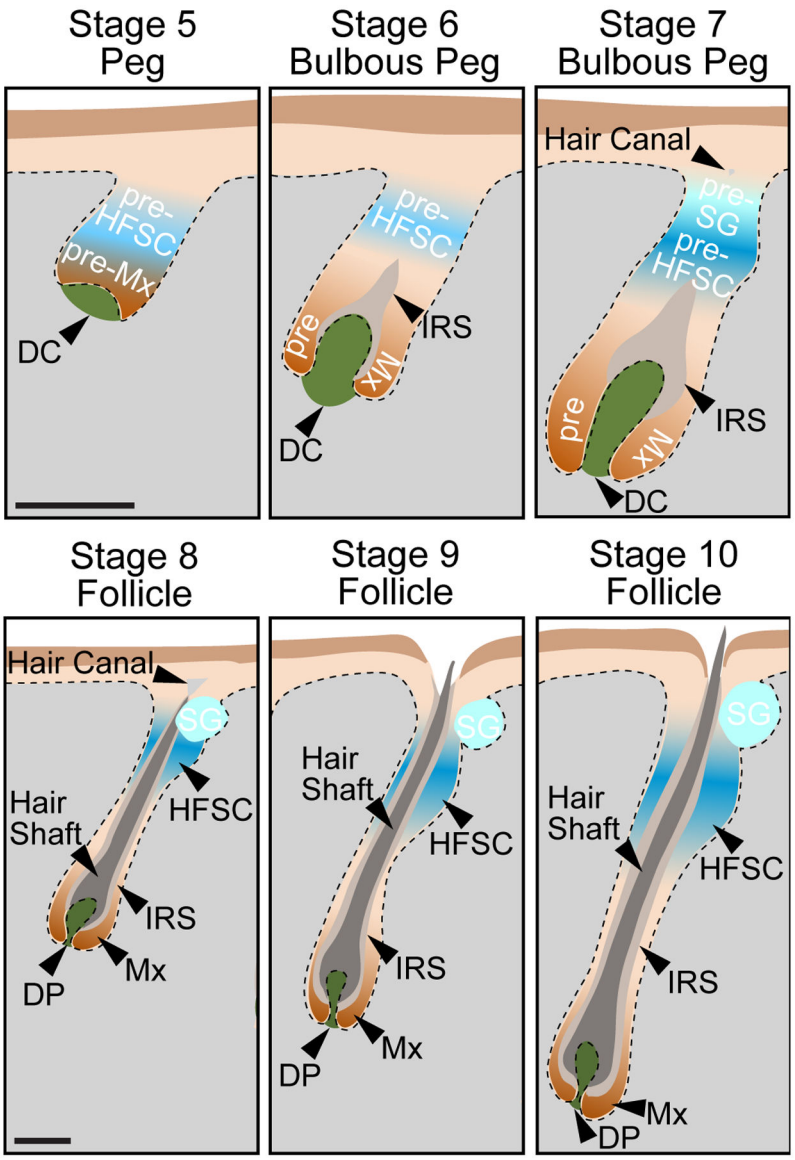

Figure 4. Stages 5–10 of Hair Follicle Morphogenesis

Sagittal view schematics for the late stages of HF morphogenesis. Pseudoscale bars represent the relative scale for stages 5–7 and stages 8–10.

Stage 5 – Peg. Downgrowth of the hair peg, comprised of Mx precursors at the leading edge, and HFSC precursors above them. The DC is beginning the be engulfed.

Stage 6 – Bulbous Peg. At the bulbous peg stage, Mx precursors engulf the DC by > 50% in the bulb region. HFSC precursors remain in the upper ORS. The IRS is beginning to form.

Stage 7 – Bulbous Peg. At this later bulbous peg stage, Mx precursors almost entirely engulf the DC. Bipotent HFSC precursors bifurcate into unipotent HFSC precursors, which reside in the presumptive bulge area, and sebocytes that will make up the sebaceous gland (pre-SG), which reside in the upper part of the follicle. The hair canal is first becoming visible.

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Stage 8 – Follicle. At the follicle stage, the Mx now completely engulfs the DP. The IRS reaches the hair canal, and the lineages of the hair shaft are being produced. HFSC reside in the bulge. The sebaceous gland (SG) is formed.

Stage 9 – Follicle. The follicle further elongates into the dermis. The tip of the hair shaft leaves the IRS and enters the hair canal. The DP is even more fully narrowed.

Stage 10 – Follicle. The hair shaft emerges from the epidermis. The follicle has reached its maximum length expanding into the subcutaneous adipose layers.

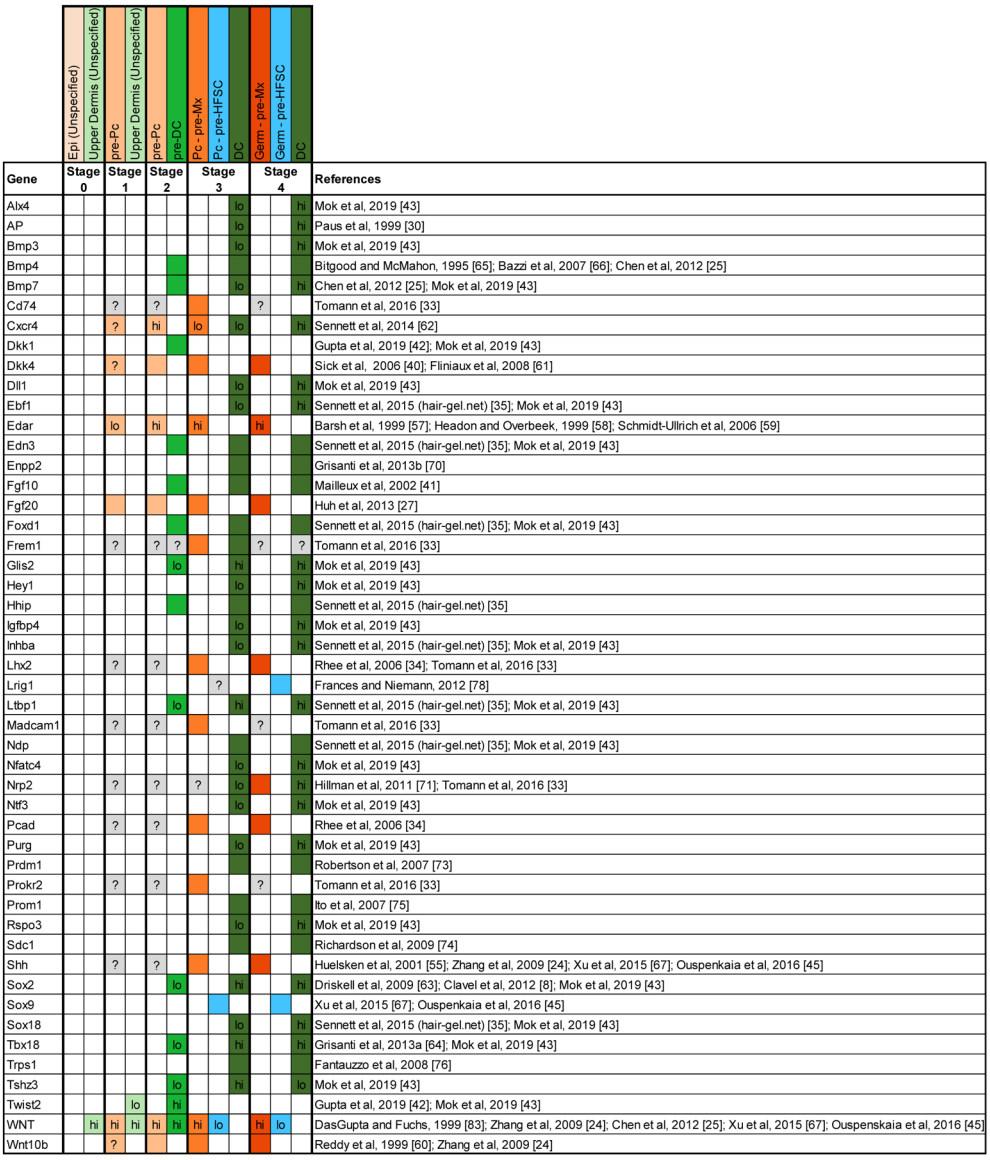

Figure 5. Molecular Markers for Early Hair Follicle Morphogenesis Cell States and Types

Marker expression for cell types at the key stages of early hair follicle morphogenesis. Genes are color-coded for expression in respective compartments; high (hi) and low (lo) expression is noted where it is known. Grey boxes with question marks refer to unknown expression by *in situ* hybridization and/or protein staining, at related neighboring stages. Color boxes with question markers (Cxcr4, Dkk4, Wnt10b) in Stage 1 pre-Pc refers to known expression by *in situ* hybridization or protein staining at E13.5, where the presence of recently discovered pre-DC (stage 2) cannot be ruled out. References for each marker are on the right.

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