Molecular principles of hair follicle induction and morphogenesis

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Summary

Hair follicle (HF) development is the result of neuroectodermal–mesodermal interactions, and can be divided into morphologically distinguishable stages (induction, organogenesis and cytodifferentiation). The spacing, polarity and differentiation patterns of HFs are driven by interacting, self-assembling gradients of inhibitors and activators, which are established jointly by the skin epithelium and mesenchyme. For HF development to occur, the dominant-negative influence of inhibitors of the HF differentiation pathway must be locally counteracted by specific antagonists and/or overriden by stimulators of hair placode formation. Once a mesenchymal condensate of inductive fibroblasts has formed, it takes over control of most subsequent steps of HF organogenesis and of epithelial stem cell differentiation into distinct lineages. In this review we introduce the morphological characteristics, major underlying principles and molecular key players that control HF development. The focus is on recent insights into the molecular interactions leading to hair follicle induction, and we close with synthesizing a corresponding working hypothesis. *BioEssays* 27:247–261, 2005.

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Abbreviations: HF, hair follicle; DP, follicular dermal papilla, ORS, outer root sheath; IRS, inner root sheath; BMP 2/4, bone morphogenic protein 2/4; Dkk-1, dickkopf-1; EdaA1 or A2, ectodysplasin A1 or A2, = *tabby*; EdaR, ectodysplasin receptor, = *downless*; EdaRADD, EdaR-associated death domain, = *crinkled*; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; FS, Follistatin; Gli, transcription factor glioblastoma; IGF, insulin-like growth factor; IKKs, IκB kinases Ikkα, Ikkβ and Ikkγ; Lef-1, lymphocyte enhancer binding protein 1; NCAM, neural cell adhesion molecule; NF-κB, nuclear factor-κB; NGF, nerve growth factor; NT-3, neurotrophin-3; $p75^{NTR}$, neurotrophin receptor; PDGF-A, -Rα, platelet-derived growth factor A, receptor α; Ptc1/2, patched 1/2; Shh, sonic hegdehog; Tcf, T cell factor; TGFβ, transforming growth factor β; TNF, tumor necrosis factor; TRAF2/6, tumor necrosis factor receptor-associated factor 6; Troy, TNF receptor superfamily expressed on the mouse embryo; Wnt, wingless-related; XEDAR, X-linked ectodysplasin A2 receptor.

Introduction

The hair follicle (HF) is the most prominent miniorgan of the skin and one of the defining features of mammalian species. It represents a prototypic neuroectodermal–mesodermal interaction system that is provided by three different stem cell sources: epithelial, neural crest and mesenchymal.[1–3] Depending on their origin, these stem cells differentiate into distinct epithelial lineages (i.e. sebocytes and keratinocytes of the hair matrix, which go on to develop into outer and inner root sheath cells, or into trichocytes), or mesenchymal lineages, such as specialized inductive fibroblasts of the follicular dermal papilla and the HF connective tissue sheath.[4] Others (neural crest cells), in turn, give rise to melanocytes of the HF pigmentary unit.[2]

Together with its associated structures (sebaceous and apocrine gland, arrector pili muscle), the HF forms an integral component of the pilosebaceous unit. Primarily, the HF serves as a factory for pigmented, multifunctional and extremely durable proteinaceous fibers—hair shafts. However, due to its nature as a stem cell repository, its life-long cycling behavior (see below), and its characteristics as a major intracutaneous source for numerous growth factors, cytokines, neuropeptides, neurotransmitters and hormones, the HF also has an unusually high regenerative potential.[5] It is functioning as the "bone marrow of the skin" and continuously remodels its cutaneous microenvironment, including skin innervation and vasculature.[5] The HF is the *only* organ in the mammalian body which, for its entire life-time, undergoes cyclic transformations, from periods of organ regeneration and rapid growth (anagen), where key aspects of its embryonic development are recapitulated, to apoptosis-driven regression (catagen). From catagen, the HF moves back into anagen via an interspersed period of relative quiescence (telogen).[4,5] These processes make the developing and the continuously cycling mature HF an exquisite, abundantly available, easily accessible and experimentally well-manipulable model for studying a huge spectrum of key problems in modern biology.[5–9]

Here, we first give a résumé on the morphological characteristics and the molecular mechanisms that control

HF development. We then go on to discuss the cross-talks between the various signaling pathways leading to hair follicle formation in mice and present a summarizing working hypothesis.

The morphological and cytodynamic basis of HF development: an overview

HF formation has been divided into eight distinct developmental stages (0–8), whose fine morphological details in man and mice are reviewed elsewhere.[9,10] Morphologically, the initiation of HF development begins with what has been termed "crowding" of epidermal keratinocytes, which become enlarged and elongated, and get organized in microscopically easily recognizable *hair placodes* (stage 1; see Fig. 1).[10] Placode formation is succeeded by a condensation of specialized fibroblasts with inductive properties in the underlying mesenchyme. The signaling cross-talk between this mesenchymal condensate and the epithelial hair placode

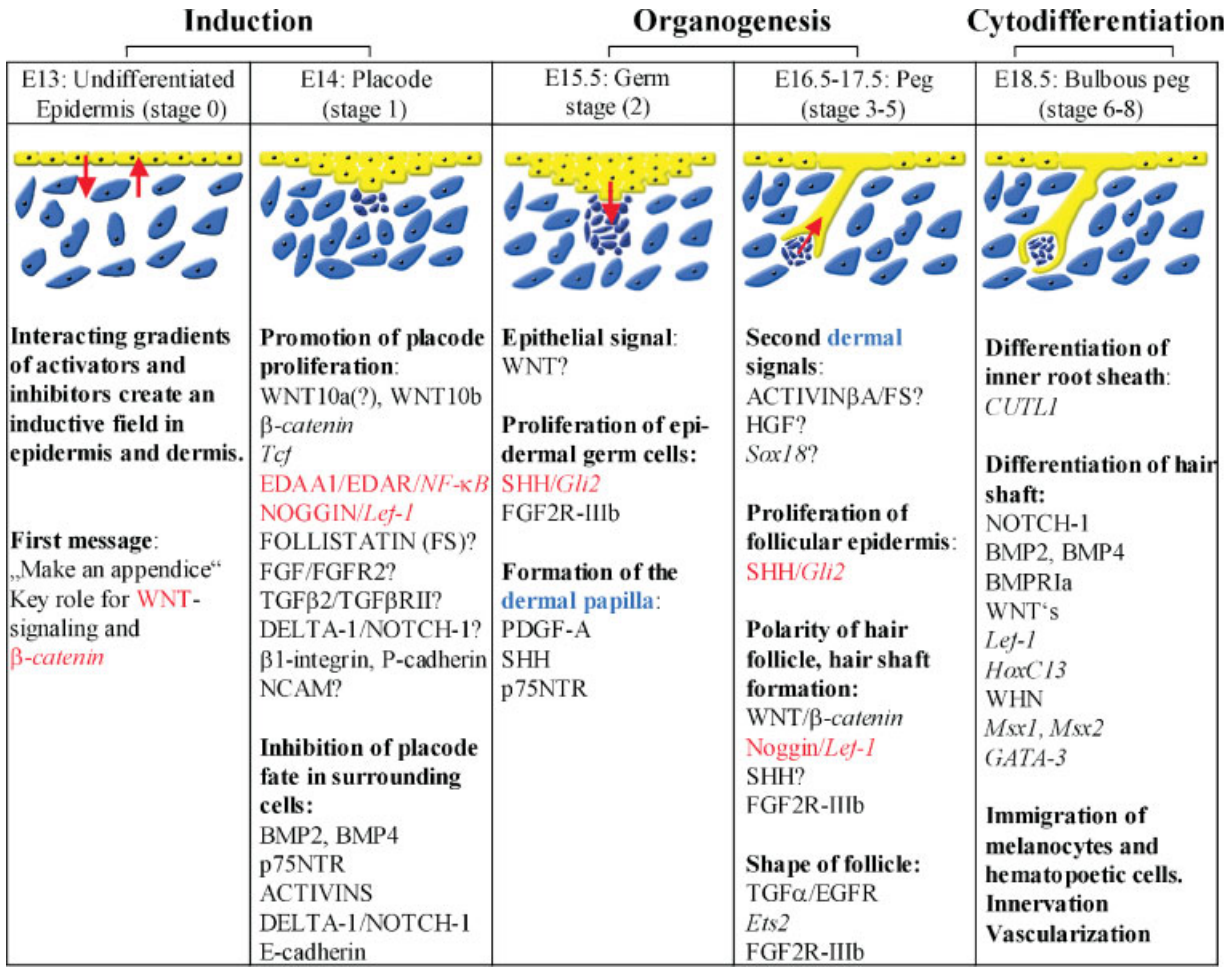


Figure 1. Upper part: The most-important developmental stages of mouse pelage hair follicles divided in induction, organogenesis and cytodifferentiation. **Below:** The best-studied molecular players of each stage. In red are the essential pathways for hair follicle development, which, when blocked, end at that particular stage. Hair follicles form due to the interaction between the embryonal epithelium (in yellow) and the underlying mesenchyme (in blue). In the beginning, gradients of activators and inhibitors of hair follicle development generate a well-circumscribed inductive field (stage 0). Subsequent signals are needed to organize the underlying mesenchyme giving rise to the dermal papilla (stage 1–2). The dermal papilla itself sends back morphogenic and growth signals to the ectoderm to eventually form the hair follicle (from stage 3 on, organogenesis). Note that, with the exception of Wnt10a and Wnt10b, Wnt stands for probably different Wnts in each case whose identity is still unknown. The EdaA1/EdaR/NF-κB pathway is activated at stage 1 (induction; placode formation) at E14 only in primary guard (tylotrich) hair follicles. Since the current review focuses on hair follicle induction and very early organogenesis, only molecular players involved in stages 0–2 of hair follicle development are discussed in the text. For reference see GATA3,[91] Fgfr2-IIIb;[95] homeobox (HMG box) genes HoxC13,[96,97] Sox18,[98,99] Msx1,[100] Msx2;[101,102] and reviews about Hox genes (see Ref. 103 and references therein). CAPITALS: signaling molecules; *italic*: transcription factors.

drives proliferation in both structures, shaping the *follicular dermal papilla* (DP) in the mesoderm and starting the downgrowth of the ectodermal placode. Already the earliest events of murine HF development are characterized by intensive proliferative activity.[11]

The following stage of massive keratinocyte proliferation leads to the formation of the *hair germ* (stage 2, Fig. 1), during which cyclin D1 becomes upregulated.[12] Further downgrowth progresses to the *peg* stage (stage 3–4; Fig. 1), where the most-proximally located keratinocytes begin to enwrap the DP, followed by the *bulbous peg* stage (stage 5–8) when distinct strata of epithelial differentiation within the HF become morphologically noticeable. As a critical event during stage 5, HF keratinocytes begin to form the *inner root sheath* (IRS). These are the first epithelial cells in the follicle to terminally differentiate, slowly forming a rigid tube. In the center of this tube, terminally differentiated trichocytes, which will form the hair shaft, become organized and compacted. The IRS itself is surrounded by increasingly distinct, cylindric layers of *outer root sheath* (ORS) cells. IRS formation is a crucial step in HF morphogenesis, since it heralds that the follicle prepares to serve its key function: that of a hair shaft factory.[9,10]

During stage 5, melanin begins to be generated in the newly formed *HF pigmentary unit* and individual keratinocytes in the distal HF epithelium differentiate into sebocytes or precursors of the apocrine gland. Around this time, the distal follicle epithelium is populated by a wave of immigrating Langerhans cells and T cells. In the mouse, almost all of these lymphocytes are gamma/delta TCR+; in humans, they are largely CD4+ or CD8+ alpha/beta TCR+ cells. An increasing number of mast cells and macrophages homes in onto the HF as well, accumulating in the perifollicular mesenchyme.[13] It remains to be clarified how these important events are controlled, but two likely key phenomena are the secretion of chemoattractants like IL-1, TNF-α or IFNγ and distinct adhesion molecule expression patterns by follicular keratinocytes.

The murine pelage consists of four different hair types developing at different time points.[14] The major pelage hair types include primary or tylotrich (guard) HFs and secondary or non-tylotrich HFs. Primary guard HFs make up approximately 2–10% of the coat and their development starts around E14. They consist of extra-large straight sensory hair fibres that protrude above the coat surface, often equipped with two sebaceous glands and associated with a ''Haarscheibe'', an epithelial pad enriched with Merkel cells.[10,15] Secondary, non-tylotrich HFs include awls, auchene and zig-zags. Awls are straight hair fibres, usually only half the length of the guard hair and comprise 28% of the pelage, developing at E15.5–E16. Auchenes are similar to awls, but have a single bend in the hair fibre, while zig-zag hairs have more than one bend in the fibre. The latter make up 70% of the fibres in the pelage, start to develop at E17 and are the last HFs to reach stage 8 of HF morphogenesis (at the latest around day 8 of postnatal life, P8). In addition to the pelage fibres, the vibrissae or whiskers are produced by extremely large and densely innervated follicles, surrounded by a large blood sinus, which serve an important sensory function. Vibrissae are the first follicles to develop, their formation starting around E12. Additional special HF subpopulations in mice are found around the body orifices and on the tail.

Molecular principles of murine HF development

The molecular communication between epidermis and dermis that drives HF development reflects ancient principles of epithelial induction and morphogenesis (Fig. 1).[6,8,9] These principles predate the appearance of mammals and are evident in the development of evolutionarily older, closely related epithelial–mesenchymal interaction systems such as scale, tooth, feather or nail: skin appendage formation at a given location and time is the net result of *interacting, self-organizing gradients* of stimulatory and inhibitory signals. These consist of secreted molecules and changes in the expression of cognate receptors, topobiological alterations in the expression of key adhesion molecules and integrins and underlie epigenetic controls.[16–18] The evolutionary ancestry of these signaling partners can be traced back millions of years to molecules like Wnt/wingless and members of the hedgehog family of secreted glycoproteins, which govern the development of simple organisms such as *Drosophila*. In addition, many other key regulatory molecules involved in proliferation, morphogenesis and patterning during embryonic development of metazoan organisms are also re-utilized in the control of HF development, such as members of the TGF/BMP and FGF families, homeobox proteins and even TNF family members.[8,16,18,19]

Mice have long provided exceptionally instructive hair research models and many basic principles of HF biology were first established in the murine system.[6,8] An ever-increasing number of novel mouse mutants, where the genetically engineered knockout or overexpression of a defined gene product is associated with a hair phenotype (Table 1), has allowed instructive insights into the molecular controls of hair development and growth.[20] In fact, our current understanding of the molecular controls of HF induction and morphogenesis is almost entirely based on the analysis of mutant mice. Therefore, caution is advised in naively assuming that exactly the same controls apply to human HF development.

Below, we shall delineate key signaling pathways involved in murine HF development, focussing on the controls of HF induction. We shall discuss several recently proposed competing theories that attempt to explain the hierarchy of signaling events during HF induction and morphogenesis. Yet, none of these theories can so far claim to reflect accurately what exactly happens in normal life and quite likely there are several signaling pathways (some perhaps even redundant),

Table 1. Selected knock-out, knock-in and transgenic mice with defects in HF development

	Mutant name	Symbol	Ch	Type	Defects in initiation	Defects in morphogenesis	Hair cycle	Reference
Dickkopf 1, under K14 promoter control. Protent Inhibitor of Wnt (wingless related)		K14-Dkk 1	19	Tg	No Wnt activity in epidermis. No placode formation of any hair follicle or other appendices at all.			Andl et al. 2002[30]
EdaR (Eda A1 receptor)	Downless	dl	10	S	No placode formation of guard hairs, zigzags or auchenes. No hairs on tail, behind the ears. Vibrissae mostly unaffected.	For awl hairs unaffected		Lyon et al., 1996 Headon and Overbeek, 1999[22] Monreal et al., 1999[61]
Eda A1	Tabby	ta	X	S	Identical to EdaR	For awl hairs unaffected		Falconer, 1952[62] Srivastava et al., 2001[69]
EDARADD	Crinkled	cr	13	S	Identical to EdaR	Not analysed		Falconer et al., 1952[62]; Headon et al., 2001[67]
Traf6			2	Tm	Identical to EdaR	Not analysed		Naito et al., 2002[109]
NF-κB p50/p65 inhibition		IκBαΔN		Tm	Identical to EdaR	For awl hairs unaffected		Schmidt-Ullrich et al., 2001[23]
β-Catenin, floxed, × K14-Cre			9	Tm	Strongly reduced placode formation.	Needed for fate decision of epidermal stem cells residing in bulge to differentiate into hair follicle keratonocytes.	Hair loss after first cycle.	Huelsken et al., 2001[54]
Lef-1		Lef1(tm1Rug)	3	Tm	No	Lack of teeth, whiskers and most body hair. Initiation normal. Arrest at stage corresponding to E17 for guard hairs.		Van Genderen et al., 1994[57]
Noggin		Nog(tm1Amc)	11	Tm	No	Retarded hair follicle development. Reduced number of hair follicles.		Botchkarev et al., 1999[24]
Shh		Shh(tm1Chg)	5	Tm	No	Initiation normal, but progression through subsequent stages is blocked. Shh is needed for morphogenesis beyond hair germ stage. Reduced size of dermal papilla. Also needed for tooth and whisker morphogenesis.		Chiang et al., 1999[79]; St.-Jacques et al., 1998[78]
Gli2		Gli2(tm1Alj)	1	Tm	No	Identical to $Shh^{-/-}$ mice.		Grachtchouk et al., 2000[81]

This table summarizes some of the knockout, knockin and transgenic mice that are discussed more in detail in the text. All these genetically engineered mice have clear defects in hair follicle induction and prenatal hair follicle morphogenesis. In most of these mice, early placode formation of the different pelage hair types, tail hair or vibrissae is absent. In others, development is arrested at peg stage as in $Shh^{-/-}$ and $Gli2^{-/-}$ mice. For a more detailed list, see also Nakamura et al., 2001.[20] Growth factors such as fibroblast growth factors (FGF) 2, 7 and 10, transforming growth factor β1 and β2 (TGFβ1 and TGFβ2), insulin-like growth factor (IGF) epidermal growth factor receptor (EGFR) and members of the nerve growth factor (NGF) family and their receptors also play a role in hair follicle development and morphogenesis.[20] However, their role in initiation is unclear and there may be redundancy for some of them, or they rather play a role at later morphogenic stages, such as EGFR and NGF. Furthermore, their target genes in hair follicle development are yet unknown, as well as how they integrate into the other better known pathways (Wnt and Eda). Tm, targeted mutation; S, spontaneous mutation; Tg, transgenic; Ch, chromosome.

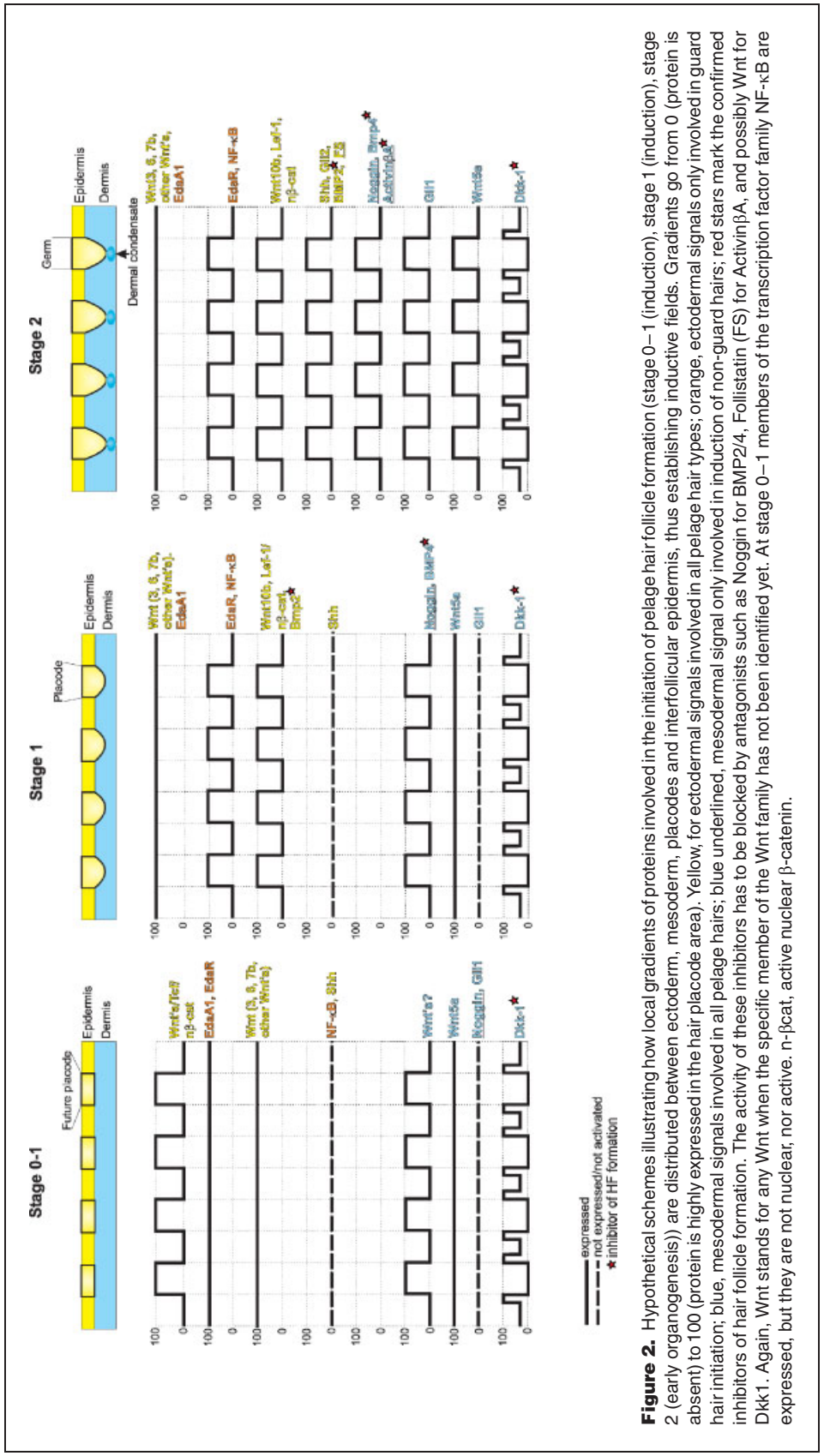


Figure 2. Hypothetical schemes illustrating how local gradients of proteins involved in the initiation of pelage hair follicle formation (stage 0–1 (induction), stage 1 (induction), stage 2 (early organogenesis)) are distributed between ectoderm, mesoderm, placodes and interfollicular epidermis, thus establishing inductive fields. Gradients go from 0 (protein is absent) to 100 (protein is highly expressed in the hair placode area). Yellow, for ectodermal signals involved in all pelage hair types; orange, ectodermal signals only involved in guard hair initiation; blue, mesodermal signals involved in all pelage hairs; blue underlined, mesodermal signal only involved in induction of non-guard hairs; red stars mark the confirmed inhibitors of hair follicle formation. The activity of these inhibitors has to be blocked by antagonists such as Noggin for BMP2/4, Follistatin (FS) for ActivinβA, and possibly Wnt for Dkk1. Again, Wnt stands for any Wnt when the specific member of the Wnt family has not been identified yet. At stage 0–1 members of the transcription factor family NF-κB are expressed, but they are not nuclear, nor active. n-βcat, active nuclear β-catenin.

acting either in parallel, in synergy or in succession, but all eventually leading to HF development.

Most of the signaling pathways proposed in the literature, for instance, do not take into account that there are several types of HFs (vibrissae, tylotrich/guard and non-tylotrich/non-guard pelage hair) which all develop in a slightly different way and at different time points during fetal and perinatal life. In fact, mounting evidence suggests that they differ significantly in their molecular controls, as exemplified by guard and zig-zag hairs, whose development specifically depends on the signaling of the new TNF receptor and ligand family members Eda/EdaR, and downstream transcription factor NF-κB (see Fig. 4).[21–23] Another example are guard versus non-guard pelage HFs, where the former need Noggin for hair shaft formation, while the latter depend on Noggin for induction.[24,25]

The molecular controls of HF development remain riddled with unresolved quandaries, especially with respect to where exactly the crucial follicle *initiation signal* arises. In addition, the *relative* functional importance and hierarchy of the bewildering number of regulatory molecules now implicated in the control of murine HF morphogenesis is still unclear, (e.g. compared to tooth development, where the molecular pathways are already rather well defined.[26,27]) Moreover, the situation is complicated by the *redundancy* of many signals and the possibility of bypassing physiological signaling cascades by "override signals".[8,9] These general caveats must always be kept in mind when contemplating how the signaling cascade resulting in HF induction is organized.

Initiation of HF development

Conventional wisdom, based on classical epithelial–mesenchymal tissue recombination studies, claims that the first signal for follicle induction arises in the dermis.[6] However, the establishment of a morphogenetic field during stage 0 may well reflect gradient patterns that are jointly generated by both dermis and epidermis (see Fig. 2).

In order to initiate the development of ectodermal appendages, as for all other organs, the boundaries of the structure-to-be have to be defined by spontaneously established, self-assembling, interacting gradients of regulatory proteins, which generate a morphogenetic field (see Fig. 2, stage 0; example given for guard hair initiation).[17,18] These regulatory proteins (receptors and their ligands, transcription factors, adhesions molecules) function as activators or inhibitors of follicle induction (Fig. 2), quite possibly following the principles of a cellular reaction/diffusion system.[18,28] Though it remains to be demonstrated unequivocally that this is really the case, it is reasonable to assume that HF initiation operates in a similar fashion to feather development, which has been dissected in great detail:[18] A positive adjacent ectodermal (horizontal) or dermal (vertical) regulator is concentrated locally, providing the initial signal. This leads to the formation of a circumscribed placode of functionally distinct keratinocytes (Fig. 2, stage 1).

In mice, activation of Wnt signaling in the skin is absolutely required for the initiation of hair placode formation and even seems to precede HF initiation (see Figs. 2 and 3).[29,30] However, up to date, it is not known exactly which member of the Wnt family is responsible for initiation and whether it arises intradermally or intraepidermally. In guard hairs, EdaA1/EdaR and, as recently was shown, downstream transcription factor NF-κB activity will evoke local epidermal cell survival and proliferation.[21–23,31,32] Interestingly, Wnt10b expression is abolished in mEdaA1 (mutant EdaA1, tabby) mice, indicating that EdaA1 signaling itself stimulates, directly or indirectly, Wnt10b expression, thereby activating the canonical Wnt pathway via β-catenin.[30] Therefore, proliferation of ectodermal cells to produce, for instance, a guard hair placode is in need of both, EdaA1/EdaR, NF-κB *and* Wnt/β-catenin.[21–23,30] Together, Wnt/β-catenin and Noggin/Lef-1 are also responsible for inhibiting the expression of the critical cell–cell adhesion molecule E-cadherin.[33,34] Inhibition of E-cadherin protein production decreases the local cell adhesion that is necessary for normal placode downgrowth.[11,33,34]

The initiation signals change for the four major different pelage HF types. The first round starting at E13/E14 gives rise to guard hairs and is regulated by EdaA1/EdaR and NF-κB.[21–23] The second round of HF initiation at E16/E17, when awl and auchene hairs develop, is independent of Eda/EdaR/NF-κB signaling, but requires Wnt/β-catenin and Noggin/Lef-1.[25,33] The molecular dependence of zig-zag hair is as yet unclear. Initiation may be identical to other secondary follicles (awl, auchene) but, in order to develop actual zigzag hairs, EdaA1/EdaR/NF-κB signaling is required, possibly downstream of Wnt/β-catenin and Noggin/Lef-1.

For HF development negative regulators must be counteracted

The gradients displayed in Fig. 2 are greatly influenced by negative regulators, which are either expressed directly in the developing HF structure or by the surrounding cells. Current evidence suggests that the signaling balance is tilted towards a suppression of HF development and that follicle induction does not occur unless placode inhibitors, such as BMP-2, BMP-4 and activinβA, are sufficiently counteracted by noggin or follistatin, respectively. In palmoplantar skin, this counteraction may never occur spontaneously, thus explaining the complete absence of pilosebaceous units at this site, while it can still be overcome even in adult rodent palmoplantar skin by the implantation of DP cells. Importantly, a dominant inhibitory control of HF induction would seem best-suited to safeguard against malignant degeneration, in particular once follicular epithelial stem cells, residing in the bulge region of the mature HF, have appeared on the scene (reviewed in Ref. 1): overexpression of genetically engineered β-catenin or Shh in

mice leads to skin tumor formation.[35,36] Moreover, β-catenin and several other key regulators of HF development (Shh/patched/smoothened) are also abnormally expressed or overactive in human basal cell carcinomas, most of which may arise from follicular epithelial stem cells.[35,36]

Dickkopf-1 (Dkk1) is a potent inhibitor of Wnt signaling.[30] In early development at E14.5 and E15.5, Dkk1 expression is low in the interfollicular dermis, but prominent in the dermis surrounding the placode and dermal condensate (see Fig. 2).[30] One could speculate that Dkk1 is responsible for concentrating Wnt activity to the placode and dermal condensate where Wnt has to overcome the HF-inhibitory Dkk1 action. In contrast, in interfollicular skin Wnt/β-catenin signaling is negatively regulated by dickkopf.[30] Other mechanisms of inhibiting the Wnt pathway are negative regulators known to associate with the Tcf/Lef-1 transcription factor family, altering its activity. For example, CtBP binds to Tcf3 and members of the Groucho family of repressors can interact with all members of the Tcf/Lef-1 family ([37] and reviewed in Ref. 38). Groucho, when binding to Tcfs can recruit histone deacetylases packaging the chromatin into an inactive state.[39] Therefore, additional counteracting signals are likely to be identifed that contribute to relieving Wnt signaling from all the dominant negative controls that repress HF induction to originate a positive, HF-inductive Wnt signal.

Conceptually, members of the TGFβ family are the most prominent inhibitors of HF formation. Hence, special factors have to become locally active to counteract this inhibition in order to develop a HF. Noggin is a potent inhibitor of the TGFβ family member BMP. It prevents BMP-4 in the dermal condensate from binding to its receptor and is expressed in the dermal condensate from stage 1 on.[24] Thus, two signals are needed for follicular morphogenesis and polarity: Wnt itself, which produces stabilized β-catenin molecules, and Noggin for BMP inhibition and subsequent Lef-1 activation.[24,25,33]

Follistatin is an antagonist of the TGFβ family member activinβA, and is also expressed in the DP.[40] Follistatin inhibits activinβA binding to its receptor in the DP.[40] Follistatin-null mice and activinβA overexpressing mice have a similar phenotype showing significant retardation of HF morphogenesis.[40–42] However, initiation seems to take place.[40–42]

Recently, activinβA has been proposed to also function as an inducer of EdaR in HF placodes.[43] Apart from the fact that activinβA expression is strictly confined to the dermal papilla while EdaR protein is only detected in the epidermis, the HF phenotype of activinβA$^{-/-}$ mice is quite different from that of mutant EdaR (= *downless*) mice.[40,41] Mutant EdaR mice do not have any guard and zigzag hairs, miss the third molar and display defective molar cusp fomation.[22,44] ActivinβA$^{-/-}$ mice lack lower incisors and vibrissae, while molars and pelage hair seem to develop normally.[40,41] In addition, morphogenesis, but not initiation of incisors and vibrissae is affected in activinβA$^{-/-}$ mice.[41] Therefore, signals other than activinßA must be responsible for local EdaR upregulation.

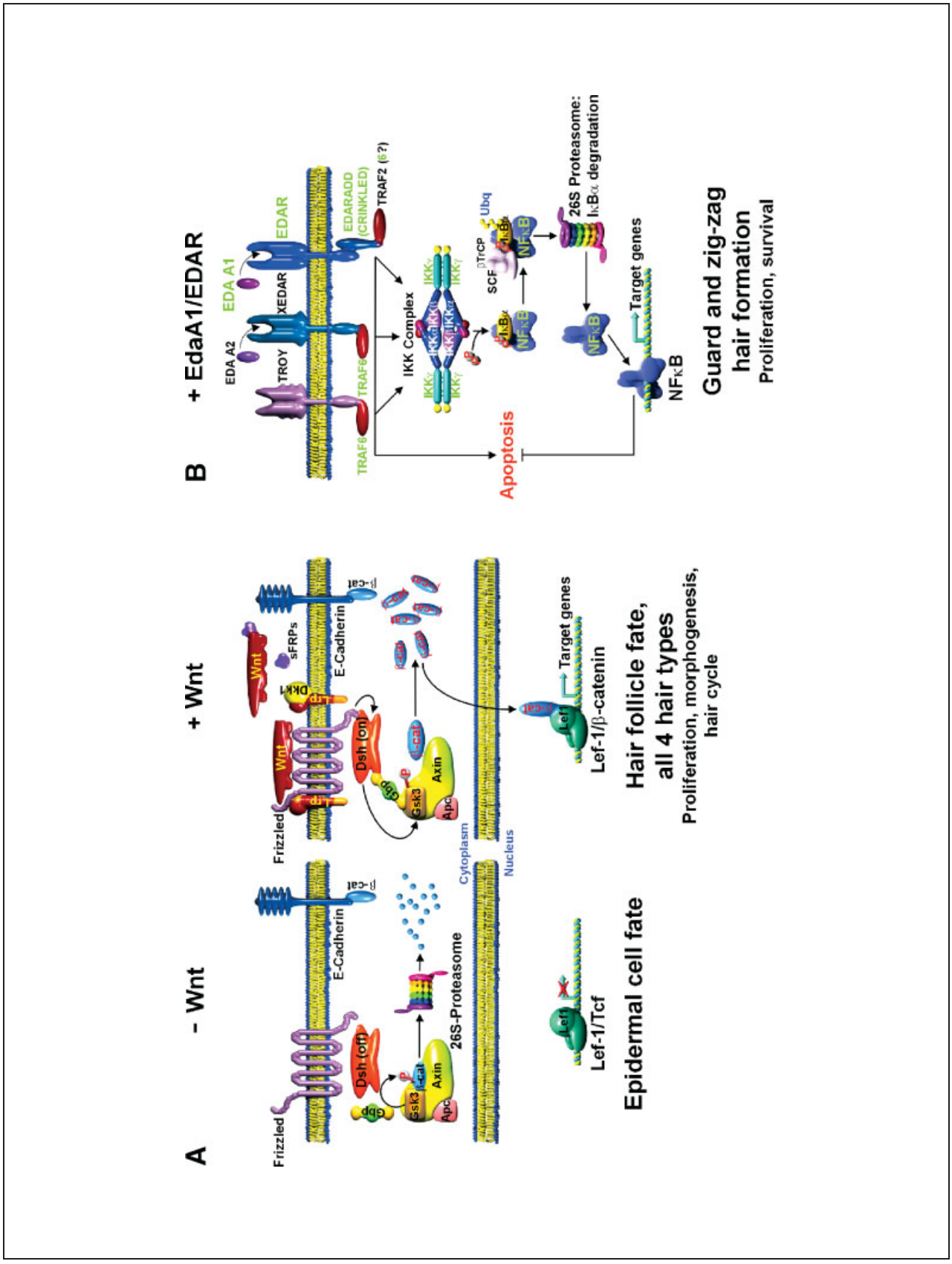
A quite different way of controlling hair follicle induction consists of regulating the transcriptional activity of NF-κB. Three recent publications demonstrated that NF-κB activity is controlled by downregulation via the de-ubiquitination enzyme CYLD, a tumor suppressor gene.[45–47] In humans, somatic mutations in CYLD lead to familial cylindromatosis causing benign neoplasms of skin appendages like HFs and sweat glands. Cylindromas are not derived from epidermal keratinocytes, but from HFs and exocrine glands;[48] this already hints at a connection with EdaR signaling and subsequent, abnormally increased NF-κB activity. Thus, CYLD presents, an intriguing novel mechanism of negative regulation of NF-κB activity and shows that signaling downstream of EdaR needs to be stringently controlled, if appendageal tumor formation is not to be risked.

Wnt signaling operates as key stimulator of HF development

Paracrine Wnt signaling (Fig. 3A) has been implicated in many developmental processes as well as in tumorigenesis, and is active in a whole variety of animals, from worms to mammals (reviewed in[49,50]). Multiple knockout and transgenic mouse models have shown by now that Wnt signaling also transfers essential signals for HF morphogenesis and patterning (reviewed in[51,52]). Canonical Wnt signals are conducted by the transcriptionally active complex between members of the Tcf/Lef-1 family of DNA-binding proteins and stabilized β-catenin (Fig. 3A).[52]

Several Wnt genes are expressed in the epidermis during mouse development, among them Wnt3, Wnt3a, Wnt4, Wnt6, Wnt7a, Wnt7b, Wnt10a and Wnt10b.[29,30] Studies using an enhancer responsive to canonical Wnt signaling to drive the β-galactosidase gene revealed Wnt activity in the embryonal ectoderm and in the underlying mesenchymal condensates.[53] In the dermal condensate, especially upregulated from germ stage on, we also find Wnt 5a, which activates an alternative pathway, independent of β-catenin and Tcf/Lef-1.[29,50] However, the absolute necessity of Wnt signaling for the initiation and morphogenesis of all HF types was nicely demonstrated by Andl et al. who produced mice expressing the Wnt repressor Dkk-1 ectodermally.[30] In these mice, the complete lack of hairs may actually be due to additional diffusion of Dkk1 into the underlying mesenchymal layer, thereby inhibiting the dermal messages *as well*.[30] The possible need to inhibit ectodermal *and* dermal Wnt in order to obtain such a null-hair mouse may explain why solely ectodermal β-catenin$^{-/-}$ mice do have some placode formation, and still express EdaR and Lef-1.[54]

Wnt signaling stabilizes β-Catenin protein, which enables it to translocate to the nulceus (Fig. 3A). β-catenin is expressed ubiquitously, including the ectoderm, but its mRNA and protein


A
- Wnt
+ Wnt
Frizzled
Dsh (off)
Gbp
Gsk3
β-cat
Axin
Apc
26S-Proteasome
E-Cadherin
Wnt
sFRPs
DKK1
LRP
Dsh (on)
Cytoplasm
Nucleus
Lef1
Lef-1/Tcf
Target genes
Lef-1/β-catenin
Epidermal cell fate
Hair follicle fate,
all 4 hair types
Proliferation, morphogenesis,
hair cycle
B
+ EdaA1/EDAR
EDA A2
EDA A1
TROY
XEDAR
EDAR
EDARADD
(CRINKLED)
TRAF2 (6?)
TRAF6
IKK Complex
IKKγ
IKKα
IKKβ
P
IκBα
NFκB
SCF
βTrCP
Ubq
26S Proteasome:
IκBα degradation
Target genes
NFκB
Apoptosis
Guard and zig-zag
hair formation
Proliferation, survival

are upregulated in hair placodes from very early on,[54,55] hinting that, in some circumstances, β-catenin is also regulated at the transcriptional level. Several studies have proven the importance of β-catenin for HF development and cycling, like a mouse model, where overexpression of β-catenin led to ectopic hair formation, or ectodermal β-catenin$^{-/-}$ mice, which showed reduced placode formation, and helped to discover that in adult animals β-catenin is required for HF fate decision and production of follicular keratinocytes.[35,54]

Stabilized nuclear β-catenin can form complexes with the various members of the Tcf/Lef-1 DNA-binding proteins, leading to the activation of different target gene sets (for review see[52]). Lef-1 is expressed from E11 on in vibrissae follicles, from E13.5 on in future pelage hair placodes, and later also in the dermal condensates.[56] In addition, several mouse models have demonstrated an important role for Tcf/Lef-1 factors in HF development. Lef-1-null mice, for instance, have normal placode formation of primary guard hairs, while development is interrupted for primary and secondary HFs around E17.5.[56,57] These findings indicate a role for guard hair patterning and morphogenesis, but for initiation of secondary HF formation.[22,23,56,57] However, there may be redundancy between the different members of the Tcf/Lef-1 family for early guard hair development, as was demonstrated for Lef-1 and Tcf-1 in limb development.[58] The exact role of Lef-1 in the initiation and morphogenesis of the three secondary hair types has not been clarified yet, but recent data disclosed that Lef-1 is indeed essential for initiation of secondary hair follicle development.[25,33] It was shown that expression of Lef-1 is induced by the BMP4 antagonist Noggin, and not by Wnt.[33] BMP4 binding to its receptor has to be inhibited by Noggin in order to produce a functional Lef-1/β-catenin transcription complex by Wnt, and Noggin is known to play an important role in secondary hair follicle induction.[24,25,33] Last but not least, members of the Tcf/Lef family also play a role in cell fate decisions in the adult HF.[59,60]

EdaA1/EdaR/NF-κB signaling is needed for placode formation of guard and zig-zag hairs

Another important signaling pathway in HF development involves EdaA1 and EdaR, members of the growing TNF super-family and its receptors (Fig. 3B).[21,22,61] EdaA1 (called *tabby* in the mouse) was the first sex-linked gene discovered in the mouse.[62] Mice carrying a spontaneous mutation in the

Figure 3. A: The Wnt signaling pathway. **Left panel:** Lack of Wnt stimulation leads to epidermal cell fate. Epithelial cell in the absence of Wnt. The downstream effector of the canonical Wnt pathway, β-Catenin, the vertebrate homologue of *Drosophila* Armadillo, is a major structural protein in the adherens junctions of the cell where it forms complexes with cadherins. In the absence of Wnt, if not integrated into the adherens junctions, uncomplexed β-catenin is phosphorylated by the Gsk3β (glycogen synthase kinase 3β) kinase complex and degraded via the ubiquitin–proteasome pathway. The DNA-binding proteins of the Lef-1/Tcf family will not start transcription without complexing β-catenin. **Right panel:** Activation of the Wnt pathway and subsequent transcription of target genes leads to hair follicle fate. Epithelial cell in the presence of Wnt. Secreted Wnt binds to a specific family of seven-transmembrane receptors, the Frizzled (FZ) proteins, and to its coreceptors from the low-density lipoprotein receptor-related protein (LPR) family, LPR5/6 (for review see Ref. 50). Presence of Wnt leads to stabilization of cytoplasmic β-catenin by inactivation of the Gsk3β kinase complex via the now activated dishevelled (Dsh) protein. Subsequently, stabilized hypophosphorylated β-catenin is translocated into the nucleus and, complexed with the DNA-binding proteins Tcf/Lef, it carries out its transactivational activity. Target genes of the canonical Wnt pathway include key cell cycle regulators such as cyclin D and c-Myc, but also hair-specific keratins (for review see[51]). Wnt signaling is alterated by Dkk (Dickkopf, Dkk1), secreted frizzled-related proteins (sFRPs) and transmembrane co-receptors like other Lrps. Apc, adenomatous polyposis coli; Gsk3β, glycogen synthase kinase 3β; Gbp, Gsk3β-binding protein. **B:** Activation of the EdaA/EdaR/NF-κB signaling pathway leads to formation of guard and zig-zag hairs. Three members of the TNF receptor superfamily are depicted: TROY, XEDAR and EDAR (also called *downless*). All three receptors are expressed in the skin. The function of XEDAR and its ligand Eda A2, and TROY in epidermal development has not been studied yet. However, binding of the TNF family member EdaA1 (also called *tabby*) to EdaR is essential for ectodermal guard hair placode formation. Members of the TNF family and its receptors are known to activate the transcription factor family NF-κB/Rel (for review see Ref. 104). So far NF-κB/Rel was known to regulate the expression of a large number of genes essential for innate and adaptive immunity, for regulation of apoptosis and cellular proliferation.[105] NF-κB is composed of heterodimers or homodimers of the related p50, p52, p65 (RelA), c-Rel, or RelB proteins, which are sequestered in the cytoplasm by its inhibitor IκB (inhibitors of κB) proteins.[106] Cellular stimulation by TNFs activates the IκB kinase complex (IKKα, β and γ) to phosphorylate the IκBs (in particular IκBα), triggering their degradation and release of NF-κB.[107] Recently it was shown that EdaA1-EdaR interaction also leads to the activation of the IκB kinase complex (IKK) complex.[31,32] The EdaR associated death domain protein EDARADD (also: *crinkled*) and TRAF2 (tumor necrosis factor-associated factor2) interact with the death domain of EdaR and link it to the downstream IKK signaling pathway.[32,67] The same is true for the adapter protein TRAF6, associated with XEDAR and Troy. Subsequently, phosphorylated IκBα is ubiquitylated by the SCF$^{\beta TRCP}$ complex and degraded by the 26S proteasome. NF-κB (here: the ubiquitous p50/p65 heterodimers), now free to enter the nucleus, starts transcription. Targets include genes involved in proliferation and inhibition of apoptosis.[108] In the absence of IKK or NF-κB activity, stimulation of cells by TNF family members eventually leads to activation of caspases and, thus, to cell cycle arrest and/or apoptosis. All proteins depicted in green letters (EdaA1, EdaR, Traf6, EDARADD, IKKγ and NF-κB) show the same phenotype, when inactivated by mutation in mice or humans.[22,2365,67,109] The phenotype in mice is analog to the rare human hereditary disease HED (Hypohydrotic Ectodermal Dysplasia) which in humans is mostly caused by mutations in either EdaA1, EdaR or IKKγ. SCF, Skp1–cullin–F-box-protein complex; βTRCP, β-transducin repeat-containing protein.

EdaA1 gene locus have an analogous phenotype to a rare human hereditary disorder first described by Charles Darwin in 1875, termed Hypohidrotic Ectodermal Dysplasia (HED).[21,63] Spontaneous mutations in its receptor, EdaR (*downless* in the mouse), equally cause the clinical symptoms of HED in humans and mice.[22,61] HED is characterized by absence of hair, most exocrine glands and abnormal tooth formation.[64]

Recently, null mutations in the loci of several other genes were described to cause HED in mice and humans, and led to the identification of proteins directly involved in the EdaA1/EdaR pathway (Fig. 3B). As members of the TNF family and its receptors are known to activate the transcription factor family NF-κB/Rel, a mouse model with systemically suppressed NF-κB activity (IκBαΔN knock-in mice) also showed a phenotype analogous to human HED, and identical to *tabby*, *downless* and *crinkled* (= EDARADD) mice (see Fig. 3B).[23] These mice made clear for the first time that NF-κB is required for early HF development and that it must be activated downstream of EdaA1 and EdaR.[23] Furthermore, biochemical studies emphasize that EdaA1 binding to EdaR directly activates NF-κB via the IKK complex (Fig. 3B).[31,32] Interestingy, some rare male patients with HED-ID (HED with immunodeficiency) show missense mutations in the C-terminal zinc-finger domain of the regulatory subunit of the IKK (IκB kinase) complex, the X-linked IKKγ (Fig. 3B).[65]

Mutant EdaA1 (= *tabby*), EdaR (= *downless*), EDARADD (= *crinkled*) and IκBαΔN knock-in mice all have a very early defect in HF development.[21–23,66,67] There is no placode formation for primary guard hairs, and secondary zig-zag hairs are also absent in these mice.[23] In the end, they only develop intermediate awl hairs.[23] In addition, all mutant mice with the HED-analogous phenotype lack hairs behind the ears and on the tail, but vibrissae are mostly unaffected.[68] The importance for the EdaA1/EdaR pathway in HF formation was further stressed by transgenic models overexpressing EdaA1 ligand, and by rescue studies of the pathway.[69–71] Overexpression of EdaA1 under the control of the K14 promoter showed expected consequences like additional hairs, because placode formation takes place continously in these mice.[70] The rescue experiments, on the other hand, made clear that EdaA1 signaling is essential for guard hair, but not for zigzag hair initiation.[69,71] Rather, EdaA1 signaling may, at a later time point, influence the morphology of zigzag hairs, giving it the characteristic two kinks. If EdaA1 signaling is absent the mice grow awl hairs instead.

Two other TNF family members are expressed in the epidermis: Troy and XEDAR (Fig. 3B). XEDAR was recently shown to be dispensable for ectoderm-derived appendages.[72] But Troy may be an additional regulator of NF-κB in HF and teeth.[73,74]

Since NF-κB is important for cell proliferation and survival, one can speculate that the EdaA1/EdaR pathway is needed for inducing placode growth once the first dermal or epidermal signal via Wnt has been emitted. Laurikkala et al. proposed Wnt6 to be an upstream regulator of EdaA1.[43] The signal that brings on localized EdaR upregulation remains to be revealed.

Shh signaling is essential for hair germ downgrowth and DP maturation

Sonic hedgehog (Shh) is a member of the mammalian hedgehog family of secreted signaling molecules that play a key role in embryogenesis and organogensis (for review see Ref. 75). The signal of Shh is transmitted via the combined receptor complex of patched (Ptc) and the multipass transmembrane protein Smoothened (Smo) leading to the activation of members of the zinc finger transcription factor family Ci/Glioblastoma, Gli1, Gli2 and Gli3.[75]

Shh is known to play an important role in HF biology and also in skin cancer (for review see Ref. 75). It is expressed exclusively in the skin epithelium.[76,77] Yet, its effectors and targets (Ptc1 and Ptc2, Gli1, Gli2 and Gli3, Smo) are expressed in the skin epithelium and the underlying mesenchyme. Therefore, Shh signaling is operative in the epithelium and the mesenchyme. In normal developmental processes Shh is essential for hair growth and morphogenesis.[78–80] $Shh^{-/-}$ and $Gli2^{-/-}$ mice both present an identical phenotype comprising developmental arrest at early peg stage.[12] Initiation of HF development proceeds normally. These two proteins are, therefore, needed for placode downgrowth.[12] Maturation of the DP also depends on Shh signaling, since $Shh^{-/-}$ mice have smaller DPs.[78–80] However, this seems to be an indirect effect, which was demonstrated by Gli2 rescue experiments.[12] Shh and Gli2 are necessary for the proliferation of epidermal and dermal cells.[12] It was recently shown that Gli2 regulates the expression of the cell cycle regulators cyclin D1 and D2.[12] This may explain why abnormal activation of Shh or Gli2 in skin leads to certain hair tumors and basal cell carcinoma.[81]

The signaling hierarchy and choreography of later stages of HF morphogenesis remain to be dissected

Once a placode has formed, epithelial signals are required to induce the development of the future "mesenchymal command center" of the HF (Fig. 2, stage 2),[5,6,9] giving rise to the follicular DP. The nature of this epithelial signal remains unclear, but may be provided by members of the Wnt family, PDGF-A (platelet derived growth factor-A), and especially by Shh[30,78,80] (see above). Interestingly, dermal Wnt5a expression is not found in $Shh^{-/-}$ mice, indicating that it is downstream or even a direct target of Shh signaling.[29] Thus, during stages 2–4 of HF development (Fig. 1), Shh induces dermal Wnt5a and transcription factor Gli1, which are both needed for normal DP proliferation. At the same time, the DP begins to express Versican, HGF and NCAM and upregulates

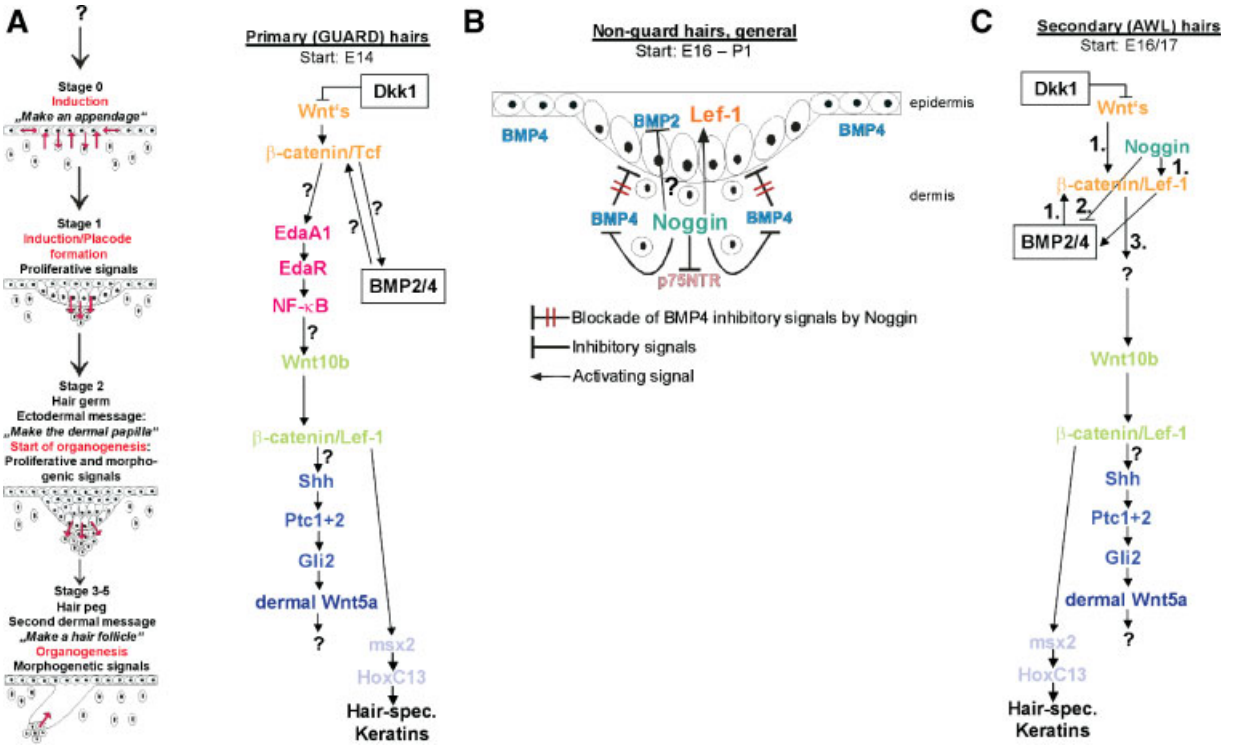


Figure 4. Our present working hypothesis, for induction of primary (guard; **A**) and secondary (non-guard, awl; **B** and **C**) pelage hair follicles. **A:** On the left side, the different stages of early hair follicle development are depicted (stage 0–5), according to the original hypothesis of M. Hardy.[6] In mammals, it is not yet known how inductive fields are created prior to initiation of hair follicle placode formation. Thus, the question mark above stage 0. To the right the currently known activation pathways involved in primary guard hair development are shown. Essential inducers are Wnt and EdaA1/EdaR/NF-κB. **B:** Secondary hair induction calls for Wnt and Noggin. A model concerning inhibition of BMP4 in the dermal papilla and BMP2 in the ectoderm by BMP anatgonist Noggin was proposed by Botchkarev et al.[24] Localized Noggin expression in the dermal papilla antagonizes the potent placode-growth-inhibitory action of the two BMPs and leads to Lef-1 expression. Noggin also inhibits $p75^{NTR}$ expression, which functions as a receptor that negatively regulates hair follicle growth.[94] Jamora et al. go a step further in proposing that Noggin is directly responsible for activation of nuclear Lef-1 expression in secondary hair follicles.[33] They suggest that Noggin must counteract any BMP action in the developing follicle because BMP is the key inhibitor of Lef-1 expression. Figure adapted from Botchkarev et al.[24] **C:** Scheme of the currently known signaling pathways involved in secondary awl hair induction. As shown in B, apart from Wnt signaling, Noggin is the second essential inducer. The numbers (1.–3.) indicate the possible order of events occuring to initiate awl hair development. One major difference of primary guard hairs compared to secondary awl hairs is the EdaA1/EdaR/NF-κB pathway, only activated during initiation of guard hairs. Inhibition of any component of this pathway leads to absence of guard hairs.[22,23] Wnt in both pathways stands for any Wnt, the exact one being unknown. The best-defined so-called "inhibitors" of hair follicle development, Dkk1 and BMPs, are boxed and marked in black. Dkk1 is a true inhibitor and, ectopically expressed in the ectoderm, it prevents formation of all hair follicle types by complete inhibition of Wnt signaling.[30] However, BMPs do not have a clear inhibitory role. They are in part activators and inhibitors, depending on the developmental stage. BMPs are expressed from early on in both hair types, and at least in secondary HFs they seem to be needed for active β-catenin complexes and downstream target gene expression of Wnt/β-catenin/Lef-1.[110,111] The exact role of BMPs in early guard hair follicles remains unknown. However, BMP action may have to be anatgonized by Noggin at later stages of guard hair follicle morphogenesis.[25,112] Shh/Gli2 signaling is activated at a later time point at stage 2–3 (hair germ—peg), and is only to a minor degree responsible for dermal papilla formation of both hair types, but seems to be required for dermal Wnt5a expression.[29] Shh/Gli2 is mostly essential for further epidermal downgrowth of the germ and the subsequent peg formation.[12] The formation of secondary zigzag hairs remains to be studied in detail. They seem to demand EdaA1/EdaR/NF-κB signaling later than in guard hairs and further downstream of Wnt/β-catenin and Noggin/Lef-1. Importantly, note that the arrows do not necessarily mean that the protein is directly regulated by a particular pathway, with the exception of Wnt/β-catenin, Eda/NF-κB and Shh/Gli2. In most cases, it means that the indicated protein is activated temporarily downstream. The multiple question marks already imply that there are still many processes that are currently unknown and need to be investigated in the future.

its alkaline phosphatase activity as indicators of its increasing inductive power.[82–84]

Eventually, further downgrowth of the rapidly proliferating epithelial cone and hair-specific epithelial differentiation are regulated by Shh/patched/Gli1/Gli2, Wnt/β-catenin, Noggin/Lef-1 and others to finally form the hair germ and peg (Figs. 1 and 2, stage 2 and 3).[8] TGFβ2/TGFR-II signaling also seems to play a role in regulating the downgrowth of keratinocytes committed to the follicular differentiation pathway.[85,86] The precise role of HGF/SF (Hepatocyte growth factor/scatter factor) and signaling through its receptor tyrosine kinase (c-Met), and of activinβA/follistatin in controlling the subsequent downgrowth remains to be defined.[8,20,40,84]

Target-specific innervation of the developing HF, which becomes detectable by stage 2 and gradually progresses thereafter in discernable developmental waves, is likely induced by neurotrophins that are produced by the budding follicle epithelium.[87–89] Similarly, the developing follicle epithelium releases promoters of angiogenesis like VEGF (vascular endothelial growth factor) to induce a proper HF vasculature. While TGFα/EGFR signaling and the transcription factor and proto-oncogene ETS2 are considered as important denominators of HF shape, it is not yet clear how HF polarity and angle are determined.[8] Again, this may be established by morphogenetic gradients.

During the remaining stages 5–8 of follicle development (cytodifferentiation phase, see Fig. 1), a multitude of different factors is being recruited in distinct tissue compartments of the developing follicle. Recent evidence suggests a key role for the transcriptional repressor CCAAT displacement protein (CDP, Cutl1) and the zinc-finger transcription factor GATA3 in committing epithelial cells to the inner root sheath (IRS) lineage.[90,91] GATA-3-null mice fail to form the IRS altogether.[91] Furthermore, the asymmetrical IRS differentiation seen in cathepsin L null mice suggests a role for this lysosomal protease in normal IRS differentiation.[92] Given the current scarcity of convincing data on the relative importance and exact hierarchy of signaling events during later steps of pilosebaceous differentiation, we shall subsequently focus on a discussion of stages 0–2 of HF development.

Theories of early HF development

We conclude this review with a brief summary of current, competing concepts that attempt to put in place the presently recognized key players in early HF development.

The still most influential general model, summarized by Hardy,[6] postulates a first dermal message {"make an appendage"} that does not yet commit the epidermis to a particular structure, but still can give rise to hair, teeth or an exocrine gland. According to this model, the subsequent message is produced by the epidermis in order to induce a mesenchymal condensate underneath the epithelial placode. Ectodermal placode and mesodermal condensate cells proliferate, the ectoderm starts to grow down into the mesenchyme. A second dermal message for the epidermis is a structure-specific command: "make a HF", committing the epithelium to a HF-specific differentiation pathway (Fig. 4A, left panel).[6]

More recent models suggest concrete molecules in the interactions between ectoderm and mesenchyme. Huelsken and colleagues proposed that EdaR (downless), and not Wnt, regulates high levels of β-catenin expression in early placodes.[54] Considering the fact that Wnt10b expression is absent in EdaA1-mutant (*tabby*) mice, β-catenin is, indeed, likely to be activated downstream of EdaA1/EdaR and NF-κB.[30] FGFs were proposed as signals upstream of β-catenin, leading to localized EdaR upregulation, and Shh and BMP2/4 signaling were thought to be located downstream of EdaR and β-catenin in this model.[54] However, Andl et al. showed with the help of ectopic Dkk1 expression that "nothing happens without Wnt" in terms of epithelial appendage formation—no HF, no teeth, no glands,[30] thus placing Wnt signaling at the start of HF development. These studies demonstrate that Wnt/β-catenin signaling is upstream also of EdaR (Fig. 4A,C). As a result of their findings, Andl et al. propose two models for HF initiation.[30] (1) Dermal Wnt is the first dermal message. This is supported by the fact that in chick feather tracts β-catenin is expressed in the dermis one day before it becomes visible in the epidermis.[93] (2) A first, yet unknown dermal message induces initiating Wnt signaling in the epithelium.[30] However, instead of the first dermal message still proposed in both hypotheses, there could as well be a *first epidermal message*. In tooth development, for instance, there is a first epidermal message sent to the underlying mesoderm.[26,27] Ectodermal Wnt and Shh play an important role in this process.

In the "Noggin model" (see Fig. 4B), first proposed by Botchkarev et al. and extended by Jamora et al.,[24,33] noggin is pictured as key antagonist for the inhibitory effects of BMP4 and BMP2 action on HF development, resulting in Lef-1 expression. Noggin, which is required for the induction of secondary HF development is not expressed at stage 0, but at stage 1.[24,25] Hence, it is needed for initiation, but also for subsequent downgrowth and morphogenesis, just like Lef-1. This model also incorporates another inhibitor of HF development, the signaling via the low affinity-neurotrophin receptor, p75NTR,[94] whose expression is downregulated by noggin.[24] Thus, noggin releases two potent, independent molecular "brakes" on HF development, BMPR1A- and p75NTR-mediated signaling (Fig. 4B).

In an attempt to integrate key features of the previously proposed models described above, we suggest a "working model", which distinguishes between the molecular controls for primary (guard) and secondary non-guard (here: awl) HFs (Fig. 4A and C). It depicts the establishment of an inductive morphogenetic field as the result of inhibitor gradients, inhibitory-neutralizing agents and stimulators of HF

development, just as illustrated in Fig. 2, which are jointly generated by skin epithelium and mesenchyme to ultimately drive stages 0–2 of HF development.

In view of the immensely increased insight into the molecular controls of HF development that we have witnessed during the past five years, the next challenge is to define how the different signaling pathways depicted in Fig. 4 regulate each other in order to develop such a complex miniorgan as the HF. Moreover, the—still elusive—events located most up-stream of the initiating Wnt signal must be identified, i.e. those events that cause the inductive field around the future placode. Furthermore, it will be important to further dissect the molecular signaling that favors the formation of an HF instead of a sweat gland, a tooth, a nail or a mammary gland in a given location of the integument. One conceivable answer may lie in the local balance of competing transcriptional co-activators or repressors that can directly interact with β-catenin.[52] Last but not least, we need to understand much better how each HF subtype differs in its controls of induction (compare Fig. 4A + C), and to which extent the current concepts derived from the study of murine HF development are transferable to human skin.

In this ongoing and exciting research endeavour, which ultimately could facilitate the generation of human HFs de novo from appropriately interacting epithelial and mesenchymal cell populations in vitro, our simplistic working model (Fig. 4) offers some theoretical guidance for the design of corresponding future experiments.

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