The ectodysplasin pathway in feather tract development

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Summary

The ectodysplasin pathway, comprising the ligand ectodysplasin, its receptor Edar and a dedicated death domain adaptor protein Edaradd, plays an important role in epidermal organ formation in mammals. Mutations in the genes encoding these proteins cause dysplasia or absence of teeth, sweat glands and hair follicles. However, the relative position of this pathway in the regulatory hierarchy directing follicle formation remains unclear. In this work, the chicken orthologs of *Eda*, *Edar* and *Edaradd* were cloned to exploit the temporal precision of the feather tract system in order to study the role of the ectodysplasin pathway. We find that these genes are expressed in a similar pattern during feather and hair development, with the notable difference that the ligand Eda, which is expressed in the epidermis of the mouse, is expressed in the dermis of the feather tract. Contrary to conclusions reached from the analysis of mutant mice, we find that localization of *Edar* expression to the nascent placode is coincident or subsequent to the local expression of other markers of placodal differentiation, and not an upstream event in tract patterning. Furthermore, forced expression of BMP and activated β-catenin demonstrate that local expression of *Edar* is dictated by the interaction between these two pathways. These results suggest that activation of the ectodysplasin pathway may be permissive for activating signals to overcome signals that inhibit placode formation, but the function of this pathway in the specification of follicle initiation lies downstream of other patterning events.

Key words: Ectodysplasin, Edar, Edaradd, Feather bud, Pattern formation

Introduction

Cutaneous appendages provide a powerful model system for the study of budding morphogenesis and pattern formation. Hair or feather follicle formation is initiated by signals from the dermis that lead to the formation of an epidermal signaling center, the epidermal placode, that then recruits dermal cells to form an underlying dermal condensation that acquires inductive properties as well (Sengel, 1976). Coordinated signaling between these two centers is required for the maintenance and continued morphogenesis of the follicle rudiment. In addition, discrete buds are generated in part by signals from the nascent follicle that prevent surrounding cells from adopting follicular fates (Jung et al., 1998; Noramly and Morgan, 1998). Inductive signaling in the process of appendage formation is conserved between birds and mammals (Dhouailly, 1973), and the manipulation of gene expression in both chicken and mouse embryos has been used to establish the importance of several signaling pathways in follicle development. The feather tract is particularly amenable to the analysis of the early steps in follicle induction, and has been used to demonstrate roles for the Wnt/β-catenin pathway and for BMP signaling in promoting and inhibiting placode formation, respectively (Jung et al., 1998; Noramly et al., 1999; Noramly and Morgan, 1998). Activation of the β-catenin pathway in the epidermis also results in induction of *Bmp2* expression (Noramly et al., 1999). These observations led to the model that, after initial activation of the β-catenin pathway in response to a more generally distributed signal from the dermis, positive feedback on the Wnt/β-catenin pathway acts locally to promote placode formation, while BMP expressed in the forming placode inhibits this fate in surrounding cells. This interaction between BMP and β-catenin signaling is necessary for the formation of a periodic array of feather buds in the tract. In conjunction with a wave of inductive activity propagating through the dermis, it is formally sufficient for the generation of the periodic array of buds in a wavefront and inhibitory field model (Held, 1992).

Studies of hair follicle development in the mouse have also identified analogous roles for the Wnt/β-catenin and BMP pathways (Pispa and Thesleff, 2003). However, another signaling pathway that plays a crucial role in follicle formation has been identified by study of the underlying causes of the human anhidrotic ectodermal dysplasia syndromes (HED). These syndromes are characterized by the dysmorphology or absence of structures that result from epidermal-mesenchymal interactions, including hair follicles, sweat glands and teeth. Mutations in the genes for *ectodysplasin* (*ED1*), a secreted signaling molecule of the TNF family, its receptor (*EDAR*), and the downstream death domain adaptor *ectodysplasin receptor associated death domain* (*EDARADD*) are responsible for the various forms of HED (Wisniewski et al., 2002) Spontaneous mutations in the murine genes for *Eda*, *Edar* and *Eadaradd* define the analagous mouse mutants *tabby*, *downless*, and *crinkled*, all

of which exhibit abnormal hair follicle development (Thesleff and Mikkola, 2002).

Analysis of primary follicle formation in the mouse mutants suggested that the ectodysplasin pathway plays a very early role in this process. In mice, hair follicle formation occurs in three distinct waves (Mann, 1962). The primary wave occurs around E14 and produces the tylotrich or guard hairs, which are ultimately larger than the hairs produced in succeeding waves. Second and third waves of follicles arise at E17 and after birth in the intervening spaces between follicles formed in previous waves, and will generate three types of hairs – awls, zigzags and auchenes. Mutations in the mouse genes encoding *Eda*, *Edar* and *Edaradd* all result in failure of the first wave of hair follicle formation, and the mice ultimately lack guard hairs (Headon et al., 2001; Headon and Overbeek, 1999; Laurikkala et al., 2002; Mikkola et al., 1999). However, follicles do form in what are thought to be abnormal versions of the subsequent waves. Although the resulting hairs are structurally abnormal and characterized as abnormal awls, this may be due in part to later roles for ectodysplasin signaling in follicle morphogenesis as both *Eda* and its receptor are expressed in the hair follicle bulb. Despite these abnormalities, it is clear that much of the follicle formation process per se can occur in the absence of Eda, Edar or Edaradd.

During skin and follicle development in the mouse, all three genes are initially expressed throughout the basal layer of forming epidermis prior to the initial differentiation of the primary follicle placodes. However, as follicle specification progresses, ectodysplasin expression becomes repressed in the epidermal placode of the follicle and thereby restricted to the interfollicular epidermis. By contrast, *Edar* and *Edaradd* transcript levels are increased in the placode and decreased in interfollicular epidermis. Like *Edar*, a number of genes become expressed in a punctate pattern in E14 skin, reflecting their preferential expression in the forming epidermal placodes. However, this punctate pattern was not observed for a battery of markers tested in the Eda pathway mutants, suggesting that the earliest steps in follicle formation may be blocked in the absence of Eda activity.

Particular attention has been paid to the localization of *Edar* expression to the forming placodes. This is in part because the localization of *Eda* expression to the interfollicular epidermis is not crucial for pattern formation. Guard hair follicle formation in the *tabby* mutant can be rescued either by expression of *Eda* under the control of the *keratin14* promoter, which results in expression throughout the basal epidermis and epidermal placode, or by injection of pregnant mice with a soluble Eda/IgGFC fusion protein that crosses the placenta (Gaide and Schneider, 2003; Mustonen et al., 2003). It is possible that the Eda pathway may play an unlocalized role upstream of initial patterning events in achieving the competence to form a placode. However, if localized signaling through the Eda pathway is required for placode formation, the restriction of receptor or other transduction components to the nascent follicle is likely to be a crucial step in the function of this pathway. Localization is in one sense dependent on Eda signaling; Edar remains expressed throughout the epithelium at E15 in the *tabby* (*Eda*) mutant mouse when expression is largely restricted to the epidermal placodes in wild-type skin (Laurikkala et al., 2002). However, when the abnormal awl follicles form at E17, *Edar* is preferentially expressed in them, demonstrating that Eda activity is not directly required for its localization and that it can occur as a consequence of follicle formation in the absence of Eda signaling.

From the perspective of pattern formation, a crucial question is whether localized Eda/Edar pathway signaling lies upstream or downstream of the interaction between the β-catenin and BMP pathways postulated to direct early patterning events. Examination of the Eda pathway components in several different transgenic and knockout lines designed to alter β-catenin pathway signaling in the skin have led to inconsistent answers, in part because of the difficulties of evaluating the precise timing of altered gene activity in the mouse embryo. This lack of temporal precision in the genetic interventions in the mouse, and the difficulty in working with cultured murine skin prior to patterning, have hampered efforts to establish the roles of the Eda pathway in pattern formation and to place them relative to other signaling pathways important for follicle specification. Although these genes are clearly crucial to follicle formation, the nature and timing of that requirement remain unclear. Is the localization of *Edar* expression and the consequent asymmetry in pathway activation a crucial first step in placode specification and pattern formation? If so, is this upstream and independent of the interactions between the β-catenin pathway and BMP that have been postulated to direct this early patterning decision? The forming feather tract of the chicken allows examination of the sequence of changes in gene expression during feather rudiment formation because each feather rudiment is added in a defined sequence so that the tract contains an ordered developmental series displayed in a precise spatial array. The feather tract is also amenable to retroviral-mediated alteration of gene activity that is targeted to specific stages of development in vivo. To exploit these advantages, we identified the components of the chicken Eda pathway to investigate whether they are expressed during feather bud formation in a pattern similar to that observed in hair follicle formation, and if so, to place the timing of localized receptor expression relative to other patterning events. Finally, we sought to test the roles of the β-catenin and BMP signaling pathways postulated to direct pattern formation in the regulation of expression of Eda pathway components.

Materials and methods

Isolation of cDNAs for chicken Edar, Eda and Edaradd

Sequences for *cEda* and *cEdaradd* were amplified from chicken skin cDNA using primers based on sequences in the BBSRC Chick EST Database. Primers for *cEda* (clone ID ChEST427a20), *tabby* (5-1) ACATACTTCATCTATAGTCAGGTA and *tabby* (3-1) TAGTGCAA-CATAAAAGCAGAGA amplified a 612 bp fragment encoding the last 92 amino acids and 333 base pairs of the 3′UTR. Primers for *cEdarADD*, Crink (5-1) TGATATGGCAGATCATGCAAC and Crink (3-1) GTCTTCTAGCAGGCAGAACA (clone ID ChEST196m22) amplified a 695 bp fragment encoding the last 203 amino acids, and 174 bp of the 3′UTR. *Edar* was amplified using the degenerate primers Dless (5-1) CTICCIGGITAC/TTAC/TATG and Dless (3-1) C/TTGCATICCA/GTCIGTCAT, which amplified sequence encoding amino acids 117 to 397, based on the mouse protein. Additional coding sequences of *cEdar* were amplified using primers from the chicken genomic sequence. Additional 5′ sequences for *cEdaradd* and *cEda* were amplified using the Clontech Marathon RACE-PCR kit.

Genomic and cDNA sequence analysis

Sequence homologies were determined using the BLAST function of the NCBI Chicken Genome Resources. Synteny was analyzed using the alignments of the draft version of the chicken genome to the human genome provided by Ensembl, a project of the Wellcome Trust and the Sanger Institute. The protein sequences inferred from the chicken cDNAs for *Edar*, *Eda* and *Edaradd* were compared with their mouse orthologs using the Clustal W function of MegAlign (DNAstar).

Riboprobe templates and in situ hybridization

The initial cDNAs isolated for *cEdar* (843 base pairs), *cEda* (945 base pairs) and *cEdaradd* (869 base pairs) described above, a *Wnt6* clone (675 base pairs) derived from BBSRC Chick EST clone ID ChEST810f19, and clones described previously (Noramly et al., 1999; Morgan et al., 1998; Noramly and Morgan, 1998) were used as templates to generate riboprobes and perform whole-mount in situ hybridization as described (Morgan et al., 1998). For comparison of gene expression changes, the following numbers of embryos were used: *Edar/Edaradd*, *n*=18; *Edar/Bmp2*, *n*=17; *Edar/Wnt6*, *n*=5; *Edar/β-catenin*, *n*=20; *Edar/Bmp4*, *n*=6; *Edar/Shh*, *n*=5; *Eda/Edar*, *n*=13; *Eda /Edaradd*, *n*=7; *Eda/β-catenin*, *n*=7; *Eda/Wnt6*, *n*=7; *Eda/Bmp2*, *n*=7; *Eda/Bmp4*, *n*=6; *Eda/Shh*, *n*=5; *Edaradd/Bmp4*, *n*=6; *Edaradd/Shh*, *n*=5). Section in situ hybidization was performed as described (Morgan et al., 1988), and images presented are a composite of a phase contrast image (30% opacity) overlying a brightfield image.

Viral infection of chicken embryos

Chicken embryos were infected at day 4 of incubation with retroviruses expressing truncated β-catenin or BMP4 prepared as described (Noramly et al., 1999; Noramly and Morgan, 1998). Embryos were harvested between 7.5 and 9.0 days of incubation, and processed for in situ hybridization with riboprobes for *Eda* (BMP infected, *n*=9; β-catenin infected, *n*=41), *Edar* (BMP infected, *n*=5; β-catenin infected, *n*=26) or *Edaradd* (BMP infected, *n*=7; β-catenin infected, *n*=16), as well as a riboprobe for the detection of the extent of viral infection. Representative samples were dehydrated and sectioned.

Results

The chicken Eda pathway genes

Sequences from partial cDNA clones in EST databases (*Eda*, *Edaradd*) and degenerate primer PCR (*Edar*) were employed in conjunction with rapid amplification of cDNA ends (RACE) to obtain the complete coding sequences for these genes (Fig. 1). Chicken Edar is 448 amino acids in length and shares 83% identity with its mouse ortholog. Its death domain exhibits 97% identity with the death domain of the mouse protein. Edaradd is 214 amino acids long and shares only 56% identity with its mouse ortholog, although the death domain exhibits 76% identity with the death domain of mouse Edaradd. In mammals, the *Eda* gene encodes two alternatively spliced proteins that differ by the presence (Eda-A1) or absence (Eda-A2) of two amino acids. Eda-A1 acts through the Edar receptor and is required for primary follicle formation, whereas Eda-A2 is mediated by a distinct receptor, Xedar, and cannot rescue primary follicle formation in *tabby* (*Eda*) mutant mice. RNAs encoding both isoforms were detected in chick skin. The last 356 amino acids of cEda-A1 share 80% identity with its mouse ortholog and 94% identity in the TNF core (Fig. 1). Consistent with the designation of these sequences as the orthologs of the mammalian genes, the *cEda* and *cEdar* sequences map to regions of chicken chromosomes 4 and 1, respectively, that are syntenic with the regions of human chromosomes X and 2 that encompass the human *ED1* and *EDAR* loci. The contig containing the *cEdaradd* gene has not yet been assigned to a chromosome, but within the currently available chicken genomic sequence, it is the only sequence that exhibits strong homology with the murine *Edaradd* gene.

Expression during feather tract development

Feather buds form in discrete tracts in the chicken embryo beginning at approximately day 7 of incubation. In each tract, feather rudiments are added sequentially in a wave that spreads progressively through the tract. Bud formation in the dorsal tract initiates at the dorsal midline and spreads laterally, whereas rudiments arise initially at the distal edge of the femoral tract and are added more medially as development progresses. Several genes thought to be induced in the epidermis in response to the 'primary' inducing signal from the dermis are expressed in a broad stripe at the leading edge of these morphogenetic waves, and then become restricted to the forming placodal or interplacodal epidermis (Fig. 2A, and data not shown) (see also Noramly and Morgan, 1998).

Whole-mount in situ hybridization analysis of expression of Eda pathway genes in the chicken reveals patterns that are superficially similar to those observed in the mouse. Expression of *Eda*, *Edar* and *Edaradd* was not detected in the presumptive feather tracts by whole-mount in situ hybridization at day 6 of incubation (data not shown). All three genes are first detected in a diffuse stripe at the initiating border of the tract at day 7 of incubation and this band of expression is observed ahead of the wave of placodal differentiation in older embryos (Fig. 2B-G). Behind the wave of differentiation, *Edar* and *Edaradd* expression is restricted to the forming feather rudiments, while *Eda* expression is observed in interfollicular skin at high levels. At later stages of bud morphogenesis, *Eda* is expressed in a spot near the center of the feather bud, while *Edar* and *Edaradd* continue to be expressed in the distal feather bud (Fig. 2H,I and data not shown).

Analysis of Eda pathway gene expression in sections revealed a striking difference from the pattern observed in the mouse. Although low levels of *Eda* expression are observed in the epidermis prior to tract patterning and appear to persist in the interfollicular epidermis at the onset of feather bud patterning (Fig. 3A,F), the robust interfollicular pattern of expression observed represents strong expression in the interfollicular dermis (Fig. 3F). Moderate levels of *Eda* transcripts are observed in the dense dermis as patterning begins (Fig. 3A), while *Edar* and *Edaradd* are expressed in the overlying epidermis (Fig. 3B and data not shown). *Eda* expression is lost in the dermal condensation, while it is increased in the surrounding dermis (Fig. 3F). *Edar* and *Edaradd* both become preferentially expressed in the epidermal placode, but the difference between expression levels in placodal and interplacodal epidermis is greater for *Edar* than *Edaradd* (Fig. 3C,D,H and data not shown). The later expression of *Eda* within the feather bud is also in the mesenchyme and persists in the distal mesenchyme of the maturing feather filament, whereas *Edar* and *Edaradd* expression remain confined to the bud epithelium (Fig. 3G and data not shown). RT-PCR analysis of isolated epidermis and dermis at different stages of development confirm these tissue

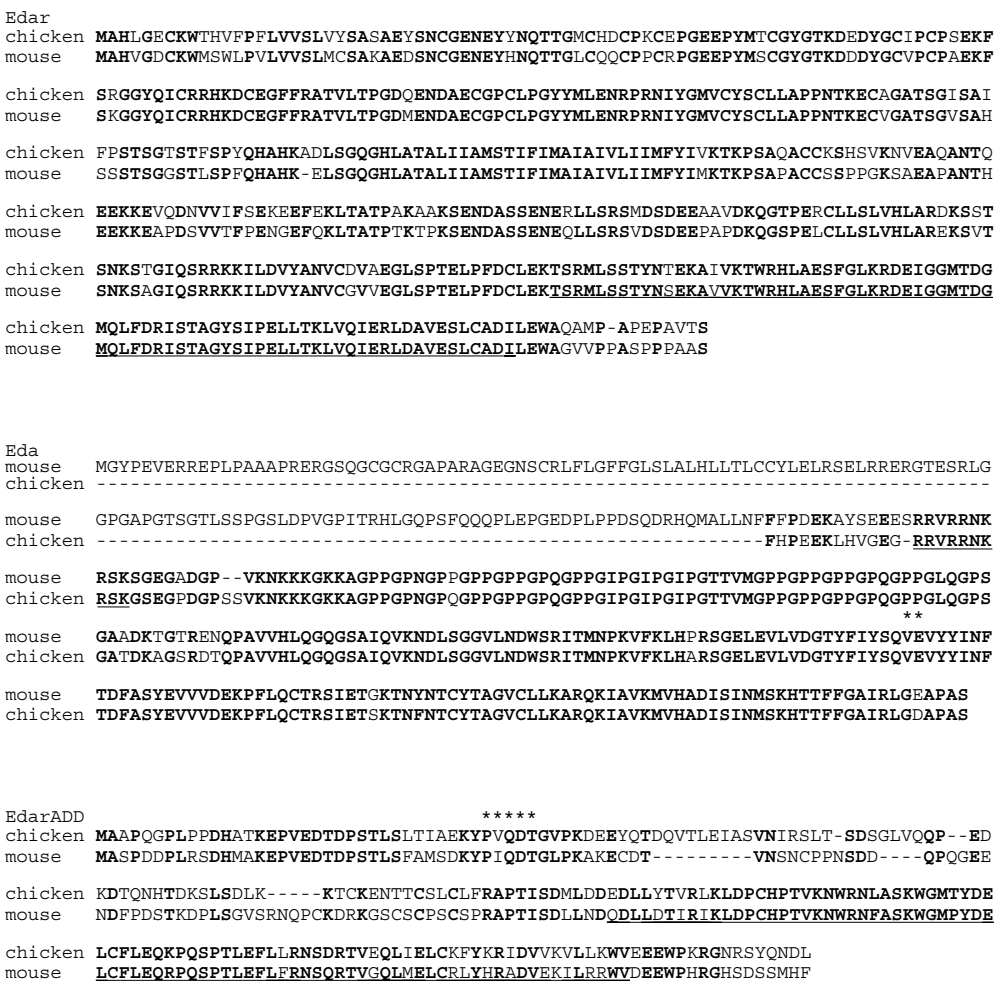

Fig. 1. Amino acid sequences of chicken Edar, Eda and Edaradd. The inferred amino acid sequences of the chicken proteins are compared with their mouse orthologs. The valine and glutamine present in Eda-A1 and absent from Eda-A2, and the TRAF interaction consensus sequence (PXQXT) of Edaradd, are marked by asterisks. Death domains of Edar and Edaradd, and the furin cleavage site of Eda, are underlined.

distributions, and indicate that both the A1 and A2 isoforms are expressed at significant levels in the dermis throughout this period of development (see Fig. S1 in the supplementary material).

Timing of Eda pathway gene expression changes

As a consequence of the sequential addition of feather rudiments, the feather tract exhibits an array of buds, each at a slightly later developmental stage than its immediate neighbors, at more proximal or anterior positions within the hexagonal lattice within which the buds arise. The femoral feather tracts on the left and right sides of the embryo develop synchronously, so that it is possible to compare changes in gene expression over a broad developmental series by examining the patterns of expression of two different genes in the corresponding buds of the left and right halves of the embryo. Using this type of analysis, a sequence of gene expression events corresponding to specific steps in feather bud development has been established. Notably, *Bmp2* exhibits the pattern described previously of diffuse expression that then resolves to the forming placode, and the localization of *Bmp2* expression is an early marker of epidermal placode specification (Noramly and Morgan, 1998). *Wnt6* is expressed in the epidermis from an earlier stage, but becomes restricted to the placode at about the same time as *Bmp2* (data not shown). *Bmp4* is expressed in the dermal condensation as it forms and *Shh* expression is detected shortly thereafter in the epidermal placode.

The expression patterns of *Edar* and *Edaradd* were similar and became observable as discrete spots in the corresponding rudiments of the left and right femoral tracts (data not shown). However, the augmented expression of *Eda* in interfollicular dermis occurs later in bud development, only after *Edar* expression has been localized to the epidermal placode, so that spots of *Edar* expression are observed in the right tract without corresponding holes in the *Eda* expression pattern in the left tract (Fig. 4A,B). The preferential expression of *Edar* in the placode is an early event in bud specification and is roughly synchronous with the corresponding change in *Bmp2* and *Wnt6* expression (Fig. 4C,D, and data not shown). In 12 out of 15 embryos, an equal number of resolved spots of *Edar* and *Bmp2* expression could be observed; in 3 out of 15 embryos an additional spot of *Bmp2* expression was observed, indicating that these patterning events occur slightly earlier in the

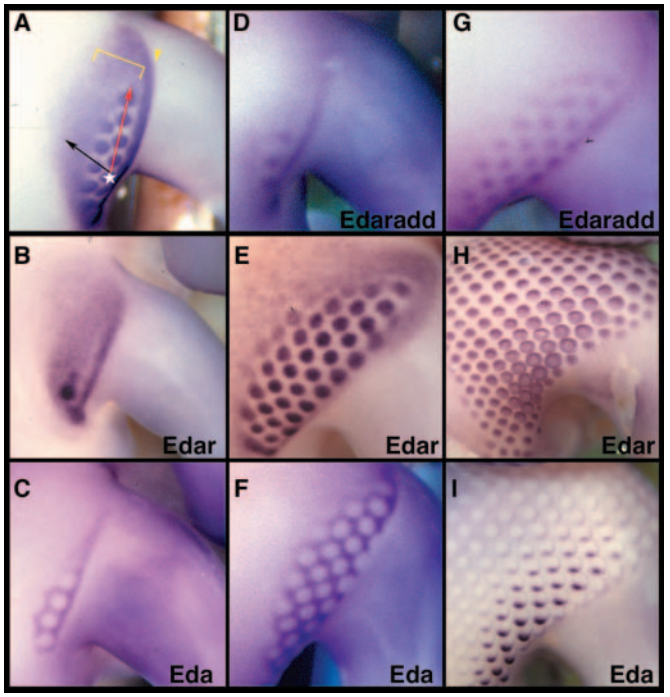

Fig. 2. Expression of *Edar*, *Edaradd* and *Eda* in the developing feather tract. (A) In situ detection of *β-catenin* transcripts shown as an example of the pattern exhibited by a class of 'early genes' during development of the femoral tract. Expression begins as a band (yellow bracket) at the distal border (yellow arrowhead) and spreads towards the trunk. It is repressed in interfollicular epidermis and increased in the epidermal placodes. The first bud to form is marked by an asterisk. Younger buds are added sequentially within a row (red arrow) and additional rows arise progressively closer to the trunk (black arrow) as development progresses so that rows closer to the trunk have buds at earlier stages of development than those more distal on the leg. (B,E,H) *Edar* is expressed in a similar pattern. Initial diffuse expression (B) becomes restricted and upregulated in the nascent placode (B,E). (C,F) Initial diffuse *Eda* expression (C) becomes restricted to interfollicular skin (F). (D,G) *Edaradd* expression resembles that of *Edar* (B,E) at all stages analyzed. In older embryos, *Edar* expression persists in feather bud epithelium (H), whereas *Eda* is detected in the posterior and distal regions of the most mature buds (I). Embryos were harvested at day 7.5 (A), day 7.25 (B-D), day 8 (E-G) and day 10 (H,I).

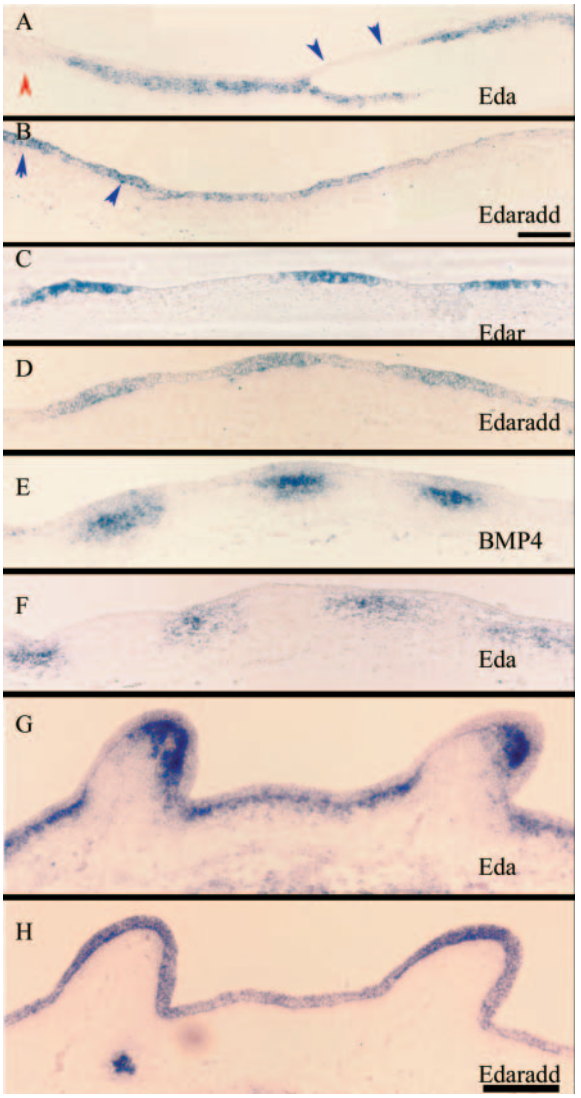

Fig. 3. Eda pathway expression in tissue sections. In situ hybridization to tissue sections from embryos at day 7.5 (A,B), day 8 (C-F) and day 10.25 (G,H) of incubation. (A) *Eda* expression is detected in the epidermis of the presumptive feather tract at the extreme right of the section, but drops below the level of detection in the more developed epidermis of the medial tract (left). Note the lack of signal in the epidermis where it has separated from dermis (blue arrowheads). Expression is observed throughout the dermis at this stage, with the exception of the extreme left, where a dermal condensation has formed (red arrowhead). (B) *Edaradd* expression is observed in an adjacent section throughout the tract epidermis, although expression is augmented in the epidermal placodes (arrowheads). (C) *Edar* expression is largely restricted to epidermal placodes as buds are formed. (D-F) Adjacent sections show placodal expression of *Edaradd* (D) overlying dermal condensations that express *Bmp4* (E) but do not express *Eda* (F). *Eda* expression is observed in the interfollicular dermis. (G,H) Adjacent sections show *Eda* expression persists in the interfollicular dermis at lower levels and is highly expressed in the posterior distal mesenchyme of the feather bud, while *Edaradd* expression is confined to the epidermis.

developmental sequence. Consistent with this observation, additional spots of *Edar* expression were observed when compared with later markers of bud development, including *Shh* and *Bmp4*, indicating that local *Edar* expression occurs early in the process of placode development (Fig. 4E,F, and data not shown). The robust expression of *Eda* in the interfollicular dermis occurs rather late, after the dermal condensation has begun to differentiate and *Shh* expression has been initiated in the epidermis. Consistent with this observation, localized expression of earlier markers such as *Wnt6*, *Bmp2* and *Cek3* is detected in additional rudiments of one tract when compared with the number of rings of dermal *Eda* arising around forming rudiments in the corresponding tract (Fig. 4G,H, and data not shown).

These observations suggest that the local expression of ectodysplasin pathway signal transduction components is an early event, but the timing of localization is consistent with the hypothesis that localization is directed by the β-catenin and BMP pathways. The expression of *Edar* and *Edaradd* resembles the previously described pattern of β-catenin

pathway activation during tract development (Noramly et al., 1999). In serial section comparisons, *Edar* and *Edaradd* expression correlate with the nuclear localization of β-catenin in the epidermis. This correlation is observed in the extreme lateral edges of the presumptive tract where no nuclear localization is observed and expression of neither gene is detected. In more medial regions where pathway activation is evident as a broad swath of cells with moderate levels of nuclear β-catenin accumulation, both *Edar* and *Edaradd* are expressed in a corresponding swath. In the medial regions, augmented expression of *Edar* is observed in regions of high-level nuclear accumulation of β-catenin and expression is lost in the regions where no nuclear accumulation is observed (Fig. 5A,B, and data not shown). By contrast, there is no correlation between *Eda* expression and β-catenin pathway activation at these early stages (data not shown). However, the *Eda* expression pattern within the bud mesenchyme at later stages correlates well with β-catenin pathway activation, whereas *Edar* and *Edaradd* expression in the bud epithelium do not (Fig. 5C-E, and data not shown).

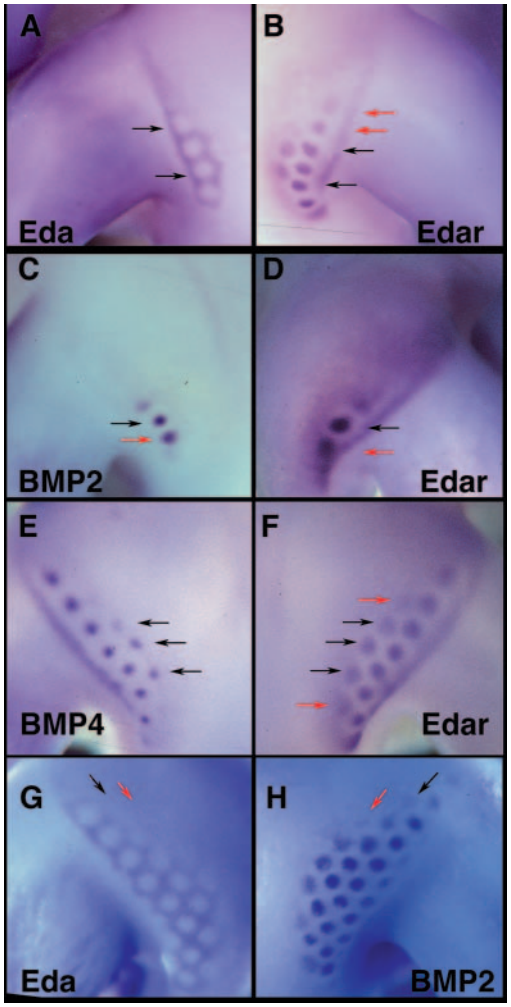

Fig. 4. Timing of gene expression changes. Gene expression was compared in the left and right femoral tracts. (A) Four rudiments lacking *Eda* expression are observed in the left femoral tract, (B) six rudiments with localized *Edar* expression are observed in the corresponding row of the right tract and additional rudiments are observed in the next row. Corresponding rudiments are noted by black arrows; rudiments with local *Edar* expression but without a corresponding region of suppressed *Eda* expression are marked by red arrows. The left femoral tract (C) shows three well-resolved spots of *Bmp2* expression, while the right femoral tract (D) shows a single well-resolved spot of *Edar* expression (black arrow). While local *Bmp2* expression in the next rudiments to form is well resolved (C, red arrow), *Edar* expression in the corresponding rudiments is not (D, red arrow). (E,F) By contrast, three well-defined spots of *Bmp4* expression are observed in the second row of follicles on the right leg (E, black arrows) when, in addition to the corresponding spots of *Edar* expression (F, black arrows), two additional spots are observed in the corresponding row on the right leg (F, red arrows). (G,H) Comparison of *Eda* expression (G) and *Bmp2* expression (H) reveals additional rudiments with local *Bmp2* expression within the corresponding row (black arrows), and an additional row of rudiments expressing *Bmp2* (red arrows).

To test the significance of this correlation, retroviruses were employed to alter the activity of the BMP and β-catenin pathways in vivo during the early stages of tract patterning, and the effects on expression of Eda pathway genes were assessed. Infection with a retrovirus expressing a stabilized form of β-catenin promotes the generation of feather follicles in both the presumptive tract and in regions that are normally featherless (Noramly et al., 1999). Embryos infected with this virus at day 4 of incubation have small patches of infection, and consequent forced activation of the β-catenin pathway, in the epidermis at day 7.5, when tract patterning has begun along the dorsal midline. In uninfected control embryos, local expression of *Edar* is observed in the rudiments along the dorsum of the embryo, but local expression is not observed in the lateral regions of the presumptive dorsal tract. By contrast, the infected embryos exhibit multiple spots of ectopic *Edar* expression in an irregular pattern in the lateral tract and pseudoapterium (Fig. 6A, see Fig. S2 in the supplementary material). Ectopic *Edar* may be observed in small clusters of cells, as well as in ectopic placodes. Subsequent detection of viral transcripts reveals that this ectopic expression closely resembles the pattern of viral infection, although it is slightly more restricted in extent (Fig. 6B). This is due in part to the fact that there is a significant lag between infection and activation of the β-catenin pathway. At day 7, viral transcripts reveal both sites of primary infection, where sufficient time for expression of the encoded transgene and activation of the β-catenin pathway have occurred, and sites of secondary infection after production and spread of this replication-competent virus, where the lag between initial appearance of viral transcripts and the accumulation of the transduced β-catenin make it unlikely that the pathway has been activated. Thus the upregulation of *Edar* in the contiguous patches of cells, which are likely to represent primary foci of infection, suggest that this is a proximal response to β-catenin pathway activation (Fig. 6B). By contrast, infected embryos show no obvious induction of *Eda* expression in the epidermis (Fig. 6E). Rather, rings and arcs of *Eda* expression in the dermis are observed, despite the fact that sectioning of sibling embryos from this injection run confirm that viral infection is restricted to the epidermis (data not shown). Subsequent detection of viral transcripts reveals that these rings surround foci of infection in the epidermis (Fig. 6G,H). Unlike *Edar*, the ectopic *Eda* is not observed in the infected cells, and ectopic *Eda* expression is an indirect consequence of bud induction. Infection within the epidermal placodes of the rudiments in the

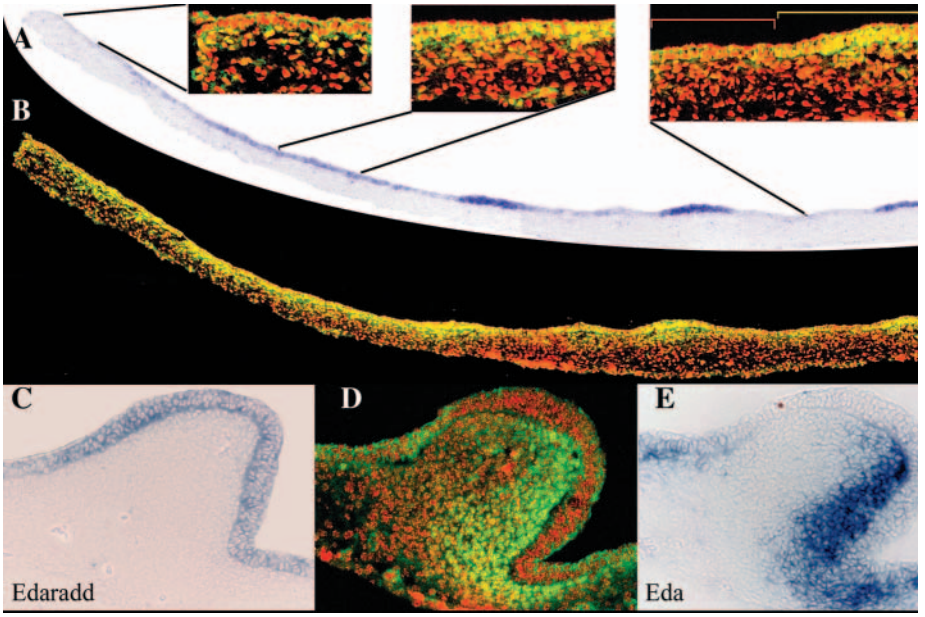

Fig. 5. *Edar* expression correlates with β-catenin pathway activation. (A,B) Detection of *Edar* transcripts (A) and β-catenin protein (B) on adjacent sections of skin from an embryo at day 8 of incubation. Nuclei are stained red and nuclear β-catenin appears yellow. In lateral regions (left), *Edar* is not detected and no accumulation of β-catenin in the nuclei is observed (left inset). In more medial regions, a band of *Edar* expression correlates with moderate nuclear accumulation of β-catenin (middle inset). At the dorsal midline, nascent buds (right inset, yellow bar) express high levels of *Edar* and nuclear accumulation of β-catenin, whereas adjacent interplacodal epidermis does not (right inset, red bar). (C-E) Adjacent sections from skin harvested at day 10 show that *Eda* expression in the bud dermis (E) corresponds to regions of nuclear β-catenin accumulation (D), whereas *Edaradd* expression in the epidermis of the bud does not (C).

dorsal tract also fails to induce *Eda* expression within the placodal epithelium (Fig. 6G,H, and data not shown).

Forced expression of BMP4 in the epidermis at the onset of tract patterning suppresses feather bud development in the surrounding region (Noramly and Morgan, 1998). Extensive expression of BMP4 in the epidermis ahead of the morphogenetic wave had no discernible effect on either *Eda* or *Edar* expression (Fig. 7A,C). However, localized *Edar* or *Edaradd* expression is not observed in regions where placode development is blocked by forced expression of BMP (Fig. 7A,B, and data not shown). Where feather bud development is blocked by BMP, expression of *Eda* expression persists in the epidermis, but is not induced in the honeycomb pattern normally observed in the dermis as rudiments are formed (Fig. 7C,D).

Discussion

Distinctive aspects of Eda pathway gene expression during feather bud formation

These results confirm that the components of the ectodysplasin pathway are expressed during feather tract patterning. Aspects of these expression patterns are similar to those observed during primary follicle formation in the mouse, where these genes play a crucial role in follicle development. In particular, the early expression of all three genes throughout the basal epidermis, and the subsequent reduction of *Eda* expression and the augmentation of *Edar* and *Edaradd* expression in forming placodes is very similar to the pattern observed during hair follicle formation. The localization of *Eda* expression to interfollicular skin is also similar in both species. However, the expression of *Eda* in the dense dermis at the onset of tract patterning and corresponding reduction of *Eda* expression in the ectoderm is quite different from the pattern reported in the mouse. The later expression of *Eda* in the mesenchyme of the feather bud is also different from the late intra-follicular expression of *Eda* as well. Although the significance of these later differences remains to be tested, there are several noteworthy distinctions in the biology of feather tract and mouse pelage hair development that are associated with them.

In the mouse, follicle formation occurs in multiple waves. Both epidermis and dermis between the primary follicles must remain competent to assume either follicular or interfollicular fates in subsequent waves. By contrast, follicle formation occurs in a single wave in the feather tract, so after the morphogenetic wave has passed through the tract, the developmental plasticity of either layer need not be maintained. In addition, the pattern of follicle formation is determinate in the chicken. Each follicle forms in a predictable time and position relative to its neighbors. By contrast, in the mouse the precise position and timing of follicle formation varies and patterning is indeterminate. The determinate patterning of the chicken is likely to be achieved by coordinating the development of the dense dermis, and consequent generation of inductive signals, with the development of the epidermis and the competence to respond to those signals. This allows inductive activity to reach competent ectoderm in a temporally regulated fashion, as each new bud is only initiated after the adjacent bud has been established and can exert its inhibitory influence. Disruption of this coordinated development by combining competent epidermis with a dermis composed of dispersed and re-aggregated dermal cells leads to formation of buds with minimal spacing, but the determinate patterning and morphogenetic wave are abolished (Jiang et al., 1999) (B.A.M., unpublished). It is therefore intriguing to speculate that expression of *Eda* in the dermis rather than the epidermis could serve to help ensure the coordinated development of epidermis and dermis and serve as a component of the primary inductive signal from the dermis that initiates feather bud development.

Most patterning events that rely on Eda signaling entail expression of both ligand and receptor in the epithelial layer (Pispa and Thesleff, 2003). The exception is the developing murine salivary gland, where expression of *Eda* in the mesenchyme of the gland is thought to act on the receptor

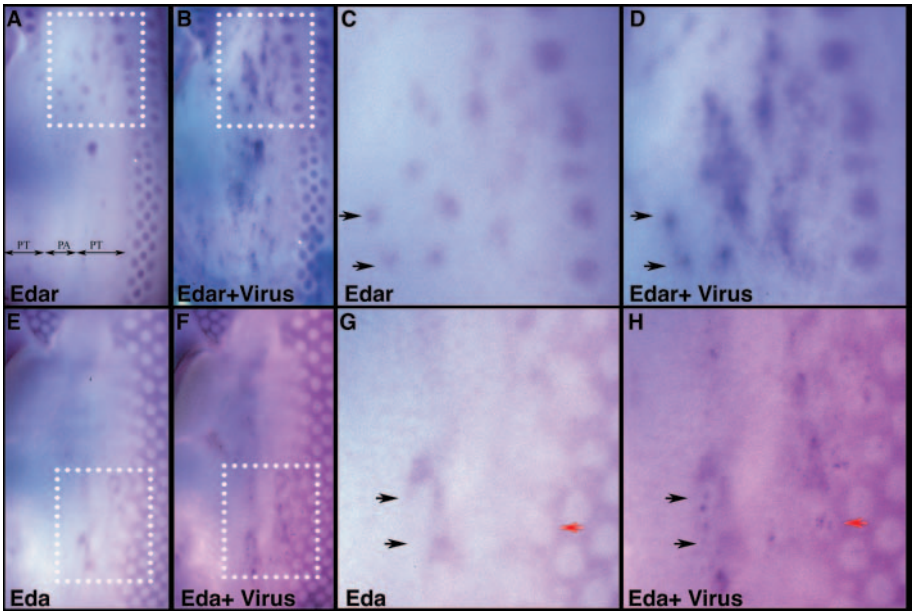

Fig. 6. β-Catenin pathway activation induces *Edar* but not *Eda*. (A) Embryos infected with a virus that activates the β-catenin pathway show ectopic and precocious expression of *Edar* in the presumptive feather tract (PT) ahead of the morphogenetic wave, as well as in the pseudoapteria (PA). (B) Subsequent detection of viral transcripts in the same embryo reveals infection in the areas of ectopic expression. (C,D) Higher magnification of the boxed areas in A and B reveals a correspondence between infection (D) and ectopic *Edar* expression (C) in coherent patches of infection (arrows). (E) Embryos infected with this virus also show ectopic expression of *Eda* ahead of the morphogenetic wave. (F) Subsequent detection of viral transcripts shows infection in the areas of ectopic expression. (G,H) higher magnification views of the boxed areas in E and F show the induced expression of *Eda* (black arrows, G) is in dermis surrounding the dermal condensations induced indirectly by foci of infection with the β-catenin virus in the epidermis (black arrows, H). Foci of infection within the bud (red arrow, H) do not alter *Eda* expression.

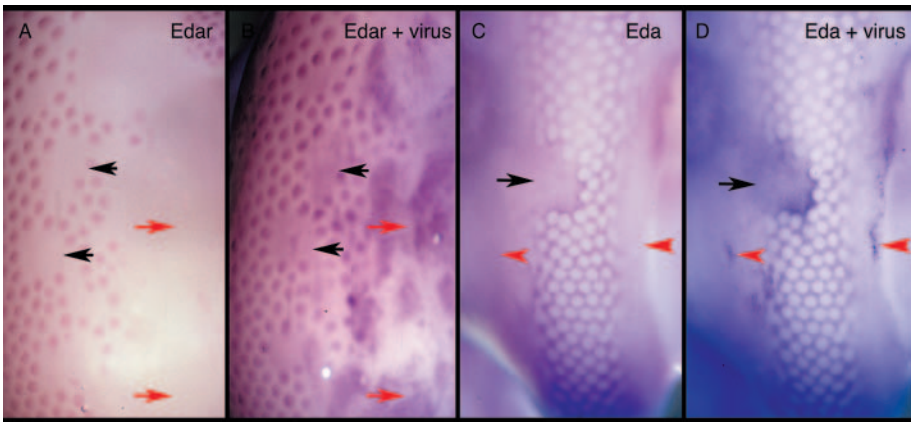

Fig. 7. BMP suppresses *Edar* but not *Eda*. (A) An embryo infected with a virus that encodes BMP4 shows suppression of *Edar* in the feather tract where bud formation is suppressed (black arrows), and no effect ahead of the morphogenetic wave or in the pseudoapteria (red arrows). (B) Detection of viral transcripts reveals infection in the areas where bud formation is suppressed (black arrows), as well as in the lateral regions of the flank (red arrows). (C) An infected embryo shows maintenance of the diffuse expression of *Eda* in an area of suppressed bud formation that would normally exhibit the honeycomb pattern of expression at this stage (black arrow). No alteration of *Eda* expression is observed ahead of the morphogenetic wave or in the pseudoapteria (red arrows). (D) Subsequent detection of viral transcripts demonstrates infection in the areas of bud suppression (black arrow), as well as in the lateral regions indicated in C (red arrows).

expressed in the gland epithelium to promote branching morphogenesis (Jaskoll et al., 2003; Pispa et al., 2003). The late expression of *Eda* within the bud mesenchyme arises as the feather makes the transition towards the radial subdivision of the epithelium that is ultimately manifested as the barbules of the down feather. It is possible that mesodermal Eda acting on Edar expressed in the epithelium plays a similar morphogenetic role in both tissues.

The role of Eda signaling in pattern formation in the feather tract

The Eda pathway components were cloned from the chicken in order to exploit the temporal precision of the feather tract system to place them within the regulatory hierarchy directing pattern formation in the skin. We find that the localization of *Edar* expression to the forming epidermal placode is an early event in tract patterning, but it does not occur prior to the localization of other early markers of placode specification. Instead, it seems to lag slightly behind the localization of *Bmp2* and *Wnt6*, two events that presage the morphological differentiation of the placode. This pattern is consistent with a role in promoting placodal fates subsequent to patterning events that create the initial asymmetry between the future placode and interplacodal cells, but is less consistent with a role in generating that asymmetry.

Two signaling pathways thought to be crucial in directing prior patterning events are the Wnt/β-catenin and BMP pathways. The normal expression pattern of *Edar* and *Edaradd* during early tract formation mirrors the pattern of β-catenin pathway activation throughout early tract patterning. Furthermore, direct activation of the β-catenin pathway by forced expression of a truncated form of the β-catenin protein induces the expression of *Edar* and *Edaradd* in the infected cells. Finally, forced expression of BMP2, which

blocks the local activation of the β-catenin pathway, also prevents the local expression of *Edar* and *Edaradd*. All these observations suggest that the initial localization of *Edar* and *Edaradd* expression to the nascent placode is directed by the Wnt/β-catenin pathway partly in response to inhibitory influences of BMPs. However, the expression of both genes persists in the epidermis of the feather bud after the β-catenin pathway is no longer active, so other input is required for the maintenance of expression.

The *Eda* gene has been reported to be a target of Wnt/β catenin signaling based on the activity of a Lef1 binding site in the promoter revealed in co-transfection studies (Durmowicz et al., 2002). The pattern of *Eda* expression in chicken skin is consistent with initial expression in the epidermis as a consequence of β-catenin pathway activation, but *Eda* expression is reduced in the epidermis and extinguished in placodes at times when the β-catenin pathway is still activated. When exogenous BMP expression suppressed bud development, *Eda* expression persisted in the epidermis despite a reduction in β-catenin pathway activation. Forced BMP expression in lateral regions where tract patterning has not begun does not induce *Eda* expression, suggesting that BMP acts by blocking signals that normally repress Eda expression in the epidermal placode. Neither forced activation of the β-catenin pathway, nor forced expression of BMP, appear to directly affect *Eda* expression in the epidermis during the early stages of tract patterning. Where forced activation of the β-catenin pathway results in ectopic feather bud formation, *Eda* is induced in the surrounding mesenchyme, but this is an indirect consequence of bud formation and does not correlate with the pattern of β-catenin pathway activation. At later stages of bud development, the robust expression of *Eda* in the posterior distal mesenchyme correlates well with β-catenin pathway activation. In total, these observations suggest that β-catenin signaling may well play important roles in *Eda* gene regulation at the earliest stages of tract development and later bud morphogenesis, but the expression changes during the early stages of pattern formation in both layers are regulated by other inputs.

Conserved function of Eda signaling in the development of cutaneous appendages

The analysis of *Eda* and *Edar* expression in the developing feather tract suggests that the localized function of this pathway in promoting bud development is downstream of an initial patterning event directed by interaction between the β-catenin and BMP signaling pathways. Although this may reflect a difference in the role of this pathway in mammals and birds, the apparent discrepancies between these conclusions and those based on the interpretation of experiments performed in the mouse can be reconciled in a model of conserved Eda pathway function.

Two results from the mouse studies seem inconsistent with this model. The first is that the local expression of placodal markers is not detected in the analysis of *tabby* (*Eda*) mutant skin, and the second is that local expression of *Edar* is observed in epidermis lacking a functional β-catenin gene while the local expression of other placodal markers was not observed. Together, these observations have been interpreted as evidence that local ectodysplasin signaling is a prerequisite to patterning events directed by β-catenin signaling and/or BMP2 in the skin. However, the interpretation of both experiments is complicated by the fact that the maintenance of the epidermal placode is dependent on its continued interaction with the dermal condensation. Disruption of signaling between the epidermal placode and dermal condensation leads to regression of the placode and extinction of most placode-specific gene expression. Thus analysis of mutant skin may fail to detect initial patterning events because the corresponding gene expression is not maintained in the absence of subsequent events to induce and maintain the dermal condensation. The experiments that conditionally inactivated β-catenin in skin clearly demonstrated that signaling through this pathway is not required to maintain the asymmetric expression of *Edar* (Huelsken et al., 2001). However, the conclusion that β-catenin signaling was blocked prior to pattern formation was based largely on the failure to detect the local expression of other markers at later time points and did not consider the requirement for continued signaling to maintain other placodal gene expression. If, as we propose, β-catenin signaling actually directs the localized expression of *Edar*, the maintenance of localized *Edar* expression in the absence of β-catenin signaling in the mouse is consistent with our observation in the chicken, where β-catenin signaling appears important for the early activation and localization of *Edar* expression, but not for the subsequent maintenance of expression in the placode.

The lack of placodal marker expression in the mutants could also be explained by a failure to maintain placodes after initial specification. Gain-of-function experiments have demonstrated that augmented Eda signaling increases the size of existing placodes, so an abortive placode specified in the absence of Eda might express lower levels of placodal markers that are more difficult to detect. However, it would seem likely that the extensive characterization of these mutants would have detected at least some evidence of an ephemeral placode population predicted by this model. Thus, we favor the alternative explanation that there is a requirement for Eda pathway activity to promote competence to form a placode in the epidermis prior to initial patterning and localization of *Edar* expression to the nascent placode. This requirement is not absolute, as it is ultimately bypassed in time for subsequent waves of follicle development, but may be crucial to achieving competence to make a placode during a crucial period for primary follicle induction. Early Eda signaling may promote the competence to form placodes in the epidermis, and as patterning directs *Edar* expression and Eda signaling preferentially to the forming placode, it could continue to promote that fate in the placodal cells and counteract placode inhibiting signals. The phenotypes of *Eda* overexpression under the control of the *keratin 14* promoter include enlarged follicles but no apparent change in the timing or density of primary follicles formed (Mustonen et al., 2003). However precocious generation of follicles was observed after the primary wave (Mustonen et al., 2003). These phenotypes are all consistent with this model, as precocious Eda signaling would be expected to be permissive but not sufficient for initiation, and the subsequent enlargement of placodes and precocious formation of secondary follicles are both expected of a signal that tips the balance between follicle promoting and inhibiting signals towards the adoption of follicular fates.

The role of the Eda pathway in feather formation remains to be tested. Nevertheless, this examination of the expression and

regulation of the components of this pathway during feather tract development has provided important refinements to the model of Eda pathway function during cutaneous appendage development.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/5/863/DC1

Note added in proof

While this manuscript was in review, a report by Mustonen et al. examining the effects of exogenous Eda concluded that 'Eda-A1 appears to act downstream of the primary inductive signal required for placode initiation during skin patterning' in the mouse (Mustonen et al., 2004). This conclusion is consistent with our analysis of Eda function in feather bud patterning.

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