The American Journal of Pathology, Vol. 169, No. 6, December 2006

DOI: 10.2353/ajpath.2006.060227

Growth Factors, Cytokines, Cell Cycle Molecules

Involvement of the Edar Signaling in the Control of Hair Follicle Involution (Catagen)

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Ectodysplasin (Eda) and its receptor (Edar) are required for normal development of several ectodermal derivatives including hair follicles (HFs). Here, we show that during the murine hair cycle the expression of Eda A1, Edar, Edaradd, and TRAF6 transcripts are minimal in the resting phase and maximal during HF transition from active growth to regression (catagen). Eda A1 mRNA and Edar proteins were expressed in the hair matrix and outer and inner root sheaths of anagen HFs. During catagen, Eda A1 mRNA and Edar protein were expressed in the outer and inner root sheaths and later in the secondary hair germ. Catagen development accompanied by increased apoptosis in the outer root sheath was significantly accelerated in *downless* mice or after treatment of wild-type mice by a fusion protein that inhibits Edar signaling, compared with the corresponding controls. Microarray, real-time polymerase chain reaction, and immunohistochemical analyses of skin of *downless* mice revealed a strong decrease of expression of X-linked inhibitor of apoptosis protein (XIAP), compared with the controls, suggesting XIAP as a target for Edar signaling. Thus, our data demonstrate that in addition to its well-established role in HF morphogenesis, Edar signaling is also involved in hair cycle control and regulates apoptosis in HF keratinocytes during catagen. *(Am J Pathol 2006, 169:2075–2084; DOI: 10.2353/ajpath.2006.060227)*

The hair follicle (HF) is a skin appendage that develops as a result of epithelial-mesenchymal interactions between epidermal keratinocytes committed to hair-specific differentiation and a cluster of dermal fibroblasts that forms the follicular papilla.[1–5] During postnatal life, HFs show patterns of cyclic activity with periods of active growth and hair production (anagen), apoptosis-driven involution (catagen), and relative resting/hair shedding (telogen/exogen).[6–8] HF development and cycling are controlled by similar signaling networks within and between the follicular epithelium and mesenchyme, using the molecules that belong to the bone morphogenetic protein (BMP)/transforming growth factor (TGF)-β, epidermal growth factor, fibroblast growth factor, Hedgehog, insulin-like growth factor, Notch, neurotrophin, tumor necrosis factor, and Wnt families.[5,6,8–12]

Ectodysplasin receptor (Edar) and two other structurally similar receptors Xedar and Troy are members of the tumor necrosis factor receptor superfamily that signals predominantly via the nuclear factor (NF)-κB transcription factors.[13] Edar ligand Eda A1, a product of the Ectodysplasin (*Eda*) gene, differs from the Xedar ligand Eda A2 by the presence of only two additional amino acids in the tumor necrosis factor motif.[14–16] Despite these minor structural differences, Eda A1 and Eda A2 show very high specificity to their corresponding receptors Edar and Xedar.[15,16] Eda A1 binding to Edar results in the recruitment of the auxiliary protein Edaradd, which binds the selected TRAF proteins (TRAF1/2/3 and 5/6).[15,17,18] TAB2 is an adaptator protein that bridges TRAF6 to TAK1 (TGF-β-activated kinase 1), allowing TAK1 activation and subsequent activation of NF-κB transcription factors, their translocation into the nucleus, and modulation of the activity of the corresponding target genes.[18,19]

Defects in the genes that encode proteins of the Edar signaling pathway cause hypohidrotic ectodermal dysplasias in humans and similar conditions in mice.[20] Hypohidrotic ectodermal dysplasia is characterized by severe defects in ectodermal appendage development, including hairs, teeth, and exocrine glands. In mice, hy-

Supported by the National Institutes of Health (grants AR48573 and AR49778 to V.A.B.) and the North-American Hair Research Society (mentorship to M.Y.F. and A.A.S. and research grant to A.A.S.).

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Accepted for publication September 6, 2006.

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Table 1. PCR Primers

cDNA	Forward primer	Reverse primer
Eda A1	5′-ATTCCAGGAACAACTGTTATG-3′	5′-AAGTTGATGTAGTAGACTTC-3′
Edar	5′-ACCAGGAGATGGAAAA-3′	5′-GCTGGATGAGTGTGCTGA-3′
Edaradd	5′-ATTACCACGCAAGAGGTTGG-3′	5′-TCTCCCTGAGGTTGGTCATC-3′
TRAF6	5′-CTTAGCTGCTGGGGTGTCTC-3′	5′-TCTCCAGAGGTGGGTCAAAC-3′
HPRT	5′-CTTTCCCTGGTTAAGCAGTACAG-3′	5′-CATATCCAACAACAAACTTGTCTGG-3′
XIAP	5′-TGGACTCTACTACACAGGTATTGG-3′	5′-AACTCACAGCATCAGATTCACTTC-3′

pohidrotic ectodermal dysplasia is caused by the defects in *Eda* (*Tabby*), *Edar* (*Downless*), *Edarrad* (*Crinkled*), and *TRAF6* genes.[17,21–23] Hair phenotypes seen in these mice include the absence of the guard and zig-zag hairs and presence of two intermediate hair types (awl and auchene), which show lack of normal arrangement of the air cells in the hair medulla.[24,25] In contrast to Edar deficiency, genetic Xedar ablation is not accompanied by any visible skin or HF abnormalities.[26]

A critical role for Edar pathway in the molecular signaling network that regulates HF development is also evident from the facts that gain of Edar signaling leads to partial rescue of the *Tabby* phenotype and also alters cell fate in the epidermis. In particular, it was shown that pharmacological administration of soluble Eda A1-Fc chimeric protein to pregnant *Tabby* mice or overexpression of Eda A1 under control of the CMV promoter on *Tabby* background result in the appearance of guard HFs, tail hairs, and exocrine glands.[27–29] However, neither Eda A1-Fc treatment nor transgenic expression of Eda A1 resulted in the appearance of zig-zag hairs in *Tabby* mice.[27]

Gain of the Eda A1 levels in the epidermis of transgenic mice (promoters: K14 or involucrin) also results in alterations in the HF patterning and formation of fused HFs because of the loss of proper spacing between neighboring HFs.[30,31] Fused HFs are joined together at their permanent portions by the outer epithelial layers, and within a fusion, each individual HF possesses its own hair bulb, dermal papilla, hair shaft, and sebaceous gland.[30,31] In addition, some K14-Eda A1 mice show formation of only curly hairs, whereas others have only straight hairs that resemble guard and awl hairs seen in wild-type mice.[32] In Inv-Eda A1 transgenic mice, guard, awl, and auchene hairs look normal, whereas zig-zag hairs are replaced by curly hairs that show a single column of air cells in the medulla.[31]

However, little is known about the involvement of Edar signaling in the control of postnatal HF cycling. It was shown that in K14-Eda A1 transgenic mice, the first anagen is prolonged,[32] suggesting that Edar signaling could play a role in postnatal hair cycle control in addition to its well-established role in HF morphogenesis. To explore this hypothesis, we demonstrate here that the expression of the components of Edar signaling pathways in mouse skin fluctuate in a hair cycle-dependent manner and reach their maximum during the late anagen-early catagen phases. We also show that genetic loss or pharmacological inhibition of Edar signaling results in significant catagen acceleration. Furthermore, we demonstrate that Edar deletion is accompanied by a significant decrease in the expression of X-linked inhibitor of apoptosis protein (XIAP) in skin and HFs, suggesting XIAP as a target of Edar signaling. Thus, our data demonstrate that Edar signaling interferes with the cyclic regeneration of postnatal HFs at least in part by regulating apoptosis during catagen.

Materials and Methods

Animal Models and Tissue Collection

Eight-week-old C57BL/6J and FVB mice were purchased from Charles River (Wilmington, MA) and housed in the community cages at the Laboratory Animal Science Center of the Boston University School of Medicine. *Downless* (*dl*) mice were kindly provided by Prof. P. Overbeek (Baylor College of Medicine, Houston, TX). All mice were fed water and murine chow *ad libitum* and kept under 12-hour light/dark cycles. Active hair growth (anagen) was induced in the back skin during telogen phase of the hair cycle by depilation as previously described.[33] Skin samples were harvested at distinct hair cycle stages (telogen, anagen II, IV, and VI, catagen II to VI; days 0, 3, 5, 12, 16, and 18 after depilation, respectively; three to five animals per stage). After harvesting, skin samples were processed for biochemical or immunohistochemical studies, as described below. For immunohistochemical studies, the back skin was harvested parallel to the vertebral line and was embedded for subsequently obtaining longitudinal cryosections through the HF.[33]

Microarray Analysis, Semiquantitative and Real-Time Polymerase Chain Reaction (PCR)

RNA was isolated from the snap-frozen skin samples using Trizol reagent (Invitrogen, Carlsbad, CA), followed by DNase I treatment and purification on RNeasy columns (Qiagen, Carlsbad, CA). Two μg of each RNA sample were converted to first strand cDNA using a first strand cDNA synthesis kit (Amersham, Piscataway, NJ). The resulting cDNAs were amplified with Eda A1-, Edar-, Edaradd-, TRAF6-, and HPRT-specific primers (Table 1) using *Taq* polymerase under the following cycling conditions: 94°C for 3 minutes, followed by denaturing at 94°C for 30 seconds, annealing at 56 to 65°C for 15 seconds, and amplification at 72°C for 30 seconds, repeated for 36 to 40 cycles. RNA dilutions without reverse transcription reaction were included as controls. The PCR products

were analyzed by standard agarose gel electrophoresis with ethidium bromide staining. RNA samples from at least two different animals were used for each experimental condition with similar results.

For microarray analysis, 5 μg of RNA isolated from full-thickness *dl* or control FVB mouse skin collected on days 16 to 17 after depilation was converted into ^{32}P-labeled probes using Ampo-Labeling-LPR kit (SuperArray Bioscience, Frederick, MD). The probes were used for microarray analysis using the Mouse Apoptosis GEArrays kit (SuperArray Bioscience, Frederick, MD; *http://www.superarray.com*). The resulting filters were analyzed using Cyclone Phosphor System (Perkin-Elmer Life Sciences, Boston, MA). For real-time reverse transcriptase (RT)-PCR, 1 μg of each RNA sample from *dl* or FVB mouse skin collected on days 16 to 17 after depilation was used as a template for cDNA synthesis using SuperScript III first-strand synthesis system and poly dT primer (InVitrogene, San Diego, CA). PCR primers were designed on Beacon Designer software (Premier Biosoft Int., Palo Alto, CA; Table 1). PCR was performed using the iCycler thermal cycler (Bio-Rad Corp., Hercules, CA), as described previously.[34] Real-time PCR was performed using iQ SYBR green supermix and MyiQ single-color real-time PCR detection system (Bio-Rad Corp.). Differences between samples and controls were calculated using the Gene Expression Macro program (Bio-Rad Corp.) based on the $\Delta\Delta C_t$ equitation method and normalized to the corresponding GAPDH values, as described before.[34]

Western Blot Analysis

Total tissue proteins obtained from full thickness back skin extracts of mice on days 0, 12, and 18 after depilation were collected in lysis buffer, and protein concentrations were determined as described previously.[34] Western analysis was performed with goat polyclonal anti-Edar antiserum (R&D Systems, Minneapolis, MN). For control of specificity, protein lysates from the anagen mouse skin (12 days after depilation) were analyzed with anti-Edar antiserum preincubated with Edar-Fc protein (2 μg/ml; R&D Systems) for 2 hours at 37°C. Horseradish peroxidase-tagged donkey anti-goat IgG was used as secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

In Situ *Hybridization, Immunofluorescence, and Morphological Analyses*

In situ hybridization using Dig-labeled anti-sense riboprobe for Eda spanning nucleotides 161 to 1997 of Eda cDNA (gift of Dr. I. Thesleff, Institute of Biotechnology, University of Helsinki, Finland) was performed as previously described.[35,36] Immunofluorescent detection of Edar, Fc-fragment of human IgG_1, Shh, activated caspase 3, and XIAP was performed using the corresponding primary antisera (R&D Systems, and Jackson ImmunoResearch Laboratories, West Grove, PA) according to the protocols described previously.[37,38] For analyses of apoptotic cells, terminal dUTP nick-end labeling (TUNEL) staining was performed, as described elsewhere.[39–42] For morphological analyses of the HFs, frozen sections of the mouse skin were stained for alkaline phosphatase activity as previously described.[33]

Pharmacological Manipulations in Vivo

To inhibit the Edar signaling in postnatal murine skin, recombinant mouse Edar protein fused with the Fc-fragment of human IgG (Edar-Fc; R&D Systems) or Fc-fragment of human IgG_1 (Fc-IgG) as a control was administered into the skin of 12-week-old C57BL/6J mice. Four μg of Edar-Fc or 2.5 μg of Fc-IgG were injected subcutaneously to each animal once a day starting on days 10 or 14 after depilation and skin samples were collected on days 18 to 19 after depilation. At least three animals of each strain were used for every experimental condition, and the obtained results were essentially identical in all animals.

Histomorphometry and Statistical Analysis

Immunoreactivity patterns were scrutinized by studying at least 50 different HFs per mouse, and five mice were assessed per hair cycle stage. The percentage of HFs at distinct catagen stages was assessed and compared between *dl* and wild-type mice at P14.5 to P17.5, as well as between mice treated by Edar-Fc or Fc-IgG. All evaluations were performed on the basis of accepted morphological criteria of HF classification.[33] Only every 10th cryosection was used for analysis to exclude the repetitive evaluation of the same HF, and two to three cryosections were assessed from each animal. Altogether, 250 to 300 HFs in 50 to 60 microscopic fields, derived from six animals of the experimental group (~40 to 50 follicles per animal) were analyzed and compared with those with corresponding numbers of HFs from the control mice. The number of TUNEL-positive cells was assessed in the hair matrix, outer and inner root sheaths of catagen II to III HFs in *dl* mice, Edar-Fc-treated mice, and corresponding controls as described previously.[41–44] In total, 40 to 50 such measurements were performed in 50 to 60 microscopic fields derived from three animals per experimental and control group. All sections were analyzed at $\times$200 to $\times$400 magnifications, and means and SEM were calculated from the pooled data. Differences were judged as significant if the *P* value was lower than 0.05, as determined by the independent Student's *t*-test for unpaired samples.

Results

Expression of the Components of the Edar Signaling Pathway Is Maximal in Late Anagen-Early Catagen Skin

To obtain the first clues about a possible role of Edar signaling in postnatal hair cycling, we analyzed the expression of the components of the Edar pathway in nor-

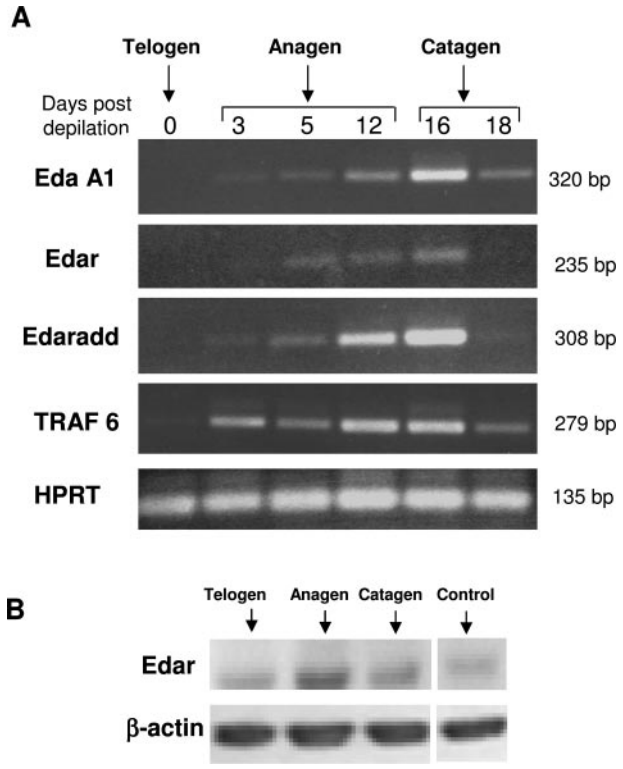

Figure 1. RT-PCR and Western blot analysis of the components of Edar pathway in full thickness mouse back skin during the depilation-induced hair cycle. Total RNA and proteins were isolated from mouse telogen back skin or mouse back skin at different time points (3, 5, 12, 16, and 18 days) of the depilation-induced hair cycle. **A:** RT-PCR. Expression of Eda A1, Edar, Edaradd, and TRAF6 during the depilation-induced hair cycle. HPRT-specific RT-PCR was used as internal control. Representative gels from one of three experiments each are shown. **B:** Western blot analysis of 48-kd Edar protein in telogen, anagen, and catagen skin. Western blot of proteins isolated from anagen skin using anti-Edar antiserum preabsorbed with Edar-Fc protein was performed as a control. Representative gels from one of three experiments each are shown.

mal mouse back skin during the depilation-induced hair cycle by semiquantitative RT-PCR and Western blot analysis. mRNAs encoding ligand Eda A1, its receptor Edar, and auxiliary molecule Edaradd were undetectable in telogen skin, whereas TRAF6 mRNA was expressed at low levels (Figure 1A). By Western blot analysis, Edar protein expression was seen in telogen skin, reached a maximum in late anagen skin, and then decreased during mid-late catagen (Figure 1B). The expression of Eda A1, Edar, Edaradd, and TRAF6 transcripts was increased during anagen development, reaching maximal levels in late anagen-early catagen skin (days 12 to 16 after depilation). However, expression levels of Eda A1, Edar, Edaradd, and TRAF6 transcripts were decreased during mid-catagen (day 18 after depilation), compared with early catagen skin (Figure 1A).

Patterns of expression for Eda mRNA and Edar protein were also examined in normal mouse skin during the depilation-induced hair cycle by *in situ* hybridization and immunofluorescence, respectively (Figure 2). Consistent with the RT-PCR data, Eda transcripts were undetectable in telogen and anagen IV HFs (Figure 2A, data not shown). However, Eda transcripts were strongly expressed in outer and inner root sheaths, as well as in the hair matrix of late anagen and early catagen HFs (days 12 and 17 after depilation, respectively; Figure 2, B and C). During mid-catagen (day 18 after depilation), expression of the Eda mRNA was markedly reduced and was only visible in the secondary hair germ (Figure 2D).

By immunofluorescence, Edar protein was seen in the epidermis as well as in the secondary hair germ of telogen HFs (Figure 2E), which is consistent with the results obtained by Western blot analysis (Figure 1B). Edar expression was detected in the inner root sheath and hair matrix of mid-anagen HFs (data not shown), and was significantly increased in the outer and inner root sheaths of late anagen HFs (Figure 2F). Interestingly, in late anagen HFs Edar expression and co-localization with Shh were seen in the unilateral cluster of hair matrix keratinocytes (Figure 2F, inset). In early catagen HFs, Edar protein was strongly expressed in the inner and outer root sheaths, whereas its expression disappeared from the hair matrix (Figure 2G). In late catagen HFs, Edar expression was seen in the secondary hair germ, whereas cells in the epithelial strand showed only weak expression (Figure 2H). Thus, these expression patterns suggested the involvement of Edar signaling in regulation of the hair shaft formation, as well as in the control of anagen-catagen transition.

Downless Mice Are Characterized by Acceleration of Catagen and Increased Apoptosis in the HF

To explore a role for Edar signaling in the control of catagen development, we compared the dynamics of catagen between *dl* and wild-type mice on days 15 to 18 after depilation, as described previously.[43] After hair cycle induction by depilation, *dl* mice showed apparently normal anagen development and formation of new hairs (data not shown). However, in contrast to wild-type mice, *dl* mice showed accelerated entry into catagen already visible by day 15 after depilation, as well as significantly ($P < 0.05$) accelerated catagen progression (Figure 3, A–C). On day 17 after depilation, most of the HFs in *dl* mice had reached mid- to late catagen stages, whereas the majority of HFs in wild-type mice were still at the beginning of catagen (Figure 3, A–C). On days 19 to 20 after depilation, the majority of the HFs in *dl* mice were already in telogen, whereas in wild-type mice most HFs were still in late catagen (data not shown).

Consistent with histomorphometry results, *dl* mice were also characterized by a significant ($P < 0.01$) increase in TUNEL-positive cells in the outer root sheath of catagen II to III HFs, whereas the number of TUNEL-positive cells in the inner root sheath and hair matrix was not changed compared with wild-type mice (Figure 3, D–F). Furthermore, expression of activated (cleaved) caspase 3 was increased in the follicular outer root sheath of catagen III HFs of *dl* mice compared with wild-type controls (Figure 3, G and H). These data suggested that Edar signaling may control catagen development at least in part by regulating apoptosis in the outer root sheath.

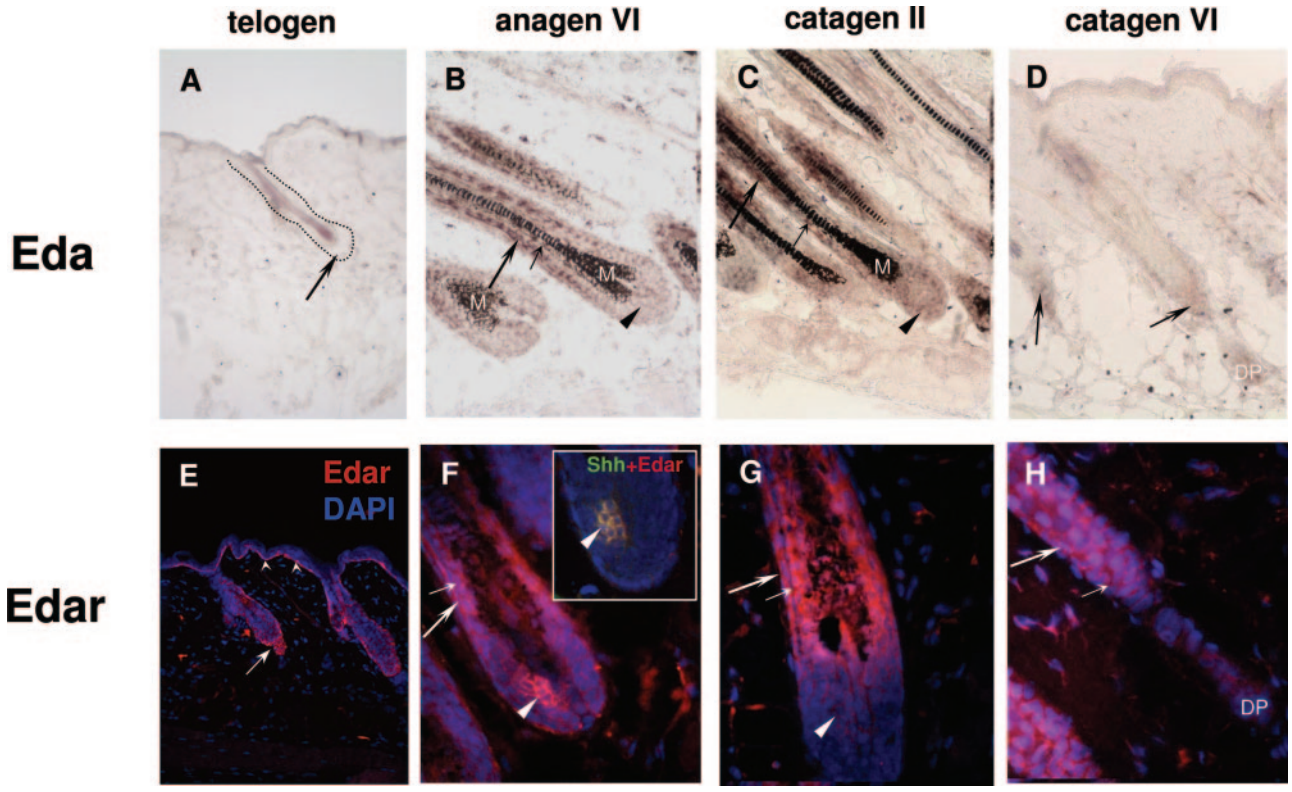

Figure 2. Expression of Eda mRNA and Edar protein in the HFs during the hair cycle. Cryosections of the dorsal skin of 10-week-old C57BL/6 mice harvested before the depilation (telogen) or 8 to 18 days after depilation (days 8 to 12, late anagen; days 17 and 18, early and mid/late catagen, respectively) were processed for visualization of the Eda mRNA by *in situ* hybridization (**A–D**) and for detection of the Edar protein by immunofluorescence (**E–H**). Cryosections were counterstained by DAPI (**E–H**, blue fluorescence). **A:** No specific signal (**arrow**) is seen in the telogen skin. HF is indicated by dotted line. **B** and **C:** Eda mRNA is seen in the outer root sheath (**large arrows**), inner root sheath (**small arrows**), and in the hair matrix (**arrowheads**) of late anagen (**B**) and early catagen HFs (**C**). **D:** Weak Eda mRNA expression is seen in the secondary hair germ of catagen VI HFs (**arrows**). **E:** Expression of the Edar protein in the secondary hair germ of the HF (**arrow**) and in the basal epidermal layer (**arrowheads**) of telogen skin. **F:** Edar expression in the outer root sheath (**large arrow**), inner root sheath (**small arrow**), and unilateral cluster of hair matrix cells (**arrowhead**) of anagen VI HF. Co-localization of Edar and Shh in the cluster of matrix cells (**inset**, **arrowhead**). **G:** Increase of Edar expression in the outer root sheath (**large arrow**) and inner root sheath (**small arrow**) and decrease of expression in the hair matrix (**arrowhead**) of catagen II HF. **H:** Edar expression in the outer root sheath (**large arrow**) and secondary hair germ (**small arrow**) of catagen VI HF. DP, dermal papilla; M, melanocytes.

Pharmacological Inhibition of Edar Signaling Causes Acceleration of Catagen

To further explore involvement of Edar signaling in the control of catagen and HF apoptosis, we tested the effects of recombinant mouse Edar protein fused with the Fc-fragment of human IgG (Edar-Fc), which inhibits Edar signaling, on the dynamics of catagen in C57BL/6 mice. Animals were treated with Edar-Fc, or Fc-IgG as a control, from days 10 or 14 to day 17 after depilation (ie, at time points when catagen is normally developed), and skin was harvested at days 18 to 19 (Figure 4A). Distribution of the mEdar-hFc protein was monitored by immunostaining for human IgG_1, which revealed positive staining of the HFs, suggesting Edar-Fc penetration into the HF epithelium (Figure 4, B and C). Histomorphometric analysis revealed that Edar-Fc administration into anagen skin (starting from day 10 after depilation) did not result in the premature entry of anagen VI HFs into catagen (data not shown). However, Edar-Fc treatment on days 14 to 17 after depilation caused a significant increase in number of HFs in more advanced catagen stages ($P < 0.05$) compared with the control (Figure 4, D–F). Although differences in catagen progression between Edar-Fc-treated and control mice were less prominent compared with that seen between *dl* and wild-type mice (Figure 3, A–C), in striking similarity to *dl* mice, Edar-Fc treatment resulted in the marked increase ($P < 0.01$) of TUNEL-positive cells in the outer root sheath compared with the control, whereas the number of TUNEL-positive cells in the inner root sheath and hair matrix remained unchanged (Figure 4, G–I). Thus, pharmacological blockade of Edar signaling in wild-type mice resulted in phenotype strikingly similar to *dl* mice, further supporting a role for Edar signaling in the control of physiological HF involution and apoptosis.

Edar Deficiency Is Accompanied by Decreased Expression of XIAP in the HF

To define mechanisms of Edar involvement in the control of HF apoptosis, expression of genes implicated in apoptosis regulation was compared between full-thickness skin of *dl* and wild-type mice on day 16 after depilation using the Mouse Apoptosis Array kit. Among 88 apoptotic genes, the most striking difference in expression of the X-linked inhibitor of apoptosis protein (XIAP) was de-

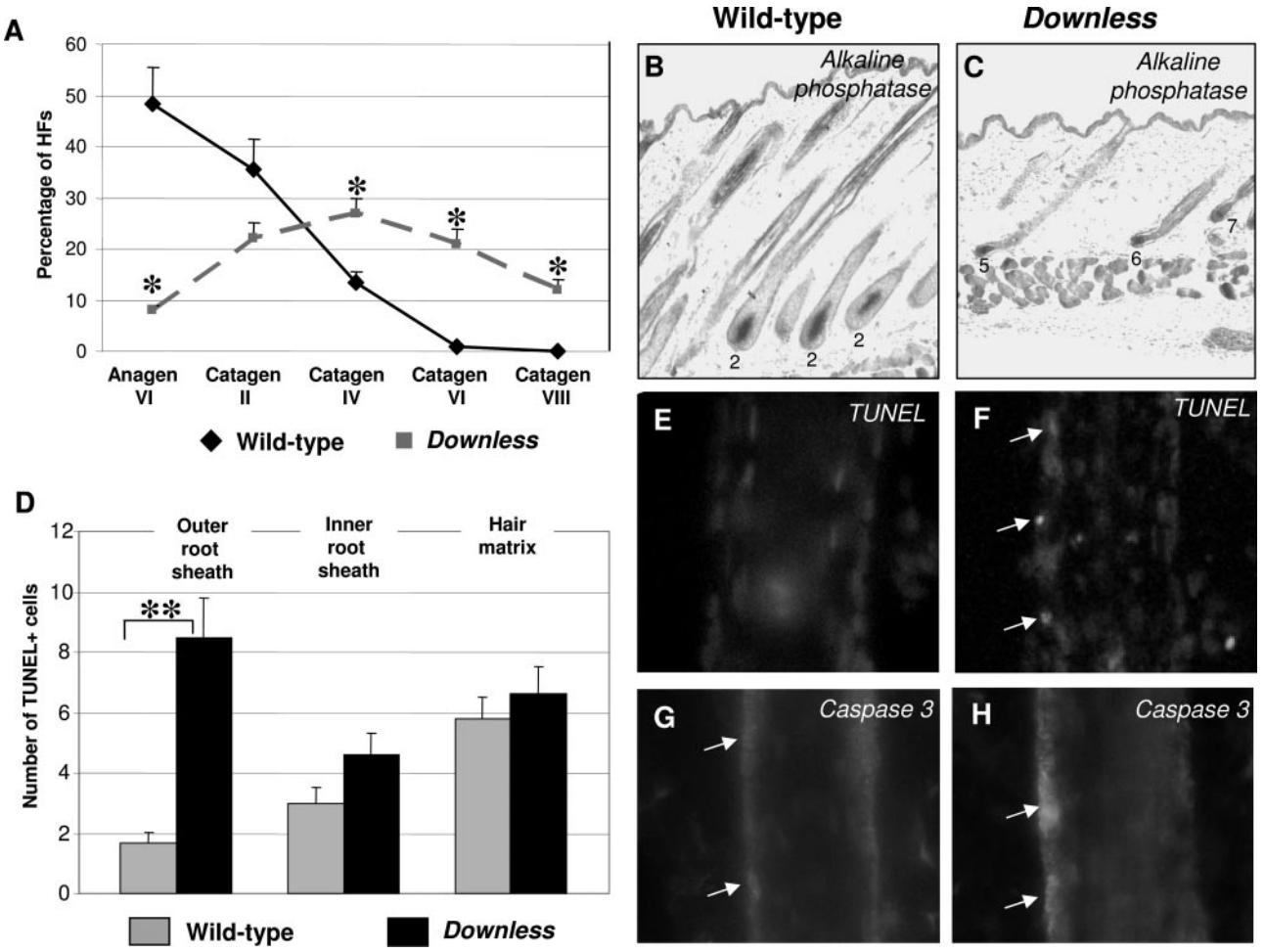

Figure 3. Catagen acceleration in *dl* mice. Hair cycle was induced in the back skin of 8- to 10-week-old FVB or *dl* mice by depilation, and skin was collected on day 17 after depilation. Sections were stained for the detection of endogenous alkaline phosphatase activity to visualize catagen stages (**A–C**) or processed for immunodetection of TUNEL (**D–F**) and activated caspase 3 (**G**, **H**). Number of TUNEL-positive cells was assessed in the hair matrix and outer and inner root sheaths of catagen II to III HFs in *dl* and wild-type mice ($n = 40$ to 50 HFs per each mouse strain). **A:** Significant acceleration of catagen development in *dl* mice compared with control. Student's *t*-test, $^{*}P < 0.05$. **B** and **C:** HFs in *dl* mice show more advanced catagen stages (**C**) compared with the control HFs (**B**). Distinct catagen stages are shown by Arabic numbers. **D:** Increase of TUNEL-positive cells in the HF outer root sheath of *dl* mice compared with the control HFs. Student's *t*-test, $^{**}P < 0.01$. **E** and **F:** HFs in *dl* mice contain numerous TUNEL-positive cells in the outer sheath (**F**, **arrows**), whereas control HFs do not show TUNEL-positive cells (**E**). **G** and **H:** HFs in *dl* mice show increased expression of caspase 3 in the outer root sheath (**H**, **arrows**) compared with wild-type mice (**G**, **arrows**).

tected between skin of *dl* and wild-type mice. XIAP is a member of the inhibitors of apoptosis protein (IAP) family and is also described as a target for NF-κB signaling.[45,46] By real-time PCR, expression of XIAP transcripts showed more than a fivefold decrease in skin of *dl* mice compared with wild-type mice, on days 16 to 17 after depilation (Figure 5A). By immunohistochemistry, expression of XIAP was strongly decreased in the hair matrix and outer root sheath of catagen II HFs in *dl* mice while remaining strong in the follicular papilla compared with wild-type mice (Figure 5, B and C). These data suggested that Edar signaling may regulate catagen development, at least in part, via controlling XIAP expression in the HF keratinocytes.

Discussion

Data obtained within the last decade reveal the essential roles for Edar signaling in the control of cell fate decision in embryonic epidermis and in the regulation of cell differentiation during HF morphogenesis.[13] Here, we show that Edar signaling also plays important roles in the control of HF cycling in postnatal skin. 1) Expression of the components of Edar signaling pathways in mouse skin fluctuates in a hair cycle-dependent manner, reaching maximum during the late anagen/early catagen phase (Figures 1 and 2). 2) Constitutive deletion or pharmacological inhibition of Edar signaling results in catagen acceleration and increased apoptosis in the HF compared with the corresponding controls (Figures 3 and 4). 3) Catagen acceleration in *dl* mice is accompanied by strong down-regulation of XIAP expression in the HF epithelium, suggesting that Edar signaling may control apoptosis in HF keratinocytes, at least in part, via positive regulation of XIAP expression (Figure 5).

Our data show that expressions of Eda A1, Edar, and two downstream components of this signaling pathway (Edaradd, TRAF6) are minimal in telogen skin, while

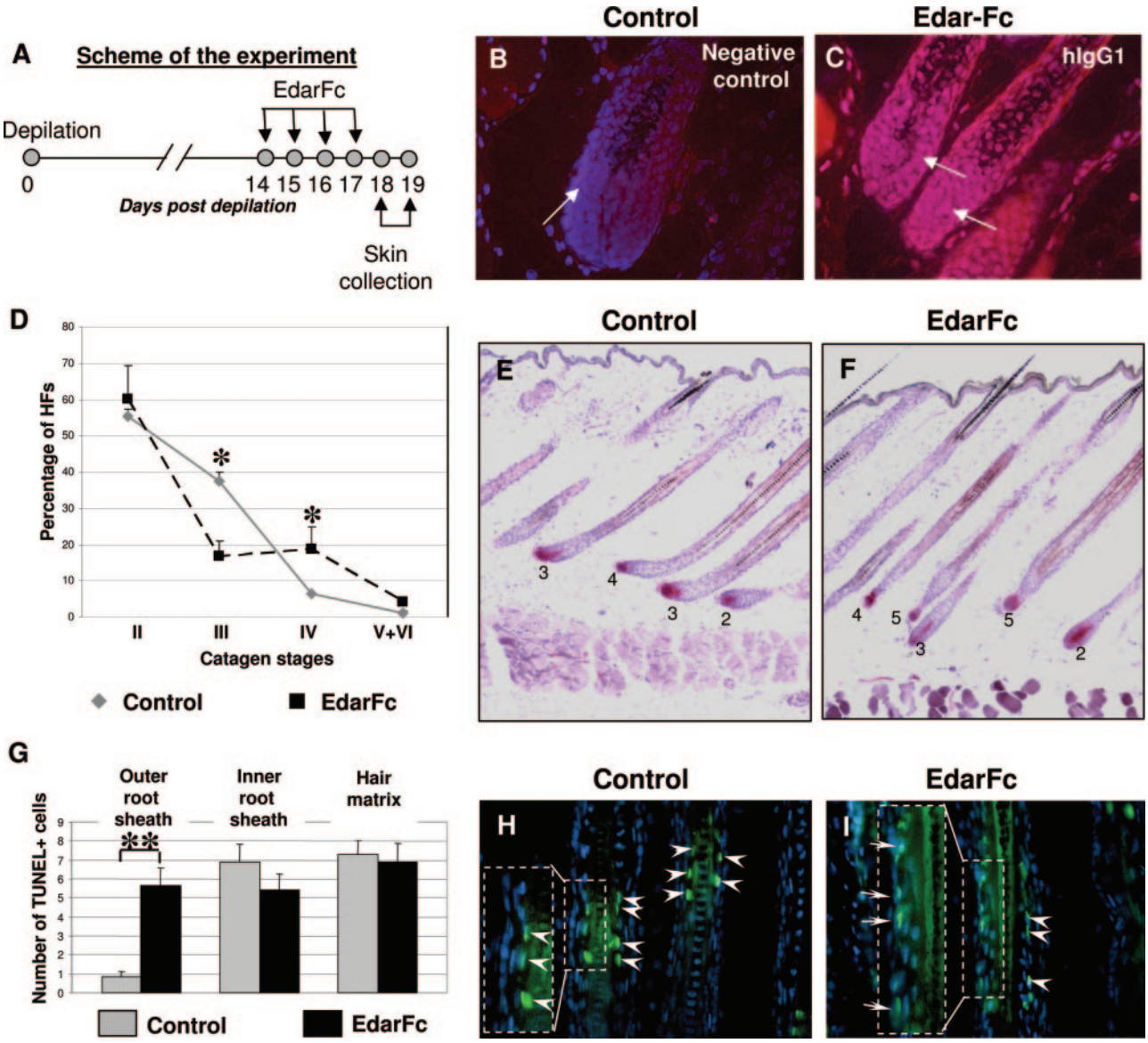

Figure 4. Pharmacological inhibition of the Edar signaling results in catagen acceleration. Hair cycle was induced in the back skin of 10-week-old C57BL/6J mice by depilation, and Edar-Fc or Fc-IgG (as control) were injected subcutaneously once a day starting on day 14 after depilation and skin samples were collected on days 18 to 19 after depilation. Sections were stained for the detection of endogenous alkaline phosphatase activity to visualize the catagen stages (**D–F**) or processed for immunodetection of TUNEL (**G–I**). Number of TUNEL-positive cells was assessed in the hair matrix and outer and inner root sheaths of catagen II to III HFs in Edar-Fc-treated mice and corresponding controls (n = 40 to 50 HFs per experimental or control group). **A:** Scheme of the experiment. **B** and **C:** Human IgG_1. Red fluorescence is seen in the HFs of Edar-Fc-treated mice (**C**, **arrows**) and suggests Edar-Fc penetration into the HFs. Lack of fluorescence in the control skin stained only with secondary antibody (**B**, **arrow**). **D:** Significant acceleration of catagen development in mice treated by Edar-Fc compared with control mice. Student's t-test, $^{*}P < 0.05$. **E** and **F:** HFs in mice treated by Edar-Fc show more advanced catagen stages (**F**) compared with the control HFs (**E**). Distinct catagen stages are shown by Arabic numbers. **G:** Increase of TUNEL-positive cells in the HF outer root sheath of mice treated by Edar-Fc compared with the control HFs. Student's t-test, $^{**}P < 0.01$. **H** and **I:** In the HFs treated by Edar-Fc, numerous TUNEL-positive cells are seen in the outer and inner root sheaths (**I**, **arrows** and **arrowheads**, respectively). Control HFs show TUNEL-positive cells only in the inner root sheath (**H**, **arrowheads**). Insets show high magnifications of the labeled areas.

strongly increased during late anagen/early catagen (Figures 1 and 2). Although Edar protein is expressed in the secondary hair germ of telogen HFs (Figure 2), the lack of expression of Eda A1, Edaradd, and TRAF6 transcripts in telogen skin raises the question of whether Edar signaling may indeed contribute significantly to the process of anagen initiation. It is well accepted that HF initiation in embryos and onset of the HF growth phase in postnatal skin are controlled by similar mechanisms.[3–6,9] Because Edar signaling is required for the initiation of guard and zig-zag HFs,[22,47] it is logical to suppose that anagen onset in these HF types may also be regulated by Edar. However, guard and zig-zag HFs are missing in *dl* mice, suggesting that a conditional knockout mouse model is required as a tool to fully understand a role of Edar signaling in controlling the anagen onset in these HF types.

In late anagen HFs, Edar is expressed in the unilateral cluster of hair matrix cells and in differentiating keratinocytes of the hair shaft and inner root sheath, whereas Eda

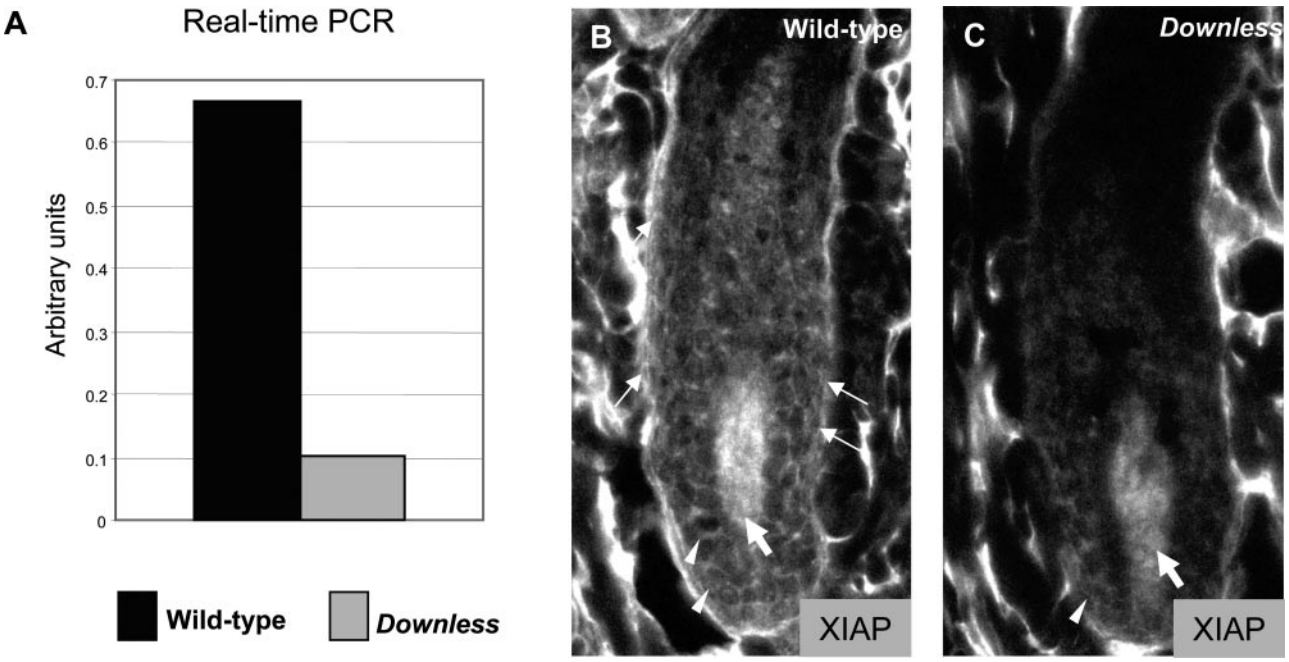

Figure 5. Decreased expression of XIAP in skin and HFs of *dl* mice. **A:** Total RNA from back skin of *dl* and control mice on days 16 and 17 after depilation were analyzed for XIAP mRNA expression by real-time PCR. **B** and **C:** Expression of XIAP protein was assessed by immunohistochemistry. **A:** XIAP mRNA level is significantly reduced in back skin of *dl* mice on days 16 to 17 after depilation compared with control skin. Expression levels were normalized to the corresponding levels of GAPDH transcripts. **B** and **C:** Decrease of XIAP expression in the outer root sheath (**small arrows**) and hair matrix (**arrowheads**) of catagen II HFs in *dl* mice (**C**) compared with control (**B**). XIAP expression in the follicular papilla (**large arrows**) does not show differences between *dl* and control mice.

transcripts are seen over the entire cyclic portion of the HF epithelium (Figure 2). Interestingly, Edar and Shh are co-expressed in the unilateral cluster of hair matrix cells (Figure 2). This expression pattern for Shh suggests its involvement in the control of cell proliferation in the hair matrix.[36,48–50] Given that Edar-mediated NF-κB signaling is required for Shh expression during early stages of HF development,[47] our data suggest a possible link between these signaling pathways in regulation of proliferation and differentiation of hair matrix keratinocytes.

Data obtained from transgenic mice overexpressing Eda A1 under control of K14 or Involucrin promoters show that Edar signaling is indeed required for hair shaft formation: in both transgenic strains, gain of Edar signaling results in an increase in hair fiber thickness and in alterations of hair shape.[31,32] In *Tabby* mice, which contain only abnormal awl and auchene HFs, hair fibers show variable defects in number and arrangement of columns of air cells in the medulla.[51] These observations strongly support the idea that the timing and magnitude of Edar signaling are important for proper control of cell differentiation in the HF and for hair shaft formation.

We also show here that Edar is expressed in the HF outer and inner root sheath during spontaneous HF involution (catagen) (Figure 2) and that genetic ablation or pharmacological inhibition of the Edar signaling results in catagen acceleration (Figures 3 and 4). These data are consistent with the findings demonstrating catagen retardation in K14-Eda A1 transgenic mice[32] and reveal a previously undiscovered role for this pathway in the regulation of apoptosis during catagen.

Indeed, catagen acceleration in *dl* mice or after Edar-Fc treatment is accompanied by a significant increase in the number of TUNEL-positive cells and the expression of activated caspase 3 in the outer root sheath, whereas the number of TUNEL-positive cells in the inner root sheath is unchanged compared with the corresponding controls (Figures 3 and 4). Our data demonstrate that Edar serves as an important signaling pathway inhibiting apoptosis in the outer root sheath and provide evidence that this inhibitory activity may, at least in part, be mediated by XIAP (Figure 5).

Apoptosis in distinct HF cell populations is controlled by different mechanisms.[7,52] During catagen, outer root sheath keratinocytes express a number of death-domain receptors, as well as TGF-β receptor type II, and data obtained from genetically engineered mice reveal that neurotrophins and TGF-β1 promote apoptosis in the outer root sheath.[39,43,53–55] However, signaling pathways that contribute to inhibition of apoptosis in the outer root sheath are not well defined.[52]

XIAP belongs to the family of inhibitors of apoptosis proteins (IAP) that are located in the cytosol and prevent activation of procaspases, thus suppressing the stage of intracellular response of apoptosis.[45,52] In wild-type mice, XIAP is expressed in the hair matrix, outer root sheath, and follicular papilla of early catagen HFs, and its expression is decreased in the HF keratinocytes of *dl* mice while remaining strong in the follicular papilla (Figure 5). Because Edar is also expressed in the hair matrix and outer root sheath keratinocytes of early catagen HFs (Figure 2), it could be suggested that Edar signaling may control XIAP expression in the HF epithelium during catagen. This hypothesis is supported by data describing XIAP as a target for NF-κB signaling that inhibits apopto-

sis in a number of epithelial and nonepithelial cells.[45,46,56]

XIAP serves as an important component of the BMP-MAPK pathway,[57] and cross-talk between BMP and Edar signaling pathways has been recently demonstrated in embryonic skin.[58,59] Recent data also demonstrate XIAP involvement in inhibiting TGF-β-mediated apoptosis via polyubiquitination of TAK1 kinase, which links TGF-β signaling with JNK kinase.[46] TGF-β signaling is important for promoting apoptosis during catagen in murine and human HFs, and mutant mice with deletion of TGF-β1 show decreased levels of apoptosis in the outer root sheath and catagen retardation.[55,60–62] It remains to be determined, however, whether cross-talk between BMP/TGF-β and Edar signaling pathways indeed takes place in keratinocytes of postnatal HFs and whether Edar signaling may inhibit catagen-promoting effects of TGF-β via upregulation of XIAP.

Taken together, our data reveal that in addition to its established role in the control of HF development, Edar signaling is also required for the proper control of postnatal HF cycling. By regulating the apoptosis in keratinocytes during catagen, Edar signaling plays important roles in the cyclic regeneration of postnatal HFs. These data also suggest a potential application of Edar agonists/antagonists for the pharmacological correction of hair growth abnormalities seen in clinical practice and cosmetology.[11,63]

Acknowledgments

We thank Prof. B.A. Gilchrest for her continuous support, Dr. P. Overbeek and Dr. I. Thesleff for providing *dl* mice and reagents for this study, and B. Shander for critical reading the manuscript.

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