# Desmogleins 1 and 3 in the Companion Layer Anchor Mouse Anagen Hair to the Follicle

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**Anchorage of the hair to its follicle is of paramount importance for survival of rodents in the wild, and is aberrant in some human alopecias. Little is understood about the mechanisms responsible for hair shaft anchorage. Desmoglein (Dsg)3−/− (knockout) mice lose hair during telogen, but their anagen hairs remain anchored to the follicle. We hypothesized that Dsg1 compensates for the loss of Dsg3 in the anagen hair follicles of these Dsg3−/− mice. Consistent with this hypothesis, we found Dsg1 and Dsg3 expression overlapping in the companion layer. To functionally address this hypothesis, we used exfoliative toxin A (ETA) to inactivate Dsg1 in Dsg3−/− mice. Four hours after injection of ETA, Dsg3−/− mice, but not Dsg3+/+ or Dsg3+/− mice, showed striking loss of anagen hair, which was confirmed and quantitated by gentle tape stripping. Histology of the skin of these mice as well as of the tape-stripped hair showed separation between the outer root sheath and inner root sheath of the hair follicle, at the plane of the companion layer. Immunostaining for trichohyalin and K6, which highlights the companion layer, in skin and stripped hair confirmed the plane of separation. Labeling of proliferating cells with bromodeoxyuridine demonstrated that the matrix keratinocytes responsible for producing the hair shaft were below the split and remained in the follicle after loss of the anagen hair. These findings demonstrate the importance of the companion layer, and particularly the Dsg1 and Dsg3 in this layer, in anchoring the anagen hair to the follicle.**

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Little is understood about the mechanisms responsible for hair anchorage during the different stages of the hair follicle cycle when follicles undergo complex changes in their morphology. During the resting stage, telogen, the hair shaft remains stationary and anchored to the follicle, which is at its shortest length (Cotsarelis and Millar, 2001; Milner *et al*, 2002). The normal growing anagen hair follicle penetrates deeply into the subcutaneous fat and consists of concentric cylinders of cells surrounding a central hair shaft. The hair shaft and surrounding inner root sheath (IRS) and companion layer glide through the skin as they grow, yet remain firmly anchored to the follicle (Orwin, 1971). Cells within the companion layer attach to the IRS centrally and to the outer root sheath (ORS) peripherally, but the companion layer cells migrate upward as the adjacent ORS cells remain stationary. The molecules responsible for the dynamic adhesion within this slippage plane during anagen are poorly understood.

In the epidermis, desmoglein (Dsg)1 and Dsg3 are critical for maintaining the structural integrity of the epidermis through cell–cell adhesion. Dsg3 is expressed in the basal layer and Dsg1 is expressed in the suprabasal layer of the epidermis. Dsg3 is an autoantigen in pemphigus vulgaris and Dsg1 is an autoantigen in pemphigus foliaceus (Udey and Stanley, 1999). Antibodies in patient serum of pemphigus vulgaris and pemphigus foliaceus attack Dsg, and cause blister formation in the epidermis. In bullous impetigo and staphylococcus scalded skin syndrome, exfoliative toxin A (ETA), a serine protease produced by *Staphylococcus aureus*, specifically attacks Dsg1and causes blister formation in the superficial layer of the epidermis (Amagai *et al*, 2000, 2002; Hanakawa *et al*, 2002b; Yamaguchi *et al*, 2002). In areas where both Dsg1 and Dsg3 are expressed, one Dsg1 can compensate for loss of the other, and prevent loss of adhesion, an explanation for the pathophysiology of pemphigus that has been termed the desmoglein compensation hypothesis (Mahoney *et al*, 1999). This hypothesis also explains the blister localization in bullous impetigo and staphylococcal scalded skin syndrome (Mahoney *et al*, 1999; Amagai *et al*, 2000). For example, in pemphigus foliaceus and bullous impetigo/staphylococcal scalded skin syndrome Dsg1 is inactivated by an autoantibody or protease, respectively. The blister localizes in the superficial epidermis only, where Dsg1 is present without any Dsg3. In the deep epidermis and in mucous membrane, however, Dsg3 is also present, preventing blister formation.

The desmoglein compensation hypothesis can also explain the anchorage of mouse telogen hair in the follicle. Dsg3, without Dsg1, is present in the club where the telogen hair is anchored to the follicle (Koch *et al*, 1998). In Dsg3−/− mice, the telogen hair falls out due to loss of this anchorage. Further validating this hypothesis, the transgenic expression of Dsg1 in this area protects against hair loss in Dsg3−/−

Abbreviations: BrdU, bromodeoxyuridine; Dsg, desmoglein; ETA, exfoliative toxin A; IRS, inner root sheath; ORS, outer root sheath

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mice(Hanakawa *et al*, 2002a). These data show that Dsg3 is critical in anchoring normal telogen hair in the follicle. These Dsg3−/− mice however, only lose telogen, not anagen hair. They first lose hair at 18–20 d after birth, in a "wave" from head to tail as the hair follicles synchronously enter telogen. When the hair follicle re-enters anagen, the hair re-grows and remains firmly anchored until the next telogen stage (Koch *et al*, 1998).

In this study, we examined the dependence of anagen hair anchorage on Dsg3 and Dsg1. We showed that if Dsg1 is inactivated by ETA in Dsg3−/− mice (i.e., both Dsg3 and Dsg1 do not function), then anagen hair lost its anchorage. The loss of anchorage occurred in the companion layer, showing a critical role for desmogleins in anagen hair anchorage and the importance of the companion layer in maintaining the anagen hair in the follicle.

## Results

**Expression of Dsg1 and Dsg3 in normal anagen hair follicles colocalizes in the companion layer** By immunofluorescence staining, Dsg1 was expressed in the ORS, companion layer, and IRS (Fig 1*A*, *C*, *E*). Dsg3 was present in the companion layer, medulla, precortical cells, and matrix cells (Fig 1*B*, *D*, *F*). Thus, Dsg1 and Dsg3 expression overlapped in the companion layer.

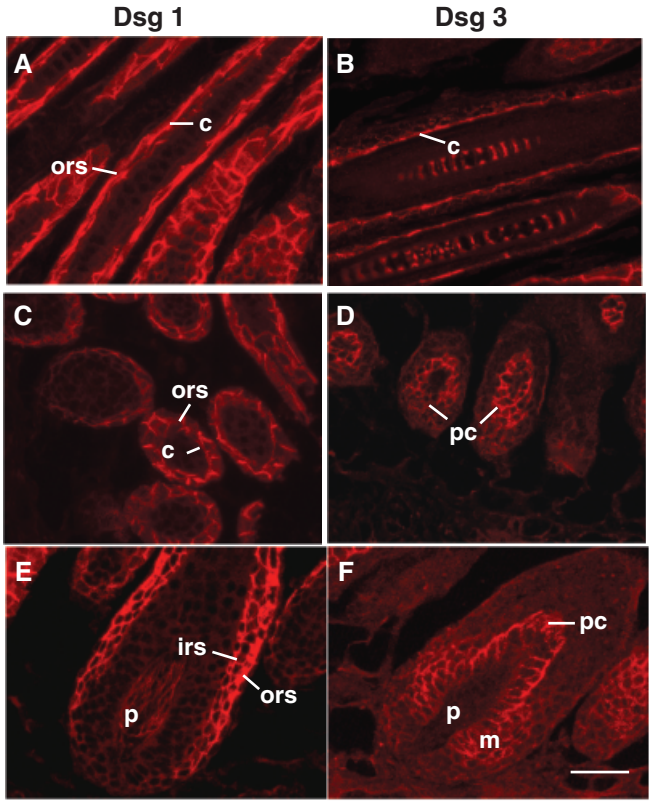



**Figure 1**
**Companion layer expresses desmoglein (Dsg)1 and Dsg3 in normal anagen hair follicles.** Longitudinal (*A*, *E*) and transverse (*C*, *D*) sections through the suprabulbar (*A*–*D*) and bulbar (*E*, *F*) portions of anagen hair follicles immunostained with antibodies against Dsg1 and Dsg3. Dsg1 was present in the outer root sheath (ORS), inner root sheath (IRS) and companion layer (c). Dsg3 was detected in the precortical cells (PC) and companion layer (*B*, *D*, *F*). The dermal papilla (p) stains weakly for DSG1 and not for DSG3 (*scale bar* = 50 μm).

**Inactivation of Dsg1 by injection of ETA causes loss of anagen hair in Dsg3−/− mice** To investigate the role of Dsg1 and Dsg3 in anchoring anagen hair, we injected ETA, which specifically cleaves Dsg1, into 13-d-old Dsg3−/− mice. Four hours after injection, mice were subjected to an adhesive tape test (Koch *et al*, 1998). Dsg3−/− mice injected with ETA easily lost hair, but Dsg3+/+ or Dsg3+/− mice injected with ETA did not show hair loss (Fig 2*A*). Dsg3−/− or Dsg3+/− mice injected with phosphate-buffered saline (PBS) also did not show hair loss. To semiquantitate the hair loss, the plucked hairs were weighed. Dsg3−/− mice injected with ETA showed a mean of 3.3 mg of hair loss (N = 15) compared to control mice (N = 56), which did not lose hair (Fig 2*B*). Control mice included wild type and Dsg3+/− mice injected with PBS or ETA, and Dsg3−/− mice injected with PBS. To exclude the possibility of a contaminant in the ETA preparation causing the hair loss, we injected ETAS195A, an inactive form of ETA with the enzymatically active serine mutated to alanine, into Dsg3−/− mice, but this did not result in hair loss (data not shown).

**Dsg3−/− mice treated with ETA develop clefts between the ORS and IRS** Histological examination of skin from Dsg3−/− mice treated with ETA showed cleft formation between the ORS and IRS of the hair follicle (Fig 3*B*, *D*), whereas control mouse skin (PBS injected Dsg3+/+ mouse) did not (Fig 3*A*, *C*). Clefts were only evident in skin fixed with 70% ethanol as opposed to formalin. The cleavage plane was likely made apparent due to the shrinkage of tissue caused by dehydration from ethanol fixation.

Histology of depilated skin from Dsg3−/− mice treated with ETA showed ORS cells continuous with the basal cell layer of the epidermis, as well as intact matrix and dermal papilla cells (Fig 3*F*, *H*). Dsg3+/− and Dsg3+/+ mice treated with ETA (Fig 3*E*, *G*) showed blister formation around the granular layer of the epidermis, as previously reported (Amagai *et al*, 2000), but did not show any significant changes in the hair follicle.

**Special stains and immunohistochemical staining confirm the level of the split as between the companion layer and ORS** Toluidine blue staining of the depilated hair showed a pale smooth surface, typical of the companion layer, surrounding the densely stained IRS in the upper hair shaft. A fractured area was present at the base of the plucked hair (Fig 4*A*). Transverse cryosections confirmed the presence of the hair shaft, IRS, and companion layer (Fig 4*B*). These results showed that the anagen hair separated from the follicle between the companion layer and ORS.

To confirm the level of the split, we performed double immunofluorescence staining of plucked hair using antibodies AE13, AE15, and anti-K6. AE13 marks hard keratins, which are found in the hair shaft (Lynch *et al*, 1986). AE15 recognizes trichohyalin, a marker for the IRS and medulla (O'Guin *et al*, 1992). Anti-K6 stains the companion layer, the medulla of the hair shaft, and the medulla precursors within the matrix (Wang *et al*, 2003). Double staining of plucked hairs with AE13 and anti-K6 antibodies highlighted the AE13-positive hair shaft in the center, and the peripheral

and central staining of anti-K6 in the companion layer and medulla (Fig 4*C–E*). Double staining with AE15 and anti-K6 of plucked hair demonstrated AE15 positive cells in the IRS adjacent to peripheral staining of the companion layer with antibody against K6, as well as positive anti-K6 staining of the medulla (Fig 4*F–H*).

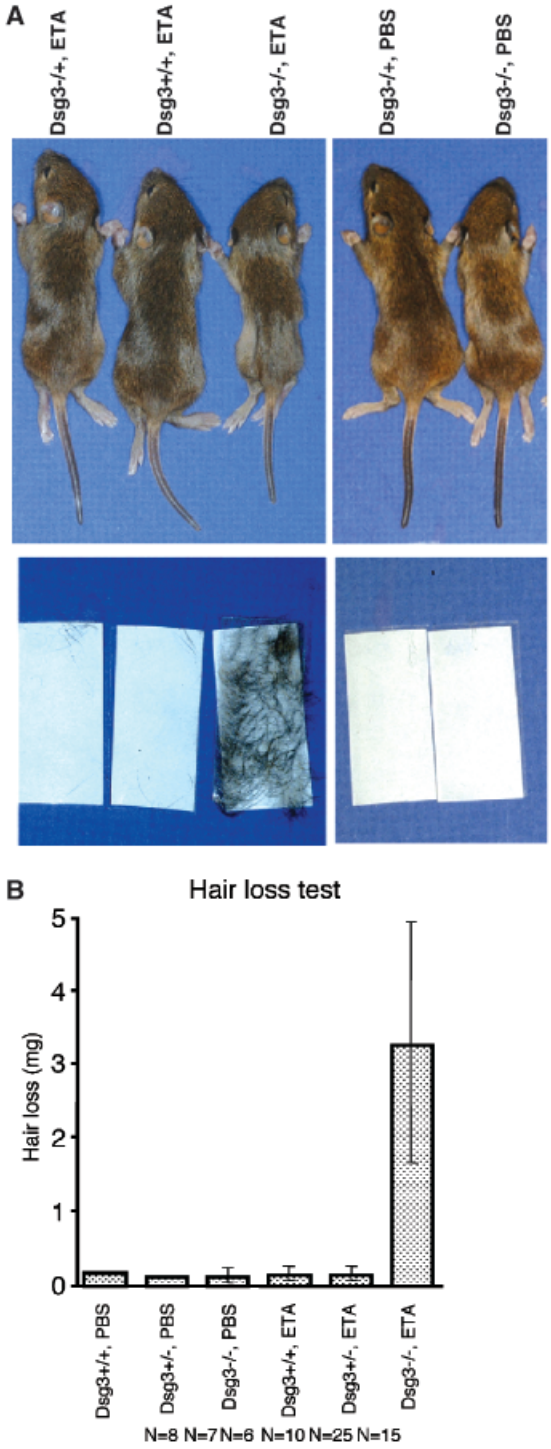



**Figure 2**
**Desmoglein (Dsg)3−/− mice lose hair after injection with exfoliative toxin A (ETA) during anagen.** (*A*) Mice as labeled were injected with ETA or phosphate-buffered saline (PBS), and 4 h later were subjected to an adhesive tape test. Only Dsg3−/− mice treated with ETA showed hair loss (seen on tape in bottom row). (*B*) Semiquantitation of hair loss on adhesive tape by weight. Dsg3−/− mice treated with ETA showed statistically significant loss of hair compared to Dsg3+/− mice treated with ETA ($p < 0.01$).

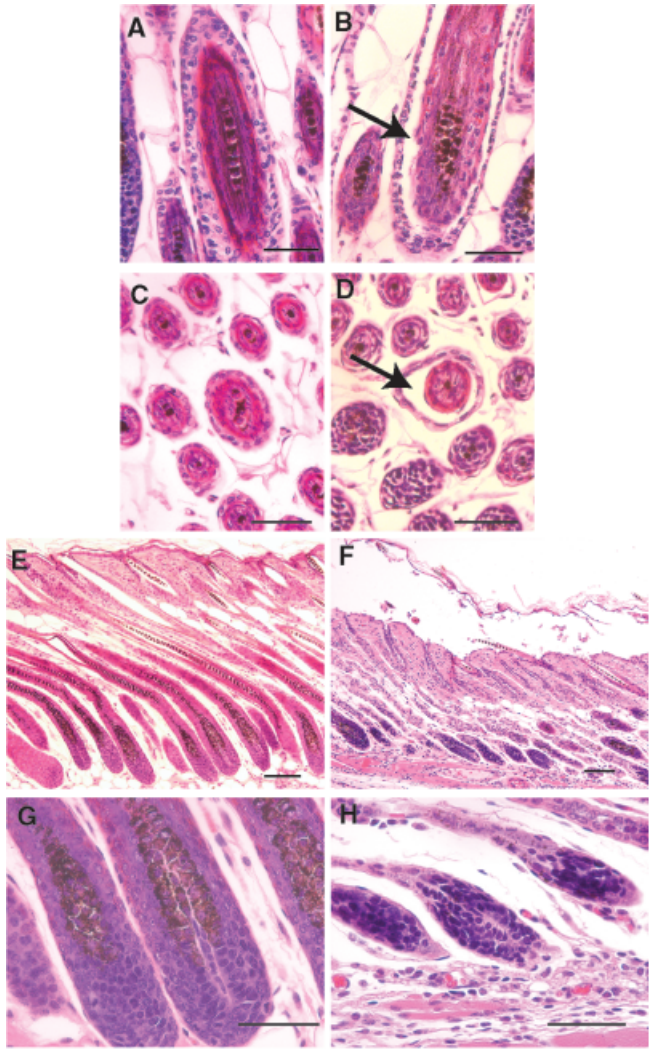



**Figure 3**
**Desmoglein (Dsg)3−/− mice injected with exfoliative toxin A (ETA) develop a split between the outer root sheath (ORS) and inner root sheath (IRS).** Histology of skin from Dsg3−/− mouse treated with ETA (*B*, *D*, *F*, *H*) demonstrates a split between the IRS and ORS (arrow), compared to skin from control Dsg3+/− mouse treated with ETA (*A*, *C*, *E*, *G*). Hair matrix cells in the bulb remain in both the treated (*G*) and control (*H*) follicles (*scale bars* = 50 μm).

Staining of depilated skin with K14 antibody and AE13 showed K14-positive cells in the ORS, but no AE13-positive cells, whereas control skin was positive for both (Fig 5*A–D*). AE15 and anti-K6 antibodies did not detect any cells in the depilated skin but control sections were positive (data not shown). These results confirm the presence of the IRS and companion layer around the plucked hair.

To detect the level of the horizontal split in the lower portion of the follicle, three hours after ETA injection, we pulse labeled for 1 h with bromodeoxyuridine (BrdU), then removed the hair by plucking. Pulse labeling results in labeling of the matrix cells in the bulb (Wilson *et al*, 1994). BrdU-labeled matrix cells were only found in the depilated skin whereas the depilated hair and surrounding layers were negative (Fig 5*E–G*). These data show that the lower split occurs between the matrix cells and precortical cells.

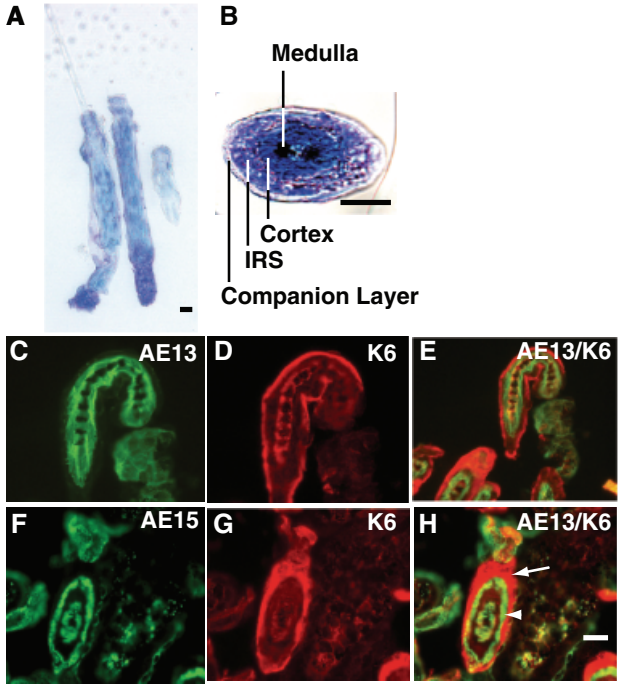



**Figure 4**
**The hair shaft, inner root sheath (IRS). and companion layer are removed after exfoliative toxin A (ETA) treatment of Dsg3−/− mice.** (*A*) Toluidine blue staining of hair and surrounding cells removed from a Dsg3−/− mouse after injection with ETA shows a smooth surface and fractured base. (*B*) Transverse cryosection of removed hair and surrounding cells stained with toluidine blue shows deep blue staining of the hair shaft (center), blue staining of the IRS (middle), and a peripheral clear companion layer. Double staining of removed hairs with AE13 (stains hard keratins in the hair shaft) and anti-K6 (stains companion layer and medulla) antibodies shows presence of hair shaft (green; *C*, *E*) and companion layer and medulla (red; *D*, *G*). Double staining with AE15 (detects trichohyalin in IRS and medulla) and anti-K6 antibodies shows IRS ([arrowhead] green; *F*, *H*) encircled by companion layer ([arrow] red; *G*, *H*) (*scale bars* = 50 μm).

## Discussion

Hair normally remains anchored to the follicle during anagen, catagen, and telogen. The hair sheds at the end of telogen during a stage termed "exogen." (Milner *et al*, 2002) In normal human scalp, exogen occurs just as the new hair shaft grows in. In the mouse pelage follicle, the hair shaft either is not shed, or if exogen occurs, the club hair is shed after the new hair has reached its near maximal length (Milner *et al*, 2002). Dsg3−/− mice lose hair at the onset of telogen, before a new hair forms, thus, Dsg3−/− mice go through periods of alopecia, hence the term "balding" to describe the phenotype of these mice (Koch *et al*, 1998). Remarkably, however, Dsg3−/− mice re-grow their anagen hairs, and these hairs remain firmly anchored to the follicle. This suggested that either Dsg3 does not play a role in anchoring the anagen follicle or that another Dsg compensates for the loss of Dsg3.

Our current studies indicate that simultaneous loss of Dsg3 and Dsg1 results in loss of anchorage of the anagen hair to its follicle. The loss of both of these Dsg causes a separation between the ORS and the companion layer. The companion layer was appreciated by Orwin (1971) as a morphologically distinct layer sandwiched between the ORS and the IRS. Although originally considered as "the innermost layer of the ORS" Winter *et al* (1998) demonstrated that this morphologically and biochemically distinct layer resembles IRS rather than the ORS (Winter *et al*, 1998). Tight adhesion of the companion layer to the IRS is mediated by "Flugelzellen," which are specialized structures that originate in Huxley's layer, pierce through Henle's layer and attach directly to the companion layer (Langbein *et al*, 2002). The structural organization of the IRS and companion layer suggests that they combine to form a rigid cone shape that molds the trichocytes produced by the bulb matrix cells into the shape

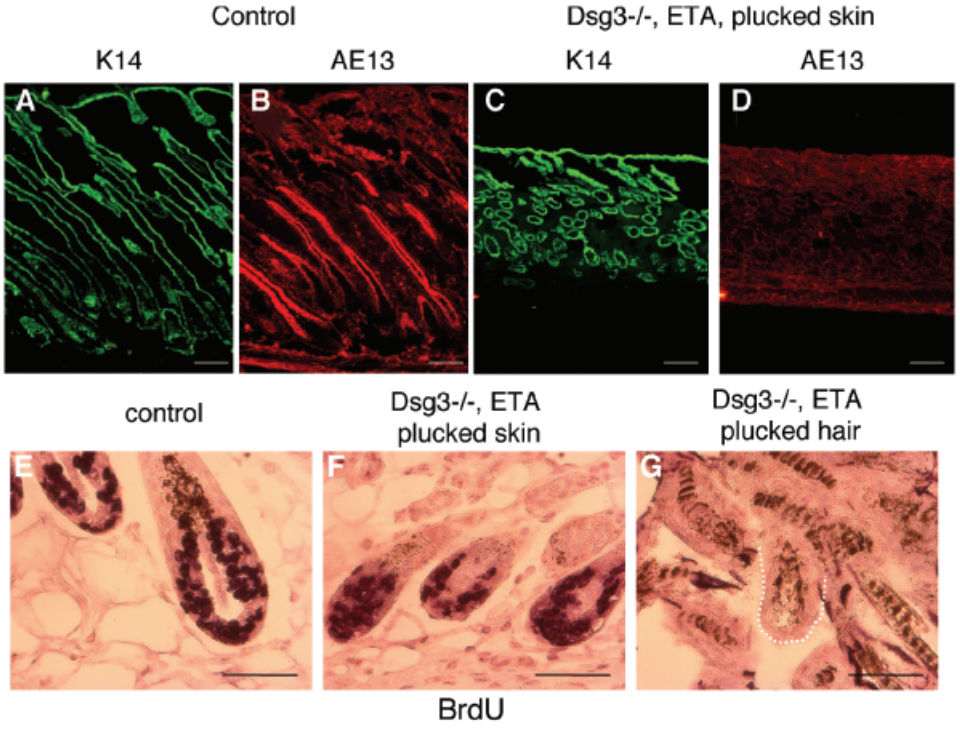



**Figure 5**
**The outer root sheath (ORS) and bulb matrix remain in the skin of desmoglein (Dsg)3−/− mice after exfoliative toxin A (ETA) treatment.** Immunofluorescence staining using anti-K14 and AE13 antibodies on Dsg3−/− mouse skin after ETA treatment and gentle hair removal (*C, D*). K14 antibody staining detects the basal epidermis and ORS in both control (*A*) and treated (*C*) mice. Lack of AE13 staining in depilated Dsg3−/− mice treated with ETA is consistent with the loss of the hair shaft (*D*). Pulse labeling of bromodeoxyuridine (BrdU) detects matrix keratinocytes in the hair bulb. Control (Dsg3−/− with phosphate-buffered saline (PBS) and BrdU) showed positive incorporation of BrdU in matrix cells (*E*). Depilated skin from Dsg3−/− mice treated with ETA showed staining for BrdU (*F*), while plucked hair was negative (*G*) suggesting a split above the matrix within the precortical cells in the ETA-treated Dsg3−/− mice. Scale bar = 50 μm

of the hair (Langbein *et al*, 2002). Orwin noted extensive connections of the companion layer to the IRS, and a dearth of attachments between the companion layer and ORS (Orwin, 1971). These findings were compatible with the idea that the companion layer, along with the IRS and hair shaft, all slid past the ORS as they moved upward during anagen.

Our findings suggest that Dsg are responsible for maintaining the integrity of the slippage plane within the growing anagen follicle. The junctions between the companion layer and ORS must undergo rapid remodeling during hair growth so that they maintain the anchorage of the hair and its surrounding migrating cell layers whereas allowing the hair shaft to exit the skin. The IRS and companion layers eventually disintegrate at the level of the isthmus. Within the isthmus, ORS cells undergo trichilemmal keratinization, in which the suprabasal cells keratinize abruptly without forming a granular layer. We previously showed that Dsg expression within the ORS correlates with the type of differentiation in human skin (Wu *et al*, 2003). For example, in areas of epidermal type keratinization, such as the infundibulum, Dsg3 is confined to the basal cell layers while Dsg3 is expressed throughout all layers of the ORS in areas of trichilemmal keratinization (Wu *et al*, 2003).

Here, we find differences between expression patterns of Dsg in the follicle of the mouse compared with those reported in the human (Wu *et al*, 2003). In particular, Dsg1 expression in the human correlates with more differentiated cells in the IRS and suprabasal superior ORS; however, in the mouse, this correlation is not apparent since Dsg1 is expressed by ORS cells in the lower follicle that presumably are relatively undifferentiated. Alternatively, this may suggest that ORS cells within the mouse pelage follicle are "more committed" than ORS cells within the human follicle. The plausibility of this notion is supported by the entirely different growth characteristics of mouse and human follicles. Human follicles stay in anagen for years, and may require constant replenishment of bulb matrix cells by bulge cells that migrate down the follicle. We proposed this based on keratin 15 staining patterns (Lyle *et al*, 1998) and later this concept was supported by Oshima who presented data suggesting that bulge cells, at least in specialized vibrissa follicles, migrate downward during anagen (Oshima *et al*, 2001). Mouse pelage hair follicles stay in anagen for 2–3 wk, and their ORS is only 1–2 cell layers thick, thus they may only require bulge cell movement downward at anagen onset. This is consistent with the idea that ORS cells in the mouse lower follicle are relatively more differentiated compared to human ORS cells.

Although we show here that Dsg1 and Dsg3 are critical for cell adhesion in the hair follicle, other Dsg are present. This is similar to the situation in epidermis and oral mucous membrane. Although Dsg2 is found in basal layers of stratified squamous epithelia (Schafer *et al*, 1994; Schafer *et al*, 1996), patients with pemphigus vulgaris with antibodies against Dsg3 and Dsg1 only, develop blisters deep in the epidermis, and Dsg3−/− mice develop acantholysis in the basal layer of the oral mucous membrane (Koch *et al*, 1997; Udey and Stanley, 1999). Similarly, although Dsg4 has recently been reported to be present in the superficial epidermis, anti-Dsg1 antibodies from pemphigus foliaceus patients and ETA, which cleaves Dsg1, cause a superficial blister, implying either that Dsg4 does not compensate for loss of function of Dsg1 in this area or that both pemphigus foliaceus antibodies and ETA inactivate Dsg4. The latter explanation has been recently shown not to be the case (Dr Amagai, personal communication). Similarly the hair follicle expresses Dsg4, but loss of Dsg4 causes hair shaft breakage, and does not cause the hair to fall out of the follicle (Kljuic *et al*, 2003). Our data, then, suggest that Dsg1 and Dsg3, as in epidermis, are important in maintaining cell adhesion in the follicle, particularly that adhesion responsible for anchoring the hair shaft in the follicle. Our results do not, however, rule out that other Dsg may also contribute to this adhesion or to adhesion in other areas (such as within the hair shaft itself).

Recently it has been reported that there are actually three different Dsg1 isoforms in mice, termed Dsg1$\alpha$, Dsg1$\beta$ (also called Dsg5), and Dsg1$\gamma$ (also called Dsg6) (Kljuic and Christiano, 2003; Pulkkinen *et al*, 2003; Whittock, 2003). Previous data regarding ETA cleavage of Dsg1, desmoglein compensation, and the data in this paper are characteristic of Dsg1$\alpha$, mainly because the antibodies used were raised against the EC5 domain of that isoform. It is unlikely that the antibody cross reacts with Dsg1$\gamma$ because its EC5 domain is not homologous to that of Dsg1$\alpha$. Similarly, it is highly unlikely that ETA cleaves Dsg1$\gamma$ because the critical glutamic acid in the cleavage site in Dsg1$\alpha$ is mutated to a basic lysine residue in Dsg1$\gamma$. We also know that the other isoforms are either cleaved by ETA or cannot compensate for loss of Dsg1$\alpha$ because blisters form in the superficial epidermis when Dsg1$\alpha$ is cleaved. This is likely the case in the hair follicle as well.

Loose anagen hair syndrome refers to a sporadic or familial disorder mainly seen in children (Price and Gummer, 1989). Patients with loose anagen hair show dysfunction in anchorage of anagen hair to the follicle, and slow growing hair that is easily removed, usually by a sibling. Most cases result in removal of hair shafts without the surrounding IRS, however, mutations in K6HF, the gene encoding a keratin found in the companion layer, have been associated with some patients with loose anagen hair syndrome (Chapalain *et al*, 2002). Loose anagen hair likely represents a common pathway that could theoretically result from mutations in a variety of genes. In addition to the hair keratin genes, of which over 50 have been described, other candidate genes for this disorder would include those encoding proteins involved in adhesion (Baden *et al*, 1992). Further studies are needed to determine whether Dsg1 and Dsg3 dysfunction causes loss of anagen hairs in any patients with loose anagen syndrome.

## Materials and Methods

**Immunohistochemistry** Preparation of mouse skin for histology and indirect immunofluorescence was performed as previously described (Hanakawa *et al*, 2002a). Non-fixed cryosections of mouse skin or plucked hair were used for immunofluorescence staining with AE13, AE15, anti-K6, and anti-K14. DAPI (Sigma, St Louis, Missouri) or PI (Sigma) were used as a nuclear counter stain. Plucked hair was used directly or cryosectioned and stained with 0.1% toluidine blue in sodium acetate buffer (pH 5.0).

**DSG3−/− (knockout) mice** All animal protocols were approved by the University of Pennsylvania IACUC. The generation of DSG3−/− mice has been previously described (Koch *et al*, 1997). Genotyping was done by PCR as previously described (Mahoney *et al*, 1999).

**Recombinant ET and injection into mice** Recombinant wild-type ETA, and ETAS195A (an inactive mutant) with a V5 and His tag on the C-terminus (these tagged ET will be abbreviated ETA and ETAS195A, respectively) were purified on Ni-NTA columns (Qiagen, Valencia, California) according to the manufacturer's protocol, then dialyzed against PBS (Hanakawa *et al*, 2002b). Protein concentrations of ET were estimated with a Protein Assay Kit (Bio-Rad Laboratories, Hercules, California). Purified ETA or ETAS195A (15 μg per gram body weight) in PBS or PBS alone was injected subcutaneously in the back skin of mice around 13 d after birth. Four hours after injection mice were killed and back skin was processed for histology and immunostaining.

**Adhesive tape hair loss test** A 1 in × 1/2 in piece of adhesive tape was pressed on the back skin of injected mice, then gently pulled off in the direction of hair growth (Koch *et al*, 1998). The tape was photographed and weighed before and after stripping. Weights were compared using the Student's *t* test.

**Antibodies** Rabbit antibodies against the extracellular domain of mouse Dsg1 (Mahoney *et al*, 1999), and the extracellular domain of mouse Dsg3 (Koch *et al*, 1998), were produced as previously reported. Mouse anti-human hard keratin, AE13 (Lynch *et al*, 1986), and mouse anti-human trichohyalin, AE15 (O'Guin *et al*, 1992), were provided by Dr Tung-Tien Sun (New York University). Rabbit antibodies against mouse K6, which recognizes mouse K6α, K6β, and K6hf (Wang *et al*, 2003) were provided by Dr Pierre Coulombe (Johns Hopkins University). Rabbit antibodies against mouse K14 (AF 64; CRP, Richmond, California) were also used.

**BrdU labeling** Three hours after ETA or PBS injection, 100 μg of BrdU in PBS was injected intraperitoneally. One hour after BrdU injection, the hair was plucked, mice were killed and skin was fixed in 70% EtOH, then embedded in paraffin. Paraffin sections were microwaved after deparafinization and used for BrdU staining with a BrdU Detection Kit II (Roche Applied Science, Indianapolis, Indiana) according to the manufacturer's instructions.

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