Desmoglein Isotype Expression in the Hair Follicle and its Cysts Correlates with Type of Keratinization and Degree of Differentiation

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Within stratified squamous epithelia, such as the epidermis, desmogleins are generally expressed in a differentiation-specific manner. Similar to the epidermis, the hair follicle is compartmentalized into a hierarchy of cell types based on their level of differentiation. Relatively undifferentiated stem cells in the bulge can generate epidermis, sebaceous gland, and hair bulb matrix cells. The latter give rise to at least six different cell types that keratinize as they move up the hair shaft and inner root sheath. Here, we examined expression patterns of the desmoglein isotypes, desmogleins 1, 2, and 3 in the cutaneous epithelium, and discovered that desmoglein 1 and 2 expression correlated with the state of differentiation of defined populations within the hair follicle. Desmoglein 2 was highly expressed by the least differentiated cells of the cutaneous epithelium, including the hair follicle bulge of the fetus and adult, bulb matrix cells, and basal layer of the outer root sheath. In contrast, desmoglein 1 defined more differentiated cell populations, and was expressed in epidermal suprabasal cells, the inner root sheath, and the innermost layers of the outer root sheath. We found that the expression pattern of desmoglein 3 correlated with different types of keratinization. In areas of trichilemmal keratinization in the follicle, and in cysts arising from these areas, desmoglein 3 was expressed throughout all layers of the outer root sheath and cyst wall. In areas of epidermal-like keratinization, such as in the infundibulum and in epidermal inclusion cysts, desmoglein 3 expression was limited mainly to the basal layer. We conclude that desmoglein expression patterns define compartments of cells in similar states of differentiation within the cutaneous epithelium, and reveal a hierarchy of differentiation among these compartments. ***Key words: desmogleins/differentiation/hair follicle/keratinization/stem cells. J Invest Dermatol 120:1152–1157, 2003***

Squamous epithelia, such as the epidermis, characteristically form desmosomes that contain desmogleins (Dsg). Dsg are transmembrane glycoproteins belonging to the cadherin superfamily of calcium-dependent adhesion molecules (Buxton *et al*, 1993). Three subtypes of Dsg, products of different genes, have been identified (Dsg1, Dsg2, and Dsg3). Although Dsg are cell adhesion molecules, many studies indicate that the distribution of the different subtypes in tissue correlates with degrees of differentiation, and more recent data show their distribution can correlate with specific types of keratinization (Koch *et al*, 1992; Arnemann *et al*, 1993; Koch and Franke, 1994; Amagai *et al*, 1996; Garrod *et al*, 1996; Shirakata *et al*, 1998; Elias *et al*, 2001). Dsg2 is generally thought to be the predominant Dsg in simple epithelia. In stratified squamous epithelia, if found at all, Dsg2 is only in the basal cells, and may be a marker of less differentiated cells. Dsg1 is present throughout the stratified squamous epithelia of epidermis and oral mucosa, with increasing amounts as the cells differentiate toward the stratum corneum, and very little detectable in the basal layer. This distribution suggests that the amount of Dsg1 correlates with the degree of differentiation, the converse of the distribution of Dsg2. The distribution of Dsg3 seems to correlate with the type of keratinization seen in the tissue in which it is expressed. In epidermis, which has a typical "basket-weave" stratum corneum on routine histology, Dsg3 is localized to the basal and immediate suprabasal layers. In contrast, in oral mucous membrane, which has a characteristic compact lamellar stratum corneum on routine histology, Dsg3 is expressed throughout the epithelium (Amagai *et al*, 1996; Wu *et al*, 2000). In transgenic mice in which there is forced expression of Dsg3 throughout the epidermis, the stratum corneum resembles that of mucosa, suggesting that Dsg3 can affect the type of keratinization (Wu *et al*, 2000).

In the epidermis, proliferating basal cells differentiate and move upward to form the spinous layer and subsequently the granular layer, which then generates the stratum corneum. Similarly, hair matrix cells in the lower follicle bulb proliferate, and their progeny differentiate and keratinize into six distinct concentric cell layers of the hair follicle as they migrate upward. The ultimate source of stem cells for both the epidermis and hair follicle resides in the bulge area of the follicle (Oshima *et al*, 2001). The bulge, which is a prominent structure during fetal development (Akiyama *et al*, 1995), contains the least differentiated keratinocytes within the cutaneous epithelium and is part of the outer root sheath (ORS), which joins the epidermis at the infundibulum near the surface of the skin (Pinkus, 1958; Lyle *et al*, 1998). Unlike the epidermis, the ORS remains nonkeratinized throughout its entire lower portion where it abuts the inner root sheath (IRS), and only begins to keratinize in the isthmus, immediately

Manuscript received November 26, 2002; revised January 26, 2003; accepted for publication February 3, 2003

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above the bulge area. In the isthmus, the ORS keratinizes abruptly without a granular layer in a so-called "trichilemmal" manner. Trichilemmal keratinization, and the differentiation of ORS keratinocytes below the bulge, have not been well characterized.

There have been few detailed studies of the distribution of Dsg in the hair follicle (Kurzen *et al*, 1998), and none of their distribution in the characteristic cysts derived from the hair follicle that undergo its two major types of keratinization. We particularly wanted to determine if the correlations of Dsg distribution with differentiation and keratinization was apparent in the various regions of the follicle.

MATERIALS AND METHODS

Normal human scalp and facial skin was obtained from the Cooperative Human Tissue Network. All protocols were approved by the University of Pennsylvania IRB. Fresh tissues were either frozen in OCT compound for cryosectioning or fixed in 4% buffered formalin and embedded in paraffin. After brief air drying, cryosections were fixed in either 100% acetone or a 1 : 1 mixture of methanol and acetone at − 20°C for 10 min. Sections from paraffin-embedded tissues were deparaffinized by incubating twice in Autodewax (Research Genetics, Huntsville, AL) at 105°C for 3 min, and followed by washing and rehydration in Autoalcohol and Universal buffer (Research Genetics), following the manufacturer's specifications. Deparaffinized sections were treated with pepsin (2.5 mg per ml; Research Genetics) at 50°C for 6–8 min. Prior to primary antibody incubation, all sections were blocked in phosphate-buffered saline with 1% normal goat serum, 1% bovine serum albumin, and 0.01% Triton X100 for 1 h. All primary antibodies were used at 1 : 50–1 : 100 dilutions of either commercial antibody solution or hybridoma culture supernatant in the blocking buffer, and incubated at 4°C overnight. Monoclonal antibody 10G11 (American Research Products, Inc., Belmont, MA) was used to detect Dsg2; monoclonal antibody 5G11 to detect Dsg3 and monoclonal antibodies 27B2 and 18D4 to detect Dsg1 (generous gifts from Dr Margaret Wheelock, Toledo, OH). The primary antibodies were detected by either immunofluorescent or immunohistochemical stains. For immunofluorescent staining, Cy^{3TM}-conjugated affiniPure goat anti-mouse IgG (H + L; Jackson Immunoresearch Laboratories. Inc., West Grove, PA) was used at a 1 : 400 dilution and incubated at room temperature for 1 h. Nuclear counterstain was performed by incubating sections with 1 : 1000 dilution of Hoechst solution for 5 min. For immunohistochemical staining, the sections were incubated with biotinylated anti-mouse IgG (H + L; Vector laboratories, Inc., Burlingame, CA) for 1 h at room temperature, followed by incubation with streptavidin–horseradish peroxidase (Life Technologies, Inc., Gaithersburg, MD) for 1 h at room temperature. The signal was generated using diaminobenzidine. The slides were counterstained with hematoxylin, coverslipped, and examined by light and fluorescence microscopy.

RESULTS

Dsg3 expression correlates with type of differentiation within the ORS In the infundibulum, Dsg3 immunopositivity was present predominantly in the basal cells, similar to the adjacent epidermis (**Fig 1*A***). Below the isthmus, basal cells of the ORS showed weak staining (**Fig 1*B–D***). In contrast, Dsg3 staining was present uniformly in all suprabasal cells of the ORS, except the innermost cell layer (companion layer of ORS; **Fig 1*B–D***). In the isthmus, Dsg3 immunopositivity in the suprabasal cells of the ORS was gradually lost toward the infundibulum (**Fig 1*A***). This transition in Dsg3 staining of the ORS correlated with the histologic onset of trichilemmal keratinization, which is characterized by abrupt keratinization lacking a granular layer, and a corrugated luminal surface (**Fig 1*A***).

We detected no Dsg3 immunostaining in the IRS or fully differentiated keratinocytes of the hair shaft (**Fig 1*B–D***); however, strong Dsg3 expression was present in the medulla of the hair shaft (**Fig 1*C,D***). Varying intensities of Dsg3 immunopositivity were seen in the suprabasal matrix and precortical cells (**Fig 1*D***), similar to the distribution pattern previously reported (Kurzen *et al*, 1998).

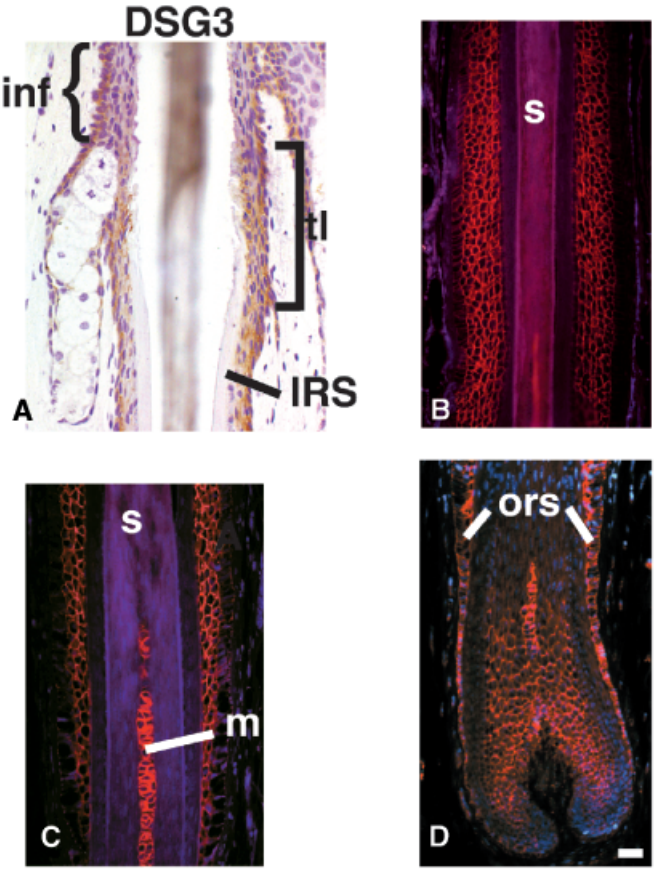

Figure 1. Expression pattern of Dsg3 in anagen scalp hair follicles. (*A*) In the isthmus, Dsg3 expression (brown) transitions from the basal pattern found it the infundibulum (inf) to the basal and suprabasal pattern at the level of trichilemmal keratinization (tl). Section was counterstained with hematoxylin. (*B*) In the upper suprabulbar follicle, Dsg3 expression (red) remains detectable throughout the ORS, although staining of the basal layer is weak or absent. (*C*) In the lower suprabulbar follicle, Dsg3 remains in the ORS. Intense staining for Dsg3 is present in the medulla (m) of the hair shaft (s). (*D*) In the bulb. suprabasal matrix and precortical cells express Dsg3 (nuclear Hoechst counterstain is blue). Panels are from representative follicles. *Scale bar* = 50 μm.

To define further the relationship between Dsg3 expression and trichilemmal keratinization, we analyzed its expression in two types of common cutaneous cysts, epidermal inclusion (infundibular), and trichilemmal (isthmus–catagen) cysts. Histologically, the wall of an epidermal inclusion cyst shows epidermal type keratinization with the presence of a granular cell layer and laminated keratin cyst contents (Krikham, 1997). In contrast, the trichilemmal cyst wall shows an absence of a granular cell layer and abrupt keratinization, analogous to trichilemmal keratinization (Krikham, 1997). Presumably, these patterns of keratinization reflect the areas from which these cysts arise. In the cyst wall of epidermal inclusion cysts, Dsg3 immunopositivity was present predominantly in the basal cells, with weak staining extending into the immediate one or two suprabasal cells, similar to that seen in the epidermis and infundibulum (**Fig 2*A***). Dsg3 immunopositivity in trichilemmal cyst walls was seen throughout the entire suprabasal cells, similar to that observed in the ORS below the infundibulum (**Fig 2*B***). Interestingly, this pattern is similar to that seen in mucous membranes (Elias *et al*, 2001).

Dsg1 expression delineates the most differentiated hair follicle cells The distribution pattern of Dsg1 in the ORS of the infundibulum was similar to that of the epidermis. In both

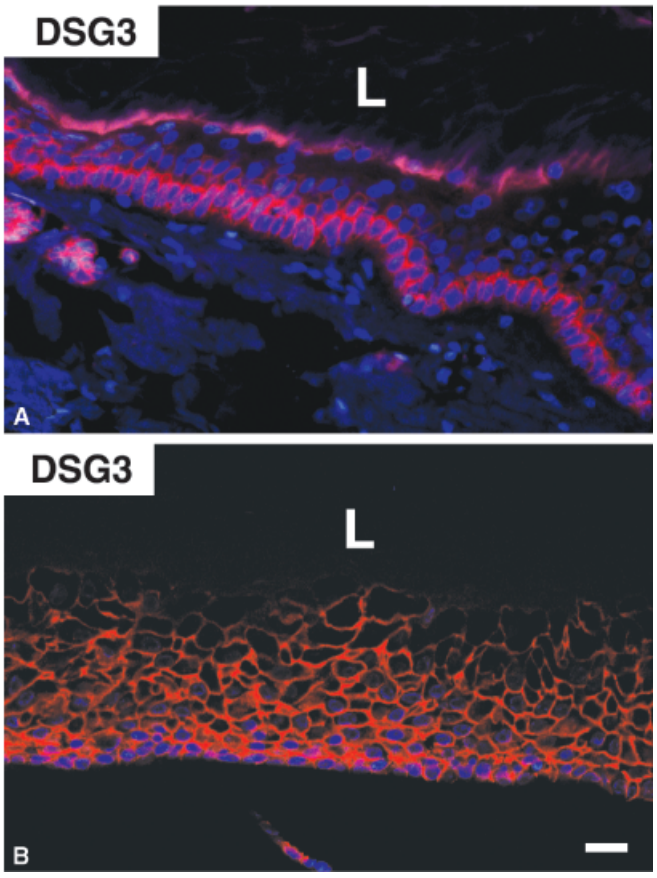

Figure 2. Expression pattern of Dsg3 in epidermal inclusion (*A*) and trichilemmal (*B*) cysts. (*A*) In the wall of an epidermal inclusion cyst, Dsg3 (red) is expressed predominantly in the basal layer, similar to the epidermis. L, lumen (*B*). Dsg3 (red) is expressed in basal and suprabasal cells of the trichilemmal cyst lining, as in the isthmus of the hair follicle. *Scale bar* = 20 μm.

of these areas, Dsg1 was expressed in the suprabasal cells with weak expression in the basal cells. At the level of the bulge, however, Dsg1 expression was undetectable in the basal layer, and was confined to the suprabasal cells only (**Fig 3*A***). Interestingly, towards the base of the follicle, Dsg1 expression gradually became restricted to the most suprabasal layers of the ORS, and then became confined to the companion layer before eventually disappearing in the ORS of the lowermost follicle (**Fig 3*A,C,E,G***). This contrasted to its expression in the IRS, in which the immunoreactivity was present in the lower follicle, then stopped abruptly at the level of the hair shaft where Huxley's layer lost trichohyalin granules and became enucleated (**Fig 3*E,G***). The overall distribution of Dsg1 within the follicle resembled the shape of a funnel (**Fig 3*I***).

Dsg2 expression marks the least differentiated follicular cells We found very weak or no expression of Dsg2 in the infundibulum and epidermis. In contrast, staining for Dsg2 was intense in the basal layer of the bulge area (**Fig 3*B***). There was a sharp transition between the weakly stained basal cells in the upper isthmus and the strongly staining basal cells of the lower isthmus/bulge region (**Fig 3*B***). This transitional point was also demarcated by Dsg1 staining, but in the opposite pattern (**Fig 3*A***).

We also found Dsg2 immunopositivity in suprabasal cells of the ORS in the lower follicle (**Fig 3*D,F,H***). In the suprabulbar region, Dsg2 staining in the suprabasal ORS cells diminished gradually towards the isthmus (**Fig 3*B,D,F,H***). This complemented Dsg1 expression, which spared the basal layer and became more suprabasal towards the base of the follicle (summarized in **Fig 3*I***).

In contrast to Dsg1, no detectable Dsg2 immunostaining was present in the IRS or hair shaft (**Fig 3*D,F,H***). The relatively undifferentiated matrix keratinocytes in the bulb, however, were positive for Dsg2 (**Fig 3*H***). The staining gradually diminished in the precortical area as trichocytes differentiated and keratinized (**Fig 3*H***).

Dsg expression in adult telogen and fetal hair follicles To analyze further the correlation between level of differentiation and Dsg expression we immunostained fetal skin and adult telogen follicles, which both contain immature keratinocytes. We previously defined the ORS surrounding the club hair as rich in epithelial stem cells (Lyle *et al*, 1998), and fetal cutaneous epithelium by definition is considered less differentiated than adult epithelium. The three Dsg isotypes showed strikingly different immunostaining patterns in adult telogen follicles. Dsg1 was weakly expressed in ORS cells immediately surrounding the club hair, and not in the basal cells of the ORS (**Fig 4*A***). Dsg2 immunostaining was seen strongly in the basal cells of the bulge, but was absent in the suprabasal ORS cells (**Fig 4*B***). Dsg3 immunopositivity was present in all ORS cells, but was especially concentrated at the apices of the basal cells as has been previously reported (**Fig 4*C***) (Koch *et al*, 1998).

In the human fetal follicle, Dsg2 was expressed with relative equal intensity throughout the basal layer of the epidermis, ORS, and bulb matrix (**Fig 5*B***). Dsg2 was also highly expressed in the upper and lower fetal bulges, which contain the sebaceous gland primordium and future site of hair follicle stem cells, respectively. Interestingly, Dsg1 was present only in the suprabasal layers of the sebaceous gland primordium and not in the lower bulge (**Fig 5*A***), whereas Dsg3 immunopositivity was present in both the sebaceous gland bulge and the lower bulge (**Fig 3*C***).

DISCUSSION

Using Dsg1 and Dsg2 expression patterns, we divided the follicle into compartments, and discovered a correlation between levels of differentiation and Dsg expression throughout the cutaneous epithelium (**Table I**). The hair follicle bulge contains the least differentiated keratinocytes within the follicle and epidermis. Ultrastructurally, bulge cells have fewer, less ordered, and thinner intermediate filament bundles compared with other basal cells (Cotsarelis *et al*, 1990; Akiyama *et al*, 1995). This ultrastructural morphology indicates a more immature phenotype, as filament bundles generally become denser with maturation (Coulombe *et al*, 1989). Biochemically, bulge cells are distinct and express keratin (K)15 and K19, which are associated with epithelial stem cell populations (Michel *et al*, 1996; Lyle *et al*, 1998, 1999). Functionally, bulge cells have the ability to generate epidermis and all of the epithelial cells of the hair follicle and sebaceous gland (Lenoir *et al*, 1988; Oshima *et al*, 2001). In contrast, under normal situations or after wounding, no evidence exists that adult epidermal cells regenerate ORS or other hair follicle cells. Kinetically, bulge cells are quiescent and the bulge is the exclusive site of long-term label-retaining cells in both mouse and human (Cotsarelis *et al*, 1990; Lyle *et al*, 1998; Morris and Potten, 1999). Thus, the bulge contains epithelial stem cells with an undifferentiated phenotype. Here we show that Dsg2 expression and absence of Dsg1 expression correlate with undifferentiated bulge cells (**Table I**).

In contrast to the bulge, the ORS below the level of the bulge has not been as well characterized. This area also possesses ultrastructurally primitive cells, but keratin expression in the lower ORS cells is variable with K15-positive cells only present about one-third of the time (Lyle *et al*, 1998), and K19-positive cells present throughout the basal layer of the lower follicle (Michel *et al*, 1996; Commo *et al*, 2000). The lower ORS also has a high colony forming efficiency, consistent with the idea that some cells in this

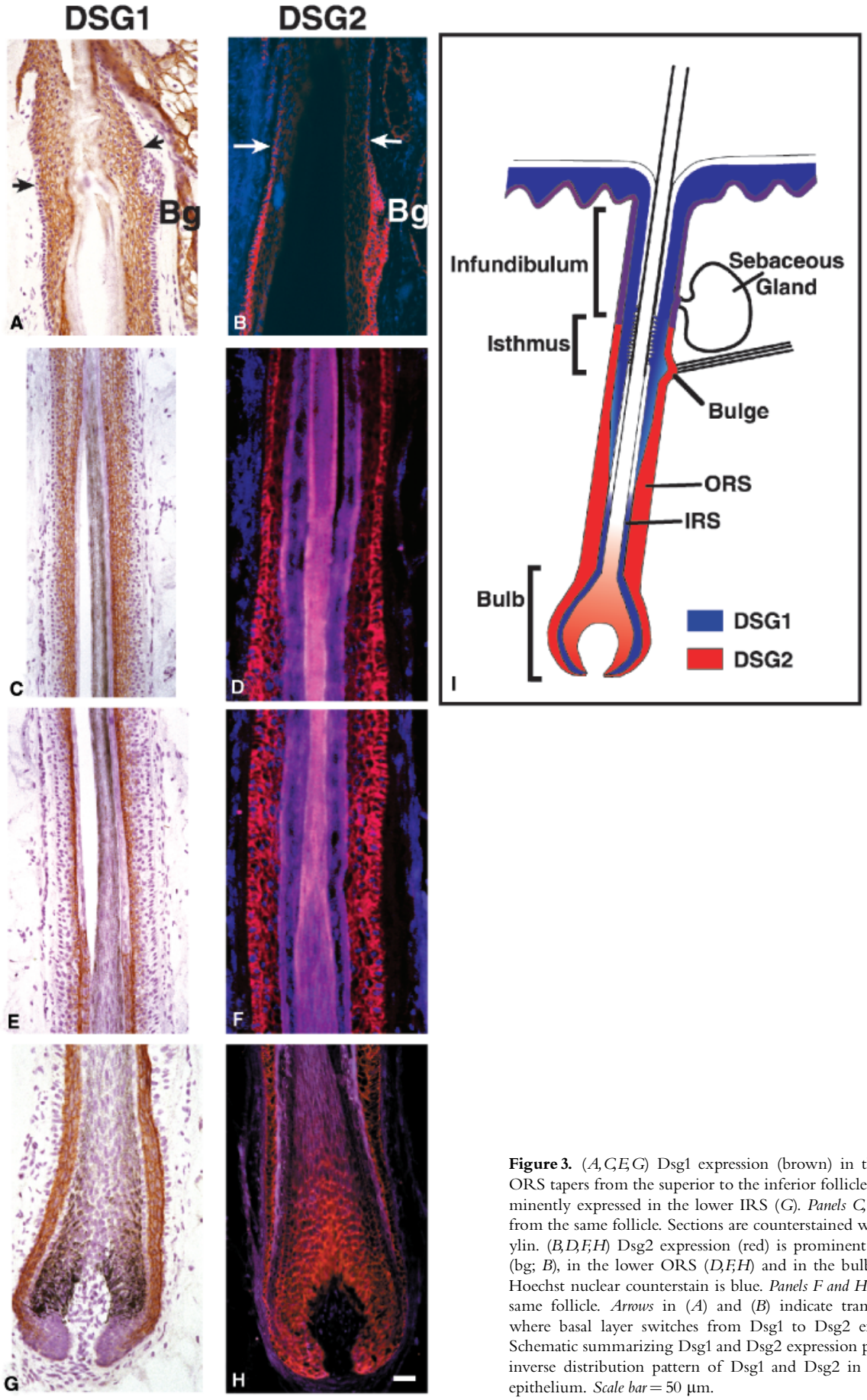

Figure 3. (*A,C,E,G*) Dsg1 expression (brown) in the suprabasal ORS tapers from the superior to the inferior follicle. Dsg1 is prominently expressed in the lower IRS (*G*). *Panels C, E, and G* are from the same follicle. Sections are counterstained with hematoxylin. (*B,D,F,H*) Dsg2 expression (red) is prominent in the bulge (bg; *B*), in the lower ORS (*D,F,H*) and in the bulb matrix (*H*). Hoechst nuclear counterstain is blue. *Panels F and H* are from the same follicle. *Arrows* in (*A*) and (*B*) indicate transitional zone where basal layer switches from Dsg1 to Dsg2 expression. (*I*) Schematic summarizing Dsg1 and Dsg2 expression patterns. Note inverse distribution pattern of Dsg1 and Dsg2 in the follicular epithelium. *Scale bar* = 50 μm.

area are less differentiated and have a high proliferative potential (Rochat *et al*, 1994; Panteleyev *et al*, 2001). Oshima *et al* (2001) have shown that basal keratinocytes migrate down the follicle from the bulge during anagen in mouse vibrissa follicles. These migrating cells appear to repopulate the matrix keratinocytes, which then generate the IRS and hair shaft (Oshima *et al*, 2001). Basal cells of the bulge and lower follicle showed strong Dsg2 expression and a lack of Dsg1 expression. This contrasted sharply with the decreased basal layer expression of Dsg2 and the onset of Dsg1 expression in the upper isthmus and infundibulum. This relatively sharp transition is consistent with the concept that bulge basal cells are distinct from the infundibular and epidermal basal cells. The strong expression of Dsg2 in the basal bulge cells, follicle ORS cells, and fetal bulge cells, suggests that these cells share a relatively primitive phenotype, and supports the notion that bulge cells migrate down the ORS in the human hair follicle.

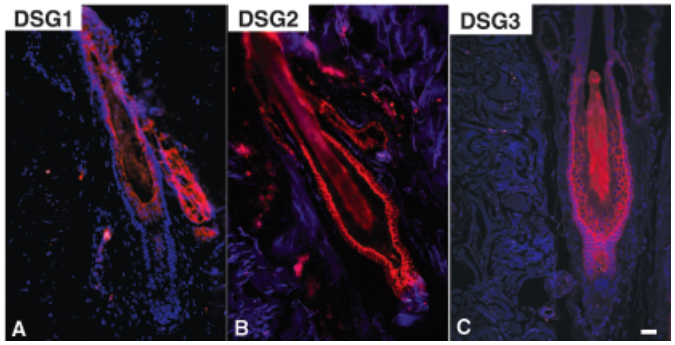

Figure 4. Dsg expression in telogen hair follicles. (*A*) Dsg1 expression (red) is present in suprabasal ORS cells surrounding the club hair in a late catagen/telogen follicle. (*B*) In contrast, Dsg2 immunoreactivity is present in the basal cells and secondary germ, and is absent in the suprabasal ORS cells. (*C*) Dsg3 immunoreactivity (red) is seen throughout the ORS. Nuclei are counterstained with Hoechst dye (blue). *Scale bar* = 50 μm.

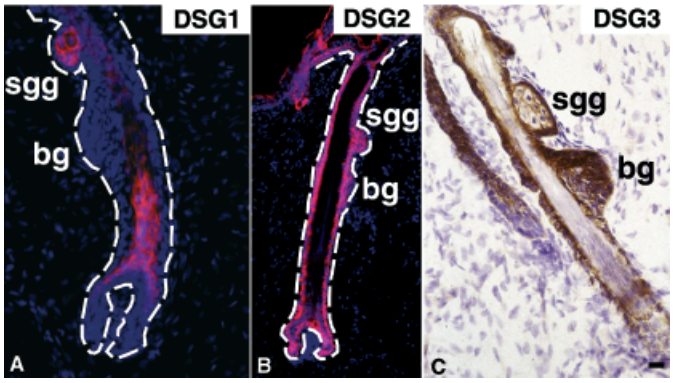

Figure 5. Dsg expression in fetal hair follicles. (*A*) Dsg1 expression (red) is present in the sebaceous gland germ (sgg), but not in the bulge (bg). (*B*) Dsg2 expression (red) is present throughout the basal layer of the ORS, including the sgg and bg, and in the bulb matrix cells. (*C*) Dsg3 expression is present throughout most of the follicle, including both the bulge and the sebaceous gland germ. Dashed line indicates junction of dermis and epithelium. *Scale bar* = 40 μm.

The expression of Dsg2 in the suprabasal ORS of the lower follicle distinguishes this area from the remainder of the ORS, and indicates that the suprabasal cells of the ORS are not a homogeneous population. Morphologically, suprabasal ORS cells are different from suprabasal cells of the epidermis or infundibulum. They are larger cells with clear cytoplasm due to abundant glycogen, the amount of which increases from the upper to lower ORS. Ultrastructurally, the keratin bundles in these cells appear to be similar to those seen in basal epidermal cells (Coulombe *et al*, 1989). Although biochemically these cells contain basal type keratins, K5 and K14 (Coulombe *et al*, 1989), ORS keratinocytes can be distinguished from epidermal keratinocytes by their expression of K6, K16, and K17 and simple epithelial K8 and K18 (Stark *et al*, 1987; Heid *et al*, 1988; Coulombe *et al*, 1989). Intriguingly, suprabasal ORS cells also proliferate, much like basal epidermal keratinocytes. In contrast, basal ORS cells rarely proliferate (Lyle *et al*, 1998; Commo *et al*, 2000). In addition, Dsg3, which is only present in the basal and first or second suprabasal layer of the epidermis is expressed throughout the multiple layers of the suprabasal ORS. Thus, the level of differentiation of suprabasal ORS cells seems similar to basal epidermal cells (**Table I**).

Our observations support the notion that there is an upward and inward maturation of suprabasal ORS cells. Proliferation studies have also shown that the lower part of the ORS is more mitotically active than the upper part (Moll, 1995; Commo and Bernard, 1997). Histologically, in the superior suprabulbar follicle, the suprabasal ORS cells gradually lose clear cytoplasm, flatten, and become eosinophilic, eventually undergoing trichilemmal keratinization at the level of the isthmus (Pinkus *et al*, 1981). This morphologic transition is marked by the onset of Dsg1 expression and the loss of Dsg2 expression, and parallels the phenotypic switch between basal and suprabasal cells in the epidermis. This expression pattern also suggests that suprabasal ORS cells move upward and inward.

Dsg, members of the cadherin family of cell adhesion proteins, clearly maintain the integrity of desmosomes, which are vital for cell adhesion; however, why different Dsg are necessary in different cells is not clear. The role of these different Dsg in hair follicle biology has been elucidated in the case of Dsg3 using studies in knockout mice. Mice carrying mutations in Dsg3 lose their telogen hairs because of a defect in cell adhesion within the keratinocytes surrounding the club hair (Koch *et al*, 1998). The role of Dsg1 and Dsg2 in the follicle is not yet known. Dsg1 and Dsg2

Table I. Summary of Dsg expression in human hair follicle and epidermis. Dsg1 expression correlates with differentiation. Dsg2 expression inversely correlates with differentiation and delineates the least differentiated cells

	Dsg1	Dsg2	Dsg3
Bulge	−	+ + +	+
Basal cells of ORS, below bulge	−	+ + to + + +	+/−
Matrix	−	+ +	+/−
Suprabasal cells of ORS, from suprabulbar to bulge region	− to + +	+ + to −	+ + + to + +
Basal cells of interfollicular epidermis and infundibulum		+	+ +
Trichocytes (precortical cells)		+	+
Suprabasal cells of ORS at isthmus, from lower to upper isthmus	+ + + +	+ to −	+ + + to −
Medulla	+ (Kurzen *et al*, 1998)	−	+ + +
Suprabasal cells of epidermis	+ + +	−	+/−
IRS	+ + +	−	−

knockout mice are not yet available. Based on the expression patterns defined here, one would predict that the loss of Dsg2 in the skin would result in major structural problems in the anagen and telogen hair follicle, especially in the basal layer of the ORS at the level of the bulge and below. The preferential expression of Dsg2 over the other Dsg proteins in these cells may indicate that, functionally, Dsg2 possesses unique characteristics required by more undifferentiated cells, including stem cells.

IRS cells gradually acquire trichohyaline granules and eventually become corneocytes. Biochemical analysis shows that a major portion of the IRS structure is insoluble (Stark *et al*, 1987). High levels of transglutaminase have been detected by immunohistochemistry in IRS (Rothnagel and Rogers, 1984). Suprabasal keratin, K1 and K10, and involucrin, all markers of differentiated cells in the epidermis are also present in the IRS (Stark *et al*, 1990; de Viragh *et al*, 1994). These results, together with our findings of strong Dsg1 expression and negativity for either Dsg2 or Dsg3 suggest that the IRS is more differentiated than any part of the ORS. This is consistent with its highly specialized role in shaping the hair shaft.

Overall, our findings indicate that desmosomal cadherin expression patterns may be important markers for different epithelial compartments within the hair follicle and epidermis based on their level of differentiation. This analysis provides a novel approach to categorizing the differentiation status of cutaneous epithelial cells, based on Dsg1 and Dsg2 expression patterns, and supports the notion that Dsg isoform expression patterns may impact on the differentiation of epithelial tissues. Future studies using transgenic and knockout studies will address these issues.

We thank Dorothy Campbell and the histology staff of the Department of Dermatology at the University of Pennsylvania for preparation of tissue sections. This project was supported by research grants from NIAMS/NIH George Cotsarelis and John Stanley, as well as by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute.

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