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Expression of Desmoglein 1 Compensates for Genetic Loss of Desmoglein 3 in Keratinocyte Adhesion

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The desmoglein compensation hypothesis, namely that one desmoglein can compensate for loss of function of another, has been proposed to explain the tissue specificity of the autoantibody-induced loss of cell adhesion in pemphigus. To validate this hypothesis genetically, we used desmoglein-3 knockout mice (DSG3–/–) that lose their telogen hair prematurely due to loss of adhesion between keratinocytes of the telogen hair club and the outer root sheath, where the only desmoglein expressed in normal mice is desmoglein-3. To determine if desmoglein-1 could substitute for the function of desmoglein-3 in telogen hair, we produced transgenic mice that express desmoglein-1 driven off the keratin 14 promoter, and then bred the transgene (TG) into DSG3–/– mice. Immunoblotting showed transgene expression in skin, and immunofluorescence showed desmoglein-1 in the telogen club of DSG3–/–TG+ but not DSG3–/–TG– mice. DSG3–/–TG– mice lost telogen hair with each wave of telogen, whereas DSG3–/–TG+ mice had markedly delayed and decreased hair loss. DSG3–/– mice also show low weights due to blisters in the oral mucosa. Surprisingly, DSG3–/–TG+ mice showed similar low weights, because the transgene, although expressed in skin, was not well expressed in oral mucous membranes. These studies show that desmoglein-1 can compensate for loss of desmoglein-3-mediated adhesion, and provide genetic evidence confirming the desmoglein compensation hypothesis. ***Key words: cadherin/desmosome/hair. J Invest Dermatol 119:27–31, 2002***

Desmosomes are multiprotein complexes that mediate epithelial tissue structure and integrity. Two types of transmembrane glycoproteins, desmogleins (Dsg) and desmocollins, play important roles in the formation and maintenance of desmosomes (Kowalczyck *et al*, 1999; Green and Gaudry, 2000). Both glycoproteins exist in three isoforms encoded by separate genes that are members of the cadherin supergene family.

Evidence from antibody and genetic studies suggest that Dsg play critical roles in the maintenance of cell adhesion by desmosomes. The first line of evidence comes from studies of pemphigus patients. These patients, whose disease is manifest as blisters due to loss of keratinocyte cell adhesion, have anti-Dsg autoantibodies fixed in their epidermis and circulating in their blood (Udey and Stanley, 1999). These anti-Dsg autoantibodies have been shown to cause loss of cell adhesion in organ culture of normal skin, without the addition of inflammatory cells or complement, suggesting that they directly inactivate the adhesion function of Dsg (Schiltz and Michel, 1976). Furthermore, passive transfer of these anti-Dsg autoantibodies into neonatal mice causes blisters due to loss of keratinocyte cell–cell adhesion (Anhalt *et al*, 1982; Amagai *et al*, 1994, 1995).

If, as suggested by the above findings, anti-Dsg autoantibodies actually inactivate Dsg to cause loss of cell adhesion, then genetic loss of Dsg should mimic antibody-induced disease. This later finding has been shown with DSG3–/– mice (Koch *et al*, 1997). These mice show loss of cell adhesion and blisters in the basal and immediate suprabasal layer of mucous membrane and skin, similar to that seen in pemphigus vulgaris patients who have anti-Dsg3 autoantibodies.

A complicating issue in trying to understand the pathophysiology of pemphigus has been that there are two major types of disease, called pemphigus vulgaris and pemphigus foliaceus, in which there are distinct anti-Dsg antibody profiles and in which blisters develop in different, but characteristic, tissue distributions (Mahoney *et al*, 1999; Udey and Stanley, 1999). For example, pemphigus foliaceus patients have anti-Dsg1 antibodies and develop blisters only in the superficial epidermis, but not the deep epidermis and not in mucous membranes at all, even though the anti-Dsg1 antibodies bind to all these regions. An explanation put forward to explain this apparent paradox has been termed the Dsg compensation hypothesis. This hypothesis states that both Dsg1 and Dsg3 maintain adhesion in the desmosome, and, in areas where both are expressed, one can compensate for antibody-induced loss of function of the other. Therefore, although anti-Dsg1 antibodies may inactivate the function of Dsg1 in deep epidermis and mucous membrane, as the Dsg3 isoform is also expressed in those locations no spontaneous blister forms. In normal superficial epidermis, however, only Dsg1 is expressed so that antibody-induced inactivation causes a blister there.

Although the correlation of blister localization, Dsg distribution, and anti-Dsg profiles in pemphigus is consistent with the Dsg compensation hypothesis, we sought more direct genetic proof that one Dsg could substitute in adhesion for another. To do so, we used the DSG3–/– mouse. The most striking phenotype of this

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Abbreviations: Dsg, desmoglein; TG, transgene.

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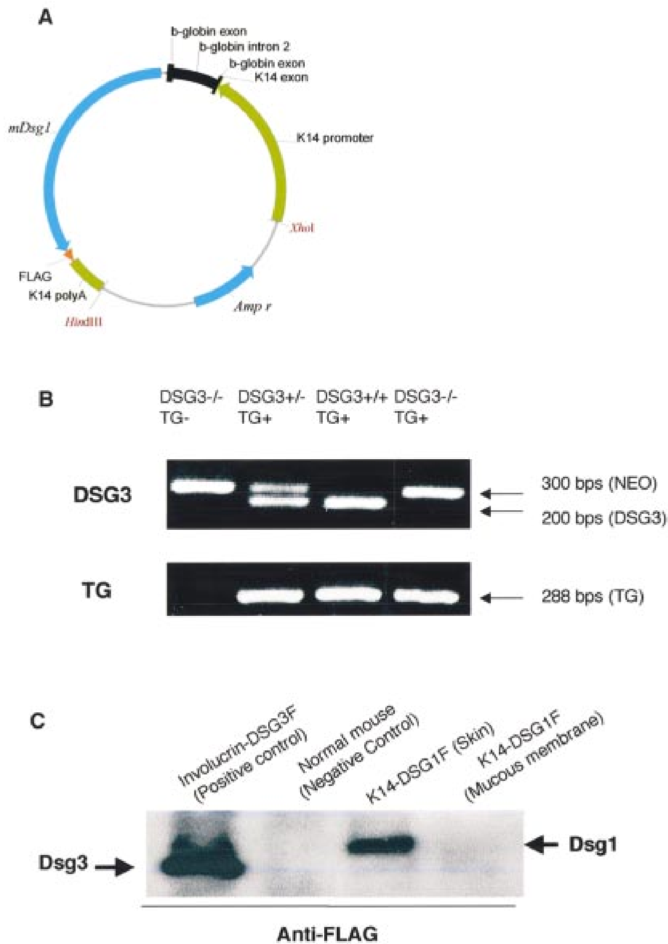

Figure 1. Genetic and expression analysis of DSG3–/– and K14-DSG1FLAG TG mice. (*A*) The TG placed mouse Dsg1 cDNA under the control of keratin 14 promoter. Nucleotides encoding the FLAG peptide sequence were added to the 3′ terminus of the coding sequence. The TG was excised from the pGEM 3Z vector with restriction enzymes of *XhoI* and *HidIII*. (*B*) Genotype analysis by PCR with three sets of primers: DSG3 exon 1 (200 bp product), neomycin DNA (300 bp product), and TG (288 bp product). (*C*) Expression of the TG was detected by Western blotting for the FLAG-tagged mouse Dsg1 from extracts of skin from transgenic 20-d-old mouse. Skin extract from an involucrin-DSG3FLAG transgenic mouse was used as a positive control. Extracts from tongue of TG mice did not show FLAG staining.

mouse is cyclical baldness from premature loss of telogen hair due to loss of cell adhesion of keratinocytes between the telogen hair club and the outer root sheath of the hair follicle (Koch *et al*, 1998). This area of the hair follicle normally contains only Dsg3 so that in the Dsg3–/– mouse there are no Dsgs, resulting in spontaneous loss of keratinocyte adhesion. The result is that as soon as the hair goes into synchronized telogen in the first hair cycle, the hair, which is normally firmly anchored until the next anagen hair replaces it, is lost. Subsequent hair cycles also show loss of telogen hair. The other phenotype of these DSG3–/– mice is poor weight gain presumably due to blisters in the basal layer of the mucous membranes where suckling causes trauma that, due to the loss of the normally expressed Dsg3, in turn causes loss of cell adhesion. We used this DSG3–/– mouse, with its easily assessed phenotypes of loss of cell–cell adhesion, to determine whether Dsg1 could substitute for Dsg3 in the basal layer by breeding in a Dsg1 transgene (TG) expressed off a keratin 14 promoter. The results of these genetic studies directly validate the Dsg compensation hypothesis.

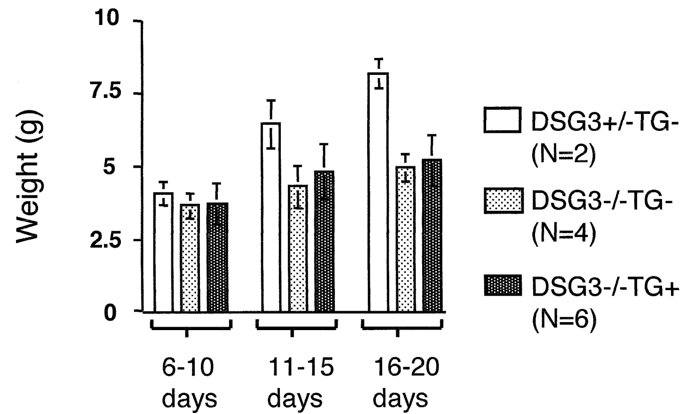

Figure 2. Weight gain of DSG3+/–TG–, DSG3–/–TG–, and DSG3–/–TG+ mice. The TG did not protect DSG3–/– mice from poor weight gain.

MATERIALS AND METHODS

Construction and genotyping of K14-DSG1 transgenic mice and DSG3 knockout mice carrying K14-DSG1 The keratin 14 promoter vector (pG32K14), which also contains the rabbit β-globin intron and K14 polyadenylation site, was a gift from Drs Elaine Fuchs and Toshihiro Tanaka (Vassar *et al*, 1989; Saitou *et al*, 1995). First, the keratin 14 promoter cassette was subcloned into pGEM-3Z (Promega, Madison, WI) vector (pGEM-K14). Mouse cDNA encoding Dsg1 was cloned as previously described (Amagai *et al*, 2000). Polymerase chain reaction (PCR) of the 3′ end was used to add 27 nucleotides that encode the FLAG octapeptide epitope, and the resultant cDNA was cloned into *BamHI* sites of the pGEM-K14. The final sequence was confirmed by nucleotide sequencing. The TG containing the K14 promoter and mouse DSG1-FLAG was excised from pGEM-3Z with *HindIII and XhoI* (**Fig 1*A***), and then microinjected into the male pronuclei of B6SJLF1/J mice zygotes prior to implantation into pseudopregnant foster CD–1 mice.

DNA extracted from tails of mice (Puregene Genomic DNA Isolation Kit, Gentra Systems, Minneapolis, MN) was used for PCR and Southern blotting to establish genotypes. PCR primers from the rabbit β-globin intron and Dsg1 were used to identify transgenic DNA (288 bp, **Fig 1*B***). After confirmation with Southern blotting, two independent TG lines were bred to the DSG3–/– line (Koch *et al*, 1997). Genotyping by PCR for DSG3–/– mice was described previously (Mahoney *et al*, 1999). PCR for the TG and for detecting the DSG3–/– phenotype were done simultaneously (**Fig 1*B***).

Antibodies Rabbit antibodies against the extracellular domain of mouse Dsg1 (Mahoney *et al*, 1999), the extracellular domain of mouse Dsg3 (Koch *et al*, 1998), and the FLAG epitope (Abcam, U.K.) were used for immunofluorescence staining. A monoclonal antibody against the FLAG epitope (M2, Sigma, St. Louis, MO) was used for immunoblotting.

Immunohistochemistry and immunoblotting Indirect immunofluorescence of formalin-fixed mouse skin with rabbit antibodies against Dsg1 and Dsg3 was described previously (Wu *et al*, 2000). Non-fixed cryosections of mouse skin were used for indirect immunofluorescence to detect FLAG epitope staining.

For immunoblotting, mouse back skin or mucous membrane (tongue) was homogenized on dry ice and then extracted with Laemmli sample buffer (Bio-Rad, Hercules, CA). Samples with equal protein amounts (Protein assay kit, Bio-Rad) were electrophoresed on 6% Tris–glycine polyacrylamide gels (Novex, San Diego, CA) and then transferred to nitrocellulose sheets (Trans blot, Bio-Rad). The sheets were incubated for 1 h in blocking buffer, 5% skim milk powder in Tris-buffered saline (50 mM Tris–HCl, pH 7.4, 150 mM NaCl). The FLAG M2 antibody conjugated with horseradish peroxidase (Sigma), diluted in blocking buffer, was applied for 1 h at room temperature. After two washes with 0.1% Tween-20 in Tris-buffered saline, the signals were detected with chemiluminescence (ECL, Amersham Pharmacia, Arlington Heights, IL).

Adhesive tape hair loss test A 1 cm^2 piece of adhesive tape was gently pressed on the nape of the neck of approximately 23-d-old mice

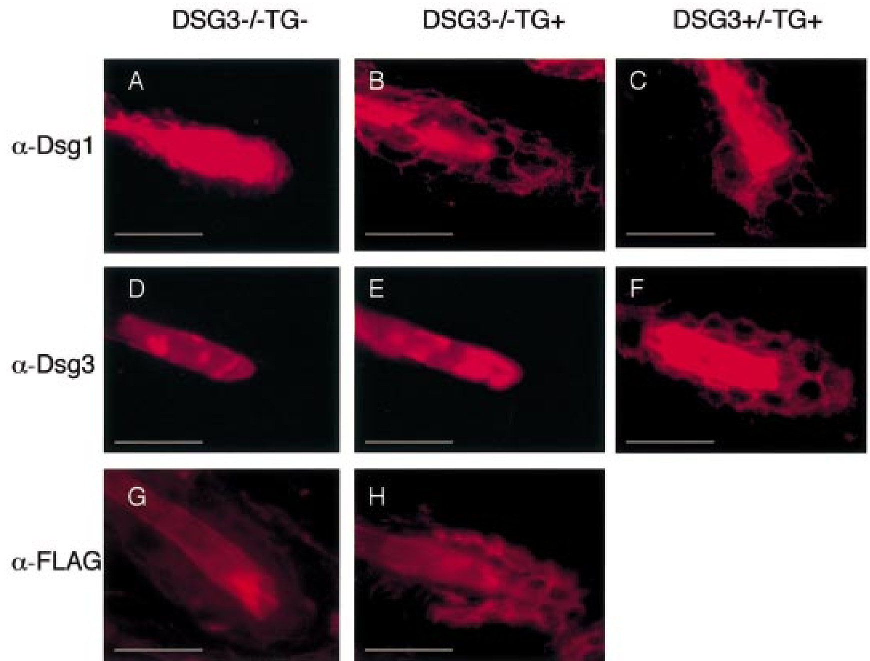

Figure 3. Immunofluorescence for TG expression in the telogen hair club. DSG3–/–TG+ mice showed positive staining of Dsg1 and FLAG at cell–cell contacts in the telogen hair club but Dsg3 is absent. DSG3–/–TG– mouse skin showed no staining of Dsg1, Dsg3, or FLAG, and DSG3+/–TG+ mice showed intense staining of both Dsg1 and Dsg3 in the telogen hair club. *Bar*: 50 μm.

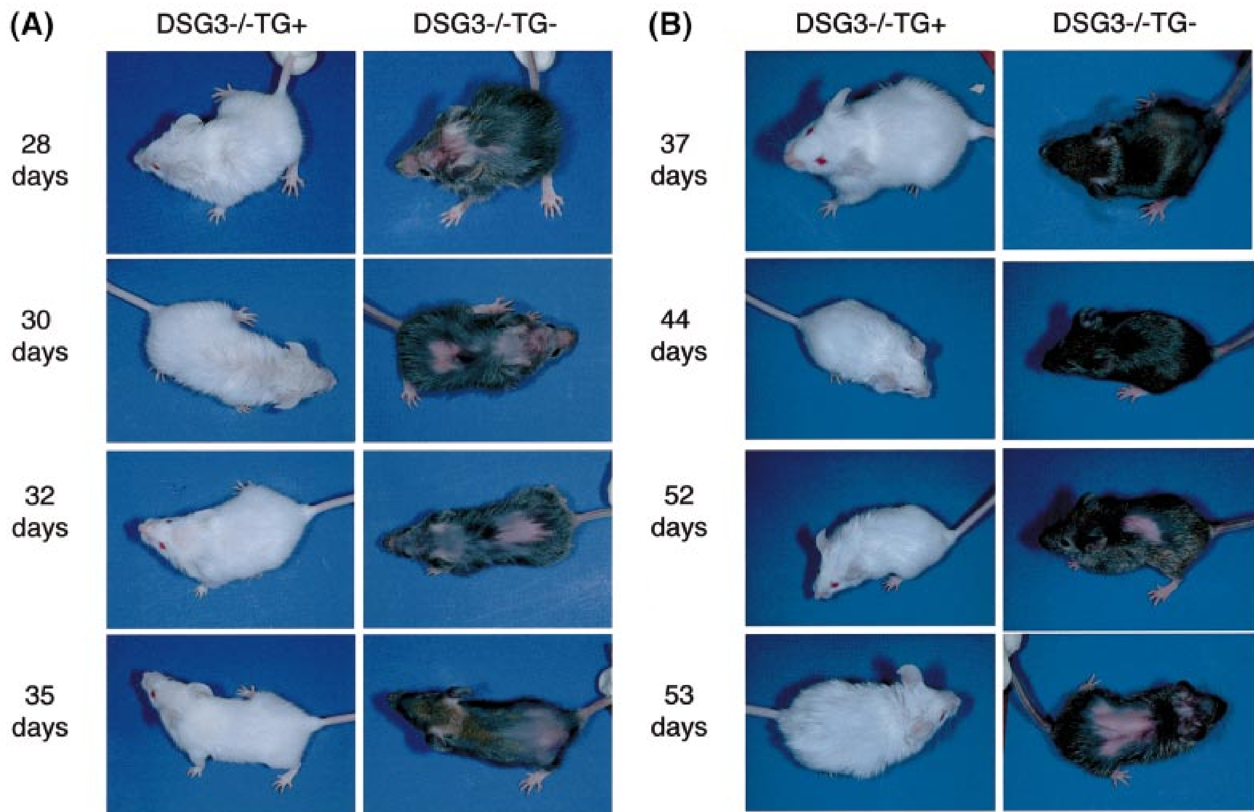

Figure 4. K14-DSG1 reversed the phenotype of telogen hair loss in DSG3–/– mice. The DSG3–/–TG– mouse lost hair from head to tail during the first (*A*) and second (*B*) telogen cycles, whereas the DSG3–/–TG+ littermate retains its hair.

(in the first telogen phase of hair growth), and then gently pulled off in the direction of hair growth (i.e., toward the tail). Tape was photographed and weighed (before and after stripping).

RESULTS

Genetic and expression analysis of DSG3–/– and K14-DSG1FLAG transgenic mice To determine whether Dsg1 expression could compensate for the genetic loss of Dsg3 in mice, we first generated transgenic mouse expressing mouse Dsg1 in the deep epidermis, where Dsg3 is normally expressed, by using mouse Dsg1 cDNA under the control of the keratin 14 promoter. In addition, nucleotides encoding the FLAG peptide sequence were added to the 3′ terminus of the coding sequence for mouse Dsg1 to create an epitope for detecting expression (**Fig 1*A***).

Analysis by PCR with primers in the rabbit β-globin intron and 5′ region of mouse Dsg1 cDNA identified a band of 288 bp in TG+ mice (**Fig 1*B***). This genotyping was confirmed by Southern blotting (data not shown). Two independent transgenic mouse lines were obtained, and both showed grossly normal phenotypes.

These transgenic mice were then bred to the DSG3–/– line to generate DSG3–/–TG+ mice by first generating DSG3+/–TG+/– mice that were interbred to generate DSG3–/–TG+ mice. Analysis by PCR with three sets of primers, in DSG3 exon 1, neomycin DNA, and the β-globin intron sequence in the TG, were used to identify DSG3+/+, DSG3+/–, and DSG3–/– mice with and without the TG (**Fig 1*B***).

Expression of the TG was detected by Western blotting for the FLAG-tagged mouse Dsg1 of extracted skin from transgenic 20-d-old mice (**Fig 1*C***). Skin extracts from involucrin-DSG3FLAG transgenic mouse (Wu *et al*, 2000) were used as positive controls. Immunofluorescence staining for the FLAG epitope tag showed expression of the TG in the deep epidermis (data not shown).

TG expression did not reverse wasting of DSG3 knockout mice DSG3–/– mice show poor weight gain, presumably due to blisters in the mucous membranes secondary to trauma from suckling. To determine whether the presence of the TG in DSG3–/– mice could reverse poor weight gain, we compared the weights of DSG3–/–TG– and DSG3–/–TG+ mice from 6 d to 20 d (**Fig 2**). This analysis indicated that the TG did not reverse poor weight gain in DSG3–/– mice. To confirm that the TG was actually expressed in oral mucous membranes, as it was in epidermis, we performed immunofluorescence (not shown) and Western blotting of tongue extracts with anti-FLAG antibodies (**Fig 1*C***). Surprisingly, the TG product was not detected, suggesting it was either expressed very weakly compared to skin or rapidly degraded at least in tongue, and indicating that compensation in oral mucous membrane could not be evaluated using these mice.

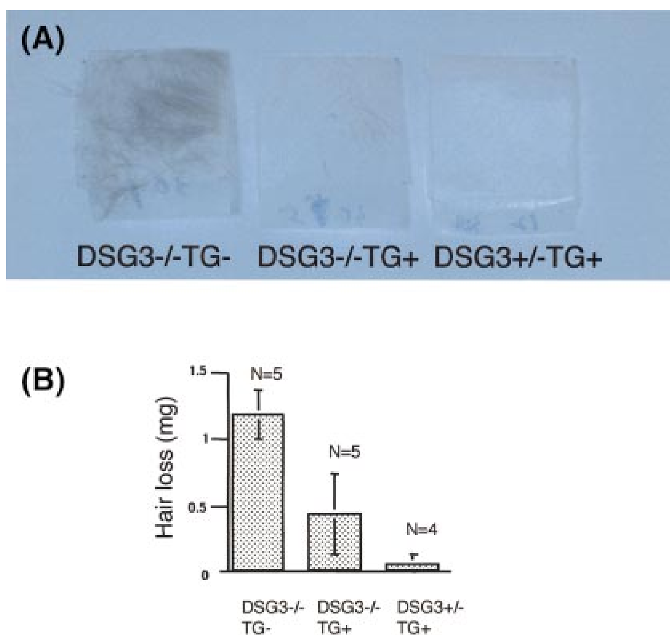

Figure 5. Adhesive tape hair pull test. On days 21–23, just before clinical hair loss in DSG3–/– mice, 1 cm^2 adhesive tape was applied to and gently removed from the nape of the neck. The hair on the adhesive tape was photographed (*A*) and weighed (*B*). DSG3–/–TG+ mice had much less hair loss than DSG3–/–TG– mice but still more than DSG3+/–TG+ mice.

TG expression reversed loss of telogen hair in DSG3 knockout mouse As compensation in mucous membrane could not be evaluated, we analyzed the other prominent phenotype of DSG3–/– mice, telogen hair loss. In normal mice, Dsg3 is the only Dsg in the telogen hair club, which is normally anchored in the outer root sheath of the hair follicle until the next anagen hair grows in. In DSG3–/– mice, the telogen club is not anchored properly and is prematurely shed due to loss of cell adhesion between the keratinocytes in the club and the outer root sheath.

First we determined if the transgenic Dsg1 was expressed in the telogen hair club. **Figure 3** confirms that transgenic mice, whether they express Dsg3 or are Dsg3 knockouts, express Dsg1 on the cell surface of keratinocytes in the telogen hair club. Furthermore the FLAG epitope is detected in the telogen hair club, confirming that the TG is expressed. These data are consistent with the finding of K14 expression in the keratinocytes of the hair club (Bowden *et al*, 1998).

Because Dsg1 replaced Dsg3 in the telogen hair club of transgenic Dsg3 knockout mice, we could determine if it compensated for telogen hair loss by comparing hair loss in DSG3–/–TG– and DSG3–/–TG+ mice. As previously described (Koch *et al*, 1998), DSG3–/–TG– mice lose hair from head to tail in the first wave of synchronized telogen starting about age 25–28 d (**Fig 4*A***). With the second anagen wave, the hair grows back from head to tail, but then is lost again in the second telogen wave (**Fig 4*B***). In later cycles the hair is less synchronized so that DSG3–/– mice show patchy hair loss (Koch *et al*, 1998). Strikingly, DSG3–/–TG+ mice show little, if any, hair loss in the first and second telogen waves (**Fig 4*A*, *B***). Eight DSG3–/–TG+ mice were analyzed and seven mice showed reduced hair loss and delayed starting of hair loss compared to DSG3–/–TG– mice. One DSG3–/–TG+ mouse showed extensive hair loss like DSG3–/–TG– mice.

To try to semiquantitate this telogen hair loss, we used an adhesive tape hair pull assay (Koch *et al*, 1998; and see *Materials and Methods*). Adhesive tape was applied and removed from the nape of the neck of 21–23-d-old mice, just at the onset of telogen. Gross inspection of the tape indicates that DSG3–/–TG– mice lose much more hair than DSG3–/–TG+ mice. DSG3–/–TG+ mice do show

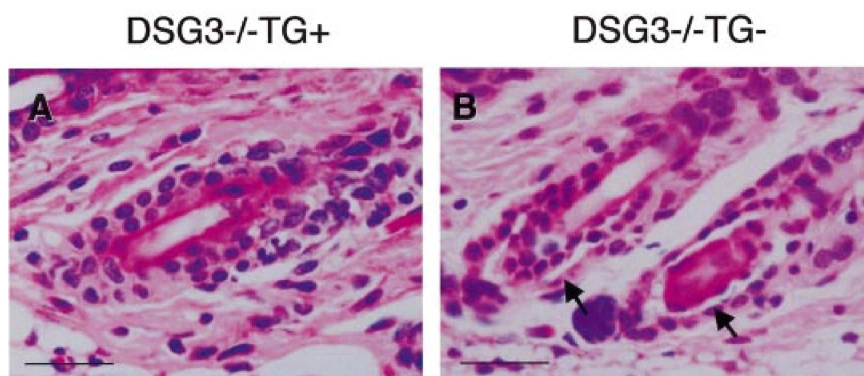

Figure 6. Histology of telogen hair in DSG3–/–TG+ compared to DSG3–/–TG– mice. Hematoxylin and eosin staining of the telogen hair follicle of DSG3–/–TG– mice showed acantholysis in the club; however, we could not detect acantholysis in the telogen hair club of DSG3–/–TG+ mice. *Scale bar*: 50 μm.

more hair loss than mice that express Dsg3, however (**Fig 5*A***). Quantitation of these results by weights of hair pulls is shown in **Fig 5(*B*)**.

Finally, we confirmed by histology that, whereas DSG3–/–TG– mice showed acantholysis (loss of cell adhesion) in the telogen hair club (*N* = 6), DSG3–/–TG+ mice did not (*N* = 7) (**Fig 6**).

Interestingly, after the second telogen hair cycle DSG3–/–TG+ mice showed increasing hair loss correlating with decreased TG expression as determined by Western blotting. By this time the hair loss was difficult to evaluate comparatively between DSG3–/–TG– and DSG3–/–TG+ mice, however, because the hair cycle was no longer synchronized and the hair loss was sporadic and patchy.

DISCUSSION

The idea that one Dsg isoform can substitute for another in adhesion is suggested by studies of the autoimmune blistering diseases pemphigus vulgaris and foliaceus. In these diseases antibody inactivation of Dsg1 or Dsg3 does not result in a blister if the other Dsg is coexpressed in the same area (Mahoney *et al*, 1999; Wu *et al*, 2000). This correlation is consistent with the so-called Dsg compensation hypothesis. More direct proof comes from studies in transgenic mice in which forced expression of Dsg3 in the superficial epidermis prevents anti-Dsg1 pemphigus foliaceus antibodies from causing a superficial epidermal blister (Wu *et al*, 2000). The latter study suggested that Dsg3 could compensate for adhesion from antibody-induced loss of function of Dsg1. The exact pathway in which antibodies might inactivate Dsg is not known, however; therefore it is possible that expression of Dsg3 could somehow interfere with the pathway of inactivation rather than directly compensate for function.

We therefore sought direct genetic proof of the Dsg compensation hypothesis. In this study we show that forced expression of Dsg1 in the telogen hair club prevents loss of cell adhesion due to absence of Dsg3 in this area. This result demonstrated that Dsg1 can compensate, at least partially, for the adhesive function of Dsg3 in the desmosome. The compensation, however, was not perfect in that there was some increased hair loss in some DSG3–/–TG+ mice compared to normal mice even in the first two telogen cycles. This is not unexpected and may be due to the fact that the expression levels of the TG Dsg1 are not regulated properly because the K14 promoter will not mimic perfectly the Dsg3 promoter. Another possibility is that although Dsg1 can compensate for Dsg3 under normal conditions, in some areas one Dsg may be superior to another if there is mechanical stress. Until we know much more about the biophysical mechanisms of adhesion in the desmosome, however, the clarification of this possibility will remain elusive.

In this study, we could not show Dsg1 compensation for absence of Dsg3 in the mucous membranes. Although K14 should be expressed in the basal layer of the mucous membranes, in our K14-DSG1 TG, in two founders, DSG1 was not expressed strongly, as determined by immunofluorescence and immunoblotting. We have no explanation for these findings, although they could be due to either inactivation of the TG, integration effects, and/or degradation of the product. Level of expression correlated grossly with the ability to compensate for loss of Dsg3 function, however.

If one Dsg isoform can compensate for another in adhesion, could there be a function other than adhesion that requires the various isoforms? A recent study indicates that the distribution of Dsg isoforms affects the epidermal barrier (Elias *et al*, 2001). Normal mice only express Dsg3 in the deep epidermis, and not in the more superficial epidermis, whereas they express Dsg3 throughout the mucous membranes. Transgenic mice with mouse Dsg3 controlled by the involucrin promoter express the Dsg3 isoform in the same distribution in epidermis as in mucous membrane. These mice show a severe barrier defect with markedly increased transepidermal water loss, probably due to premature degradation of corneodesmosomes in epidermal stratum corneum.

With these findings in mind, it is likely that Dsg isoforms have more than one function. Adhesion is a major function, however, and its importance is underscored by the loss of this function causing the life-threatening blistering diseases pemphigus vulgaris and foliaceus. This study confirms that Dsg compensation in adhesion can occur, and validates the Dsg compensation hypothesis as an explanation for blister localization in these diseases.

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