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ORIGINAL ARTICLE

Consequences of Depleted SERCA2-Gated Calcium Stores in the Skin

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Sarco(endo)plasmic reticulum Ca^{2+}-ATPase isoform 2 (SERCA2) pumps belong to the family of Ca^{2+}-ATPases responsible for the maintenance of calcium in the endoplasmic reticulum. In epidermal keratinocytes, SERCA2-controled calcium stores are involved in cell cycle exit and onset of terminal differentiation. Hence, their dysfunction was thought to provoke impaired keratinocyte cohesion and hampered terminal differentiation. Here, we assessed cultured keratinocytes and skin biopsies from a canine family with an inherited skin blistering disorder. Cells from lesional and phenotypically normal areas of one of these dogs revealed affected calcium homeostasis due to depleted SERCA2-gated stores. In phenotypically normal patient cells, this defect compromised upregulation of $p21^{WAF1}$ and delayed the exit from the cell cycle. Despite this abnormality it failed to impede the terminal differentiation process in the long term but instead coincided with enhanced apoptosis and appearance of chronic wounds, suggestive of secondary mutations. Collectively, these findings provide the first survey on phenotypic consequences of depleted SERCA-gated stores for epidermal homeostasis that explain how depleted SERCA2 calcium stores provoke focal lesions rather than generalized dermatoses, a phenotype highly reminiscent of the human genodermatosis Darier disease.

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INTRODUCTION

Sarco(endo)plasmic reticulum Ca^{2+}-ATPase isoform 2 (SERCA2) calcium pumps are Ca^{2+}-ATPases that belong to the family of P-type cation pumps coupling ATP hydrolysis to calcium transport across membranes (Shull, 2000). The pumps are located at the sarco/endoplasmic reticulum (ER) and contribute to the maintenance of ER calcium levels by actively transporting surplus calcium from the cytosol into the lumen of their organelle.

Prime evidence for the importance of SERCA2-gated calcium stores was provided by the lethal phenotype of SERCA2 knockout mice (Periasamy *et al.*, 1999) and by loss of calcium regulatory ability in heterozygous SERCA2 null mutant cells. For instance in heterozygous murine cardiomyocytes, the amplitude of calcium transients, known to require mobilization of calcium from intracellular stores (Thomas, 2000; Putney *et al.*, 2001), was significantly decreased whereas the resting level of cytosolic calcium was unchanged (Ji *et al.*, 2000). The use of the drug thapsigargin and its unique specificity for SERCA pump inhibition (Treiman *et al.*, 1998) had helped to obtain insight into consequences of depleted SERCA2-gated calcium stores in various biological processes, specifically during keratinocyte terminal differentiation (eg Hakii *et al.*, 1986; Lowry *et al.*, 1996). In these epidermal skin cells, thapsigargin impaired terminal differentiation and junction assembly. This is in line with the current knowledge that the Ca^{2+}/inositol trisphosphate pathway drives proliferating keratinocytes to exit the cell cycle and commit to terminal differentiation, requiring mobilization of calcium from ER stores (Bikle *et al.*, 2001), which in these cells are mostly controlled by SERCA2 pumps (Shull, 2000). Generation of cytosolic calcium transients then triggers among others the upregulation of $p21^{WAF1}$ (Santini *et al.*, 2001). $P21^{WAF1}$ inhibits cyclin-dependent kinases and is therefore a key factor in controlling cell cycle progression and the exit from the cell cycle into quiescence (Lee and Yang, 2001). This function of $P21^{WAF1}$ is essential not only under normal conditions but also in response to DNA damage.

The importance of SERCA2 pumps in the skin was underscored by the finding that mutations in *ATP2A2*, the gene encoding SERCA2, can cause the dominantly inherited skin blistering disorder Darier disease (Sakuntabhai *et al.*, 1999). However, mutations in *ATP2A2* were identified in

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Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; Dsg, desmoglein; ER, endoplasmic reticulum; SERCA2, Sarco(endo)plasmic reticulum Ca^{2+}-ATPase isoform 2

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only up to 50% of Darier disease patients (Ringpfeil *et al*., 2001; Ikeda *et al*., 2003). This highlights that different, so far unidentified, regulatory genes governing similar processes or alternatively non-coding gene regions of *ATP2A2* when mutated lead to the same pathogenesis. The histopathological features of the disease (or syndrome in case different genes are affected) include focal epidermal lesions exhibiting premature differentiation of single cells (dyskeratosis), loss of intercellular adhesion (acantholysis), and hyperkeratosis (hyperproliferation) often resulting in keratotic papules (Hovnanian, 2004). Characteristically, the disease is exacerbated by tissue damage, such as wounding or mechanical friction (Burge and Wilkinson, 1992). Although Darier disease is histopathologically very similar to Hailey–Hailey disease (Burge and Schomberg, 1992), the two forms can be clearly distinguished by one physiological feature; in contrast to Dories disease, Hailey–Hailey keratinocytes have normal calcium stores in the ER evidenced by their release after thapsigargin treatment (Hu *et al*., 2000).

According to effects observed in studies using thapsigargin, it was discussed that in Darier disease patients carrying *ATP2A2* mutations, loss of calcium regulatory ability due to impaired SERCA2 function might have a major impact on intercellular junction assembly and keratinocyte differentiation (Peacocke and Christiano, 1999; Periz and Fortini, 1999; Sakuntabhai *et al*., 1999). Intriguingly, if the deregulation of intracellular calcium stores controlled by SERCA2 pumps was crucial for the establishment of intercellular adhesion and the integrity of the epidermal architecture, the histopathological features of Darier disease would be expected to manifest as a generalized dermatosis and not as focal lesions. Moreover, the finding that under some experimental settings thapsigargin treatment provoked cell cycle arrest and apoptosis (McConkey and Orrenius, 1996), as opposed to sustained proliferation (eg Hakii *et al*., 1986; Lowry *et al*., 1996), contributed to obscure the primary consequences of depleted SERCA2-gated stores in the patient keratinocytes. It is further noteworthy that to date mostly lesional skin has been investigated in Darier disease patients, which does not allow any inference on the primary consequences of affected SERCA2-gated stores for the normal skin. Consequently, the initial cellular mechanisms that provoke focal lesions in uninvolved skin have remained unresolved.

Some years ago, a canine family with an inherited skin disorder (genodermatosis) was described (Shanley *et al*., 1992; Sueki *et al*., 1997). Based on clinical and histopathological features, the disease was, at the time, considered similar to human Hailey–Hailey disease, or alternatively Darier disease, (Hailey and Hailey, 1939; Burge, 1992). Our initial finding that SERCA2-gated stores were depleted in keratinocytes of the canine patient using thapsigargin-induced release, excluded the possibility of Hailey–Hailey disease (because this disease does not present with abnormalities in ER calcium levels (Sudbrak *et al*., 2000)). It further identified the disorder under study as an interesting model to elucidate the phenotypic consequences of depleted SERCA2-controled calcium stores in the skin. Our analysis focusing on phenotypically normal skin and cultured keratinocytes from one of the canine patients aimed in providing a broad survey of the consequences of depleted SERCA2-gated calcium stores for epidermal tissue homeostasis. Because canine SERCA2 pumps have comparable physiological properties than SERCA2 pumps from other species including humans (Autry and Jones, 1997), the results likely have broader implications as they now offer a novel insight into the mechanism by which depleted SERCA2 calcium stores create a predisposition for focal lesions, like the ones described in Darier disease patients.

RESULTS

Characterization of long-term keratinocyte cultures as compared to the *in vivo* situation

Keratinocyte cultures were established from a persistent keratotic papule (lesional) and phenotypically normal (non-lesional) skin from one of the dogs with inherited skin blistering disease (Shanley *et al*., 1992; Sueki *et al*., 1997) and from the skin of corresponding body sites of two control dogs. Initially, we evaluated whether the *in vivo* phenotype of these keratinocytes persisted in culture. To compare the proliferation and terminal differentiation properties between biopsies and cultured keratinocytes, we prepared cultures grown at the air–liquid interface for 14 days. These cultures reproduced the stratification patterns observed *in vivo* (Suter *et al*., 1991a). As seen in Figure 1a, and noted previously (Shanley *et al*., 1992; Sueki *et al*., 1997), lesional epidermis was hyperplastic with marked hyper- and parakeratosis compared with non-lesional epidermis. In contrast, non-lesional epidermis was visually indistinguishable from normal control tissue with its 2–5 cell layers. In non-lesional epidermis from the affected dog, immunofluorescence staining for Ki67, a marker for cycling cells, was limited to the basal layer identical to the controls (Figure 1b). Hyperplasia in the lesional skin correlated with enhanced staining for Ki67 in basal as well as suprabasal epidermis. Concomitantly the basal cell-specific keratin 14 was detected in both the basal and suprabasal layers of the lesional epidermis, suggesting that certain basal cell characteristics were retained suprabasally in the lesional epithelium. This was consistent with weak staining for differentiation-associated proteins keratin 1 and involucrin.

Both, cultured control and non-lesional keratinocytes grown at the air–liquid interface developed an epidermis-like structure of approximately 2–5 cell layers with a thin stratum corneum (Figure 1c) analogous to the *in vivo* phenotype. Consistent with the *in vivo* situation, lesional keratinocytes formed a much thicker epithelium with nearly 20 vertical layers, and a thick, partially parakeratotic stratum corneum. Immunofluorescence staining for Ki67 was rare in the basal layer of non-lesional and control cultures a few days after lifting (data not shown), and was no more detectable after 14 days (indicating that cells had ceased to proliferate under these *in vitro* conditions). It was still apparent, however, in basal and also several suprabasal layers in the culture of lesional cells. In addition, keratin 14 staining was detectable throughout the hyperplastic lesional cultures, and staining for keratin 1 and involucrin was in general weaker than in the controls and exhibited some dyskeratosis. In agreement with

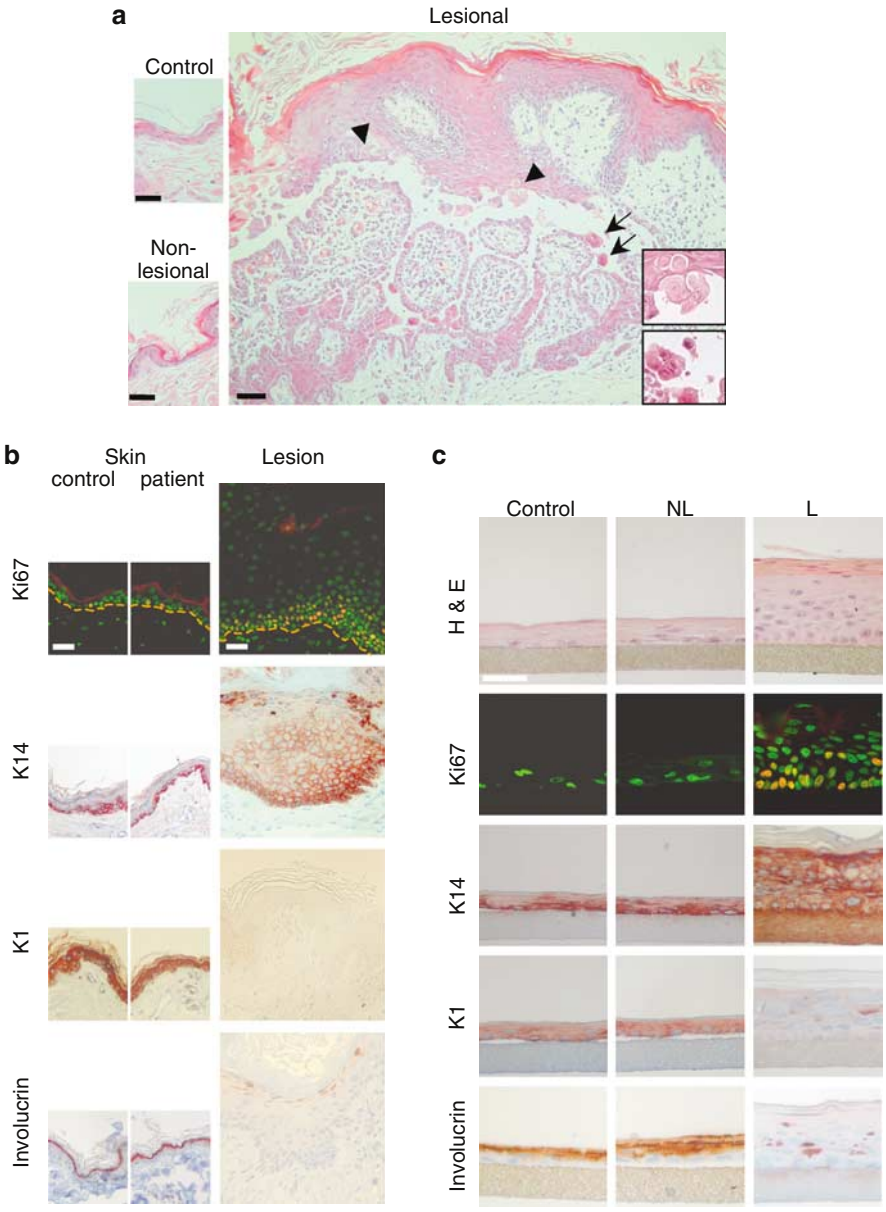

Figure 1. Lesional phenotype *in vivo* and *in vitro*. (**a**) Hematoxilin staining of a keratotic papule from the lower thigh of the affected dog compared with non-lesional skin of the same dog and control skin. Arrows point to acantholytic cells and arrowheads to dyskeratotic cells. Scale bars: 50 μm. Insets represent two times higher magnification of these cells. (**b**) Immunofluorescence and histochemical analysis of proliferation and differentiation markers of lesional and non-lesional epidermis of the affected dog and a control dog. Ki67 staining (red) also shows counterstained nuclei (green). Dotted line: basement membrane. Same magnification for all pictures, scale bars: 30 μm. (**c**) Immunohistochemical analysis of cultured keratinocytes grown at the air–liquid interface: hematoxilin and eosin-stained sections and immunohistochemical staining for indicated proliferation and terminal differentiation markers. Nuclei of Ki67-stained cells (red) were counterstained. Bar = 30 μm. Abbreviations: K14, keratin 14; K1, keratin 1; NL, non-lesional, and L, lesional keratinocytes; H&E, hematoxilin and eosin.

these data, extracts of differentiating submerged cultures, analyzed by Western blot analysis, revealed readily detectable keratin 1 and involucrin expression in non-lesional and control cells, but not detectable in keratin 1 and involucrin in lesional keratinocytes (see Figure 3f).

Collectively, these results demonstrate that the cultured keratinocytes reproduce the major characteristics seen in the original *in vivo* phenotype. In particular, they show that the lesional phenotype was propagated in culture, suggesting that it is irreversibly induced.

Phenotypic disease-related alterations

Histopathologically, the genodermatosis under study resembled Hailey–Hailey-like disease or Darier disease (Hailey and Hailey, 1939; Burge, 1992; Shanley *et al.*, 1992; Sueki *et al.*, 1997). As these diseases can be caused by calcium pump mutations (Sakuntabhai *et al.*, 1999; Hu *et al.*, 2000; Sudbrak *et al.*, 2000), we assessed the generation of calcium transients by confocal microscopy. Keratinocytes from the control dog held in calcium-free hyperfusion solution responded to the addition of increasing extracellular calcium

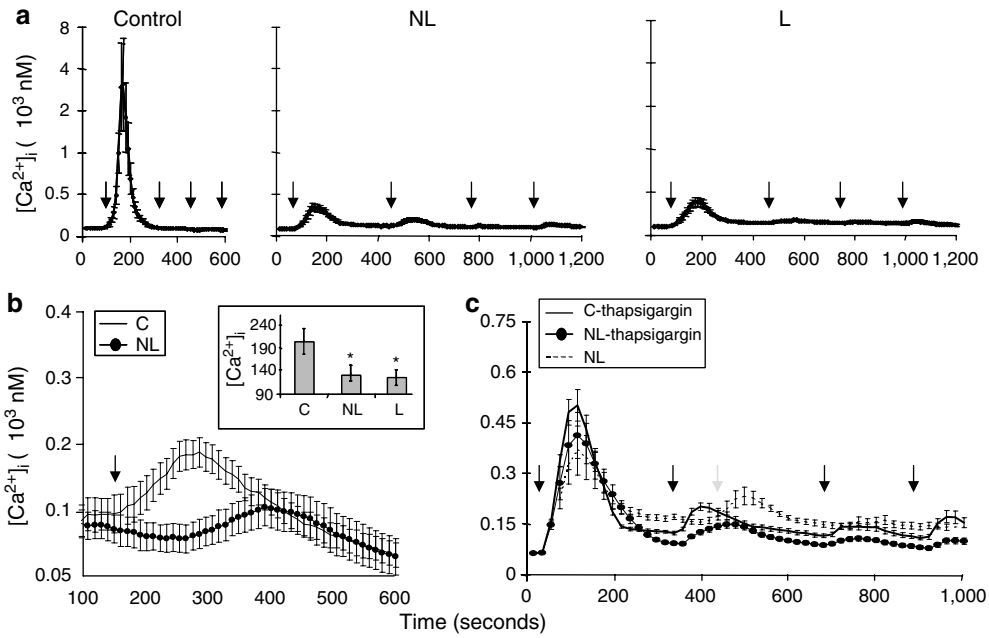

Figure 2. Generation of calcium transients in Fluo 3AM-loaded, cultured keratinocytes. (**a**) Increasing concentrations of extracellular calcium (arrows; 1, 2, 4, 10 mM) were injected into the calcium-free perfusion solution of keratinocyte cultures. The mean rise in fluorescence expressed as $[Ca^{2+}]_i$ is presented. Note that in control cells these values reproducibly were in the micromolar range similar to that reported for HaCat cells using the same buffer system (Biro *et al.*, 1998). (**b**) Thapsigargin was injected into the perfusion solution of the culture chamber (single arrow) and $[Ca^{2+}]_i$ assessed subsequently as in (**a**). The amount of calcium leakage was 2.3 times larger in control than in non-lesional cells as calculated from the surface area $t = 166–436$, base 0.095 for control cells and $t = 286–516$, base 0.08 for non-lesional cells. The inset shows the mean of the maximal calcium leakage from thapsigargin-sensitive stores into the cytosol. $^*P < 0.002$ (Bonferroni-corrected KruskalWallis test). (**c**) The same experiment was performed as in (**a**) except that the cells were incubated in thapsigargin before addition of 1 mM $CaCl_2$ (arrows). The dotted line is an overlay of the result obtained in panel a for non-lesional cells. Note that the second addition of $CaCl_2$ (light gray arrow) occurred at a later time point in (**a**) than in (**c**). (**a**–**c**) Measurements of 8–10 cells per treatment were made in duplicate. Results show one representative experiment repeated three times ± standard error. Abbreviations: NL, non-lesional; L, lesional keratinocytes; C, control.

on average with a 10-fold higher increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) than cultured keratinocytes from both lesional and non-lesional areas of the affected dog (Figure 2a).

To address whether the difference in the amplitude of calcium transients was due to depleted intracellular SERCA2-gated stores (as discussed in Darier disease (Sakuntabhai *et al.*, 1999)), we assessed their calcium level using the drug thapsigargin. Nanomolar concentrations of thapsigargin specifically inhibit SERCA pumps (Treiman *et al.*, 1998). Furthermore, the addition of thapsigargin to the cell culture induces an initial and transient increase in $[Ca^{2+}]_i$, which is representative for the level of stored calcium in the ER. In the absence of extracellular calcium, 50 nM thapsigargin caused a release of up to 100 nM calcium from the ER stores into the cytoplasm in control keratinocytes (Figure 2b and inset). In both non-lesional and lesional keratinocytes from the affected dog, the release of calcium was significantly lower (Figure 2b and inset), indicative of substantially depleted SERCA2 stores. To demonstrate that the difference in generating calcium transients was due to depleted SERCA2-gated calcium stores, we depleted them by pre-incubation with thapsigargin before addition of extracellular calcium (Figure 2c). In control keratinocytes, the $[Ca^{2+}]_i$ response to extracellular calcium was almost 9-fold lower than the response seen without thapsigargin pre-treatment (compare Figures 2c and a). In contrast, thapsigargin pre-incubation had virtually no effect on the response of the diseased dog keratinocytes (Figure 2c). Collectively, these results demonstrate that thapsigargin-sensitive, SERCA2-gated calcium stores were depleted in keratinocytes from the affected dog, and they further suggest that this depletion substantially reduced responsiveness to extracellular calcium in terms of $[Ca^{2+}]_i$ mobilization through presumably store-operated calcium channels (Parekh and Putney, 2005).

Consequences of depleted SERCA2-gated stores on intercellular junction assembly and terminal differentiation

The consequences of low intracellular calcium stores on the kinetics of junction assembly was addressed using a modified version of the adhesion assay described previously (Caldelari *et al.*, 2001), which was designed to visualize minimal differences in the establishment of intercellular adhesion (Figure 3a). Briefly, during the course of establishing intercellular adhesion, cells were incubated with dispase II, a protease that digests cell–substrate adhesion, and cellular sheets were inspected visually. In this assay, no or weak intercellular adhesion was characterized by round single cells as seen in low calcium medium (0, Figure 3a), which coincided with the absence of membrane staining for

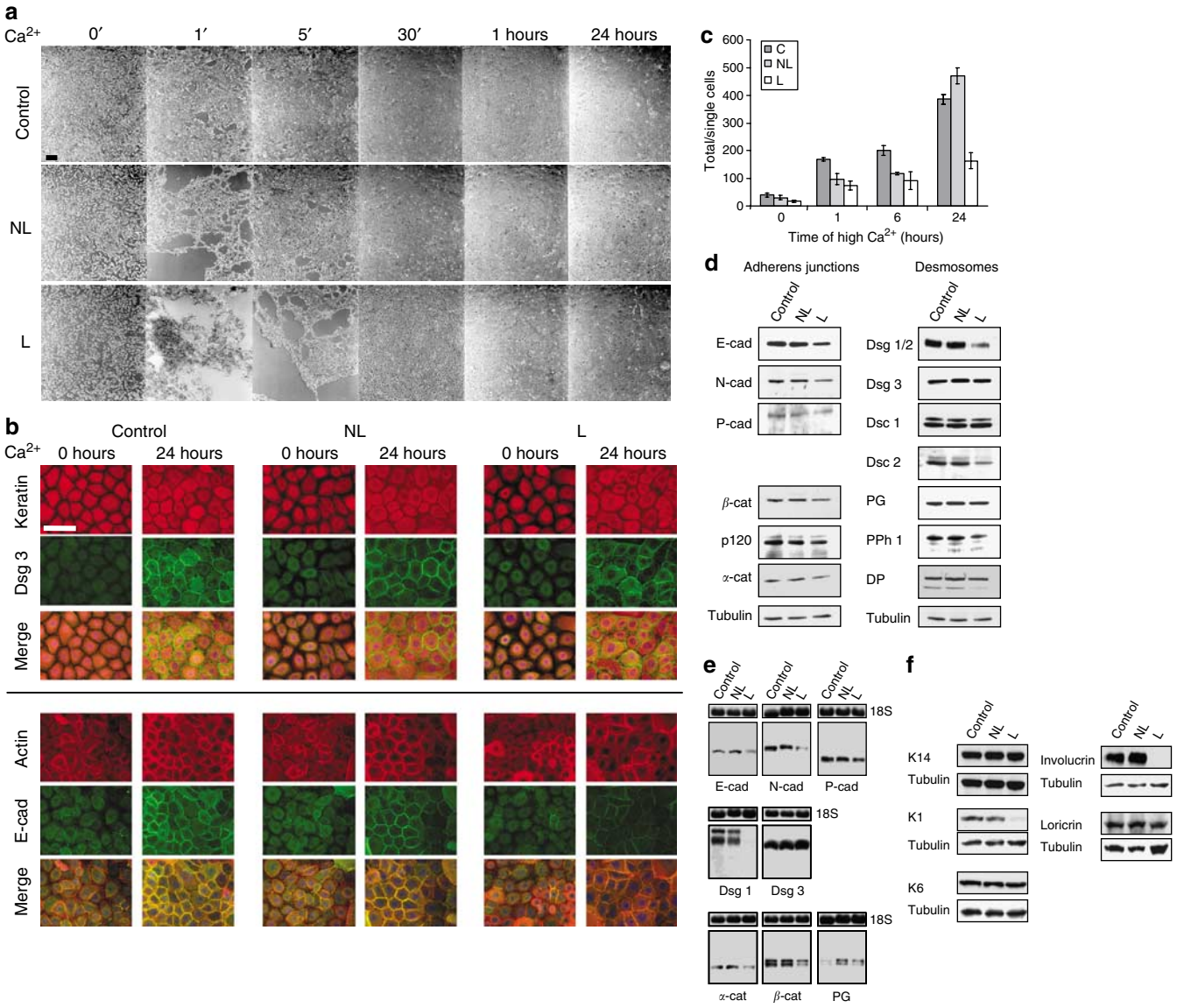

Figure 3. Establishment of intercellular adhesion in response to elevated extracellular calcium. (**a**) Cells incubated for the indicated length of time in high calcium medium are shown after dispase II treatment for 15 minutes. Scale bar: 200 μm. (**b**) Double immunofluorescence labeling demonstrates distribution of adhesion molecules and cytoskeletal components after a 24-hour incubation in high calcium medium (green: desmoglein 3 or E-cadherin, red: keratin or actin). Scale bar: 20 μm. (**c**) Intercellular adhesiveness was quantified during the 24 hours after incubation in high calcium medium using the adhesion assay. Results show the mean ratio of total over single cells (high values indicate high intercellular adhesive strength) from two independent experiments performed in triplicates. Bars represent standard error (L *vs* NL and C after 24 hours, $P<0.001$ using a Bonferroni-corrected Kruskal–Wallis test). (**d**) The steady-state levels of adherens junction and desmosomal proteins were assessed by Western blot analysis in total cell lysates from cultures after 3 days in high calcium medium. (**e**) In parallel to the experiment in (**d**), the steady-state level of junctional mRNA was analyzed by Northern blot analysis. (**f**) Western blots with total lysates from cells after 5 days in high calcium were assessed for steady-state expression of differentiation markers. Experiments depicted in (**d–f**) were performed at least four times. Abbreviations as in Figure 1b and Dsg 3 and Dsg 1, 2, desmoglein 3 and 1/2; E-, N-, P-cad, E-, N- and P-cadherin; Dsc 1, 2, 3, desmocollin 1, 2, 3; PG, plakoglobin; PPh 1, 3, plakophilin 1, 3; DP, desmoplakin; α-cat, α-catenin; β-cat, β-catenin; tub, tubulin; NL, non-lesional cells; L, lesional.

intercellular adhesion components in double-labeling immunofluorescence analyses (0 hour; Figure 3b). Incubation of the different cell cultures in high calcium medium for 24 hours followed by dispase II treatment resulted in an intact cellular sheet in all three cultures (24 hours, Figure 3a), indicative for establishment of intercellular adhesion between most cells in the monolayer. This positively correlated with a continuous linear staining pattern for adherens junction (E-cadherin) and desmosomal (desmoglein 3) proteins at cell contacts, and the belt-like or radial organization of actin and keratin filaments, respectively, as seen by immunofluorescence analysis (24 hours, Figure 3b). Between the time point of no and established adhesion, prominent holes formed in the monolayer (Figure 3a). Compared with

control cells, non-lesional and lesional cell layers from the diseased dog had consistently more holes after 1 and 5 minutes of high calcium treatment, indicating that establishment of intercellular adhesion occurred, however, with some delay. In cells of lesional origin, the delay was more pronounced. Nevertheless, after 1 hour of high calcium incubation, no holes were detectable in any cellular sheet.

To quantify adhesive strength, we performed an adhesion assay in which mechanical stress is applied to the dispase-treated cellular sheet (Caldelari *et al.*, 2001). After 24 hours in high calcium medium, the adhesive capacity of confluent non-lesional and control cells was similar, despite an initially slower development of intercellular adhesion in the non-lesional cells (Figure 3c). These results correlated with the finding that at a time point of presumably fully established adhesion (3 days in high calcium medium), the steady-state levels of the main adherens junction and desmosomal proteins were indistinguishable between non-lesional and normal control keratinocytes (Figure 3d). In contrast, adhesive strength in keratinocytes of lesional origin was nearly three times weaker than the control cells after 24 hours of incubation (Figure 3c). This correlated with decreased steady-state levels of most adherens junction and desmosomal proteins (with exception of desmoglein (Dsg) 3, Dsc 1, and plakoglobin). We also confirmed that the distribution of membrane *versus* cytoplasmic adhesion components in non-lesional cells was the same as in control cells while the levels of those proteins found to be altered were overall lower in lesional cells (data not shown). These results indicated that impairment of adhesion was restricted to lesional cells at both the adherens junction and desmosomal level (Figure 3c). It is also noteworthy that prolonged incubation in high calcium medium failed to further upregulate adhesion molecule expression in these cells (data not shown).

Interestingly, junctional protein expression fully correlated with the steady-state levels of the corresponding mRNAs as demonstrated by Northern blot analysis, that is, no difference in non-lesional cells *versus* controls, and major differences in lesional cells (Figure 3e). For lesional cells, this suggested altered regulation at the transcriptional or post-transcriptional level. Of all the junctional components for which both protein and mRNA were measured, only plakoglobin exhibited some discrepancy. The plakoglobin protein levels were overall similar, but its mRNA was lower in control cells compared to non-lesional and lesional cells.

It was already demonstrated in this study that cells of lesional origin form a thick hyperplastic sheet when grown at the air–liquid interface, and that the expression of differentiation markers was low in these cultures (see Figures 1b and c). To gain further insights into the capacity of cells from lesional and non-lesional sites of the affected dog to undergo terminal differentiation, we evaluated expression of the major differentiation markers after 5 days of submerged culture in high calcium medium (Figure 3f). The steady-state level of keratin 1 was investigated along with several products that constitute the cornified envelope, such as involucrin and loricrin. We also addressed expression of keratin 14, a marker of proliferating cells, and keratin 6, which is also expressed to some extent in cell cultures. Similar to the expression of adhesion molecules, there was no difference between control cells and cells of non-lesional origin for any of these markers. In contrast, in lesional cells the expression of differentiation markers keratin 1 and involucrin was substantially decreased while loricrin was slightly reduced. Collectively, these data demonstrate that the altered calcium homeostasis, and an initial delay in the assembly of cell–cell adhesion, failed to affect the capability of non-lesional cells from the affected dog to assemble intercellular adhesion in the long term, to undergo terminal differentiation, and to finally, form an intact epidermal sheet. In contrast, despite a comparable low level of stored intracellular calcium (Figure 2b), the lesional cells showed an irreversibly impaired ability to form intercellular junctions and severely affected differentiation both *in vivo* and *in vitro* (Figures 1a and b). These profound differences corroborate the hypothesis that irreversible changes, possibly additional somatic mutations, have occurred in the lesional areas.

Consequences of depleted ER stores on cell cycle regulation

So far our results demonstrate that lesional cells are dramatically perturbed in the terminal differentiation process, whereas the changes in non-lesional keratinocytes with similarly low generation of cytosolic calcium transients were restricted to some delay in establishing intercellular adhesion. We expected the primary molecular consequences of depleted ER stores to manifest initially in non-lesional keratinocyte function. Hence, we focused our interest on alterations in the cellular processes of non-lesional cells.

First, we addressed the consequences of depleted ER stores on the exit from the cell cycle *in vivo*. Various sections from the skin, lip, and esophagus were stained with Ki67 (which is expressed in all phases of the cell cycle) and compared with the corresponding sections of the control dog (Table 1). As described above, Ki67-positive cells are restricted to the basal cell layer of the epidermis in both control and non-lesional skin (Figure 1b). In spite of this common feature, the number of Ki67-positive cells in the non-lesional epidermis and lip epithelium (data not shown) from the affected dog was found to significantly exceed those in controls by approximately three times (Table 1). Biopsies from two other control dogs exhibited the same difference to the non-lesional cells (data not shown). To address whether the higher number of proliferative cells was counterbalanced by enhanced cell death, we evaluated the apoptotic cells by a TUNEL assay (Table 1). TUNEL-positive cells were only rarely seen throughout the epidermis, but those in non-lesional skin (and also lesional skin used for comparison) of the affected dog exceeded those in control skin significantly. Collectively, these tangible differences between non-lesional and control skin indicated some alterations in cell cycle regulation, which in particular appeared to lead to enhanced apoptosis and a delayed exit of basal keratinocytes from the cell cycle into the quiescent stage.

The latter finding was further addressed by monitoring BrdU incorporation in non-lesional *versus* control keratinocyte cultures during their proliferative phase, and the

Table 1. Ki67-positive and apoptotic cells *in vivo*

Skin	Ki67$^+$ (%)	Count (*n*)	χ^2	Apoptotic$^+$ (%)	Count (*n*)	χ^2
Control	2	1,216		0.25	5,195	
Non-lesional	7	737	$P<0.001$	1.06	10,830	$P<0.001$
Lesional	32	600	$P<0.001$	1.50	3,000	$P<0.001$

The χ^2 test was used to compare the percentage of Ki67-positive and apoptotic cells in lesional and non-lesional skin with control skin. Note that percentage values are based on the number (*n*) of counted cells.

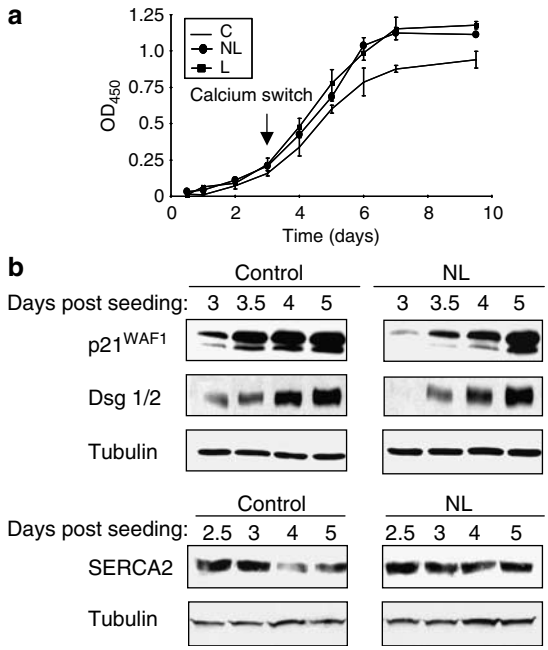

Figure 4. Proliferation and exit from cell cycle in cultured keratinocytes. (**a**) BrdU incorporation during proliferation and calcium-induced differentiation (calcium switch) is shown over time. Bars represent standard error (C *vs* NL from day 5 onwards, $P<0.004$ using a Bonferroni-corrected Kruskal–Wallis test). (**b**) Western blot analysis of lysates at the transition between proliferation to onset of terminal differentiation (at 2.5–5 days post seeding). Despite comparable cell densities (**a**), a delay of 1–1.5 days was observed with respect to p21^{WAF1} and Dsg 1 upregulation in non-lesional cells. For each individual antibody, the blots were incubated together and exposed for the same length of time. Tubulin was used as a loading control and is shown in the upper panel for the p21^{WAF1} blot. One of three representative experiments is shown. Abbreviations: NL, non-lesional; L, lesional keratinocytes; C, control.

subsequent exit from the cell cycle (Figure 4a). Lesional cells were used for comparison. In the linear phase (proliferation and the last mitotic divisions before cell cycle exit), BrdU incorporation in lesional and non-lesional cells did not significantly differ from control cells. However, between days 5 and 6, BrdU incorporation started to cease in control cells, but continued in lesional and non-lesional cells in a linear fashion for another day before reaching the plateau. The net result was a significantly higher BrdU incorporation in cells of lesional and non-lesional origin. Consistent with the results obtained *in vivo*, this indicated a delayed exit of cells from the canine patient into the quiescent stage.

To substantiate these findings, we assessed the steady-state level of p21^{WAF1} protein, a cyclin-dependent kinase inhibitor, during proliferation and subsequent onset of terminal differentiation (Figure 4b). During the proliferative phase, the steady-state level of p21^{WAF1} seen in Western blot analysis of a cycling cell population was low (Figure 4b, day 2.5–3 post seeding; see Figure 4a for kinetics). Consistent with our previous findings in canine cells (Kolly *et al.*, 2005), p21^{WAF1} protein increased when cells start to exit the cell cycle (Figure 4b, day 3–3.5 post seeding). However, in non-lesional cells, the steady-state level of p21^{WAF1} reached that seen in control cells with a delay of 1–1.5 days in spite of the fact that no significant difference was observed at that stage in the proliferation rate between the different cell types (Figure 4a). This delay was further paralleled by retarded stabilization of Dsg 1/2 protein assessed in the same lysates (Figure 4b, upper panel). SERCA2 protein, which was found to be mutated in 50% of Darier disease patients (Ringpfeil *et al.*, 2001; Ikeda *et al.*, 2003), showed comparable levels between control and non-lesional cells at days 2.5 and 3, but its decline was delayed thereafter in non-lesional cells (Figure 4b, lower panel).

DISCUSSION

Collectively, our results on non-lesional skin demonstrate that calcium depletion of the ER has a primary effect on cell cycle regulation including impaired upregulation of p21^{WAF1}. During keratinocyte differentiation, this defect postpones the exit of keratinocytes into the quiescent stage both *in vitro* and *in vivo*. Past this initial delay, the terminal differentiation process proceeds unperturbed, consistent with a normal epidermal morphology as seen in the patient outside of focal lesions. These findings rule out any major primary consequences of calcium-depleted ER stores on the establishment of normal tissue architecture. However, in case of trauma such as mechanical stress, heat or UV irradiation, compromised cell cycle control and in particular delayed upregulation of p21^{WAF1} is known to be detrimental as it permits cell cycle progression without repair (Shapiro and Harper, 1999; Lee and Yang, 2001; Weinberg and Denning, 2002). In case of DNA damage, this can lead to an accumulation of secondary somatic mutations, and ultimately formation of persistent lesions. Alternatively, if the microtubular and DNA damages are too important, uncontrolled progression through the cell cycle results in cell death.

It is possible that persistent lesions observed in the dog family are due to a higher incidence in somatic mutations. Although this remains to be demonstrated, such a hypothesis is supported by the fact that the lesional phenotype persisted in cell culture. It is also in line with the finding that lesional cells were indistinguishable from non-lesional cells with respect to their altered calcium homeostasis, but showed dramatic changes in adhesion molecule (both at the mRNA and protein level) and differentiation marker expression. In fact, genomic instability in conjunction with defective SERCA2 function was reported in a long-term study with SERCA2$^{+/-}$ mice (Liu *et al.*, 2001). Aged SERCA2$^{+/-}$ mice suffered from haploinsufficiency, and were found to have a

high incidence of papillomas or squamous cell tumors that are known to arise from somatic mutations (Yuspa, 1998). Similarly, ectopic long-term treatment of mice with thapsigargin together with non-12-*O*-tetradecanoylphorbol-13-acetate increased the incidence of mainly papillomas, but also squamous cell carcinomas (Hakii *et al*., 1986; Lowry *et al*., 1996). Moreover, squamous cell carcinomas and papillomas have been described in some Darier disease patients (Orihuela *et al*., 1995; Downs *et al*., 1997; Shimizu *et al*., 2000), of which the frequently observed keratotic papules might be the precursors.

The importance of $p21^{WAF1}$ in G1 and at the G2/M check point arrest following DNA and cellular damage has been well documented in keratinocytes (Maeda *et al*., 2002; Weinberg and Denning, 2002). In contrast, the mode of $p21^{WAF1}$ regulation during these processes is less well understood. Evidence for an involvement of ER calcium stores in upregulating $p21^{WAF1}$ at the G1 and G2/M check points is indirect and warrants further investigations. However, their involvement is compatible with the consequences of altered calcium homeostasis we observed in the diseased dog. First, the genomic instability discussed above suggests compromised activation of the tumor suppressor p53. p53 upregulates $p21^{WAF1}$ in case of DNA damage at the G1 and in particular at the G2/M check point (Maeda *et al*., 2002; Hoogervorst *et al*., 2005). Upstream of $p21^{WAF1}$, p53 is activated by $p38^{MAPK}$ in UVB-irradiated human keratinocytes and osmotic shock-exposed epithelial cells and fibroblasts (Kishi *et al*., 2001; Chouinard *et al*., 2002). $p38^{MAPK}$ activation, in turn, can critically depend on Ca^{2+}/calmodulin as was demonstrated for instance in ischemic PC12 cells (Conrad *et al*., 2000). This sequence of events provides a potential link between ER calcium stores and damage-induced check-point arrest via $p53/p21^{WAF1}$, which, if compromised, could promote genomic instability. Second, in irradiated human keratinocytes, $p38^{MAPK}/p53/p21^{WAF1}$ induction was found to confer resistance to apoptosis (Chouinard *et al*., 2002), and keratinocytes with disrupted check-point control such as p53 knockout keratinocytes consistently are more susceptible to apoptosis in response to DNA and microtubular damage (Shapiro and Harper, 1999; Maeda *et al*., 2002). In fact, even without apparent trauma apoptosis was significantly enhanced in the skin of the canine patient reported here, thereby corresponding with a recently described feature in human Darier disease patients (Aronchik I, Racs E, LLi L, Behne MJ, Mauro T (2004) Apoptotic signaling pathways in Darier's disease. *J Invest Derm (Ed)*, Society for Investigative Dermatology, Rhode Island Convetion Center, Providence, RI Abstract 541). This provides further support of compromised $p38^{MAPK}/p53/p21^{WAF1}$-mediated cell cycle arrest and consequent enhanced apoptosis, which could explain the susceptibility to trauma described in the canine patient (Shanley *et al*., 1992) as well as in human Darier disease patients (Burge and Wilkinson, 1992). Together, these collective studies suggest that the ER calcium stores are not only involved in the G1/G0 transition preceding keratinocyte terminal differentiation (Santini *et al*., 2001), but are also critical for the $p53/p21^{WAF1}$-mediated check point controls, which when compromised are known to support accumulation of secondary mutations or alternatively apoptosis in response to DNA and cellular damage.

Finally, the question arises which gene is mutated in this canine dermatosis. As the disease pattern, histologically and physiologically, resembles Darier disease, one possibility is a mutation in the *ATP2A2* gene that affects protein function rather than protein production (as the SERCA2 levels were not substantially altered). Alternatively, in around half of all Darier disease patients, the Darier disease phenotype could not be attributed to a mutation in the *ATP2A2* gene (Ringpfeil *et al*., 2001; Ikeda *et al*., 2003). It is therefore possible that the genodermatosis in the dog family, as seen in these other Darier disease patients, affects another gene whose product regulates the ER calcium levels, like IP3 receptors (Berridge *et al*., 2000) or calreticulin, the major calcium-binding chaperon in the ER (Groenendyk *et al*., 2004). Malfunction of calreticulin would be highly compatible with the depletion and delayed release of ER calcium stores described here in response to thapsigargin while that of both IP3 receptor and calreticulin would be consistent with impaired generation of cytoplasmic calcium transients.

In conclusion, our in-depth analysis of epidermal keratinocytes both *in vivo* and *in vitro* from the uninvolved skin of a patient with depleted SERCA2-gated calcium stores proposes a novel model for the molecular consequences of this defect in epidermal keratinocytes. Rather than having a harmful effect on keratinocyte terminal differentiation and establishment of intercellular adhesion, calcium-depleted ER stores, via, for example, a delay in $p21^{WAF1}$ upregulation, may primarily manifest during tissue damage. Although unproved, such a mechanism could increase susceptibility to trauma due to enhanced apoptosis, and alternatively lead to a higher incidence of secondary somatic mutations and finally chronic lesions, phenotypes that are compatible with focal lesions as opposed to a generalized dermatosis.

MATERIALS AND METHODS

Subjects and cell cultures

One of two related male English setters with persistent keratotic paules (Shanley *et al*., 1992; Sueki *et al*., 1997) was bred to a laboratory Beagle to enable investigations on disease pathogenesis. Two out of five offsprings developed analogous focal lesions with marked papillomatous acanthosis, hyperkeratosis and localized dyskeratosis and acantholysis on light microscopy. For further investigations, a typical lesion from the upper lateral thigh of the one affected dog kept at the animal facility was selected (Figure 1a). Biopsies from lesional skin and the collateral non-lesional thigh were used for immunohistochemical analysis as well as for the establishment of two independent keratinocyte cultures of each lesional and non-lesional sites (Suter *et al*., 1991b; Kolly *et al*., 2005) using the canine keratinocyte medium CnT-09 (CELLnTEC advanced cell systems AG, Bern, Switzerland; termed hereafter as high calcium medium). Biopsies from the same body site of two non-related Beagle and one Bernese Mountain dog were used as *in vivo* controls and for the establishment of control cultures. The Bernese Mountain dog was chosen to ensure that parameters of cell cycle control and apoptosis obtained in control Beagles matched those of normal

animals of an unrelated genetic background. Note that results obtained from matching cultures were comparable. Before experimentation, cells were seeded at 3×10^4 in low calcium medium Cnt-02 (0.09 mM $CaCl_2$; CELLnTEC advanced cell systems AG, Bern, Switzerland) containing 5% fetal calf serum and switched to CnT-09 (high calcium medium) without fetal calf serum at confluency, except if stated otherwise. To produce ''lifted cultures'', cells were grown in CnT-09 at the air–liquid interface for 14 days as described earlier (Suter *et al.*, 1991b). The ethical committee of the University of Bern, Vetsuisse Faculty and the Swiss office for biosafety approved all the described studies.

Antibodies, reagents, and probes

Primary antibodies were against desmoplakin I and II (for Western blot analysis: NW 161, K. Green, Northwestern University, Chicago, IL; for immunofluorescence: multi-epitope cocktail 2.15, 2.17, 2.20 Progen, Heidelberg, Germany), plakophilin 1 (11C6), plakoglobin (Pg5.1) and β-catenin (10C4; the latter three from M. Wheelock, Nebraska Medical Center, Omaha, NE), α-catenin (Zymed, San Francisco, CA), p120 (clone 98, Transduction Laboratories, Lexington, KY, Germany), E-cadherin (DECMA, R. Kemler, Max-Planck Institute, Freiburg i.Br., Germany), P-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), N-cadherin and Dsc2 (the latter two from P. Wheelock, Nebraska Medical Center, Omaha, NE), Dsg 1/2 (DG 3.10, Progen, Heidelberg, Germany), Dsg 3 (RDI, Flanders, Netherlands), Dsc 1 (D.R. Garrod, University of Manchester, Manchester, UK), pan-keratin (LP34, Dako, Glostrup, Denmark), keratin 14 (BioGenex, San Ramon, CA), keratin 6 (P. Coulombe, John Hopkins University School of Medicine, Baltimore, NH), keratin 1 (AF 109), filaggrin (AF 111) and loricrin (AF 62; the latter three from Covance, Richmond, CA), involucrin (SY5, F. Watt, Imperial Cancer Research Fund, London, UK), tyrosine tubulin (TUB1A2, Sigma, Saint Louis, MO), Ki67 (7B11, Zymed, San Francisco, CA), SERCA2 (VE121G9, gifted by Novo Castra Laboratories, Newcastle uponTyne, UK), $p21^{WAF1}$ (SC J97, Santa Cruz Biotechnology, Santa Cruz, CA), and TexasRed-conjugated reagent phalloidin (A. Hemphill, University of Bern, Bern, Switzerland). The antibody specificity was tested by omitting the primary antibody and testing various dilutions of primary antibodies. The cDNAs used were: canine Dsg 1 and 3 (our laboratory: (Müller *et al.*, 2000), Dsg 3 unpublished), canine E-cadherin (L. Rubin, EISAI London Research, London, UK), human P-cadherin (S. Hirohashi, National Cancer Center Research Institute, Tokyo, Japan), human N-cadherin (A. Ben-Ze'ev, Weizmann Institute for Science, Rehovot, Israel), mouse α-catenin, β-catenin, and human plakoglobin (R. Kemler, Max-Planck Institute, Freiburg, Germany), and rat 18S rDNA (R. Levine, Cornell University, Ithaca, NY).

Immunohistochemistry and immunofluorescence

Formalin-fixed and paraffin-embedded tissue and ''lifted culture'' sections were de-paraffinized and antibodies retrieved by 10–15 minutes at 100°C before incubation with antibodies for 1 hour in phosphate-buffered saline containing 2 mM $MgCl_2$/2 mM $CaCl_2$ (phosphate-buffered saline+) and 1% BSA. The assessment of apoptotic cells was performed on paraffin sections using a TUNEL kit (Appligene Oncor, Illkirch Graffenstaden, France) according to the manufacturer's protocol.

For double labeling of Dsg 3 and keratin-cultured keratinocytes grown on Lab-teks® chamber slides (Nalge Nunc International, Naperville, IL) were incubated with Dsg 3 antibodies in CnT-09 for 1 hour on ice before fixation and permeabilization with pre-cooled 100% methanol for 7 minutes at −20°C. This was followed by incubation with pan-keratin antibodies followed by secondary antibodies. For labeling of E-cadherin and the actin cytoskeleton with phalloidin, cells were fixed with 1% paraformaldehyde in phosphate-buffered saline+ for 10 minutes and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline+ for 10 minutes.

Calcium measurements using confocal microscopy

The experimental design was according to Hu *et al.* (2000). Briefly, keratinocytes were seeded in duplicates on collagen-coated glass cover slips. At 70% confluency, they were incubated with 1 μM cell-permeant Fluo-3 acetoxymethyl ester (Fluo-3 AM; TefLabs, Austin, TX) for 30 minutes at 37°C in calcium-free superfusion solution containing (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 10 glucose, pH 7.4. Cells were then viewed in an observation chamber with a ×40 oil-immersion objective and inverted microscope (Nikon TMD) (Fluor, NA = 1.3; Nikon). Fluo-3 was excited with the 488 nm line of an argon-ion laser at 150 μW intensity and detected at 540±15 nm. Fluorescence images were acquired with a confocal laser-scanning system (MRC 1000, Bio-Rad, Hercules, CA). The changes in intracellular fluorescence were monitored every 10 seconds in 8–12 marked cells after addition of increasing extracellular Ca^{2+} concentrations (1, 2, 4, 10 mM) or 50 nM thapsigargin into the superfusion solution at 37°C. Alternatively, cells were pre-treated with 50 nM thapsigargin for 1 hour at 37°C before addition of 1 mM extracellular calcium. Maximal fluorescence was measured after addition of 10 μM ionomycin and saturating calcium, minimal fluorescence for Fluo-3 is approximately zero. Readings of single cells were averaged after conversion of the fluorescence signals to cytoplasmic calcium concentration $[Ca^{2+}]_i$ using a Kd of 325 nM (Weinberg and Denning, 2002). The experiments were repeated three times.

Adhesion assay

The assay was performed in triplicates as previously described (Caldelari *et al.*, 2001) and in one case without applying mechanical stress (Figure 3a). As cell density is critical for the adhesive capacity, cells were processed at equal density, that is, at 100% density and $1 \times 10^5 \pm 0.1$ cells/cm^2. Each experiment was repeated two times.

Northern and Western blot analysis

Total RNA was isolated according to Chomczynski and Sacchi (1987) and total protein lysates generated by directly scraping the cells into radioimmunoprecipitation assay buffer. Northern and Western blot analyses were performed according to standard protocols.

BrdU measurement

The assay was performed with the Cell Proliferation ELISA (BrdU) from Roche diagnostics GmbH (Mannheim, Germany) according to the manufacturer's protocol. Briefly, 1 hour after seeding at 3×10^4, 10 μM BrdU was added to all wells, and refreshed every day during the entire experiment. Cells were harvested for assessment of BrdU incorporation in a 24-hour interval between 1 and 10 days, air-dried and kept at 4°C. The BrdU detection was performed simultaneously

for all samples within one experiment; the substrate reaction was stopped with H_2SO_4 and absorbance measured with a microplate reader (Molecular Devices) at 450 nm. Unlabeled cells were used as reference. The assay was repeated two times.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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