Dermatology Research and Practice

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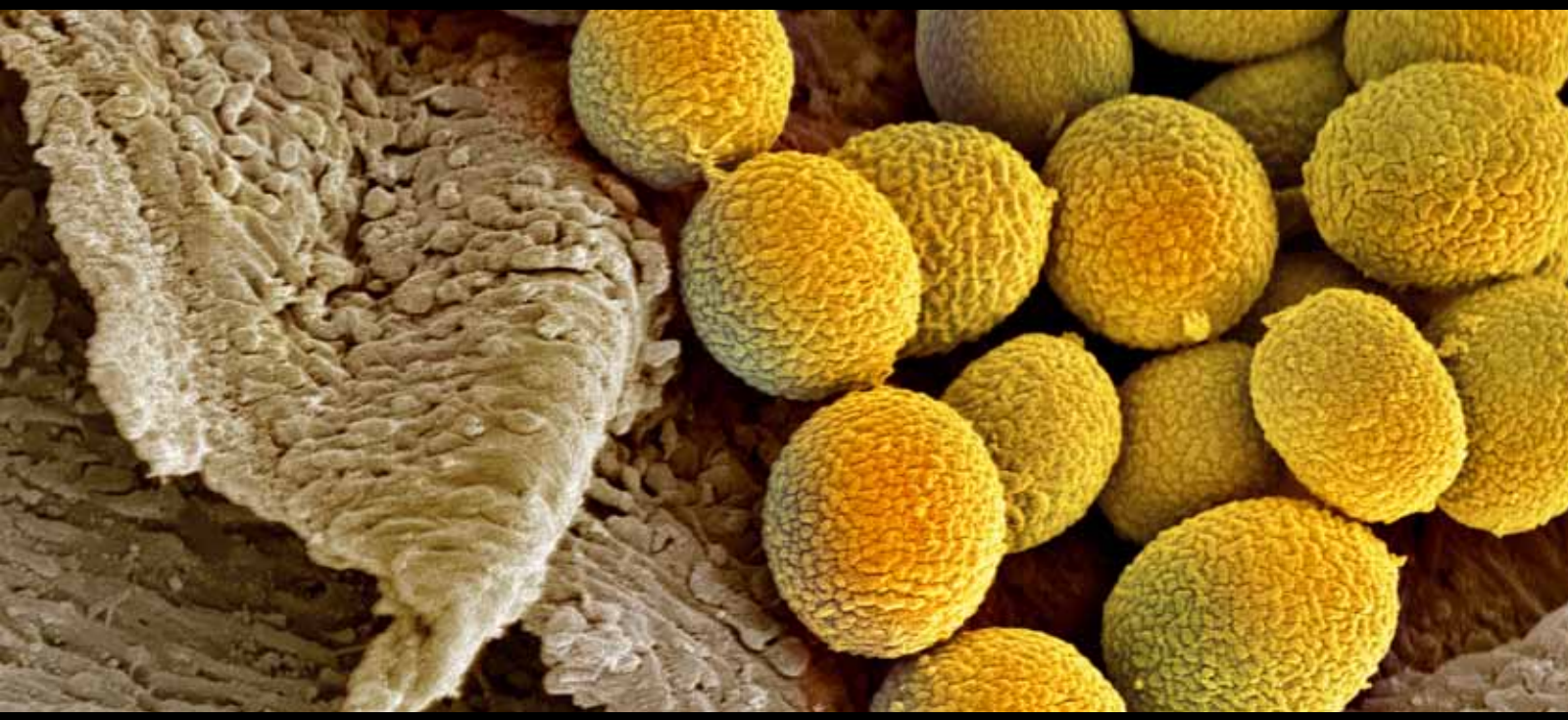
Guest Editors: Mỹ G. Mahoney, Eliane J.Müller, and Peter J. Koch



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Editorial Board

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Editorial

Desmosomes and Desmosomal Cadherin Function in Skin and Heart Diseases—Advancements in Basic and Clinical Research

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In the last two decades desmosomal research has developed from a highly specialized area of cell biology to an area of biomedical research aimed at elucidating the role of these cell junctions in tissue and organ development and homeostasis.

Until the late 1980s, desmosomal research focused, to a large extent, on the morphological characterization of desmosomes and the biochemical identification of desmosomal proteins. Towards the end of that decade, the first desmosomal transmembrane proteins (desmogleins and desmocollins) were cloned, demonstrating that these proteins are sequence related to cadherins, a family of calcium-dependent cell adhesion proteins. Subsequent knockout experiments in mice, published in the 1990s, demonstrated that at least some of the desmosomal cadherins (e.g., desmoglein 3) are required to maintain tissue integrity in the oral mucosa (see the paper of Ganeshan et al.). Around the same time, it was shown that patients who suffer from autoimmune diseases characterized by skin and mucous membrane blistering produce autoantibodies against desmogleins 1 and 3 (DSG1, DSG3). These two observations suggested that the loss of normal desmosome function could lead to tissue fragility disorders.

Subsequent to the identification of pemphigus as a desmosomal disease, reports began to emerge suggesting that mutations in desmosomal genes can cause a variety of skin blistering disorders and cardiomyopathy (see the paper by J. Li and G. L. Radice).

Recently the question has emerged: Are all desmosomal diseases caused by a loss of cell adhesion? Mounting evidence suggests that abnormal cell signaling might contribute to the pathophysiology of at least certain types of desmosomal diseases, such as pemphigus (see the paper of M. Bektas et al. and D. Brennan et al.). The putative contributions of cell adhesion defects and abnormal cell signaling in diseases like pemphigus are still under intense debate. We can expect to see a continued influx of exciting new findings in this area.

The present special issue is a collection of reviews and original research articles that focus on fundamental aspects of desmosome biology and on clinical research relating to desmosomal diseases of the skin, mucous membranes, and heart. These papers are also examples of how a synergistic approach that utilizes the tools of genetic engineering in mice, human genetics, cell and molecular biology and immunology have led to a significant advancement of our understanding of the pathological mechanisms underlying desmosomal diseases. We sincerely thank the authors for their high-quality and exciting contributions. We hope that these provocative papers will stimulate discussions and promote future collaborations.

Basic Biological Features

The paper entitled "*Desmosomes in vivo*" D. Garrod discusses the importance of desmosome assembly and disassembly

during development and in disorders such as blistering diseases of the skin. Most of our understanding of the underlying regulatory mechanisms has been gathered from investigations on cultured cells where desmosomes are in a calcium-dependent as opposed to a calcium-independent or hyperadhesive state. This thought-provoking review from a leader in the desmosomal field critically discusses the state of desmosomes in cell culture and in vivo, the properties of calcium-independent desmosomes, and their reversion to a calcium-dependent state during wound healing. Highly instructive for researchers interested in disorders affecting desmosomal structure and function, this paper is a "must" for young investigators in the field (Editor: E. J. Müller).

The paper entitled "*Exploring the nature of desmosomal cadherin associations in 3D*", by G. R. Owen and D. L. Stokes, states that the expression of desmosomal proteins is highly regulated in both a spatial and temporal manner, thus establishing an intricate, dynamic network of proteins that must provide both structural stability as well as flexibility to cells and tissues. This paper highlights the use of a state-of-the-art technique known as high-resolution electron tomography, to examine the interactions between cadherins within the desmosome structure. The findings from these studies offer us an architectural perspective of cadherin interactions and how the binding of pathogenic pemphigus antibodies to these cadherins could disrupt desmosome adhesion and induce intra-epithelial blistering (Editor: M. G. Mahoney).

"*Leaving the desmosome—the desmosomal plaque proteins of the plakophilin family*" by S. Neuber et al. reviews the diverse cell adhesion-dependent and adhesion-independent functions of junctional proteins. In this paper, the authors discuss the plakophilin family of proteins and the complexity of their roles, which ranges from cell signaling to organization of the cytoskeleton and control of protein biosynthesis. Aberrant expression and disrupted function of these proteins can result in abnormal tissue and organ development (Editor: M. G. Mahoney).

"*Desmosomes in developing human epidermis*" by S. Peltonen et al. tackles a rare topic: the distribution of desmosomes and other adhesive structures in the developing epidermis of human embryos. The authors encourage investigators in skin to complement these existing data by mechanistic analyses that can link up with current knowledge obtained from animal models. Of great value for the reader, this paper provides a comprehensive insight into the time line of epidermal morphogenesis from the surface ectoderm to the periderm and stratified epithelium (Editor: E. J. Müller).

Disease Models

The paper entitled "*Desmosomal molecules in and out of adhering junctions—normal and diseased states of epidermal, cardiac and mesenchymally derived cells*" by S. Pieperhoff et al. explores the heterogeneity of cell junctions in terms of morphology and composition and how these factors relate to the development of diseases. Special emphasis is given to cell-cell junctions of the mammalian heart, a target organ for severe diseases caused by impaired desmosome function (Editor: P. J. Koch).

"*A new prospective on intercalated disc organization—implications for heart disease*" by J. Li and G. L. Radice reviews arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D), an inherited fibrotic heart muscle disease resulting from mutations in many desmosomal proteins including plakoglobin, desmoplakin, plakophilin-2, and desmoglein-2. The skin and the heart are two tissues that undergo tremendous mechanical stress. Unlike in the skin, where the adherens junctions are distinct from the desmosomes, in the heart they are integrated into a specialized hybrid structure called the area composita, localized in the cardiac intercalated discs. In their paper, Li and Radice discuss the crosstalk among different junctions and their implications in the pathophysiology of ARVC/D (Editor: M. G. Mahoney).

The paper entitled "*Desmosomal component expression in normal, dysplastic and oral squamous cell carcinoma*" by N. Narayana et al. shows that during malignant transformation, cell-cell adhesion is often reorganized with dramatic changes in various junctional proteins. The authors showed that the desmomal plaque proteins desmoplakin and plakophilin-1 are downregulated in dyplasias and squamous cell carcinomas as compared to control epithelia. The results identify these proteins as potential markers for neoplastic lesions of the oral cavity (Editor: M. G. Mahoney).

In the paper entitled "*Loss of the desmosomal component perp impairs wound healing in vivo*" by V. G. Beaudry et al., the authors investigated cutaneous wound healing in mice with a conditional null mutation in the desmosomal Perp gene. The mice showed a delay in cutaneous wound healing, suggesting that desmosome formation or desmosome remodeling might play an important role during reepithelialization of skin wounds (Editor: P. J. Koch).

Wier et al.'s "*Experimental human cell and tissue models of pemphigus*" discusses the advantages and disadvantages of various in vitro skin models (keratinocyte cultures, raft cultures, reconstructed skin, and human and mouse keratinocyte grafts) used to study the pathophysiology of pemphigus, a group of human autoimmune bullous diseases (Editor: P. J. Koch).

"*Mouse models for blistering skin disorders*" by R. Ganeshan et al. discusses the important roles that autoantibodies play in autoimmune diseases such as pemphigus vulgaris, with antibodies targeting several proteins including the desmosomal cadherins, desmoglein-3, and desmocollin-3. In this paper the authors explore the expression patterns of desmoglein-3 and desmocollin-3 in various stratified epithelial tissues including skin. Furthermore, the authors discuss genetically engineered Dsg3-null and Dsc3-null mice, which develop blistering phenotypes similar to human pemphigus vulgaris patients, thus providing insights into the roles of these proteins in cell-cell adhesion and intra-epidermal blister formation (Editor: M. G. Mahoney).

The paper entitled "*Suprabasal Dsg2 expression limits epidermal blister formation mediated by pemphigus foliaceus antibodies and exfoliative toxins*" by D. Brennan et al. demonstrates that, upregulation of Dsg2 in the upper epidermis of mice can compensate for a loss of Dsg1 function induced

by pathogenic pemphigus foliaceus antibodies or exfoliative toxins, thus preventing blistering. This supports the idea that compensatory upregulation of desmogleins could be a therapeutic approach to suppress skin blistering in PF (Editor: P. J. Koch).

"*Apoptotic pathways in pemphigus*" by M. Bektas et al. is a comprehensive review of pemphigus, a group of devastating human autoimmune blistering diseases of the skin and oral mucosa. Although the major uncontested culprits of the disease are the autoantibodies to desmogleins, the pathomechanism of pemphigus is still a heavily debated topic. In this review, they explore the role of cellular apoptosis in pemphigus and summarize substantial evidence suggesting that blister formation can occur independent of apoptosis. However, the authors suggest that the activation of proapoptotic proteins may play important roles in sensitizing the keratinocytes to the acantholytic effects of pemphigus IgG (Editor: M. G. Mahoney).

In "*Targeted immunotherapy with rituximab leads to transient alteration of the IgG autoantibody profile in pemphigus vulgaris*" by Ralf Müller et al., the authors present a clinical evaluation of the B-cell-depleting antibody rituximap in pemphigus patients. The results support the notion that this treatment can lead to a reduction of pathogenic antibodies and, consequently, a temporary disappearance of disease symptoms (Editor, P. J Koch).

In "*Hypothesis concerning a potential involvement of ceramide in apoptosis and acantholysis induced by pemphigus autoantibodies*" by W. B. Bollag, the author presents an exciting and provocative new hypothesis linking certain types of pemphigus to abnormal ceramide metabolism (Editor: P. J. Koch).

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Review Article

Desmosomes In Vivo

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The structure, function, and regulation of desmosomal adhesion in vivo are discussed. Most desmosomes in tissues exhibit calcium-independent adhesion, which is strongly adhesive or "hyperadhesive". This is fundamental to tissue strength. Almost all studies in culture are done on weakly adhesive, calcium-dependent desmosomes, although hyperadhesion can be readily obtained in confluent cell culture. Calcium dependence is a default condition in vivo, found in wounds and embryonic development. Hyperadhesion appears to be associated with an ordered arrangement of the extracellular domains of the desmosomal cadherins, which gives rise to the intercellular midline identified in ultrastructural studies. This in turn probably depends on molecular order in the desmosomal plaque. Protein kinase C downregulates hyperadhesion and there is preliminary evidence that it may also be regulated by tyrosine kinases. Downregulation of desmosomes in vivo may occur by internalisation of whole desmosomes rather than disassembly. Hyperadhesion has implications for diseases such as pemphigus.

1. Introduction

Desmosomes are intercellular junctions that are uniquely able to provide very strong intercellular adhesion [1, 2]. This is especially important in tissues that are subject to mechanical stress such as epidermis and cardiac muscle. They are present in the lowliest, jawless vertebrates, hagfishes, but appear to be absent from our nearest chordate ancestors [3–7]. It is probable, therefore, that they have played a key role in vertebrate evolution by contributing to a strong integument and a powerful heart.

Desmosomes are composed of a small number of well-defined molecular components (Figure 1). These are their adhesion molecules, the desmosomal cadherins desmoglein and desmocollin, the plakin desmoplakin that links the adhesion molecules to the intermediate filaments (IFs), and the armadillo proteins plakoglobin and plakophilin that link the adhesion molecules to desmoplakin and appear to regulate desmosomal assembly and size. For details please see recent reviews in [1, 9–16].

2. Epithelial Cells In Vivo: Some Myths and Specific Junctional Considerations

The major topic of this review will be a consideration of how desmosomes function in vivo with particular reference to their adhesive properties. It is relatively easy to study cell behaviour and function in culture. Cellular organelles are readily visualized, extracted, and subjected to a wide range of analytical techniques. The results obtained are often striking, but are they relevant in vivo? At worst they may be complete artefacts; at best they may suggest a relevant in vivo mechanism, but this needs to be demonstrated. An ideal but perhaps unrealistic goal would be for all discoveries made in tissue culture to be followed by attempts to show their relevance in vivo.

We have a striking example of this in the field of cell adhesion. Focal contacts are small, usually elongated structures of the order of 1 μm in length where the basal surfaces of cells spread on solid surfaces in culture approach the substratum to a separation of about 15 nm [17]. Cytoplasmically they provide the insertion points for actin stress fibers. They are easily visualized by interference refection microscopy, electron microscopy, or fluorescence imaging of any one of a prodigious number of proteins that are associated with them [17, 18]. They are fascinating structures but what is their relevance in vivo? This issue seems hardly to have been addressed, though it is beginning to be [19, 20]. An extreme view, hopefully incorrect, would be that they are culture artifacts and that their study does little to elucidate relevant in vivo mechanisms of cell adhesion.

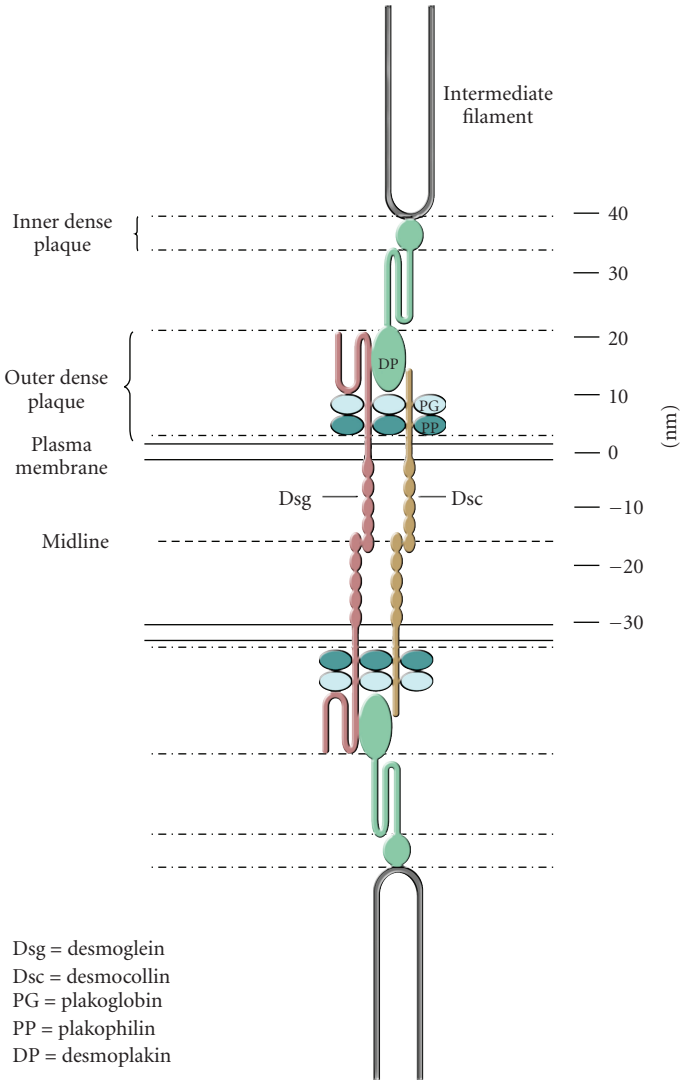

Figure 1: Schematic model of a desmosome showing the relative positions of the major desmosomal components. (See Figure 4 for an electron micrograph of desmosomes.) The scale on the right-hand side indicates distance in nanometres. The figure is largely based on the immunogold labelling experiments of North et al. [8]. Reproduced from the study by Garrod and Chidgey in [1].

Desmosomes occur predominantly in epithelial cells. In vivo these cells contribute to epithelia, which are continuous or confluent cell sheets providing dynamic barriers between body compartments. The confluence of the sheet is vital; without it the barrier function of the epithelium cannot be maintained. If confluence is broken in a healthy individual, the epithelium repairs itself to restore the barrier as rapidly as possible, as most easily and dramatically seen in epidermal wound healing.

Maintenance of confluence and barrier function appears to be a compulsive behaviour of epithelial cells. Intercellular junctions, including desmosomes, are key contributors to this process. Two commonly held myths in cell biology are that (i) cells lose adhesion during cell division and (ii) cell division is inhibited by intercellular contact, the so-called "contact inhibition of cell division". The former notion seems to have arisen from watching isolated fibroblasts round up when they divide in culture. Certainly cell division necessitates a change of shape and some remodelling of cell contacts. But epithelial cells in confluent sheets, both in culture and in vivo, retain tight and adherens junctions and desmosomes throughout cell division [21] (Figure 2). If this were not the case, the barrier function of the epithelium would be compromised every time a cell divided. The drive to maintain confluence and barrier function is also manifest when cells in epithelial cell sheets undergo apoptosis. The dying cell is often extruded from the sheet and this would leave a hole if nothing were done about it. In fact neighbours of the extruded cell move to fill the gap, zipping up junctions as they go and presumably enabling a seamless continuity of barrier function [22]. Although junctions are maintained throughout these processes, it is likely that modulation of junctions in localised regions of the cell surface may be required in order to facilitate cell shape changes.

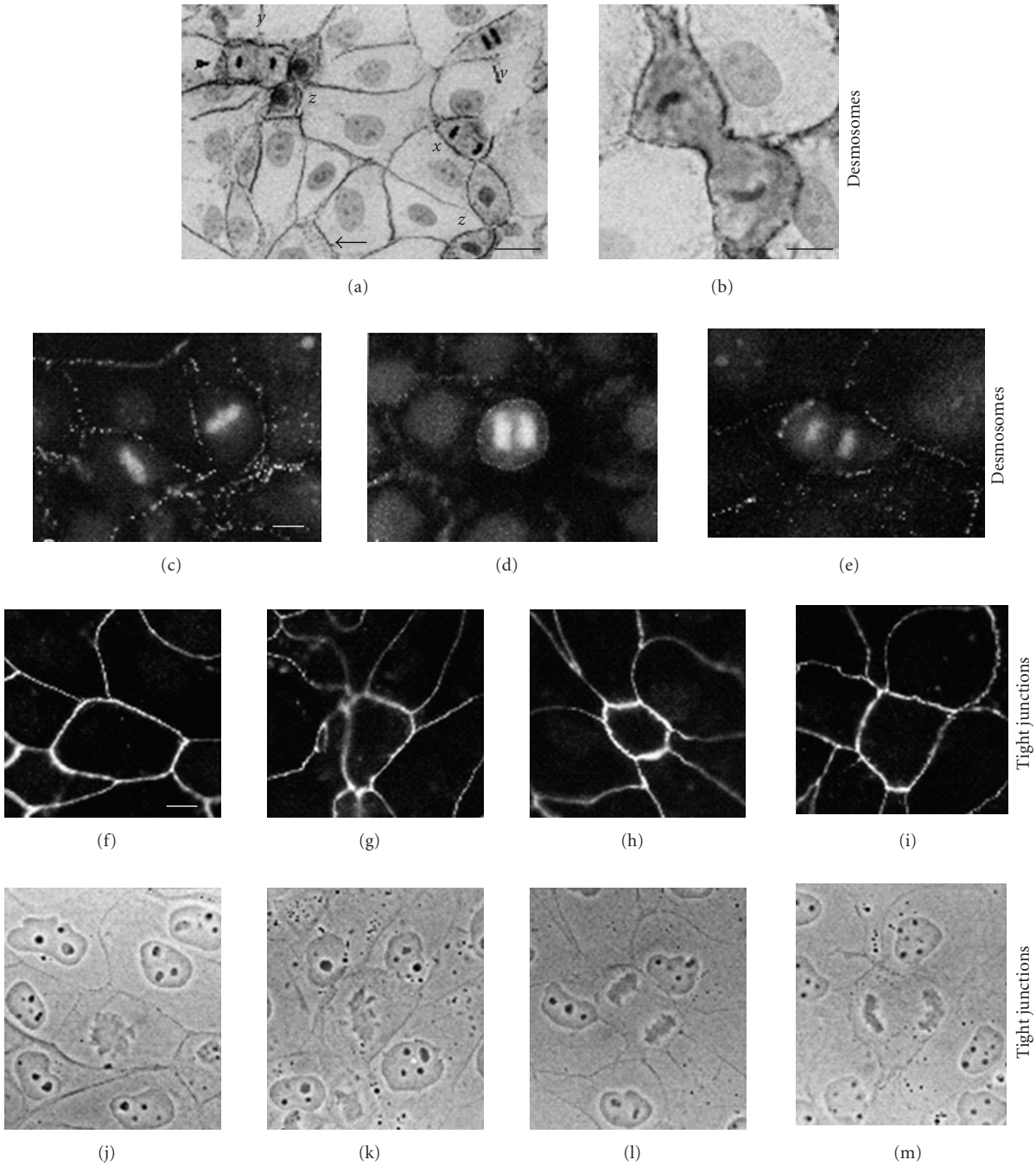

Figure 2: Epithelial cells retain junctional contact during cell division. (a) and (b) MDCK cells stained with monoclonal antibody to desmoplakin by the ABC technique and counterstained with haematoxylin. (a) Cells at various stages of division are shown: *w*, anaphase; *x*, telophase; *y*, early cytokinesis; *z*, advanced cytokinesis. All dividing cells show prominent peripheral staining for desmoplakin. In some cases (e.g., small arrowhead), the desmoplakin staining is punctate and not obviously confined to the cell periphery. However, similar staining is commonly seen in nondividing cells (e.g., large arrowhead) and appears to be due to oblique viewing of the cell interfaces. (b) High-power photographs of similar cell in advanced cytokinesis. Note the prominent peripheral desmoplakin staining, even at the borders of the cleavage furrow. Although the cytoplasm is generally more darkly staining than that of neighbouring non-dividing cells, this does not indicate desmosome internalisation (see following fluorescence micrographs). Bars: 20 m (a) and 10 m (b) (c)–(e) MDCK cells stained with monoclonal antibody to desmoplakin and propidium iodide to show chromosomes. (c) Two cells in metaphase, (d) cell in early anaphase, and (e) cell in late anaphase. All dividing cells show peripheral punctate staining for desmoplakin, comparable to that seen in non-dividing neighbours. However, no punctate staining indicative of desmosome internalisation is present in the cytoplasm of the dividing cells. Bar: 10 m. (f)–(i) MDCK cells stained with monoclonal antibody to tight junction protein ZO-1. (j)–(m) Corresponding phase-contrast images. The cell in (F,J) is in prophase, that in (g) and (k) in metaphase and those in the remaining pictures in telophase. Each dividing cell is surrounded by a complete ring of ZO-1 staining and shows no evidence of junction internalization or disruption. Dividing cells in epidermis and intestine also showed retention of junctions by electron microscopy. Bar: 10 m. Reproduced from the study by Baker and Garrod in [21].

Maintenance of cell-cell contact during cell division graphically demonstrates that division of epithelial cells is not inhibited by intercellular contact. A careful analysis of fibroblast division has shown that this is also not inhibited by contact [23]. A moment's thought about cells in vivo suffices to conclude that the very existence of multicellular organisms would be impossible if there were such a thing as "contact inhibition of cell division". In vivo all epithelial cells are in continuous or confluent cell sheets, yet many epithelia exhibit rapid cell turnover involving continuous cell division to replenish lost cells. How would this be possible if cell division were inhibited by intercellular contact? In saying this it is also recognised that cell-cell adhesion can contribute to the regulation of the plane and, in some cases, the rate of cell division and the latter may be particularly important in some situations, for example, in cancer [24–27].

The key reason for retention of junctions during cell division is that epithelial barriers must be maintained at all costs. Small skin wounds can be tolerated temporarily provided that the hole is rapidly plugged by a clot and that the epidermal covering is restored with reasonable alacrity. When the epidermal barrier is severely compromised, as in extensive burning, the consequences are lethal. Intercellular junctions are vital for the barrier as illustrated by some human mutations and engineered defects in mice [28–32] in which loss of junctional function compromises the epidermal barrier causing rapid neonatal death. Less obvious but equally important is the maintenance of internal epithelial barriers. Diminished barrier function in the gut gives rise to inflammatory bowel disease and airway epithelial barrier is compromised both in asthma and by invading allergens, leading to enhanced allergen delivery and respiratory allergy [33, 34].

When interpreting studies on cultured epithelial cells careful attention should be given to the degree of culture confluence under which the observations were made. Many such observations are made on subconfluent cells. This is fine provided it is recognised that subconfluent epithelial cells are in an abnormal or "activated" state broadly equivalent to cells at the edge of a wound. The behaviour of such cells is fundamentally different from the behaviour of epithelial cells in cell sheets in culture or, most important, in vivo. A simple example will illustrate this. Clumps of subconfluent cultured epithelial cells such as MDCK treated with hepatocytes growth factor (HGF) (scatter factor) will scatter, undergoing epithelial-mesenchymal transition (EMT) and downregulating their intercellular junctions [35, 36]. Other growth factors produce similar effects with other types of epithelial cell. However, treatment of confluent epithelial cells with HGF or a range of other growth factors has no effect whatsoever on cell-cell adhesion [37]. Thus, although scattering by growth factors and EMT are fascinating behaviours, some change in the epithelium equivalent to wounding or "activation" would appear necessary for them to become relevant to an epithelium in vivo. However, the essential role for such signalling mechanisms in specific in vivo aspects of cell migration, for example, limb bud development, is clear [38].

For many purposes confluent cells are less easy to study than subconfluent cells. However, confluence is essential if it is desired to approach in culture the "normal" situation for epithelial cells in vivo.

3. Calcium-Induced Desmosome Assembly and Its Significance

It is frequently stated that desmosomal adhesion is calcium dependent. This is because of the following. (a) The desmosomal adhesion molecules, desmocollin and desmoglein, are members of the cadherin family and bind calcium; (b) desmosomes (and other junctions) do not form when cells are cultured at an extracellular calcium concentration of <0.1 mM; (c) when the calcium concentration is raised, desmosomes form rapidly between cells cultured as in (b); (d) desmosomal adhesion is lost by subconfluent or early confluent cells when extracellular calcium is depleted.

This is all indisputable but it must be realised that desmosomes in tissues do not behave and are not regulated in this way. In the first place the calcium concentration of tissue fluid is rigorously maintained at about 1 mM. Variations from this level have serious consequences for many bodily functions including nerve conduction and muscle contraction. It is therefore unlikely in the extreme that variations in extracellular calcium concentration have anything to do with the regulation of cell adhesion in vivo. At worst, observations on junctional changes induced by "calcium switching" in culture may be completely artefactual. On the other hand "calcium switching" may be a suitable model for junction assembly in vivo, but this needs to be rigorously tested.

Calcium-induced desmosome assembly appears to be a simple process of stabilisation of adhesion. Cells cultured in low-calcium medium (LCM) synthesise desmosomal components [39, 40]. The desmosomal cadherins, or assemblies of desmosomal proteins in the form of half-desmosomes, are transported to the cell surface where they are unable to participate in adhesive binding [39, 41]. They are therefore internalised and degraded, so in LCM they have short half-lives. Desmoplakin also has a short half life in LCM but usually appears to remain in the cytoplasm in punctate form associated with the cytoskeleton, or in a more diffuse pool [42, 43]. When the calcium concentration is raised, adhesion is triggered and desmosomes assemble at cell interfaces, a process that continues for up to 36 hours in MDCK cells [42–46]. Such desmosomes become relatively stable structures; nevertheless desmocollin remains fairly mobile [47].

Adhesion is triggered because a calcium concentration of ca. 1 mM is necessary to maintain cadherin extracellular domains in an extended configuration such that they are adhesion competent [48]. In vivo every cadherin molecule emerging onto the cell surface is instantly exposed to such a concentration of calcium (intracellular calcium is in the M range) and therefore will immediately become adhesion competent. Thus the only calcium switch in vivo probably occurs when a molecule emerges from the intracellular into the extracellular environment.

A similar argument indicates that calcium depletion does not induce loss of junctional adhesion in vivo. On the other

hand, depletion or chelation of extracellular calcium can be used as an experimental assay of desmosomal adhesiveness. Calcium depletion applied to subconfluent or early confluent epithelial cells in culture induces a genuine loss of desmosomal adhesion, which is a splitting of desmosomes through the intercellular region, resulting in the formation of half desmosomes that are subsequently internalized by the cells [49, 50]. This presumably occurs because of the reverse of the process described above; the cadherin extracellular domains lose their extended, adhesion-competent configuration. However, this type of "calcium-dependent" desmosomal adhesion, though commonly encountered with cells in culture, appears to be rare in vivo, only being encountered in special situations where cells are "activated" for tissue modelling or remodelling, such as embryonic development or wound closure.

4. Desmosomes Form an Insoluble Complex with Intermediate Filaments

Fey et al. sequentially extracted confluent epithelial cells to generate the nuclear matrix-intermediate filament (NM-IF) scaffold [51]. This is the cytoskeletal network remaining after extraction with the nonionic detergent Triton X-100, high salt, RNase, and DNase. The NM-IF contains junctional components including desmosomes, as verified by electron microscopy. To dissolve the NM-IF it was necessary to treat it with sodium dodecyl sulphate (SDS) and a reducing agent or urea. Even when isolated from intermediate filaments, desmosomes remain highly insoluble. Thus desmosomes isolated from bovine nasal epithelium by treatment with citric acid-sodium citrate buffer at pH2.6, which dissolves keratin filaments, have to be dialysed for hours against SDS-containing buffer in order to dissolve them [52, 53].

Such insolubility is an indicator of the toughness of desmosome that fits them for their role of extremely strong adhesion. From the biologist's viewpoint it makes it extremely difficult to study protein-protein interactions in desmosomes because dissolving them disrupts such interactions. Many biochemical procedures such as pull-down assays carried out on various soluble fractions of cultured cells must therefore be interpreted with extreme caution when applied to desmosomal components because they do not provide information relating to intact desmosomes. The interactions that they reveal apply to upstream events, that is, prior to desmosome assembly, or downstream events, that is, after proteins have left the desmosome. They may also reflect interactions within desmosomes but it should not be assumed that they do without further proof.

5. Desmosome "Modulation" Requires Epithelial Confluence

Using the calcium chelation assay it has been shown that, while the maintenance of desmosomal adhesion is initially calcium dependent, it matures to become functionally calcium independent [37, 45, 46, 54] (Figure 3). Mature, calcium-independent desmosomes do not lose adhesion and split in the intercellular space when extracellular calcium is depleted. Although other types of junction, the tight and adherens junctions, may break down leading to substantial loss of intercellular adhesion, the desmosomes remain adherent and continue to bind cells together [37, 50, 54]. This can cause the cells to adopt a stellate appearance since, although largely rounded, they remain attached at points where desmosomes accumulate during the retraction process [37] (Figure 3).

Development of calcium independence requires culture confluence. If cells are maintained at subconfluent density, calcium independence does not develop [37]. In confluent epithelial cell cultures the onset of calcium independence is gradual, in the sense that it takes several days for all of the cells to acquire mature desmosomes [37, 54]. Slow junctional maturation is also found in tight junctions, which generally take several days of confluent culture to develop so that transepithelial electrical resistance is maximal [57–59].

In order to maintain desmosomal calcium-independence, culture confluence must also be maintained. If a cell sheet in which calcium-independence has reached 100% is scratch wounded, the cells at the wound edge reacquire calcium dependence within about an hour [37]. This effect is then propagated away from the wound edge through the cell sheet. No evidence could be found for the involvement of extracellular diffusible factors or for the release of cytoskeletal tension in this propagation. The most likely explanation is that some form of cell-cell communication via gap junctions, such as intercellular calcium signals, is involved, but this has yet to be demonstrated.

6. Calcium-Independence Is the Normal State for Desmosomes In Vivo

Epithelia of animal tissues are confluent cell sheets and remain so unless wounded or diseased. If the above observations on the adhesive properties of desmosomes in cell culture are of in vivo relevance, it would be expected that desmosomes in vivo should generally be calcium independent. That this is the case has been rigorously demonstrated for the epidermis by exposing small pieces of excised mouse epidermis to EGTA for several hours and examining junctional structure by electron microscopy [55]. It was found that all 200 desmosomes examined retained adhesion and apparently unaltered structure after 6 hours of incubation in EGTA (Figure 4). Previously it had been found in a wider survey of adult mouse tissues by immunofluorescence that desmosome calcium-independence was the norm [37]. In addition to epiderm are these included trachea, oesophagus, tongue, liver, and cardiac muscle. Here it was shown that adherens junctions (E-cadherin staining) were internalised upon EGTA treatment, so there is no doubt that the chelating agent was able to penetrate the tissues.

It was surprising to find that calcium-independence was so all-pervading; no calcium-dependent desmosomes were found, even in the basal layer of epidermis. Presumably desmosome assembly is likely to be a continuous process in epidermis because of cell turnover. This may indicate that desmosomal adhesion matures very rapidly in vivo or that

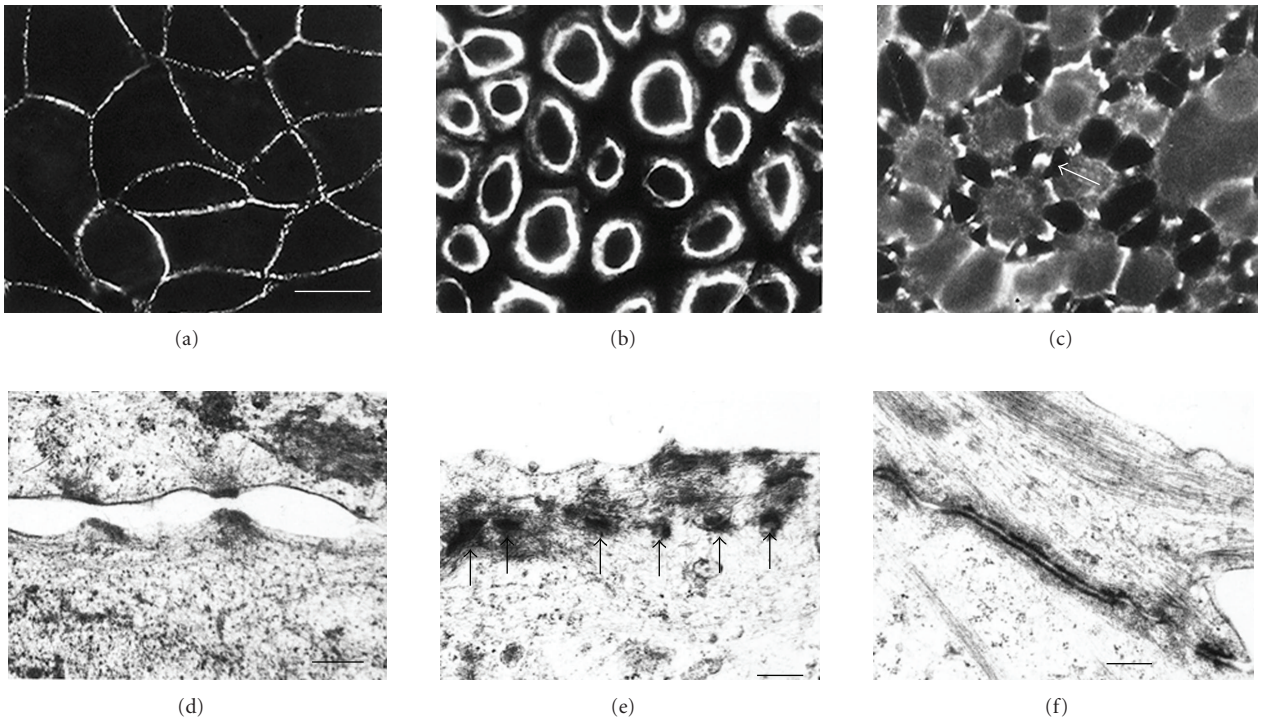

Figure 3: Desmosomes of MDCK cells can be calcium-dependent or calcium-independent. (a) Desmosomes of MDCK cells in confluent culture stained with monoclonal antibody to desmoplakin. Note that the desmosomes are located at the cell peripheries and that the staining is generally punctate. (b) A monolayer that has been cultured at confluent density for 24 hours in SM and then treated with LCM-EGTA for 90 minutes, showing loss of intercellular contact and of desmosomal staining from the cell peripheries. This is indicative of calcium-dependent desmosomes. (c) A 6-day-confluent monolayer treated with LCM-EGTA for 90 minutes, showing partial loss of intercellular contact but persistence of joining processes with intense desmosomal staining (e.g., arrow). This is indicative of calcium-desmosomes. Bar, 20 m. (d) When subconfluent or 1-day-confluent cells are treated with LCM-EGTA adhesion is lost and desmosomal halves separate. The half desmosomes are then internalised ((e), arrows). (f) By contrast when 6-day-confluent cells are treated with LCM-EGTA desmosomes remain adherent and accumulate at the cell surface between interconnecting cell processes. Bars: 0.3 m in (d) and (f); 0.4 m in (e) (a)–(c). Reproduced from the study by Wallis et al. in [37]. The isoform of protein kinase (c) is involved in signalling the response of desmosomes to wounding in cultured epithelial cells. (d)–(f) Reproduced from the study by Mattey and Garrod in [50].

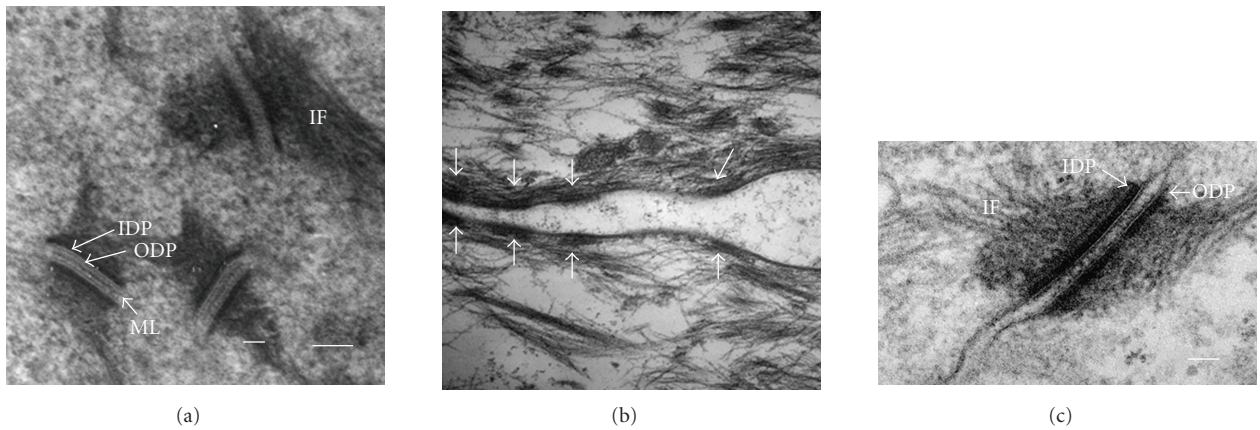

Figure 4: Transmission electron micrographs of desmosomes in mouse epidermis. Normal (a) and wound-edge (b) mouse epidermis after exposure to LCM-EGTA for 6 hours (a) and 1 hour (b). Note that the desmosomes in (a) are intact and two that are sectioned precisely transversely exhibit entirely unaltered structure, whereas in (b) the desmosomal halves (arrows) have lost adhesion and separated (compare with Figure 3(d)). Thus the desmosomes in (a) are calcium independent and those in (b) are calcium dependent. (c) An example of transversely sectioned desmosomes from untreated wound-edge epidermis showing absence of midline but plaque structure appears unchanged at this resolution. ML, midline; ODP, outer dense plaque; IDP, inner dense plaque; IF, intermediate filaments. Bar: 0.1 m. Reproduced from the study by Garrod et al. in [55].

newly formed desmosomes are extremely rare and therefore difficult to find. In order to determine whether desmosomal calcium-dependence occurs at any stage in vivo we have examined desmosomes in developing mouse epidermis (T. E. Kimura, A. J. Merritt, and D. R. Garrod; unpublished). Using both immunofluorescence and electron microscopy to assay the results, we found that epidermal desmosomes remained entirely calcium dependent until E12 (embryonic day 12) but by E14 > 75% of epidermal cells possessed calcium-independent desmosomes. By E19 this had increased to almost 100%. In contrast, adherens junctions remained calcium dependent throughout. Thus it appears that desmosomal adhesiveness is developmentally upregulated.

Our results should be compared with earlier results on tissues of the frog Rana pipiens that were exposed to the nonspecific chelating agent EDTA and examined by electron microscopy [60]. Borysenko and Revel showed that desmosomes of stratified squamous and glandular epithelia were insensitive to EDTA but that those of simple columnar epithelia were EDTA-sensitive. The latter result was in agreement with that of a study on oxyntic cells of the gastric glands, in which it was found that EDTA induced desmosomal changes were reversed by readdition of calcium [61]. Since our studies have shown that mammalian simple epithelial cells such as MDCK readily develop calcium-independent desmosomes, it appears that mammals and frogs may differ in this respect. It is interesting that the oxyntic cell desmosomes studied by Sedar and Forte showed no intercellular midlines, whereas those of the frog epidermis, shown by Borysenko and Revel, had very distinct midlines (see below for the significance of this observation).

7. Desmosomes Revert to Calcium-Dependence in Wounded Epidermis

Culture experiments suggest that wounding a confluent cell sheet causes desmosomes to revert from calcium independence to calcium dependence. In order to determine whether similar changes occur in vivo, wound-edge epithelium from mouse epidermis was incubated in EGTA and the desmosomes examined by electron microscopy [55]. It was found that 53.3% of such desmosomes were calcium dependent by 48 hours after wounding and 63.8% by 72 hours (Figure 4). Thus the change in desmosomal adhesiveness shown on wounding by simple epithelial cells in tissue culture is also an in vivo phenomenon in the epidermis indicating that cell culture potentially provides a good model for desmosome regulation in tissues.

8. Calcium-Independent Desmosomes Are Hyperadhesive

Because calcium independence makes desmosomes difficult or impossible to dissociate, it seemed likely that it represents a strongly adhesive state. In order to test this we compared the adhesiveness of dispase-detached sheets of epidermal keratinocytes (HaCaT) with calcium-dependent and calcium-independent desmosomes using a fragmentation assay [54]. So as to be sure that we, really were comparing adhesiveness we confirmed by electron microscopy that the fragmentation was occurring by splitting desmosomes in the intercellular region rather than by breaking cells. Differential cell breaking could also increase the number of sheet fragments in response to mechanical stress, but would indicate differences in other cellular properties, such as cytoskeletal changes, rather than adhesiveness (see below for disease situations where cell breaking occurs). The results showed that sheets with calcium-independent desmosomes were considerably more cohesive than those with calcium-dependent desmosomes. Moreover, this greater cohesiveness persisted in the presence of EGTA, indicating that it was a truly calcium-independent phenomenon.

The greater adhesiveness of calcium-independent desmosomes has been termed "hyperadhesion" because it is a unique and very important property. It is unique because it does not appear to be a feature of the other major adhesive junction, the adherens junction, which is always calcium dependent according to all reports and to our experience with cultured cells and tissues. It is very important because it is fundamental to the strength and integrity of vertebrate tissues. In contrast to hairy animals human beings are surrounded by an extremely thin epithelial layer, the epidermis, and this is their only protection against severe environmental stresses such as physical abrasion and dehydration. It would not be able to do this without desmosomal hyperadhesion, which binds its cells tightly together into a tough, cohesive epithelium. In addition they have cardiac muscle that can generate a pressure of almost 1/3 atmospheric, again demanding tight cohesion between cardiac myocytes.

It has long been axiomatic that desmosomes provide strong adhesion but it has never before actually been demonstrated that this strong adhesion is a regulable property dependent on desmosome maturation and requiring calcium-independent adhesive binding by cadherins. The assumption of strong adhesiveness has been based on a number of considerations. Firstly, desmosomes are most abundant in tissues such as epidermis where resistance to physical stress is essential. Secondly, desmosomal adhesion is the intercellular link in the desmosome-intermediate filament complex, which forms a cytoskeletal network extending throughout tissues that contain it. Such a bracing network needs equivalent strength at every point or it would not function [1]. Thirdly, several human diseases that affect desmosomal components weaken the tissues that are most affected, such as the epidermis and the heart [16, 62, 63]. Fourthly, several constitutive or conditional deletions of desmosomal genes from mice also result in weakening of the epidermis and/or the heart [32, 64–67]. The demonstration of hyperadhesion provides experimental confirmation of a long-held belief.

9. Acquisition of Hyperadhesion Involves No Change in the Composition of Desmosomes

A simple explanation of hyperadhesion and its regulation might be that one or other of these components is recruited to or lost from desmosomes to regulate this adhesive

change. We had previously found that inhibition of protein synthesis with cycloheximide did not prevent the conversion of desmosomes making this explanation unlikely. Because the extent of hyperadhesion in HaCaT cell sheets was easily quantifiable and the desmosomes in these cells could be readily and rapidly converted between the two states (see below), we made qualitative and quantitative comparison between the desmosomal components in cells expressing one state and the other. The cells express plakoglobin, desmoplakin, two isoforms each of desmoglein and desmocollin, and three isoforms of plakophilin. There was no qualitative or quantitative difference in any of the components between cells in the two adhesive states, nor in the localisation of any component to desmosomes at the cell periphery [54].

10. Desmosome Structure Suggests a Basis for Hyperadhesion

A striking and unique feature of the structure of desmosomes is the presence of an electron-dense midline between the plasma membranes in the intercellular space [60, 68, 69]. The midline lies at the centre of the adhesive interface between adjacent cells and thus may represent a structural specialisation for hyperadhesion. The other major adhesive junction, the adherens junction, which does not appear to adopt hyperadhesion, notably lacks a midline. A significant finding is that desmosomes in epidermal wound-edge epithelium, which are generally calcium dependent, lack a midline and their intercellular space is ca.10% narrower than that of hyperadhesive, midline-possessing desmosomes of unwounded epidermis [55]. The intercellular space of the calcium-dependent adherens junction is generally reported as being narrower than that of the desmosome. The calcium-dependent desmosomes of embryonic epidermis also lack midlines, but midlines are acquired together with hyperadhesion (T. E. Kimura et al., unpublished). Thus hyperadhesion seems to go together with a wider intercellular space and the presence of a midline, while calcium-dependence seems to be accompanied by a narrower intercellular space with no midline.

A short paper by Rayns et al. provided the only significant insight into the structure of the desmosomal interspace until very recently [70]. The most noteworthy feature of this structure was its regularity. Infiltration of guinea-pig heart muscle with the electron-dense tracer, lanthanum, revealed a highly regular structure in which the midline appeared as a zigzag that was connected to the plasma membranes by alternating cross-bridges (alternating light and dark parallel lines) of 70–75 periodicity. Tangential sections through these structures revealed arrays of dense particles of 75 periodicity or quadratic arrays of dense particles of 55 centre-to-centre spacing [70].

In order to form crystals, molecules must pack into regular arrays. When the crystal structure of the partial EC domain of the classical cadherin, N-cadherin, was published, it was described as an "adhesion zipper" that resembled the ultrastructure of the desmosome rather than the adherens junction [71, 72]. The later publication of the crystal structure of the full-length EC domain of Xenopus C-cadherin produced a model that seemed even more to resemble a desmosome [56]. We were struck by the remarkable similarity between the periodicity found in the C-cadherin structure and that previously reported by Rayns et al. [70]. Homology models for Dsc2 and Dsg2 were generated using the C-cadherin ectodomain as a template. Modelling of the desmosomal cadherins (Dsc2 and Dsg2) in the crystal packing observed for C-cadherin shows that it is feasible to produce a similar array, despite the modest sequence identity between them (ca.30%) [55]. However, some differences in detail between the intermolecular and intramolecular interactions in the desmosomal cadherins may be expected. The final quality of the models for Dsc2 and Dsg2 was compared with the C-cadherin structure. The root mean square deviation for all equivalent carbons was 1.03 for Dsc2 and 1.04 for Dsg2.

In an attempt to model the Ca^{2+}-independent desmosome structure we computed a 3D array of Dsc2 molecules generated according to the crystallographic cell symmetry of C-cadherin [55]. A similar array was generated for Dsg2. In these arrays, the adhesion interfaces are aligned to the x-axis (along the crystallographic x-axis) (Figure 5 shows the Dsc2 array). The structure seemed to account very well for the desmosomal midline and showed remarkable agreement with the repeating periodicity shown by Rayns et al. [70] Therefore, we proposed that this 3D array is a good model for the highly ordered, quasicrystalline desmosome structure observed in ultrastructural studies.

A most exciting development has been the study of the extracellular domains of tissue desmosomes by cryoelectron tomography of vitreous sections [73]. This technique enables the visualization of three-dimensional molecular structure under close-to-native conditions. Three-dimensional reconstruction showed a regular array of densities at 70 intervals along the midline, with a curved shape resembling the X-ray structure of C-cadherin.

How is it possible for desmosomes to alternate between calcium-independent hyperadhesion and calcium dependence without changing their molecular composition? We suggest that the ordered arrangement of the EC domains of the desmosomal cadherins is dynamically variable. In support of this are our observations that the midline structure is not evident in calcium-dependent embryonic and wound-edge epithelial desmosomes. It is probable that such desmosomes have a less ordered arrangement of the EC domains, which therefore appear more diffuse in the electron microscope. Thus we suggest that a type of locking mechanism operates; in the ordered arrangement, the EC domains are locked together giving strong adhesion that cannot be readily dissociated, while in the less ordered arrangement, adhesion is weaker and dissociation is possible. We also suggested that the locked form may involve entrapment of calcium ions, a possible explanation of "calcium independence".

An alternative view of desmosome structure is that the desmosomal cadherins in the desmosomal interspace form a series of tangled knots rather than a regular structure [74]. It seems to the author that such an arrangement is less likely to account for the dynamic properties of desmosomes and

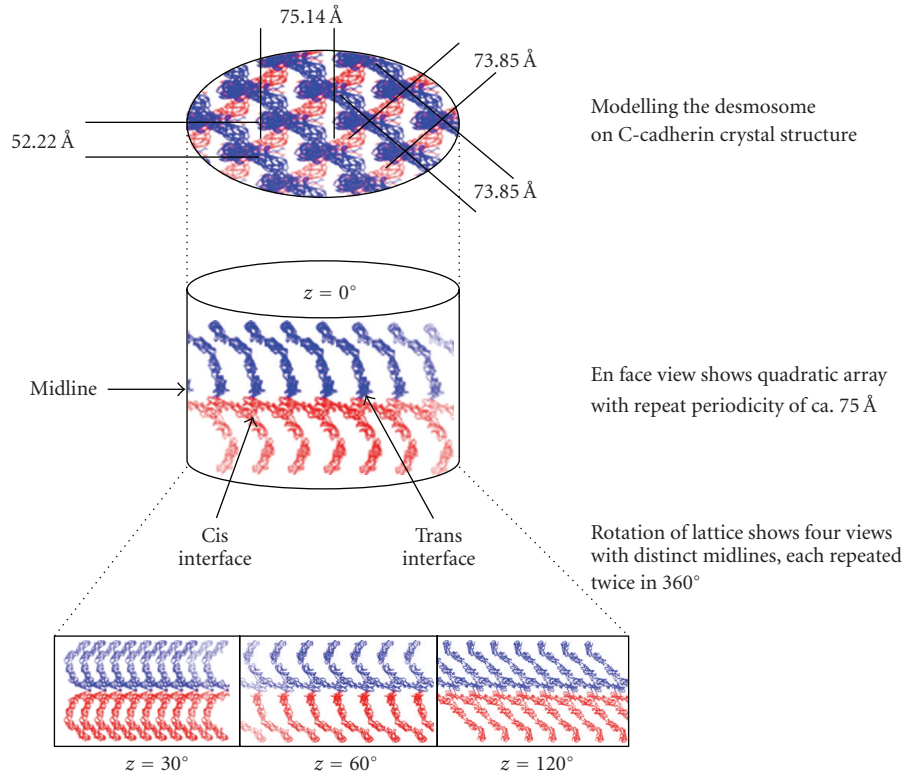

Figure 5: Schematic representation of the Dsc2 ectodomain model 3D array, generated from the crystallographic structure of C-cadherin [56]. In the centre we represented the desmosomal interspace as a cylinder, showing the midline formed by trans and cis interactions between molecules on opposed cell surfaces. Monomers from one cell surface are coloured in red and monomers from the opposed cell surface are coloured in blue. The midline is aligned with the x-axis of the cylinder (coincident with the x-axis in the crystallographic lattice). Rotation of the 3D array around the x-axis by 90° (top) shows a regular lattice with distances between rows of molecules of 73.85 and distances of 75.14 between layers. Rotations around the z-axis produce four different views, all of them with a midline dense zone (bottom). These are strand dimer1 at $z = 0°$, "boat" at $z = 30°$, strand dimer 2 (inverse form strand dimer 1) at $z = 60°$, and "zipper" at $z = 120°$. The cis and trans interfaces between distal domains are indicated by arrows. Reproduced from the study by Garrod et al. in [55].

for their great adhesive strength. The desmosomes studied in this paper were from neonatal mouse epidermis. It is possible that their cadherins appeared irregular because the desmosomes were calcium dependent, though this seems unlikely for two reasons. Firstly they had midlines, which in our experience are invariably associated with calcium independence. Secondly, a study of the developmental regulation of desmosomal adhesion in the mouse epidermis has shown a major change to calcium independence by E14 and by E19: 100% of keratinocytes have calcium independent adhesion, as in the adult (Kimura at al., unpublished) [55]. A more probable explanation is that the desmosomes studied were calcium independent and that the structure was distorted during preparation, which involved freeze substitution and resin embedding.

11. An Ordered Intercellular Zone Requires an Ordered Plaque

The cytoplasmic domains of the desmosomal cadherins lie in the desmosomal plaque. An ordered arrangement of their extracellular domains would seem to require an equally ordered arrangement of their cytoplasmic domains and therefore of the plaque in general. Several ultrastructural studies have provided preliminary evidence that the plaque has a lamellar structure parallel to the membrane and a periodic structure at right angle to it.

Thus, the desmosomal plaque shows filamentous or periodic organisation perpendicular to the plasma membrane and lamellar organisation parallel to it. The former was revealed by early freeze-fracture studies [75–78]. Cryosections of tissue prepared by the Tokyasu method revealed an electron opaque lamina ca. 17 nm from the plasma membrane while negative staining of polyvinyl alcohol-embedded material revealed the presence of at least two laminae in the outer dense plaque [8, 79]. Miller et al. [79] noted transverse periodicity of approximately 2.6 nm in a region probably corresponding to the inner dense plaque (IDP) and North et al. [8] observed fine filaments perpendicular to the membrane in the outer dense plaque (ODP). A remarkably similar picture has emerged from cryoelectron microscopy of rapidly frozen, fully hydrated human epidermis [80, 81]. The desmosomal plaque showed a 10-11 nm region of medium electron density adjacent to the plasma membrane (PM) followed by a 7-8 nm thick electron dense layer showing a transverse periodicity of about 7 nm.

The major challenge to elucidating the structure of the desmosomal plaque is its density. The most effective method of analysis thus far has been immunogold electron microscopy [79, 82–84]. The most detailed study used quantitative analysis of immunogold labelling of the N- and C-termini of DP, PG, and PKP 1 and the C-termini of Dsg 3, Dsc 2a, and Dsc 2b to produce a molecular map of the desmosomal plaque [8]. This showed that the ODP is the region where the armadillo proteins probably interact with the N-terminus of DP and the cytoplasmic domains of the desmosomal cadherins while the C-terminus of DP lies in the IDP in accordance with its known function of binding the IFs. Mapping the localisation data from the study of North et al. in [8] onto the structure reported by Al-Amoudi et al. [80, 81] suggests that the density Al-Amoudi et al. reported in the plaque corresponds to this same region of substantial protein-protein interaction.

12. Protein Kinase C Modulates Desmosomal Adhesiveness

Since modulation of desmosomal adhesion involves no change in protein composition, an alternative mechanism might involve some type of inside-out signalling such as has been found to modulate the adhesiveness of, for example, integrins [85, 86]. That such is also the case for desmosomes was indicated by the observations that treatment of MDCK and HaCaT cells with protein kinase C (PKC) activators rapidly converted desmosomes from hyperadhesive to calcium dependent [37, 54]. Conversely, PKC inhibitors induced hyperadhesiveness in calcium-dependent desmosomes. Because (i) MDCK cells contain relatively few PKC isozymes, (ii) an inhibitor of conventional PKC isozymes was effective in calcium-dependence/hyperadhesion conversion, and (iii) PKC was localised to the membranes of cells with calcium-dependent desmosom es but diffuse cytoplasmic in cells with hyperadhesive desmosomes, it was concluded that PKC plays an important role in this conversion [37]. Is this also the case in vivo?

In normal mouse epidermis where all desmosomes appear to be hyperadhesive, PKC is most strongly expressed in the basal layer, has a diffuse cytoplasmic distribution, and shows no colocalisation with desmosomes. (N. B. PKC expression may differ between layers of the epidermis in humans and mice [87].) However, in wound-edge epidermis where the desmosomes are predominantly calcium dependent, PKC is abundantly localised to desmosomal plaques [55]. This strongly suggests that PKC plays a role in regulating desmosomal adhesiveness in vivo but does not constitute definitive functional evidence.

During epidermal development calcium-dependent desmosomes at E12 could be converted to hyperadhesiveness by treatment with the conventional PKC isozymes inhibitor Gö 6976 suggesting that in vivo, as in culture, PKC is involved in desmosomal regulation (Kimura et al., unpublished). However, PKC$\alpha -/-$ mice developed normal desmosomes that showed developmental regulation to hyperadhesion with the same timing as wild-type mice, suggesting either that additional regulatory mechanisms are involved or that other isozymes compensate for the absence of PKC. If the above observations on the relocalisation of PKC in epidermal wound healing have functional significance, inhibition/activation of this isozyme should have predictable effects on wound healing and this is currently being tested.

We were surprised to find recently that treatment of MDCK cells with the general tyrosine phosphatase inhibitor sodium pervanadate caused a substantial conversion of calcium-dependent desmosomes of subconfluent MDCK cells to hyperadhesion [88]. This treatment caused increased tyrosine phosphorylation of plakoglobin and desmoglein 2, which nevertheless remained in complex in the soluble cell fraction. The observation also clearly suggested that protein tyrosine kinases in addition to PKC may be involved in regulating desmosomal adhesiveness.

How could protein kinases regulate desmosomal adhesiveness? Given the clear localisation of PKC to the desmosomal plaque during conversion of desmosomes, the most likely mechanism would seem to involve phosphorylation of one or more of the desmosomal plaque components. This could then cause a configurational change within the plaque that could in turn lead to a disordering of the plaque and a consequent disordering of the extracellular domains of the desmosomal cadherins leading to calcium dependence. Dephosphorylation would then restore order and lead to hyperadhesion. Such a mechanism would require no change in the major components of desmosomes, in accordance with our results, though it may require the recruitment and/or loss of protein kinases and phosphatases. The difficulty is in determining which desmosomal protein(s) is the key phosphorylation target.

We and others have shown by metabolic labelling and immunoprecipitation that all major desmosomal components in cultured cells are phosphorylated under normal conditions [89, 90]. The phosphorylation may be on serine/threonine or tyrosine in the case of plakoglobin and desmoglein [88, 91–94]. The difficulty with such experiments lies in knowing the origin of the desmosomal components concerned; only phosphorylation changes that occur within desmosomes themselves can account for the adhesive changes. Changes that occur in soluble cell fractions are not relevant, though they may be associated with desmosome assembly.

An alternative approach is mutagenesis of known or potential phosphorylation sites. Quantitative analysis of the location of PKC in the plaques of epidermal wound-edge desmosomes suggested a biphasic distribution, one peak lying within a few nanometers of the inner leaflet of the plasma membrane and the other within the outer dense plaque [55]. Based on our previous molecular mapping of the desmosomal plaque, no desmosomal component could be eliminated on the basis of colocalisation with PKC [8]. Accordingly we undertook mutagenesis of conserved consensus phosphorylation sites in the cytoplasmic domains of desmocollin 2 and desmoglein 2, the desmosomal cadherins with the widest tissue distributions, reasoning that sites involved in such a key regulatory process would be likely to be conserved (A. Smith, S. Haddad, Z. Nie, and D. Garrod; unpublished) (Figure 6). Mutant proteins were

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hDsg2   TESLNASIGCCSFIEGELDDRFLDDLGLKFKTLAEVCLGQKIDINKEIEQRQKPATETSMNT
mDsg2   TASLRGSVGCCSFIEGELDDLFLDDLGLKFKTLAEVCLGRKIDLDVDIEQRQKPVTEASVSA
bDsg1   AGSPAGSVGCCSFIGEDLDDSFLDTLGPKFKKLADISLG--------KDVEPFPDSDPSWPP
hDsg1   VGSPAGSVGCCSFIGEDLDDSFLDTLGPKFKKLADISLG--------KESYPDLDPSWPP
hDsg3   TGSPVGSVGCCSFIADDLDDSFLDSLGPKFKKLAEISLG--------VDGEGKEVQPPSKDS
          810  814                                                  866
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(a)

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hDsg2   AVG-QNVTVTERVLAPASTLQSS
mDsc2   AAGGQNVTVTERILTPASTLQSS
bDsg1   LSNTHNVIVTERVVSGSGITGSS
hDsg1   LANAHNVIVTERVVSGAGVTGIS
hDsg3   LRGSHTMLCTEDPCS---------
              1051
```

(b)

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mDsc1a  KVYLCGQAEEHKHCEDYVRPYNYEGKGSMAGSVGCCSDRQEEEGL
hDsc1a  KVYLCGQDEEHKHCEDYVCSYNYEGKGSLAGSVGCCSDRQEEEGL
bDsc1a  KVYLCGQDEEHKLCEDYVRSYSYEGKGSVAGSVGCCSDRQEEEGL
mDsc2a  KVQFCHTDDNQKLAQDYVLTYNYEGKGSAAGSVGCCSDLQEEDGL
hDsc2a  KVYLCNQDENHKHAQDYVLTYNYEGRGSVAGSVGCCSERQEEDGL
bDsc3a  KLHLCNQDEEHMPSQDYVLTYNYEGRGSPAGSVGCCSEKQEEDGL
hDsc3a  KLHRCNQNEDRMPSQDYVLTYNYEGRGSPAGSVGCCSEKQEEDGL
                                  729  733   738
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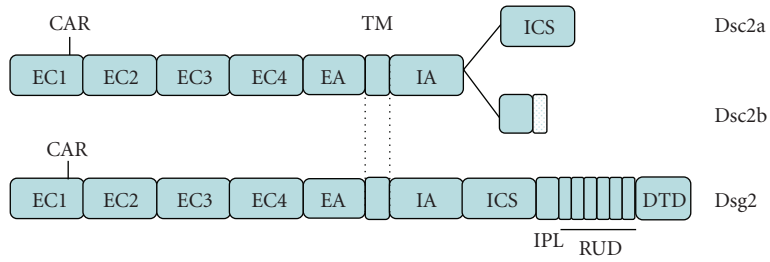
(c)



(d) Desmosomal cadherins

Figure 6: Mutations of phosphorylation sites in desmoglein and desmocollin produce slight increases in desmosomal hyperadhesion. (a) and (b) show conserved predicted phosphorylation site (in red) in the cytoplasmic domains of desmogleins and (c) shows similar sites in desmocollins. These were mutated to alanine and the mutant proteins expressed in MDCK cells. The effects on desmosomal adhesiveness are described in the text. (d) shows stick models of the desmosomal cadherins desmocollin 2 (Dsc2) and desmoglein 2 (Dsg2). Sites S810 and S814 are located towards the C-terminal end of the ICS (intracellular cadherin-specific) region of Dsg2, S866 towards the C-terminal end of the IPL (intracellular proline-rich) region, and S1051 in the sixth repeat of the specific repeat unit domain (RUD). Sites S729, S733, and S738 are towards the C-terminal end of the ICS region of Dsc2a but are absent from the shorter C-terminal region of Dsc2b because alternative splicing truncates this region. (d) is reproduced from the study by Garrod and Chidgey in [1].

expressed in MDCK cells and the cells assessed at an early stage of confluent culture for increased hyperadhesiveness. It was found that the combined mutations S729A and S738A in Dsc2 and S810A and T1051A in Dsg2 increased the percentage of sub-confluent/early confluent cells with hyperadhesive desmosomes to 22% compared with 6% for cells transfected with plasmids encoding wild-type proteins. RNAi depletion of endogenous Dsg2 increased this figure to about 37%. Furthermore, both mutations significantly slowed the rate of healing in a scratch wound assay. These rather modest effects probably indicate that regulation of desmosomal adhesiveness is a complex process involving multiple sites on several desmosomal proteins.

13. Downregulation of Desmosomes In Vivo: Internalisation versus Disassembly

A vital aspect of tissue homeostasis is the turnover of molecules and organelles. In the case of desmosomes in epithelial tissues such as the epidermis, the need for such turnover is clear. As keratinocytes migrate upwards through the epidermal layers, the protein composition of individual

desmosomes changes, the more basally expressed desmosomal cadherin and plakophilin isoforms being gradually replaced by others that are upregulated in the upper layers [1, 10], while the other components, desmoplakin and plakoglobin, which do not have multiple isoforms, continue to be expressed. It is not known how this change is achieved; it could be by molecular exchange within existing desmosomes, as seems possible in cultured cells [47], or by downregulation of existing desmosomes and replacement with new ones of differing composition. Of these two alternatives, there is no evidence for the former in vivo, while there is evidence for the latter, not as far as I know from intact, normal epidermis, but from situations where desmosome downregulation would be expected to be increased, such as wound healing and disease.

Downregulation of desmosomes in wound-edge epidermis appears to have been first reported by Croft and Tarin [95]. It seems intuitively obvious that for multilayered keratinocytes to form a single migrating layer they must reduce their intercellular adhesions. Reduce but not abolish, because maintenance of cell-cell adhesion appears essential for coordinated migration of epithelial cell sheets [96]. When examining wound-edge epidermis we were surprised that we never found half desmosomes [55]. (Apparently desmosome splitting occurs when calcium is chelated but does not appear to be the "normal" methods of downregulation.) Instead, whole, apparently intact desmosomes were frequently observed inside wound-edge cells. This suggests that engulfment of entire desmosomes may be the mechanism of downregulation, consistent with a mechanism for desmosomal engulfment previously proposed by Allen and Potten [97]. Membrane processes originating from one of a pair of cells were suggested to engulf the desmosome enclosing it in a cytoplasmic vesicle within the protagonist. What determines which member of the cell pair gets the desmosome is not known. Such a mechanism would be consistent with the finding that the actin cytoskeleton is involved in internalisation of half desmosomes in cultured cells [98]. The puzzle in our studies was that the majority of internalized desmosomes did not appear to be enclosed in vacuoles, and the reason for this is not clear. Internalised desmosomes, usually with at least a partial associated vacuole, have been reported from a variety of skin lesions (see [55] for references) but it is not clear whether this is the general mechanism for desmosome downregulation in vivo.

Desmosome downregulation is frequently referred to as desmosome "disassembly", largely on the basis of research on cultured cells. "Disassembly" implies the dissolution or disruption of an object into its component parts or in this case its component molecules. In order for this to be conclusively demonstrated it has to be shown that molecules appearing in the soluble fraction of cells were previously incorporated into desmosomes. The author submits that this is rarely, if ever, the case. Comparison between the non-ionic detergent-soluble and insoluble fractions is a start but is in itself inadequate because some desmosomal components enter the insoluble fraction shortly after synthesis and probably before incorporation into desmosomes [99]. Thus the difference between the soluble and insoluble fractions could represent prevention of assembly rather than promotion of disassembly. Furthermore, in the lack of any convincing evidence to the contrary, such studies have invariably been carried out on immature, calcium-dependent desmosomes, the in vivo relevance of which is doubtful. There is no evidence that the author is aware of that desmosome disassembly ever occurs in vivo.

Even when calcium-dependent desmosomes are disrupted in culture by chelation of extracellular calcium, there is no evidence that they disassemble. Under these circumstances desmosomes split in the midline (Figure 4) and the resulting half desmosomes are internalised into cytoplasmic vacuoles [49, 50]. Such half desmosomes are gradually transported to the perinuclear region of the cell and continue to be so even if a new round of desmosome assembly is initiated by once again restoring extracellular calcium [47, 50]. This suggests that desmosomes are not disassembled and their components reutilised for the assembly of new desmosomes. Our unpublished studies based on immunofluorescent colocalisation of all major components of internalised desmosomes and their insolubility in internalising cells reaffirm that internalised desmosomal halves do not disassemble (McHarg, Hopkins, Lin, and Garrod; unpublished).

14. Some Implications for and Suggestions from Human Disease

In pemphigus vulgaris (PV), a potentially fatal blistering disease of skin and mucous membranes, autoantibodies to the desmosomal cadherins Dsg3 (and Dsg1 in some patients) cause loss of cell-cell adhesion or acantholysis [100, 101]. Suggested alternative but not necessarily mutually exclusive mechanisms for acantholysis include direct disruption of desmosomal adhesion due to steric hindrance by the autoantibodies and activation of outside-in signalling by autoantibody binding [16, 102].

A review of the literature on the ultrastructural analysis of PV appears to show that direct disruption of desmosomal adhesion is not the primary event [103–107]. Rather there is extensive loss of cell-cell adhesion in interdesmosomal regions and possible intracellular cleavage behind the desmosomal plaque that might indicate a weakening of the cytoskeleton, perhaps through a signalling mechanism involving plakoglobin [102, 103]. By contrast abundant split desmosomes with inserted keratin filaments were found in a mouse model of PV [108]. This model involves immunising desmoglein 3 null mice against desmoglein 3 and then transferring splenocytes from the immunised mice to Rag2 null mice, which then produce antidesmoglein 3 and develop symptoms of PV [109]. Ingenious though this model is, the above results on disruption of desmosomal adhesion should be regarded with caution because there is no guarantee that the antibodies produced function by the same mechanism as PV autoantibodies.

A series of publications reporting work in which keratinocytes were treated with PV autoantibodies in culture showed that desmosomes are downregulated and that

desmoglein 3 is depleted and internalised [110–115]. However, all of this work was presumably carried out with keratinocytes possessing calcium-dependent desmosomes, which, as we have shown, do not represent the in vivo situation. A recent study in which comparison was made between keratinocytes with calcium-dependent desmosomes and those with hyperadhesive desmosomes showed that hyperadhesion substantially inhibited PV autoantibody-induced acantholysis and internalisation of adhesion molecules including desmoglein 3 and E-cadherin [116]. Further work is needed to determine the extent to which hyperadhesive keratinocytes in culture provide a suitable model for PV but the results of Cirillo et al. are extremely encouraging.

While the primary effect of PV autoantibodies does not appear to be direct disruption of existing desmosomal adhesion, they could inhibit de novo desmosome assembly, which must be a continuous process in stratified epithelia [117]. During the progress of disease this would be expected to result in gradual downregulation of desmosomes and loss of cell-cell adhesion. It would therefore be interesting to know whether the abovementioned results obtained with calcium-dependent keratinocytes in culture have any counterpart in vivo. It could also be the case that the primary loss of inter-desmosomal adhesion found in PV might resemble epidermal wounding and thus cause activation of PKC and consequent weakening of desmosomal adhesion as we have described [55]. In this case inhibition of PKC could provide a novel therapy for Pemphigus, as Cirillo et al. and others have suggested [116, 118].

Does human disease provide any suggestions as to how hyperadhesion might be regulated? A certain amount of speculation on this point appears possible.

A single case of lethal acantholytic epidermolysis bullosa has been described [119]. This was caused by the occurrence of two different recessive mutations in the carboxy-terminal domain of desmoplakin resulting in the loss of IF attachment to desmosomes, a consequence that is entirely consistent with molecular cell biological studies on the role of desmoplakin in IF attachment [32, 120–123]. This resulted in massive epidermal acantholysis during birth, not because of loss of desmosomal adhesion, but because of cellular disruption behind desmosomal plaques [119]. Careful examination of the electron micrographs in the latter paper suggests that, though lacking IFs, the desmosomes were otherwise perfectly formed including a normal plaque structure and a clear midline in the intercellular space. If we are correct in our suggestion that the presence of a midline is consistent with hyperadhesion, this would indicate that hyperadhesion does not depend on desmosomal interaction with the IF cytoskeleton.

Ectodermal dysplasia-skin fragility syndrome, the first human disease shown to result from a mutation in a desmosomal component, results from a mutation in the gene for plakophilin-1 [124]. Effectively a plakophilin-1 knockout, this condition results in skin blistering, complete absence of hair, and pachyonychia and is nonlethal probably because of compensation by other plakophilin isoforms. There are small epidermal desmosomes with absence of midline and inner dense plaque, and detachment of IFs. Keratinocytes isolated from diseased epidermis fail to develop desmosomal resistance to depletion of extracellular calcium and thus hyperadhesion [125]. This suggests that plakophilin-1 contributes to the development of desmosomal hyperadhesion. Similarly, mouse keratinocytes lacking plakoglobin do not develop hyperadhesion (McHarg, Müller, and Garrod; unpublished observations).

The above observations together with our aforementioned mutagenesis of desmosomal cadherins suggest that the establishment and regulation of hyperadhesion may be a complex process involving more than one desmosomal component and more than one kinase. An experimental investigation of the molecular basis of hyperadhesion and its regulation is currently in progress.

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References

[1] D. Garrod and M. Chidgey, "Desmosome structure, composition and function," *Biochimica et Biophysica Acta*, vol. 1778, no. 3, pp. 572–587, 2008.

[2] D. Garrod and T. E. Kimura, "Hyper-adhesion: a new concept in cell-cell adhesion," *Biochemical Society Transactions*, vol. 36, no. 2, pp. 195–201, 2008.

[3] H. Bartels, "Intercellular junctions in the gill epithelium of the Atlantic hagfish, Myxine glutinosa," *Cell and Tissue Research*, vol. 254, no. 3, pp. 573–583, 1988.

[4] M. Elger, "The branchial circulation and the gill epithelia in the Atlantic hagfish, Myxine glutinosa L," *Anatomy and Embryology*, vol. 175, no. 4, pp. 489–504, 1987.

[5] J. Mallatt and C. Paulsen, "Gill ultrastucture of the Pacific hagfish Eptatretus stouti," *American Journal of Anatomy*, vol. 177, no. 2, pp. 243–269, 1986.

[6] D. C. Pfeiffer and A. W. Vogl, "Actin-related intercellular adhesion junctions in the germinal compartment of the testis in the hagfish (Eptatretus stouti) and lamprey (Lampetra tridentatus)," *Tissue and Cell*, vol. 34, no. 6, pp. 450–459, 2002.

[7] Y. Sasakura, E. Shoguchi, N. Takatori, et al., "A genomewide survey of developmentally relevant genes in Ciona intestinalis. X. Genes for cell junctions and extracellular matrix," *Development Genes and Evolution*, vol. 213, no. 5-6, pp. 303–313, 2003.

[8] A. J. North, W. G. Bardsley, J. Hyam, et al., "Molecular map of the desmosomal plaque," *Journal of Cell Science*, vol. 112, no. 23, pp. 4325–4336, 1999.

[9] A. E. Bass-Zubek, L. M. Godsel, M. Delmar, and K. J. Green, "Plakophilins: multifunctional scaffolds for adhesion and signaling," *Current Opinion in Cell Biology*, vol. 21, no. 5, pp. 708–716, 2009.

[10] R. L. Dusek, L. M. Godsel, and K. J. Green, "Discriminating roles of desmosomal cadherins: beyond desmosomal adhesion," *Journal of Dermatological Science*, vol. 45, no. 1, pp. 7–21, 2007.

[11] K. J. Green and C. L. Simpson, "Desmosomes: new perspectives on a classic," *Journal of Investigative Dermatology*, vol. 127, no. 11, pp. 2499–2515, 2007.

[12] M. Hatzfeld, "Plakophilins: multifunctional proteins or just regulators of desmosomal adhesion?" *Biochimica et Biophysica Acta*, vol. 1773, no. 1, pp. 69–77, 2007.

[13] B. Holthofer, R. Windoffer, S. Troyanovsky, and R. E. Leube, "Structure and function of desmosomes," *International Review of Cytology*, vol. 264, pp. 65–163, 2007.

[14] M. D. Kottke, E. Delva, and A. P. Kowalczyk, "The desmosome: cell science lessons from human diseases," *Journal of Cell Science*, vol. 119, no. 5, pp. 797–806, 2006.

[15] A. Sonnenberg and R. K. H. Liem, "Plakins in development and disease," *Experimental Cell Research*, vol. 313, no. 10, pp. 2189–2203, 2007.

[16] J. Waschke, "The desmosome and pemphigus," *Histochemistry and Cell Biology*, vol. 130, no. 1, pp. 21–54, 2008.

[17] J. P. Heath and G. A. Dunn, "Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflexion and high-voltage electron-microscope study," *Journal of Cell Science*, vol. 29, pp. 197–212, 1978.

[18] R. Zaidel-Bar, S. Itzkovitz, A. Ma'ayan, R. Iyengar, and B. Geiger, "Functional atlas of the integrin adhesome," *Nature Cell Biology*, vol. 9, no. 8, pp. 858–867, 2007.

[19] P. Friedl and K. Wolf, "Tumour-cell invasion and migration: diversity and escape mechanisms," *Nature Reviews Cancer*, vol. 3, no. 5, pp. 362–374, 2003.

[20] S. Kumar and V. M. Weaver, "Mechanics, malignancy, and metastasis: the force journey of a tumor cell," *Cancer and Metastasis Reviews*, vol. 28, no. 1-2, pp. 113–127, 2009.

[21] J. Baker and D. Garrod, "Epithelial cells retain junctions during mitosis," *Journal of Cell Science*, vol. 104, no. 2, pp. 415–425, 1993.

[22] B. M. Corfe, C. Dive, and D. R. Garrod, "Changes in intercellular junctions during apoptosis precede nuclear condensation or phosphatidylserine exposure on the cell surface," *Cell Death and Differentiation*, vol. 7, no. 2, pp. 234–235, 2000.

[23] E. Martz, "Contact inhibition of speed in 3T3 and its independence from postconfluence inhibition of cell division," *Journal of Cellular Physiology*, vol. 81, no. 1, pp. 39–48, 1973.

[24] M. S. Balda and K. Matter, "Tight junctions and the regulation of gene expression," *Biochimica et Biophysica Acta*, vol. 1788, no. 4, pp. 761–767, 2009.

[25] M. Perrais, X. Chen, M. Perez-Moreno, and B. M. Gumbiner, "E-cadherin homophilic ligation inhibits cell growth and epidermal growth factor receptor signaling independently of other cell interactions," *Molecular Biology of the Cell*, vol. 18, no. 6, pp. 2013–2025, 2007.

[26] K. Raymond, M.-A. Deugnier, M. M. Faraldo, and M. A. Glukhova, "Adhesion within the stem cell niches," *Current Opinion in Cell Biology*, vol. 21, no. 5, pp. 623–629, 2009.

[27] Y. Takai, J. Miyoshi, W. Ikeda, and H. Ogita, "Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation," *Nature Reviews Molecular Cell Biology*, vol. 9, no. 8, pp. 603–615, 2008.

[28] P. M. Elias, N. Matsuyoshi, H. Wu, et al., "Desmoglein isoform distribution affects stratum corneum structure and function," *Journal of Cell Biology*, vol. 153, no. 2, pp. 243–249, 2001.

[29] M. Furuse, M. Hata, K. Furuse, et al., "Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice," *Journal of Cell Biology*, vol. 156, no. 6, pp. 1099–1111, 2002.

[30] M. Matsuki, F. Yamashita, A. Ishida-Yamamoto, et al., "Defective stratum corneum and early neonatal death in mice lacking the gene for transglutaminase 1 (keratinocyte transglutaminase)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 3, pp. 1044–1049, 1998.

[31] J. Uitto, G. Richard, and J. A. McGrath, "Diseases of epidermal keratins and their linker proteins," *Experimental Cell Research*, vol. 313, no. 10, pp. 1995–2009, 2007.

[32] V. Vasioukhin, E. Bowers, C. Bauer, L. Degenstein, and E. Fuchs, "Desmoplakin is essential in epidermal sheet formation," *Nature Cell Biology*, vol. 3, no. 12, pp. 1076–1085, 2001.

[33] S. Prasad, R. Mingrino, K. Kaukinen, et al., "Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells," *Laboratory Investigation*, vol. 85, no. 9, pp. 1139–1162, 2005.

[34] H. Wan, H. L. Winton, C. Soeller, et al., "Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions," *Journal of Clinical Investigation*, vol. 104, no. 1, pp. 123–133, 1999.

[35] P. Savagner, K. M. Yamada, and J. P. Thiery, "The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition," *Journal of Cell Biology*, vol. 137, no. 6, pp. 1403–1419, 1997.

[36] M. Stoker and M. Perryman, "An epithelial scatter factor released by embryo fibroblasts," *Journal of Cell Science*, vol. 77, pp. 209–223, 1985.

[37] S. Wallis, S. Lloyd, I. Wise, G. Ireland, T. P. Fleming, and D. Garrod, "The alpha isoform of protein kinase C is involved in signaling the response of desmosomes to wounding in cultured epithelial cells," *Molecular Biology of the Cell*, vol. 11, no. 3, pp. 1077–1092, 2000.

[38] F. Bladt, D. Riethmacher, S. Isenmann, A. Aguzzi, and C. Birchmeier, "Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud," *Nature*, vol. 376, no. 6543, pp. 768–771, 1995.

[39] E. J. Penn, I. D. J. Burdett, C. Hobson, A. I. Magee, and D. A. Rees, "Structure and assembly of desmosome junctions: biosynthesis and turnover of the major desmosome components of Madin-Darby canine kidney cells in low calcium medium," *Journal of Cell Biology*, vol. 105, no. 5, pp. 2327–2334, 1987.

[40] E. J. Penn, C. Hobson, D. A. Rees, and A. I. Magee, "Structure and assembly of desmosome junctions: biosynthesis, processing, and transport of the major protein and glycoprotein components in cultured epithelial cells," *Journal of Cell Biology*, vol. 105, no. 1, pp. 57–68, 1987.

[41] M. P. Demlehner, S. Schafer, C. Grund, and W. W. Franke, "Continual assembly of half-desmosomal structures in the absence of cell contacts and their frustrated endocytosis: a coordinated sisyphus cycle," *Journal of Cell Biology*, vol. 131, no. 3, pp. 745–760, 1995.

[42] M. Pasdar and W. J. Nelson, "Kinetics of desmosome assembly in Madin-Darby canine kidney epithelial cells: temporal and spatial regulation of desmoplakin organization and stabilization upon cell-cell contact. I. Biochemical analysis," *Journal of Cell Biology*, vol. 106, no. 3, pp. 677–685, 1988.

[43] M. Pasdar and W. J. Nelson, "Kinetics of desmosome assembly in Madin-Darby canine kidney epithelial cells: temporal and spatial regulation of desmoplakin organization

and stabilization upon cell-cell contact. II. Morphological analysis," *Journal of Cell Biology*, vol. 106, no. 3, pp. 687–695, 1988.

[44] D. L. Mattey, G. Burdge, and D. R. Garrod, "Development of desmosomal adhesion between MDCK cells following calcium switching," *Journal of Cell Science*, vol. 97, part 4, pp. 689–704, 1990.

[45] D. L. Mattey and D. R. Garrod, "Calcium-induced desmosome formation in cultured kidney epithelial cells," *Journal of Cell Science*, vol. 85, pp. 95–111, 1986.

[46] F. M. Watt, D. L. Mattey, and D. R. Garrod, "Calcium-induced reorganization of desmosomal components in cultured human keratinocytes," *Journal of Cell Biology*, vol. 99, no. 6, pp. 2211–2215, 1984.

[47] R. Windoffer, M. Borchert-Stuhltrager, and R. E. Leube, "Desmosomes: interconnected calcium-dependent structures of remarkable stability with significant integral membrane protein turnover," *Journal of Cell Science*, vol. 115, no. 8, pp. 1717–1732, 2002.

[48] B. Nagar, M. Overduin, M. Ikura, and J. M. Rini, "Structural basis of calcium-induced E-cadherin rigidification and dimerization," *Nature*, vol. 380, no. 6572, pp. 360–364, 1996.

[49] J. Kartenbeck, E. Schmid, W. W. Franke, and B. Geiger, "Different modes of internalization of proteins associated with adhaerens junctions and desmosomes: experimental separation of lateral contacts induces endocytosis of desmosomal plaque material," *EMBO Journal*, vol. 1, no. 6, pp. 725–732, 1982.

[50] D. L. Mattey and D. R. Garrod, "Splitting and internalization of the desmosomes of cultured kidney epithelial cells by reduction in calcium concentration," *Journal of Cell Science*, vol. 85, pp. 113–124, 1986.

[51] E. G. Fey, K. M. Wan, and S. Penman, "Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: three-dimensional organization and protein composition," *Journal of Cell Biology*, vol. 98, no. 6, pp. 1973–1984, 1984.

[52] C. J. Skerrow and A. G. Matoltsy, "Chemical characterization of isolated epidermal desmosomes," *Journal of Cell Biology*, vol. 63, no. 2, pp. 524–530, 1974.

[53] C. J. Skerrow and A. G. Matoltsy, "Isolation of epidermal desmosomes," *Journal of Cell Biology*, vol. 63, no. 2, pp. 515–523, 1974.

[54] T. E. Kimura, A. J. Merritt, and D. R. Garrod, "Calcium-independent desmosomes of keratinocytes are hyper-adhesive," *Journal of Investigative Dermatology*, vol. 127, no. 4, pp. 775–781, 2007.

[55] D. R. Garrod, M. Y. Berika, W. F. Bardsley, D. Holmes, and L. Tabernero, "Hyper-adhesion in desmosomes: its regulation in wound healing and possible relationship to cadherin crystal structure," *Journal of Cell Science*, vol. 118, no. 24, pp. 5743–5754, 2005.

[56] T. J. Boggon, J. Murray, S. Chappuis-Flament, E. Wong, B. M. Gumbiner, and L. Shapiro, "C-cadherin ectodomain structure and implications for cell adhesion mechanisms," *Science*, vol. 296, no. 5571, pp. 1308–1313, 2002.

[57] J. L. Madara and K. Dharmsathaphorn, "Occluding junction structure-function relationships in a cultured epithelial monolayer," *Journal of Cell Biology*, vol. 101, no. 6, pp. 2124–2133, 1985.

[58] M. Pinto, S. Robine-Leon, and M. D. Appay, "Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture," *Biology of the Cell*, vol. 47, no. 3, pp. 323–330, 1983.

[59] G. Ranaldi, R. Consalvo, Y. Sambuy, and M. L. Scarino, "Permeability characteristics of parental and clonal human intestinal Caco-2 cell lines differentiated in serum-supplemented and serum-free media," *Toxicology In Vitro*, vol. 17, no. 5-6, pp. 761–767, 2003.

[60] J. Z. Borysenko and J. P. Revel, "Experimental manipulation of desmosome structure," *American Journal of Anatomy*, vol. 137, no. 4, pp. 403–421, 1973.

[61] A. W. Sedar and J. G. Forte, "Effects of calcium depletion on the junctional complex between oxyntic cells of gastric glands," *Journal of Cell Biology*, vol. 22, pp. 173–188, 1964.

[62] M. C. Bolling and M. F. Jonkman, "Skin and heart: une liaison dangereuse," *Experimental Dermatology*, vol. 18, no. 8, pp. 658–668, 2009.

[63] J. E. Lai-Cheong, K. Arita, and J. A. McGrath, "Genetic diseases of junctions," *Journal of Investigative Dermatology*, vol. 127, no. 12, pp. 2713–2725, 2007.

[64] C. Bierkamp, K. J. McLaughlin, H. Schwarz, O. Huber, and R. Kemler, "Embryonic heart and skin defects in mice lacking plakoglobin," *Developmental Biology*, vol. 180, no. 2, pp. 780–785, 1996.

[65] G. I. Gallicano, C. Bauer, and E. Fuchs, "Rescuing desmoplakin function in extra-embryonic ectoderm reveals the importance of this protein in embryonic heart, neuroepithelium, skin and vasculature," *Development*, vol. 128, no. 6, pp. 929–941, 2001.

[66] K. S. Grossmann, C. Grund, J. Huelsken, et al., "Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation," *Journal of Cell Biology*, vol. 167, no. 1, pp. 149–160, 2004.

[67] P. Ruiz, V. Brinkmann, B. Ledermann, et al., "Targeted mutation of plakoglobin in mice reveals essential functions of desmosomes in the embryonic heart," *Journal of Cell Biology*, vol. 135, no. 1, pp. 215–225, 1996.

[68] M. A. Listgarten, "The ultrastructure of human gingival epithelium," *The American Journal of Anatomy*, vol. 114, pp. 49–69, 1964.

[69] G. F. Odland, "The fine structure of the interrelationship of cells in the human epidermis," *The Journal of Biophysical and Biochemical Cytology*, vol. 4, no. 5, pp. 529–538, 1958.

[70] D. G. Rayns, F. O. Simpson, and J. M. Ledingham, "Ultrastructure of desmosomes in mammalian intercalated disc; appearances after lanthanum treatment," *Journal of Cell Biology*, vol. 42, no. 1, pp. 322–326, 1969.

[71] L. A. Lasky, "From sticky zippers to morphology," *Nature Structural Biology*, vol. 2, no. 4, pp. 258–261, 1995.

[72] L. Shapiro, A. M. Fannon, P. D. Kwong, et al., "Structural basis of cell-cell adhesion by cadherins," *Nature*, vol. 374, no. 6520, pp. 327–337, 1995.

[73] A. Al-Amoudi, D. C. Diez, M. J. Betts, and A. S. Frangakis, "The molecular architecture of cadherins in native epidermal desmosomes," *Nature*, vol. 450, no. 7171, pp. 832–837, 2007.

[74] W. He, P. Cowin, and D. L. Stokes, "Untangling desmosomal knots with electron tomography," *Science*, vol. 302, no. 5642, pp. 109–113, 2003.

[75] D. E. Kelly and A. M. Kuda, "Traversing filaments in desmosomal and hemidesmosomal attachments: freeze-fracture approaches toward their characterization," *Anatomical Record*, vol. 199, no. 1, pp. 1–14, 1981.

[76] D. E. Kelly and F. L. Shienvold, "The desmosome. Fine structural studies with freeze fracture replication and tannic acid staining of sectioned epidermis," *Cell and Tissue Research*, vol. 172, no. 3, pp. 309–323, 1976.

[77] R. Leloup, L. Laurent, and M. F. Ronveaux, "Desmosomes and desmogenesis in the epidermis of calf muzzle," *Biologie Cellulaire*, vol. 34, no. 2-3, pp. 137–152, 1979.

[78] L. A. Staehelin, "Structure and function of intercellular junctions," *International Review of Cytology*, vol. 39, pp. 191–283, 1974.

[79] K. Miller, D. Mattey, H. Measures, C. Hopkins, and D. Garrod, "Localisation of the protein and glycoprotein components of bovine nasal epithelial desmosomes by immunoelectron microscopy," *EMBO Journal*, vol. 6, no. 4, pp. 885–889, 1987.

[80] A. Al-Amoudi, J.-J. Chang, A. Leforestier, et al., "Cryo-electron microscopy of vitreous sections," *EMBO Journal*, vol. 23, no. 18, pp. 3583–3588, 2004.

[81] A. Al-Amoudi, J. Dubochet, and L. Norlen, "Nanostructure of the epidermal extracellular space as observed by cryo-electron microscopy of vitreous sections of human skin," *Journal of Investigative Dermatology*, vol. 124, no. 4, pp. 764–777, 2005.

[82] W. W. Franke, R. Moll, and D. L. Schiller, "Desmoplakins of epithelial and myocardial desmosomes are immunologically and biochemically related," *Differentiation*, vol. 23, no. 2, pp. 115–127, 1982.

[83] A. Shimizu, A. Ishiko, T. Ota, et al., "In vivo ultrastructural localization of the desmoglein 3 adhesive interface to the desmosome mid-line," *Journal of Investigative Dermatology*, vol. 124, no. 5, pp. 984–989, 2005.

[84] M. S. Steinberg, H. Shida, G. J. Giudice, M. Shida, N. H. Patel, and O. W. Blaschuk, "On the molecular organization, diversity and functions of desmosomal proteins," *Ciba Foundation Symposium*, vol. 125, pp. 3–25, 1987.

[85] R. O. Hynes, "Integrins: bidirectional, allosteric signaling machines," *Cell*, vol. 110, no. 6, pp. 673–687, 2002.

[86] R. C. Liddington and M. H. Ginsberg, "Integrin activation takes shape," *Journal of Cell Biology*, vol. 158, no. 5, pp. 833–839, 2002.

[87] A. Jerome-Morais, H. R. Rahn, S. S. Tibudan, and M. F. Denning, "Role for protein kinase C-alpha in keratinocyte growth arrest," *Journal of Investigative Dermatology*, vol. 129, no. 10, pp. 2365–2375, 2009.

[88] D. R. Garrod, C. Fisher, A. Smith, and Z. Nie, "Pervanadate stabilizes desmosomes," *Cell Adhesion & Migration*, vol. 2, no. 3, pp. 161–166, 2008.

[89] E. P. Parrish, J. E. Marston, D. L. Mattey, H. R. Measures, R. Venning, and D. R. Garrod, "Size heterogeneity, phosphorylation and transmembrane organisation of desmosomal glycoproteins 2 and 3 (desmocollins) in MDCK cells," *Journal of Cell Science*, vol. 96, part 2, pp. 239–248, 1990.

[90] M. Pasdar, Z. Li, and H. Chan, "Desmosome assembly and disassembly are regulated by reversible protein phosphorylation in cultured epithelial cells," *Cell Motility and the Cytoskeleton*, vol. 30, no. 2, pp. 108–121, 1995.

[91] C. A. Gaudry, H. L. Palka, R. L. Dusek, et al., "Tyrosine-phosphorylated plakoglobin is associated with desmogleins but not desmoplakin after epidermal growth factor receptor activation," *Journal of Biological Chemistry*, vol. 276, no. 27, pp. 24871–24880, 2001.

[92] J. H. Lorch, J. Klessner, J. K. Park, et al., "Epidermal growth factor receptor inhibition promotes desmosome assembly and strengthens intercellular adhesion in squamous cell carcinoma cells," *Journal of Biological Chemistry*, vol. 279, no. 35, pp. 37191–37200, 2004.

[93] S. Miravet, J. Piedra, J. Castano, et al., "Tyrosine phosphorylation of plakoglobin causes contrary effects on its association with desmosomes and adherens junction components and modulates beta-catenin-mediated transcription," *Molecular and Cellular Biology*, vol. 23, no. 20, pp. 7391–7402, 2003.

[94] T. Yin, S. Getsios, R. Caldelari, et al., "Mechanisms of plakoglobin-dependent adhesion: desmosome-specific functions in assembly and regulation by epidermal growth factor receptor," *Journal of Biological Chemistry*, vol. 280, no. 48, pp. 40355–40363, 2005.

[95] C. B. Croft and D. Tarin, "Ultrastructural studies of wound healing in mouse skin. I. Epithelial behaviour," *Journal of Anatomy*, vol. 106, no. 1, pp. 63–77, 1970.

[96] Y. Danjo and I. K. Gipson, "Actin 'purse string' filaments are anchored by E-cadherin-mediated adherens junctions at the leading edge of the epithelial wound, providing coordinated cell movement," *Journal of Cell Science*, vol. 111, no. 22, pp. 3323–3332, 1998.

[97] T. D. Allen and C. S. Potten, "Desmosomal form, fate, and function in mammalian epidermis," *Journal of Ultrastructure Research*, vol. 51, no. 1, pp. 94–105, 1975.

[98] P. K. Holm, S. H. Hansen, K. Sandvig, and B. Van Deurs, "Endocytosis of desmosomal plaques depends on intact actin filaments and leads to a nondegradative compartment," *European Journal of Cell Biology*, vol. 62, no. 2, pp. 362–371, 1993.

[99] M. Pasdar and W. J. Nelson, "Regulation of desmosome assembly in epithelial cells: kinetics of synthesis, transport, and stabilization of desmoglein I, a major protein of the membrane core domain," *Journal of Cell Biology*, vol. 109, no. 1, pp. 163–177, 1989.

[100] M. Amagai, V. Klaus-Kovtun, and J. R. Stanley, "Autoantibodies against a novel epithelial cadherin in Pemphigus vulgaris, a disease of cell adhesion," *Cell*, vol. 67, no. 5, pp. 869–877, 1991.

[101] X. Ding, L. A. Diaz, J. A. Fairley, G. J. Giudice, and Z. Liu, "The anti-desmoglein 1 autoantibodies in pemphigus vulgaris sera are pathogenic," *Journal of Investigative Dermatology*, vol. 112, no. 5, pp. 739–743, 1999.

[102] E. J. Muller, L. Williamson, C. Kolly, and M. M. Suter, "Outside-in signaling through integrins and cadherins: a central mechanism to control epidermal growth and differentiation?" *Journal of Investigative Dermatology*, vol. 128, no. 3, pp. 501–516, 2008.

[103] G. F. H. Diercks, H. H. Pas, and M. F. Jonkman, "The ultrastructure of acantholysis in pemphigus vulgaris," *British Journal of Dermatology*, vol. 160, no. 2, pp. 460–461, 2009.

[104] K. Hashimoto and W. F. Lever, "An electron microscopic study on pemphigus vulgaris of the mouth and the skin with special reference to the intercellular cement," *Journal of Investigative Dermatology*, vol. 48, no. 6, pp. 540–552, 1967.

[105] C. H. Hu, B. Michel, and J. R. Schiltz, "Epidermal acantholysis induced in vitro by pemphigus autoantibody. An ultrastructural study," *American Journal of Pathology*, vol. 90, no. 2, pp. 345–362, 1978.

[106] Y. Takahashi, H. P. Patel, and R. S. Labib, "Experimentally induced pemphigus vulgaris in neonatal BALB/c mice: a time-course study of clinical, immunologic, ultrastructural, and cytochemical changes," *Journal of Investigative Dermatology*, vol. 84, no. 1, pp. 41–46, 1985.

[107] G. F. Wilgram, J. B. Caufield, and W. F. Lever, "An electron microscopic study of acntholysis in pemphigus vulgaris," *Journal of Investigative Dermatology*, pp. 373–382, 1961.

[108] A. Shimizu, A. Ishiko, T. Ota, K. Tsunoda, M. Amagai, and T. Nishikawa, "IgG binds to desmoglein 3 in desmosomes

and causes a desmosomal split without keratin retraction in a pemphigus mouse model," *Journal of Investigative Dermatology*, vol. 122, no. 5, pp. 1145–1153, 2004.

[109] M. Amagai, K. Tsunoda, H. Suzuki, K. Nishifuji, S. Koyasu, and T. Nishikawa, "Use of autoantigen-knockout mice in developing an active autoimmune disease model for pemphigus," *Journal of Clinical Investigation*, vol. 105, no. 5, pp. 625–631, 2000.

[110] Y. Aoyama and Y. Kitajima, "Pemphigus vulgaris-IgG causes a rapid depletion of desmoglein 3 (Dsg3) from the Triton X-100 soluble pools, leading to the formation of Dsg3- depleted desmosomes in a human squamous carcinoma cell line, DJM-1 cells," *Journal of Investigative Dermatology*, vol. 112, no. 1, pp. 67–71, 1999.

[111] C. C. Calkins, S. V. Setzer, J. M. Jennings, et al., "Desmoglein endocytosis and desmosome disassembly are coordinated responses to pemphigus autoantibodies," *Journal of Biological Chemistry*, vol. 281, no. 11, pp. 7623–7634, 2006.

[112] N. Cirillo, G. Campisi, F. Gombos, L. Perillo, F. Femiano, and A. Lanza, "Cleavage of desmoglein 3 can explain its depletion from keratinocytes in pemphigus vulgaris," *Experimental Dermatology*, vol. 17, no. 10, pp. 858–863, 2008.

[113] N. Cirillo, F. Femiano, F. Gombos, and A. Lanza, "Serum from pemphigus vulgaris reduces desmoglein 3 half-life and perturbs its de novo assembly to desmosomal sites in cultured keratinocytes," *FEBS Letters*, vol. 580, no. 13, pp. 3276–3281, 2006.

[114] E. Delva, J. M. Jennings, C. C. Calkins, M. D. Kottke, V. Faundez, and A. P. Kowalczyk, "Pemphigus vulgaris IgG-induced desmoglein-3 endocytosis and desmosomal disassembly are mediated by a clathrin- and dynamin-independent mechanism," *Journal of Biological Chemistry*, vol. 283, no. 26, pp. 18303–18313, 2008.

[115] Y. Yamamoto, Y. Aoyama, E. Shu, K. Tsunoda, M. Amagai, and Y. Kitajima, "Anti-desmoglein 3 (Dsg3) monoclonal antibodies deplete desmosomes of Dsg3 and differ in their Dsg3-depleting activities related to pathogenicity," *Journal of Biological Chemistry*, vol. 282, no. 24, pp. 17866–17876, 2007.

[116] N. Cirillo, A. Lanza, and S. S. Prime, "Induction of hyper-adhesion attenuates autoimmune-induced keratinocyte cell-cell detachment and processing of adhesion molecules via mechanisms that involve PKC," *Experimental Cell Research*, vol. 316, no. 4, pp. 580–592, 2010.

[117] X. Mao, E. J. Choi, and A. S. Payne, "Disruption of desmosome assembly by monovalent human pemphigus vulgaris monoclonal antibodies," *Journal of Investigative Dermatology*, vol. 129, no. 4, pp. 908–918, 2009.

[118] I. Sanchez-Carpintero, A. Espana, B. Pelacho, et al., "In vivo blockade of pemphigus vulgaris acantholysis by inhibition of intracellular signal transduction cascades," *British Journal of Dermatology*, vol. 151, no. 3, pp. 565–570, 2004.

[119] M. F. Jonkman, A. M. G. Pasmooij, S. G. M. A. Pasmans, et al., "Loss of desmoplakin tail causes lethal acantholytic epidermolysis bullosa," *American Journal of Human Genetics*, vol. 77, no. 4, pp. 653–660, 2005.

[120] E. A. Bornslaeger, C. M. Corcoran, T. S. Stappenbeck, and K. J. Green, "Breaking the connection: displacement of the desmosomal plaque protein desmoplakin from cell-cell interfaces disrupts anchorage of intermediate filament bundles and alters intercellular junction assembly," *Journal of Cell Biology*, vol. 134, no. 4, pp. 985–1001, 1996.

[121] E. A. Smith and E. Fuchs, "Defining the interactions between intermediate filaments and desmosomes," *Journal of Cell Biology*, vol. 141, no. 5, pp. 1229–1241, 1998.

[122] T. S. Stappenbeck, E. A. Bornslaeger, C. M. Corcoran, H. H. Luu, M. L. A. Virata, and K. J. Green, "Functional analysis of desmoplakin domains: specification of the interaction with keratin versus vimentin intermediate filament networks," *Journal of Cell Biology*, vol. 123, no. 3, pp. 691–705, 1993.

[123] T. S. Stappenbeck and K. J. Green, "The desmoplakin carboxyl terminus coaligns with and specifically disrupts intermediate filament networks when expressed in cultured cells," *Journal of Cell Biology*, vol. 116, no. 5, pp. 1197–1209, 1992.

[124] J. A. McGrath, J. R. McMillan, C. S. Shemanko, et al., "Mutations in the plakophilin 1 gene result in ectodermal dysplasia/skin fragility syndrome," *Nature Genetics*, vol. 17, no. 2, pp. 240–244, 1997.

[125] A. P. South, H. Wan, M. G. Stone, et al., "Lack of plakophilin 1 increases keratinocyte migration and reduces desmosome stability," *Journal of Cell Science*, vol. 116, no. 16, pp. 3303–3314, 2003.

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Review Article

Exploring the Nature of Desmosomal Cadherin Associations in 3D

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Desmosomes are a complex assembly of protein molecules that mediate adhesion between adjacent cells. Desmosome composition is well established and spatial relationships between components have been identified. Intercellular cell-cell adhesion is created by the interaction of extracellular domains of desmosomal cadherins, namely, desmocollins and desmogleins. High-resolution methods have provided insight into the structural interactions between cadherins. However, there is a lack of understanding about the architecture of the intact desmosomes and the physical principles behind their adhesive strength are unclear. Electron Tomography (ET) studies have offered three-dimensional visual data of desmosomal cadherin associations at molecular resolution. This review discusses the merits of two cadherin association models represented using ET. We discuss the possible role of sample preparation on the structural differences seen between models and the possibility of adaptive changes in the structure as a direct consequence of mechanical stress and stratification.

1. The Desmosome-a Historical Perspective

The desmosome ultrastructure has been the topic of many investigations since it was first described by the Italian pathologist Bizzozero in 1864 [1]. Schaffer [2] later introduced the term "desmosome" from the greek "desmos" meaning bond and "soma" meaning body. During this era the desmosome was thought to be a cytoplasm-filled intercellular bridge. This hypothesis was dismissed by the electron microscopy (EM) study of desmosomes by Porter [3], which was the first to display the desmosome as contacts between adjacent cells. A novel staining approach for EM by Rayns and coworkers [4] suggested that discrete dense particles of 4 nm in diameter provided the connections between cells. These particles were arranged in a staggered pattern with respect to the opposing membrane establishing a zipper-like mechanism of intercellular adhesion. Later on in the 1970's ultrastructural work focused on desmosome formation [5] and was complemented by biochemical and molecular biology approaches. The latter techniques were applied to identify the desmosome components and characterize their interactions [6–10] enabling the production of antibodies against each component. The spatial relationships between individual desmosome components were then identified, at the EM level, by immunogold localization [11]. Once the individual components involved in intercellular adhesion were established, high resolution EM [12], X-ray crystallography [13], Nuclear Magnetic Resonance (NMR) [14], and molecular force approaches [15] were used to study the extracellular associations between transmembrane glycoproteins named desmosomal cadherins, thus shedding light on the ability of desmosomes to resist mechanical shear force.

At present the main focus of investigations involve the role of desmosomes in cell differentiation [16–18], cancer [19] and inherited diseases of the heart and skin [20]. However, a revived interest in the structural mechanism of desmosomal function has become more prominent in the last few years with the advent of electron tomography (ET). ET provides high-resolution imaging of cellular complexes in their natural cellular environment, thus bridging the gap between structural studies at the single molecule

(NMR, X-ray crystallography) and cellular (Transmission Electron Microscopy (TEM), Light Microscopy) levels. ET studies have offered three-dimensional visual data of desmosomal cadherin associations at molecular resolution [21–23] complementing biochemical and molecular data. However, differences have been observed in the organization of the desmosomal cadherins, raising questions about the preservation of native structure during specimen preparation.

The purpose of this review is to discuss (1) the desmosome structure and its importance in maintaining tissue integrity; (2) high-resolution models of cadherin associations; and (3) current ET models of desmosomal cadherin associations *in situ*.

2. Desmosome Structure and Tissue Integrity

Today, desmosomes are widely recognized as an adhesive junction that are prevalent in tissue subjected to shear force (e.g., skin and heart and many epithelia). Desmosomes maintain tissue organization and provide mechanical strength by linking intermediate filaments (IF) networks of neighboring cells to each other, thus producing a scaffold that propagates across the entire tissue [24].

Structurally desmosomes resemble rivets at cell borders with a diameter of approximately 300 nm. They have two principal domains: the extracellular core domain (ECD) or "desmoglea" which is ~30 nm wide and bisected by a dense mid-line region, and a cytoplasmic dense plaque which lies parallel to the plasma membrane and separated from it by a less dense zone. The ECD is made up of extracellular domains of transmembrane glycoproteins belonging to the cadherin super family, which generally mediate calcium-dependent cell-cell adhesion in vertebrate tissue [25–27]. The desmosomal cadherins are called desmocollin (Dsc) and desmoglein (Dsg) and their cytoplasmic tails bind to a heterogeneous assembly of cytoplasmic components (desmoplakin (DP) I and II, plakoglobin (PG) and plakophilin (PKP)) to form the cytoplasmic dense plaque at the intracellular surface of the membrane. By providing the anchoring site for IF, this plaque indirectly connects the intercellular cadherins to the IF network of the cell (Figure 1).

Disruption of the desmosome structure generally has devastating consequences to the integrity of the tissue and the viability of the organism. For example, knockout of Dsc-1 causes epidermal fragility, barrier defects, abnormal differentiation, hyperproliferation, and hair loss [28] whereas the knockout of Dsc-3 causes permeability barrier defects of the stratum corneum and mice die shortly after birth with severe dehydration [29]. The targeted Dsc-3 null mutation is lethal before implantation in homozygous mutants [30] whereas the targeted Dsg-2 null mutation is lethal shortly after implantation. In the case of Dsg-2, defects are thought to be desmosome independent during early development when Dsg-2 is needed for survival of both embryonic stem cells and the early embryo [31].

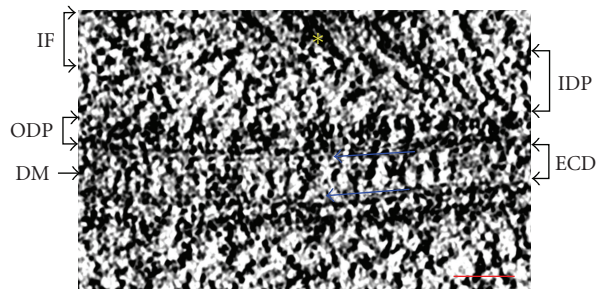


Figure 1: A tomographic slice of a desmosome bridging two opposing keratinocytes (delineated by two blue arrows) from the epidermis of a newborn mouse. Intermediate filaments (labeled IF and marked by a yellow asterix) from neighboring cells are interconnected to each other via the transmembrane cadherin proteins. Intracellular bridging proteins of the inner dense plaque (labeled IDP is a structural framework that includes cadherin intracellular domains, plakoglobin, plakophillin, and the N-terminal domain of desmoplakin) and the outer dense plaque (labeled ODP is a structural framework consisting of the rod domain and C-terminal domain of desmoplakin) link the intermediate filaments to the extracellular core domain (ECD). The ECD is composed of extracellular domains of desmosomal cadherins, namely, desmocollins and desmogleins. The area of cadherin interaction appears as a dense mid-line bisecting the ECD due to heterophilic associations between desmocollins and desmogleins. Scale bar represents 50 nm.

The importance of cytoplasmic plaque proteins such as DP or PG is also evident from mutational studies. Dsp mutant embryos die very early in E5.5–6.5 owing to defects in the extraembryonic tissues causing the expansion failure of the developing embryo egg cylinder [32]. Most PG-null embryos die of heart failure from E10.5–12.5 onwards [33]. However those PG-null mice that survive birth exhibit skin blistering and heart abnormalities [34]. Mutations to the desmosome components, such as DP and PG found in human genetic diseases, can lead to heart [16], skin [35], and hair defects [36]. Even Dsg-2 mis-expression has been associated with human squamous cell carcinomas for example, gastric cancer, where it is underexpressed, overexpressed [37] or upregulated [38]. However, the dependence of tissues on desmosome integrity is most evident in the autoimmune disease pemphigus vulgaris [39] and the exfoliative toxin in staphylococcal scalded skin syndrome [40]. Pemphigus vulgaris gives rise to acantholysis (i.e., the loss of cell-cell adhesion between keratinocytes) due to binding of autoantibodies to the EC1 domain of Dsg-1 and Dsg-3 [41], thus inhibiting cell-cell adhesion through steric hindrance [42]. In staphylococcal scalded skin syndrome superficial epidermal splitting is caused by the exfoliative toxin serine protease, which cleaves Dsg-1 between EC3 and EC4 again disrupting the cell-cell adhesion between keratinocytes [43]. Recent evidence suggests that antibody binding to desmosomal cadherins trigger external cascades, which may indeed amplify the disease pathogenesis [44] supporting the premise that the desmosome is a dynamic rather than a static structure.

3. High-Resolution Models of Cadherin Associations

When first formed, desmosome cadherin associations are relatively weak, but eventually they are capable of locking themselves in a hyperadhesive state [45]. Unlike the early desmosomes, this hyperadhesive junction is resistant to disruption by the calcium chelator, ethylene glycol bis (2-aminoethyl ether)-N,N,N′,N′- tetraacetic acid (EGTA) and is termed calcium independent [45]. In wound healing situations it has been demonstrated that desmosomes can revert to the weaker, calcium dependent state, when cell migration is necessary for regeneration of the epithelium. These observations indicate that the desmosome is not a static entity and is able to respond to environmental cues. The mechanism for controlling these adhesive states is believed to involve protein kinase C signaling, directly affecting cadherin association in the ECD [46]. Although no high-resolution structural work on desmsomes has yet addressed these states, there is evidence that cadherin organization may be involved. Understanding the mode of cadherin associations in these adhesive states would be a significant step in determining how signaling can alter adhesivity and would provide possible targets for treating conditions related to compromised desmosome integrity.

Cadherin association in the ECD is a calcium dependent process and defines this family of adhesion molecules. Cadherins are composed of an extracellular portion with five tandem Ig-like domains (Figures 2(a) and 2(b)), a single transmembrane helix and a cytosolic domain designed to interact with various proteins that compose the intracellular plaque. Each extracellular domain consists of ∼110 amino acids [47] that adopt a β-sandwich fold with the topology of a Greek key [14, 48] of which the dimensions are $4.5 \times 2.5 \times 2.5\,\text{nm}^3$. Three calcium ions are bound to the loop connecting successive domains via conserved sequence motifs [49, 50]. X-ray crystallographic data reveal that the three calcium ions bind between successive domains and are coordinated by conserved amino acids at the base of one domain and the top of the next and this coordination is similar in each of the four interdomain interfaces. In this way calcium binding confers rigidity to the domain interface and has been shown to induce extension of the entire extracellular portion of the molecule [12]. In the fully extended state, the molecule assumes a curved structure [51] with an angle of ∼100° between the N-terminal EC1 domain and the juxtamembrane EC5 domain [50].

The majority of the structural studies on cadherin-cadherin interactions have been carried out on the classical cadherins or type I cadherins. A conserved tryptophan residue near the N-terminus has been shown to be critical for adhesion in this type of cadherin (Figures 2(c) and 2(d)). The side chain of Trp^2 has been suggested to bind within a hydrophobic pocket in EC1 of a crystallographic neighbor, presumably corresponding to a partner cadherin from the opposing cell membrane [13, 48, 50] (Figure 2(e)). This interaction is known as the strand dimer (Figure 2(f)). Although desmosomal cadherins engage in heterophilic interactions whereas classic cadherins engage in homophilic interactions, interestingly despite their functional distinctions, Trp^2 is also conserved in desmosomal cadherins and is believed to be critical for heterophilic interactions. Heterophilic interactions can be formed by strand (otherwise known as cis) or lateral (otherwise known as trans) interactions. Lateral interactions describe cadherin domain associations from the same cell surface whereas strand interactions describe cadherin domain associations between opposite cell surfaces. Both lateral, trans, strand and cis interactions will be used in this review in accordance to the terms used in the original work. Due to the greater binding partner options available to desmosomal cadherins alternative models to the strand dimer model (Figure 3(a)) have been suggested as seen in Figures 3(b)–3(e). These being as follows.

(1) Cadherin adhesive dimerization by both lateral and strand associations via the surface of EC1 domain containing $\text{His}^{233}/\text{Val}^{235}$ residues [13] (Figure 3(b)).

(2) Lateral dimerization via the EC1/EC2 calcium binding sites [49, 51] (Figure 3(c)).

(3) Lateral interaction between the EC1 and EC2 domains [50] (Figure 3(d)).

(4) Adhesive interactions between antiparallel cadherin molecules along their full length-interdigitation model [52] (Figure 3(e)).

The strand dimer and the interdigitation models are the most plausible candidates for cadherin heterophilic interactions. This is because there is no evidence for adhesive interactions via the $\text{His}^{233}/\text{Val}^{235}$ containing EC1 domain surface or for lateral dimerization via the EC1–EC2 calcium-binding site [54–57]. However, the strand dimer model emphasizes symmetric interaction between EC1 domains and the interdigitation model implies very different interactions of EC1 with EC2–EC5. A recent study using intramolecular force microscopy may resolve the discrepancies between the two models [58]. This study confirms the existence of multiple binding sites for cadherins and proposes that as cadherins are pushed together, multiple bonds are formed as they form a parallel alignment in the middle of the extracellular gap. An increase in the curvature in the EC2-4 domains would be required to accommodate this parallel interaction at the mid-point between cells allowing for the formation of a mid-line bisecting the ECD [59]. Such cadherin associations would allow for the domain swap between EC1 domain proposed by Shapiro et al. [13] and Boggon et al. [50] and could explain the existence of associations between other extracellular domains proposed Zhu et al. [52].

4. Electron Tomography of Desmosomal Cadherin Associations

The models, described in the previous section, provide evidence for possible modes of desmosomal cadherin associations. However, an explanation for the ability of cadherins to recognize specific binding partners is still unresolved. The

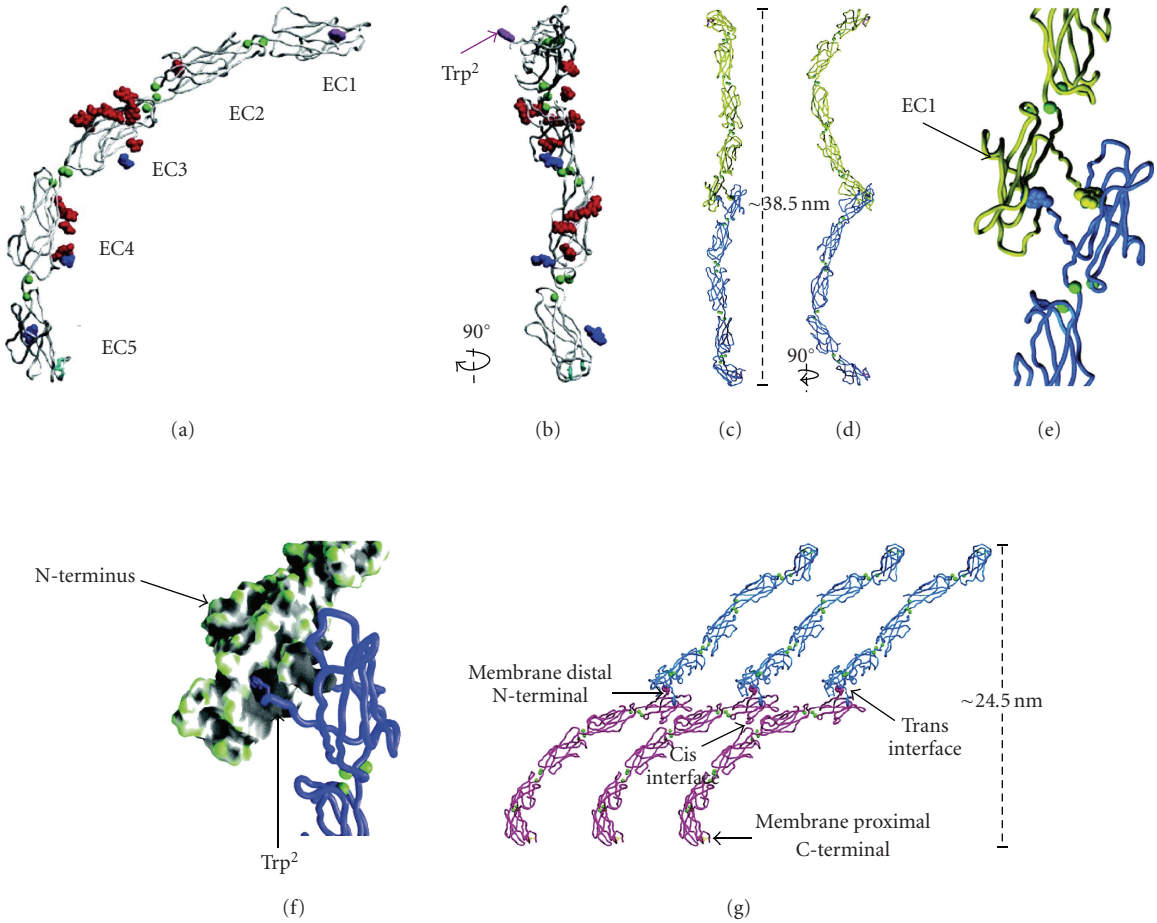


Figure 2: Type I or classical cadherin structure obtained from X-ray crystallographic studies. Structurally the extracellular portion of the cadherin molecule (also known as ectodomains and abbreviated EC) appears as five tandem Ig-like domains, each domain adopting a β-sandwich fold with the topology of a Greek key. Between each domain reside three calcium ions (green spheres) conferring rigidity to the domain interface and inducing the entire structure to assume an extended curve with a defined tilt of ~100° between the long axes of EC1, and 5. Disulfide bonds, O-linked sugars, and N-linked sugars are represented in cyan, red, and blue, respectively (a). In classical cadherins a conserved tryptophan residue near the N-terminus of EC1 has been shown to be crucial in the process of adhesion and is also conserved in desmosomal cadherins (abbreviated to Trp^2 and is depicted in purple by space filling representation in both a, and b. This Trp^2 is visibly protruding from the cadherin molecule when viewed at 90° rotation from a (b)) The tip-to-tip associations between the EC1 domains of cadherin molecules from opposing cells membranes form the strand dimer interface (c and at 90° rotation in d). EC1 domain binding interaction between opposing cadherins is highlighted in the magnified view (e). The side chain of Trp^2 has been suggested as a possible binding site within a hydrophobic pocket in EC1 of a corresponding partner cadherin from the opposing cell membrane. Trp^2 side chains (displayed as a backbone worm trace) can be seen inserting into a large concave cavity (depicted as grey shading in the molecular surface model) of the opposing EC1 domain (f). As the Trp^2 is conserved in desmosomal cadherins it is assumed that the Trp^2 interaction from classical cadherin studies is a compelling model for desmosomal cadherin association, forming a symmetric interdigitation interaction between cadherins of opposing cell membranes, and is known as the strand dimer model (g). Cadherin ectodomains from opposing cells are represented by blue or pink colors. Trp^2 side chains (space filling representation) are depicted in the color representing the cell. Yellow represents disulfide bonds. Modified from [50] reprinted with permission from AAAS.

difficulty lies in the artificial molecular constructs required for NMR or X-ray crystallography. In contrast, ET allows the study of desmosome structure in the native cellular environment.

Over the past decade technical advances in TEM hardware, imaging software, and sample preparation methods have significantly increased the resolving power of such instruments. The technique of ET has gained popularity as a high resolution imaging method because it provides three-dimensional visual data from any given asymmetric object and bridges the gap between structural studies at the molecular level and the cellular level [60]. Most importantly ET allows the investigation of the molecular architecture of cellular complexes in their natural cellular environment and therefore can be used to elucidate their structure-function relationships. Currently, a significant amount of data investigating desmosomal cadherin associations has been collected by ET. Before elaborating on the findings we will briefly introduce the concept of ET and the methodology involved. This understanding should then allow the reader

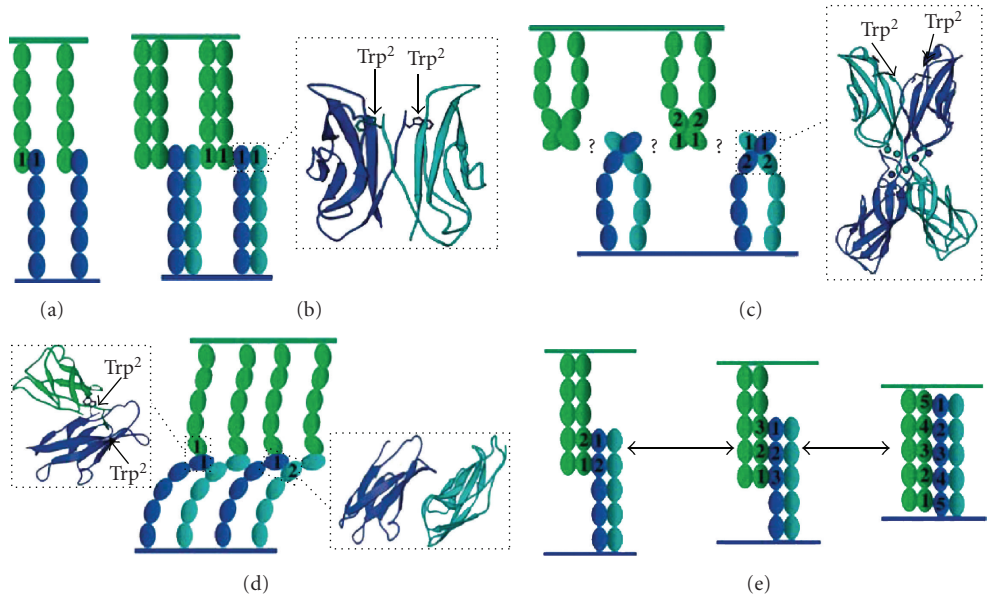


Figure 3: A visual representation of alternative desmosomal cadherin association models from the strand dimer (a), that consider the heterophilic interactions of desmosomal cadherins. Individual desmosomal cadherin ectodomains (1 to 5) are portrayed as ovals and are colored accordingly to clearly separate lateral from strand interactions. Identical color-coding is used to separate lateral and strand interactions in the ribbon representations. Cadherin ectodomains spanning from one cell surface are colored dark or light blue respectively and ectodomains spanning from an opposing cell surface are shown in green. (b) Cadherin dimerization via the surface of EC1 domains containing His^{233}/Val^{235} residues (residues not shown). Lateral dimers interact with strand dimers from an opposing cell. The insert shows a ribbon representation of the Trp^2-mediated lateral-contact. (c) Lateral dimerization via EC1/EC2 calcium binding sites facilitated by the rod-like shape of cadherin ectodomains. The inset shows a ribbon representation of the domain pair dimer from the same cell surface, the position of the calcium ions are represented by spheres and are color coded dark or light blue to represent the cadherin ectodomains in c. (d) Lateral interaction at EC1 between strand dimers bound together by EC1 and EC2 interactions. The left inset is a ribbon representation of Trp^2 mediated contact between N-terminal domains; the right inset is a ribbon representation of lateral interactions between EC1 and EC2 domains. (e) Adhesive interactions between antiparallel cadherin molecules along their full length. Strand interactions occur between more than two EC domains resulting in the formation of three types of adhesive complexes of different length. Modified from [53] with permission from Birkhäuser Verlag AG.

to follow the discussion on the differences seen in each desmosomal cadherin association model and the possible reasons for those differences.

4.1. Electron Tomography. The principle of creating a three-dimensional (3D) object from two-dimensional (2D) projections was conceived by Radon [61]. Theoretical predictions were then put into practice much later by DeRosier [60] when computing technology became available. The steps of creating a 3D object involve collecting 2D projection images at different tilt angles. The 2D projections are then represented as a 2D Fourier transform, which corresponds to the central section of the objects' 3D Fourier transform, that is, normal to the imaging angle. By tilting the object to different angles, the objects' entire Fourier transform can conceivably be sampled. The resolution of the technique is dependent on range and increment of the tilt angles. Due to practical limits of using a planar sample, the tilt range is limited to 160°, whereas the tilt increment is dependent on the electron dose that can be tolerated by the sample. Practically, the resolution of the technique depends upon the method of sample preservation and data collection parameters [62]. These two points will be briefly discussed in the next section.

4.2. Sample Preservation. Water is important in biological samples because the surfaces of nearly all biological macromolecules, macromolecular assemblies, and biological membranes are hydrophilic. Due to the nature of the exposed chemical groups transient hydrogen bonds are formed with water, forming a hydration shell. This shell prevents molecules from aggregating and maintains protein conformation. For this reason, native biological samples are incompatible with the high vacuum conditions maintained in the EM and are generally dehydrated prior to being embedded in a hard plastic and sectioned.

This process is innately destructive to the components of the tissue and a major goal of specimen preparation is to preserve cellular structure at a scale finer than the desired resolution. Preserving the specimen with cross-linking chemicals helps it withstand aggregation and extraction during dehydration. However, the conformation of the molecular constituents are often affected by this fixation and do not represent the native state.

Cryofixation is an alternative that exploits the high water content in cells and utilizes it as a physical fixative. Theoretically, it has the potential to preserve biological structures at the atomic level. Amorphous ice, a non-crystalline vitreous state of water, is the goal of cryofixation. Two techniques are currently used to cryofix samples for ET and are chosen depending on the size of the sample. High pressure freezing (HPF) is used to preserve bulk samples. This technique subjects the sample to a pressure of 2045 bar, thus decreasing the melting point of water to a minimum of 251 K. Within a few milliseconds a jet of liquid nitrogen cools the sample below the vitrification temperature. Plunge freezing is the technique normally used for smaller samples normally present in an aqueous suspension. Samples in aqueous suspension are spread thinly onto a glow discharged holey carbon grid and plunged into a cryogen at 1 m/s, embedding them in vitrified ice. Samples preserved with both methodologies can be imaged in their native frozen state at low temperature. Both methodologies serve a purpose in cryofixation, namely, to preserved samples of differing sizes. Samples preserved in vitrified thin films created by plunge freezing are generally isolated macromolecular complexes that are small enough to fit in a 200–300 nm thick layer of water. Objects larger than 1 μm are too thick to be observed by normal TEM and also too thick to vitrify by plunge freezing. Both problems can be overcome by HPF followed by physically cutting the vitrified sample into thin sections that can be observed with the TEM. This method is known as cryoelectron microscopy of vitreous sections (CEMOVIS) and has been shown to be applicable for tissue preservation in electron tomography [63]. Although cryoultramicrotomes are commercially available for cutting these sections, practical difficulties in handling these thin flakes of tissue and, specifically, in establishing their adherence to EM specimen support films make this method very technically demanding and its application is therefore limited to a few specialized laboratories worldwide.

An alternative technique known as freeze substitution has been developed so that cryofixed specimens can be imaged at room temperature with minimal changes from the vitrified state. The process of freeze substitution involves substituting the amorphous ice produced by HPF with solvents while maintaining the sample in the vitrified (frozen) state. Fixatives can optionally be added to the solvent so that cell constituents are preserved as the hydration shell is slowly removed by the dehydrating action of the solvent. Such mild conditions for fixation and dehydration minimize artifacts associated with aggregation and extraction. When the sample eventually reaches room temperature, after a stepwise increase in temperature, it is dehydrated and can then be embedded in epoxy resin and processed for imaging.

4.3. Data Acquisition. Whether the goal is to image the biological sample embedded in vitreous ice or in epoxy resin the irradiation of both samples by electrons can damage the specimen and alter the structures of interest. Low dose microscopy is a technique used during ET data acquisition to minimize the electron dose on the specimen while maximizing the signal-to-noise ratio in the resulting image [64].

4.4. In Situ Desmosome Cadherin Association Models in 3D. Desmosomes are readily identifiable in 2D TEM micrographs by their lamellar structure, prominent dense mid-line between opposing cell surfaces, and intermediate filaments inserting into an electron dense cytoplasmic plaque. Only two models of desmosomal cadherin associations in 3D have been described and their findings remain controversial. In the next section each model will be described and their differences discussed.

He and coworkers [21] investigated the desmosomal cadherin interaction in epidermis from newborn mice. The epidermis was processed by high pressure freezing followed by freeze substitution and embedding at room temperature in epoxy resin. Ultrathin sections were cut and stained with the conventional lead and uranium stains. Tomograms were collected by tilting about two orthogonal axes and tomograms were generated with isotropic resolution of approximately 2 nm. The authors' observed finger-like projections spanning across the intercellular space (28 nm wide) forming a dense mid-line believed to correspond to the area of cadherin associations (Figure 4(a)). These finger-like projections were tracked through the 3D volume and were found to be arranged in an irregular manner with tip-to-tip interaction only (Figure 4(b)i). These observations are inconsistent with the interdigitation model, though bore some resemblance to the molecular packing observed in the x-ray crystal of C-cadherin. In particular, the curved nature of the cadherin molecule seen by electron tomography did resemble C-cadherin ectodomains [50]. Since C-cadherins have 30–35% sequence identities to those of desmosomal cadherin ectodomains [59] the C-cadherin crystal structure was fitted into individual densities of cadherins to follow their interactions. Three distinct geometries were found resembling the letters W (Figure 4(b) ii), S (Figure 4(b) iii), and the greek letter λ (Figure 4(b) iv). Trans interactions occurred in W (23%) and S (43%) shaped cadherins pairs, whereas the addition of a third molecule with cis interactions produced a λ shape (40%) (Figure 4(b) v). The W shape closely resembled the two-fold symmetric strand dimer observed in the x-ray structure, which creates an exclusive molecular pairing due to the mutual binding of Trp^2 side chains to the hydrophobic pocket of the dimeric partner. However, the S and λ shapes distorted this symmetry to allow nonsymmetric interactions amongst larger groups of molecules. In particular, the S-shape also formed trans interactions and was related to the W-shape by rotation of the lower molecule relative to the upper one by 90°. This rotation had the effect of pulling one of the Trp^2 side chains out of its neighbors hydrophobic pocket, thereby making available for binding of a third molecule. Addition of this third molecule produced the λ-shape, which used the free Trp^2 to form a cis interaction. Interestingly this strategy of adding additional molecules in both cis and trans appeared to generate interacting networks of up to 6 molecules (Figure 4 vi). In such networks, a stochastic

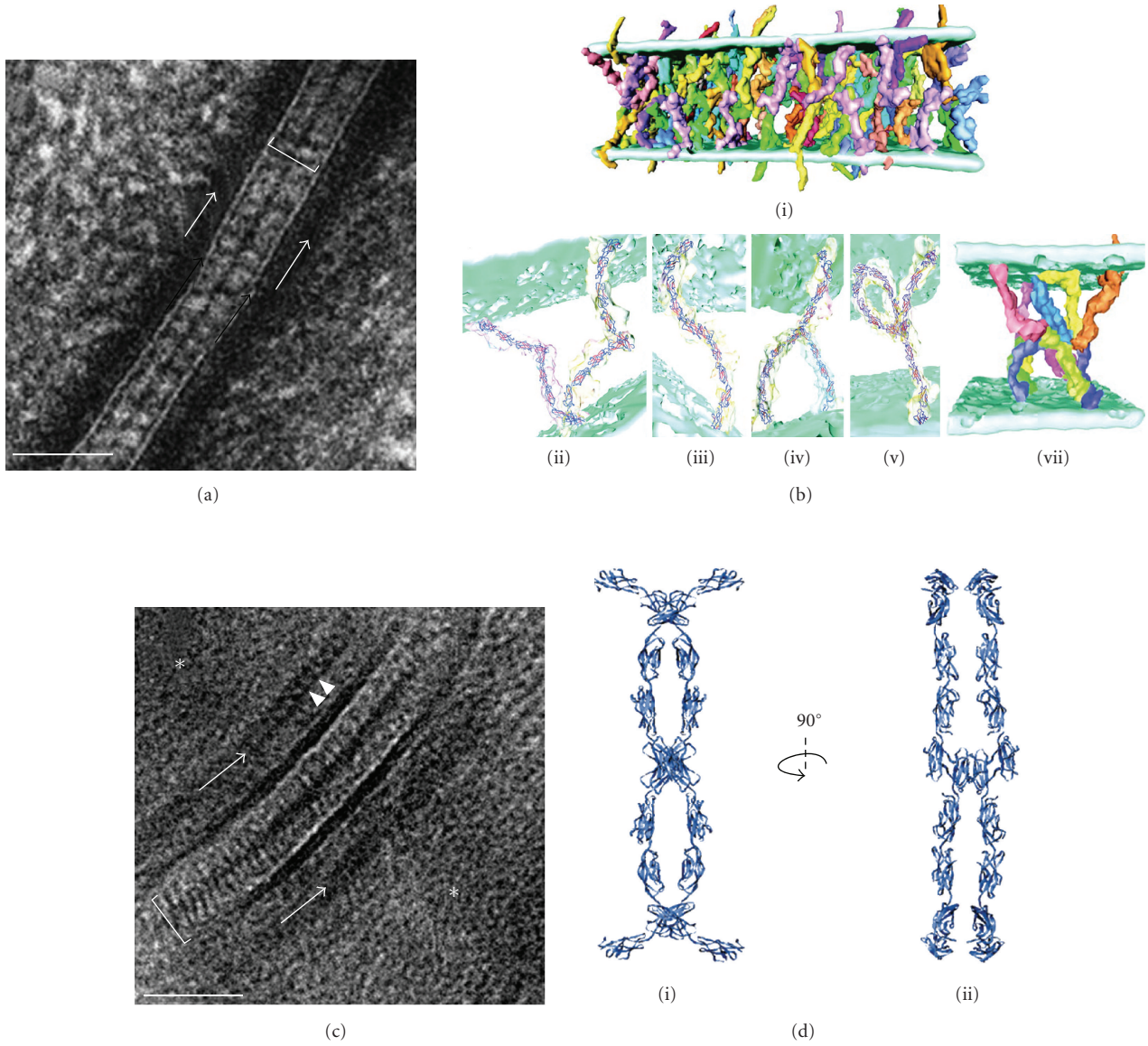


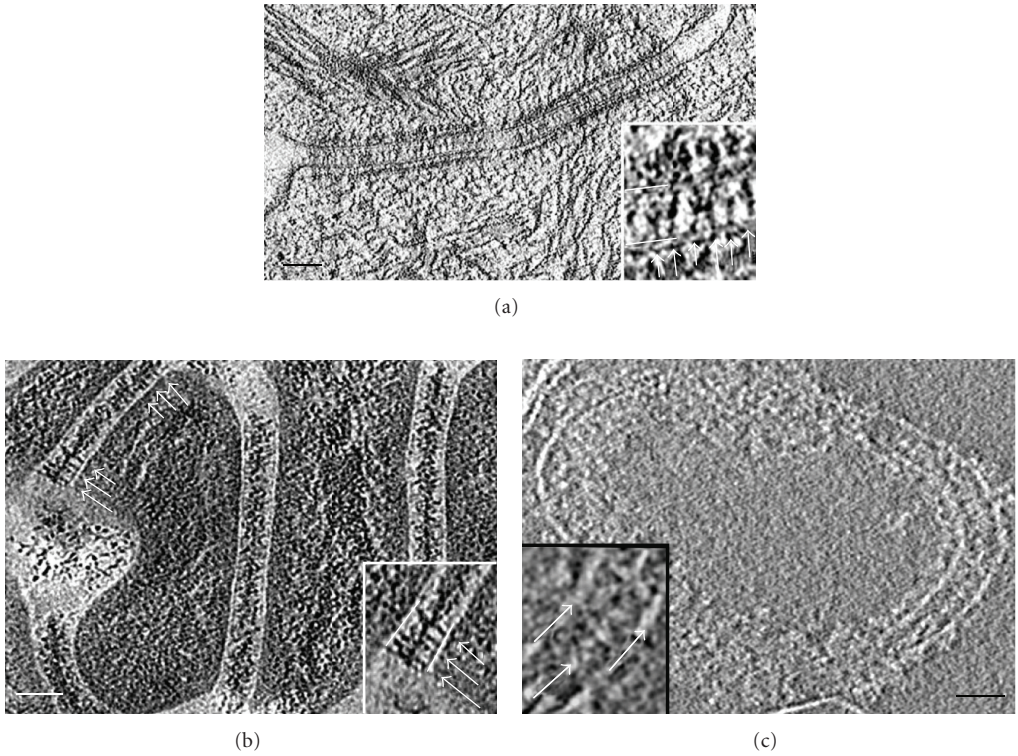
Figure 4: Two models of desmosomal cadherin associations generated from two methods of preparation. A single TEM projection of a newborn mouse epidermis desmosome preserved by high pressure freezing followed by freeze substitution (a). The dense midline is visible bisecting the extracellular core domain (ECD) (white line). Cadherins spanning the extracellular space are separated in an irregular nonsymetrical fashion. A model of cadherin association generated from a dual axis tomogram of newborn mouse epidermis (b i) preserved as in a. C-cadherin crystal structure has been traced onto densities in the ECD believed to be cadherins. The model displays the irregularity of cadherin associations however a dense mid-line is created by tip-to-tip association. The arrangement of cadherins differs to that of the strand dimer model. Three distinct geometries were found resembling the letters W (b ii), S (b iii), and the greek letter λ (b iv). Trans interactions mostly occurred in W and S whereas with λ, cis interactions mostly with S occurred if a third molecule was added (b v). An addition of a third molecule in cis could form a network in associations between up to 6 molecules (b vi). A single TEM projection of a desmosome from vitrified human epidermis processed by cryoelectron microscopy of vitreous sections (c). Densely packed and periodically arranged cadherins can be seen spanning the extracellular space in a straight manner (white line) and associating to form a dense mid-line. A model of cadherin association generated from a single axis tomogram of vitrified human epidermis processed as in c (d). The ribbon representation of the cadherin association model shows a periodic arrangement of curved cadherin densities that are organized with alternating trans and cis associations tip-to-tip. Two distinct geometries were visible in the model. The W shape formed the trans interactions (see d i) whereas the V-shape formed cis interactions resulting in a highly packed regular zipper-like organization. The organization is better visualized when then model is rotated at 90° from d i, revealing two trans interactions and one cis interaction (d ii). Images 4 B modified from [21] reprinted with permission from AAAS. Images 4 a & c modified from, [65] reprinted with permission from Elsevier, and Images 4D from [22] by permission from Macmillan Publishers Ltd. Scale bars 50 nm.

arrangement of cadherins interwoven at the midline formed a tangle of molecules (Figure 4 vi). Despite the distortion of the strand dimer interface seen in the x-ray structure, the authors concluded Trp^2 insertion into the hydrophilic pocket of a neighboring molecule was responsible for many of the interactions within the network. Such interactions were possible because of flexibility in the peptide linking Trp^2 to the EC1 domain, and to potential flexibility between successive EC2–EC5 domains. Calcium binding at the EC domain linkers is likely to control the overall flexibility of the cadherin molecule and perhaps to control the propensity of these various observed shapes. Flexibility was also observed in the orientation of the EC5 domain relative to the membrane. These EC5 domains were observed to form pairs or triplets with other EC5 domains. Interestingly, the molecules involved in EC5 groups near the membrane were engaged with different molecules at the midline, providing a mechanism for propagating interactions along the plane of the membrane. In summary, this investigation stressed the crucial role of Trp^2 and its hydrophobic pocket suggesting that Trp^2 can mediate both cis and trans interactions. Such interactions are possible due to the flexibility of the cadherin maximizing interactions between cadherin forming the junctions. As a result the tangle arrangement of cadherins at the midline may form physical bonds between cells whereas the intracellular domain may be responsible for the lateral stabilization of the groups.

A new model of cadherin associations in 2D by Al-Amoudi et al. [65] contradicted the irregular or stochastic cadherin associations observed by He et al. [21]. Desmosomes from human epidermis were imaged by CEMOVIS. The extracellular domains of desmosomal cadherins were visualized as densely packed and periodically arranged but protruding in a straight manner from the opposing cell membranes (Figure 4(c)). Cadherin periodicity at the dense mid-line was 5 nm within the 33 nm wide ECD. Differences in cadherin associations were attributed to the deleterious effect of conventional specimen preparation, citing that aggregation forces during dehydration may have caused the cadherins to form the observed stochastic associations [66]. However, such conclusions were derived from a single 2D projection and from a preparation technique, which is known to create sections that have been compressed between 30–45% along the cutting direction [67].

Recently, a 3D model of the human epidermis desmosome was presented by Al-Amoudi et al. [22]. The epidermis was processed by CEMOVIS and ultrathin cryosections of tissue were then imaged in low dose mode. Tomograms were generated by tilting about a single axis, thus producing an anisotropic resolution of approximately 3.4 nm. The study verified the periodic arrangement of the cadherins observed in 2D but at ~7 nm intervals along the mid-line rather than the 5 nm previously reported. In 3D, these periodic array of densities were curved (at an angle of 20° with the cell membrane), resembling the X-ray structure of C-cadherin and adopting an organization with alternating trans and cis interactions. The authors state that the highly conserved Trp^2 was most likely involved in adhesive binding and two distinct geometries were seen. The W-shape (as described in the He et al. model [21]) formed the trans interactions between molecules emanating from opposing cell surfaces. However, differing from the He et al. model [21] V-shape interactions between molecules emanating from the same cell membrane formed the cis interactions. These interactions were likely to be restricted to the EC1 domain when their concave surfaces faced each other. Such arrangement at the midline formed alternate cis and trans dimers resulting in a highly packed regular zipper-like organization (Figure 4(d) i–iii).

Of the two 3D models that exist describing cadherin interactions, they differ in that one describes the cadherin interaction with periodicity and the other with irregularity. Explanations for the disparity in these models are that cadherin packing varies in different tissue or that the preparative methodologies affect the results. A study by Owen et al. [23] attempted to evaluate the effects of the preparation methods on cadherin interactions in two types of tissues from two different species in 3D. In this study high pressure frozen, freeze substituted epoxy resin ultrathin sections of neonatal mouse epithelium and the well-characterized bovine snout *stratum spinosium* were first compared with electron tomography. Desmosome structure displayed similar features in both samples although the densities of cadherins and IF were much lower in the mouse epithelium (Figure 5(a)) compared to bovine snout (Figure 5(b)). A remarkable difference in ECD distances was seen between both tissues. Mouse skin ECD distances of 32.70 ± 1.97 nm ($n = 4$) confirmed existing results by He et al. [21], whereas the bovine snout ECD distances of 42.7 ± 2.63 nm ($n = 8$) were notably larger than the mouse. In order to compare the effects of preparation methods on the desmosome structure, purified isolated desmosomes from bovine snout were used as the sample because of their well-documented biochemical constitution [68, 69] and because milligram quantities of desmosomes cores were readily isolated, consisting of their plasma membrane and intracellular components as well as some intracellular filamentous material. For tomography, isolated desmosomes were vitrified in suspension by plunge freezing and imaged at low temperature in low-dose mode. In this way the need for sectioning was unnecessary therefore eliminating any possibility of compression artifacts that may have been caused by sectioning. In 3D the basic trilaminar features as seen in stained desmosomes *in situ* were observed that is, apposed membranes separated by a distinct midline and flanked by remnants of intracellular material. Irregular patterns of globular densities within the intracellular space were consistent with the presence of unresolved groups of cadherin molecules along the mid-line (Figure 5(c)). Such irregular groupings of cadherin molecules were observed previously in freeze substituted mouse epidermis. Therefore, this study suggested that the protocol used for freeze substitution did not cause major rearrangement of cadherin packing within the intercellular region. The authors hypothesized that, if any possibility of compression artifacts were eliminated then, the differences reported between cadherin associations in freeze substituted resin-embedded new-born mouse epidermis and frozen hydrated sections of human forearm epidermis might be due to different mechanical conditions experienced by the respected tissues. In tissues



(a)

(b)

(c)

Figure 5: Electron tomography of desmosomes from freeze-substituted resin-embedded epidermis of newborn mouse (a). Cadherin densities can be seen spanning the extracellular space and individual intermediate filaments projecting towards the cell membrane in the cytoplasm. The inset provides a magnified view of the irregular nature of cadherin association creating the dense mid-line. (b) In the cow snout *stratum spinosium*, cadherin densities can be seen spanning the extracellular space but the density of cadherin molecules is appreciably much higher. The dense mid-line can be observed by viewing the slice at a glancing angle however, in some areas, the irregular nature of cadherin association is visible (arrows) and can be seen magnified in the inset. Membranes are highlighted by lines and the cadherins by arrows in both a and b. (c) Cryoelectron tomography of isolated desmosomes in the frozen unstained state. A slice from a tomogram showing a desmosome which has curled up at the edge to provide a direct view of cadherin organisation. The two bilayers are clearly visible and a midline composed of globular densities can be seen bisecting the extracellular space (inset). These globular densities (arrow) probably correspond to the groups of cadherin molecules seen previously in freeze-substituted samples. Reproduced with permission from, [23] the Biochemical Society (http://www.biochemsoctrans.org/). Scale bars 50 nm.

placed under repeated, directional stress, irregular groups of cadherin molecules might be remodeled to form a quasi-ordered array to provide the greatest directional adhesive strength.

5. Conclusion, Perspectives, and Prospects for the Future

Desmosomes have an intrinsic mechanical role in maintaining the integrity of epithelium and this characteristic has been well documented. The desmosome also plays a role in directing tissue morphogenesis, sensing environmental signals, regulating tissue homeostasis, and as being a phenotypic determinator. On a structural level, these processes are controlled by the interconnection of the proteins composing the desmosome. Of particular interest is how desmosomal cadherin isoforms contribute to tissue morphogenesis and phenotypic determination. These processes are almost certainly controlled by intercellular interactions between desmosomal cadherins, which has been the topic of this review. In particular, cadherin interaction models using isolated cadherin domains have provided evidence for various modes of cadherin associations. In electron tomograms of desmosomes from both mouse skin and human skin samples, the highly conserved Trp^2 and its hydrophobic pocket has been deemed the most likely mechanism of adhesive binding. Neither structure shows any evidence for the interdigitation model proposed on the basis of intermolecular force measurements. However, these two models differ in that one observed cadherin interactions with periodicity and the other with irregularity. Although differences in preparation methods may have contributed to the observed differences, a study comparing freeze substituted samples with native vitrified samples of bovine snout desmosomes suggested that freeze substitution is capable of preserving molecular detail in the tissue. Another explanation for the structural differences may be the different mechanical conditions experienced in the various tissues: newborn mouse skin, cow snout, and the human forearm skin. The ~30% increase in Dsg-3 and Dsc-3 expression in thick versus thin skin may well signify how

differing mechanical differences can affect desmosome composition [70, 71] and, indirectly, cadherin association patterns. Therefore, further analyses of desmosomal cadherin associations are warranted to unequivocally demonstrate the adaptive response of desmosomal cadherin associations to mechanical stress. Indeed, although the basic organization of desmosomes is conserved across different species and tissues, they differ in their polypeptide composition [72, 73]. In fact, variation in desmosome frequency, diameter [74] and ECD thickness [75] have all been observed within a given tissue. Distinct expression profiles of cadherins across the epidermis is also well documented [71]. Such changes are thought to reflect the adhesive requirements of the epidermis as stratification proceeds [70], but might also reflect that desmosomes are less dynamic and able to form a more stable structure whilst allowing keratinocytes to maintain their adhesive properties [76, 77]. Understanding the effects of differentiation and mechanical shear on the structural associations within desmosomes represents a challenging, but important task for future investigations.

References

[1] G. Bizzozero, "Delle cellule cigliate, del reticulo Malpighiani d'ell epiderme," *Annali Universali di Medicina*, vol. 190, pp. 110–118, 1864.

[2] J. Schaffer, *Vorlesungen über Histologie und Histogenese*, Willhelm Engelman, Leipzig, Germany, 1920.

[3] K. P. Porter, "Observations on the fine structure of animal epidermis," in *Proceedings of the International Congress on Electron Microscopy*, p. 539, Royal Microscopical Society, London, UK, 1956.

[4] D. G. Rayns, F. O. Simpson, and J. M. Ledingham, "Ultrastructure of desmosomes in mammalian intercalated disc; appearances after lanthanum treatment," *Journal of Cell Biology*, vol. 42, no. 1, pp. 322–326, 1969.

[5] J. Overton, "Experimental manipulation of desmosome formation," *Journal of Cell Biology*, vol. 56, no. 3, pp. 636–646, 1973.

[6] C. J. Skerrow and A. G. Matoltsy, "Chemical characterization of isolated epidermal desmosomes," *Journal of Cell Biology*, vol. 63, no. 2, pp. 524–530, 1974.

[7] P. Drochmans, C. Freudenstein, J. C. Wanson, et al., "Structure and biochemical composition of desmosomes and tonofilaments isolated from calf muzzle epidermis," *Journal of Cell Biology*, vol. 79, no. 2 I, pp. 427–443, 1978.

[8] R. Moll and W. W. Franke, "Intermediate filaments and their interactions with membranes. The desmosome-cytokeratin filament complex and epithelial differentiation," *Pathology Research and Practice*, vol. 175, no. 2-3, pp. 146–161, 1982.

[9] G. Gorbsky and M. S. Steinberg, "Isolation of the intercellular glycoproteins of desmosomes," *Journal of Cell Biology*, vol. 90, no. 1, pp. 243–248, 1981.

[10] P. Cowin and D. R. Garrod, "Antibodies to epithelial desmosomes show wide tissue and species cross-reactivity," *Nature*, vol. 302, no. 5904, pp. 148–150, 1983.

[11] A. J. North, W. G. Bardsley, J. Hyam et al., "Molecular map of the desmosomal plaque," *Journal of Cell Science*, vol. 112, no. 23, pp. 4325–4336, 1999.

[12] S. Pokutta, K. Herrenknecht, R. Kemler, and J. Engel, "Conformational changes of the recombinant extracellular domain of E-cadherin upon calcium binding," *European Journal of Biochemistry*, vol. 223, no. 3, pp. 1019–1026, 1994.

[13] L. Shapiro, A. M. Fannon, P. D. Kwong et al., "Structural basis of cell-cell adhesion by cadherins," *Nature*, vol. 374, no. 6520, pp. 327–336, 1995.

[14] M. Overduin, T. S. Harvey, S. Bagby et al., "Solution structure of the epithelial cadherin domain responsible for selective cell adhesion," *Science*, vol. 267, no. 5196, pp. 386–389, 1995.

[15] S. Sivasankar, W. Brieher, N. Lavrik, B. Gumbiner, and D. Leckband, "Direct molecular force measurements of multiple adhesive interactions between cadherin ectodomains," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 21, pp. 11820–11824, 1999.

[16] K. J. Green and C. L. Simpson, "Desmosomes: new perspectives on a classic," *Journal of Investigative Dermatology*, vol. 127, no. 11, pp. 2499–2515, 2007.

[17] D. Garrod and M. Chidgey, "Desmosome structure, composition and function," *Biochimica et Biophysica Acta*, vol. 1778, no. 3, pp. 572–587, 2008.

[18] C. Jamora and E. Fuchs, "Intercellular adhesion, signalling and the cytoskeleton," *Nature Cell Biology*, vol. 4, no. 4, pp. E101–E108, 2002.

[19] M. Chidgey and C. Dawson, "Desmosomes: a role in cancer?" *British Journal of Cancer*, vol. 96, no. 12, pp. 1783–1787, 2007.

[20] O. Huber, "Structure and function of desmosomal proteins and their role in development and disease," *Cellular and Molecular Life Sciences*, vol. 60, no. 9, pp. 1872–1890, 2003.

[21] W. He, P. Cowin, and D. L. Stokes, "Untangling desmosomal knots with electron tomography," *Science*, vol. 302, no. 5642, pp. 109–113, 2003.

[22] A. Al-Amoudi, D. C. Díez, M. J. Betts, and A. S. Frangakis, "The molecular architecture of cadherins in native epidermal desmosomes," *Nature*, vol. 450, no. 7171, pp. 832–837, 2007.

[23] G. R. Owen, D. Acehan, K. D. Derr, W. J. Rice, and D. L. Stokes, "Cryoelectron tomography of isolated desmosomes," *Biochemical Society Transactions*, vol. 36, no. 2, pp. 173–179, 2008.

[24] B. M. Gumbiner, "Cell adhesion: the molecular basis of tissue architecture and morphogenesis," *Cell*, vol. 84, no. 3, pp. 345–357, 1996.

[25] R. S. Buxton and A. I. Magee, "Structure and interactions of desmosomal and other cadherins," *Seminars in Cell Biology*, vol. 3, no. 3, pp. 157–167, 1992.

[26] P. J. Koch and W. W. Franke, "Desmosomal cadherins: another growing multigene family of adhesion molecules," *Current Opinion in Cell Biology*, vol. 6, no. 5, pp. 682–687, 1994.

[27] F. Nollet, P. Kools, and F. van Roy, "Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members," *Journal of Molecular Biology*, vol. 299, no. 3, pp. 551–572, 2000.

[28] M. Chidgey, C. Brakebusch, E. Gustafsson et al., "Mice lacking desmocollin 1 show epidermal fragility accompanied by barrier defects and abnormal differentiation," *Journal of Cell Biology*, vol. 155, no. 5, pp. 821–832, 2001.

[29] P. M. Elias, N. Matsuyoshi, H. Wu et al., "Desmoglein isoform distribution affects stratum corneum structure and function," *Journal of Cell Biology*, vol. 153, no. 2, pp. 243–249, 2001.

[30] Z. Den, X. Cheng, M. Merched-Sauvage, and P. J. Koch, "Desmocollin 3 is required for pre-implantation development of the mouse embryo," *Journal of Cell Science*, vol. 119, no. 3, pp. 482–489, 2006.

[31] L. Eshkind, Q. Tian, A. Schmidt, W. W. Franke, R. Windoffer, and R. E. Leube, "Loss of desmoglein 2 suggests essential functions for early embryonic development and proliferation of embryonal stem cells," *European Journal of Cell Biology*, vol. 81, no. 11, pp. 592–598, 2002.

[32] G. I. Gallicano, P. Kouklis, C. Bauer et al., "Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage," *Journal of Cell Biology*, vol. 143, no. 7, pp. 2009–2022, 1998.

[33] G. I. Gallicano, C. Bauer, and E. Fuchs, "Rescuing desmoplakin function in extra-embryonic ectoderm reveals the importance of this protein in embryonic heart, neuroepithelium, skin and vasculature," *Development*, vol. 128, no. 6, pp. 929–941, 2001.

[34] C. Bierkamp, H. Schwarz, O. Huber, and R. Kemler, "Desmosomal localization of β-catenin in the skin of plakoglobin null-mutant mice," *Development*, vol. 126, no. 2, pp. 371–381, 1999.

[35] J. A. McGrath, "Inherited disorders of desmosomes," *Australasian Journal of Dermatology*, vol. 46, no. 4, pp. 221–229, 2005.

[36] H. Bazzi and A. M. Christiano, "Broken hearts, woolly hair, and tattered skin: when desmosomal adhesion goes awry," *Current Opinion in Cell Biology*, vol. 19, no. 5, pp. 515–520, 2007.

[37] M. Yashiro, N. Nishioka, and K. Hirakawa, "Decreased expression of the adhesion molecule desmoglein-2 is associated with diffuse-type gastric carcinoma," *European Journal of Cancer*, vol. 42, no. 14, pp. 2397–2403, 2006.

[38] H. Kurzen, I. Münzing, and W. Hartschuh, "Expression of desmosomal proteins in squamous cell carcinomas of the skin," *Journal of Cutaneous Pathology*, vol. 30, no. 10, pp. 621–630, 2003.

[39] W. F. Lever, "Pemphigus," *Medicine*, vol. 32, no. 1, pp. 1–123, 1953.

[40] M. E. Melish and L. A. Glasgow, "The staphylococcal scalded skin syndrome: development of an experimental model," *New England Journal of Medicine*, vol. 282, no. 20, pp. 1114–1119, 1970.

[41] M. Sekiguchi, Y. Futei, Y. Fujii, T. Iwasaki, T. Nishikawa, and M. Amagai, "Dominant autoimmune epitopes recognized by pemphigus antibodies map to the N-terminal adhesive region of desmogleins," *Journal of Immunology*, vol. 167, no. 9, pp. 5439–5448, 2001.

[42] A. Shimizu, A. Ishiko, T. Ota, K. Tsunoda, M. Amagai, and T. Nishikawa, "IgG binds to desmoglein 3 in desmosomes and causes a desmosomal split without keratin retraction in a pemphigus mouse model," *Journal of Investigative Dermatology*, vol. 122, no. 5, pp. 1145–1153, 2004.

[43] K. Nishifuji, M. Sugai, and M. Amagai, "Staphylococcal exfoliative toxins: "molecular scissors" of bacteria that attack the cutaneous defense barrier in mammals," *Journal of Dermatological Science*, vol. 49, no. 1, pp. 21–31, 2008.

[44] R. L. Dusek, L. M. Godsel, and K. J. Green, "Discriminating roles of desmosomal cadherins: beyond desmosomal adhesion," *Journal of Dermatological Science*, vol. 45, no. 1, pp. 7–21, 2007.

[45] D. Garrod and T. E. Kimura, "Hyper-adhesion: a new concept in cell-cell adhesion," *Biochemical Society Transactions*, vol. 36, no. 2, pp. 195–201, 2008.

[46] D. Garrod, M. Y. Berika, W. F. Bardsley, D. Holmes, and L. Tabernero, "Hyper-adhesion in desmosomes: its regulation in wound healing and possible relationship to cadherin crystal structure," *Journal of Cell Science*, vol. 118, no. 24, pp. 5743–5754, 2005.

[47] K. Hatta, A. Nose, A. Nagafuchi, and M. Takeichi, "Cloning and expression of cDNA encoding a neural calcium-dependent cell adhesion molecule: its identity in the cadherin gene family," *Journal of Cell Biology*, vol. 106, no. 3, pp. 873–881, 1988.

[48] L. Shapiro, P. D. Kwong, A. M. Fannon, D. R. Colman, and W. A. Hendrickson, "Considerations on the folding topology and evolutionary origin of cadherin domains," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 15, pp. 6793–6797, 1995.

[49] B. Nagar, M. Overduin, M. Ikura, and J. M. Rini, "Structural basis of calcium-induced E-cadherin rigidification and dimerization," *Nature*, vol. 380, no. 6572, pp. 360–364, 1996.

[50] T. J. Boggon, J. Murray, S. Chappuis-Flament, E. Wong, B. M. Gumbiner, and L. Shapiro, "C-cadherin ectodomain structure and implications for cell adhesion mechanisms," *Science*, vol. 296, no. 5571, pp. 1308–1313, 2002.

[51] O. Pertz, D. Bozic, A. W. Koch, C. Fauser, A. Brancaccio, and J. Engel, "A new crystal structure, Ca^{2+} dependence and mutational analysis reveal molecular details of E-cadherin homoassociation," *EMBO Journal*, vol. 18, no. 7, pp. 1738–1747, 1999.

[52] B. Zhu, S. Chappuis-Flament, E. Wong, I. E. Jensen, B. M. Gumbiner, and D. Leckband, "Functional analysis of the structural basis of homophilic cadherin adhesion," *Biophysical Journal*, vol. 84, no. 6, pp. 4033–4042, 2003.

[53] A. W. Koch, K. L. Manzur, and W. Shan, "Structure-based models of cadherin-mediated cell adhesion: the evolution continues," *Cellular and Molecular Life Sciences*, vol. 61, no. 15, pp. 1884–1895, 2004.

[54] Y. Shimoyama, H. Takeda, S. Yoshihara, M. Kitajima, and S. Hirohashi, "Biochemical characterization and functional analysis of two type II classic cadherins, cadherin-6 and -14, and comparison with E-cadherin," *Journal of Biological Chemistry*, vol. 274, no. 17, pp. 11987–11994, 1999.

[55] M. Kitagawa, M. Natori, S. Murase, S. Hirano, S. Taketani, and S. T. Suzuki, "Mutation analysis of cadherin-4 reveals amino acid residues of EC1 important for the structure and function," *Biochemical and Biophysical Research Communications*, vol. 271, no. 2, pp. 358–363, 2000.

[56] O. Y. Laur, J. Klingelhöfer, R. B. Troyanovsky, and S. M. Troyanovsky, "Both the dimerization and immunochemical properties of E-cadherin EC1 domain depend on Trp(156) residue," *Archives of Biochemistry and Biophysics*, vol. 400, no. 1, pp. 141–147, 2002.

[57] R. B. Troyanovsky, E. Sokolov, and S. M. Troyanovsky, "Adhesive and lateral E-cadherin dimers are mediated by the same interface," *Molecular and Cellular Biology*, vol. 23, no. 22, pp. 7965–7972, 2003.

[58] Y. Tsukasaki, K. Kitamura, K. Shimizu, A. H. Iwane, Y. Takai, and T. Yanagida, "Role of multiple bonds between the single cell adhesion molecules, nectin and cadherin, revealed by high sensitive force measurements," *Journal of Molecular Biology*, vol. 367, no. 4, pp. 996–1006, 2007.

[59] D. L. Stokes, "Desmosomes from a structural perspective," *Current Opinion in Cell Biology*, vol. 19, no. 5, pp. 565–571, 2007.

[60] D. J. DeRosier and A. Klug, "Reconstrucution of three dimensional structures from electron micrographs," *Nature*, vol. 217, no. 5124, pp. 130–134, 1968.

[61] J. H. Radon and A. Klug, "Über die Bestimmng von Funktionen durch ihre Integralwerte längs gewisser Mannigfaltigkeiten. Berichte über die Verhandlungen der Königlich Sächsische Gesellschaft der Wissenschaften zu Leipzig,"

Mathematisch-Physikalischen Klasse, vol. 69, pp. 262–277, 1917.

[62] G. Owen and D. L. Stokes, "An introduction to low dose electron tomography- from specimen preparation to data collection," in *Modern Research and Educational Topics in Microscopy*, vol. 2 of *Microscopy Series*, no. 3, pp. 939–950, Formatex, Badjadoz, Spain, 2007.

[63] A. Al-Amoudi, J.-J. Chang, A. Leforestier, et al., "Cryo-electron microscopy of vitreous sections," *EMBO Journal*, vol. 23, no. 18, pp. 3583–3588, 2004.

[64] K. Dierksen, D. Typke, R. Hegerl, and W. Baumeister, "Towards automatic electron tomography. II. Implementation of autofocus and low-dose procedures," *Ultramicroscopy*, vol. 49, no. 1–4, pp. 109–120, 1993.

[65] A. Al-Amoudi, L. P. O. Norlen, and J. Dubochet, "Cryo-electron microscopy of vitreous sections of native biological cells and tissues," *Journal of Structural Biology*, vol. 148, no. 1, pp. 131–135, 2004.

[66] J. Dubochet and N. Sartori Blanc, "The cell in absence of aggregation artifacts," *Micron*, vol. 32, no. 1, pp. 91–99, 2001.

[67] A. Al-Amoudi, J. Dubochet, H. Gnaegi, W. Lüthi, and D. Studer, "An oscillating cryo-knife reduces cutting-induced deformation of vitreous ultrathin sections," *Journal of Microscopy*, vol. 212, no. 1, pp. 26–33, 2003.

[68] G. Gorbsky, S. M. Cohen, H. Shida, G. J. Giudice, and M. S. Steinberg, "Isolation of the nonglycosylated proteins of desmosomes and immunolocalization of a third plaque protein: desmoplakin III," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 82, no. 3, pp. 810–814, 1985.

[69] U. A. Nuber, S. Schafer, A. Schmidt, P. J. Koch, and W. W. Franke, "The widespread human desmocollin Dsc2 and tissue-specific patterns of synthesis of various desmocollin subtypes," *European Journal of Cell Biology*, vol. 66, no. 1, pp. 69–74, 1995.

[70] J. Arnemann, K. H. Sullivan, A. I. Magee, I. A. King, and R. S. Buxton, "Stratification-related expression of isoforms of the desmosomal cadherins in human epidermis," *Journal of Cell Science*, vol. 104, no. 3, pp. 741–750, 1993.

[71] H. Wan, P. J. C. Dopping-Hepenstal, M. J. Gratian, M. G. Stone, J. A. McGrath, and R. A. J. Eady, "Desmosomes exhibit site-specific features in human palm skin," *Experimental Dermatology*, vol. 12, no. 4, pp. 378–388, 2003.

[72] G. J. Giudice, S. M. Cohen, N. H. Patel, and M. S. Steinberg, "Immunological comparison of desmosomal components from several bovine tissues," *Journal of Cellular Biochemistry*, vol. 26, no. 1, pp. 35–45, 1984.

[73] S. M. Jones, J. C. Jones, and R. D. Goldman, "Fractionation of desmosomes and comparison of the polypeptide composition of desmosomes prepared from two bovine epithelial tissues," *Journal of cellular biochemistry*, vol. 36, no. 3, pp. 223–236, 1988.

[74] C. J. Skerrow, D. G. Clelland, and D. Skerrow, "Changes to desmosomal antigens and lectin-binding sites during differentiation in normal human epidermis: a quantitative ultrastructural study," *Journal of Cell Science*, vol. 92, no. 4, pp. 667–677, 1989.

[75] A. Konohana, I. Konohana, G. P. Roberts, and R. Marks, "Biochemical changes in desmosomes of bovine muzzle epidermis during differentiation," *Journal of Investigative Dermatology*, vol. 89, no. 4, pp. 353–357, 1987.

[76] E. A. Smith and E. Fuchs, "Defining the interactions between intermediate filaments and desmosomes," *Journal of Cell Biology*, vol. 141, no. 5, pp. 1229–1241, 1998.

[77] E. Candi, E. Tarcsa, J. J. Digiovanna, et al., "A highly conserved lysine residue on the head domain of type II keratins is essential for the attachment of keratin intermediate filaments to the cornified cell envelope through isopeptide crosslinking by transglutaminases," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 5, pp. 2067–2072, 1998.

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Review Article

The Desmosomal Plaque Proteins of the Plakophilin Family

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Three related proteins of the plakophilin family (PKP1_3) have been identified as junctional proteins that are essential for the formation and stabilization of desmosomal cell contacts. Failure of PKP expression can have fatal effects on desmosomal adhesion, leading to abnormal tissue and organ development. Thus, loss of functional PKP 1 in humans leads to ectodermal dysplasia/skin fragility (EDSF) syndrome, a genodermatosis with severe blistering of the epidermis as well as abnormal keratinocytes differentiation. Mutations in the human PKP 2 gene have been linked to severe heart abnormalities that lead to arrhythmogenic right ventricular cardiomyopathy (ARVC). In the past few years it has been shown that junctional adhesion is not the only function of PKPs. These proteins have been implicated in cell signaling, organization of the cytoskeleton, and control of protein biosynthesis under specific cellular circumstances. Clearly, PKPs are more than just cell adhesion proteins. In this paper we will give an overview of our current knowledge on the very distinct roles of plakophilins in the cell.

1. Introduction

Cellular adhesion is mediated by distinct protein complexes at the cytoplasmic membrane, termed junctions, that have been characterized by their morphology on the ultrastructural level [1]. Desmosomes reveal a characteristic appearance and anchor different types of intermediate filaments (IF) to the cell membrane. The fundamental functional importance of desmosomal cell contacts for cellular and tissue architecture, differentiation, development, and tissue stability is generally accepted and has previously been described [2–4]. Experimental evidence for the importance of desmosomal adhesion for specific tissues and organs has been established by knockout experiments of desmosomal genes in mice (see, e.g., [5]). Moreover, examination of a variety of human diseases characterized by a loss, or impairment of desmosomal adhesion—regardless of genetical, autoimmune, or infectious etiology—advanced our understanding of desmosomal function [6]. Desmosomes are formed by all epithelial tissues and tumors derived therefrom as well as by specific nonepithelial tissues such as heart muscle cells. Desmosomal cadherins (i.e., desmogleins DSGs and desmocollins DSCs) located on adjacent cells mediate intercellular connection via interactions of their extracellular domains (for review see [7]). On the cytoplasmic side of the plasma membrane, IF are linked to the desmosomal cadherins via desmosomal plaque proteins. Besides the constitutive desmosomal plaque proteins desmoplakin (DSP) and plakoglobin (JUP), at least one of the three classical members of the plakophilin family (PKP 1 to PKP 3) is required for the formation of functional desmosomes [8–10]. The role of PKPs in cellular adhesion have been analyzed in detail during the past decade [8–10]. However, additional functions of the plakophilins that are not directly linked to desmosomal adhesion have recently been described. In this review we want to provide insights not only into the known properties and functions of plakophilins in desmosomes, but also into cellular functions not related to adhesion.

2. Common Features of the Plakophilins

Plakophilins are probably the most basic proteins identified in cellular adhesion complexes so far with an isoelectric point

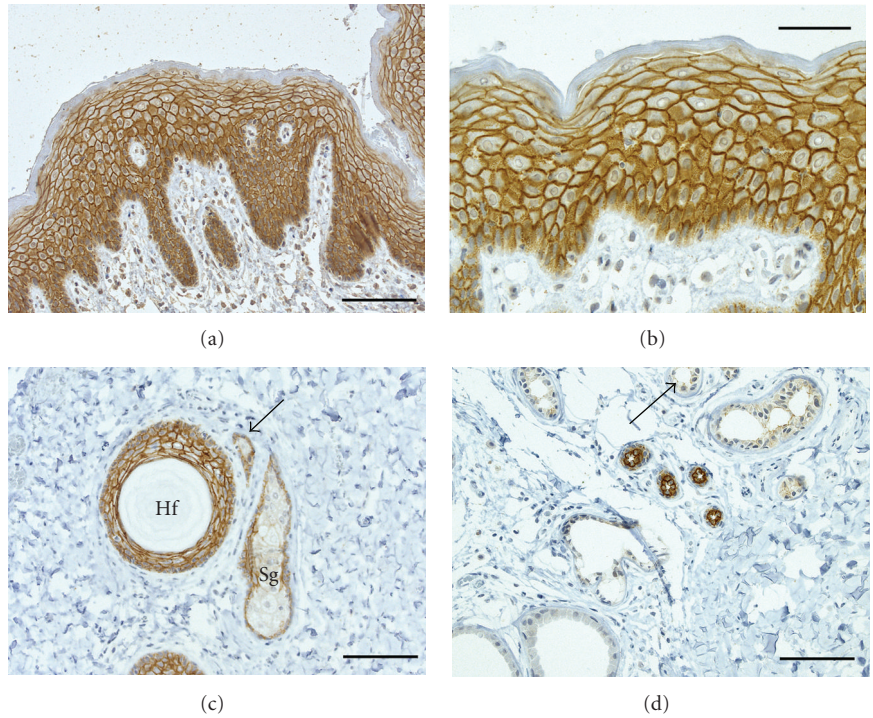

Figure 1: Immunohistochemical staining of sections of human skin with antibodies against PKP 1. Sections of formaldehyde-fixed tissue samples of human skin were stained with a monoclonal antibody (clone PP1 5C2; Progen, Heidelberg; for methods see [18]) against PKP 1 a to d. (a) Overview of epidermis showing a strong reaction of the antibodies at the desmosomes of all layers. (b) At a higher magnification, the basal layers exhibit a somewhat weaker desmosomal staining that can be resolved occasionally into individual spot-like desmosomes containing PKP 1. During keratinocyte differentiation, desmosomal labeling is getting more pronounced. (c) Cross-section of a hair follicle (Hf) with desmosomal staining of the outer root sheath while the hair-shaft is not stained (Sg, sebaceous gland). Arrow marks the duct of a sebaceous gland. (d) Eccrine sweat ducts are marked intensively by antibodies while the secretory portions of eccrine glands show a distinct but weaker staining (arrow). Apocrine sweat glands (lower left corner) are negative. Scale bars: 100 μm (b); 200 μm a, c, and d.

(pI) of about pH 9.3. Based on their primary sequences, PKPs have been classified as a distinct subfamily of the armadillo repeat proteins (for review see [11]). The carboxyl-terminal part of the proteins includes nine armadillo repeats which contain a spacer sequence between the fifth and sixth repeat that leads to a characteristic kink in the domain structure as determined by crystallography of the armadillo domain of PKP 1 [12]. The amino-terminal parts (head domain) of the three plakophilins are rather diverse and exhibit no obvious homology to themselves or other proteins. Only a small sequence near the amino-terminus, designated homology region (HR) 2, shows some degree of homology between the plakophilins. An analysis of amino acid sequence homology reveals that the PKPs are related to the catenin proteins of the $p120^{ctn}$-group, which are associated with classical cadherins, such as E-cadherin, in adherens junctions. The PKPs are more distantly related to the classical catenins, β-catenin and plakoglobin [8, 13]. PKPs show complex but overlapping expression patterns in mammalian tissues. Certain cells and tissues express only one type of PKP. Mutations affecting the corresponding PKPs thus can lead to severe diseases in these tissues since compensatory PKP isoforms are not expressed or may not substitute for all functional aspects. This probably explains the severe skin diseases caused by PKP 1 mutations and the heart diseases caused by PKP 2 mutations.

2.1. Plakophilin 1. PKP 1 is the smallest of the plakophilins, with a calculated molecular weight of 80.497 Da and an apparent molecular weight of approximately 75 kDa as judged by SDS-PAGE [14]. This protein is localized in the desmosomes of stratified, complex, and transitional epithelia but is absent in simple epithelia [14–16]. In stratified epithelia, PKP 1 is synthesized in all cell layers, with an increase in expression from the basal to the granular compartment as determined by quantifications of PKP 1-specific immunofluorescence signal intensity in human epidermis [17]. This indicates that PKP1 is a marker for keratinocyte differentiation. PKP 1 appears to be absent in the *stratum corneum* of stratified squamous epithelia though (Figure 1).

The human PKP 1 gene is expressed as two different splice variants which differ with respect to cell-biological behavior, molecular weight, and abundance. PKP1a is the smaller isoform while the larger PKP1b isoform (predicted molecular weight: 82.860 kDa) is less abundant in stratified epithelia. The additional amino acid sequence contained in PKP1b is encoded by exon 7 which is spliced out of the PKP1a mRNA [19]. The PKP1b-specific amino acid sequence is located at the end of the fourth armadillo repeat and has a distinct effect on the cell biological activities of the protein. In addition to its desmosomal localization, PKP 1 has been detected in the nucleus of a broad range

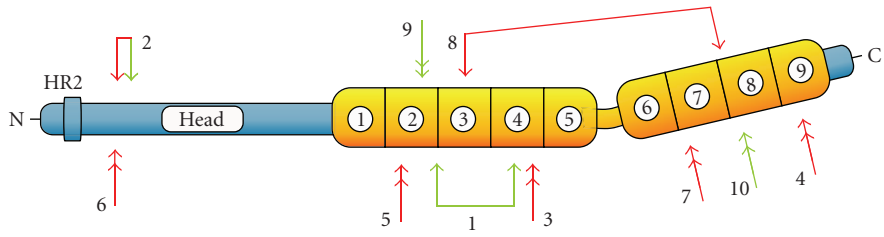

Figure 2: Position of mutations in human PKP 1 gene. Schematic representation of the protein structure of PKP 1 with head domain "Head" in blue color containing the homologous region 2 "HR2" near the amino-terminus which is followed by nine armadillo repeats (yellow boxes; numbered in circles from 1 to 9). Finally, a short domain (blue) at the carboxyl-terminus is shown. Positions of homozygous mutations are marked by double arrows, positions of compound heterozygous mutations by connected arrows. Green arrows designate mutations affecting the coding region, red arrows denote splice-site mutations. For numbering and references of the mutations see Table 1.

of cell types, even in those that do not incorporate PKP 1 in desmosomes such as simple epithelial cells [14, 19]. This distinct subcellular distribution has been observed for both variants of PKP 1. While the smaller PKP 1a may also be present in desmosomes, PKP 1b localization is restricted to the nucleus and not detectable in desmosomes. This conclusion is supported by transfection of cDNAs into cultured cells, where PKP 1a accumulates in desmosomes and is also rapidly transferred into the nucleus, while PKP 1b is only nuclear (own observations). Nevertheless, neither the way PKP 1 enters the nucleus nor the functions of this protein therein are yet known.

Both the nuclear and desmosomal PKP 1 pool are degraded by caspases rapidly during apoptosis of keratinocytes suggesting that this protein is involved in the remodeling of the cytoskeleton under these conditions [20]. Signaling functions, as shown for some of the related catenins such as β-catenin, plakoglobin, and p120^{ctn}, have been postulated for PKP 1, but proof is still lacking [21, 22]. A typical nuclear localization signal has not been identified in the protein so far, but cDNA transfection studies of the complete protein or individual parts of the protein into cells have shown that the head domain on its own, and to some extent the armadillo domain, are able to enter the nucleus [23]. The mechanism of the PKP 1 nuclear migration is currently unknown, but may utilize a piggyback mechanism.

Various in vitro approaches revealed that the binding of desmosomal PKP 1 to other desmosomal proteins such as DSP, DSG 1, DSC 1, and different keratins is mediated by its head domain sequence [24–28]. The armadillo repeat domain of PKP 1 alone is sufficient to localize the protein to the plasma membrane [23]. The PKP 1 binding partner at the plasma membrane has not been determined but might be one of the desmosomal proteins or even cortical actin. In particular, it has been observed that the armadillo domain coaligns with actin microfilaments under certain circumstances and may be involved in the reorganization of this cytoskeletal component [26]. Nevertheless, the carboxyl-terminal part, in particular the last 40 amino acids, seems to be essential for the recruitment of the entire PKP 1 to the plasma membrane as shown by transfection studies of mutant cDNA constructs into A431 keratinocytes [28].

Important clues for the understanding of PKP 1 function came from a report of an autosomal-recessive genodermatosis that is caused by mutations in the PKP 1 gene [29]. The ectodermal dysplasia/skin fragility (EDSF) syndrome (OMIM 604536; the collection of known mutations in the PKP 1 gene is shown in Figure 2 and published cases of EDSF syndrome are listed in Table 1) clinically manifests in the skin and its appendages. Patients suffer from blistering with erosions of their skin upon mechanical stress. Nails are dystrophic and the epidermis of soles and palms displays hyperkeratosis. The hair density on the scalp, eyebrows, and eyelashes is reduced. In severe cases, hair might be completely absent from these body regions. Impaired sweating has occasionally been observed. All other epithelial tissues that express PKP 1, including mucous membranes, seem to be normal in these patients, suggesting functional compensation by the other PKPs. Histological examination of affected skin reveals that the intercellular space is widened and epidermal keratinocytes are acantholytic from the suprabasal layers upwards, suggesting loss of cell-cell adhesion. Cell rupture, as noticed for epidermolytical bullous dermatosis, has not been observed. Immunofluorescence microscopy analyses of patients' skin biopsies showed that certain desmosomal components such as desmogleins, desmocollins, and plakoglobin are still localized at the plasma membrane. In contrast, PKP 1 is completely absent or drastically reduced [30]. As a consequence, desmoplakin is no longer localized in the desmosomes but instead is dispersed throughout the cytoplasm. On the ultrastructural level, desmosomes appear smaller and are numerically reduced in the affected epidermal layers. Additionally, keratin filaments have lost contact to desmosomal junctions and are collapsed around the nucleus. Biochemical analysis of patients' skin revealed that the other PKPs are upregulated to some extent and may compensate in part for the loss of PKP 1 in nonaffected epidermal layers [17]. Interestingly, it does not seem to matter for the development of the clinicopathological findings of EDSF syndrome to what extent the protein is truncated due to the mutations in PKP 1 gene. In a case reported by McGrath and colleagues the mutations occurred close to the amino-terminus of the protein, which could result either in a severely truncated protein or—more likely—in complete loss of the protein (i.e., a functional null mutation) as

Table 1: Published cases of EDSF syndrome with clinical features and observed mutations in PKP 1 gene.

Case[1]	Clinicopathological findings					Observed mutations[2]	Reference
	Epidermal fragility	Hyperkeratosis on palms/soles	Alopecia	Nail dysplasia	Hypohidrosis		
1	yes	yes	yes	yes	yes	(a) p.Q304X (b) c.1132ins28	[29]
2	yes	yes	yes	yes	yes	(a) p.Y71X (b) IVS1−1G>A	[31]
3	yes	yes	yes	yes	no	IVS6−2A>T	[32]
4	yes	yes	yes	yes	yes	IVS11+1G>A	[30]
5	yes	no	yes	yes	no	IVS4−2A>G	[33]
6	yes	yes	yes	yes	not observed	IVS1−1G>A	[33]
7	yes	yes	no	yes	no	IVS9+1G>A	[34]
8	yes	yes	yes	yes	no	(a) c.1053T>A +IVS5+1G>A (b) IVS10−2G>T	[35]
9	yes	yes	yes	yes	no	c.888delC	[36]
10	yes	yes	yes	yes	not observed	p.R672X	[37]

[1] Numbering of the case correlates to the positions of mutations shown in Figure 2.
[2] For compound heterozygosity, mutations of both alleles are given as (a) and (b).

judged by immunofluorescence microscopy [29]. In contrast, the mutations in the PKP 1 gene reported by Hamada et al. occurred near the carboxyl-terminus resulting in the expression of a truncated protein. Based on the mild phenotype of the ESDF syndrome in these patients, it can be assumed that this truncated protein is at least partially functional but clinicopathology of ESDF still manifests [30]. Surprisingly, most of the EDSF-related mutations in human PKP 1 gene involve splice-site mutations (8 out of 13 known mutated alleles) leading to impaired splicing products and subsequent mRNA degradation or the generation of truncated proteins. The reason for the prevalence of splice-site mutations in EDSF is not known.

These findings in conjunction with cell biological data obtained in transfection studies convincingly illustrate that PKP 1 is essential for the recruitment of desmoplakin to the desmosomal plaque and probably is involved into lateral enlargement of the plaque structure in skin, explaining the structural and functional defects in epidermal desmosomes lacking PKP 1. Evidently, integration of PKP 1 in the desmosomes provides the epidermal keratinocytes with stability against mechanical stress. A sequence stretch in the HR2 domain of PKP 1 is thought to be essential for the recruitment of DSP and represents a conserved motif of all the PKPs, suggesting that DSP recruitment is a common function of all PKPs [28].

Although a direct interaction of PKP 1 with keratins has been demonstrated frequently in vitro, it is not clear whether this protein alone is sufficient to connect the intermediate filament cytoskeleton to the desmosome. Specific inactivation of DSP in the skin of mice demonstrates the necessity of both proteins, DSP and PKP 1 (in cooperation with plakoglobin), for anchorage of keratins [38] suggesting that all three components are required. This is further demonstrated by the fact that failure of either PKP 1 or DSP can lead to loss of cell-cell adhesion and acantholysis in the epidermis. The mechanism underlying the failure of epidermal desmosomes without PKP 1 to maintain adhesion is not known. It is tempting to speculate that besides structural defects cell signaling defects could contribute to this phenomenon, similar to the disease mechanisms postulated for the autoimmune blistering diseases of the *pemphigus* group in which autoantibodies target desmosomal cadherins. Binding of autoantibodies to the desmosomal cadherins seems to trigger intracellular signaling pathways that lead to the reorganization of the cytoskeleton involving the disconnection of desmosomal cadherins of adjacent cells (for the mechanisms of this outside-in signaling see [39]). The same pathways may be involved in the dissolution of desmosomal adhesion when PKP 1 is lost. Given that patients with PKP 1 null mutations show defects in differentiation pathways affecting skin appendage formation and homeostasis, it is unlikely that adhesion defects can account for the entire spectrum of disease phenotypes.

Analysis of keratinocytes derived from patients suffering from EDSF syndrome exhibits some interesting properties. Quantitative analyses of the desmosome size in cultured cells revealed that reintroduction of PKP 1 increases the lateral extent of desmosomes. As proposed by others [25, 40], desmosomal cohesiveness might be increased by lateral interactions of PKP 1 with DSP, making additional linkage between desmosomal proteins and keratin network accessible [41]. It is noteworthy that PKP 1 null keratinocytes show increased cell migration, which has implications for tumor biology.

2.2. Plakophilin 2. PKP 2 is, with a predicted mass of 92.756 Da and an apparent molecular weight of 100 kDa (estimated from Western blot analysis), the largest of the

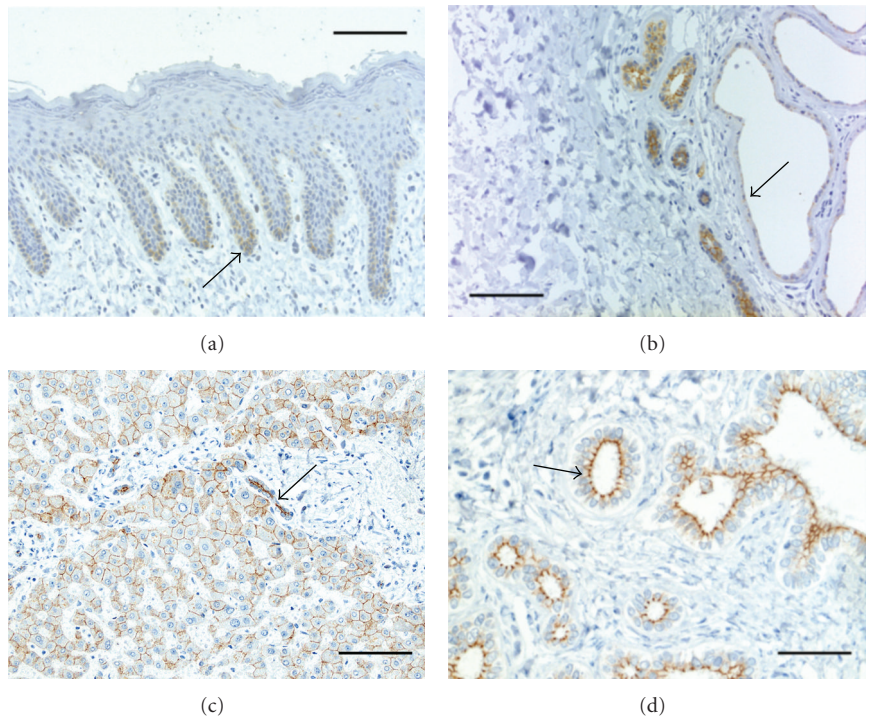

Figure 3: Immunohistochemical staining of sections of human skin a, and b and liver c, and d with antibodies against PKP 2. (a) The staining of samples of human skin with a monoclonal antibody against PKP 2 (clone PP2-150; Progen, Heidelberg) demonstrates a weak and delicate desmosomal staining as well as cytoplasmic staining in the basal layer of the interfollicular epidermis (arrow). Suprabasal keratinocytes remain unstained. (b) Eccrine sweat glands and ducts show a strong reaction with PKP 2-specific antibodies while apocrine sweat glands exhibit an apical, distinct but weak desmosomal reaction (arrow). (c) Hepatocytes as well as bile ductules are marked at the cell-cell contacts by PKP 2-specific antibodies (arrow). (d) Bile ducts also show a sharp and apical staining of desmosomal structure by the PKP 2-antibodies. The samples shown in (c) and (d) are derived from liver tissue in the vicinity of a metastasis of a gastrointestinal stromal tumor with portal and periportal fibrosis and ductal and ductular proliferation. Scale bars: 100 μm (d), 200 μm (a, b, c).

three plakophilins and it is also the prevailing isoform since it is expressed in all cell types with desmosomal junctions [42]. PKP 2 is found in the basal cells of certain stratified epithelia while more differentiated keratinocytes are negative for desmosomal PKP 2 (Figure 3). Moreover, PKP 2 has recently also been found in new types of cell junction which differ in terms of their biochemical composition from both classical desmosomes and conventional adherens junctions (reviewed in [43]). Similar to PKP 1, PKP 2 also occurs as two different splice variants. An additional exon coding for 44 amino acids is integrated into PKP 2 b close to the border of the second to third armadillo repeat of the protein [42]. The two PKP 2 splice variants appear to be coexpressed in all cell types analyzed thus far, and it is not known whether these two proteins have different functions.

Like PKP 1, PKP 2 has been detected in the nucleus of many cell types [42]. Its presence in the nucleus is independent of its presence in desmosomes. Some nonepithelial cell types, which do not assemble desmosomes, show only nuclear localization of PKP 2 (e.g., fibroblasts [42]). In stratified epithelia, nuclear and desmosomal localization of PKP2 is regulated independently. In the differentiated layers of stratified epithelia, PKP 2 is excluded from desmosomes and accumulates in the nuclei of keratinocytes. Recently, Müller and colleagues identified a molecular pathway that appears to regulate nuclear accumulation of PKP 2 [44]. The Cdc25C-associated kinase 1 (C-TAK 1) emerges to be involved in cell-cycle regulation and Ras-signaling. It was shown that C-TAK 1 phosphorylates Cdc25C and KSR1, a scaffold protein for mitogen-activated protein kinase (MAPK) and Raf-1 kinase. Müller et al. demonstrated that PKP 2 is also a substrate for C-TAK 1 [44]. This phosphorylation of PKP 2 enforces an interaction of PKP 2 with 14-3-3 proteins, which prevents the nuclear accumulation of PKP 2. Consequently, mutation of the C-TAK 1 phosphorylation site or the 14-3-3 binding domain in PKP 2 increases nuclear accumulation of PKP 2. The pathways that trigger C-TAK 1-mediated phosphorylation of PKP 2 and its retention in the cytoplasm have not been analyzed so far.

What does PKP 2 do in the nucleus? Recent experiments by Mertens and colleagues provided some insights [45]. Immunoprecipitation experiments revealed an association of PKP 2 with the largest subunit of RNA-polymerase-III holoenzyme, protein RPC155, as well as other components such as RPC82 and RPC39. The PKP 2-positive complexes also contain RNA-polymerase-III-associated transcription factor TFIIIB but not TFIIIC. The colocalization of PKP 2 and RPC155 in particles in the interchromatin space has

been shown by immunofluorescence microscopy. Mertens and colleagues [45] postulated that these particles do not represent active forms of polymerase-III, because the PKP 2-positive particles do not contain transcription factor TFIIIC, a factor required for the formation of an active RNA polymerase III complex. Thus, the actual function of these complexes remains unclear. Nevertheless, the almost general appearance of PKP 2, as well as PKP 1, in the nucleus seems to differ fundamentally from the nuclear localization of other related catenins such as β-catenin or p120^{ctn}, which are translocated into the nucleus upon specific signals and have been shown to be involved in gene regulation [21, 22].

Besides these nuclear functions, PKP 2 may be involved in cytoplasmic signaling, which is based on the observation that it can bind β-catenin [46], a key downstream effector protein of the canonical Wnt-signaling pathway [21]. Using two-hybrid and immunoprecipitation assay, it was shown that PKP 2 can bind to β-catenin. However, when bound to PKP 2, β-catenin cannot associate to E-cadherin, which may reduce the pool of β-catenin available to function in cell adhesion. Overexpression of PKP 2 in colon carcinoma cells leads to an increase in β-catenin/TCF signaling suggesting a regulatory role of PKP 2 in Wnt signaling and providing a potential functional link between desmosomal adhesion and signaling [46].

PKP 2 also seems to be involved in the assembly of the desmosomal components into desmosomes. siRNA-mediated depletion of PKP 2 in keratinocytes leads to changes in the subcellular localization of DSP which mimics the behavior of a DSP mutant deficient for a PKCα (i.e., protein kinase C) phosphorylation site. Different isoforms of PKC have been implicated in the regulation of cellular processes such as migration, cellular adhesion, or cytoskeletal reorganization (for review see [47]). Bass-Zubeck et al. investigated the connection between PKP 2, DSP, and PKC [48]. The authors found that PKP 2 binds to PKCα and DSP via its head domain. A detailed analysis revealed that PKP 2 simultaneously binds DSP and PKCα, which facilitates the subsequent phosphorylation of DSP at its IF-binding domain by PKC [48]. This increases DSP integration into the desmosomes and the subsequent attachment of IFs to desmoplakin.

Insights into the function of PKP 2 also came from gene knockout experiments in mice, as well as the analysis of an autosomal-dominant human hereditary disease linked to PKP 2 mutations [49, 50]. Ablation of the PKP 2 gene in mice leads to a lethal phenotype around mid-gestation (E10.5) [49]. Homozygous PKP 2-null embryos died because of severe alterations of the heart structure resulting in the outflow of blood into the pericardium and subsequent collapse of the embryonic blood circulation. On the microscopic level, PKP 2 deficient hearts display reduced trabeculation as well as abnormally thin cardiac walls. The reason for the instability of cell contacts between cardiomyocytes is apparent on the ultrastructural level. The junctional complexes of the *areae compositae* (formerly designated as intercalated disks; see [43]) that connect cardiomyocytes include at least two types of junctions in an amalgamated fashion, desmosomes and adherens junctions. The *areae compositae* are altered significantly in PKP 2-mutant mice. Associated with the deficiency of PKP 2, DSP is depleted from the desmosomal junctions and accumulates in the cytoplasm. Additionally, DSG 2 expression seems to be reduced in PKP 2-null cardiomyocytes and desmosomal components were less resistant to detergent extraction, suggesting impaired function of cell junctions. Therefore, PKP 2 seems to be essential for the regular subcellular distribution of desmoplakin and its accumulation in the *areae compositae* of cardiomyocytes. Interestingly, Grossmann et al. found no alteration in other PKP 2-expressing epithelia in the mutant animals [49]. This is likely due to the expression of multiple PKP isoforms in many cell types (except for the heart which expresses only PKP 2), providing functional compensation in case one isoform is not functional.

The essential function of PKP 2 in the heart was also demonstrated by the identification of a haplo-insufficiency of PKP 2 in a hereditary human disease, autosomal-dominant arrhythmogenic right ventricular cardiomyopathy (ARVC; [50]). In ARVC, cardiomyocytes are progressively replaced by fibro-fatty tissue, especially in the right ventricle (for a recent review see [51]). This replacement leads to abnormal electrical conductance with syncopes and tachycardia and an often lethal failure in the mechanical capability of the heart (e.g., "sudden cardiac death" of young athletes). The mechanism leading to ARVC may include apoptosis of cardiomyocytes due to the weak and disrupted intercellular adhesion of cardiomyocytes caused by haplo-insufficiency of PKP 2 and subsequent insufficient anchorage of DSP [52]. The decline of cardiomyocytes may therefore lead to the development of scar tissue in the right ventricle. Moreover, transdifferentiation of cardiomyocytes into fibro- or adipocytes may take place, probably caused by disturbed Wnt/β-catenin-signaling [53, 54]. This is supported by further observations. The decrease of DSP in cultured atrial myocytes by siRNA results in the redistribution of plakoglobin to the nucleus and the suppression of the canonical Wnt/β-catenin-signaling pathway [54]. Genes inducing adipogenesis and fibrogenesis were upregulated in these DSP-deficient cells. Decrease of DSP was also noticed in cardiomyocytes of PKP 2-deficient mice [49], suggesting that a cellular transdifferentiation may also occur in ARVC. At least 12 different genes or chromosomal loci have been associated with the autosomal-dominant or recessive types of ARVC so far, including all five known desmosomal genes expressed in cardiomyocytes (i.e., DSG 2, DSC 2, DSP, JUP, and PKP 2).

The loss of PKP 2 may also contribute to the abnormal electrical conductance of the heart [55]. Gap junctions play an essential role in the electrical coupling of cardiomyocytes and the coordinated heart contraction (reviewed in [56]). Downregulation of PKP 2 in primary cardiomyocytes of rat heart leads to reduced expression of the gap junction protein connexin 42. In addition, a decrease of cellular coupling via gap junctions is also detectable, which may result in the disturbed transmission of electrical impulses in the ventricle. Therefore, it appears that PKP 2 can influence the organization of different types of cellular junctions such as gap junctions and *areae compositae* in heart muscle cells.

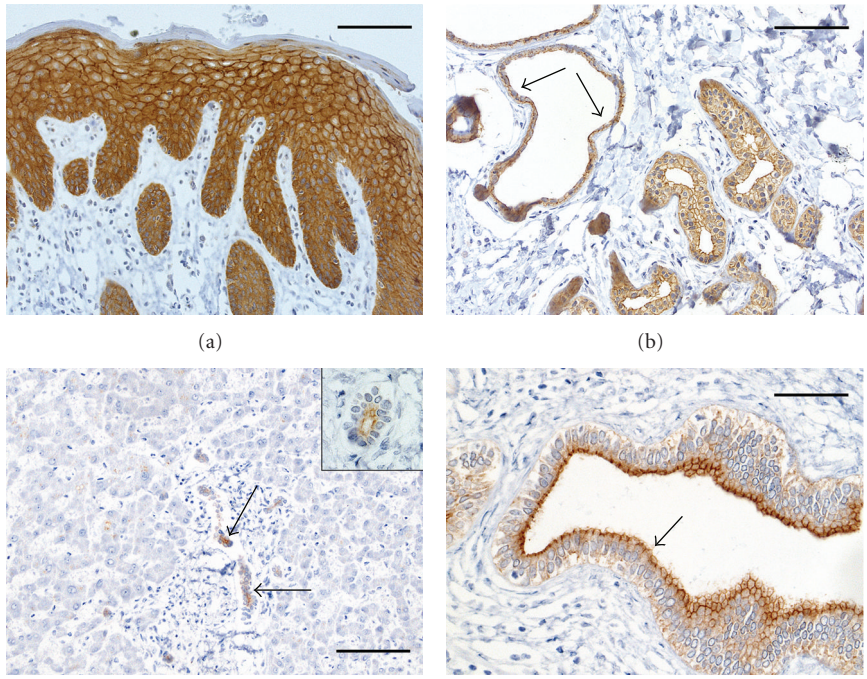

Figure 4: Immunohistochemical staining of sections of human skin a, and b and liver c, and d with antibodies against PKP 3. (a) Intensive reaction of desmosomes and cytoplasm is visible by staining sections of human skin with a monoclonal antibody against PKP 3 (clone PKP3 310.9.1; Progen, Heidelberg). Basal and lower suprabasal keratinocytes exhibit a strong cytoplasmic staining while desmosomal staining is less prominent. With ongoing differentiation, the desmosomal labeling is increasing. (b) Eccrine and apocrine (arrows) sweat glands show strong desmosomal labeling with PKP 3-specific antibodies. (c) Reaction of PKP 3-specific antibodies on liver is restricted to bile ductules (arrow; see description of liver tissue in the legend to Figure 3) while hepatocytes are completely negative for PKP 3. The insert presents a magnification of a bile ductule of human liver stained with antibodies against PKP 3, exhibiting a labeling of the desmosomal junctions. (d) Bile ducts (here in a large portal field) show a clear desmosomal reaction at the apical pole of cells (arrow). Scale bars: 100 μm (d), 200 μm a, b, c.

2.3. Plakophilin 3. PKP 3 has a calculated mass of 87,081 Da and is detected with an apparent molecular weight of approximately 87 kDa on Western blot analysis [57, 58]. Strikingly, in contrast to the other PKP gens, PKP3 gene seems not to encode for different splice variants. PKP 3 is present in the desmosomes of all cell layers of stratified epithelia and in almost all simple epithelia, with the exception of hepatocytes (Figure 4). In epidermal cells, PKP 3 is expressed in a homogeneous pattern. Furthermore, it is detectable in the desmosomes of some nonepithelial cells with the notable exception of cardiomyocytes. This fact may explain the severe heart phenotype of PKP 2 loss, since PKP 2 is the only PKP expressed in cardiomyocytes and its loss of function cannot be compensated by the other PKPs. Although PKP 3 is mainly located in desmosomes, a significant proportion of the protein remains soluble in the cytoplasm. In contrast to the other PKPs, PKP 3 has not been detected in the nucleus.

A better understanding of the functions of PKP 3 came from the analyses of PKP 3 knockout mice [59]. In contrast to the other two PKPs, the PKP 3 knockout phenotype is fairly mild. PKP 3-null animals are viable and exhibit defects in the morphogenesis and morphology of specific hair follicles. Moreover, alterations in density and spacing of desmosomes and adherens junctions in PKP 3-null epidermis and oral cavity were observed (own unpublished observations). Consequently, PKP 3 is involved in the development or maintenance of skin appendages. Other PKP 3-positive epithelia appear normal in PKP 3-null animals. In addition, an upregulation of the expression of specific junctional proteins, such as the other PKPs, was noticed. In comparison to the other two PKPs, the PKP 3 knockout phenotype is modest, which may in part be due to the fact that an additional PKP is coexpressed in most epithelia and may compensate for at least some of the PKP 3 functions. Diseases in associated with the loss or heterozygosity of PKP 3 have not been reported so far.

Surprisingly, among the three plakophilins, PKP 3 exhibits the most extensive binding repertoire to other desmosomal components [60] and it demonstrates *in silico* the most extensive interaction rate of desmosomal proteins, as predicted for keratinocytes by Cirillo and Prime [61]. It is capable to bind to most of the desmosomal proteins such as all DSG and DSC isoforms, JUP and DSP and furthermore, it is the only PKP that interacts with the smaller DSC-b isoforms that are missing the binding site for plakoglobin [60]. This implicates an apparent binding site for PKP 3 at the juxtamembrane domain of desmosomal cadherins.

Both the PKP 3 head domain and the arm-repeats seem to be crucial for these interactions, since most of the interactions to other desmosomal proteins occur in yeast two-hybrid assay only using the entire PKP 3 but not using the individual domains [60].

Further PKP 3 interaction partners are emerging, that are not linked to cell adhesion, suggesting a broader biological role of PKP 3. PKP 3 has been shown, for example, to interact with RNA-binding proteins such as poly-A binding protein C1 (PABPC1), FXR1 (Fragile X mental redartation-1), and G3BP (GAP SH3 domain-binding protein) in stress granules [62]. Stress granules develop when cells respond to diverse environmental stress conditions and these particles represent stalled translational complexes (for a recent review of stress granules see [63]). The function of PKP 3 in stress granules and the basis for the integration into stress granules remain unclear, but it seems likely that this is not a general function of all PKPs, since in addition to PKP 3, only PKP 1 but not PKP 2 has the ability to integrate into the stress granules.

Another PKP 3-binding protein identified is dynamin-like protein DNM-1L [64]. DNM-1L is involved in the peroxisomal and mitochondrial fission and fusion as well as mitochondrial-dependent apoptosis of cells [65, 66]. Although the biological significance for this interaction is not clear, it is tempting to speculate that the PKP 3 could affect the apoptotic response of cells.

2.4. Plakophilins in Tumors. Cellular adhesion molecules, especially components of the adherens junctions such as E-cadherin and β-catenin, have been shown to be important in the development, progression, and metastasis of tumors [67]. Likewise, several desmosomal proteins have also been linked to malignant processes (reviewed in [68]). Reliable data demonstrating a causal link between plakophilins and tumor development are still forthcoming. Thus far, most published studies focused on the expression of PKPs in tumors and a correlation of PKP expression and tumor prognosis. Well and moderately differentiated squamous cell carcinomas (SqCC) of skin express PKP 1, whereas in poorly differentiated tumors, PKP 1 is downregulated [69]. Tumor cells of basal cell carcinomas (BCC) exhibit a more heterogeneous expression of PKP 1, being confined to small patchy areas [69]. In solid nodular BCCs, PKP 1 expression has been found to be reduced in comparison to normal overlaying epidermis and was hardly detectable in nodules growing close to the basal epidermis. Immunohistochemical analysis of the expression of PKP 1 in oral SqCCs revealed similar results to those obtained with skin tumors [18, 70]. This is, however, conflicting with observations made by others [71], who found that PKP 1 is strongly expressed only in a small proportion of well-differentiated SqCCs. Furthermore, these authors found that most of the well-differentiated tumors are negative for PKP 1. Interestingly, using cells derived from oral SqCCs, Sobolik-Delmaire et al. [70] could demonstrate that cell lines expressing low levels of PKP 1 exhibit increased cell mobility which is reduced by ectopic expression of PKP 1. In contrast, another cell line of an oral SqCC that expresses comparably high levels of PKP 1 becomes more mobile and invasive in vitro when PKP 1 is diminished by a shRNA knock-down approach.

Interestingly, in a part of oral and pharyngeal SqCCs analyzed by Schwarz et al. [18], nuclear localization of PKP 1 in tumor cells was noticed. This is remarkable since adjacent non-neoplastic squamous epithelium did not show nuclear PKP 1. In contrast to PKP 1, immunostaining for PKP 2 in histological sections of SqCC is low and often restricted to peripherally located tumor cells or is even completely absent [18], whereas PKP 3 expression patterns are similar to PKP 1 in SqCC. The expression of PKP 3 seems to correlate inversely with the degree of malignancy of tumors.

An analysis of adenocarcinomas from different organs such as colon and pancreas revealed that PKP 1 is not detected whereas PKP 2 and PKP 3 are frequently expressed [18, 72], sometimes associated with a change from an apical desmosomal staining to a staining of almost complete lateral surface. The only exceptions were prostate adenocarcinomas which displayed a low level of PKP 1 immunoreactivity. Interestingly, in non-small cell lung carcinomas (NSCLC; adenocarcinomas and SqCC) and cultured cells derived thereof, Furukawa et al. observed an elevated expression of PKP 3 [64]. Inhibition of PKP 3 expression by siRNA approach in NSCLC cultured cells led to reduced colony formation and less viability of cells. Moreover, over-expression of PKP 3 in COS-cells caused enhanced proliferation rate and elevated activity in in vitro invasion assay. The authors postulated that PKP 3 may have an oncogenic function when localized in the cytoplasm under certain conditions. It thus appears that PKP 3 can potentially both, advance tumorigenesis (as seen in some NSCLC) or suppress it (as noticed for some SqCCs). Recent observations suggest that PKP 3 may be involved in epithelial-mesenchymal transition (EMT) that is of relevance especially for metastasis of tumor cells [73]. Analysis of PKP 3 expression in invasive cancer cells revealed that PKP 3 expression seems to be repressed by the transcription factor ZEB 1, a potent repressor of E-cadherin expression that is also involved in EMT, at least in breast cancer cells. Nuclear accumulation of ZEB 1 (i.e., Zinc finger E-box-binding homeobox-1) correlated with a loss of membrane staining for PKP 3. Similar observations have been reported for PKP 2-repression by ZEB 2 in colon cancer cells [74]. In conclusion, the precise role of PKPs in tumor development and tumor progression is not clear. It is possible that some of these proteins can function both, as oncogenes or as tumor suppressors, depending on the cell type studied. Further research is needed to establish a causal link between PKP expression (or loss of expression) and cancer.

In summary, in the past few years PKPs have been recognized to be essential for desmosomal adhesion and tissue integrity. Nevertheless, recent data suggest that PKPs exert cellular functions unrelated to cell adhesion. Further questions like the ability of individual PKPs to compensate for the loss of one isoform and the role of PKPs in cell signaling and in tumor development need to be further investigated.

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References

[1] M. G. Farquhar and G. E. Palade, "Junctional complexes in various epithelia," *The Journal of Cell Biology*, vol. 17, pp. 375–412, 1963.

[2] X. Cheng, Z. Den, and P. J. Koch, "Desmosomal cell adhesion in mammalian development," *European Journal of Cell Biology*, vol. 84, no. 2-3, pp. 215–223, 2005.

[3] B. Holthöfer, R. Windoffer, S. Troyanovsky, and R. E. Leube, "Structure and function of desmosomes," *International Review of Cytology*, vol. 264, pp. 65–163, 2007.

[4] A. Schmidt and P. J. Koch, "Desmosomes in development and disease," in *Cell Junctions: Adhesion, Development, and Disease*, S. E. LaFlamme and A. P. Kowalczyk, Eds., pp. 235–249, Wiley-VCH, Weinheim, Germany, 2008.

[5] K. J. Green and C. L. Simpson, "Desmosomes: new perspectives on a classic," *Journal of Investigative Dermatology*, vol. 127, no. 11, pp. 2499–2515, 2007.

[6] J. E. Lai-Cheong, K. Arita, and J. A. McGrath, "Genetic diseases of junctions," *Journal of Investigative Dermatology*, vol. 127, no. 12, pp. 2713–2725, 2007.

[7] R. L. Dusek, L. M. Godsel, and K. J. Green, "Discriminating roles of desmosomal cadherins: beyond desmosomal adhesion," *Journal of Dermatological Science*, vol. 45, no. 1, pp. 7–21, 2007.

[8] A. Schmidt and S. Jäger, "Plakophilins-hard work in the desmosome, recreation in the nucleus?" *European Journal of Cell Biology*, vol. 84, no. 2-3, pp. 189–204, 2005.

[9] M. Hatzfeld, "Plakophilins: multifunctional proteins or just regulators of desmosomal adhesion?" *Biochimica et Biophysica Acta*, vol. 1773, no. 1, pp. 69–77, 2007.

[10] A. E. Bass-Zubek, L. M. Godsel, M. Delmar, and K. J. Green, "Plakophilins: multifunctional scaffolds for adhesion and signaling," *Current Opinion in Cell Biology*, vol. 21, no. 5, pp. 708–716, 2009.

[11] M. Hatzfeld, "The armadillo family of structural proteins," *International Review of Cytology*, vol. 186, pp. 179–224, 1999.

[12] H.-J. Choi and W. I. Weis, "Structure of the armadillo repeat domain of plakophilin 1," *Journal of Molecular Biology*, vol. 346, no. 1, pp. 367–376, 2005.

[13] M. Hatzfeld, "The p120 family of cell adhesion molecules," *European Journal of Cell Biology*, vol. 84, no. 2-3, pp. 205–214, 2005.

[14] H. W. Heid, A. Schmidt, R. Zimbelmann, et al., "Cell type-specific desmosomal plaque proteins of the plakoglobin family: plakophilin 1 (band 6 protein)," *Differentiation*, vol. 58, no. 2, pp. 113–131, 1994.

[15] H.-P. Kapprell, K. Owaribe, and W. W. Franke, "Identification of a basic protein of M_r 75,000 as an accessory desmosomal plaque protein in stratified and complex epithelia," *Journal of Cell Biology*, vol. 106, no. 5, pp. 1679–1691, 1988.

[16] A. Schmidt, H. W. Heid, S. Schafer, U. A. Nuber, R. Zimbelmann, and W. W. Franke, "Desmosomes and cytoskeletal architecture in epithelial differentiation: cell type-specific plaque components and intermediate filament anchorage," *European Journal of Cell Biology*, vol. 65, no. 2, pp. 229–245, 1994.

[17] J. R. McMillan, M. Haftek, M. Akiyama, et al., "Alterations in desmosome size and number coincide with the loss of keratinocyte cohesion in skin with homozygous and heterozygous defects in the desmosomal protein plakophilin 1," *Journal of Investigative Dermatology*, vol. 121, no. 1, pp. 96–103, 2003.

[18] J. Schwarz, A. Ayim, A. Schmidt, et al., "Differential expression of desmosomal plakophilins in various types of carcinomas: correlation with cell type and differentiation," *Human Pathology*, vol. 37, no. 5, pp. 613–622, 2006.

[19] A. Schmidt, L. Langbein, M. Rode, S. Prätzel, R. Zimbelmann, and W. W. Franke, "Plakophilins 1a and 1b: widespread nuclear proteins recruited in specific epithelial cells as desmosomal plaque components," *Cell and Tissue Research*, vol. 290, no. 3, pp. 481–499, 1997.

[20] J. Weiske, T. Schöneberg, W. Schröder, M. Hatzfeld, R. Tauber, and O. Huber, "The fate of desmosomal proteins in apoptotic cells," *Journal of Biological Chemistry*, vol. 276, no. 44, pp. 41175–41181, 2001.

[21] K. Willert and K. A. Jones, "Wnt signaling: is the party in the nucleus?" *Genes and Development*, vol. 20, no. 11, pp. 1394–1404, 2006.

[22] J. M. Daniel, "Dancing in and out of the nucleus: p120ctn and the transcription factor Kaiso," *Biochimica et Biophysica Acta*, vol. 1773, no. 1, pp. 59–68, 2007.

[23] M. W. Klymkowsky, "Plakophilin, armadillo repeats, and nuclear localization," *Microscopy Research and Technique*, vol. 45, no. 1, pp. 43–54, 1999.

[24] E. A. Smith and E. Fuchs, "Defining the interactions between intermediate filaments and desmosomes," *Journal of Cell Biology*, vol. 141, no. 5, pp. 1229–1241, 1998.

[25] A. P. Kowalczyk, M. Hatzfeld, E. A. Bornslaeger, et al., "The head domain of plakophilin-1 binds to desmoplakin and enhances its recruitment to desmosomes. Implications for cutaneous disease," *Journal of Biological Chemistry*, vol. 274, no. 26, pp. 18145–18148, 1999.

[26] M. Hatzfeld, C. Haffner, K. Schulze, and U. Vinzens, "The function of plakophilin 1 in desmosome assembly and actin filament organization," *Journal of Cell Biology*, vol. 149, no. 1, pp. 209–222, 2000.

[27] E. A. Bornslaeger, L. M. Godsel, C. M. Corcoran, et al., "Plakophilin 1 interferes with plakoglobin binding to desmoplakin, yet together with plakoglobin promotes clustering of desmosomal plaque complexes at cell-cell borders," *Journal of Cell Science*, vol. 114, no. 4, pp. 727–738, 2001.

[28] T. Sobolik-Delmaire, D. Katafiasz, and J. K. Wahl III, "Carboxyl terminus of plakophilin-1 recruits it to plasma membrane, whereas amino terminus recruits desmoplakin and promotes desmosome assembly," *Journal of Biological Chemistry*, vol. 281, no. 25, pp. 16962–16970, 2006.

[29] J. A. McGrath, J. R. McMillan, C. S. Shemanko, et al., "Mutations in the plakophilin 1 gene result in ectodermal dysplasia/skin fragility syndrome," *Nature Genetics*, vol. 17, no. 2, pp. 240–244, 1997.

[30] T. Hamada, A. P. South, Y. Mitsuhashi, et al., "Genotype-phenotype correlation in skin fragility-ectodermal dysplasia syndrome resulting from mutations in plakophilin 1," *Experimental Dermatology*, vol. 11, no. 2, pp. 107–114, 2002.

[31] J. A. McGrath, "A novel genodermatosis caused by mutations in plakophilin 1, a structural component of desmosomes," *Journal of Dermatology*, vol. 26, no. 11, pp. 764–769, 1999.

[32] N. V. Whittock, M. Haftek, N. Angoulvant, et al., "Genomic amplification of the human plakophilin 1 gene and detection of a new mutation in ectodermal dysplasia/skin fragility syndrome," *Journal of Investigative Dermatology*, vol. 115, no. 3, pp. 368–374, 2000.

[33] E. Sprecher, V. Molho-Pessach, A. Ingber, E. Sagi, M. Indelman, and R. Bergman, "Homozygous splice site mutations in PKP1 result in loss of epidermal plakophilin 1 expression and underlie ectodermal dysplasia/skin fragility syndrome in two consanguineous families," *Journal of Investigative Dermatology*, vol. 122, no. 3, pp. 647–651, 2004.

[34] P. M. Steijlen, M. A. M. van Steensel, B. J. H. Jansen, et al., "Cryptic splicing at a non-consensus splice-donor in a patient with a novel mutation in the plakophilin-1 gene," *Journal of Investigative Dermatology*, vol. 122, no. 5, pp. 1321–1324, 2004.

[35] R. Zheng, D.-F. Bu, and X.-J. Zhu, "Compound heterozygosity for new splice site mutations in the plakophilin 1 gene (PKP1) in a Chinese case of ectodermal dysplasia-skin fragility syndrome," *Acta Dermato-Venereologica*, vol. 85, no. 5, pp. 394–399, 2005.

[36] S. Ersoy-Evans, G. Erkin, H. Fassihi, et al., "Ectodermal dysplasia-skin fragility syndrome resulting from a new homozygous mutation, 888delC, in the desmosomal protein plakophilin 1," *Journal of the American Academy of Dermatology*, vol. 55, no. 1, pp. 157–161, 2006.

[37] A. Tanaka, J. E. Lai-Cheong, M. E. M. Café, et al., "Novel truncating mutations in PKP1 and DSP cause similar skin phenotypes in two Brazilian families," *British Journal of Dermatology*, vol. 160, no. 3, pp. 692–697, 2009.

[38] V. Vasioukhin, E. Bowers, C. Bauer, L. Degenstein, and E. Fuchs, "Desmoplakin is essential in epidermal sheet formation," *Nature Cell Biology*, vol. 3, no. 12, pp. 1076–1085, 2001.

[39] J. Waschke, "The desmosome and pemphigus," *Histochemistry and Cell Biology*, vol. 130, no. 1, pp. 21–54, 2008.

[40] A. P. Kowalczyk, E. A. Bornslaeger, J. E. Borgwardt, et al., "The amino-terminal domain of desmoplakin binds to plakoglobin and clusters desmosomal cadherin-plakoglobin complexes," *Journal of Cell Biology*, vol. 139, no. 3, pp. 773–784, 1997.

[41] A. P. South, H. Wan, M. G. Stone, et al., "Lack of plakophilin 1 increases keratinocyte migration and reduces desmosome stability," *Journal of Cell Science*, vol. 116, no. 16, pp. 3303–3314, 2003.

[42] C. Mertens, C. Kuhn, and W. W. Franke, "Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque," *Journal of Cell Biology*, vol. 135, no. 4, pp. 1009–1025, 1996.

[43] W. W. Franke, S. Rickelt, M. Barth, and S. Pieperhoff, "The junctions that don't fit the scheme: special symmetrical cell-cell junctions of their own kind," *Cell and Tissue Research*, vol. 338, no. 1, pp. 1–17, 2009.

[44] J. Müller, D. A. Ritt, T. D. Copeland, and D. K. Morrison, "Functional analysis of C-TAK1 substrate binding and identification of PKP2 as a new C-TAK1 substrate," *The EMBO Journal*, vol. 22, no. 17, pp. 4431–4442, 2003.

[45] C. Mertens, I. Hofmann, Z. Wang, et al., "Nuclear particles containing RNA polymerase III complexes associated with the junctional plaque protein plakophilin 2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 14, pp. 7795–7800, 2001.

[46] X. Chen, S. Bonné, M. Hatzfeld, F. van Roy, and K. J. Green, "Protein binding and functional characterization of plakophilin 2. Evidence for its diverse roles in desmosomes and β-catenin signaling," *Journal of Biological Chemistry*, vol. 277, no. 12, pp. 10512–10522, 2002.

[47] C. Larsson, "Protein kinase C and the regulation of the actin cytoskeleton," *Cellular Signalling*, vol. 18, no. 3, pp. 276–284, 2006.

[48] A. E. Bass-Zubek, R. P. Hobbs, E. V. Amargo, et al., "Plakophilin 2: a critical scaffold for PKCα that regulates intercellular junction assembly," *Journal of Cell Biology*, vol. 181, no. 4, pp. 605–613, 2008.

[49] K. S. Grossmann, C. Grund, J. Huelsken, et al., "Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation," *Journal of Cell Biology*, vol. 167, no. 1, pp. 149–160, 2004.

[50] B. Gerull, A. Heuser, T. Wichter, et al., "Mutations in the desmosomal protein plakophilin-2 are common in arrhythmogenic right ventricular cardiomyopathy," *Nature Genetics*, vol. 36, no. 11, pp. 1162–1164, 2004.

[51] T. Herren, P. A. Gerber, and F. Duru, "Arrhythmogenic right ventricular cardiomyopathy/dysplasia: a not so rare "disease of the desmosome" with multiple clinical presentations," *Clinical Research in Cardiology*, vol. 98, no. 3, pp. 141–158, 2009.

[52] Z. Mallat, A. Tedgui, F. Fontaliran, R. Frank, M. Durigon, and G. Fontaine, "Evidence of apoptosis in arrhythmogenic right ventricular dysplasia," *The New England Journal of Medicine*, vol. 335, no. 16, pp. 1190–1196, 1996.

[53] G. d'Amati, C. R. T. di Gioia, C. Giordano, and P. Gallo, "Myocyte transdifferentiation: a possible pathogenetic mechanism for arrhythmogenic right ventricular cardiomyopathy," *Archives of Pathology and Laboratory Medicine*, vol. 124, no. 2, pp. 287–290, 2000.

[54] E. Garcia-Gras, R. Lombardi, M. J. Giocondo, et al., "Suppression of canonical Wnt/β-catenin signaling by nuclear plakoglobin recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy," *Journal of Clinical Investigation*, vol. 116, no. 7, pp. 2012–2021, 2006.

[55] E. M. Oxford, H. Musa, K. Maass, W. Coombs, S. M. Taffet, and M. Delmar, "Connexin43 remodeling caused by inhibition of plakophilin-2 expression in cardiac cells," *Circulation Research*, vol. 101, no. 7, pp. 703–711, 2007.

[56] G. G. Hesketh, J. E. van Eyk, and G. F. Tomaselli, "Mechanisms of gap junction traffic in health and disease," *Journal of Cardiovascular Pharmacology*, vol. 54, no. 4, pp. 263–272, 2009.

[57] A. Schmidt, L. Langbein, S. Prätzel, M. Rode, H.-R. Rackwitz, and W. W. Franke, "Plakophilin 3—a novel cell-type-specific desmosomal plaque protein," *Differentiation*, vol. 64, no. 5, pp. 291–306, 1999.

[58] S. Bonné, J. van Hengel, F. Nollet, P. Kools, and F. van Roy, "Plakophilin-3, a novel armadillo-like protein present in nuclei and desmosomes of epithelial cells," *Journal of Cell Science*, vol. 112, no. 14, pp. 2265–2276, 1999.

[59] T. Sklyarova, S. Bonné, P. D'Hooge, et al., "Plakophilin-3-deficient mice develop hair coat abnormalities and are prone to cutaneous inflammation," *Journal of Investigative Dermatology*, vol. 128, no. 6, pp. 1375–1385, 2008.

[60] S. Bonné, B. Gilbert, M. Hatzfeld, X. Chen, K. J. Green, and F. van Roy, "Defining desmosomal plakophilin-3 interactions," *Journal of Cell Biology*, vol. 161, no. 2, pp. 403–416, 2003.

[61] N. Cirillo and S. S. Prime, "Desmosomal interactome in keratinocytes: a systems biology approach leading to an understanding of the pathogenesis of skin disease," *Cellular and Molecular Life Sciences*, vol. 66, no. 21, pp. 3517–3533, 2009.

[62] I. Hofmann, M. Casella, M. Schnölzer, T. Schlechter, H. Spring, and W. W. Franke, "Identification of the junctional plaque protein plakophilin 3 in cytoplasmic particles containing RNA-binding proteins and the recruitment of plakophilins 1 and 3 to stress granules," *Molecular Biology of the Cell*, vol. 17, no. 3, pp. 1388–1398, 2006.

[63] P. Anderson and N. Kedersha, "RNA granules: post-transcriptional and epigenetic modulators of gene expression," *Nature Reviews Molecular Cell Biology*, vol. 10, no. 6, pp. 430–436, 2009.

[64] C. Furukawa, Y. Daigo, N. Ishikawa, et al., "Plakophilin 3 oncogene as prognostic marker and therapeutic target for lung cancer," *Cancer Research*, vol. 65, no. 16, pp. 7102–7110, 2005.

[65] M. Yan, N. Rayapuram, and S. Subramani, "The control of peroxisome number and size during division and proliferation," *Current Opinion in Cell Biology*, vol. 17, no. 4, pp. 376–383, 2005.

[66] A. Santel and S. Frank, "Shaping mitochondria: the complex posttranslational regulation of the mitochondrial fission protein DRP1," *IUBMB Life*, vol. 60, no. 7, pp. 448–455, 2008.

[67] F. H. Brembeck, M. Rosário, and W. Birchmeier, "Balancing cell adhesion and Wnt signaling, the key role of β-catenin," *Current Opinion in Genetics and Development*, vol. 16, no. 1, pp. 51–59, 2006.

[68] M. Chidgey and C. Dawson, "Desmosomes: a role in cancer?" *British Journal of Cancer*, vol. 96, no. 12, pp. 1783–1787, 2007.

[69] I. Moll, H. Kurzen, L. Langbein, and W. W. Franke, "The distribution of the desmosomal protein, plakophilin 1, in human skin and skin tumors," *Journal of Investigative Dermatology*, vol. 108, no. 2, pp. 139–146, 1997.

[70] T. Sobolik-Delmaire, D. Katafiasz, S. A. Keim, M. G. Mahoney, and J. K. Wahl III, "Decreased plakophilin-1 expression promotes increased motility in head and neck squamous cell carcinoma cells," *Cell Communication and Adhesion*, vol. 14, no. 2-3, pp. 99–109, 2007.

[71] S. Papagerakis, A.-H. Shabana, J. Depondt, P. Gehanno, and N. Forest, "Immunohistochemical localization of plakophilins (PKP1, PKP2, PKP3, and p0071) in primary oropharyngeal tumors: correlation with clinical parameters," *Human Pathology*, vol. 34, no. 6, pp. 565–572, 2003.

[72] C. Mertens, C. Kuhn, R. Moll, I. Schwetlick, and W. W. Franke, "Desmosomal plakophilin 2 as a differentiation marker in normal and malignant tissues," *Differentiation*, vol. 64, no. 5, pp. 277–290, 1999.

[73] K. Aigner, L. Descovich, M. Mikula, et al., "The transcription factor ZEB1 (δEF1) represses plakophilin 3 during human cancer progression," *FEBS Letters*, vol. 581, no. 8, pp. 1617–1624, 2007.

[74] C. Vandewalle, J. Comijn, B. De Craene, et al., "SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell-cell junctions," *Nucleic Acids Research*, vol. 33, no. 20, pp. 6566–6578, 2005.

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Review Article

Desmosomes in Developing Human Epidermis

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Desmosomes play important roles in the cell differentiation and morphogenesis of tissues. Studies on animal models have greatly increased our knowledge on epidermal development while reports on human developing skin are rare due to the difficult accessibility to the samples. Although the morphology of periderm cells and the process how the epidermis develops very much resemble each other, the timetable and the final outcome of a mature human epidermis markedly differ from those of murine skin. Even the genetic basis of the junctional components may have profound differences between the species, which might affect the implementation of the data from animal models in human studies. The aim of this review is to focus on the development of human skin with special emphasis on desmosomes. Desmosomal development is mirrored in perspective with other simultaneous events, such as maturation of adherens, tight and gap junctions, and the basement membrane zone.

1. Introduction

The literature on developing human epidermis is limited, collectively not exceeding 100 cases in the reports covering the fetal age. The development of human skin has been studied at the morphological level in quite detail by electron microscopy [1–3]. The timetable for the formation of epidermal architecture is based on the evaluation of sixty human fetuses, age 7–20 weeks [2]. Sparsely located desmosomes are detected already in the samples from the youngest fetuses, and during the maturation the density of desmosomes increases [2, 4].

Since desmosomes are relatively easily identifiable by their ultrastructural appearance, they were the first specific cell junctions recognized in human skin by electron microscopy. The other cell junctions, adherens, tight, and gap junctions, were originally identified and named in simple epithelia using electron microscopy, but their ultrastructural characteristics in simple epithelia are not directly applicable to those of stratified epithelia, such as epidermis. The recognition of a number of biomarkers of cell junctions and subsequent availability of specific antibodies at 80's and 90's enabled investigation of the junctional proteins of epidermis using immunohistochemical approaches at light and electron microscopic levels. Immunolocalization studies thus helped the identification of desmosomal, adherens junction, gap junction and tight junction, components in developing epidermis. Although some studies regarding regulation of development of human skin have been published, very little is known about the regulatory signals regarding developmental regulation of human skin.

2. Morphological Development of Human Epidermis

Human epidermis is derived from a single layer of embryonic surface ectoderm. The ectoderm proliferates in the 4th week of development and produces two layers of cells [1, 2] (Figure 1). The inner layer of cells is the basal layer while the outer layer is called the periderm, and proliferation takes place in both cell layers [2]. In the 11th week of EGA, the basal layer produces a new intermediate cell layer between itself and the periderm which marks the beginning of stratification and more complicated differentiation of the epidermis. The periderm cells in contrast, cease dividing in the first trimester, become larger and elevated, and exhibit rounded blebs on their outer surfaces [2]. The periderm cells

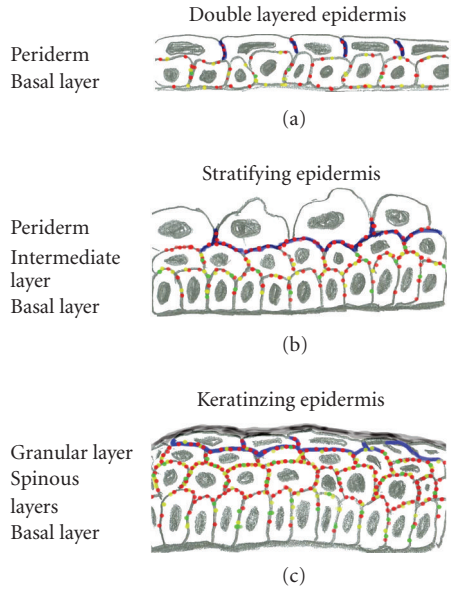

Figure 1: Schematic representation of developing human epidermis. (a) Two cell layers, peridermal cells and basal cells at the first trimester (8 weeks). (b) Three cell layers at 11 weeks. The periderm cells become elevated. (c) By 21–24 weeks all the cell layers of mature epidermis are present. Tight junctions are shown in blue, desmosomes in red, adherens junctions in yellow, and green indicates gap junctions. The density of junctions increases during the maturation.

form a cornified cell envelope in the three-layered stage of development [5, 6]. By 21–24 weeks EGA, the intermediate cell layer has apparently given rise to the definitive three layers of the outer epidermis: the spinous, the granular, and the cornified cell layers. As the keratinization proceeds, the periderm is gradually shed into the amniotic fluid by the beginning of the last trimester [3]. The periderm cells display characteristics consistent with apoptosis prior to being sloughed off [7]. Cornified cell envelope is formed in the upper cell layers of epidermis after the shedding of the periderm cells [5, 6]. Mice and man show clear differences in the development of the epidermis including the time schedule, maturity at newborn and differences at the genetic level. For example, mice posses three dsg1 genes with distinct epidermal expression patterns whereas there is only a single human DSG1 gene.

3. Formation of Intercellular Junctions in Early Two-Layered Epidermis: 4–9 Weeks

Desmosomes are easily detectable in transmission electron microscopy. Ultrastructural studies have revealed desmosomes at 5 weeks of EGA between the basal and periderm cells [2]. It is possible that desmosomes exist earlier but this has not been verified because of lack of samples representing earlier time points. Formation of desmosomes is thus a very early event well preceding, for example, the maturation of the basement membrane zone. In the youngest fetal samples investigated, the desmosomes are widely separated [2] and evaluation of the electron microscopic images suggests that the desmosomal plaque is considerably thinner and less prominent than in later developmental stages, although this was not highlighted in the original publication.

The protein composition of early fetal desmosomes has been studied at 5 weeks using serum from pemphigus patients, but no intercellular fluorescence was detected at that time [8]. Correspondingly, pemphigus sera revealed positive immunoreaction only after 11 weeks in the study by Lane et al. [9]. Thus, the presence of desmogleins could not be proved in the samples of earliest developmental points studied. However, at 8 weeks indirect immunofluorescence with antibodies to desmoplakin, pan-desmocollin, and pan-desmoglein showed punctate labeling associated with plasma membranes of peridermal and basal cells [4] (Figures 1 and 2), indicating that developing desmosomes have the elements for transmembrane and plaque parts. The suggestive intermediate filaments binding to the desmosomal plaques in basal cells are CK5 and CK14 which can be first detected between 8 and 10 weeks [10] while the periderm cells contain CK19 and CK8 [11].

At eight weeks of EGA, desmosomal proteins were also localized to the basal plasma membrane of the basal cells suggesting that separation of cell membranes to basal and apicolateral compartments had not taken place at this time. At this time, immunolabeling for β4 integrin shows widely distributed spots [4], instead of a linear labeling of mature basement membrane zone. Structural hemidesmosomes are also not seen in the electron microscopy [2, 12, 13]. These findings support the view that the polarity of the basal cells has not developed yet. It is however known that at 5 weeks EGA, the basement membrane zone is composed of a basal cell plasma membrane, lamina lucida, and lamina densa [12] which contain laminin and type IV collagen [9, 14, 15]. β1 integrin can also be seen in the periphery of the basal cells, including the basal and apicolateral plasma membranes [4, 16, 17].

In vitro studies on human primary keratinocytes have shown that adherens junctions precede the development of desmosomes [18]. Classical cadherins are important in the initiation of intercellular junction formation, and regulation of desmosome *assembly depends at least to some extent, on expression of classical cadherins* [19], Tinkle et al. [20]. In two-layered developing human epidermis of eight weeks, E-cadherin is expressed in the periphery of basal cells, including the basal aspect, and in the periphery of peridermal cells. E-cadherin and P-cadherin are also present in the intercellular junctions of the basal and peridermal cells [4, 10, 21]. The same localization was also noted for α catenin, vinculin, and α actinin [4]. This indicates that the prerequisite for desmosome formation in the form of adherens junction components is available and thus the formation of desmosomes may follow the same sequence of events as has been described in vitro. It should be noted that the adherens junctions were not *reported* in the ultrastuctural studies. This is, however, not surprising since the plaque of the adherens junctions is much less prominent than the desmosomal plaque and is difficult to visualize even in the adult epidermis [22].

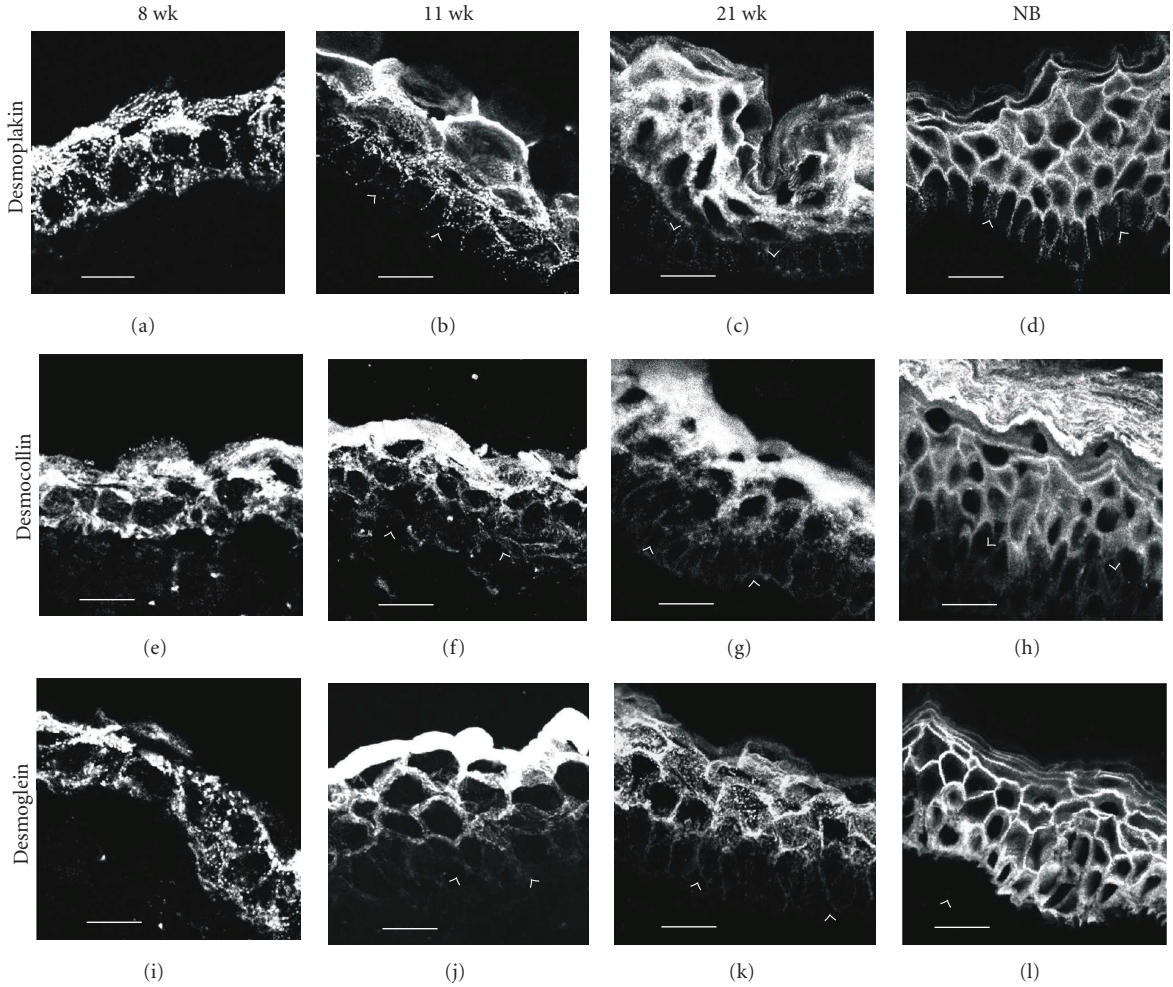

Figure 2: Expression and localization of desmosomal proteins in developing human skin at 8, 11, and 21 weeks of EGA and at newborn age (NB). Note the gradual increase in the density of desmosomes. At 8 weeks EGA, the epidermis is composed of basal and peridermal cell layers (a, e, i). Antibodies to desmoplakin (a), desmocollin (e), and desmoglein (i) label cell membranes of basal and peridermal cells. Note also immunolabeling in the dermal-epidermal junction. At 11 weeks EGA, the intermediate cell layer has developed in the epidermis (b, f, j). An intense signal for desmoplakin (b), pan-desmocollin (f), and pan-desmoglein (j) is apparent in the peridermal cells. Intermediate cells also show desmosomal antigens, while lateral membranes of basal cells are almost devoid of these desmosomal proteins. At 21 weeks EGA, the peridermal cell layer has been shed and the epidermis is composed of the four definitive layers of epidermis (c, g, k). Desmoplakin (c), desmocollin (g), and desmoglein (k) antibodies label all the cell layers, the basal cells being only weakly labelled (*arrowheads point to the* dermal-epidermal junction; *bars* (a, e, i) 10 μm, (b, c, d, f, g, h, j, k, i) 20 μm).

It should also be noted that tight junctions are visible between the neighboring peridermal cells [23]. Of the components of tight junctions, ZO-1 and occludin have been demonstrated in the cell junction complexes of peridermal cells between 8 and 21 weeks of EGA [23]. Tight junctions are responsible for the epidermal diffusion barrier at this age when mature stratum corneum does not exist. Providing diffusion barrier for the epidermis might also be an important basic function of the periderm.

Gap junctions, composed of connexin (Cx) subunits, are channels that allow intercellular communication between adjacent cells. They are considered to play a key role in the regulation of cell proliferation and differentiation. The major Cx subtypes in human skin are Cx26 and Cx43 [24]. The first connexin type expressed already at 7 weeks is Cx26 [25] and sparse gap junctions containing connexin 43 are present at eight weeks [4]. Formation of gap junctions increases while the epidermis develops and matures, suggesting that gap junctions may play an important role in fetal skin development.

4. Initiation of Stratification and Differentiation of the Basal Cells between 9 and 20 Weeks

Between 9 and 20 weeks, the intermediate filament bundles associated with desmosomes become larger and more prominent, and the number of desmosomes increases [2, 11]. The new intermediate layer of cells contains more

desmosomes and more prominent keratin filaments than the basal and periderm cells. The putative binding partners for desmosomal plaque proteins are CK5 and CK14 in the basal cells, while the intermediate cells change the expression to CK1 and CK10 [11]. As the stratification proceeds, CK1 and CK10 are expressed in all suprabasal cell layers. CK8 and CK19 are still expressed in both the basal and periderm cell layers at this stage of development, but disappear with keratinization, by about 24 weeks EGA [11].

When the stratification takes place, labeling of the basal cells for desmoplakin, pan-desmocollin, and pan-desmoglein becomes very weak and only some distinct spots of desmosomes are visible between the basal cells [4] (Figures 1 and 2). The basal cells have acquired their polarity and the desmosomal proteins have disappeared from the dermal-epidermal junction. Both of these characteristics resemble those of mature epidermis. The intermediate cells express all the desmosomal proteins studied which is in accordance with the presence of numerous desmosomes in the mature spinous cell layers. A study using pemphigus sera suggests that at this developmental state desmoglein3 is present in the basal and intermediate layers [9] while the peridermal cells merely show diffuse cytoplasmic labeling for many proteins studied [4, 9]. By 21 weeks, EGA the labeling pattern for desmosome components becomes more continuous which indicates the presence of more numerous desmosomes at the cell-cell contacts (Figure 2).

During stratification the expression profiles of adherens junction proteins undergoes minor changes. The basal cells continue to express both E- and P-cadherins, while the intermediate cells express only E-cadherin [21]. The α-actinin disappears from the peridermal cells already by 11 weeks, while being prominently expressed in the junctions connecting basal and intermediate cells throughout the development [4]. α-catenin and vinculin are expressed in all the three epidermal layers. Between 13 and 21 weeks, as the number of intermediate cell layers increases, the expression pattern remains essentially the same and by 21 weeks EGA, the labeling patterns of adherens junction antigens resemble that of neonatal epidermis. The expression of β1 integrin continues in the basal cell layer as described earlier [16, 26].

Simultaneously with the initiation of stratification, the basement membrane zone goes through major changes as hemidesmosomes and anchoring fibrils begin to shape [12, 13]. Between 9 and 15 weeks (EGA), the number of hemidesmosomes is increased by about fourfold, they are matured and become increasingly associated with intermediate and anchoring filaments [9, 14]. By 20 weeks (EGA), the expression of $\alpha 6\beta 4$ integrin becomes mostly concentrated at the basal surface of the basal cells [4, 26, 27]. The basement membrane becomes continuous and thicker.

5. Keratinization of the Epidermis and Shedding of the Periderm Cells after 20 Weeks of EGA

By approximately 21–24 weeks EGA, the intermediate cell layer has proliferated and produced the definitive three layers of the outer epidermis: the spinous, the granular, and the cornified cell layers [28] (Figure 1). As the keratinization proceeds, the periderm is gradually shed into the amniotic fluid [3]. After 20 gestational weeks the morphology of the epidermis increasingly resembles that of a newborn. The expression patterns of the cell junction and basement membrane components remain essentially the same and little alterations have been noted during this period of epidermal development. Desmosomes become more densely located in the spinous cell and granular cell layers. This is also shown in the immunofluorescence labeling for desmosomal antibodies which gradually reveal more continuous pattern in the cell-cell contacts [4] (Figures 1 and 2). The uppermost granular cell layer and the lowest layer of the stratum corneum, as well as the lateral plasma membranes of the granular layer are interconnected with tight junctions that are *intermingled* with numerous desmosomes [23, 29]. The density of gap junctions increases [4].

6. Regulation of the Development of Human Epidermis

Even though some of the signaling molecules and pathways are universally conserved, marked differences between human and mouse exist. Thus, findings in mice are not directly applicable for human development, or diseases. Yet, only few reports concerning the regulation of the differentiation of fetal human skin are available, and selected ones of those are reviewed here.

Wnt/β catenin signaling is known to play important roles in the development of skin and its appendages [30]. One study which was based on five fetal skin samples aged over 20 weeks, showed expression of Wnt3a, active β catenin and Dkk1 in fetal epidermis [31]. The authors suggest that Wnt/β catenin, signaling thus plays a role in human fetal skin development and homeostasis. Further studies would however be needed in order to investigate this pathway in more detail and at earlier time points.

Desmosome assembly and disassembly are regulated, for example, by calcium and cross-talk with adherens junctions (for review see [32]). Adherens junctions and tight junctions are also regulated by calcium [33]. The effect of calcium is at least in part mediated by the epidermal calcium gradient which results in typical calcium concentrations in different epidermal cell layers [34]. However, no evidence on the epidermal calcium levels in fetal skin is available.

The epidermal growth factor (EGF) family comprises multiple mediators such as transforming growth factor α, amphiregulin, heparin binding-EGF, and epiregulin, which are crucially involved in the tissue-specific proliferation/differentiation homeostasis [35]. TGFα is believed to play a role in cell proliferation and differentiation via an autocrine mechanism. It exerts its effects on cells through binding to the epidermal growth factor receptor (EGFR) [35]. TGFα has showed a vertical progressive increase in expression in the fetal skin of 14, 20, and 34 weeks [36]. In contrast to normal adult human skin in which the EGFR is primarily restricted to the basal and immediately

suprabasal keratinocytes, the fetal epidermis showed a persistent expression of EGFR in all cell layers [37]. Based on these observations it has been suggested that TGFα and EGFR interact strictly to promote skin development during fetal period.

Periderm is an embryonic- and fetal-specific transient cell layer which is destined to detach into the amniotic fluid. During human skin development periderm cells and incompletely keratinized cells are replaced by differentiating keratinocytes. The fate of the peridermal cells has been shown to take place via apoptosis [7]. Immunohistochemical localization of transglutaminases in fetal periderm and intermediate epidermal cells coincides with DNA fragmentation indicating that apoptosis is involved in deletion of these stage-specific cells. The detachment of periderm cells also has to involve disassembly of the desmosomes.

7. Conclusions

Studies on human skin are needed to relate the findings of animal studies with human development, physiology, and pathological conditions. Detailed timetables of the expression of several cell junction components are available, and based on these studies it is likely that the development of desmosomes is synchronized with the maturation of other junction types. However, studies on even the most profound mechanisms of differentiation of human skin are still lacking.

Abbreviations

EGA: Estimated gestational age = the time from fertilization
EGF: Epidermal growth factor
TNF: tumor necrosis factor.

References

[1] A. S. Breathnach and L. M. Wyllie, "Fine structure of cells forming the surface layer of the epidermis in human fetuses at fourteen and twelve weeks," *Journal of Investigative Dermatology*, vol. 45, no. 3, pp. 179–189, 1965.

[2] K. A. Holbrook and G. F. Odland, "The fine structure of developing human epidermis: light, scanning, and transmission electron microscopy of the periderm," *Journal of Investigative Dermatology*, vol. 65, no. 1, pp. 16–38, 1975.

[3] K. A. Holbrook and G. F. Odland, "Regional development of the human epidermis in the first trimester embryo and the second trimester fetus (ages related to the timing of amniocentesis and fetal biopsy)," *Journal of Investigative Dermatology*, vol. 74, no. 3, pp. 161–168, 1980.

[4] M. Hentula, J. Peltonen, and S. Peltonen, "Expression profiles of cell-cell and cell-matrix junction proteins in developing human epidermis," *Archives of Dermatological Research*, vol. 293, no. 5, pp. 259–267, 2001.

[5] M. Akiyama, L. T. Smith, K. Yoneda, K. A. Holbrook, D. Hohl, and H. Shimizu, "Periderm cells form cornified cell envelope in their regression process during human epidermal development," *Journal of Investigative Dermatology*, vol. 112, no. 6, pp. 903–909, 1999.

[6] M. Akiyama, L. T. Smith, and H. Shimizu, "Expression of transglutaminase activity in developing human epidermis," *British Journal of Dermatology*, vol. 142, no. 2, pp. 223–225, 2000.

[7] R. R. Polakowska, M. Piacentini, R. Bartlett, L. A. Goldsmith, and A. R. Haake, "Apoptosis in human skin development: morphogenesis, periderm, and stem cells," *Developmental Dynamics*, vol. 199, no. 3, pp. 176–188, 1994.

[8] A. Delpech-Stewart, "An immunofluorescent study of antigenic structures in the development of human epidermis," *Journal of Histochemistry and Cytochemistry*, vol. 19, no. 10, pp. 627–629, 1971.

[9] A. T. Lane, K. F. Helm, and L. A. Goldsmith, "Identification of bullous pemphigoid, pemphigus, laminin, and anchoring fibril antigens in human fetal skin," *Journal of Investigative Dermatology*, vol. 84, no. 1, pp. 27–30, 1985.

[10] S. V. Lourenço, L. Kamibeppu, J. D. Fernandes, M. N. Sotto, and M. M. S. Nico, "Relationship of adhesion molecules expression with epithelial differentiation markers during fetal skin development," *Journal of Cutaneous Pathology*, vol. 35, no. 8, pp. 731–737, 2008.

[11] B. A. Dale, K. A. Holbrook, J. R. Kimball, M. Hoff, and T. T. Sun, "Expression of epidermal keratins and filaggrin during human fetal skin development," *Journal of Cell Biology*, vol. 101, no. 4, pp. 1257–1269, 1985.

[12] L. T. Smith, L. Y. Sakai, R. E. Burgeson, and K. A. Holbrook, "Ontogeny of structural components at the dermal-epidermal junction in human embryonic and fetal skin: the appearance of anchoring fibrils and type VII collagen," *Journal of Investigative Dermatology*, vol. 90, no. 4, pp. 480–485, 1988.

[13] J. R. McMillan and R. A. J. Eady, "Hemidesmosome ontogeny in digit skin of the human fetus," *Archives of Dermatological Research*, vol. 288, no. 2, pp. 91–97, 1996.

[14] J. D. Fine, L. T. Smith, K. A. Holbrook, and S. I. Katz, "The appearance of four basement membrane zone antigens in developing human fetal skin," *Journal of Investigative Dermatology*, vol. 83, no. 1, pp. 66–69, 1984.

[15] M. Malminen, S. Peltonen, J. Koivunen, and J. Peltonen, "Functional expression of NF1 tumor suppressor protein: association with keratin intermediate filaments during the early development of human epidermis," *BMC Dermatology*, vol. 2, article 10, 2002.

[16] J. Peltonen, H. Larjava, S. Jaakkola, et al., "Localization of integrin receptors for fibronectin, collagen, and laminin in human skin: variable expression in basal and squamous cell carcinomas," *Journal of Clinical Investigation*, vol. 84, no. 6, pp. 1916–1923, 1989.

[17] H. Larjava, J. Peltonen, S. K. Akiyama, et al., "Novel function for β_1 integrins in keratinocyte cell-cell interactions," *Journal of Cell Biology*, vol. 110, no. 3, pp. 803–815, 1990.

[18] V. Vasioukhin, C. Bauer, M. Yin, and E. Fuchs, "Directed actin polymerization is the driving force for epithelial cell-cell adhesion," *Cell*, vol. 100, no. 2, pp. 209–219, 2000.

[19] C. Michels, T. Buchta, W. Bloch, T. Krieg, and C. M. Niessen, "Classical cadherins regulate desmosome formation," *Journal of Investigative Dermatology*, vol. 129, pp. 2072–2075, 2009.

[20] C. L. Tinkle, H. A. Pasolli, N. Stokes, and E. Fuchs, "New insights into cadherin function in epidermal sheet formation and maintenance of tissue integrity," *Proceedings of the National Academy of Sciences of the USA*, vol. 105, no. 40, pp. 15405–15410, 2008.

[21] M. Fujita, F. Furukawa, K. Fujii, Y. Horiguchi, M. Takeichi, and S. Imamura, "Expression of cadherin cell adhesion

molecules during human skin development: morphogenesis of epidermis, hair follicles and eccrine sweat ducts," *Archives of Dermatological Research*, vol. 284, no. 3, pp. 159–166, 1992.

[22] M. Haftek, M. U. Hansen, H. W. Kaiser, H. W. Kreysel, and D. Schmitt, "Interkeratinocyte adherens junctions: immunocytochemical visualization of cell-cell junctional structures, distinct from desmosomes, in human epidermis," *Journal of Investigative Dermatology*, vol. 106, no. 3, pp. 498–504, 1996.

[23] K. Pummi, M. Malminen, H. Aho, S.-L. Karvonen, J. Peltonen, and S. Peltonen, "Epidermal tight junctions: ZO-1 and occludin are expressed in mature, developing, and affected skin and in vitro differentiating keratinocytes," *Journal of Investigative Dermatology*, vol. 117, no. 5, pp. 1050–1058, 2001.

[24] D. Salomon, E. Masgrau, S. Vischer, et al., "Topography in mammalian connexins in human skin," *Journal of Investigative Dermatology*, vol. 103, no. 2, pp. 240–247, 1994.

[25] K. Arita, M. Akiyama, Y. Tsuji, J. R. McMillan, R. A. J. Eady, and H. Shimizu, "Changes in gap junction distribution and connexin expression pattern during human fetal skin development," *Journal of Histochemistry and Cytochemistry*, vol. 50, no. 11, pp. 1493–1500, 2002.

[26] M. D. Hertle, J. C. Adams, and F. M. Watt, "Integrin expression during human epidermal development in vivo and in vitro," *Development*, vol. 112, no. 1, pp. 193–206, 1991.

[27] J. Ryynänen, S. Jaakkola, E. Engvall, J. Peltonen, and J. Uitto, "Expression of β4 integrins in human skin: comparison of epidermal distribution with β1-integrin epitopes, and modulation by calcium and vitamin D3 in cultured keratinocytes," *Journal of Investigative Dermatology*, vol. 97, no. 3, pp. 562–567, 1991.

[28] W. J. Larsen, "Development of the integumentary system," in *Human Embryology*, pp. 419–433, Churchill Livingstone, New York, NY, USA, 1993.

[29] H. Schlüter, R. Wepf, I. Moll, and W. W. Franke, "Sealing the live part of the skin: the integrated meshwork of desmosomes, tight junctions and curvilinear ridge structures in the cells of the uppermost granular layer of the human epidermis," *European Journal of Cell Biology*, vol. 83, no. 11-12, pp. 655–665, 2004.

[30] R. B. Widelitz, "Wnt signaling in skin organogenesis," *Organogenesis*, vol. 4, no. 2, pp. 123–133, 2008.

[31] L. Jia, J. Zhou, S. Peng, J. Li, Y. Cao, and E. Duan, "Effects of Wnt3a on proliferation and differentiation of human epidermal stem cells," *Biochemical and Biophysical Research Communications*, vol. 368, no. 3, pp. 483–488, 2008.

[32] T. Yin and K. J. Green, "Regulation of desmosome assembly and adhesion," *Seminars in Cell and Developmental Biology*, vol. 15, no. 6, pp. 665–677, 2004.

[33] E. Proksch, J. M. Brandner, and J.-M. Jensen, "The skin: an indispensable barrier," *Experimental Dermatology*, vol. 17, no. 12, pp. 1063–1072, 2008.

[34] P. T. Leinonen, P. M. Hägg, S. Peltonen, et al., "Reevaluation of the normal epidermal calcium gradient, and analysis of calcium levels and ATP receptors in Hailey-Hailey and Darier epidermis," *Journal of Investigative Dermatology*, vol. 129, pp. 1379–1387, 2009.

[35] S. Pastore, F. Mascia, V. Mariani, and G. Girolomoni, "The epidermal growth factor receptor system in skin repair and inflammation," *Journal of Investigative Dermatology*, vol. 128, no. 6, pp. 1365–1374, 2008.

[36] C. Sergi, P. Kahl, and H. F. Otto, "Immunohistochemical localization of transforming growth factor-α and epithelial growth factor receptor in human fetal developing skin, psoriasis and restrictive dermopathy," *Pathology and Oncology Research*, vol. 6, no. 4, pp. 250–255, 2000.

[37] L. B. Nanney, C. M. Stoscheck, L. E. King Jr., R. A. Underwood, and K. A. Holbrook, "Immunolocalization of epidermal growth factor receptors in normal developing human skin," *Journal of Investigative Dermatology*, vol. 94, no. 6, pp. 742–748, 1990.

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Review Article

Desmosomal Molecules In and Out of Adhering Junctions: Normal and Diseased States of Epidermal, Cardiac and Mesenchymally Derived Cells

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Current cell biology textbooks mention only two kinds of cell-to-cell adhering junctions coated with the cytoplasmic plaques: the desmosomes (*maculae adhaerentes*), anchoring intermediate-sized filaments (IFs), and the actin microfilament-anchoring adherens junctions (AJs), including both punctate (*puncta adhaerentia*) and elongate (*fasciae adhaerentes*) structures. In addition, however, a series of other junction types has been identified and characterized which contain desmosomal molecules but do not fit the definition of desmosomes. Of these special cell-cell junctions containing desmosomal glycoproteins or proteins we review the composite junctions (*areae compositae*) connecting the cardiomyocytes of mature mammalian hearts and their importance in relation to human arrhythmogenic cardiomyopathies. We also emphasize the various plakophilin-2-positive plaques in AJs (*coniunctiones adhaerentes*) connecting proliferatively active mesenchymally-derived cells, including interstitial cells of the heart and several soft tissue tumor cell types. Moreover, desmoplakin has also been recognized as a constituent of the plaques of the *complexus adhaerentes* connecting certain lymphatic endothelial cells. Finally, we emphasize the occurrence of the desmosomal transmembrane glycoprotein, desmoglein Dsg2, out of the context of any junction as dispersed cell surface molecules in certain types of melanoma cells and melanocytes. This broadening of our knowledge on the diversity of AJ structures indicates that it may still be too premature to close the textbook chapters on cell-cell junctions.

1. Introduction

Typical desmosomes (*maculae adhaerentes*) are cell-cell junctions connecting cells of epithelial, meningothelial, and myocardial nature or malignantly transformed cells derived therefrom. Over the past two decades the molecular and ultrastructural organization of diverse cell-cell-connecting plasma membrane structures has been elucidated and two novel categories and architectonic principles have been recognized: desmosomal molecules as functionally important components of special non-desmosomal junctions and the existence of certain desmosomal molecules in a non-junction-bound form.

2. The Desmosomes of Stratified and Other Epithelia

The ultrastructural organization and the high lateral packing density of desmosomes in the epidermis or other multistratified vertebrate epithelia is best seen in the electron microscope (e.g., Figure 1), and the corresponding protein compositions of the various desmosomal subtypes in the specific strata have been determined by biochemical analyses and immunolocalization techniques [1–7].

In most stratified epithelia, epidermis included, the desmosome packing density is so high that more than half of the entire cell-cell membrane contact area is represented

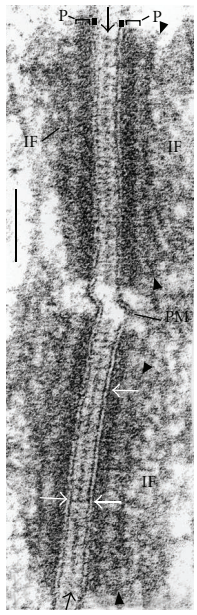

Figure 1: High-magnification electron micrograph of two cross sectioned desmosomal structures connecting *stratum spinosum* cells of human fetal (20 wk) foot-sole epidermis. Brackets and label "P": cytoplasmic dense plaque; black arrows: midline structure; white arrows: trilaminar "unit membrane" structure of the plasma membrane; arrowheads (top and bottom): secondary dense layer of the plaque; IF: intermediate-sized filaments (for further details see [7]); PM: plasma membrane. Bar: 0.1 μm.

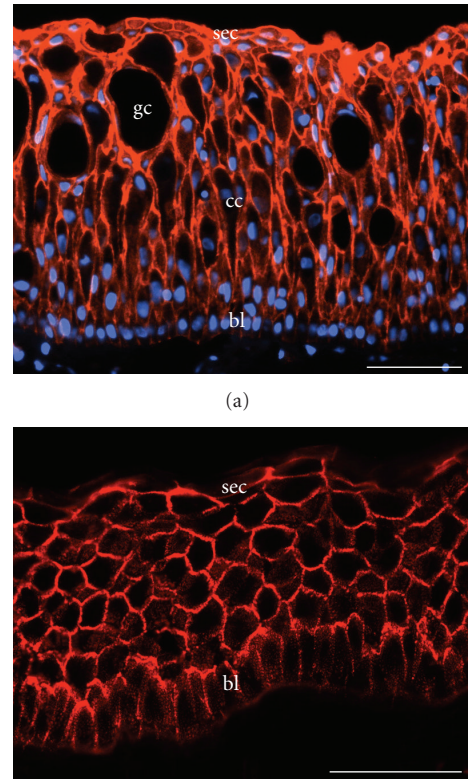

Figure 2: Immunofluorescence microscopy of cryostat sections through fish and amphibian skin, showing the localization of desmosomal components in all layers of the epidermis. (a) Immunofluorescence labeling of plakoglobin (red), in addition to nuclear DAPI staining (blue), in a section through the paraffin-embedded skin of an eel (*Anguilla anguilla*). The section is shown after antigen retrieval and immunoreaction (for methods see [8]) using a monoclonal mouse plakoglobin antibody (mAb PG 5.1; Progen Biotechnik, Heidelberg, Germany). Note the distinct basal layer (*stratum basale*) of the epidermis and the relatively large club shaped cells ("club cells") as well as the mucous goblet cells and the dense-packed apical cell layers. (b) Immunofluorescence labeling of desmoplakin on a cryostat section through the skin of the frog, *Rana pipiens*, using mAb DP447 (Progen Biotechnik). Note the continuous pattern of very closely spaced, finely punctate staining of the epidermal desmosomes. Bl: basal layer; cc: club cells; gc: goblet cells; sec: superficial epithelial cells (for histological terminology see [8]). Bar in (a): 100 μm; bar in (b): 50 μm.

by desmosomal structures. Using immunohistochemical techniques with desmosomal markers this high packing density is directly demonstrable not only for all layers of the highly cornified mammalian epidermis but also in the skin of lower vertebrates such as fishes and amphibia (Figure 2(a) shows the immunolocalization of plakoglobin on a section of fixed, paraffin-embedded skin of the eel, *Anguilla anguilla*; Figure 2(b) shows desmoplakin immunostaining on the skin of the frog, *Rana pipiens*; for details see [8]). Clearly, in stratified epithelia the desmosomal constituents are, together with the keratin filaments, among the most frequent proteins. Moreover, the molecular composition of the epidermal desmosomes—and those of similar multistratified epithelia—has been found to show fundamental strata-specific differences, notably with respect to the desmogleins, Dsg1-4, the desmocollins, Dsc1-3, and the plaque proteins plakophilins, Pkp1-3 (Table 1 and [4–6, 9]).

The stratum-specific molecular ensembles, specifically the Dsg and Dsc glycoproteins, are also of marked importance with respect to the pathogenesis of autoimmune skin diseases of the pemphigus type which show a more or less direct correlation with the specific desmosomal glycoprotein complement of the affected layer (see, e.g., [4, 6, 9, 24–28]). These autoimmune diseases are specifically dealt within other articles of this issue. Another aspect of the desmosomal arrays in the epidermis and other stratified epithelia is their frequent—in some areas almost regular—punctuation by very small "sandwich junctions" (*iuncturae structae*) containing the four times-membrane-spanning tight junction hallmark protein, occludin [29, 30].

3. The Composite Junctions of the Intercalated Disks (IDs) Connecting Mammalian Cardiomyocytes

In mammals the development of the ID junctional system connecting cardiomyocytes does not stop at birth but continues postnatally [15, 31, 32]. In particular the two types of adhering junctions originally distinguishable show further polar translocation, accumulation in the ID region

Table 1: Shown are specific cell-cell adhering junctions containing transmembrane glycoproteins and cytoplasmic plaque proteins of "classic" desmosomes and adherens junctions (AJs). Special types of adhering junctions containing desmosomal proteins in human tissues and cell cultures.

Type	Cells	Transmembrane glycoproteins	Some representative plaque proteins	References
Macula adhaerens (desmosome)	Epithelial and mesothelial cells and carcinomas derived therefrom, cardiomyocytes of immature hearts and cardiac conductive cells	Desmogleins-1-4 Desmocollins-1-3	Plakophilins-1-3 Plakoglobin Desmoplakin	Franke et al. [10, 11] Cowin et al. [12] Mertens et al. [13]
Area composita (composite junction)	Cardiomyocytes and Purkinje fiber cells	N-Cadherin Cadherin-11 Desmoglein-2 Desmocollin-2	α- and β-Catenin Proteins p120, p0071 and ARVCF Plakoglobin Plakophilin-2 Desmoplakin Afadin Myozap	Franke et al. [14] Pieperhoff and Franke [15] Goossens et al. [16] Seeger et al. [17]
Complexus adhaerens	Certain endothelia (spec. endothelial and virgultar tissues of lymph node sinus)	VE-Cadherin N-Cadherin Claudin-5 JAM-A	α- and β-Catenin Protein p120 Plakoglobin Desmoplakin, Afadin	Schmelz and Franke Hämmerling et al. [18] Moll et al. [19]
Zona limitans externa	Neural retina	N-Cadherin	Neurojungin α- and β-Catenin Plakoglobin Plakophilin-2	Paffenholz et al. [20]
Colligatio permixta	Astrocytes and astrocytoma cells	N-Cadherin Cadherin-11 VE-Cadherin	α- and β-Catenin Protein p120 Plakoglobin Plakophilin-2 Afadin	Boda-Heggemann et al. [21]
Coniunctio adhaerens	Mesenchymally derived cells of high proliferative activity in situ and in culture	N-Cadherin Cadherin-11	α- and β-Catenin Proteins p120 and p0071 Plakoglobin Plakophilin-2 [Plakophilin-3] Afadin	Rickelt et al. [22] Barth et al. [23]

and fusions of the desmosomal and the *fascia adhaerens* components, accompanied by an increasing amalgamation of the two kinds of molecular ensembles into the new mixed category of *area composita* (AC) structures (Figures 3(a)–3(c), 4(b), and 5(a); for details of molecular localizations and biochemical analyses see [14, 15, 33]; for non-mammalian species see however [8, 34]). As a typical result rather extended AC structures are seen which combine compositional and ultrastructural properties of desmosomes with those of AJs (Figures 3 and 4(b)). In addition, the "mixed AC ensembles" of the ID (Table 1; Figure 4(b)) include a series of additional proteins such as α-T-catenin [16] and the recently identified plaque protein called "myozap" [17]. The special organizational importance of certain *armadillo*-type proteins, in particular plakoglobin and plakophilin-2, for the entire ID contact of cardiomyocytes has also been demonstrated for early stages of murine heart formation in the absence of the genes encoding these proteins [35, 36] and in siRNA-downregulation experiments ([37–39], for related experiments see also [40, 41]).

The physiological and medical importance of desmosomal molecules in the composite junctions of the myocardiac IDs is most impressively demonstrated by the recent avalanche of publications that specific mutations in genes encoding desmosomal proteins can result in arrhythmogenic cardiomyopathies (ARVC/D), mostly in the right ventricle but left ventricle damages have also been reported (Table 2). Here the gene encoding plakophilin-2 appears to be especially vulnerable as defects in this gene alone seem to account for about two thirds of the cases. This category of ARVC/D-based diseases and "sudden death" events resulting from altered desmosomal proteins also includes complex hereditary syndromes such as combinations of dermatological disorders ("woolly hair", diverse patterns of striate and

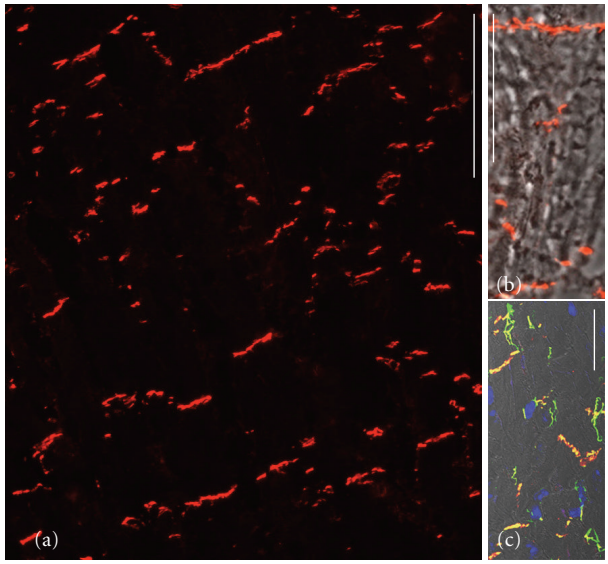

Figure 3: Micrographs showing the immunolocalization of the desmosomal plaque protein, desmoplakin, on cryostat sections through the murine myocardium. (a) Immunofluorescence micrograph showing the localization of desmoplakin (red) in all composite junction structures of the intercalated disks (IDs). (b) Immunofluorescence micrograph showing desmoplakin (red) labeling of the composite junctions on the background of phase-contrast optics. (c) Merged image showing plakophilin-2 (red), β-catenin (green) and nuclear DAPI-staining (blue), on an interference contrast microscopy background. Composite junctions (CJs) are characterized by their yellow merge colour whereas the *zonulae adhaerentes* and other AJs of the capillary endothelial cell layers here show only β-catenin-positivity. Bar in (a): 100 μm; bar in (b): 50 μm; bar in (c): 25 μm.

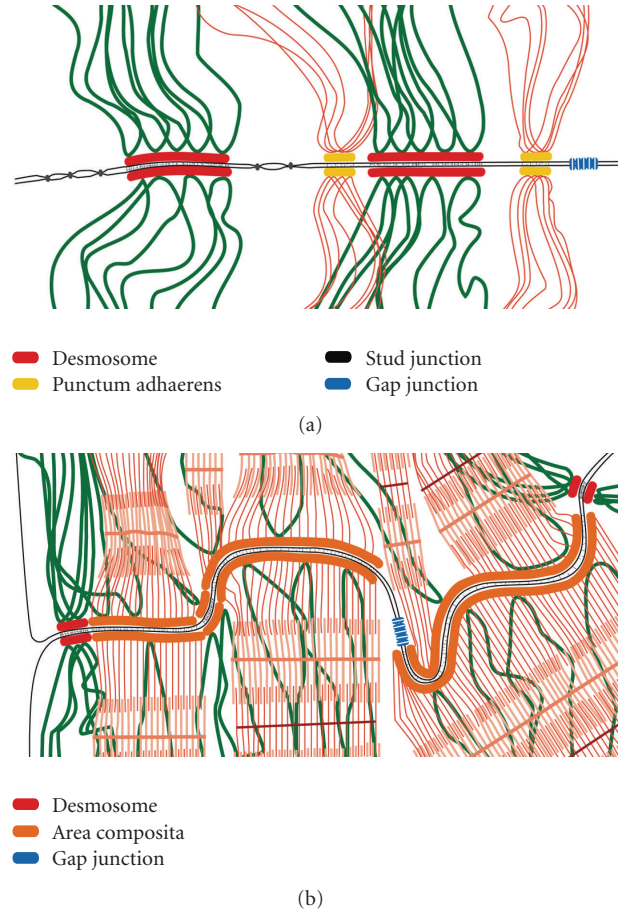

Figure 4: Schematic comparison of the different principles of organization of cell-cell junctions containing desmosomal proteins in mammalian epidermal and myocardiac tissue. (a) Junction organizations between epidermal keratinocytes as observed in the *stratum spinosum*. Note the densely spaced desmosomes (red plaques), which anchor bundles of intermediate sized filaments containing keratins (dark-green). Smaller adherens junctions (*puncta adhaerentia*, yellow plaques) anchor actin-microfilament bundles (red filaments). Furthermore, special types of tight junction-like structures, the small "stud junctions" (black dots), and channel-like connexin paracrystals (gap junctions, blue) are also generally found. For details see [29, 30]. (b) The *area composita* (composite junction, orange) structures of intercalated disks (ID) connecting cardiomyocytes of an adult mammalian heart. This amalgamated type of adhering junction is characterized by a mixture of typical desmosomal and AJ molecules. This kind of composite junction is the predominating adhering junction structure in the ID of the adult mammalian heart.

diffuse keratoderma changes, particularly in palmoplantar skin) and cardiac disease features such as in the classic "Naxos disease" type or the "Carvajal syndrome" subtype, first identified in the year 2000 in three Ecuadorian families, which may also include damages in both ventricles (for an anthology see [42]). The specific dominant and recessive forms of the human diseases ascribed to mutations of genes encoding desmosomal proteins have been dealt with in several specific recent review articles [42, 97–102].

4. Adhering Junctions in the Specialized Cells of the Cardiac Conduction System

The specialized cells of the mammalian cardiac conduction system are connected by three different types of AJs [103]: desmosomes, which in certain cell regions occur in impressively high packing density, as well as AJs and CJs (Figures 5(b)–5(d)). The conductive cells of the ovine and bovine Purkinje fiber systems have been studied in special detail with respect to their nature as modified cardiomyocytes and to the various forms of junctions of which a major proportion is located at lateral cell-cell contact sites [103–105]. These findings have led to the hypothesis that the abundance of apparently "normal-looking" desmosomes in the conductive tissue might also—and perhaps primarily—be affected by the desmosomal protein mutations in cases of human ARVC/D (Table 2), as also suggested from the much higher conduction speed of these "cell fibers" (see, e.g., [103, 106, 107]).

The various size classes of the desmosomal protein-rich junctions connecting conduction cells are presented by desmoplakin immunoelectron microscopy in Figures 5(b)–5(d), including some very small junctions (arrowheads in

Table 2: During the last few years an avalanche of publications has appeared—and is still continuing to do so—showing the involvement of mutations in desmosomal components in the development of arrhythmogenic cardiomyopathies, including "sudden death" cases (for references see [10–12, 38, 40, 42–96]). Recent references reporting that certain mutations in human genes encoding desmosomal proteins and glycoproteins contribute to arrhythmogenic ventricular cardiomyopathies (ARVC).

Protein	Reference	
Plakophilin-2	Gerull et al. [43] Antoniades et al. [44] Calkins [45] Nagaoka et al. [46] Kannankeril et al. [47] Dalal et al. [48] Syrris et al. [49] Tsatsopoulou et al. [50] Van Tintelen et al. [51] Awad et al. [60] Lahtinen et al. [52] Otterspoor et al. [53]	Fidler et al. [38] Joshi-Mukherje et al. [54] Ram and Van Wagoner, [55] Tandri et al. [56] Wu et al. [57] Qiu et al. [58] (5 cases) Hall et al. [40] Bhuiyan et al. [59] (23 cases) den Haan et al. [64] (21 cases) Xu et al. [61] (38 cases) Bauce et al. [62] (7 cases) Cox et al. [95] (58 cases)
Desmoplakin	Norgett et al. [84] Rampazzo and Danieli [97] Alcalai et al. [86] Bauce et al. [77] Norman et al. [85] Sen-Chowdhry et al. [87] Norgett et al. [75] Uzumcu et al. [74] Sen-Chowdhry et al. [88]	Tsatsopoulou et al. [50] Yang et al. [89] den Haan et al. [64] (1 case) Mahoney et al. [81] Xu et al. [61] (10 cases) Bauce et al. [62] (5 cases) Cox et al. [95] (1 case) Bolling et al. [96]
Desmoglein-2	Pilichou et al. [90] Tsatsopoulou et al. [50] Awad et al. [60] Syrris et al. [73] Yu et al. [91]	Bhuiyan et al. [59] (4 cases) den Haan et al. [64] (8 cases) Xu et al. [61] (10 cases) Bauce et al. [62] (4 cases) Cox et al. [95] (3 cases)
Desmocollin-2	Heuser et al. [83] Syrris et al. [72] Beffagna et al. [79] Bhuiyan et al. [59] (2 cases)	Simpson et al. [76] Xu et al. [61] (4 cases) Bauce et al. [62] (2 cases) Cox et al. [95] (3 cases)
Plakoglobin	McKoy et al. [80] Protonotarios et al. [78, 82] Kaplan et al. [71] Garcia-Gras et al. [92]	Asimaki et al. [93] Asimaki et al. [94] den Haan et al. [64] (1 case) Xu et al. [61] (2 cases)

Selected review articles: Bazzi and Christiano [65]; Marcus et al. (Eds.) [42]; Awad et al. [66]; Corrado et al. [67]; Herren et al. [68]; Saffitz [69]; Sen-Chowdhry et al. [63].
First animal model (boxer dog): Oxford et al. [70].

Figure 5(b)). Frequently, a number of individual desmin-containing intermediate filaments can be resolved at such junctions (e.g., Figure 5(c)), often revealing closely-parallel plaque associations (e.g., arrows in Figure 5(d)).

5. Desmosomal Plaque Proteins in Special Non-Desmosomal Adhering Junctions (*Coniunctiones* and *Complexus Adhaerentes*)

Proteins of the plakophilin-subfamily of *armadillo* proteins are constitutive, apparently necessary components of desmosomal plaques [13, 108–113]. Their special organizational role and architectonic importance has been demonstrated perhaps most convincingly in the case of plakophilin-2 by gene abrogation as well as siRNA-mediated mRNA reduction experiments [36, 37, 39–41]. Moreover, the functional importance of some plakophilins, in particular plakophilin-2, may extend beyond desmosomal plaques to gap junctions [37, 38] and into the interior of the cell, including certain cytoplasmic as well as nuclear complexes [114–116].

Recently, however, we have discovered that the occurrence of both plakophilin-2 and plakophilin-3 is not necessarily restricted to the plaques of desmosomes but that these proteins can also occur as constitutive molecules in plaques of some non-desmosomal junctions such as the *puncta adhaerentia*-like AJs of certain cell cultures [22, 23, 117] or in proliferatively active cells of certain tumors, for example in cardiac myxomata [118]. Here the rapid acquisition

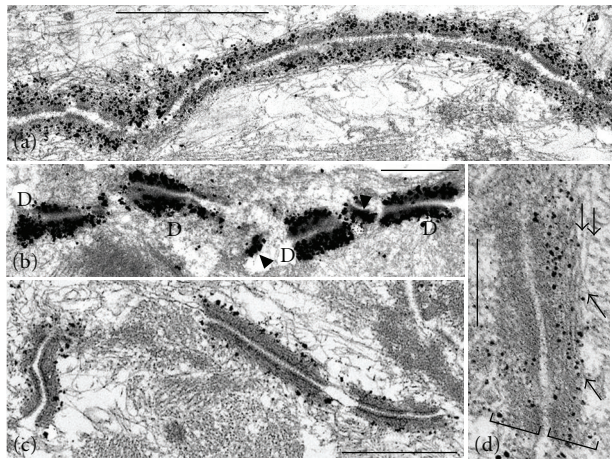

Figure 5: Immunoelectron microscopy of ultrathin sections through bovine myocardium (a) and Purkinje fibers ((b)–(d)) using antibodies against desmoplakin. (a) Immunogold labeling of a typical extended *area composita* structure in an intercalated disk (ID) of adult cardiomyocytes. (b) By contrast, note the relatively small desmoplakin-positive junctions that connect cells of the Purkinje fiber conductive system ((d), desmosomes; arrowheads denote some particularly small desmosome-like junction structures with asymmetric labeling). The desmoplakin-rich plaques of such junctions are very intensely labeled. (c) The morphology and the relatively close packing of the major type of junctions are similar to those of the *area composita* structures of adult mammalian cardiomyocytes. (d) High-magnification immunoelectron micrograph of a composite junction. Note the very close near parallel association of intermediate-sized filaments (some are denoted by arrows) with junctional plaques. Bars in (a) and (b): 1 μm; bar in (c): 0.25 μm.

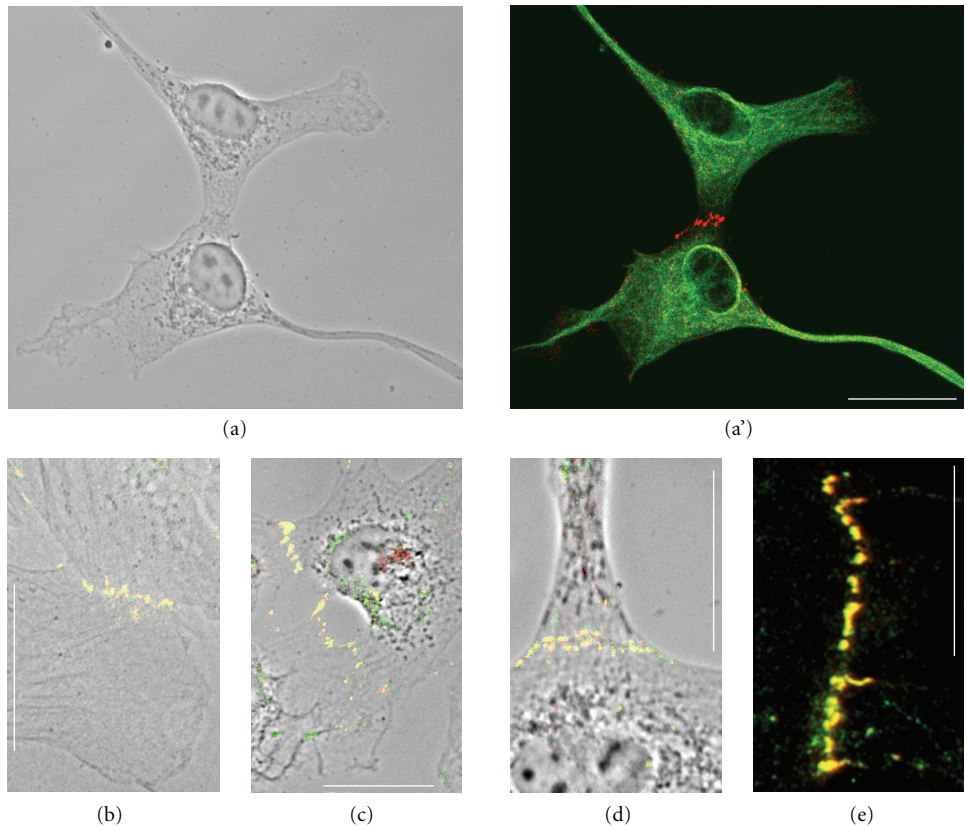

Figure 6: Double-label immunofluorescence micrographs, showing the localization of the "desmosomal protein", plakophilin-2, in mesenchymally derived cultures of valvular interstitial cells (VICs). (a), (a′) Phase contrast and immunofluorescence micrograph showing ovine VICs forming clusters of AJs positive for the typical desmosomal plaque component plakophilin-2 (red; vimentin filaments are labeled in green). (b)–(d) Represent merged images of ovine VICs, showing colocalization staining (yellow) of plakophilin-2 (red) with typical AJ proteins such as cadherin-11 (green, (b)), β-catenin (green, (c)), protein p120 (green, (d)) all on a phase contrast background. (e) Human VICs exhibit similar colocalization of plakophilin-2 (red) here with N-cadherin (green) as shown by the yellow merge colour (for further details see [23]). Bar in (a): 25 μm; bar in (b): 30 μm; bars in (c) and (d): 20 μm; bar in E: 10 μm.

of plakophilin-2 to the non-desmosomal plaques of these tumor cell AJs, in particular the fact that it appears in the earliest in vitro culture passages of cardiac valvular interstitial cells from various mammalian species [23], suggests that the addition of plakophilin-2 alone—or together with plakophilin-3—to these junctions is somehow related to the induction of proliferation and cell cycle growth. It is therefore likely that in the future the systematic examination of the presence of plakophilins in such AJs will give valuable diagnostic informations. Figures 6(a)–6(e) present the early integration of plakophilin-2 into a series of small *puncta adhaerentia* connecting interstitial cells freshly brought from cardiac valve matrix tissue into cell culture (for details see [23]).

The junctional system connecting the endothelial cells of blood and lymph vessels is obviously of great biological importance and has been studied extensively (reviews: [119, 120]). There is, however, a special category of variously-sized and -shaped AJs which connect certain types of endothelial cells in some parts of the lymphatic system, including the three-dimensionally branched "virgultar" cells of lymph node sinus as well as specific cutaneous and other peripheral lymph capillaries, which are characterized by AJs containing in addition desmoplakin as a major plaque protein, in most cases probably in combination with plakoglobin [18, 121–123]. The existence of such *complexus adhaerentes* in special parts of the vascular endothelial system has since been confirmed several times ([124–126], for a recent review see [19]). The formation of plaque complexes of VE-cadherin with desmoplakin and plakoglobin has also been demonstrated for dermal capillary endothelium in special molecular assembly experiments [127].

Beyond this role of desmoplakin as a regular constituent of the plaques of such "complex junctions" in certain lymphatic endothelia of the mature body a fundamental and general role of desmoplakin in the formation of the vascular endothelial system is also indicated by the transgene embryogenesis studies of Gallicano et al. [128, 129]. Thus, the *complexus adhaerens* junctions have to be added to the list of novel kinds of adhering junctions in their own right (see Table 1; for recent reviews see: [19, 130, 131]).

6. Dispersed, Non-Junction Bound States of Desmosomal Cadherins: Desmoglein Dsg2

Desmosomal cadherins typically associate with each other and form close-packed cis-clusters in the membranes of cytoplasmic vesicles, then exocytose to form a "half-junction" on the cell surface and under sufficient Ca^{2+}-concentration may further associate head-to-head in transform with another "half-junction", usually a domain of an adjacent cell, to a symmetrical junction [132–137]. Isolated, that is, non-junction-bound, desmosomal cadherin molecules that have not been included in desmosome structures and consequently may be dispersed over extended parts of the plasma membrane until recently had not been observed in natural cells. Only in certain cell culture lines deficient of most junction components such as the human fibrosarcoma HT-1080 cells states of the isolated desmosomal cadherin, Dsg2, have been described to occur on cell surfaces and could be integrated into junction-like structures only upon introduction of further desmosomal proteins [137, 138].

Thus it was with great surprise when we noted the occurrence of Dsg2 molecules dispersed over large portions of the surface membrane of certain cultures of human melanocytes or neval cells as well as on surfaces of a subtype of melanoma cells in situ and in culture [139, 140]. As far as it could be concluded from the biochemical analyses and immunolocalization experiments so far performed, these Dsg2 glycoprotein molecules were not stably complexed with specific other transmembrane or with any plaque molecules but nevertheless seemed to be somehow involved in close membrane-to-membrane associations. It is obvious that such stages, that is, dispersed, non-junction-bound desmosomal cadherins will have to be studied with special care as they point to the existence of yet unknown, radically different cell-cell adhesion mechanisms involving desmosomal cadherins.

Acknowledgments

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References

[1] M. G. Farquhar and G. E. Palade, "Junctional complexes in various epithelia," *The Journal of Cell Biology*, vol. 17, pp. 375–412, 1963.

[2] P. Drochmans, C. Freudenstein, J. C. Wanson, et al., "Structure and biochemical composition of desmosomes and tonofilaments isolated from calf muzzle epidermis," *Journal of Cell Biology*, vol. 79, pp. 427–443, 1978.

[3] P. Cowin, W. W. Franke, C. Grund, H. P. Kapprell, and J. Kartenbeck, "The desmosome-intermediate filament complex," in *The Cell in Contact*, G. M. Edelman and J. P. Thiery, Eds., pp. 427–460, John Wiley & Sons, New York, NY, USA, 1985.

[4] L. M. Godsel, S. Getsios, A. C. Huen, and K. J. Green, "The molecular composition and function of desmosomes," in *Cell Adhesion*, J. Behrens and W. J. Nelson, Eds., pp. 137–193, Springer, Heidelberg, Germany, 2004.

[5] D. Garrod and M. Chidgey, "Desmosome structure, composition and function," *Biochimica et Biophysica Acta*, vol. 1778, no. 3, pp. 572–587, 2008.

[6] A. Schmidt and P. J. Koch, "Desmosomes in development and disease," in *Cell Junctions*, S. E. LaFlamme and A. P. Kowalczyk, Eds., pp. 235–249, Wiley-VCH, Weinheim, Germany, 2008.

[7] W. W. Franke, "Discovering the molecular components of intercellular junctions-a historical view," in *Cell-Cell Junctions*, W. J. Nelson and E. Fuchs, Eds., Cold Spring Harbor Perspectives in Biology, pp. 1–34, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2010.

[8] S. Pieperhoff and W. W. Franke, "The area composita of adhering junctions connecting heart muscle cells of vertebrates. VI. Different precursor structures in non-mammalian species," *European Journal of Cell Biology*, vol. 87, no. 7, pp. 413–430, 2008.

[9] J. Waschke, "The desmosome and pemphigus," *Histochemistry and Cell Biology*, vol. 130, no. 1, pp. 21–54, 2008.

[10] W. W. Franke, E. Schmid, C. Grund, et al., "Antibodies to high molecular weight polypeptides of desmosomes: specific localization of a class of junctional proteins in cells and tissues," *Differentiation*, vol. 20, no. 3, pp. 217–241, 1981.

[11] W. W. Franke, R. Moll, D. L. Schiller, E. Schmid, J. Kartenbeck, and H. Mueller, "Desmoplakins of epithelial and myocardial desmosomes are immunologically and biochemically related," *Differentiation*, vol. 23, no. 2, pp. 115–127, 1982.

[12] P. Cowin, H.-P. Kapprell, and W. W. Franke, "Plakoglobin: a protein common to different kinds of intercellular adhering junctions," *Cell*, vol. 46, no. 7, pp. 1063–1073, 1986.

[13] C. Mertens, C. Kuhn, and W. W. Franke, "Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque," *Journal of Cell Biology*, vol. 135, no. 4, pp. 1009–1025, 1996.

[14] W. W. Franke, C. M. Borrmann, C. Grund, and S. Pieperhoff, "The area composita of adhering junctions connecting heart muscle cells of vertebrates. I. Molecular definition in intercalated disks of cardiomyocytes by immunoelectron microscopy of desmosomal proteins," *European Journal of Cell Biology*, vol. 85, no. 2, pp. 69–82, 2006.

[15] S. Pieperhoff and W. W. Franke, "The area composita of adhering junctions connecting heart muscle cells of vertebrates—IV: coalescence and amalgamation of desmosomal and adhaerens junction components—late processes in mammalian heart development," *European Journal of Cell Biology*, vol. 86, no. 7, pp. 377–391, 2007.

[16] S. Goossens, B. Janssens, S. Bonne, et al., "A unique and specific interaction between alphaT-catenin and plakophilin-2 in the area composita, the mixed-type junctional structure of cardiac intercalated discs," *Journal of Cell Science*, vol. 120, no. 12, part 2, pp. 2126–2136, 2007.

[17] T. S. Seeger, D. Frank, C. Rohr, et al., "Myozap, a novel intercalated disc protein, activates serum response factor-dependent signaling and is required to maintain cardiac function in vivo," *Circulation Research*, vol. 106, no. 5, pp. 880–890, 2010.

[18] B. Hämmerling, C. Grund, J. Boda-Heggemann, R. Moll, and W. W. Franke, "The complexus adhaerens of mammalian lymphatic endothelia revisited: a junction even more complex than hitherto thought," *Cell and Tissue Research*, vol. 324, no. 1, pp. 55–67, 2006.

[19] R. Moll, E. Sievers, B. Hämmerling, et al., "Endothelial and virgultar cell formations in the mammalian lymph node sinus: endothelial differentiation morphotypes characterized by a special kind of junction (complexus adhaerens)," *Cell and Tissue Research*, vol. 335, no. 1, pp. 109–141, 2009.

[20] R. Paffenholz, C. Kuhn, C. Grund, S. Stehr, and W. W. Franke, "The arm-repeat protein NPRAP (neurojungin) is a constituent of the plaques of the outer limiting zone in the retina, defining a novel type of adhering junction," *Experimental Cell Research*, vol. 250, no. 2, pp. 452–464, 1999.

[21] J. Boda-Heggemann, A. Regnier-Vigouroux, and W. W. Franke, "Beyond vessels: occurrence and regional clustering of vascular endothelial (VE-) cadherin-containing junctions in non-endothelial cells," *Cell and Tissue Research*, vol. 335, no. 1, pp. 49–65, 2009.

[22] S. Rickelt, S. Winter-Simanowski, E. Noffz, C. Kuhn, and W. W. Franke, "Upregulation of plakophilin-2 and its acquisition to adherens junctions identifies a novel molecular ensemble of cell-cell-attachment characteristic for transformed mesenchymal cells," *International Journal of Cancer*, vol. 125, no. 9, pp. 2036–2048, 2009.

[23] M. Barth, H. Schumacher, C. Kuhn, P. Akhyari, A. Lichtenberg, and W. W. Franke, "Cordial connections: molecular ensembles and structures of adhering junctions connecting interstitial cells of cardiac valves in situ and in cell culture," *Cell and Tissue Research*, vol. 337, no. 1, pp. 63–77, 2009.

[24] M. Amagai, "Desmoglein as a target in autoimmunity and infection," *Journal of the American Academy of Dermatology*, vol. 48, no. 2, pp. 244–252, 2003.

[25] J. C. R. Jones, K. M. Yokoo, and R. D. Goldman, "Further analysis of pemphigus autoantibodies and their use in studies on the heterogeneity, structure, and function of desmosomes," *Journal of Cell Biology*, vol. 102, no. 3, pp. 1109–1117, 1986.

[26] A. S. Payne, Y. Hanakawa, M. Amagai, and J. R. Stanley, "Desmosomes and disease: pemphigus and bullous impetigo," *Current Opinion in Cell Biology*, vol. 16, no. 5, pp. 536–543, 2004.

[27] M. G. Mahoney, Z. H. Wang, and J. R. Stanley, "Pemphigus vulgaris and pemphigus foliaceus antibodies are pathogenic in plasminogen activator knockout mice," *Journal of Investigative Dermatology*, vol. 113, no. 1, pp. 22–25, 1999.

[28] M. G. Mahoney, Z. Wang, K. Rothenberger, P. J. Koch, M. Amagai, and J. R. Stanley, "Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris," *Journal of Clinical Investigation*, vol. 103, no. 4, pp. 461–468, 1999.

[29] L. Langbein, C. Grund, C. Kuhn, et al., "Tight junctions and compositionally related junctional structures in mammalian stratified epithelia and cell cultures derives therefrom," *European Journal of Cell Biology*, vol. 81, no. 8, pp. 419–435, 2002.

[30] H. Schlüter, I. Moll, H. Wolburg, and W. W. Franke, "The different structures containing tight junction proteins in epidermal and other stratified epithelial cells, including squamous cell metaplasia," *European Journal of Cell Biology*, vol. 86, no. 11-12, pp. 645–655, 2007.

[31] M. Leu, E. Ehler, and J.-C. Perriard, "Characterisation of postnatal growth of the murine heart," *Anatomy and Embryology*, vol. 204, no. 3, pp. 217–224, 2001.

[32] A. Hirschy, F. Schatzmann, E. Ehler, and J.-C. Perriard, "Establishment of cardiac cytoarchitecture in the developing mouse heart," *Developmental Biology*, vol. 289, no. 2, pp. 430–441, 2006.

[33] C. M. Borrmann, C. Grund, C Kuhn, I. Hofmann, S. Pieperhoff, and W. W. Franke, "The area composita of adhering junctions connecting heart muscle cells of vertebrates. II. Colocalizations of desmosomal and fascia adhaerens molecules in the intercalated disk," *European Journal of Cell Biology*, vol. 85, no. 6, pp. 469–485, 2006.

[34] S. Pieperhoff, W. Bennett, and A. P. Farrell, "The intercellular organization of the two muscular systems in the adult salmonid heart, the compact and the spongy myocardium," *Journal of Anatomy*, vol. 215, no. 5, pp. 536–547, 2009.

[35] P. Ruiz, V. Brinkmann, B. Ledermann, et al., "Targeted mutation of plakoglobin in mice reveals essential functions of desmosomes in the embryonic heart," *Journal of Cell Biology*, vol. 135, no. 1, pp. 215–225, 1996.

[36] K. S. Grossmann, C. Grund, J. Huelsken, et al., "Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation," *Journal of Cell Biology*, vol. 167, no. 1, pp. 149–160, 2004.

[37] E. M. Oxford, H. Musa, K. Maass, W. Coombs, S. M. Taffet, and M. Delmar, "Connexin43 remodeling caused by inhibition of plakophilin-2 expression in cardiac cells," *Circulation Research*, vol. 101, no. 7, pp. 703–711, 2007.

[38] L. M. Fidler, G. J. Wilson, F. Liu, et al., "Abnormal connexin43 in arrhythmogenic right ventricular cardiomyopathy caused by plakophilin-2 mutations," *Journal of Cellular and Molecular Medicine*, vol. 13, no. 10, pp. 4219–4228, 2008.

[39] S. Pieperhoff, H. Schumacher, and W. W. Franke, "The area composita of adhering junctions connecting heart muscle cells of vertebrates. V. The importance of plakophilin-2 demonstrated by small interference RNA-mediated knockdown in cultured rat cardiomyocytes," *European Journal of Cell Biology*, vol. 87, no. 7, pp. 399–411, 2008.

[40] C. Hall, S. Li, H. Li, V. Creason, and J. K. Wahl III, "Arrhythmogenic right ventricular cardiomyopathy plakophilin-2 mutations disrupt desmosome assembly and stability," *Cell Communication and Adhesion*, vol. 16, no. 1–3, pp. 15–27, 2009.

[41] P. Y. Sato, H. Musa, W. Coombs, et al., "Loss of plakophilin-2 expression leads to decreased sodium current and slower conduction velocity in cultured cardiac myocytes," *Circulation Research*, vol. 105, no. 6, pp. 523–526, 2009.

[42] F. I. Marcus, A. Nava, and G. Thiene, *Arrhythmogenic RV Cardiomyopathy/Dysplasia*, Springer, Heidelberg, Germany, 2007.

[43] B. Gerull, A. Heuser, T. Wichter, et al., "Mutations in the desmosomal protein plakophilin-2 are common in arrhythmogenic right ventricular cardiomyopathy," *Nature Genetics*, vol. 36, no. 11, pp. 1162–1164, 2004.

[44] L. Antoniades, A. Tsatsopoulou, A. Anastasakis, et al., "Arrhythmogenic right ventricular cardiomyopathy caused by deletions in plakophilin-2 and plakoglobin (Naxos disease) in families from Greece and Cyprus: genotype-phenotype relations, diagnostic features and prognosis," *European Heart Journal*, vol. 27, no. 18, pp. 2208–2216, 2006.

[45] H. Calkins, "Arrhythmogenic right-ventricular dysplasia/cardiomyopathy," *Current Opinion in Cardiology*, vol. 21, no. 1, pp. 55–63, 2006.

[46] I. Nagaoka, K. Matsui, T. Ueyama, et al., "Novel mutation of plakophilin-2 associated with arrhythmogenic right ventricular cardiomyopathy," *Circulation Journal*, vol. 70, no. 7, pp. 933–935, 2006.

[47] P. J. Kannankeril, Z. A. Bhuiyan, D. Darbar, M. M. A. M. Mannens, A. A. M. Wilde, and D. M. Roden, "Arrhythmogenic right ventricular cardiomyopathy due to a novel plakophilin 2 mutation: wide spectrum of disease in mutation carriers within a family," *Heart Rhythm*, vol. 3, no. 8, pp. 939–944, 2006.

[48] D. Dalal, L. H. Molin, J. Piccini, et al., "Clinical features of arrhythmogenic right ventricular dysplasia/ cardiomyopathy associated with mutations in plakophilin-2," *Circulation*, vol. 113, no. 13, pp. 1641–1649, 2006.

[49] P. Syrris, D. Ward, A. Asimaki, et al., "Clinical expression of plakophilin-2 mutations in familial arrhythmogenic right ventricular cardiomyopathy," *Circulation*, vol. 113, no. 3, pp. 356–364, 2006.

[50] A. A. Tsatsopoulou, N. I. Protonotarios, and W. J. McKenna, "Arrhythmogenic right ventricular dysplasia, a cell adhesion cardiomyopathy: insights into disease pathogenesis from preliminary genotype—phenotype assessment," *Heart*, vol. 92, no. 12, pp. 1720–1723, 2006.

[51] J. P. Van Tintelen, M. M. Entius, Z. A. Bhuiyan, et al., "Plakophilin-2 mutations are the major determinant of familial arrhythmogenic right ventricular dysplasia/cardiomyopathy," *Circulation*, vol. 113, no. 13, pp. 1650–1658, 2006.

[52] A. M. Lahtinen, A. Lehtonen, M. Kaartinen, et al., "Plakophilin-2 missense mutations in arrhythmogenic right ventricular cardiomyopathy," *International Journal of Cardiology*, vol. 126, no. 1, pp. 92–100, 2008.

[53] L. C. Otterspoor, C. L. A. Reichert, M. J. M. Cramer, Z. A. Bhuiyan, A. A. M. Wilde, and R. N. W. Hauer, "Arrhythmogenic right ventricular cardiomyopathy: asymptomatic to life threatening as illustrated by the cases of two sisters," *Netherlands Heart Journal*, vol. 15, no. 10, pp. 348–353, 2007.

[54] R. Joshi-Mukherjee, W. Coombs, H. Musa, E. Oxford, S. Taffet, and M. Delmar, "Characterization of the molecular phenotype of two arrhythmogenic right ventricular cardiomyopathy (ARVC)-related plakophilin-2 (PKP2) mutations," *Heart Rhythm*, vol. 5, no. 12, pp. 1715–1723, 2008.

[55] R. Ram and D. R. Van Wagoner, "Plakophilin-2 mutations as a cause of arrhythmogenic right ventricular cardiomyopathy: progress toward linking structural with functional changes," *Heart Rhythm*, vol. 5, no. 12, pp. 1724–1725, 2008.

[56] H. Tandri, A. Asimaki, D. Dalal, J. E. Saffitz, M. K. Halushka, and H. Calkins, "Gap junction remodeling in a case of arrhythmogenic right ventricular dysplasia due to plakophilin-2 mutation," *Journal of Cardiovascular Electrophysiology*, vol. 19, no. 11, pp. 1212–1214, 2008.

[57] S.-L. Wu, P.-N. Wang, Y.-S. Hou, et al., "Mutation of plakophilin-2 gene in arrhythmogenic right ventricular cardiomyopathy," *Chinese Medical Journal*, vol. 122, no. 4, pp. 403–407, 2009.

[58] X. Qiu, W. Liu, D. Hu, et al., "Mutations of plakophilin-2 in Chinese with arrhythmogenic right ventricular dysplasia/cardiomyopathy," *American Journal of Cardiology*, vol. 103, no. 10, pp. 1439–1444, 2009.

[59] Z. A. Bhuiyan, J. D. Jongbloed, J. van der Smagt, et al., "Desmoglein-2 and desmocollin-2 mutations in dutch arrhythmogenic right ventricular dysplasia/cardiomypathy patients: results from a multicenter study," *Circulation: Cardiovascular Genetics*, vol. 2, no. 5, pp. 418–427, 2009.

[60] M. M. Awad, D. Dalal, C. Tichnell, et al., "Recessive arrhythmogenic right ventricular dysplasia due to novel cryptic splice mutation in PKP2," *Human Mutation*, vol. 27, no. 11, p. 1157, 2006.

[61] T. Xu, Z. Yang, M. Vatta, et al., "Compound and digenic heterozygosity contributes to arrhythmogenic right ventricular cardiomyopathy," *Journal of the American College of Cardiology*, vol. 55, no. 6, pp. 587–597, 2010.

[62] B. Bauce, A. Nava, G. Beffagna, et al., "Multiple mutations in desmosomal proteins encoding genes in arrhythmogenic right ventricular cardiomyopathy/dysplasia," *Heart Rhythm*, vol. 7, no. 1, pp. 22–29, 2010.

[63] S. Sen-Chowdhry, R. D. Morgan, J. C. Chambers, and W. J. McKenna,, "Arrhythmogenic cardiomyopathy: etiology, diagnosis, and treatment," *Annual Review of Medicine*, vol. 61, pp. 233–253, 2010.

[64] A. D. den Haan, B. Y. Tan, M. N. Zikusoka, et al., "Comprehensive desmosome mutation analysis in north americans with arrhythmogenic right ventricular dysplasia/cardiomyopathy," *Circulation. Cardiovascular Genetics*, vol. 2, no. 5, pp. 428–435, 2009.

[65] H. Bazzi and A. M. Christiano, "Broken hearts, woolly hair, and tattered skin: when desmosomal adhesion goes awry," *Current Opinion in Cell Biology*, vol. 19, no. 5, pp. 515–520, 2007.

[66] M. M. Awad, H. Calkins, and D. P. Judge, "Mechanisms of disease: molecular genetics of arrhythmogenic right ventricular dysplasia/cardiomyopathy," *Nature Clinical Practice Cardiovascular Medicine*, vol. 5, no. 5, pp. 258–267, 2008.

[67] D. Corrado, C. Basso, and G. Thiene, "Arrhythmogenic right ventricular cardiomyopathy: an update," *Heart*, vol. 95, no. 9, pp. 766–773, 2009.

[68] T. Herren, P. A. Gerber, and F. Duru, "Arrhythmogenic right ventricular cardiomyopathy/dysplasia: a not so rare "disease of the desmosome" with multiple clinical presentations," *Clinical Research in Cardiology*, vol. 98, no. 3, pp. 141–158, 2009.

[69] J. E. Saffitz, "Arrhythmogenic cardiomyopathy and abnormalities of cell-to-cell coupling," *Heart Rhythm*, vol. 6, no. 8, pp. S62–S65, 2009.

[70] E. M. Oxford, M. Everitt, W. Coombs, et al., "Molecular composition of the intercalated disc in a spontaneous canine animal model of arrhythmogenic right ventricular dysplasia/cardiomyopathy," *Heart Rhythm*, vol. 4, no. 9, pp. 1196–1205, 2007.

[71] S. R. Kaplan, J. J. Gard, N. Protonotarios, et al., "Remodeling of myocyte gap junctions in arrhythmogenic right ventricular cardiomyopathy due to a deletion in plakoglobin (Naxos disease)," *Heart Rhythm*, vol. 1, no. 1, pp. 3–11, 2004.

[72] P. Syrris, D. Ward, A. Evans, et al., "Arrhythmogenic right ventricular dysplasia/cardiomyopathy associated with mutations in the desmosomal gene desmocollin-2," *American Journal of Human Genetics*, vol. 79, no. 5, pp. 978–984, 2006.

[73] P. Syrris, D. Ward, A. Asimaki, et al., "Desmoglein-2 mutations in arrhythmogenic right ventricular cardiomyopathy: a genotype-phenotype characterization of familial disease," *European Heart Journal*, vol. 28, no. 5, pp. 581–588, 2007.

[74] A. Uzumcu, E. E. Norgett, A. Dindar, et al., "Loss of desmoplakin isoform I causes early onset cardiomyopathy and heart failure in a Naxos-like syndrome," *Journal of Medical Genetics*, vol. 43, no. 2, p. e5, 2006.

[75] E. E. Norgett, T. W. Lucke, B. Bowers, C. S. Munro, I. M. Leigh, and D. P. Kelsell, "Early death from cardiomyopathy in a family with autosomal dominant striate palmoplantar keratoderma and woolly hair associated with a novel insertion mutation in desmoplakin," *Journal of Investigative Dermatology*, vol. 126, no. 7, pp. 1651–1654, 2006.

[76] M. A. Simpson, S. Mansour, D. Ahnood, et al., "Homozygous mutation of desmocollin-2 in arrhythmogenic right ventricular cardiomyopathy with mild palmoplantar keratoderma and woolly hair," *Cardiology*, vol. 113, no. 1, pp. 28–34, 2009.

[77] B. Bauce, C. Basso, A. Rampazzo, et al., "Clinical profile of four families with arrhythmogenic right ventricular cardiomyopathy caused by dominant desmoplakin mutations," *European Heart Journal*, vol. 26, no. 16, pp. 1666–1675, 2005.

[78] N. Protonotarios, A. Tsatsopoulou, A. Anastasakis, et al., "Genotype-phenotype assessment in autosomal recessive arrhythmogenic right ventricular cardiomyopathy (Naxos disease) caused by a deletion in plakoglobin," *Journal of the American College of Cardiology*, vol. 38, no. 5, pp. 1477–1484, 2001.

[79] G. Beffagna, M. De Bortoli, A. Nava, et al., "Missense mutations in Desmocollin-2 N-terminus, associated with arrhythmogenic right ventricular cardiomyopathy, affect intracellular localization of desmocollin-2 in vitro," *BMC Medical Genetics*, vol. 8, article 65, 2007.

[80] G. McKoy, N. Protonotarios, A. Crosby, et al., "Identification of a deletion in plakoglobin in arrhythmogenic right ventricular cardiomyopathy with palmoplantar keratoderma and woolly hair (Naxos disease)," *Lancet*, vol. 355, no. 9221, pp. 2119–2124, 2000.

[81] M. G. Mahoney, S. Sadowski, D. Brennan, et al., "Compound heterozygous desmoplakin mutations result in a phenotype with a combination of myocardial, skin, hair, and enamel abnormalities," *Journal of Investigative Dermatology*, vol. 130, no. 4, pp. 968–978, 2010.

[82] N. I. Protonotarios, A. A. Tsatsopoulou, and K. A. Gatzoulis, "Arrhythmogenic right ventricular cardiomyopathy caused by a deletion in plakoglobin (Naxos disease)," *Cardiac Electrophysiology Review*, vol. 6, no. 1-2, pp. 72–80, 2002.

[83] A. Heuser, E. R. Plovie, P. T. Ellinor, et al., "Mutant desmocollin-2 causes arrhythmogenic right ventricular cardiomyopathy," *American Journal of Human Genetics*, vol. 79, no. 6, pp. 1081–1088, 2006.

[84] E. E. Norgett, S. J. Hatsell, L. Carvajal-Huerta, et al., "Recessive mutation in desmoplakin disrupts desmoplakin-intermediate filament interactions and causes dilated cardiomyopathy, woolly hair and keratoderma," *Human Molecular Genetics*, vol. 9, no. 18, pp. 2761–2766, 2000.

[85] M. Norman, M. Simpson, J. Mogensen, et al., "Novel mutation in desmoplakin causes arrhythmogenic left ventricular cardiomyopathy," *Circulation*, vol. 112, no. 5, pp. 636–642, 2005.

[86] R. Alcalai, S. Metzger, S. Rosenheck, V. Meiner, and T. Chajek-Shaul, "A recessive mutation in desmoplakin causes arrhythmogenic right ventricular dysplasia, skin disorder, and woolly hair," *Journal of the American College of Cardiology*, vol. 42, no. 2, pp. 319–327, 2003.

[87] S. Sen-Chowdhry, P. Syrris, and W. J. McKenna, "Genetics of right ventricular cardiomyopathy," *Journal of Cardiovascular Electrophysiology*, vol. 16, no. 8, pp. 927–935, 2005.

[88] S. Sen-Chowdhry, P. Syrris, D. Ward, A. Asimaki, E. Sevdalis, and W. J. McKenna, "Clinical and genetic characterization of families with arrhythmogenic right ventricular dysplasia/cardiomyopathy provides novel insights into patterns of disease expression," *Circulation*, vol. 115, no. 13, pp. 1710–1720, 2007.

[89] Z. Yang, N. E. Bowles, S. E. Scherer, et al., "Desmosomal dysfunction due to mutations in desmoplakin causes arrhythmogenic right ventricular dysplasia/cardiomyopathy," *Circulation Research*, vol. 99, no. 6, pp. 646–655, 2006.

[90] K. Pilichou, A. Nava, C. Basso, et al., "Mutations in desmoglein-2 gene are associated with arrhythmogenic right ventricular cardiomyopathy," *Circulation*, vol. 113, no. 9, pp. 1171–1179, 2006.

[91] C.-C. Yu, C.-H. Yu, C.-H. Hsueh, et al., "Arrhythmogenic right ventricular dysplasia: clinical characteristics and identification of novel desmosome gene mutations," *Journal of the Formosan Medical Association*, vol. 107, no. 7, pp. 548–558, 2008.

[92] E. Garcia-Gras, R. Lombardi, M. J. Giocondo, et al., "Suppression of canonical Wnt/β-catenin signaling by nuclear plakoglobin recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy," *Journal of Clinical Investigation*, vol. 116, no. 7, pp. 2012–2021, 2006.

[93] A. Asimaki, P. Syrris, T. Wichter, P. Matthias, J. E. Saffitz, and W. J. McKenna, "A novel dominant mutation in plakoglobin causes arrhythmogenic right ventricular cardiomyopathy," *American Journal of Human Genetics*, vol. 81, no. 5, pp. 964–973, 2007.

[94] A. Asimaki, H. Tandri, H. Huang, et al., "A new diagnostic test for arrhythmogenic right ventricular cardiomyopathy," *The New England Journal of Medicine*, vol. 360, no. 11, pp. 1075–1084, 2009.

[95] M. G. Cox, J. J. Vandersmagt, M. Noorman, et al., "ARVD/C diagnosis: impact of new task force criteria," *Circulation: Arrhythmia and Electrophysiology*, vol. 3, no. 2, pp. 126–133, 2010.

[96] M. C. Bolling, M. J. Veenstra, M. F. Jonkmann, et al., " Lethal acantholytic epidermolysis bullosa due to a novel homozygous deletion in DSP: expanding the phenotype and implications for desmoplakin functions in skin and heart," *British Journal of Dermatology*, vol. 162, no. 6, pp. 1388–1394, 2010.

[97] A. Rampazzo and G. A. Danieli, "Advances in genetics: dominant forms," in *Arrhythmogenic RV Cardiomyopathy/Dysplasia*, F. I. Marcus, A. Nava, and G. Thiene, Eds., pp. 7–14, Springer, Heidelberg, Germany, 2007.

[98] N. Protonotarios and A. Tsatsopoulou, "Advances in genetics: dominant forms," in *Arrhythmogenic RV Cardiomyopathy/Dysplasia*, F. I. Marcus, A. Nava, and G. Thiene, Eds., pp. 15–20, Springer, Heidelberg, Germany, 2007.

[99] B. Bauce and A. Nava, "Genotype-phenotype correlations," in *Arrhythmogenic RV Cardiomyopathy/Dysplasia*, F. I. Marcus, A. Nava, and G. Thiene, Eds., pp. 21–28, Springer, Heidelberg, Germany, 2007.

[100] C. Basso and G. Thiene, "Autopsy and endomyocardial biopsy findings," in *Arrhythmogenic RV Cardiomyopathy/Dysplasia*, F. I. Marcus, A. Nava, and G. Thiene, Eds., pp. 30–44, Springer, Heidelberg, Germany, 2007.

[101] J. Saffitz, "Cell adhesion pathology," in *Arrhythmogenic RV Cardiomyopathy/Dysplasia*, F. I. Marcus, A. Nava, and G. Thiene, Eds., Springer, Heidelberg, Germany, 2007.

[102] E. Czarnowska, M. D. Barbera, G. Thiene, M. Valente, and C. Basso, "Ultrastructural substrates," in *Arrhythmogenic RV Cardiomyopathy/Dysplasia*, F. I. Marcus, A. Nava, and G. Thiene, Eds., pp. 53–60, Springer, Heidelberg, Germany, 2007.

[103] S. Pieperhoff, C. Borrmann, C. Grund, M. Barth, S. Rizzo, and W. W. Franke, "The area composita of adhering junctions connecting heart muscle cells of vertebrates. VII. The different types of lateral junctions between the special cardiomyocytes of the conduction system of ovine and bovine hearts," *European Journal of Cell Biology*, vol. 89, no. 5, pp. 365–378, 2010.

[104] L.-E. Thornell, A. Eriksson, B. Johansson, and U. Kjorell, "Intermediate filament and associated proteins in heart Purkinje fibers: a membrane-myofibril anchored cytoskeletal system," *Annals of the New York Academy of Sciences*, vol. 455, pp. 213–240, 1985.

[105] A. Orlandi, H. Hao, A. Ferlosio, et al., "Alpha actin isoforms expression in human and rat adult cardiac conduction system," *Differentiation*, vol. 77, no. 4, pp. 360–368, 2009.

[106] D. Durrer, R. T. van Dam, G. E. Freud, M. J. Janse, F. L. Meijler, and R. C. Arzbaecher, "Total excitation of the isolated human heart," *Circulation*, vol. 41, no. 6, pp. 899–912, 1970.

[107] G. Thiene, N. Pennelli, and L. Rossi, "Cardiac conduction system abnormalities as a possible cause of sudden death in young athletes," *Human Pathology*, vol. 14, no. 8, pp. 707–709, 1983.

[108] H.-P. Kapprell, K. Owaribe, and W. W. Franke, "Identification of a basic protein of Mr 75.000 as an accessory desmosomal plaque protein in stratified and complex epithelia," *Journal of Cell Biology*, vol. 106, no. 5, pp. 1679–1691, 1988.

[109] H. W. Heid, A. Schmidt, R. Zimbelmann, et al., "Cell type-specific desmosomal plaque proteins of the plakoglobin family: plakophilin 1 (band 6 protein)," *Differentiation*, vol. 58, no. 2, pp. 113–131, 1995.

[110] A. Schmidt, L. Langbein, M. Rode, S. Prätzel, R. Zimbelmann, and W. W. Franke, "Plakophilins 1a and 1b: widespread nuclear proteins recruited in specific epithelial cells as desmosomal plaque components," *Cell and Tissue Research*, vol. 290, no. 3, pp. 481–499, 1997.

[111] A. Schmidt, L. Langbein, S. Prätzel, M. Rode, H.-R. Rackwitz, and W. W. Franke, "Plakophilin 3—a novel cell-type-specific desmosomal plaque protein," *Differentiation*, vol. 64, no. 5, pp. 291–306, 1999.

[112] S. Bonné, J. Van Hengel, F. Nollet, P. Kools, and F. Van Roy, "Plakophilin-3, a novel Armadillo-like protein present in nuclei and desmosomes of epithelial cells," *Journal of Cell Science*, vol. 161, no. 14, pp. 2265–2276, 1999.

[113] M. Hatzfeld, "Plakophilins: multifunctional proteins or just regulators of desmosomal adhesion?" *Biochimica et Biophysica Acta*, vol. 1773, no. 1, pp. 69–77, 2007.

[114] C. Mertens, I. Hofmann, Z. Wang, et al., "Nuclear particles containing RNA polymerase III complexes associated with the junctional plaque protein plakophilin 2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 14, pp. 7795–7800, 2001.

[115] I. Hofmann, M. Casella, M. Schnölzer, T. Schlechter, H. Spring, and W. W. Franke, "Identification of the junctional plaque protein plakophilin 3 in cytoplasmic particles containing RNA-binding proteins and the recruitment of plakophilins 1 and 3 to stress granules," *Molecular Biology of the Cell*, vol. 17, no. 3, pp. 1388–1398, 2006.

[116] A. Wolf, M. Krause-Gruszczynska, O. Birkenmeier, A. Ostareck-Lederer, S. Hüttelmaier, and M. Hatzfeld, "Plakophilin 1 stimulates translation by promoting eIF4A1 activity," *Journal of Cell Biology*, vol. 188, no. 4, pp. 463–471, 2010.

[117] W. W. Franke, S. Rickelt, M. Barth, and S. Pieperhoff, "The junctions that don't fit the scheme: special symmetrical cell-cell junctions of their own kind," *Cell and Tissue Research*, vol. 338, no. 1, pp. 1–17, 2009.

[118] S. Rickelt, S. Rizzo, Y. Dörflinger, et al., "A novel kind of tumor type-characteristic junction: Plakophilin-2 as a major protein of adherens junctions in cardiac myxomata," *Modern Pathology*. In press.

[119] E. Dejana, "Endothelial cell-cell junctions: happy together," *Nature Reviews Molecular Cell Biology*, vol. 5, no. 4, pp. 261–270, 2004.

[120] M. G. Lampugnani and E. Dejana, "Interendothelial junctions: structure, signalling and functional roles," *Current Opinion in Cell Biology*, vol. 9, no. 5, pp. 674–682, 1997.

[121] M. Schmelz and W. W. Franke, "A new type of intercellular junction: desmosomal proteins in the extended junctions of certain endothelial cells of the lymphatic systems," *Cell Biology International Reports*, vol. 14, p. 54, 1990.

[122] M. Schmelz, R. Moll, C. Kuhn, and W. W. Franke, "Complexus adhaerentes, a new group of desmoplakin-containing junctions in endothelial cells: II. Different types of lymphatic vessels," *Differentiation*, vol. 57, no. 2, pp. 97–117, 1994.

[123] N. Ebata, Y. Nodasaka, Y. Sawa, et al., "Desmoplakin as a specific marker of lymphatic vessels," *Microvascular Research*, vol. 61, no. 1, pp. 40–48, 2001.

[124] O. Valiron, V. Chevrier, Y. Usson, F. Breviario, D. Job, and E. Dejana, "Desmoplakin expression and organization at human umbilical vein endothelial cell-to-cell junctions," *Journal of Cell Science*, vol. 109, no. 8, pp. 2141–2149, 1996.

[125] P. Baluk, J. Fuxe, H. Hashizume, et al., "Functionally specialized junctions between endothelial cells of lymphatic vessels," *Journal of Experimental Medicine*, vol. 204, no. 10, pp. 2349–2362, 2007.

[126] F. Pfeiffer, V. Kumar, S. Butz, et al., "Distinct molecular composition of blood and lymphatic vascular endothelial cell junctions establishes specific functional barriers within the peripheral lymph node," *European Journal of Immunology*, vol. 38, no. 8, pp. 2142–2155, 2008.

[127] A. P. Kowalczyk, P. Navarro, E. Dejana, et al., "VE-cadherin and desmoplakin are assembled into dermal microvascular endothelial intercellular junctions: a pivotal role for plakoglobin in the recruitment of desmoplakin to intercellular junctions," *Journal of Cell Science*, vol. 111, no. 20, pp. 3045–3057, 1998.

[128] G. Ian Gallicano, P. Kouklis, C. Bauer, et al., "Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage," *Journal of Cell Biology*, vol. 143, no. 7, pp. 2009–2022, 1998.

[129] G. I. Gallicano, C. Bauer, and E. Fuchs, "Rescuing desmoplakin function in extra-embryonic ectoderm reveals the importance of this protein in embryonic heart, neuroepithelium, skin and vasculature," *Development*, vol. 128, no. 6, pp. 929–941, 2001.

[130] G. Jurisic and M. Detmar, "Lymphatic endothelium in health and disease," *Cell and Tissue Research*, vol. 335, no. 1, pp. 97–108, 2009.

[131] E. Dejana, F. Orsenigo, C. Molendini, P. Baluk, and D. M. McDonald, "Organization and signaling of endothelial cell-to-cell junctions in various regions of the blood and lymphatic vascular trees," *Cell and Tissue Research*, vol. 335, no. 1, pp. 17–25, 2009.

[132] H. Hennings, D. Michael, and C. Cheng, "Calcium regulation of growth and differentiation of mouse epidermal cells in culture," *Cell*, vol. 19, no. 1, pp. 245–254, 1980.

[133] H. Hennings and K. A. Holbrook, "Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture. An ultrastructural study," *Experimental Cell Research*, vol. 143, no. 1, pp. 127–142, 1983.

[134] F. M. Watt, D. L. Mattey, and D. R. Garrod, "Calcium-induced reorganization of desmosomal components in cultured human keratinocytes," *Journal of Cell Biology*, vol. 99, no. 6, pp. 2211–2215, 1984.

[135] P. Cowin, D. Mattey, and D. Garrod, "Identification of desmosomal surface components (desmocollins) and inhibition of desmosome formation by specific Fab," *Journal of Cell Science*, vol. 70, pp. 41–60, 1984.

[136] M. P. Demlehner, S. Schäfer, C. Grund, and W. W. Franke, "Continual assembly of half-desmosomal structures in the absence of cell contacts and their frustrated endocytosis:a coordinated sisyphus cycle," *Journal of Cell Biology*, vol. 131, no. 3, pp. 745–760, 1995.

[137] N. A. Chitaev and S. M. Troyanovsky, "Direct Ca^{2+}-dependent heterophilic interaction between desmosomal cadherins, desmoglein and desmocollin, contributes to cell-cell adhesion adhesion," *Journal of Cell Biology*, vol. 138, no. 1, pp. 193–201, 1997.

[138] J. Koeser, S. M. Troyanovsky, C. Grund, and W. W. Franke, "De novo formation of desmosomes in cultured cells upon transfection of genes encoding specific desmosomal components," *Experimental Cell Research*, vol. 285, no. 1, pp. 114–130, 2003.

[139] C. J. Schmitt, W. W. Franke, S. Goerdt, B. Falkowska-Hansen, S. Rickelt, and W. K. Peitsch, "Homo- and heterotypic cell contacts in malignant melanoma cells and desmoglein 2 as a novel solitary surface glycoprotein," *Journal of Investigative Dermatology*, vol. 127, no. 9, pp. 2191–2206, 2007.

[140] S. Rickelt, W. W. Franke, Y. Doerflinger, S. Goerdt, J. M. Brandner, and W. K. Peitsch, "Subtypes of melanocytes and melanoma cells distinguished by their intercellular contacts: heterotypic adherens junctions, adhesive associations, and dispersed desmoglein 2 glycoproteins," *Cell and Tissue Research*, vol. 334, no. 3, pp. 401–422, 2008.

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Review Article

A New Perspective on Intercalated Disc Organization: Implications for Heart Disease

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Adherens junctions and desmosomes are intercellular adhesive junctions and essential for the morphogenesis, differentiation, and maintenance of tissues that are subjected to high mechanical stress, including heart and skin. The different junction complexes are organized at the termini of the cardiomyocyte called the intercalated disc. Disruption of adhesive integrity via mutations in genes encoding desmosomal proteins causes an inherited heart disease, arrhythmogenic right ventricular cardiomyopathy (ARVC). Besides plakoglobin, which is shared by adherens junctions and desmosomes, other desmosomal components, desmoglein-2, desmocollin-2, plakophilin-2, and desmoplakin are also present in ultrastructurally defined fascia adherens junctions of heart muscle, but not other tissues. This mixed-type of junctional structure is termed hybrid adhering junction or area composita. Desmosomal plakophilin-2 directly interacts with adherens junction protein alphaT-catenin, providing a new molecular link between the cadherin-catenin complex and desmosome. The area composita only exists in the cardiac intercalated disc of mammalian species suggesting that it evolved to strengthen mechanical coupling in the heart of higher vertebrates. The cross-talk among different junctions and their implication in the pathogenesis of ARVC are discussed in this review.

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited heart muscle disease estimated to affect approximately 1 in 5,000 individuals [1]. The prominent features are myocytes loss, fibro-fatty tissue replacement, and life-threatening ventricular arrhythmias [2–4]. Approximately a third of patients with ARVC have one or more mutations in genes encoding cardiac desmosomal proteins; hence ARVC is referred to as "a disease of the desmosome" [5, 6].

Desmosomes and adherens junctions are intercellular adhesive junctions that anchor intermediate filaments and actin cytoskeleton, respectively, at the plasma membrane of adjoining cells, thereby provide mechanical attachment between the cells, and support the structural and functional integrity of the tissues. Desmosomes consist of three families of proteins, desmosomal cadherins, armadillo proteins, and plakins (Table 1) [7]. Desmosomal cadherins, desmogleins (DSGs), and desmocollins (DSCs) form the extracellular connections by homophilic and heterophilic binding. The cytoplasmic tails of desmosomal cadherins bind to the armadillo protein plakoglobin (PG) and plakophilins (PKP), which in turn bind to the plakin protein, desmoplakin (DP). Desmoplakin links desmosomes to intermediate filament protein. Different desmosomal proteins are differentially expressed in a tissue-specific as well as differentiation-dependent manner. Unlike DSG-1, -3, and -4 and DSC-1 and 3, which are predominantly expressed in the skin epidermis, DSG-2 and DSC-2 are highly expressed in the myocardium of the heart. PKP-1 and PKP-3 are the major epidermal PKPs, whereas PKP-2 is the sole PKP present in cardiac tissue. Desmosomal proteins DP, PG, and plectin are shared by epidermis and myocardium (Table 1). Consistent with differential expression pattern of desmosomal components between heart and skin, human mutations causing ARVC in the genes encoding PG, DP, and DSC-2 are associated with the cardiocutaneous syndrome [8]. In addition to ARVC, patients exhibit woolly hair and palmoplantar keratoderma, and they may also have skin blistering.

The classical cadherins are calcium-dependent cell adhesion receptors, located in the adherens junction. The

extracellular domain of cadherins interacts homophilically mediating strong cell-cell adhesion and plays a key role in the maintenance of tissue structure. In the classic model for adherens junction, the cytoplasmic tail of cadherin interacts in a mutually exclusive manner with either β-catenin or PG. β-catenin or PG links cadherins to α-catenin, and α-catenin interacts with the actin cytoskeleton. Gap junction is another intercellular junction in the cell responsible for cell-cell communication and electrical coupling by mediating small molecules and ion transfer between the cells. Each gap junction is composed of two hexameric structures called connexons or hemichannels that dock across the extracellular space and form a permeable pore. Each connexon consists of six transmembrane proteins called connexin (Cx). Cx43 is the most abundant connexin isotype in the heart. The different junctional complexes must be properly organized in the intercalated disc (ICD) of the myocardium to preserve normal mechanical and electrical function of the heart.

Plakoglobin, also known as γ-catenin, is the only linker protein present in both desmosomes and adherens junctions in skin and heart [9]. Plakoglobin was also the first component of the desmosome to be implicated in the pathogenesis of ARVC. Studies of individuals from the Greek island of Naxos identified an autosomal recessive inherited ARVC with palmoplantar keratoderma and woolly hair. Gene sequencing revealed a homozygous 2 bp deletion (2157-2158delGT) in the junction plakoglobin gene (*JUP*) in affected individuals [10]. A study of a German family recently reported the first dominantly inherited *JUP* mutation (S39_K40insS) to cause ARVC without cutaneous abnormalities [11]. Importantly, reduced immunoreactive signal of PG at the ICD is a consistent feature in patients with ARVC making it an important diagnostic tool for ARVC in affected individuals [12]. Most recently, studies on cardiac restricted deletion of PG in adult mice have shown similar features to ARVC, including myocytes loss, inflammation, fibrosis, and cardiac dysfunction [13].

The mixing of junctional components in the heart was first described in PG null mice. The homozygous PG null animals die between embryonic days 12–16 due to ventricular rupture and hemorrhaging into the pericardial cavity. Desmosomes are not detected in the mutant hearts. Instead extended adherens junctions develop, which contain desmosomal proteins such as desmoplakin, forming "mixed type" adhering junction [14, 15]. In the heart of PKP-2 null embryos, the desmosome-like structures are also not present, and two morphotypes of desmosomes and adherens junctions are found difficult to distinguish [16]. These observations in mutant mice suggest that the formation of hybrid adhering junctions may be compensatory response to weakened adhesion due to loss of desmosomal proteins PG or PKP-2 in the myocardium.

The typical morphological appearance of cardiac intercalated disc at the ultra structural level in the mice is submembranous plaques with electron dense material adjacent to intercellular space between the myocytes (Figure 1(A)). A relatively large junction that anchors primarily bundles of actin myofilaments is fascia adhaerens-like junction, and junction that anchors primarily intermediate filaments is a desmosomal-like junction (Figure 1(A)). For desmosomes, distinct electron dense material is often observed in the intercellular space. During embryonic development, the shape of the individual cardiomyocyte changes from more polygonal to more elongate with alignment of the myofibrils to the longitudinal axis of the cell. Accompanying the morphological change, the junctional components distribute from all round the cell to the sites of myofibril attachment and eventually restrict to the cell-cell contact of polarized adult myocytes (i.e., ICD) [17].

Table 1: The different composition of desmosomes in skin and heart.

	Skin	Heart
Desmosomal cadherins	Desmoglein (DSG) 1–4	Desmoglein (DSG)-2
	Desmocollin (DSC) 1–3	Desmocollin (DSC)-2
Armadillo proteins	Plakoglobin (PG)	Plakoglobin (PG)
	Plakophilin (PKP) 1–3	Plakophilin (PKP)-2
Plakins	Desmoplakin (DP)	
	Plectin	Desmoplakin (DP)
	Envoplakin	Plectin
	Periplakin	

By comprehensive immunoelectron microscopy with immunogold DP antibody labeling, Franke et al. observed that in normal heart muscle, DP is located in all plaques of both the desmosome-like and fascia adhaerens-type junctions. Very intensely labeled junctions with DP are seen in the more desmosome-like junctions (Figure 1(B)), whereas equal label intensity of continuous DP is seen in mixed-type junctions or hybrid adhering junctions (Figure 1(C)). Using various antibodies to desmosomal plaque proteins, Franke et al. further found that other desmosomal molecules, PG, PKP-2, DSC-2, and DSG-2, are also not restricted to the desmosome-like junctions but also can be detected in adherens junction structures. This large plaque-coated hybrid structure therefore has been termed an "area composita" [18] (Figure 2). By light and electron microscopy, the molecules known as typical components of fascia adhaerens, including N-cadherin, α-catenin, and β-catenin, also have been shown to colocalize with desmosomal proteins in the majority of the area composita junctions [19]. These studies suggest that the area composita is an unusual high molecular complexity and the elements that exist in this hybrid structure are intimately associated [19]. Consistent with late maturation of the ICD structure during development, the formation of the extended area composita junction is also a late, primarily postnatal process in mammalian heart [20]. By contrast, in nonmammalian species (fishes, amphibia, birds), adherens junctions and desmosomes remain separate and distinct structures in these adult hearts, suggesting that the formation of the area composita is not only a relatively late process in mammalian ontogenesis but also in vertebrate evolution [20, 21].

αT-catenin is a recently identified member of α-catenin family with restricted expression in testis, brain, and cardiac muscle [22]. Extensive studies from Goossens et al. provided

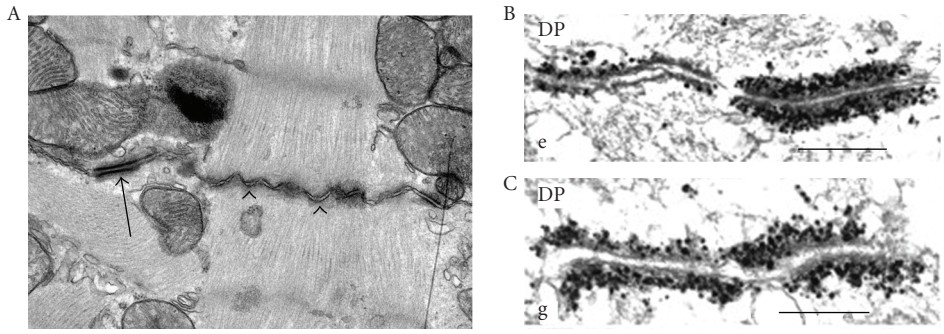

Figure 1: (A) Transmission electron microscopy of intercalated disc in the normal mouse heart, showing the plasma membranes of the adjacent cardiomyocytes with a region structurally resembling a desmosome (arrow) anchoring intermediate filaments, and fascia adhaerens-like structure anchoring predominantly bundles of actin filaments (arrowheads). (B) and (C) Immunoelectron microscopy of the myocardium of mouse heart, showing desmoplakin (DP) antibody labeling with silver amplification at cell-cell junctions. Note that DP immunogold label is enriched in plaques of the junctions. Higher label intensity of DP is desmosome-like structure (the right hand junction in Figure 1(B)), and the fascia adhaerens-like junction is shown in less intensity of DP labeling (the left hand junction in Figure 1(B)). A continuous and equal intensity of DP labeling shows the hybrid junctions (Figure 1(C)) [16]. Figures 1(B) and 1(C) was originally published in "The Journal of Cell Biology, Grossman et al., 2004. doi:10.1083/jcb.200402096" [16].

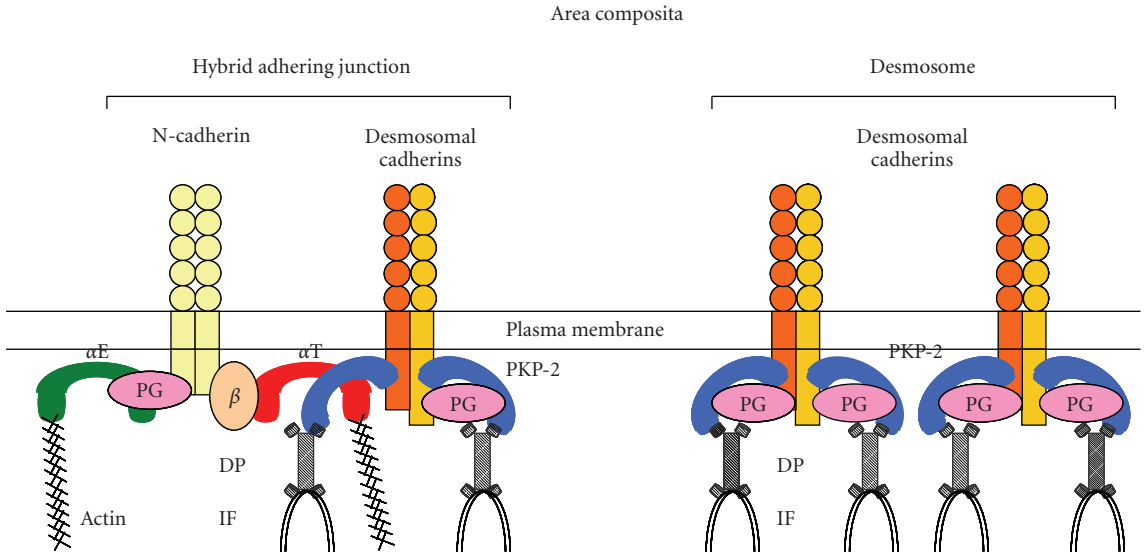

Figure 2: Model for cadherin-based area composita in the heart. αT-catenin recruits desmosomal protein, plakophilin- (PKP-) 2 to hybrid adhering junction (left drawing), thereby forming, together with desmosome (right drawing), an area composita, which is an enforced, mixed-type junctional structure attached to both the actin cytoskeleton and the intermediate filament (PG: plakoglobin; DP: desmoplakin).

molecular evidence that αT-catenin, a unique molecule of adherens junction, functions as cytoskeletal linker protein that specifically brings the desmosomes and adherens junctions together in the intercalated disc of the heart [23]. Using yeast two-hybrid and co-immunoprecipitation, αT-catenin was shown to interact specifically with desmosomal PKP-2 implicating a novel molecular linkage between the adherens junction and desmosome. By double αT-catenin/PKP-2 immunolabeling electron microscopy, αT-catenin is observed to colocalize with other molecules of cadherin/catenin complex, β-catenin, and N-cadherin at the fascia adhaerens-like junctions, but also with desmosomal proteins such as PKP-2, DSG-2, and DP. By contrast, no localization of either αE-catenin or β-catenin could be seen at desmosome-like junctions of the ICD. Based on the biochemical and morphological studies, αT-catenin is thought to recruit desmosomal proteins to hybrid adhering junctions, forming a mixed-type, reinforced junction at the ICD that is attached to both the intermediate filaments and actin cytoskeletons (Figure 2). Accordingly, interfering with αT-catenin function may impaire intercellular coupling between αT-catenin, PKP-2, and the cytoskeleton, which may subsequently result in destabilization of the ICD structure, cardiac dysfunction, as well as cardiac arrhythmia [23]. αT-catenin is coexpressed with a closely related family member, αE-catenin, in the heart. Most importantly, αE-catenin lacks the PKP-2 binding domain; therefore mutations in αT-catenin are predicted to have an adverse affect on the organization of the area composita since αE-catenin is not able to interact with PKP-2. Human αT-catenin gene *CTNN3*

has been mapped to chromosome 10q21, a region that links to autosomal dominant familial dilated cardiomyopathy (DCM) [24]. Although genetic screening has not detected any DCM-linked *CTNN3* mutations to date, αT-catenin is considered a candidate gene and may be the potential cause of DCM or ARVC [24].

It is estimated that as many as 70% of the desmosomal mutations linked to familial ARVC are in the gene coding for PKP-2 [25]. Abnormal expression of gap junction protein Cx43 has been observed in heterozygous human PKP-2 mutations [26, 27]. Similar observations have also been made in patients with mutations in plakoglobin (Naxos disease) or desmoplakin (Carvajal syndrome) genes [5, 28–30]. These studies suggest that abnormalities in intercellular adhesion caused by mutations in desmosomal proteins may promote remodeling of gap junctions, which, in turn, alters cardiac conduction and potentially leads to ventricular arrhythmogenic phenotype in this disease [5]. This hypothesis has been supported in a cellular model in which PKP-2 was knockdown by shRNA [31]. Loss of PKP-2 expression in the cells leads to a decrease in total Cx43 content, a significant redistribution of Cx43 to the intercellular space, and a decrease in dye coupling between cells. GST-pulldown assays have shown that PKP-2 and Cx43 coexist in the same macromolecular complex. Recently, siRNA-mediated reduction of PKP-2 has been shown to disintegrate area composita junction structures in cardiac myocytes [32]. These results provide a possibility that PKP-2 is directly involved in the stabilization of Cx43 within the gap junction plaques [31].

The presence of stable mechanical coupling mediated by N-cadherin/catenin adhesion complex is of paramount importance for maintaining the structural integrity of the heart. We demonstrated that cardiac-specific deletion of N-cadherin in mice (N-cad CKO) causes dissolution of the adherens junction, desmosomes, and area composita resulting in absence of ICD structure in the N-cadherin mutant heart [33]. Gap junction protein Cx43 is also markedly decreased from the ICD in the N-cad CKO mice, leading to spontaneous ventricular tachycardia and sudden cardiac death [34]. In contrast, induced deletion of Cx43 in the adult heart did not affect the structure of the ICD with respect to the spatial organization of adherens junction and desmosome [35]. To our knowledge, N-cad CKO is the first animal model with such a dramatic structural phenotype affecting all the junctional complexes in the heart. Our studies demonstrated that the integrity of ICD structure including the area composita is dependent on N-cadherin function in the adult myocardium [33].

In conclusion, the idea that anchoring junctions of the ICD are segregated into distinct domains performing independent functions needs to be reevaluated based on recent data demonstrating novel protein interactions between components from different junction complexes; for example, αT-catenin/PKP-2 and PKP-2/Cx43. The different junctional complexes must be properly localized in the ICD to mediate normal mechanical and electrical coupling between cardiomyocytes. The mixed-type, reinforced junction at the ICD may have evolved to maintain the exceptionally large gap junction plaques found in the mammalian heart, which is under high mechanical stress. Given the large number of PKP-2 mutations identified in ARVC patients, it will be imperative to understand the function of this plakophilin in maintaining electrical synchrony in the heart. Recent studies from human genetics and animal models suggest cross-talk between intercellular junctions constituting the ICD, including adherens junction, desmosome, area composita, and gap junctions. Loss-of-function studies in mice have provided important insight into the hierarchical relationship between the different junction complexes; however subtle mutations in ICD proteins will be necessary to understand the molecular mechanisms underlying ARVC. In the future, it will be interesting to know how the area composita is affected in ARVC patients and its implication in the pathogenesis of the disease.

Abbreviations

ARVC:	Arrhythmogenic right ventricular cardiomyopathy
DCM:	Dilated cardiomyopathy
ICD:	Intercalated disc
DSG:	Desmoglein
DSC:	Desmocollin
PG:	Plakoglobin
PKP:	Plakophilin
DP:	Desmoplakin
Connexin:	Cx.

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References

[1] M. M. Awad, H. Calkins, and D. P. Judge, "Mechanisms of disease: molecular genetics of arrhythmogenic right ventricular dysplasia/cardiomyopathy," *Nature Clinical Practice Cardiovascular Medicine*, vol. 5, no. 5, pp. 258–267, 2008.

[2] C. Basso, G. Thiene, D. Corrado, A. Angelini, A. Nava, and M. Valente, "Arrhythmogenic right ventricular cardiomyopathy: dysplasia, dystrophy, or myocarditis?" *Circulation*, vol. 94, no. 5, pp. 983–991, 1996.

[3] W. J. McKenna, G. Thiene, A. Nava, et al., "Diagnosis of arrhythmogenic right ventricular dysplasia/cardiomyopathy," *British Heart Journal*, vol. 71, no. 3, pp. 215–218, 1994.

[4] G. Thiene, A. Nava, D. Corrado, L. Rossi, and N. Pennelli, "Right ventricular cardiomyopathy and sudden death in young people," *The New England Journal of Medicine*, vol. 318, no. 3, pp. 129–133, 1988.

[5] J. E. Saffitz, "Arrhythmogenic cardiomyopathy and abnormalities of cell-to-cell coupling," *Heart Rhythm*, vol. 6, no. 8, supplement, pp. S62–S65, 2009.

[6] S. Sen-Chowdhry, P. Syrris, and W. J. McKenna, "Genetics of right ventricular cardiomyopathy," *Journal of Cardiovascular Electrophysiology*, vol. 16, no. 8, pp. 927–935, 2005.

[7] K. J. Green and C. A. Gaudry, "Are desmosomes more than tethers for intermediate filaments?" *Nature Reviews Molecular Cell Biology*, vol. 1, no. 3, pp. 208–216, 2000.

[8] M. C. Bolling and M. F. Jonkman, "Skin and heart: une liaison dangereuse," *Experimental Dermatology*, vol. 18, no. 8, pp. 658–668, 2009.

[9] P. Cowin, H.-P. Kapprell, and W. W. Franke, "Plakoglobin: a protein common to different kinds of intercellular adhering junctions," *Cell*, vol. 46, no. 7, pp. 1063–1073, 1986.

[10] G. McKoy, N. Protonotarios, A. Crosby, et al., "Identification of a deletion in plakoglobin in arrhythmogenic right ventricular cardiomyopathy with palmoplantar keratoderma and woolly hair (Naxos disease)," *The Lancet*, vol. 355, no. 9221, pp. 2119–2124, 2000.

[11] A. Asimaki, P. Syrris, T. Wichter, P. Matthias, J. E. Saffitz, and W. J. McKenna, "A novel dominant mutation in plakoglobin causes arrhythmogenic right ventricular cardiomyopathy," *American Journal of Human Genetics*, vol. 81, no. 5, pp. 964–973, 2007.

[12] A. Asimaki, H. Tandri, H. Huang, et al., "A new diagnostic test for arrhythmogenic right ventricular cardiomyopathy," *The New England Journal of Medicine*, vol. 360, no. 11, pp. 1075–1084, 2009.

[13] J. Li, et al., "Loss of plakoglobin leads to remodeling of the intercalated disc and activation of beta-catenin signaling in the heart," in *Proceedings of the American Heart Association Scientific Sessions*, Orlando, Fla, USA, November 2009.

[14] C. Bierkamp, K. J. McLaughlin, H. Schwarz, O. Huber, and R. Kemler, "Embryonic heart and skin defects in mice lacking plakoglobin," *Developmental Biology*, vol. 180, no. 2, pp. 780–785, 1996.

[15] P. Ruiz, V. Brinkmann, B. Ledermann, et al., "Targeted mutation of plakoglobin in mice reveals essential functions of desmosomes in the embryonic heart," *Journal of Cell Biology*, vol. 135, no. 1, pp. 215–225, 1996.

[16] K. S. Grossmann, C. Grund, J. Huelsken, et al., "Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation," *Journal of Cell Biology*, vol. 167, no. 1, pp. 149–160, 2004.

[17] A. Hirschy, F. Schatzmann, E. Ehler, and J.-C. Perriard, "Establishment of cardiac cytoarchitecture in the developing mouse heart," *Developmental Biology*, vol. 289, no. 2, pp. 430–441, 2006.

[18] W. W. Franke, C. M. Borrmann, C. Grund, and S. Pieperhoff, "The area composita of adhering junctions connecting heart muscle cells of vertebrates. I. Molecular definition in intercalated disks of cardiomyocytes by immunoelectron microscopy of desmosomal proteins," *European Journal of Cell Biology*, vol. 85, no. 2, pp. 69–82, 2006.

[19] C. M. Borrmann, C. Grund, C. Kuhn, I. Hofmann, S. Pieperhoff, and W. W. Franke, "The area composita of adhering junctions connecting heart muscle cells of vertebrates. II. Colocalizations of desmosomal and fascia adhaerens molecules in the intercalated disk," *European Journal of Cell Biology*, vol. 85, no. 6, pp. 469–485, 2006.

[20] S. Pieperhoff and W. W. Franke, "The area composita of adhering junctions connecting heart muscle cells of vertebrates. VI. Different precursor structures in non-mammalian species," *European Journal of Cell Biology*, vol. 87, no. 7, pp. 413–430, 2008.

[21] S. Pieperhoff and W. W. Franke, "The area composita of adhering junctions connecting heart muscle cells of vertebrates—IV: coalescence and amalgamation of desmosomal and adhaerens junction components—late processes in mammalian heart development," *European Journal of Cell Biology*, vol. 86, no. 7, pp. 377–391, 2007.

[22] B. Janssens, S. Goossens, K. Staes, et al., "αT-catenin: a novel tissue-specific β-catenin-binding protein mediating strong cell-cell adhesion," *Journal of Cell Science*, vol. 114, no. 17, pp. 3177–3188, 2001.

[23] S. Goossens, B. Janssens, S. Bonné, et al., "A unique and specific interaction between αT-catenin and plakophilin-2 in the area composita, the mixed-type junctional structure of cardiac intercalated discs," *Journal of Cell Science*, vol. 120, no. 12, pp. 2126–2136, 2007.

[24] B. Janssens, B. Mohapatra, M. Vatta, et al., "Assessment of the CTNNA3 gene encoding human αT-catenin regarding its involvement in dilated cardiomyopathy," *Human Genetics*, vol. 112, no. 3, pp. 227–236, 2003.

[25] J. P. van Tintelen, M. M. Entius, Z. A. Bhuiyan, et al., "Plakophilin-2 mutations are the major determinant of familial arrhythmogenic right ventricular dysplasia/ cardiomyopathy," *Circulation*, vol. 113, no. 13, pp. 1650–1658, 2006.

[26] J. L. Manias, I. Plante, X.-Q. Gong, et al., "Fate of connexin43 in cardiac tissue harbouring a disease-linked connexin43 mutant," *Cardiovascular Research*, vol. 80, no. 3, pp. 385–395, 2008.

[27] R. Joshi-Mukherjee, W. Coombs, H. Musa, E. Oxford, S. Taffet, and M. Delmar, "Characterization of the molecular phenotype of two arrhythmogenic right ventricular cardiomyopathy (ARVC)-related plakophilin-2 (PKP2) mutations," *Heart Rhythm*, vol. 5, no. 12, pp. 1715–1723, 2008.

[28] J. E. Saffitz, "Adhesion molecules: why they are important to the electrophysiologist," *Journal of Cardiovascular Electrophysiology*, vol. 17, no. 2, pp. 225–229, 2006.

[29] S. R. Kaplan, J. J. Gard, L. Carvajal-Huerta, J. C. Ruiz-Cabezas, G. Thiene, and J. E. Saffitz, "Structural and molecular pathology of the heart in Carvajal syndrome," *Cardiovascular Pathology*, vol. 13, no. 1, pp. 26–32, 2004.

[30] S. R. Kaplan, J. J. Gard, N. Protonotarios, et al., "Remodeling of myocyte gap junctions in arrhythmogenic right ventricular cardiomyopathy due to a deletion in plakoglobin (Naxos disease)," *Heart Rhythm*, vol. 1, no. 1, pp. 3–11, 2004.

[31] E. M. Oxford, H. Musa, K. Maass, W. Coombs, S. M. Taffet, and M. Delmar, "Connexin43 remodeling caused by inhibition of plakophilin-2 expression in cardiac cells," *Circulation Research*, vol. 101, no. 7, pp. 703–711, 2007.

[32] S. Pieperhoff, H. Schumacher, and W. W. Franke, "The area composita of adhering junctions connecting heart muscle cells of vertebrates. V. The importance of plakophilin-2 demonstrated by small interference RNA-mediated knockdown in cultured rat cardiomyocytes," *European Journal of Cell Biology*, vol. 87, no. 7, pp. 399–411, 2008.

[33] I. Kostetskii, J. Li, Y. Xiong, et al., "Induced deletion of the N-cadherin gene in the heart leads to dissolution of the intercalated disc structure," *Circulation Research*, vol. 96, no. 3, pp. 346–354, 2005.

[34] J. Li, V. V. Patel, I. Kostetskii, et al., "Cardiac-specific loss of N-cadherin leads to alteration in connexins with conduction slowing and arrhythmogenesis," *Circulation Research*, vol. 97, no. 5, pp. 474–481, 2005.

[35] D. E. Gutstein, F.-Y. Liu, M. B. Meyers, A. Choo, and G. I. Fishman, "The organization of adherens junctions and desmosomes at the cardiac intercalated disc is independent of gap junctions," *Journal of Cell Science*, vol. 116, no. 5, pp. 875–885, 2003.

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Research Article

Desmosomal Component Expression in Normal, Dysplastic, and Oral Squamous Cell Carcinoma

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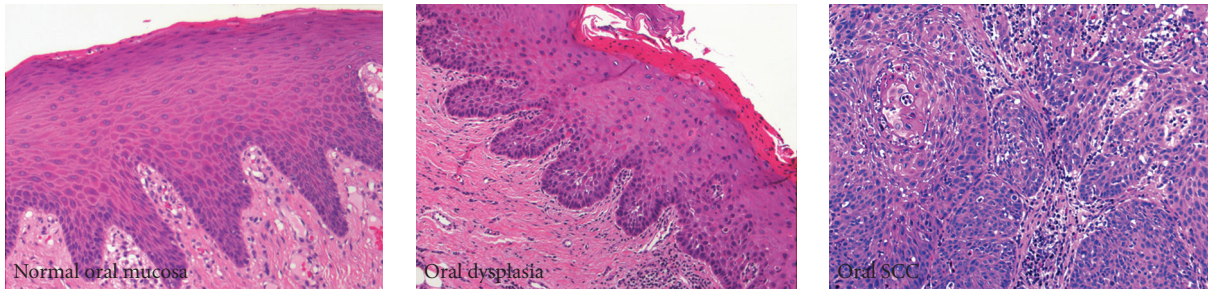
Squamous cell carcinoma (oral SCC) is the most common oral cancer in the U.S., affecting nearly 30,000 Americans each year. Despite recent advances in detection and treatment, there has been little improvement in the five-year survival rate for this devastating disease. Oral cancer may be preceded by premalignant disease that appears histologically as dysplasia. Identification of molecular markers for cellular change would assist in determining the risk of dysplasia progressing to oral squamous cell carcinoma. The goal of this study was to determine if any correlation exists between histological diagnosed dysplasia and OSCC lesions and altered expression of desmosomal cell-cell adhesion molecules in the oral epithelium. Our data showed that oral SCC tissue samples showed decreased immunoreactivity of both desmoplakin and plakophilin-1 proteins compared to normal oral epithelium. Furthermore, significant decrease in desmoplakin immunoreactivity was observed in dysplastic tissue compared to normal oral epithelium. In contrast, the level of desmoglein-1 staining was unchanged between samples however desmoglein-1 was found localized to cell borders in oral SCC samples. These data suggest that changes in expression of desmoplakin and plakophilin-1 may prove to be a useful marker for changes in tissue morphology and provide a tool for identifying pre-neoplastic lesions of the oral cavity.

1. Introduction

Oral cancer affects 3% of the United States population and it is estimated that 35,000 new cases will be diagnosed this year [1]. Despite recent advancements in detection and treatment of oral SCC, survival has only modestly improved in the past 30 years (reviewed in [2]). Changes in tumor cell migration and interactions with the extracellular environment have been demonstrated to promote the progression of many solid tumors. Alterations in adhesive characteristics of cancer cells allow rapidly growing tumor cells to detach from their neighbors, infiltrate the underlying stroma, and disseminate to distant sites in the body establishing a tumor metastasis. Therefore understanding the changes in adhesion molecule expression is important for determining the invasive capacity of cells in a tissue and predicting the likelihood of metastasis. It has been proposed that areas of oral dysplasia may progress to oral SCC over time [3, 4] and therefore can be considered premalignant lesions. However, diagnosing dysplasia using tissue morphology is subjective and depends upon the training and experience of the oral pathologist. Therefore, identification of molecular markers for cellular change would assist in recognition of premalignant lesions and assist in determining the risk of dysplasia progressing to malignancy. Characterization of novel markers would also assist in earlier diagnosis and thereby improve the prognosis of oral cancer.

Desmosomes are the most prominent cell-cell junctional complex in stratified squamous epithelial tissues. Loss of desmosomes in various types of carcinomas is associated with increased migratory capacity of the tumor cells [5–7]. The transmembrane core of the desmosome is comprised of single pass desmosomal cadherins (desmogleins and desmocollins) that are believed to interact heterotypically and homotypically in the extracellular space to mediate



(a) (b) (c)

Figure 1: Morphologic evaluation of normal oral mucosa (a), dysplastic oral mucosa (b), and oral squamous cell carcinoma (c). Representative sections were stained with Hematoxylin and eosin to verify the initial diagnosis of the tissue blocks used in the present study.

cell-cell adhesion [8, 9]. In addition to the desmosomal cadherins, the recently identified tetraspan protein, PERP has also been shown to localize to the desmosome and affect desmosome assembly in keratinocytes [10]. The cytoplasmic domain of the desmosomal cadherins associates directly with several desmosomal plaque proteins, including plakoglobin and plakophilins that in turn recruit the keratin intermediate filament cytoskeleton via interactions with desmoplakin [11]. Assembly of the desmosomal junction allows the keratin intermediate filament cytoskeleton to stretch across cells and provide epithelial tissues a mechanism to withstand mechanical stress. Inactivation of specific desmosomal adhesion complexes by autoimmune sera as seen in pemphigus vulgaris or pemphigus foliaceus results in epidermal blisters [12].

Inherited mutations in desmosomal genes have been identified that result in various skin, hair, and heart defects (reviewed in [13, 14]). Mutations in plakophilin-1 are associated with ectodermal dysplasia and skin fragility syndrome [15] while mutations in desmoglein-1 are associated with striate palmoplantar keratoderma. Mutations in desmoplakin and plakophilin-2, two genes encoding desmosomal components expressed in the heart, have been implicated in the development of arrhythmogenic right ventricular cardiomyopathy (ARVC) [16]. ARVC patients exhibit fibro-fatty replacement of the heart muscle which can result in sudden cardiac death. Often patients harboring desmoplakin mutations can also exhibit defects in hair and skin due to disruption of desmosomes in these epithelial tissues.

Given these findings, we hypothesize that altered expression and/or localization of the desmosomal proteins may result as cells become dysplastic and eventually progress to squamous cell carcinoma. While many studies have described the localization and expression of desmosomal components in skin and skin tumors, relatively little is known regarding desmosomal component expression in tumors arising from the oral mucosa. In this study we examined the expression of two desmosomal plaque proteins, desmoplakin and plakophilin-1. Desmoplakin is found in desmosomes in all the living layers of the epidermis while plakophilin-1 is most highly expressed in differentiated layers. Additionally, we examined the expression of the differentiation specific desmosomal cadherin, desmoglein-1. In this study we hypothesized that changes in differentiation specific components are more likely to exhibit changes in expression between normal and dysplastic samples. These changes are likely to be maintained in oral SCC samples.

2. Materials and Methods

2.1. Tissue Procurement and Immunostaining. Archival tissue sections from The UNMC Oral Pathology service were obtained with approval from the UNMC Institutional Review Board. Eight of normal (oral buccal mucosa) fifteen histologically confirmed dysplasia samples and fifteen oral squamous cell carcinoma samples were used for analysis. All paraffin embedded tissue sections cut into 5 μm sections and collected onto charged Superfrost slides (Electron Microscopy Sciences, Hatfield, PA.). Formalin fixed paraffin embedded sections were dewaxed using xylene and rehydrated through a graded alcohol series and water. Antigen retrieval was achieved by microwave treatment for 5 minutes in freshly prepared 10 mM Sodium citrate (pH 6.0). Tissues were incubated in blocking buffer (1 x phosphate buffered saline, 0.1% Triton x-100, and 1% bovine serum albumin) for 30 minutes prior to incubation with primary antibodies overnight at 4°C. Excess primary antibodies were removed by extensive washing with 1 x phosphate-buffered saline. Tissues were incubated with appropriate FITC-conjugated antimouse secondary antibodies and mounted in vectashield mounting media containing DAPI (Vector Laboratories, Burlingame, CA). Images were collected on a Zeiss axiovert 200 M microscope and axiocam CCD camera using SlideBook software from Intelligent Imaging Innovations (Denver, CO.)

2.2. Antibodies. Mouse monoclonal antidesmoplakin (10F6), plakophilin-1, and desmoglein-1 were generated in our laboratory as previously described [17, 18]. Antidesmoplakin antibody 10F6 recognizes the carboxy terminal domain of human desmoplakin (AA 1960-2151). Generation of antiplakophilin-1 and antidesmoglein-1 monoclonal antibodies has been described previously [18, 19].

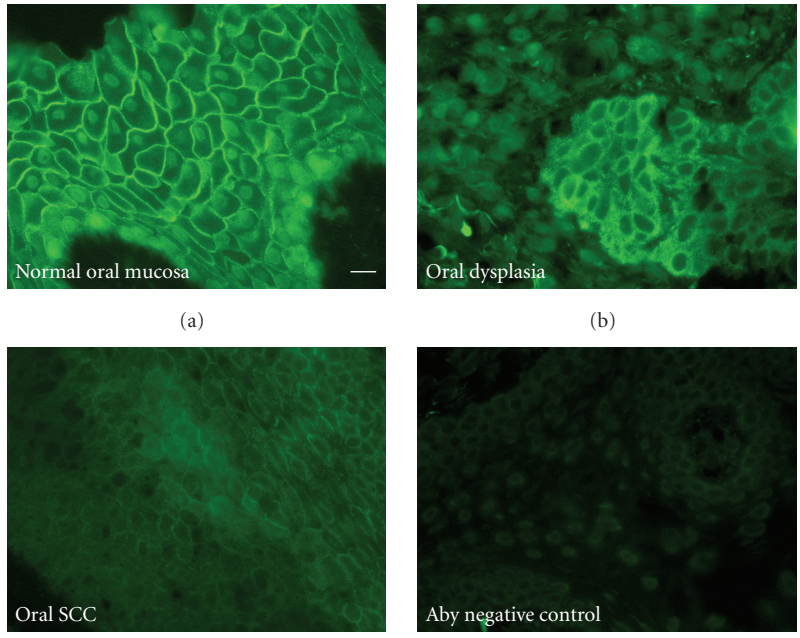


Figure 2: Representative antidesmoplakin staining of oral tissues. Antidesmoplakin monoclonal antibody (10F6) was used to stain normal oral mucosa (a), dysplastic oral mucosa (b), and oral squamous cell carcinoma (c). Normal oral mucosa processed in the absence of primary antibody serves as a negative control (d). The scale bar in panel A corresponds to 20 μm.

2.3. Evaluation of Staining Behavior. Immunostaining was evaluated in a semiquantitative system based on the scoring of at least three independent evaluators. Scoring was based on overall staining throughout the tissue rather that staining at one field of view. As a negative control, normal oral mucosa was processed without the primary antibodies and this signal intensity was determined to be background. A numerical score for each sample was assigned based on the following scale. Intense cell border signal and relatively weak cytoplasmic signal was given a score of "3", moderate cell border and cytoplasmic staining intensity was given a score of "2", overall weak staining intensity was scored a "1", and staining intensity similar to background levels was scored "0". For plakophilin-1 immunostaining, the observers were instructed to judge the nuclear signal together with the cytoplasmic signal to arrive at an overall signal intensity score since the nuclear signal was often heterogeneous throughout a given tissue sample. Statistical analysis of scores was carried out by ANOVA analysis and significant differences were determined by Kruskal-wallis multiple comparisons.

3. Results and Discussion

To begin our analysis we selected a panel of previously diagnosed dysplasia and oral SCC tissue samples available as part of the UNMC oral pathology biopsy service within the college of dentistry. We selected fifteen dysplasia samples and fifteen oral SCC samples to be compared to eight normal oral mucosa samples. Dysplastic tissues were chosen based on the presence of basal cell layer hyperplasia, cellular pleomorphism, increased mitotic figures, and disorganization of stratification within the epithelium compared to normal oral mucosa (Figure 1(b)). For the purposes of this study we chose not to further stratify the dysplasia samples due to the high degree of subjectivity in the diagnosis of these samples. Oral SCC samples corresponded to moderately differentiated squamous cell carcinomas as diagnosed by oral pathologists within the UNMC biopsy service (Figure 1(c)). Normal oral mucosa was obtained from samples exhibiting underlying fibroma with normal appearing surface epithelium (Figure 1(a)). Hematoxylin and eosin staining of the tissue was used to reconfirm the diagnosis and representative sections are shown in Figure 1.

Previous reports have demonstrated that desmosomal component expression is often reduced or absent in oral SCC when compared to normal epithelium [5, 20]. For our analysis we chose to include dysplastic samples to determine if loss of desmosomal adhesion is an early event in the progression to squamous cell carcinoma. We stained a panel of tissues using monoclonal antibodies specific for human desmosomal components (desmoplakin, plakophilin-1, and desmoglein-1) and compared the staining patterns of dysplastic and oral SCC samples to that of normal oral mucosa.

Desmoplakin is an obligatory component of the desmosomal plaque that has been shown to play an essential role in recruiting the keratin intermediate filament cytoskeleton to sites of cell-cell adhesion. As expected, desmoplakin staining in normal oral mucosa displayed an intense staining pattern present at cell-cell borders in all the differentiated layers of

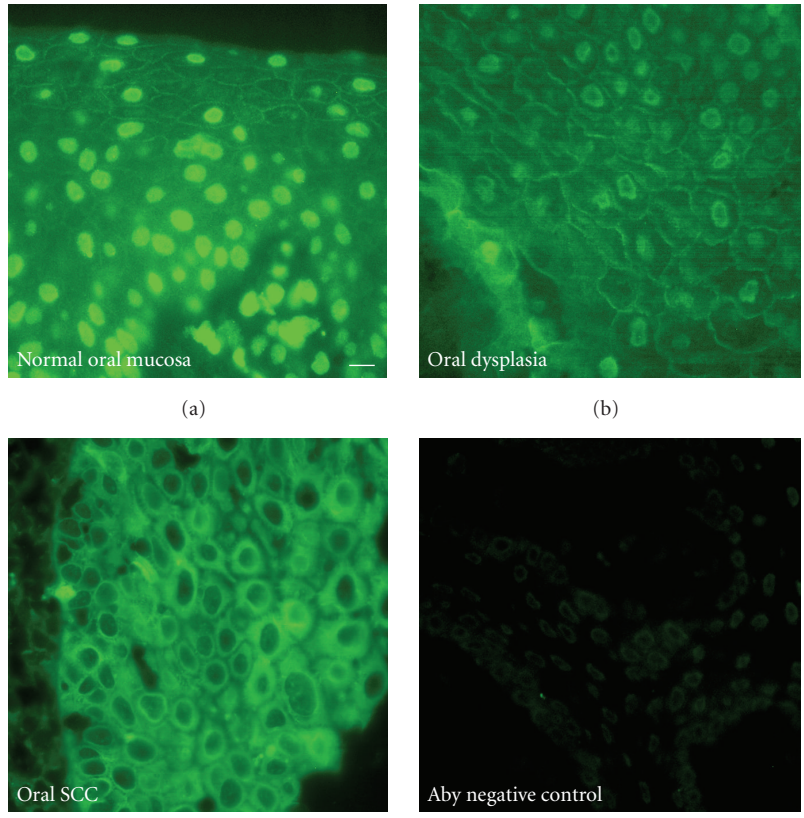


Figure 3: Representative antiplakophilin-1 staining of oral tissues. Antiplakophilin-1 monoclonal antibody (14B11) was used to stain normal oral mucosa (a), dysplastic oral mucosa (b), and oral squamous cell carcinoma (c). Normal oral mucosa processed in the absence of primary antibody serves as a negative control (d). The scale bar in panel A corresponds to 20 μm.

epidermis (Figure 2(a)). Some diffuse cytoplasmic signal was observed; however this signal was minor compared to the cell border staining. Some faint nuclear signal was also present that was determined to be background signal arising from the secondary antibody since this signal was also seen in the negative control samples in which the antibody was omitted (Figure 2(d)).

Desmoplakin immunostaining of oral dysplastic tissues revealed a disruption in the desmosomal localization of desmoplakin resulting in diffuse cytoplasmic localization. In addition, there was a decrease in the overall intensity of the antidesmoplakin signal (Figure 2(b)). Areas of relatively normal desmoplakin localization could be seen but these regions were small and did not extend throughout the dysplastic tissues (data not shown). Staining of oral SCC samples with antidesmoplakin antibodies revealed relatively low protein expression in several samples; however the small amount of desmoplakin that was present could be seen localized at cell borders in a pattern similar to that seen in normal tissues (Figure 2(c)). Most oral SCC samples displayed no antidesmoplakin immunoreactivity and were scored as negative.

Semiquantitative scoring was performed to assess the desmoplakin signal intensity across the panel of tissues. At least three independent observers were trained to recognize normal cell border-associated desmoplakin staining in normal tissues and negative background signal associated with a negative control tissue. Identifiers were removed from the slides and scores were recorded. Desmoplakin staining in normal oral mucosa samples scored highest while staining in dysplastic samples and oral SCC samples scored significantly lower (Figure 5). These data suggest that loss of antidesmoplakin immunoreactiviy is detectable during the transition from normal to dysplasia in the oral cavity.

Plakophilins are a family of armadillo repeat proteins that play an important role in assembly and maintenance of the desmosome [14, 21]. Interestingly, plakophilin-1 has also been identified as a nuclear protein in cultured cells derived from stratified epithelial tissues [22, 23]. Plakophilin-1 has been reported to be highly expressed in the most differentiated layers of stratified squamous epithelium such as skin [7, 24]. Unlike plakophilin-1 localization in the keratinizing epithelium of the skin, plakophilin-1 localized to all the living layers of the epithelium in the oral mucosa (Figure 3(a)). This difference in expression is likely to reflect a difference between keratinized and nonkeratinized tissues.

In addition to the expected cell border localization of plakophilin-1 in normal oral mucosa, we also observed

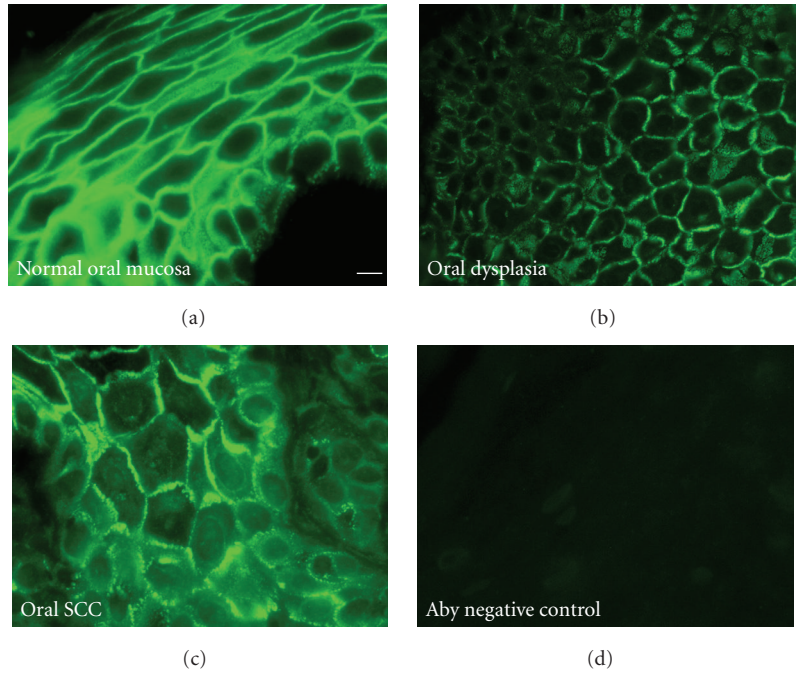


Figure 4: Representative anti-desmoglein-1 staining of oral tissues. Anti-desmoglein-1 monoclonal antibody (27B2) was used to stain normal oral mucosa (a), dysplastic oral mucosa (b), and oral squamous cell carcinoma (c). Normal oral mucosa processed in the absence of primary antibody serves as a negative control (d). The scale bar in panel A corresponds to 10 μm.

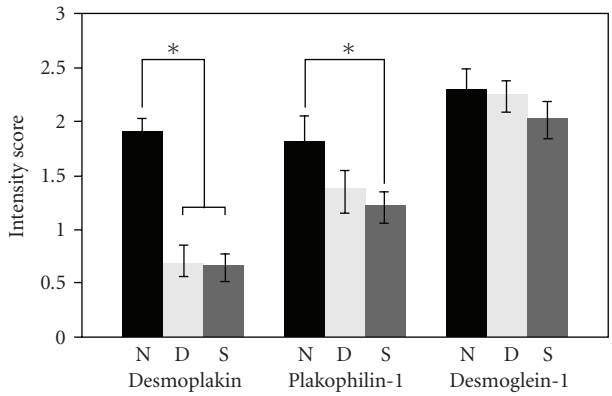


Figure 5: Semiquantitative scoring of desmosomal component expression. Relative intensity of desmoplakin, plakophilin-1, and desmoglein-1 was scored by three observers and the average score is presented (+ or − the standard error) for normal oral mucosa (N), dysplastic epithelium (D), and oral squamous cell carcinoma (S) (* : z value > 2.394 indicate significant difference).

intense nuclear localization of plakophilin-1. The cell border signal seen for plakophilin-1 resembled that of desmoplakin although the intensity was somewhat reduced. The majority of plakophilin-1 signal seen in normal samples was concentrated in the nucleus (Figure 3(a)) and often obscured the signal seen at the cell periphery. Nuclear localization of plakophilin-1 in cultured cells has been previously reported [22, 25, 26]. Nuclear localization of plakophilin-1 in formalin fixed tissues is not homogenous throughout the individual tissues. Some areas of the tissue were observed in which no nuclear plakophilin-1 was observed while strong cell border staining was present. Currently, the nuclear function of plakophilin-1 is unknown. Plakophilin-1 immunostaining of dysplasia samples often revealed a decrease in overall intensity, especially in the nuclear plakophilin-1 pool (Figure 3(b)). Nuclear plakophilin-1 was only rarely and weakly observed in dysplastic and oral SCC samples. Plakophilin-1 localization in oral SCC samples was most often diffusely localized in the cytoplasm with little to no nuclear signal (Figure 3(c)). Complete loss of cell border association of plakophilin-1 was often seen in the oral SCC samples (data not shown).

Scoring of the antiplakophilin-1 signal in these tissues revealed an overall slight decrease in the dysplastic tissues compared to normal oral mucosa although this change was not significant. Comparison of plakophilin-1 staining in oral SCC samples to staining in normal oral mucosa revealed a significant decrease in plakophilin-1 immunoreactivity between these tissue samples (Figure 5). Consistent with previous reports, plakophilin-1 staining is decreased in SCC compared to normal tissue [7, 24, 27]; however plakophilin-1 is not significantly decreased in dysplastic tissues.

Desmoglein-1 is a transmembrane desmosomal cadherin most highly expressed in the differentiated layers of the epidermis. In our oral mucosa samples, this differentiation specific desmosomal cadherin was highly expressed in all the living layers of normal oral mucosa unlike skin where desmoglein 1 expression is restricted to the most differentiated cell layers [28]. Interestingly, we observed robust desmoglein-1 signal in dysplastic tissues and in oral SCC

samples. The staining intensity of the antidesmoglein-1 signal was often slightly reduced; however we consistently observed significant desmoglein-1 protein in all of the samples we observed (Figure 4). Although desmoglein-1 localization in oral SCC was at cell borders, it was possible to also observe some cytoplasmic punctuate signal near cell borders. This altered localization was consistently observed in all the oral SCC samples. Scoring of the antidesmoglein-1 staining in these tissues did not reveal a significant change in anti-desmoglein-1 immunoreactivity between our samples.

Although our sample size is relatively small, we are able to detect clearly significant changes in desmoplakin immunoreactivity between normal and dysplastic oral epithelium suggesting that disruption of desmosomal adhesion may be an early event in the progression to oral SCC. In addition, changes in plakophilin-1 expression appear at a later stage compared to changes seen for desmoplakin immunoreactivity, possibly in response to altered keratin intermediate filament attachment at sites of cell-cell contact.

In our samples, desmoglein-1 immunoreactivity was not significantly altered between normal and oral SCC samples. This finding is in disagreement with a recent study that showed an inverse correlation of desmoglein-1 expression and poor prognosis of head and neck squamous cell carcinoma patients [29]. The relatively small size of our sample pool may explain the differences observed between the two studies. The distribution of desmoglein-1 was often disrupted and was not concentrated at cell borders but was rather diffuse throughout the cells, most likely on the cell surface (Figure 4(c)). Based on the loss of the desmosomal plaque proteins, desmoplakin and plakophilin-1, diffuse localization of desmoglein-1 is not unexpected. Examination of these cell adhesion markers, particularly desmoplakin, in dysplastic tissues may provide a good marker of tissues at increased risk for progression to oral SCC.

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References

[1] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu, and M. J. Thun, "Cancer statistics, 2007," *CA: Cancer Journal for Clinicians*, vol. 57, no. 1, pp. 43–66, 2007.

[2] E. E. W. Cohen, J. Baru, D. Huo, et al., "Efficacy and safety of treating T4 oral cavity tumors with primary chemoradiotherapy," *Head and Neck*, vol. 31, no. 8, pp. 1013–1021, 2009.

[3] D. I. Rosenthal, T. R. Mendoza, M. S. Chambers, et al., "Measuring head and neck cancer symptom burden: the development and validation of the M. D. Anderson symptom inventory, head and neck module," *Head and Neck*, vol. 29, no. 10, pp. 923–931, 2007.

[4] S. Warnakulasuriya, J. Reibel, J. Bouquot, and E. Dabelsteen, "Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses and scope for improvement," *Journal of Oral Pathology and Medicine*, vol. 37, no. 3, pp. 127–133, 2008.

[5] A. Hiraki, M. Shinohara, T. Ikebe, S. Nakamura, S. Kurahara, and D. R. Garrod, "Immunohistochemical staining of desmosomal components in oral squamous cell carcinomas and its association with tumour behaviour," *British Journal of Cancer*, vol. 73, no. 12, pp. 1491–1497, 1996.

[6] A. L. Krunic, D. R. Garrod, S. Madani, M. D. Buchanan, and R. E. Clark, "Immunohistochemical staining for desmogleins 1 and 2 in keratinocytic neoplasms with squamous phenotype: actinic keratosis, keratoacanthoma and squamous cell carcinoma of the skin," *British Journal of Cancer*, vol. 77, no. 8, pp. 1275–1279, 1998.

[7] T. Sobolik-Delmaire, D. Katafiasz, S. A. Keim, M. G. Mahoney, and J. K. Wahl III, "Decreased plakophilin-1 expression promotes increased motility in head and neck squamous cell carcinoma cells," *Cell Communication and Adhesion*, vol. 14, no. 2-3, pp. 99–109, 2007.

[8] N. A. Chitaev and S. M. Troyanovsky, "Direct Ca^{2+}-dependent heterophilic interaction between desmosomal cadherins, desmoglein and desmocollin, contributes to cell-cell adhesion," *Journal of Cell Biology*, vol. 138, no. 1, pp. 193–201, 1997.

[9] S.-E.-H. Syed, B. Trinnaman, S. Martin, S. Major, J. Hutchinson, and A. I. Magee, "Molecular interactions between desmosomal cadherins," *Biochemical Journal*, vol. 362, no. 2, pp. 317–327, 2002.

[10] R. A. Ihrie, M. R. Marques, B. T. Nguyen, et al., "Perp is a p63-regulated gene essential for epithelial integrity," *Cell*, vol. 120, no. 6, pp. 843–856, 2005.

[11] B. Holthöfer, R. Windoffer, S. Troyanovsky, and R. E. Leube, "Structure and function of desmosomes," *International Review of Cytology*, vol. 264, pp. 65–163, 2007.

[12] R. W. Groves, "Pemphigus: a brief review," *Clinical Medicine*, vol. 9, no. 4, pp. 371–375, 2009.

[13] A. E. Bass-Zubek, L. M. Godsel, M. Delmar, and K. J. Green, "Plakophilins: multifunctional scaffolds for adhesion and signaling," *Current Opinion in Cell Biology*, vol. 21, no. 5, pp. 708–716, 2009.

[14] J. E. Lai-Cheong, K. Arita, and J. A. McGrath, "Genetic diseases of junctions," *Journal of Investigative Dermatology*, vol. 127, no. 12, pp. 2713–2725, 2007.

[15] J. A. McGrath, J. R. McMillan, C. S. Shemanko, et al., "Mutations in the plakophilin 1 gene result in ectodermal dysplasia/skin fragility syndrome," *Nature Genetics*, vol. 17, no. 2, pp. 240–244, 1997.

[16] T. Herren, P. A. Gerber, and F. Duru, "Arrhythmogenic right ventricular cardiomyopathy/dysplasia: a not so rare "disease of the desmosome" with multiple clinical presentations," *Clinical Research in Cardiology*, vol. 98, no. 3, pp. 141–158, 2009.

[17] K. R. Johnson, J. E. Lewis, D. Li, et al., "P- and E-cadherin are in separate complexes in cells expressing both cadherins," *Experimental Cell Research*, vol. 207, no. 2, pp. 252–260, 1993.

[18] J. K. Wahl III, "Generation of monoclonal antibodies specific for desmoglein family members," *Hybridoma and Hybridomics*, vol. 21, no. 1, pp. 37–44, 2002.

[19] J. K. Wahl III, "A role for plakophilin-1 in the initiation of desmosome assembly," *Journal of Cellular Biochemistry*, vol. 96, no. 2, pp. 390–403, 2005.

[20] J. Depondt, A.-H. Shabana, S. Florescu-Zorila, P. Gehanno, and N. Forest, "Down-regulation of desmosomal molecules in oral and pharyngeal squamous cell carcinomas as a marker for

tumour growth and distant metastasis," *European Journal of Oral Sciences*, vol. 107, no. 3, pp. 183–193, 1999.

[21] M. Hatzfeld, "Plakophilins: multifunctional proteins or just regulators of desmosomal adhesion?" *Biochimica et Biophysica Acta*, vol. 1773, no. 1, pp. 69–77, 2007.

[22] M. Hatzfeld, G. I. Kristjansson, U. Plessmann, and K. Weber, "Band 6 protein, a major constituent of desmosomes from stratified epithelia, is a novel member of the armadillo multigene family," *Journal of Cell Science*, vol. 107, no. 8, pp. 2259–2270, 1994.

[23] H. W. Heid, A. Schmidt, R. Zimbelmann, et al., "Cell type-specific desmosomal plaque proteins of the plakoglobin family: plakophilin 1 (band 6 protein)," *Differentiation*, vol. 58, no. 2, pp. 113–131, 1994.

[24] I. Moll, H. Kurzen, L. Langbein, and W. W. Franke, "The distribution of the desmosomal protein, plakophilin 1, in human skin and skin tumors," *Journal of Investigative Dermatology*, vol. 108, no. 2, pp. 139–146, 1997.

[25] M. Hatzfeld, "The armadillo family of structural proteins," *International Review of Cytology*, vol. 186, pp. 179–224, 1999.

[26] A. Schmidt, L. Langbein, M. Rode, S. Pratzel, R. Zimbelmann, and W. W. Franke, "Plakophilins 1a and 1b: widespread nuclear proteins recruited in specific epithelial cells as desmosomal plaque components," *Cell and Tissue Research*, vol. 290, no. 3, pp. 481–499, 1997.

[27] H. Kurzen, I. Münzing, and W. Hartschuh, "Expression of desmosomal proteins in squamous cell carcinomas of the skin," *Journal of Cutaneous Pathology*, vol. 30, no. 10, pp. 621–630, 2003.

[28] J. Arnemann, K. H. Sullivan, A. I. Magee, I. A. King, and R. S. Buxton, "Stratification-related expression of isoforms of the desmosomal cadherins in human epidermis," *Journal of Cell Science*, vol. 104, no. 3, pp. 741–750, 1993.

[29] M. P. Wong, M. Cheang, E. Yorida, et al., "Loss of desmoglein 1 expression associated with worse prognosis in head and neck squamous cell carcinoma patients," *Pathology*, vol. 40, no. 6, pp. 611–616, 2008.

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Research Article

Loss of the Desmosomal Component Perp Impairs Wound Healing *In Vivo*

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Epithelial wound closure is a complex biological process that relies on the concerted action of activated keratinocytes and dermal fibroblasts to resurface and close the exposed wound. Modulation of cell-cell adhesion junctions is thought to facilitate cellular proliferation and migration of keratinocytes across the wound. In particular, desmosomes, adhesion complexes critical for maintaining epithelial integrity, are downregulated at the wound edge. It is unclear, however, how compromised desmosomal adhesion would affect wound reepithelialization, given the need for a delicate balance between downmodulating adhesive strength to permit changes in cellular morphology and maintaining adhesion to allow coordinated migration of keratinocyte sheets. Here, we explore the contribution of desmosomal adhesion to wound healing using mice deficient for the desmosomal component Perp. We find that *Perp* conditional knockout mice display delayed wound healing relative to controls. Furthermore, we determine that while loss of Perp compromises cell-cell adhesion, it does not impair keratinocyte proliferation and actually enhances keratinocyte migration in *in vitro* assays. Thus, Perp's role in promoting cell adhesion is essential for wound closure. Together, these studies suggest a role for desmosomal adhesion in efficient wound healing.

1. Introduction

A fundamental aspect of the skin's ability to maintain barrier function is its rapid response to wounding. Critical components of adult wound healing include the contraction of the dermal tissue beneath the wound, which helps draw the wound edges in proximity, and reepithelialization, in which keratinocytes become activated to proliferate and migrate as an invading sheet to resurface the wound [1–3]. Keratinocytes display a number of distinct characteristics during wound healing, including increased size, elongated polarized morphology, compromised cell-cell adhesion, and retracted keratin filaments [2, 4, 5]. In addition, they undergo enhanced proliferation several cell diameters away from the wound edge [1, 3, 6]. These changes are thought to reflect reprogramming of epidermal keratinocytes to ones dedicated to wound healing.

The dynamic regulation of cell-cell adhesion junctions, including desmosomes, is thought to be an important facet of wound healing. Desmosomal adhesion junctions are initially destabilized at the wound front, presumably to facilitate proliferation and migration, and are reassembled later during the sealing of the epithelium [7, 8]. Desmosomes are multiprotein cell-cell adhesion complexes essential for maintaining the structural integrity of tissues through connection to the intermediate filament network [9–12]. Transmembrane desmosomal cadherins, known as Desmogleins and Desmocollins, mediate contact between apposing cells, and the cytoplasmic tails of these cadherins interact with Plakoglobin and Plakophilins, which connect to the intermediate filament cytoskeleton via Desmoplakin. Recently, the Perp tetraspan membrane protein was identified as an additional member of the desmosome [13]. Perp's critical role in desmosomal adhesion was evidenced by

the presence of dramatic blisters observed in the epidermis and oral mucosa of mice lacking Perp as well as by the ultrastructural abnormalities observed in desmosomes of these mice.

The role of desmosomal adhesion junctions in wound reepithelialization has been queried through *in vitro* studies to examine the consequence of desmosome-deficiency on individual cellular functions critical for wound healing, such as proliferation, adhesion, and migration. Because of the complex nature of wound healing, however, it is unclear what effect desmosome dysfunction would have on proper wound repair *in vivo*. Loss of desmosomal components might result in enhanced keratinocyte proliferation and migration, which could facilitate wound closure. Indeed, desmosomal component loss enhances proliferation *in vivo* [13–15] and increases cell migration *in vitro* [16–19]. Furthermore, signaling cascades that drive cell proliferation and migration in response to wounding, such as EGFR signaling, induce desmosome dissolution, and therefore desmosome loss might promote wound healing by accelerating the effects of the factors activating these pathways [17, 20, 21]. Alternatively, desmosome loss might be expected to hinder wound healing, due to the lack of cell-cell junctions to enable efficient coordinated migration of epithelial sheets or the inability to seal the wound. However, the consequences of desmosomal protein deficiency in wound healing have not been addressed using an *in vivo* genetic model, in part due to the lethal phenotypes observed in many knockout mouse strains lacking individual desmosome components [22]. We circumvent this problem by using *Perp* conditional knockout mice that we generated to assess the role of desmosomes in epidermal wound healing *in vivo*. Our studies here reveal an important role for Perp in efficient wound closure.

2. Materials and Methods

All animal studies were approved by the Stanford University Administrative Panel on Laboratory Animal Care.

2.1. Wounding Study in Mice. Keratin 14CreER^{T2} mice were bred to *Perp*$^{fl/fl}$ conditional knockout mice and kept on a 129/Sv; C57BL/6 mixed background [23]. At 6 weeks of age, 0.1 mg of tamoxifen diluted in corn oil was administered to mice for 5 consecutive days via intraperitoneal injection. 4 weeks later mice were anesthetized with avertin (2,2,2-Tribromoethanol, Sigma Chemical Corp. St. Louis, MO), shaved, and subjected to 6 mm punch biopsies using a Dermal Biopsy Punch (Miltex, York, PA). The area of the wounds was measured on a daily basis for 10 days. Paraffin sections from adult skin were processed by standard methods.

2.2. Immunofluorescence Analysis. Samples were deparaffinized, rehydrated, and unmasked using Trilogy (Cell Marque, Rocklin, CA) in a pressure cooker for 15 minutes according to manufacturer's instructions. Samples were then rinsed in PBS, blocked in PBS containing 5% normal goat serum (Sigma Chemical Corp.), 2.5% BSA (Sigma Chemical Corp.), and 0.01% Triton X-100 (Fisher Scientific, Pittsburgh, PA). Sections were incubated in primary antibody overnight at 4°C, rinsed in PBS with Tween-20 (0.01%), incubated with secondary antibody and DAPI for 1 hr at 37°C, washed in PBS, and then mounted in Mowiol (Calbiochem, San Diego, CA). Antibodies used in this study were directed against Perp (1 : 150; [13]), Dsg 1 (18D4; 1 : 100 Santa Cruz Biotechnology, Santa Cruz, CA), Desmoplakin (11-5F; 1 : 50; gift of David Garrod, University of Manchester, Manchester, UK) Dsg1/3 (32-2D 1 : 50; gift of David Garrod, University of Manchester, Manchester, UK), Loricrin (1 : 500; Covance, Princeton, NJ), PCNA (1 : 100; Santa Cruz Biotechnology), and Keratin 1 & 14 (1 : 500; Covance). Secondary antibodies were FITC or AlexaFluor594 conjugated donkey anti-rabbit or anti-mouse IgG (1 : 400; Jackson Immunoresearch, West Grove, PA, and Invitrogen, Carlsbad, CA, resp.). Fluorescence images were examined using a Leica DM6000B microscope (Leica Microsystems, Bannockburn, IL), and images were acquired using a Retiga Exi Camera (Q imaging, Surrey, British Columbia, Canada) and Image Pro 6.2 software from Media Cybernetics (Silver Spring, MD).

2.2.1. Explant Assay. Skin explants from wild-type and *Perp*$^{-/-}$ newborns were grown as described [24]. A minimum of 8 biopsies per mouse per experiment was examined.

2.2.2. Transwell Migration Assay. 24 well transwell plates (Costar, Corning, NY) were coated for 1 hour with a collagen-fibronectin solution. Primary mouse keratinocytes were isolated as described [13]. 50 000 cells for each genotype were plated into each well in triplicate, and 24 hours later cells that had migrated through the transwell were fixed in 4% paraformaldehyde for 15', rinsed in PBS, and stained with 0.01% crystal violet. The number of migrated cells was counted in three separate 200× fields. The average number of cells migrated per well was averaged over three independent experiments.

2.2.3. Adhesion Assay. Mechanical dissociation assays were performed as described [25] on mouse keratinocytes grown to confluence in 0.05 mM calcium media, then switched to 0.2 mM calcium for 24 hours. Cell fragments were counted in four different 100× fields, in triplicate, using a Leica M × 6 dissecting microscope (Leica Microsystems).

3. Results

3.1. Perp-Deficiency Delays Wound Healing In vivo. To define the role of Perp and desmosomes in wound healing, we analyzed wound reepithelialization *in vivo* using Perp-deficient mice. We generated cohorts of 6-week-old control and conditional *Perp* knockout mice (*Perp*$^{fl/fl}$; *fl*=floxed) expressing a *K14CreER* transgene, which allows deletion of the *Perp* locus in the epidermis upon introduction of tamoxifen. Immunohistochemistry confirmed that Perp expression was successfully abolished in the majority of these mice 4 weeks after tamoxifen injection (Figure 1(a)). Specifically,

~70% of these mice exhibited highly efficient Perp ablation (>90%) throughout the epidermis, while the other ~30% displayed somewhat less efficient Perp loss in the epidermis (>50%). Analysis of the skin from these mice revealed specific alterations in the epidermis of *K14CreER; Perp$^{fl/fl}$* mice compared to controls (Figures 1(b)–1(e)). For example, we noted an increase in the percentage of proliferating basal cells in the *K14CreER; Perp$^{fl/fl}$* mice compared to controls (Figures 1(c), 1(d)), similar to that observed in constitutive *Perp* knockout mice. Furthermore, this enhanced proliferation led to the expansion of differentiation marker staining in the skin (Figure 1(e)), although there were no apparent aberrations in the differentiation patterns of the skin in the *K14CreER; Perp$^{fl/fl}$* mice compared to controls. Occasional blisters were also observed in *K14CreER; Perp$^{fl/fl}$* mice, and accordingly, we found that desmosomes were functionally compromised in the skin of *K14CreER; Perp$^{fl/fl}$* mice using a solubility assay (Figure 1(f)). This assay relies on the fact that stably formed desmosomal complexes can only be solubilized by chaotropic agents, whereas improperly assembled desmosomal components can be solubilized by the nonionic detergent Triton X-100. We found that Desmoglein 1 and Plakoglobin display enhanced Triton X-100 solubility in skin from *K14CreER; Perp$^{fl/fl}$* mice compared to skin from control mice, confirming that acute deletion of Perp leads to impaired desmosome formation similar to that observed in constitutive *Perp$^{-/-}$* mice [13].

To assay wound reepithelialization, we subjected mice in the Perp-deficient and control cohorts to full skin thickness punch biopsies. The experimental cohort consisted of tamoxifen-treated *K14CreER; Perp$^{fl/fl}$* mice, while the control cohorts comprised tamoxifen-treated *K14CreER; Perp$^{+/+}$*, *Perp$^{fl/+}$*, and *Perp$^{fl/fl}$* mice as well as untreated *Perp$^{fl/fl}$*, *Perp$^{fl/+}$*, *K14CreER; Perp$^{fl/fl}$*, and *K14CreER; Perp$^{fl/+}$* mice. Four 6 mm punches were performed on each mouse, and the sizes of the wounds were measured daily for approximately 10 days to determine the rate of wound closure. We found that mice lacking Perp in the epithelial compartment exhibited a delay in wound healing relative to controls, underscoring the importance of Perp in the wound healing process (Figures 2(a), and 2(b)).

To further examine the wound healing process in the cohorts, histological analysis of transverse sections of wound sites from both control and *K14CreER; Perp$^{fl/fl}$* mice was performed 1, 5, 7, and 10 days post wounding (Figure 3, data not shown). These analyses revealed that while reepithelialization typically neared completion in the control mice by day 5, the epithelial cells had not fully migrated across wounds in the *K14CreER; Perp$^{fl/fl}$* mice (Figure 3(c)). In addition, by day 7, many of the wounds in control mice were not only fully reepithelialized but had reestablished normal epidermal architecture (data not shown). In contrast, in the *K14CreER; Perp$^{fl/fl}$* mice, keratinocytes had migrated across the wound but had not yet formed a full thickness skin layer by day 7 (data not shown). By day 10, both cohorts of mice exhibited fully reepithelialized wounds (Figure 3(d)). These data reinforce our observations at the macroscopic level that Perp loss delays wound healing after cutaneous injury.

3.2. Dynamic Changes in Desmosomal Protein Expression during Wound Healing in Wild-Type and Perp-Deficient Mice. The desmosomal component Desmoplakin has been reported to exhibit decreased expression in migrating keratinocytes at the leading edge of the wound both in keratinocyte scratch wound assays and in wounded epidermis [8]. However, adhesion remains intact between more distant cells of the epithelium, presumably to allow coordinated migration of the tissue. Examining changes in desmosomal protein localization during the stages of wound healing and determining how these patterns are affected in the absence of Perp may provide a clue to how Perp and potentially the desmosome are involved in wound closure. We examined the expression pattern of Perp and the desmosomal components Desmoglein 3/1 (Dsg3/1) and Desmoplakin (Dp) at different times after wounding *in vivo*, to determine their expression at the leading edge and in regions more internal to the wound. Analysis of samples from control mice 1 day post wounding revealed a decrease in membrane staining of Perp and Dsg3/1 near the leading edge of the epithelial tongue compared to uninjured epidermis (Figures 4(b), 4(c), 4(f) and 4(g)). By day 5, expression of Perp and Dsg3/1 became uniform throughout the migrating epithelium and by day 10, expression of all desmosomal components was restored to levels similar to those observed in uninjured epidermis (data not shown, and Figures 4(d) and 4(h)). Expression pattern changes for Dp were similar to those of other components (data not shown). These data support the notion that desmosome proteins are downregulated near the leading edge during the initial stages of the wound healing process but then are quickly restored, potentially to provide adhesive strength to the migrating epithelium. Similar patterns were observed in the wounds from *K14CreER; Perp$^{fl/fl}$* mice, suggesting that gross disruptions in desmosomal component targeting to the plasma membrane do not provide an explanation for the delayed wound closure observed in the Perp-deficient mice (Figures 4(i)–4(p)). We did note a few isolated migrating cells expressing Perp during the wound healing experiment, likely reflecting incomplete Perp deletion in stem cells of the interfollicular epidermis or the bulge. However, the number of cells expressing Perp was minimal and therefore unlikely to significantly affect our results. Importantly, any incomplete deletion we observed is likely to result in an underestimation of the extent of our phenotype.

3.3. Loss of Perp Does Not Enhance Proliferation or Apoptosis during Wound Reepithelialization. Defective wound closure could reflect alterations in keratinocyte proliferation. Specifically, enhanced cellular proliferation several cell diameters away from the edge is associated with wound reepithelialization [3]. We therefore examined whether the delayed wound healing observed in the absence of Perp was attributable to decreased cellular proliferation at and near the wound margin by staining for Ki67, a proliferation marker. Uninjured regions of control skin exhibit Ki67-positive cells scattered throughout the actively dividing basal layer (Figure 5(a)). One day post wounding, Ki67-positive cells were found in the area proximal to the wound

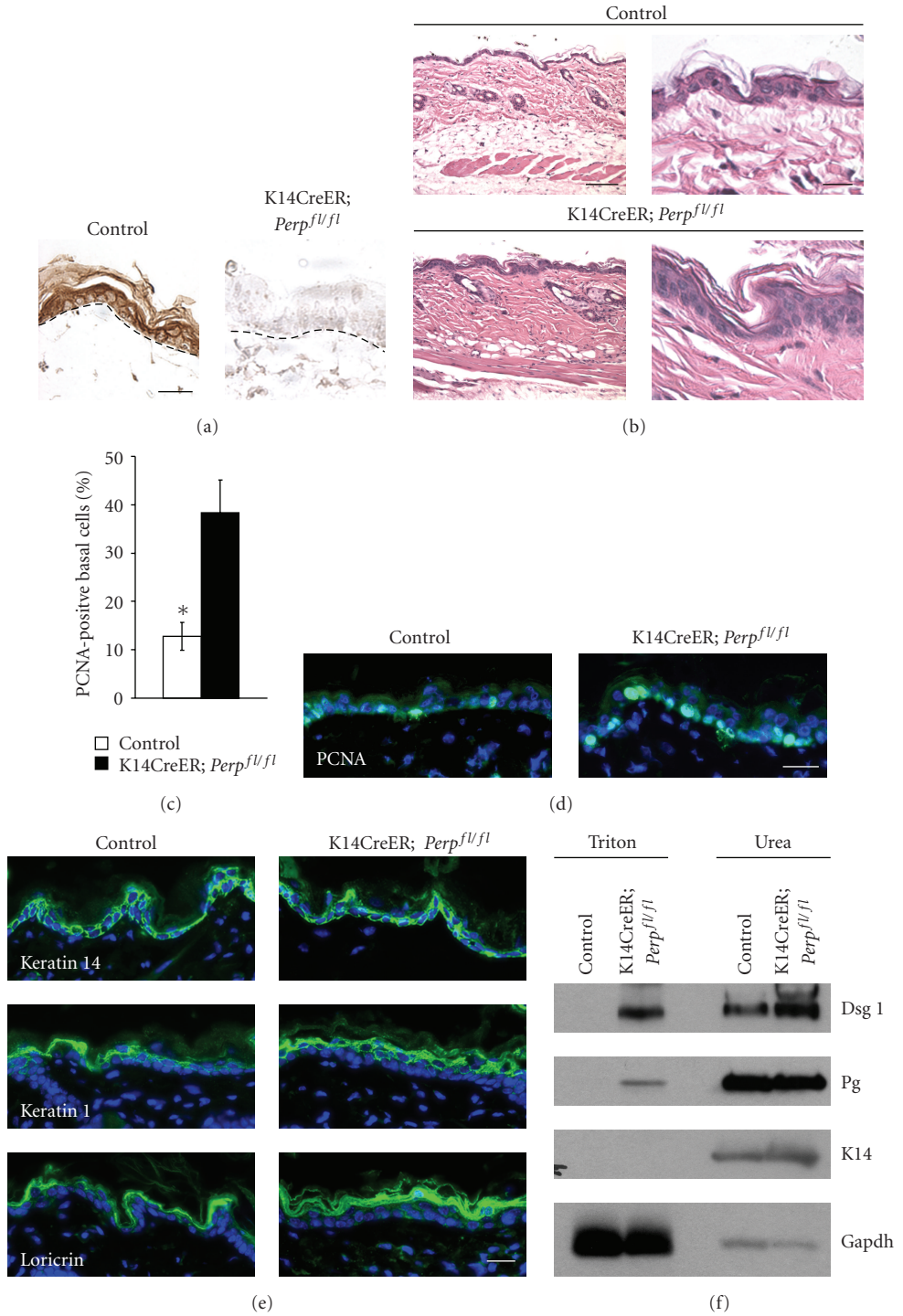

Figure 1: Acute deletion of Perp leads to desmosome defects. (a) Perp immunohistochemistry on skin samples from control (*Perp*$^{fl/fl}$) and *K14CreER; Perp*$^{fl/fl}$ mice 4 weeks post tamoxifen injection. (b) H&E analysis of control (*Perp*$^{fl/fl}$) and *K14CreER; Perp*$^{fl/fl}$ mice 4 weeks post tamoxifen injection, 200× and 400× magnification. (c) Graph displays the average percentage of PCNA positive cells in the basal cell layer in both control (*Perp*$^{fl/fl}$) and *K14CreER; Perp*$^{fl/fl}$ mice 4 weeks post tamoxifen injection. Graph represents the average of 3 separate fields from each of 4 mice +/− SEM. Statistical significance was determined using the Mann-Whitney test * = $P < .03$. (d) Representative image of the proliferation in the basal layer of the epidermis measured by PCNA immunostaining in control (*Perp*$^{fl/fl}$) and *K14CreER; Perp*$^{fl/fl}$ mice 4 weeks post tamoxifen injection. (e) Immunofluorescence analysis of differentiation markers on control (*Perp*$^{fl/fl}$) and *K14CreER; Perp*$^{fl/fl}$ mice 4 weeks post tamoxifen injection. Keratin 14, Keratin 1, and Loricrin mark the basal, the spinous, and the granular layers, respectively. DAPI is used to mark nuclei. (f) Solubility/western blot analysis of Dsg1 and Pg in *K14CreER; Perp*$^{fl/fl}$ or control (*K14CreER; Perp*$^{+/+}$) mouse skin. Both Triton X-100-soluble and Urea fractions are presented. Gapdh and Keratin 14 serve as loading controls for the Triton X-100 and urea fractions, respectively. Scale bar for panels (a, b) (right column), (d), and (e) equals 20 μm. Scale bar for panel (b) (left column) equals 100 μm.

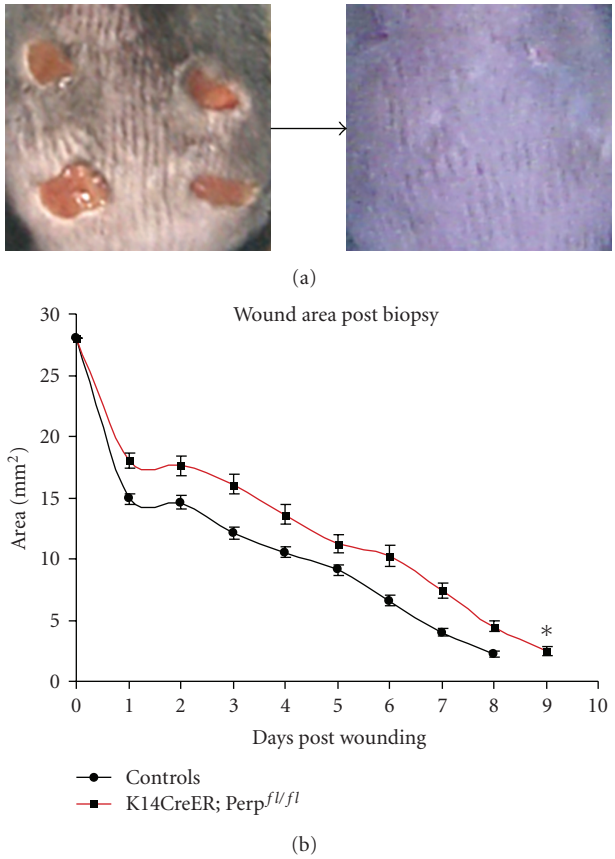

Figure 2: Loss of Perp delays wound healing *in vivo*. (a) Representative photos of dorsal skin bearing open wounds at the beginning of the study and fully closed wounds at the end of the study. (b) Graph displays the average area of all wounds as a function of time post-biopsy. Combined controls: $n = 64$ wounds amongst 16 mice. *K14CreER; Perp*$^{fl/fl}$ mice: $n = 32$ wounds amongst 8 mice. Error bars represent the SEM. Statistical significance was determined using ANOVA * = $P < .03$.

edge (Figure 5(b)). However, at day 5, the epithelial sheet migrating across the wound expressed few Ki67-positive cells (Figure 5(c)). By day 10, Ki67-positive cells were observed throughout the basal layer of the healed epithelium, as in uninjured epidermis (Figure 5(d)). Upon analysis of Perp-deficient mice, we found no clear decrease in the levels of Ki67-positivity post wounding relative to controls (Figures 5(e)–5(h)). We also performed cleaved caspase 3 staining at days 1, 3, 5, and 10 post wounding to determine if there was any depletion of cells in the absence of Perp that could contribute to delayed wound healing, but we found no significant apoptosis in mice of either genotype (data not shown). Thus, altered levels of proliferation or apoptosis do not appear to account for delayed wound healing in Perp-deficient mice.

3.4. Perp Loss Disrupts Cell-Cell Adhesion and Enhances Keratinocyte Migration. As defects in cell migration provide another potential mechanism for compromised wound healing, we next assessed effects of Perp loss on keratinocyte motility. First we confirmed that Perp loss leads to a demonstrable defect in cell-cell adhesion *in vitro*, where we planned to model migration, using a mechanical dissociation assay. This assay entails inducing desmosome formation in sheets of keratinocytes, detaching the epithelial sheet from the tissue culture dish using dispase, and incubating these sheets while rocking [25]. The number of fragments released provides a quantitative measurement of intercellular adhesive strength and the integrity of the epithelium. We found that Perp-deficient keratinocytes are indeed less adhesive than wild-type keratinocytes, as reflected by an increase in fragments seen after mechanical dissociation (Figure 6(a)).

We next used two different approaches to examine the role of Perp in regulating keratinocyte migration. We first examined cell migration using a transwell filter assay utilized previously to demonstrate that Plakoglobin-deficient cells display enhanced motility [26]. Wild-type and *Perp*$^{-/-}$ keratinocytes were grown in the upper chamber of transwell motility plates, and after 48 hours, their migration capacity was assessed by measuring the number of cells traveling through this filter to a lower chamber coated with collagen IV and fibronectin. Using this approach, we found that Perp loss does not compromise cell migration but instead augments it (Figures 6(b) and 6(c)). We also assessed migratory capacity in the context of an intact epithelial sheet using an *ex vivo* explant culture assay in which punch biopsies from mouse skin are cultured *in vitro* and the outgrowth of cells from the explant is measured, as a model for the behavior of cells at the edge of a wound [24]. We cultured punch biopsies of newborn skin from wild-type or constitutive *Perp*$^{-/-}$ mice and monitored the outward migration of keratinocytes from the "wounded" edge of the biopsy over time. Consistent with the transwell assays, these experiments demonstrated that Perp-deficiency resulted in an enhanced area of outgrowth relative to wild-type mice, suggesting that cell migration is not defective, but instead is enhanced, in the absence of Perp (Figures 6(d) and 6(e)). Thus, a defect in migration *per se* does not account for the delayed wound healing observed in the Perp-deficient epidermis. Intriguingly, however, there appeared to be a qualitative difference in the way in which keratinocytes migrated in the absence of Perp. Specifically, despite migrating further, the leading edge of the migrating sheets from the *Perp*$^{-/-}$ explants displayed a disorganized border, with cells detaching from one another and single cells transiting away. This phenotype is in contrast to that observed in the wild-type explants, in which keratinocytes appeared completely cohesive and moved as a coordinated epithelial sheet (Figure 6(f)). This disorganization, which is presumably a direct consequence of the impaired cell-cell adhesion seen in *Perp* null cells, could potentially contribute to the impaired wound healing *in vivo*, as coordinated migration is thought to be necessary for efficient wound reepithelialization.

4. Discussion

The regeneration of a functional epithelial barrier after wound healing is essential for preventing dehydration and infection [27]. Epithelial wound healing is a complex

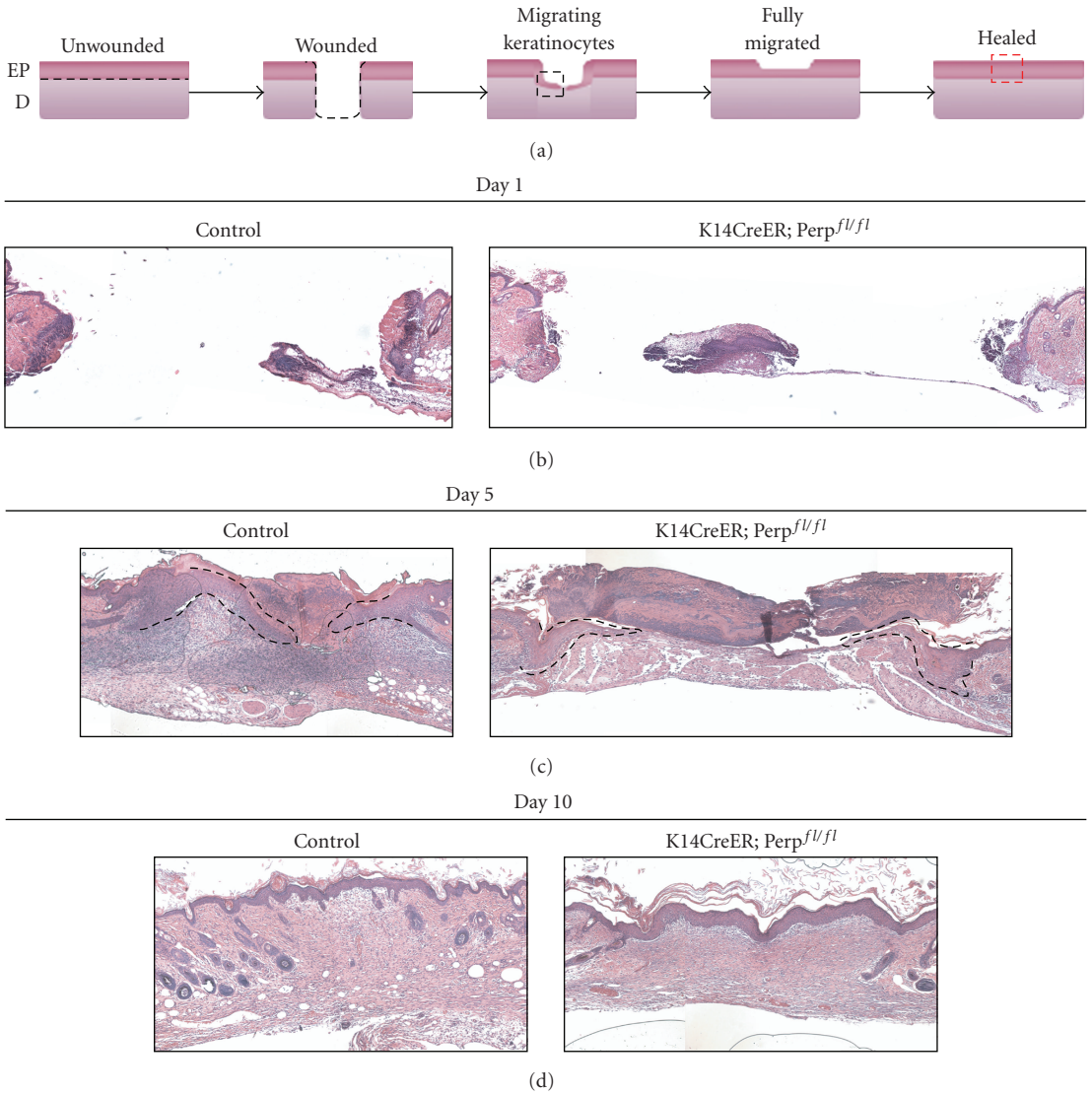

Figure 3: Wounded *K14CreER; Perp*$^{fl/fl}$ mice exhibit delays in reepithelialization. (a) Cartoon sketch depicting the different stages occurring during the wound healing process. The black dashed line marks the boundary between the epidermis and dermis. Black dashed box demarcates region with migrating keratinocytes, as shown in panel (c). Red dashed box represents region of fully closed wound, as shown in panel (d). EP signifies epidermis and D, dermis. (b) Representative H&E-stained section of open wounds from tamoxifen-injected control (*Perp*$^{fl/fl}$) and *K14CreER; Perp*$^{fl/fl}$ mice 1 day post wounding. (c) Representative H&E-stained section of wounds from tamoxifen-injected control (*Perp*$^{fl/fl}$) and *K14CreER; Perp*$^{fl/fl}$ mice 5 days post wounding. The migrating epithelial sheets are outlined by the black dashed line. (d) Representative H&E-stained section of closed wounds from tamoxifen-injected control (*Perp*$^{fl/fl}$) and *K14CreER; Perp*$^{fl/fl}$ mice 10 days post wounding. Sizes of boxes in (b) and (c) were determined by the edges of the wound, as marked by the panniculus carnosus.

process, relying on the coordinated activation of fibroblasts mediating dermal contraction and keratinocytes responsible for reepithelialization of the wound as well as communication between these compartments [2, 3]. Differentiated keratinocytes undergo profound alterations to allow them to proliferate and migrate during the resurfacing of a wound. One notable change is the weakening of cell-cell adhesion at the leading edge of the wound. Although the dynamic regulation of cell-cell adhesion junctions like desmosomes has been suggested to be relevant for wound healing, the contribution of these complexes to efficient wound reepithelialization *in vivo* has not been elucidated genetically. Here, we queried the role of desmosomal adhesion junctions in epithelial wound closure using conditional knockout mice lacking the desmosomal component Perp. We observed a clear delay in wound healing in the absence of Perp, underscoring the importance of Perp and potentially desmosome function in epidermal cells for efficient wound closure *in vivo*.

As wound healing relies on keratinocyte proliferation and migration, the delay in wound healing observed in the absence of Perp could reflect deficits in these processes. However, we found that keratinocytes lacking Perp do not

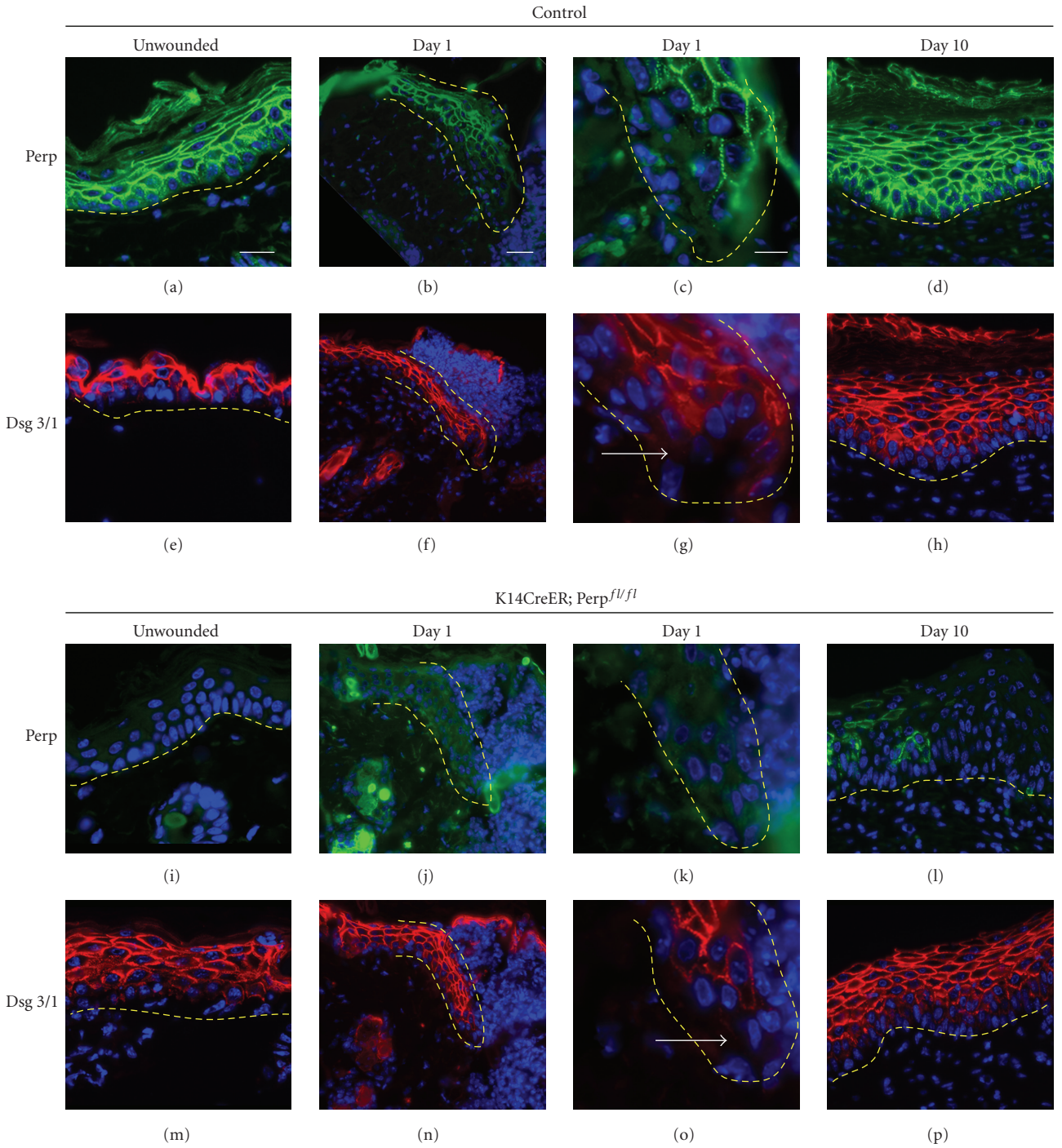

Figure 4: Migrating keratinocytes display reduced desmosomal protein expression at the wound edge. (a)–(d) Immunofluorescence images of Perp expression in the epithelium at different timepoints post wounding in control mice. The dashed yellow line represents the border between the migrating keratinocytes and underlying dermis. (e)–(h) Desmoglein 3/1 expression at different timepoints post wounding in control mice (tamoxifen-injected *Perp*$^{fl/fl}$ mice). (i)–(l) Immunofluorescence images of Perp expression in the epithelium at different timepoints post wounding in *K14CreER; Perp*$^{fl/fl}$ mice. (m)–(p) Desmoglein 3/1 expression at different timepoints post wounding in *K14CreER; Perp*$^{fl/fl}$ mice. DAPI is used to mark nuclei. Arrows indicate cells with reduced desmosomal protein staining. Scale bar in (a) equals 20 μm, scale bar in (b) equals 50 μm, and scale bar in (c) equals 10 μm.

display a defect in proliferation and, moreover, are more migratory than wild-type keratinocytes. This phenotype is consistent with other studies demonstrating that loss of desmosome components, including *PKP1, PKP3, Pg*, and *DP*, fails to affect proliferation but increases migration, either in scratch wound assays or in transwell assays [16, 18, 19, 26]. Instead of strictly affecting the rate of cell motility, the defective adhesion in *Perp* null cells could impede the ability

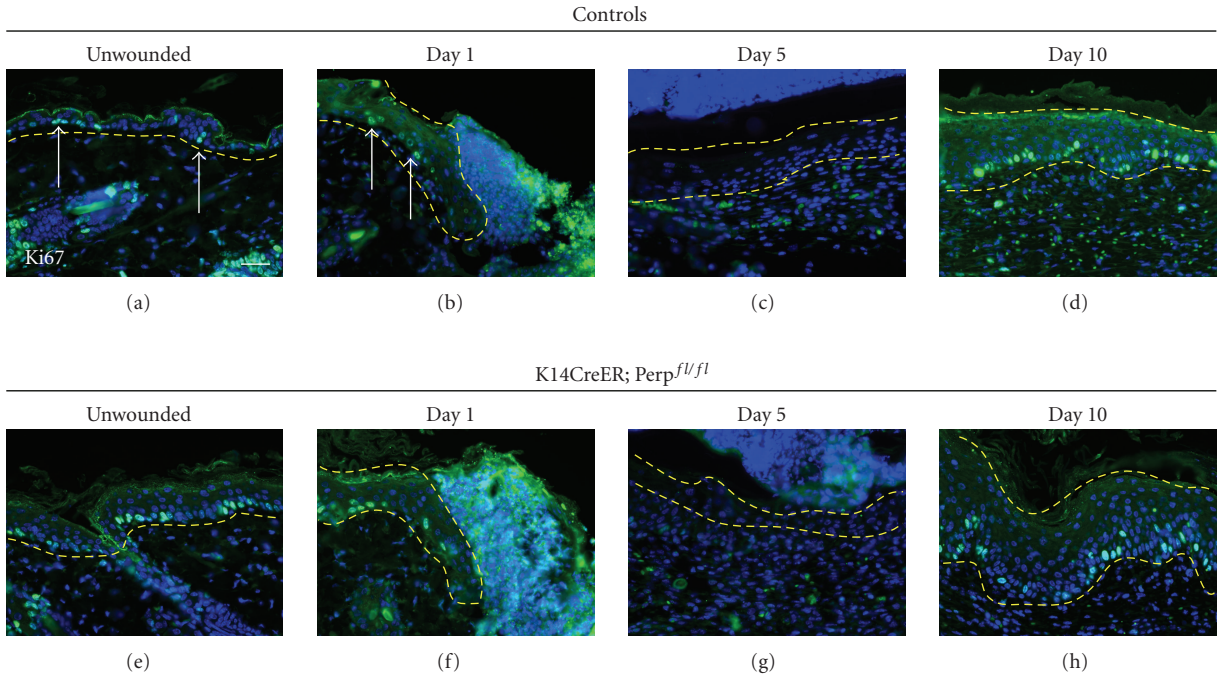

Figure 5: Migrating keratinocytes lacking Perp do not exhibit altered proliferation *in vivo*. (a)–(d) Representative images of Ki67 staining on sections of wounds at various timepoints post injury in control (*Perp*$^{fl/fl}$) mice. (e)–(h) Representative images of Ki67 staining of wounds from *K14CreER; Perp*$^{fl/fl}$ mice. Dashed yellow line surrounds epithelium. DAPI is used to stain nuclei. Arrows indicate Ki67 positive cells. Scale bar equals 50 μm.

of the migrating sheet to move efficiently in a coordinated manner, thereby potentially contributing to the delayed wound closure observed *in vivo*. This notion is consistent with the disorganized cells at the leading edge of the wound in the explant assays we performed.

The ability of desmosomes to confer strength and resiliency to the skin relies on their connection to the keratin intermediate filament network. Analysis of mice deficient for keratins has supported the notion that intermediate filaments are important for efficient wound healing. Specifically, loss of Keratin 17 results in delayed wound healing using a mouse embryonic limb amputation system [28], and inactivation of its partner Keratin 6 impairs the closure of partial thickness wounds in adult mice [29]. These findings are consistent with the observation that induction of Keratins 6, 16, and 17 occurs rapidly in response to injury to the skin at the wound edge [28]. Given the tight linkage between the keratin intermediate filament network and the desmosome, it is possible that desmosomes would be affected in the keratin knockout mice and that their loss would contribute to the observed delay in wound healing [30, 31].

Mutations in genes encoding desmosomal components or the production of autoantibodies against desmosomal constituents have been associated with several human diseases. Mutations found in *PKP1, DSG1, PG,* or *DP* give rise to symptoms typical of ectodermal dysplasias, while autoantibodies against desmosomal proteins induce Pemphigus Vulgaris or Pemphigus Foliaceus, accompanied by blisters in the oral mucosa and/or skin [32, 33]. Patients bearing mutations in *PKP1* display chronic erosions and excessive scale-crust after trauma, suggesting a deficit in wound healing [18]. The inability of wounds to fully heal in these patients is likely attributable to defects in desmosome-mediated adhesion. Understanding how the desmosome participates in the wound healing process may lead to improved treatments for these patients.

Our studies examining the role of Perp and desmosomes in epithelial wound healing have implications for other biological processes as well, as many of the events taking place during wound healing are important in other contexts. In particular, wound healing mimics the transitions occurring during a key developmental and tumorigenic process, epithelial-mesenchymal transition or EMT [34]. Consistent with this idea, Slug, a basic-helix-loop-helix transcriptional repressor required for certain characteristics of EMT, has been shown to be important for wound healing [35]. Slug is critical for wound outgrowth of cultured explants, and Slug overexpression induces desmosome dissolution, cell migration, and accelerated wound healing of keratinocytes [21, 35, 36]. Thus, studying the dynamics of desmosome behavior during wound healing may help illuminate pathways involved in development and cancer.

Our studies have highlighted a role for Perp in efficient wound closure. Not only is desmosome dissolution likely important at the leading edge of wounds early in wound healing, but intact desmosomal function may also be important for proper wound closure, potentially for efficient migration of the epithelium. These data suggest that precise spatial and temporal regulation of desmosomal complexes may be critical for wound healing. Future investigation into the signals regulating desmosome dynamics will provide further insight into both wound healing and cancer.

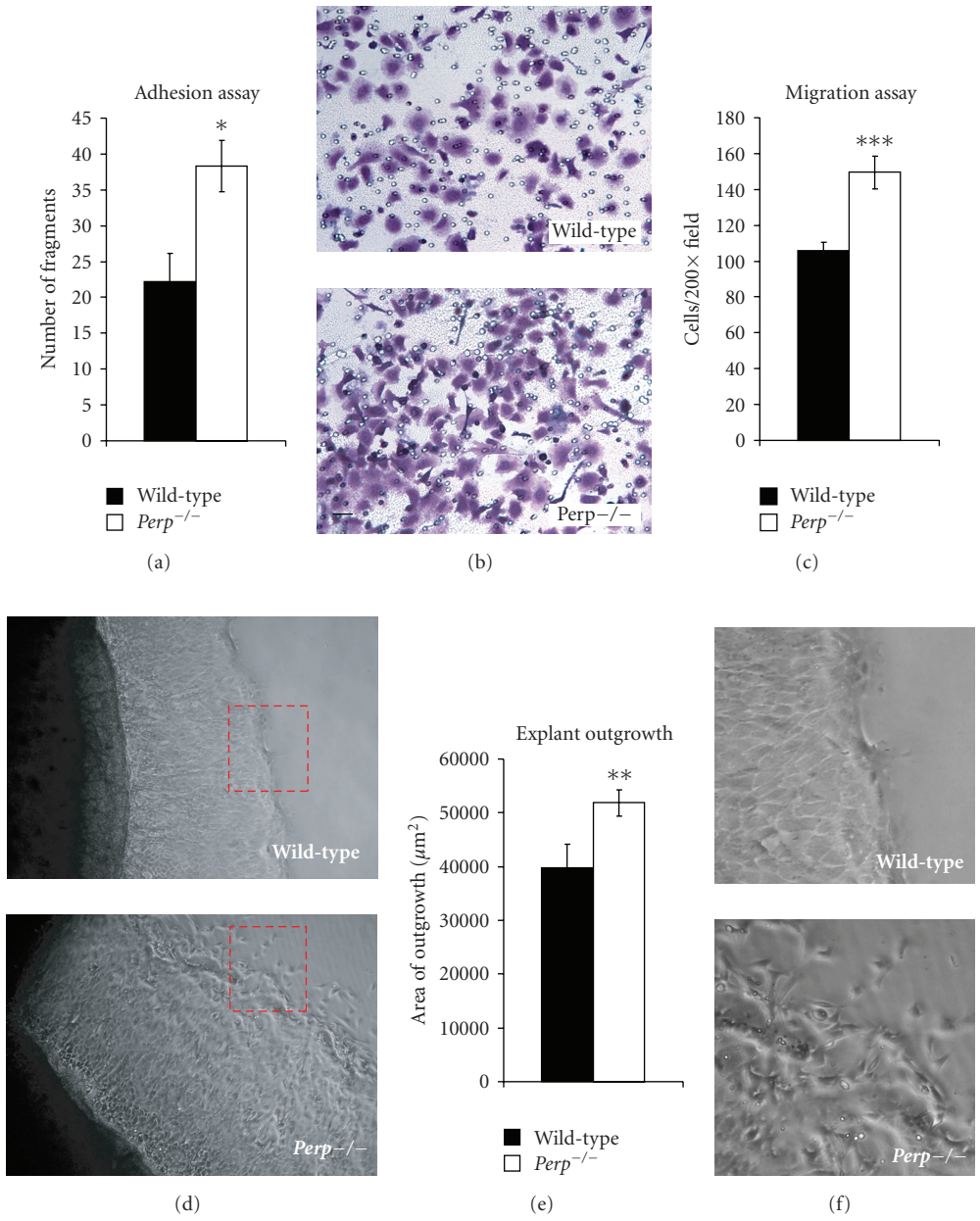

FIGURE 6: $Perp^{-/-}$ keratinocytes display enhanced migration capacity *in vitro*. (a) A mechanical dissociation assay was performed on wild-type and $Perp^{-/-}$ keratinocyte monolayers. The number of cellular fragments per 4 100× fields was counted and averaged. Graphs show the average of three independent experiments performed in triplicate and error bars represent +/− SEM. (b) A transwell migration assay was used to assess the migration potential of wild-type and $Perp^{-/-}$ keratinocytes. Representative image of migrated cells stained with crystal violet. (c) The number of cells migrated in each of 3 200× fields was counted and averaged. The results represent the average of three individual experiments +/− SEM. (d) Representative phase-contrast images of wild-type and $Perp^{-/-}$ explants. The red dashed boxes represent the area seen in (f). (e) The average areas of outgrowth after 10 days for wild-type and $Perp^{-/-}$ explants are quantified in the graph. Wild-type: $n = 26$. $Perp^{-/-}$: $n = 50$. Error bars represent the SEM. (f) Enlarged phase-contrast images of the areas boxed in red in panel (d) showing wild-type and $Perp^{-/-}$ explants. Statistical significance was determined using a Student's unpaired t-test * $= P < .04$, ** $= P < .03$, *** $= P < .003$.

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References

[1] J. Bereiter-Hahn, *Biology of the Integument*, Springer, Berlin, Germany, 1896.

[2] P. Martin, "Wound healing—aiming for perfect skin regeneration," *Science*, vol. 276, no. 5309, pp. 75–81, 1997.

[3] P. A. Coulombe, "Wound epithelialization: accelerating the pace of discovery," *The Journal of Investigative Dermatology*, vol. 121, no. 2, pp. 219–230, 2003.

[4] A. J. Singer and R. A. F. Clark, "Cutaneous wound healing," *The New England Journal of Medicine*, vol. 341, no. 10, pp. 738–746, 1999.

[5] M. Wawersik, R. D. Paladini, E. Noensie, and P. A. Coulombe, "A proline residue in the alpha-helical rod domain of type I keratin 16 destabilizes keratin heterotetramers," *Journal of Biological Chemistry*, vol. 272, no. 51, pp. 32557–32565, 1997.

[6] R. F. Clark, *Mechanisms of Cutaneous Wound Repair*, McGraw-Hill, New York, NY, USA, 1993.

[7] W. S. Krawczyk, "A pattern of epidermal cell migration during wound healing," *Journal of Cell Biology*, vol. 49, pp. 247–263, 1971.

[8] D. R. Garrod, M. Y. Berika, W. F. Bardsley, D. Holmes, and L. Tabernero, "Hyper-adhesion in desmosomes: its regulation in wound healing and possible relationship to cadherin crystal structure," *Journal of Cell Science*, vol. 118, no. 24, pp. 5743–5754, 2005.

[9] T. Yin and K. J. Green, "Regulation of desmosome assembly and adhesion," *Seminars in Cell and Developmental Biology*, vol. 15, no. 6, pp. 665–677, 2004.

[10] C. L. Leung, K. J. Green, and R. K. H. Liem, "Plakins: a family of versatile cytolinker proteins," *Trends in Cell Biology*, vol. 12, no. 1, pp. 37–45, 2002.

[11] M. Hatzfeld, "The armadillo family of structural proteins," *International Review of Cytology*, vol. 186, pp. 179–224, 1999.

[12] M. Hatzfeld, "Plakophilins: multifunctional proteins or just regulators of desmosomal adhesion?" *Biochimica et Biophysica Acta*, vol. 1773, no. 1, pp. 69–77, 2007.

[13] R. A. Ihrie, M. R. Marques, B. T. Nguyen, et al., "Perp is a p63-regulated gene essential for epithelial integrity," *Cell*, vol. 120, no. 6, pp. 843–856, 2005.

[14] T. Sklyarova, S. Bonne, P. D'hooge, et al., "Plakophilin-3-deficient mice develop hair coat abnormalities and are prone to cutaneous inflammation," *Journal of Investigative Dermatology*, vol. 128, no. 6, pp. 1375–1385, 2008.

[15] M. Chidgey, C. Brakebusch, E. Gustafsson, et al., "Mice lacking desmocollin 1 show epidermal fragility accompanied by barrier defects and abnormal differentiation," *Journal of Cell Biology*, vol. 155, no. 5, pp. 821–832, 2001.

[16] S. T. Kundu, P. Gosavi, N. Khapare, et al., "Plakophilin3 down-regulation leads to a decrease in cell adhesion and promotes metastasis," *International Journal of Cancer*, vol. 123, no. 10, pp. 2303–2314, 2008.

[17] T. Yin, S. Getsios, R. Caldelari, et al., "Mechanisms of plakoglobin-dependent adhesion: desmosome-specific functions in assembly and regulation by epidermal growth factor receptor," *Journal of Biological Chemistry*, vol. 280, no. 48, pp. 40355–40363, 2005.

[18] A. P. South, H. Wan, M. G. Stone, et al., "Lack of plakophilin 1 increases keratinocyte migration and reduces desmosome stability," *Journal of Cell Science*, vol. 116, no. 16, pp. 3303–3314, 2003.

[19] S. V. Setzer, C. C. Calkins, J. Garner, S. Summers, K. J. Green, and A. P. Kowalczyk, "Comparative analysis of armadillo family proteins in the regulation of A431 epithelial cell junction assembly, adhesion and migration," *Journal of Investigative Dermatology*, vol. 123, no. 3, pp. 426–433, 2004.

[20] C. A. Gaudry, H. L. Palka, R. L. Dusek, et al., "Tyrosine-phosphorylated plakoglobin is associated with desmogleins but not desmoplakin after epidermal growth factor receptor activation," *Journal of Biological Chemistry*, vol. 276, no. 27, pp. 24871–24880, 2001.

[21] P. Savagner, K. M. Yamada, and J. P. Thiery, "The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition," *Journal of Cell Biology*, vol. 137, no. 6, pp. 1403–1419, 1997.

[22] K. J. Green and C. L. Simpson, "Desmosomes: new perspectives on a classic," *Journal of Investigative Dermatology*, vol. 127, no. 11, pp. 2499–2515, 2007.

[23] D. Metzger, M. Li, and P. Chambon, "Targeted somatic mutagenesis in the mouse epidermis," *Methods in Molecular Biology*, vol. 289, pp. 329–340, 2005.

[24] S. Mazzalupo, M. J. Wawersik, and P. A. Coulombe, "An ex vivo assay to assess the potential of skin keratinocytes for wound epithelialization," *Journal of Investigative Dermatology*, vol. 118, no. 5, pp. 866–870, 2002.

[25] A. C. Huen, J. K. Park, L. M. Godsel, et al., "Intermediate filament-membrane attachments function synergistically with actin-dependent contacts to regulate intercellular adhesive strength," *Journal of Cell Biology*, vol. 159, no. 6, pp. 1005–1017, 2002.

[26] T. Yin, S. Getsios, R. Caldelari, et al., "Plakoglobin supresses keratinocyte motility through both cell-cell adhesion-dependent and -independent mechanisms," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 15, pp. 5420–5425, 2005.

[27] R. F. Rushmer, K. J. K. Buettner, J. M. Short, and G. F. Odland, "The skin," *Science*, vol. 154, no. 3747, pp. 343–348, 1966.

[28] S. Mazzalupo, P. Wong, P. Martin, and P. A. Coulombe, "Role for keratins 6 and 17 during wound closure in embryonic mouse skin," *Developmental Dynamics*, vol. 226, no. 2, pp. 356–365, 2003.

[29] S. M. Wojcik, D. S. Bundman, and D. R. Roop, "Delayed wound healing in keratin 6a knockout mice," *Molecular and Cellular Biology*, vol. 20, no. 14, pp. 5248–5255, 2000.

[30] G. I. Gallicano, P. Kouklis, C. Bauer, et al., "Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage," *Journal of Cell Biology*, vol. 143, no. 7, pp. 2009–2022, 1998.

[31] P. Vijayaraj, C. Kroger, U. Reuter, R. Windoffer, R. E. Leube, and T. M. Magin, "Keratins regulate protein biosynthesis through localization of GLUT1 and -3 upstream of AMP kinase and Raptor," *Journal of Cell Biology*, vol. 187, no. 2, pp. 175–184, 2009.

[32] J. A. McGrath, J. R. McMillan, C. S. Shemanko, et al., "Mutations in the plakophilin 1 gene result in ectodermal dysplasia/skin fragility syndrome," *Nature Genetics*, vol. 17, no. 2, pp. 240–244, 1997.

[33] M. D. Kottke, E. Delva, and A. P. Kowalczyk, "The desmosome: cell science lessons from human diseases," *Journal of Cell Science*, vol. 119, no. 5, pp. 797–806, 2006.

[34] R. Kalluri and R. A. Weinberg, "The basics of epithelial-mesenchymal transition," *Journal of Clinical Investigation*, vol. 119, no. 6, pp. 1420–1428, 2009.

[35] P. Savagner, D. F. Kusewitt, E. A. Carver, et al., "Developmental transcription factor slug is required for effective re-epithelialization by adult keratinocytes," *Journal of Cellular Physiology*, vol. 202, no. 3, pp. 858–866, 2005.

[36] V. Arnoux, M. Nassour, A. L'Helgoualc'h, R. A. Hipskind, and P. Savagner, "Erk5 controls Slug expression and keratinocyte activation during wound healing," *Molecular Biology of the Cell*, vol. 19, no. 11, pp. 4738–4749, 2008.

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Review Article

Experimental Human Cell and Tissue Models of Pemphigus

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Pemphigus is a chronic mucocutaneous autoimmune bullous disease that is characterized by loss of cell-cell contact in skin and/or mucous membranes. Past research has successfully identified desmosomes as immunological targets and has demonstrated that acantholysis is initiated through direct binding of IgG. The exact mechanisms of acantholysis, however, are still missing. Experimental model systems have contributed considerably to today's knowledge and are still a favourite tool of research. In this paper we will describe to what extent human cell and tissue models represent the in vivo situation, for example, organ cultures of human skin, keratinocyte cultures, and human skin grafted on mice and, furthermore, how suitable they are to study the pathogenesis of pemphigus. Organ cultures closely mimic the architecture of the epidermis but are less suitable to answer posed biochemical questions. Cultured keratinocyte monolayers are convenient in this respect, but their desmosomal make-up in terms of adhesion molecules does not exactly reflect the in vivo situation. Reconstituted skin is a relatively new model that approaches organ culture. In models of human skin grafted on mice, acantholysis can be studied in actual human skin but now with all the advantages of an animal model.

1. Introduction

Pemphigus is a chronic mucocutaneous autoimmune bullous disease, characterized by the presence of autoantibodies against the desmosomal cadherins, desmoglein 1 (Dsg1), and/or desmoglein 3 (Dsg3). There are two main forms of pemphigus: pemphigus foliaceus (PF) and pemphigus vulgaris (PV). PF presents as superficial blistering of the skin and the presence of autoantibodies against Dsg1. In the case of mucosal dominant PV, patients have suprabasal blistering of the mucous membranes and auto-antibodies against Dsg3 only. Patients with mucocutaneous PV have suprabasal blistering of both the skin and the mucous membranes, in combination with autoantibodies against both Dsg1 and 3.

Since the discovery by Beutner and Jordon in the sixties, who demonstrated by indirect immunofluorescence (IIF) microscopy that sera of pemphigus vulgaris patients contained IgG antibodies directed against a substance on the surface of keratinocytes [1], investigators have tried to answer an intriguing question: how do these antibodies cause acantholysis in skin? In the nineties, Mahoney et al. presented their theories on steric hindrance and desmoglein compensation [2] as an explanation for acantholysis. Recently, researchers are also focusing on other putative mechanisms for example, cell signalling [3, 4], apoptosis [5], desmosome assembly and disassembly [6], and endocytosis [7].

Although the exact steps in the process of acantholysis in pemphigus are still not clear, research herein has considerably benefitted from experimental models, for example, mouse models and in vitro models. Unlike the animal models, the in vitro models have been used to study the effector-phase of pemphigus and not its cell-mediated immune regulation. In this paper we will discuss the in vitro models and focus on human cell and tissue models. These models comprise organ cultures of human skin, cultured human monolayer keratinocytes, reconstituted skin, and human skin grafted on mice. We will discuss how well these human cell and tissue models represent the in vivo situation in human skin and their suitability to study the pathogenesis of pemphigus.

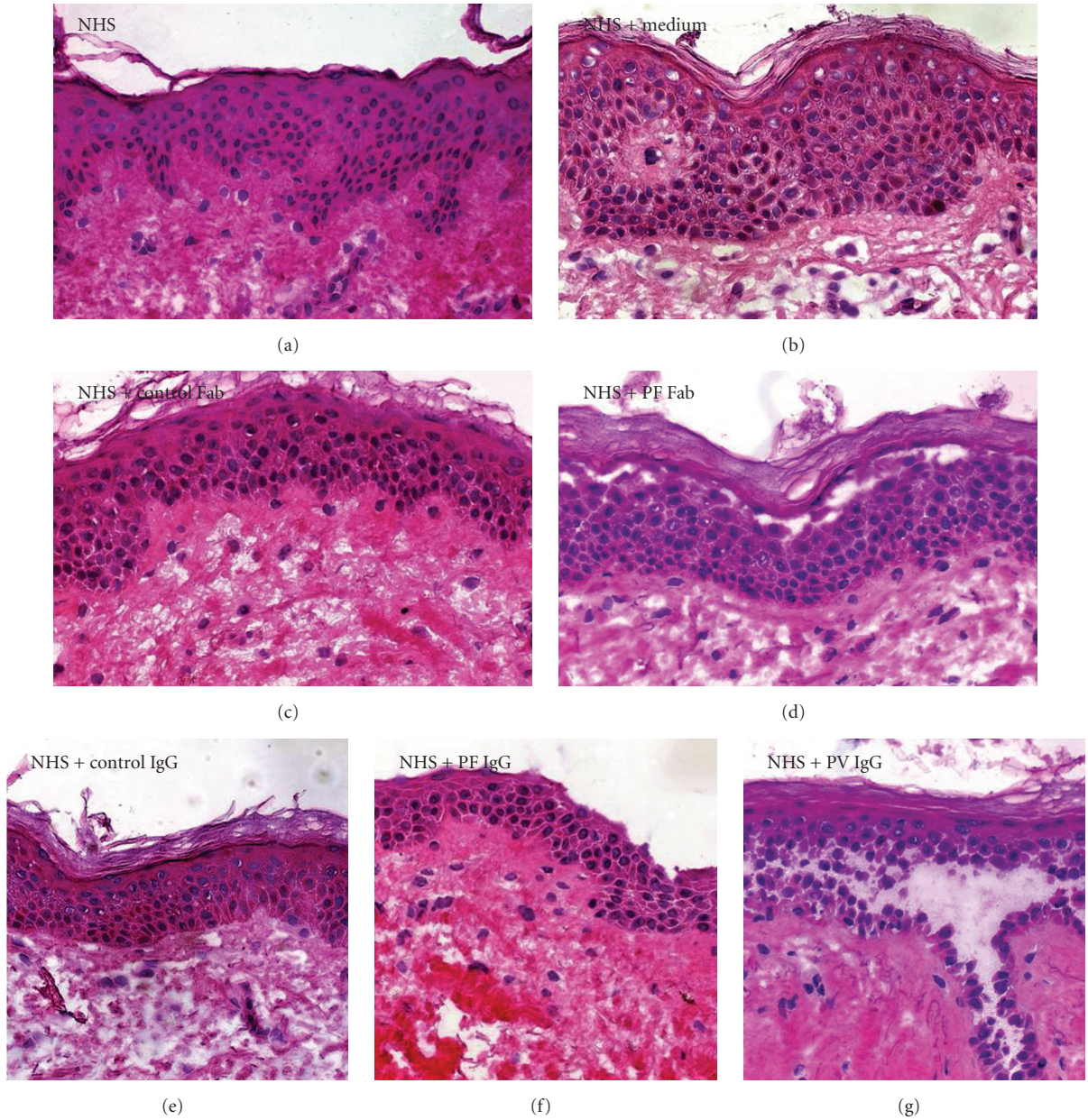

Figure 1: Incubation of normal human skin with pemphigus IgG or Fab fragments leads to suprabasal or subcorneal acantholytic blistering. (a) Normal human skin (NHS) before incubation. Incubation of NHS for 24 hours in (b) medium only or in medium with added (c) control Fab fragments or (e) control IgG leads to limited spongiosis of the epidermis. Incubation of NHS in medium with added (d) PF Fab fragments and (f) PF IgG induces a subcorneal split. Incubation of NHS in medium with (g) PV IgG induces suprabasal acantholysis.

2. Organ Cultures of Human Skin

Michel and Ko were among the first who successfully produced acantholysis in vitro by using an organ culture model [8]. They described a relatively simple and reproducible method based on the work of Sarkany et al. [9]. Michel et al. placed a skin explant on lens paper which floated on the surface of liquid that contained crude pemphigus serum. Since then, more research groups have used this organ culture model to study pemphigus [10–18]. We ourselves have recently performed experiments using an organ culture model with air-liquid interface in which a biopsy of normal human skin is not floated on lens paper but instead placed on a transwell such that the bottom of the biopsy contacts the solution containing IgG (Figure 1). In a second approach we submerged biopsies in solution. This enabled culturing more biopsies in one and the same volume of medium with added pemphigus IgG or Fab fragments. Biopsies can be easily harvested at any time and processed for light microscopy, immunofluorescence, or electron microscopy. Although submerged culturing induces shifts in the expression of the different cadherins, for

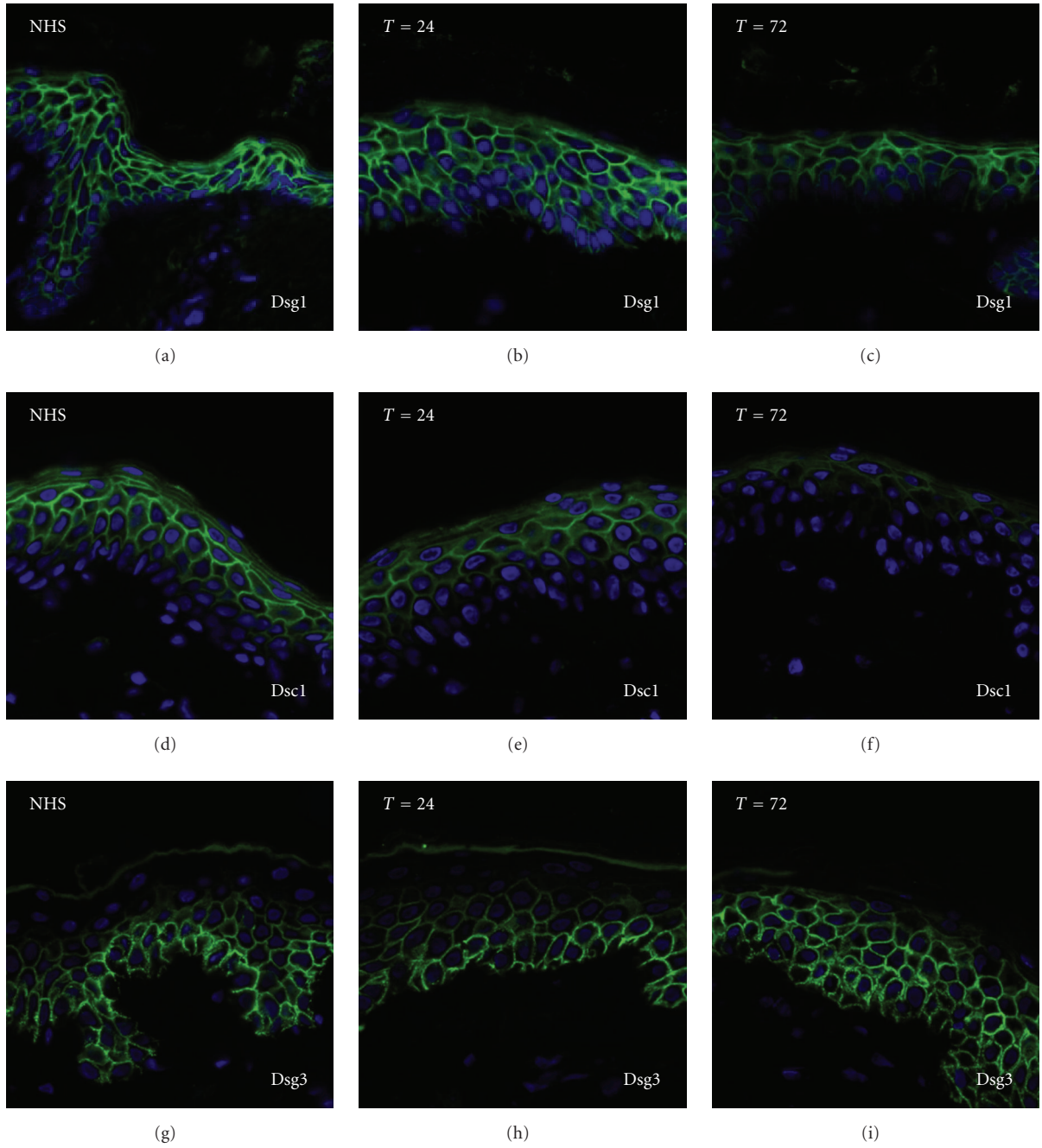

Figure 2: Shift in expression of Dsg1, Dsc1, and Dsg3 in submerged skin cultures after more than 24 hours. (a) Dsg1 is expressed throughout all the layers of NHS. (b) The expression of Dsg1 by skin incubated in medium for 24 hours is comparable to that of NHS. (c) After incubation in medium for 72 hours, Dsg1 expression is reduced. (d) Dsc1 is expressed in the upper layers of the epidermis. (e) The expression of Dsc1 by skin incubated in medium for 24 hours is comparable to that of NHS. (f) After incubation in medium for 72 hours, Dsc1 expression is reduced. (g) Dsg3 is expressed in the basal and suprabasal layers of the epidermis in NHS. (h) The expression of Dsg3 after incubation in medium for 24 hours is comparable to that of NHS. (i) After incubation in medium for 72 hours, Dsg3 is also expressed in the upper layers of the epidermis.

example, substantial loss of Dsg1 and desmocollin 1 (Dsc1) with increased expression of Dsg3 in higher cell layers, this only manifests after prolonged culturing, and their expression remains comparable to normal human skin when the experiments are limited to 24 hours (Figure 2).

Michel and Ko incubated normal human skin with undiluted sera from pemphigus patients. Direct immunofluorescence (DIF) showed intercellular staining of IgG. Light microscopy showed a split after 24-hours incubation. Unfortunately, this first attempt was not as successful as had been

hoped and both PF and PV sera induced a suprabasal split [8]. Later investigators, however, did succeed in producing correct subcorneal splits in normal human skin with PF IgG [16]. In our own organ culture model subcorneal acantholysis can be induced not only by PF IgG but also by PF Fab fragments (Figure 1(f)).

Next to whole serum, Michel and Ko also performed incubations with heated serum in order to inactivate complement [8]. Heated serum also led to acantholysis, which showed that the pathogenesis of pemphigus was not complement dependent [8, 19, 20]. The demonstration that Fab fragments of pemphigus IgG also induce acantholysis confirmed the concept that complement fixation was not a necessary step in this disease [21]. Acantholysis thus is independent of IgG subclass.

Hu et al. studied the effects of pemphigus IgG incubation on normal human skin by electron microscopy [22]. After 12-hours incubation the first changes, for example, intercellular widening, were seen. After 24 hours the intercellular widening had progressed, and dissolution of the desmosomes became visible. Also desmosome remnants could be seen on the surfaces of the keratinocytes; the tonofilaments had retracted from the cell periphery and were concentrated in a perinuclear position. After 72 hours a suprabasal split and widening between the basal cells (a row of tombstones) were seen [22]. Most of the observations described by Hu et al. are comparable to those seen in pemphigus patient skin, but whether or not the observed retraction of tonofilaments in this organ culture model is comparable to the in vivo situation remains a matter of debate. Unlike others [23], we ourselves did not observe this retraction of tonofilaments in pemphigus patient skin [24].

The human organ culture model has been very valuable in obtaining information on the mechanisms of acantholysis and, moreover, has also been used to test old and new therapeutic drugs for pemphigus, for example, hydrocortisone [13], dapsone [13], methylprednisolone [17], and protease inhibitors [15]. Although most popular in the eighties, it is still used today, often in combination with other models [16, 25–28]. The major advantage of skin explants remains that it is actual human skin with correct architecture of all epidermis layers. Layer-specific changes in morphology or protein localization can easily be studied by light microscopy, immunofluorescence, or electron microscopy. However, explants are less suitable to answer biochemical questions concerning molecular pathways, that is, the activation of receptor molecules or changes in phosphorylation state of pathway intermediates. In contrast to cultured cells, cells in the skin explant reside in layers of varied differentiation that most likely respond differently to external stimuli. Aside from this, cultured cells will instantaneously make contact with the added IgG, while in organ cultures the IgG must diffuse into the epidermis and will not reach all cells simultaneously. Therefore, more easily manageable culture models are the preferred models for biochemical and molecular biological research on acantholysis.

3. Keratinocyte Cultures

A year after the first publication on the organ culture model, Schiltz et al. incubated human keratinocytes with pemphigus IgG [29]. The results of these experiments suggested that binding of pemphigus antibodies to the keratinocytes initiates a series of events which result in the release or activation of hydrolytic enzymes by the keratinocytes with subsequent autolysis and acantholysis. This made it clear that keratinocyte cultures could serve as a model for acantholysis. Various sources of cells are now being used, with most researchers using normal human epidermal keratinocytes (NHEKs) [30–32]. These keratinocytes are often derived from neonatal foreskin [6, 7, 28, 33–37] but can also be obtained from surgical excised skin [38]. HaCaT cells, a non-tumorigenic human keratinocyte cell line, are also popular [16, 39–43]. Less commonly used is the squamous cell carcinoma (SCC) cell line DJM-1 [35, 44].

Cultured keratinocytes are mostly used as monolayers or alternatively as reconstituted skin. Keratinocytes grown in low calcium medium will proliferate until confluent and then become growth arrested. In high-calcium medium (1.2 mM or higher) cells will differentiate, form desmosomes, and stratify [45]. For reconstituted skin, it is a requisite to culture the keratinocytes on a dermal equivalent [46]. Varying the calcium concentration provides a tool to induce and study desmosome assembly and disassembly [6, 45]. Whether cultured keratinocytes are a reliable model, to study pemphigus pathogenesis, be it in the form of monolayers or reconstituted skin, will depend mainly on their ability to form mature desmosomes with correct make-up of cadherins and associated molecules. The expression and localization of the pemphigus antigens and other desmosomal adhesion molecules in cultured cells, therefore, became an early subject of research.

By IIF staining with patient sera it was shown that the PV antigen is expressed in human epidermal monolayers when cultured under high-calcium conditions, but at the same time these monolayers lack the PF antigen [47].

Low-calcium cultured monolayer cells do not express Dsg1 [47, 48] and Dsg2 [48], while high-calcium cultured monolayers express Dsg1 [47]. As shown by immunoblot, Dsg1 is detectable after 1 to 6 days of culturing [48], but the expression levels appear to be low [33]. As keratinocytes become stratified, Dsg1 expression increases and can be detected on the plasma membrane of stratified cells in a membrane-bound pattern [47]. The Dsg2 expression in high-calcium cultured monolayers is only positive after 5-6 days as shown by immunoblot [48]. Some groups report that the immunoblot does not show expression of Dsg3 by low-calcium cultured monolayers [48]. Dsg3 was detected in the cytoplasm of cells grown under low-calcium conditions while the protein is translocated to the plasma membrane when cultured under high-calcium conditions [6]. Staining of desmocollin 3 (Dsc3) in NHEKs cultured under low-calcium conditions shows a diffuse cytoplasmic and a focal desmosomal pattern, but comparable to Dsg3 the desmosomal staining intensifies after raising the calcium concentration [6].

Table 1: Expression of desmosomal components by monolayers composed of different cell types cultured in low- or high-calcium medium. −: negative, ±: weak positive, +: positive.

	Low calcium				High calcium			
Cell type	Dsg1	Dsg2	Dsg3	Dsc3	Dsg1	Dsg2	Dsg3	Dsc3
NHEK	−	−	−	+	±	+	+	+
HaCaT	−	+	+		+	+	+	
SCC	−	−	−		+	+	+	

HaCaT cells are capable of expressing Dsg1, Dsg2, and Dsg3 [43, 48], and similar to normal keratinocytes, Dsg1 expression is induced by high levels of calcium [48].

Most tested SCC cell lines have weak or focal intercellular expression of PV antigens and expression of PF antigens in localized areas [47]. Denning et al. tested several SCC cell lines and showed by immunoblot Dsg2 and Dsg3 expression by these cells when cultured in normal or high-calcium media [48]. Aoyama and Kitajima used the DJM-1 cell line and showed expression of Dsg1 and Dsg3 when cultured in high calcium [49].

From the information summarized in Table 1, we can conclude that NHEKs have limitations as an experimental model for pemphigus since these cells do not express significant amounts of Dsg1. Consequently, these experimental systems are not suitable to study acantholysis in PF and mucocutaneous PV. HaCaT cells and DJM-1 cells (Table 1), which express Dsg1 in monolayers, might be more appropriate model systems. It must be taken into account, however, that these cells might express Dsg2 [50] that is not present in most skin areas affected by pemphigus. Despite all drawbacks, monolayers have contributed much to our knowledge on acantholysis and have been at the basis of new ideas and insights. An elegant practical example of their use is the in vitro keratinocyte dissociation assay that can quantify the anti-Dsg3 acantholytic effects of patient IgG [7, 32, 33, 42, 44, 51–53]. After incubation of monolayers with IgG, dispase is used to release the cell sheet from the culture dish. This sheet is then subjected to fierce mechanical stress by means of pumping in and out of a syringe. The resulting number of cell fragments is a quantification of the acantholytic effect of the IgG [54]. An illustration of just how important the cadherin composition of the desmosomes is becomes apparent when HaCaT cells are used in the same assay and no fragments are formed. This is likely due to the high Dsg2 expression [54].

Keratinocytes cultured in reconstituted skin will differentiate and stratify. Therefore both PV and PF antigens are expressed in reconstituted skin [47, 55–57]. By culturing keratinocytes air-exposed on a dermal equivalent, it is possible to reconstruct a multilayered epidermis [46, 58]. The morphology of this reconstituted skin can be compared to that of epidermis in vivo [46, 58, 59]. Ultrastructural assessment of a skin equivalent showed mature desmosome formation [46, 59]. Unfortunately, the expression of the desmosomal proteins, the cadherin antigens, and the formation of desmosomes in these skin equivalents are not well documented. DIF or IIF of desmosomes showed intercellular staining, but in contrast to human skin, there is also strong staining at the top level or cornified layer [58, 59]. Few researchers used reconstituted skin as an in vitro model to study the pathogenesis of pemphigus [60–62].

4. Human Skin Grafted on Mice

As mentioned in the introduction, mouse models are often used in pemphigus research next to the human in vitro models. By using mouse models, however, pemphigus is induced in murine skin which might differ in its function from human skin. By grafting human skin on mice, acantholysis can be studied in human skin while at the same time providing the researcher with the advantages of a mouse model [63]. There is only limited experience with these mouse models in pemphigus. Zillikens et al. grafted full-thickness human skin onto the back of SCID mice [64]. PF and PV IgG were injected in the dermis of the graft. Histopathologic findings and DIF of the grafted human skin were comparable to histopathologic findings and DIF in PF and PV patient skin. Others used reconstituted skin grafted onto SCID mice, and subcorneal blistering was induced by injection of PF IgG [27]. These graft models therefore seem very promising.

5. Model Comparison

When studying a human disease, a model is required that approaches the in vivo human situation as closely as possible. Studying the pathogenesis of pemphigus in patients unfortunately has its limitations. For ethical reasons, biopsies cannot be taken too often making it impossible to in detail follow the time course of disease development. Mouse models have given great insight into the disease, but mice are not completely comparable to humans, so some questions remain that will have to be addressed in human models. The human in vitro models described in this paper all have their advantages and disadvantages. Therefore no single model may be preferred, but different models may be used in a complementary fashion. Organ cultures and skin equivalents have the advantage that they are most comparable to human skin in terms of desmoglein expression and mature desmosomes. Acantholysis can be evaluated easily with light microscopy, immunofluorescence or electron microscopy. To study pathways and to follow the fate of individual molecules in a narrow time frame in terms of expression level, shifts in localization, phosphorylation, or molecular interaction, easily manageable culture models are favoured. Cell lines should be chosen such that they

reflect the skin situation as closely as possible. As discussed before they are suitable to study aspects of acantholysis in PV but not PF for the simple reason that no cells so far have been cultured that express Dgs1 in absence of Dsg3. The mechanism of pemphigus acantholysis has been studied for the past forty years and has taught us which molecules are involved and that acantholysis occurs in the absence of inflammation mediators. How desmosomes split and what molecular pathways lead to acantholysis is still being debated. The use of different experimental models is required to investigate the patho-mechanism.

Abbreviations

Dsg: Desmoglein
PF: Pemphigus foliaceus
PV: Pemphigus vulgaris
IIF: Indirect immunofluorescence
IgG: Immunoglobulin G
Fab: Fragment antigen-binding
Dsc: Desmocollin
DIF: Direct immunofluorescence.

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References

[1] E. H. Beutner and R. E. Jordon, "Demonstration of skin antibodies in sera of pemphigus vulgaris patients by indirect immunofluorescent staining," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 117, pp. 505–510, 1964.

[2] M. G. Mahoney, Z. Wang, K. Rothenberger, P. J. Koch, M. Amagai, and J. R. Stanley, "Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris," *Journal of Clinical Investigation*, vol. 103, no. 4, pp. 461–468, 1999.

[3] P. Sharma, X. Mao, and A. S. Payne, "Beyond steric hindrance: the role of adhesion signaling pathways in the pathogenesis of pemphigus," *Journal of Dermatological Science*, vol. 48, no. 1, pp. 1–14, 2007.

[4] E. J. Müller, L. Williamson, C. Kolly, and M. M. Suter, "Outside-in signaling through integrins and cadherins: a central mechanism to control epidermal growth and differentiation?" *Journal of Investigative Dermatology*, vol. 128, no. 3, pp. 501–516, 2008.

[5] E. Schmidt and J. Waschke, "Apoptosis in pemphigus," *Autoimmunity Reviews*, vol. 8, no. 7, pp. 533–537, 2009.

[6] X. Mao, E. J. Choi, and A. S. Payne, "Disruption of desmosome assembly by monovalent human pemphigus vulgaris monoclonal antibodies," *Journal of Investigative Dermatology*, vol. 129, no. 4, pp. 908–918, 2009.

[7] E. Delva, J. M. Jennings, C. C. Calkins, M. D. Kottke, V. Faundez, and A. P. Kowalczyk, "Pemphigus vulgaris IgG-induced desmoglein-3 endocytosis and desmosomal disassembly are mediated by a clathrin- and dynamin-independent mechanism," *Journal of Biological Chemistry*, vol. 283, no. 26, pp. 18303–18313, 2008.

[8] B. Michel and C. S. Ko, "An organ culture model for the study of pemphigus acantholysis," *British Journal of Dermatology*, vol. 96, no. 3, pp. 295–302, 1977.

[9] I. Sarkany, K. Grice, and G. A. Caron, "Phytohaemagglutinin induced mitotic stimulation of epithelial cells in organ culture of adult human skin," *The British Journal of Dermatology*, vol. 77, pp. 65–76, 1965.

[10] T. Hashimoto, "Experimental suprabasal bulla formation in organ cultured human skin with low calcium medium," *Journal of Investigative Dermatology*, vol. 90, no. 4, pp. 501–504, 1988.

[11] T. Hashimoto, M. Sugiura, and S. Kurihara, "Experimental acantholysis by complement-fixing intercellular antibodies," *Archives of Dermatological Research*, vol. 273, no. 1-2, pp. 129–135, 1982.

[12] T. Hunziker, U. E. Nydegger, P. G. Lerch, and J.-D. Vassalli, "Platelet-derived factors enhance pemphigus acantholysis in skin organ cultures," *Clinical and Experimental Immunology*, vol. 64, no. 2, pp. 442–449, 1986.

[13] E. W. B. Jeffes III, R. P. Kaplan, and A. R. Ahmed, "Acantholysis produced in vitro with pemphigus serum: hydrocortisone inhibits acantholysis, while dapsone and 6-mercaptopurine do not inhibit acantholysis," *Journal of Clinical Immunology*, vol. 4, no. 5, pp. 359–363, 1984.

[14] S. Morioka, K. Naito, and H. Ogawa, "The pathogenic role of pemphigus antibodies and proteinase in epidermal acantholysis," *Journal of Investigative Dermatology*, vol. 76, no. 5, pp. 337–341, 1981.

[15] T. Schuh, R. Besch, E. Braungart, et al., "Protease inhibitors prevent plasminogen-mediated, but not pemphigus vulgaris-induced, acantholysis in human epidermis," *Biological Chemistry*, vol. 384, no. 2, pp. 311–315, 2003.

[16] V. Spindler, D. Drenckhahn, D. Zillikens, and J. Waschke, "Pemphigus IgG causes skin splitting in the presence of both desmoglein 1 and desmoglein 3," *American Journal of Pathology*, vol. 171, no. 3, pp. 906–916, 2007.

[17] D. L. Swanson and M. V. Dahl, "Methylprednisolone inhibits pemphigus acantholysis in skin cultures," *Journal of Investigative Dermatology*, vol. 81, no. 3, pp. 258–260, 1983.

[18] J. Waschke, V. Spindler, P. Bruggeman, D. Zillikens, G. Schmidt, and D. Drenckhahn, "Inhibition of Rho A activity causes pemphigus skin blistering," *Journal of Cell Biology*, vol. 175, no. 5, pp. 721–727, 2006.

[19] J. R. Schiltz and B. Michel, "Production of epidermal acantholysis in normal human skin in vitro by the IgG fraction from pemphigus serum," *Journal of Investigative Dermatology*, vol. 67, no. 2, pp. 254–260, 1976.

[20] J. R. Schiltz, B. Michel, and R. Papay, "Appearance of "pemphigus acantholysis factor" in human skin cultured with pemphigus antibody," *Journal of Investigative Dermatology*, vol. 73, no. 6, pp. 575–581, 1979.

[21] J. R. Schiltz, "Pemphigus acantholysis: a unique immunological injury," *Journal of Investigative Dermatology*, vol. 74, no. 5, pp. 359–362, 1980.

[22] C. H. Hu, B. Michel, and J. R. Schiltz, "Epidermal acantholysis induced in vitro by pemphigus autoantibody. An ultrastructural study," *American Journal of Pathology*, vol. 90, no. 2, pp. 345–361, 1978.

[23] J.-C. Bystryn and S. A. Grando, "A novel explanation for acantholysis in pemphigus vulgaris: the basal cell shrinkage hypothesis," *Journal of the American Academy of Dermatology*, vol. 54, no. 3, pp. 513–516, 2006.

[24] G. F. H. Diercks, H. H. Pas, and M. F. Jonkman, "The ultrastructure of acantholysis in pemphigus vulgaris," *British Journal of Dermatology*, vol. 160, no. 2, pp. 460–461, 2009.

[25] H. Dobrev, L. Popova, and D. Vlashev, "Proteinase inhibitors and pemphigus vulgaris. An in vitro and in vivo study," *Archives of Dermatological Research*, vol. 288, no. 11, pp. 648–655, 1996.

[26] M. Frusic-Zlotkin, R. Pergamentz, B. Michel, et al., "The interaction of pemphigus autoimmunoglobulins with epidermal cells: activation of the Fas apoptotic pathway and the use of caspase activity for pathogenicity tests of pemphigus patients," *Annals of the New York Academy of Sciences*, vol. 1050, pp. 371–379, 2005.

[27] S. Ortiz-Urda, A. Elbe-Bürger, J. Smolle, et al., "The plant lectin wheat germ agglutinin inhibits the binding of pemphigus 27 foliaceus autoantibodies to desmoglein 1 in a majority of patients and prevents 28 pathomechanisms of pemphigus foliaceus in vitro and in vivo," *Journal of Immunology*, vol. 171, no. 11, pp. 6244–6250, 2003.

[28] X. Wang, F. Brégégère, M. Frusic-Zlotkin, M. Feinmesser, B. Michel, and Y. Milner, "Possible apoptotic mechanism in epidermal cell acantholysis induced by pemphigus vulgaris autoimmunoglobulins," *Apoptosis*, vol. 9, no. 2, pp. 131–143, 2004.

[29] J. R. Schiltz, B. Michel, and R. Papay, "Pemphigus antibody interaction with human epidermal cells in culture. A proposed mechanism for pemphigus acantholysis," *Journal of Clinical Investigation*, vol. 62, no. 4, pp. 778–788, 1978.

[30] P. Berkowitz, P. Hu, Z. Liu, et al., "Desmosome signaling: inhibition of p38MAPK prevents pemphigus vulgaris IgG-induced cytoskeleton reorganization," *Journal of Biological Chemistry*, vol. 280, no. 25, pp. 23778–23784, 2005.

[31] B. Nguyen, R. L. Dusek, V. G. Beaudry, M. P. Marinkovich, and L. D. Attardi, "Loss of the desmosomal protein perp enhances the phenotypic effects of pemphigus vulgaris autoantibodies," *Journal of Investigative Dermatology*, vol. 129, no. 7, pp. 1710–1718, 2009.

[32] W.-M. Heupel, P. Engerer, E. Schmidt, and J. Waschke, "Pemphigus vulgaris IgG cause loss of desmoglein-mediated adhesion and keratinocyte dissociation independent of epidermal growth factor receptor," *American Journal of Pathology*, vol. 174, no. 2, pp. 475–485, 2009.

[33] C. C. Calkins, S. V. Setzer, J. M. Jennings, et al., "Desmoglein endocytosis and desmosome disassembly are coordinated responses to pemphigus autoantibodies," *Journal of Biological Chemistry*, vol. 281, no. 11, pp. 7623–7634, 2006.

[34] A. I. Chernyavsky, J. Arredondo, Y. Kitajima, M. Sato-Nagai, and S. A. Grando, "Desmoglein versus non-desmoglein signaling in pemphigus acantholysis: characterization of novel signaling pathways downstream of pemphigus vulgaris antigens," *Journal of Biological Chemistry*, vol. 282, no. 18, pp. 13804–13812, 2007.

[35] M. Kanno, Y. Isa, Y. Aoyama, et al., "p120-catenin is a novel desmoglein 3 interacting partner: identification of the p120-catenin association site of desmoglein 3," *Experimental Cell Research*, vol. 314, no. 8, pp. 1683–1692, 2008.

[36] M. Puviani, A. Marconi, E. Cozzani, and C. Pincelli, "Fas ligand in pemphigus sera induces keratinocyte apoptosis through the activation of caspase-8," *Journal of Investigative Dermatology*, vol. 120, no. 1, pp. 164–167, 2003.

[37] X. Wang, F. Brégégère, Y. Soroka, M. Frusic-Zlotkin, and Y. Milner, "Replicative senescence enhances apoptosis induced by pemphigus autoimmune antibodies in human keratinocytes," *FEBS Letters*, vol. 567, no. 2-3, pp. 281–286, 2004.

[38] A. Baroni, E. Buommino, I. Paoletti, M. Orlando, E. Ruocco, and V. Ruocco, "Pemphigus serum and captopril induce heat shock protein 70 and inducible nitric oxide synthase overexpression, triggering apoptosis in human keratinocytes," *British Journal of Dermatology*, vol. 150, no. 6, pp. 1070–1080, 2004.

[39] N. Cirillo, G. Campisi, F. Gombos, L. Perillo, F. Femiano, and A. Lanza, "Cleavage of desmoglein 3 can explain its depletion from keratinocytes in pemphigus vulgaris," *Experimental Dermatology*, vol. 17, no. 10, pp. 858–863, 2008.

[40] W.-M. Heupel, D. Zillikens, D. Drenckhahn, and J. Waschke, "Pemphigus vulgaris IgG directly inhibit desmoglein 3-mediated transinteraction," *Journal of Immunology*, vol. 181, no. 3, pp. 1825–1834, 2008.

[41] B. Pelacho, C. Natal, A. España, I. Sánchez-Carpintero, M. J. Iraburu, and M. J. López-Zabalza, "Pemphigus vulgaris autoantibodies induce apoptosis in HaCaT keratinocytes," *FEBS Letters*, vol. 566, no. 1–3, pp. 6–10, 2004.

[42] E. Schmidt, J. Gutberlet, D. Siegmund, D. Berg, H. Wajant, and J. Waschke, "Apoptosis is not required for acantholysis in pemphigus vulgaris," *American Journal of Physiology*, vol. 296, no. 1, pp. C162–C172, 2009.

[43] J. Waschke, P. Bruggeman, W. Baumgartner, D. Zillikens, and D. Drenckhahn, "Pemphigus foliaceus IgG causes dissociation of desmoglein 1-containing junctions without blocking desmoglein 1 transinteraction," *Journal of Clinical Investigation*, vol. 115, no. 11, pp. 3157–3165, 2005.

[44] Y. Yamamoto, Y. Aoyama, E. Shu, K. Tsunoda, M. Amagai, and Y. Kitajima, "Anti-desmoglein 3 (Dsg3) monoclonal antibodies deplete desmosomes of Dsg3 and differ in their Dsg3-depleting activities related to pathogenicity," *Journal of Biological Chemistry*, vol. 282, no. 24, pp. 17866–17876, 2007.

[45] F. M. Watt, D. L. Mattey, and D. R. Garrod, "Calcium-induced reorganization of desmosomal components in cultured human keratinocytes," *Journal of Cell Biology*, vol. 99, no. 6, pp. 2211–2215, 1984.

[46] G. Hinterhuber, Y. Marquardt, E. Diem, K. Rappersberger, K. Wolff, and D. Foedinger, "Organotypic keratinocyte coculture using normal human serum: an immunomorphological study at light and electron microscopic levels," *Experimental Dermatology*, vol. 11, no. 5, pp. 413–420, 2002.

[47] K. Iwatsuki, K. Sugaya, and M. Takigawa, "Dynamic expression of pemphigus and desmosomal antigens by cultured keratinocytes," *British Journal of Dermatology*, vol. 128, no. 1, pp. 16–22, 1993.

[48] M. F. Denning, S. G. Guy, S. M. Ellerbroek, S. M. Norvell, A. P. Kowalczyk, and K. J. Green, "The expression of desmoglein isoforms in cultured human keratinocytes is regulated by calcium, serum, and protein kinase C," *Experimental Cell Research*, vol. 239, no. 1, pp. 50–59, 1998.

[49] Y. Aoyama and Y. Kitajima, "Pemphigus vulgaris-IgG causes a rapid depletion of desmoglein 3 (Dsg3) from the Triton X-100 soluble pools, leading to the formation of Dsg3- depleted desmosomes in a human squamous carcinoma cell line, DJM-1 cells," *Journal of Investigative Dermatology*, vol. 112, no. 1, pp. 67–71, 1999.

[50] S. Schäfer, P. J. Koch, and W. W. Franke, "Identification of the ubiquitous human desmoglein, Dsg2, and the expression catalogue of the desmoglein subfamily of desmosomal cadherins," *Experimental Cell Research*, vol. 211, no. 2, pp. 391–399, 1994.

[51] N. Cirillo, M. Lanza, F. Femiano, et al., "If pemphigus vulgaris IgG are the cause of acantholysis, new IgG-independent mechanisms are the concause," *Journal of Cellular Physiology*, vol. 212, no. 3, pp. 563–567, 2007.

[52] W.-M. Heupel, T. M'sduller, A. Efthymiadis, E. Schmidt, D. Drenckhahn, and J. Waschke, "Peptides targeting the desmoglein 3 adhesive interface prevent autoantibody-induced acantholysis in pemphigus," *Journal of Biological Chemistry*, vol. 284, no. 13, pp. 8589–8595, 2009.

[53] V. Spindler, W.-M. Heupel, A. Efthymiadis, et al., "Desmocollin 3-mediated binding is crucial for keratinocyte cohesion and is impaired in pemphigus," *Journal of Biological Chemistry*, vol. 284, no. 44, pp. 30556–30564, 2009.

[54] K. Ishii, R. Harada, I. Matsuo, Y. Shirakata, K. Hashimoto, and M. Amagai, "In vitro keratinocyte dissociation assay for evaluation of the pathogenicity of anti-desmoglein 3 IgG autoantibodies in pemphigus vulgaris," *Journal of Investigative Dermatology*, vol. 124, no. 5, pp. 939–946, 2005.

[55] K. Iwatsuki, H. Harada, R. Yokote, and F. Kaneko, "Differences in the expression of pemphigus antigens during epidermal differentiation," *British Journal of Dermatology*, vol. 133, no. 2, pp. 209–216, 1995.

[56] J. R. Stanley, P. Hawley-Nelson, M. Poirier, S. I. Katz, and S. H. Yuspa, "Detection of pemphigoid antigen, pemphigus antigen, and keratin filaments by indirect immunofluorescence in cultured human epidermal cells," *Journal of Investigative Dermatology*, vol. 75, no. 2, pp. 183–186, 1980.

[57] D. Woodley, J. H. Saurat, M. Prunieras, and M. Regnier, "Pemphigoid, pemphigus and Pr antigens in adult human keratinocytes grown in nonviable substrates," *Journal of Investigative Dermatology*, vol. 79, no. 1, pp. 23–29, 1982.

[58] D. Asselineau, B. A. Bernard, C. Bailly, et al., "Human epidermis reconstructed by culture: is it "normal"?" *Journal of Investigative Dermatology*, vol. 86, no. 2, pp. 181–186, 1986.

[59] E. Tinois, M. Faure, and P. Chatelain, "Growth and differentiation of human keratinocytes on extracellular matrix," *Archives of Dermatological Research*, vol. 279, no. 4, pp. 241–246, 1987.

[60] T. Hunziker, C. Boillat, H. A. Gerber, U. Wiesmann, and B. U. Wintroub, "In vitro pemphigus vulgaris model using organotypic cultures of human epidermal keratinocytes," *Journal of Investigative Dermatology*, vol. 93, no. 2, pp. 263–267, 1989.

[61] M. D. Orlov, A. I. Chernyavsky, J. Arredondo, and S. A. Grando, "Synergistic actions of pemphigus vulgaris IgG, Fas-ligand and tumor necrosis factor-α during induction of basal cell shrinkage and acantholysis," *Autoimmunity*, vol. 39, no. 7, pp. 557–562, 2006.

[62] L. Williamson, N. A. Raess, R. Caldelari, et al., "Pemphigus vulgaris identifies plakoglobin as key suppressor of c-Myc in the skin," *The EMBO Journal*, vol. 25, no. 14, pp. 3298–3309, 2006.

[63] M. Garcia, M. J. Escamez, M. Carretero, et al., "Modeling normal and pathological processes through skin tissue engineering," *Molecular Carcinogenesis*, vol. 46, no. 8, pp. 741–745, 2007.

[64] D. Zillikens, E. Schmidt, S. Reimer, et al., "Antibodies to desmogleins 1 and 3, but not to BP180, induce blisters in human skin grafted onto SCID mice," *The Journal of Pathology*, vol. 193, no. 1, pp. 117–124, 2001.

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Review Article

Mouse Models for Blistering Skin Disorders

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Genetically engineered mice have been essential tools for elucidating the pathological mechanisms underlying human diseases. In the case of diseases caused by impaired desmosome function, mouse models have helped to establish causal links between mutations and disease phenotypes. This review focuses on mice that lack the desmosomal cadherins desmoglein 3 or desmocollin 3 in stratified epithelia. A comparison of the phenotypes observed in these mouse lines is provided and the relationship between the mutant mouse phenotypes and human diseases, in particular pemphigus vulgaris, is discussed. Furthermore, we will discuss the advantages and potential limitations of genetically engineered mouse lines in our ongoing quest to understand blistering skin diseases.

1. Introduction

In recent years, mouse models have become an essential tool for studying genetic diseases, especially in cases where the disease is caused by mutations in a single gene (monogenic disorders). It has been a challenge, however, to faithfully reproduce the pathophysiology of autoimmune disorders in mice, in part because the human disease is influenced by complex factors such as the genetic background, the nature of the autoantibodies (epitopes recognized and antibody subclass) and titers of the circulating autoantibodies.

Pemphigus is a class of autoimmune blistering skin diseases which manifest itself in the form of painful lesions in the skin and oral mucosa and which, in the case of pemphigus vulgaris, can be lethal if left untreated. The sera of patients with pemphigus contain autoantibodies directed against desmosomal cadherins (e.g., desmogleins and most likely desmocollins; see below) [1, 2], a group of transmembrane glycoproteins that are required to establish and maintain cell-cell adhesion between epidermal keratinocytes (reviewed in [3]). Desmogleins and desmocollins are transmembrane glycoproteins that are thought to establish cell coupling via binding of their extracellular domains. On the cytoplasmic surface of the plasma membrane, these cadherins are connected to the intermediate filament cytoskeleton via a network of desmosomal plaque proteins (desmoplakin, plakoglobin, plakophilins).

How does the binding of pathogenic pemphigus autoantibodies induce intraepithelial blistering, the characteristic histopathological feature of the disease? Do these antibodies inhibit the function of these proteins? A simple approach to test this hypothesis is to eliminate the target protein in genetically engineered mice and to determine whether the resulting loss-of-function phenotype replicates the disease.

Pemphigus vulgaris (PV) patients develop autoantibodies that target desmoglein (DSG) 3 and under certain conditions desmoglein (DSG) 1 [1]. Patients with DSG3 antibodies alone develop mucous membrane lesions whereas, in the presence of both DSG3 and DSG1 antibodies, skin lesions are observed as well [1]. Furthermore, it has been shown that injecting DSG3-specific PV antibodies into newborn mice can replicate the histopathology of the disease, demonstrating the pathogenicity of these antibodies (see [1]).

Based on the assumption that autoantibodies neutralize adhesive functions, one would predict that loss of *Dsg3* function in mice would mimic the phenotype of PV restricted to mucous membranes. To test this hypothesis, we generated *Dsg3* null mice.

2. Null Mutation in *Dsg3* Provided First Functional Link to PV

Mice with a targeted disruption of *Dsg3* exhibited phenotypes very similar to those seen in PV patients and provided direct evidence for a role of DSG3 in maintaining cell-cell adhesion between keratinocytes. A hallmark feature of PV in humans is acantholysis (loss of cell-cell adhesion) just above the basal layer in stratified epithelia. Furthermore, basal keratinocytes often lose lateral cell contact, leading to a histological finding that has been called a "row of tombstones". The *Dsg3*-null mice developed severe erosions of the oral mucosa (similar to that seen in PV patients) that prevented them from feeding thus causing runting. Suprabasal blistering was also evident in other stratified epithelia such as the vaginal epithelium (see below). Overt skin lesions were not noted in these mice, except for areas exposed to significant mechanical stress such as the skin around the snout, the nipples of nursing females, and the muco-cutaneous junctions in the eyes [4, 5]. A histological examination of other mouse tissues that express DSG3, such as the esophagus, the forestomach (which is lined by a stratified epithelium structurally similar to the epidermis), and the thymus, did not reveal abnormalities. Note that the forestomach is a characteristic feature of the mouse which is not present in humans.

A likely explanation for the absence of lesions in these tissues lies in the expression of functionally redundant desmoglein isoforms which compensate for the loss of DSG3. This idea is consistent with the compensation hypothesis, put forward by John Stanley and colleagues (see [2]), to explain the tissue-specificity of pemphigus autoantibodies. Based on this idea, one would predict that DSG3 is the predominant or the only DSG isoform expressed in the area where acantholysis occurs (e.g., in the deep layers of the oral mucosa). Further, extensive overlap between DSG3 and other DSG isoforms should exist in tissues and cell layers where no spontaneous lesions were detected. The distribution of the three major DSG isoforms (DSG1–3) in affected (mucous membranes) and unaffected tissues (e.g., skin) appears to be largely consistent with this idea as illustrated in Figure 1. In the skin of mice, DSG3 is restricted to the basal and immediate suprabasal cell layers, whereas DSG1 and 2 are present throughout the epithelium. Thus, DSG1 and 2 ensure that cell-cell adhesion is maintained in *Dsg3* null skin (Figure 1). Nevertheless, traumatizing the skin by rubbing or scratching resulted in lesions, indicating that in the absence of DSG3, the mechanical strength of the epithelial tissue was compromised. In mucous membranes, DSG1 and 2 are present throughout the epithelium but are weaker in the deep epidermis where DSG3 dominates as judged by immunohistochemical staining (Figure 1(b)), thus explaining the PV-like acantholysis observed in *Dsg3* null oral mucosa.

3. Loss of *Dsc3* and *Dsg3* Lead to Similar Histopathology but Target Different Tissues

Desmosomal adhesion is thought to be mediated both by homophilic as well as by heterophilic interactions between desmogleins and desmocollins, which form the adhesive core of desmosomes (e.g., [6, 7]). The relative contributions of homophilic (DSG-DSG; DSC-DSC) and heterophilic (DSG-DSC) interactions to establish and maintain cell adhesion are currently not known. In the case of DSG3, it had originally been speculated that a DSG3-DSC3 complex might be essential to maintain keratinocyte adhesion in the deep layers of stratified epithelia (for an alternative view see [6]). We thus hypothesized that ablating *Dsc3* in mice might mimic the effects of loss of DSG3, leading to PV-like histopathology.

Since germline deletion of the *Dsc3* gene was embryonic lethal [8], conditional *Dsc3* null mutant mouse lines were generated that lacked DSC3 expression in basal keratinocytes of stratified epithelia, including skin and mucous membranes [9]. These tissue-specific *Dsc3* null mice developed skin blisters (Figures 2(a) and 2(b)) that were histologically similar to those found in humans with muco-cutaneous PV. In contrast to skin lesions in *Dsg3*-null mice (which were restricted to areas exposed to significant mechanical trauma), *Dsc3* null skin blisters developed spontaneously and were present in all skin samples of newborn mice that were analyzed. Nevertheless, the extent of blister formation varied between individual mice, most likely due to different degrees of mechanical stress to which the animals were exposed prior to tissue harvesting. Even small trauma resulted in extensive skin blistering in *Dsc3* null skin, a typical characteristic of PV in humans (Nikolsky's sign).

Unlike the lesions observed in *Dsg3*-null mutants (Figures 2(c) and 2(d)), lesions in the *Dsc3* null mice were restricted to the skin and were not present in internal stratified epithelia, such as those of the oral cavity. As in the case of the *Dsg3*-null mice, we believe that the restriction of the blistering phenotype to the skin can be explained by the expression of compensatory proteins, in this case DSC isoforms. In the skin, DSC3 is present throughout the epithelium with weaker expression levels in the granular layers (Figure 1). DSC1 is mainly restricted to the granular cell layer of the interfollicular epidermis. The distribution of DSC2 in the mouse epidermis is currently not known, due to the lack of antibodies that recognize the mouse isoform. Nevertheless, in humans and cows, it is known that *Dsc2* is only weakly expressed or even absent in most of the interfollicular epidermis, with the notable exception of the palms and soles (see [9]). Given that the DSC1 and DSC3 expression patterns are very similar in mice and humans, it is reasonable to speculate that the distribution of DSC2 is also very similar in both species. Consequently, DSC3 would be the major DSC isoform expressed in the deep layers of the epidermis, thus explaining why loss of *Dsc3* causes acantholysis in these cell layers. How to explain the absence of lesions in the oral cavity of *Dsc3* mutants? We believe that the key to understanding the tissue specificity of these lesions is the distribution of DSC2. Data from bovine samples indicated that *Dsc2* is strongly expressed throughout all layers of internal stratified epithelia, such as tongue [10–13]. The combination of DSC1 and DSC2 is thus likely to maintain cell-cell adhesion in oral mucosa in the absence of DSC3 (Figure 1, and data not shown). Nevertheless, little is known regarding the molecular (homophilic and

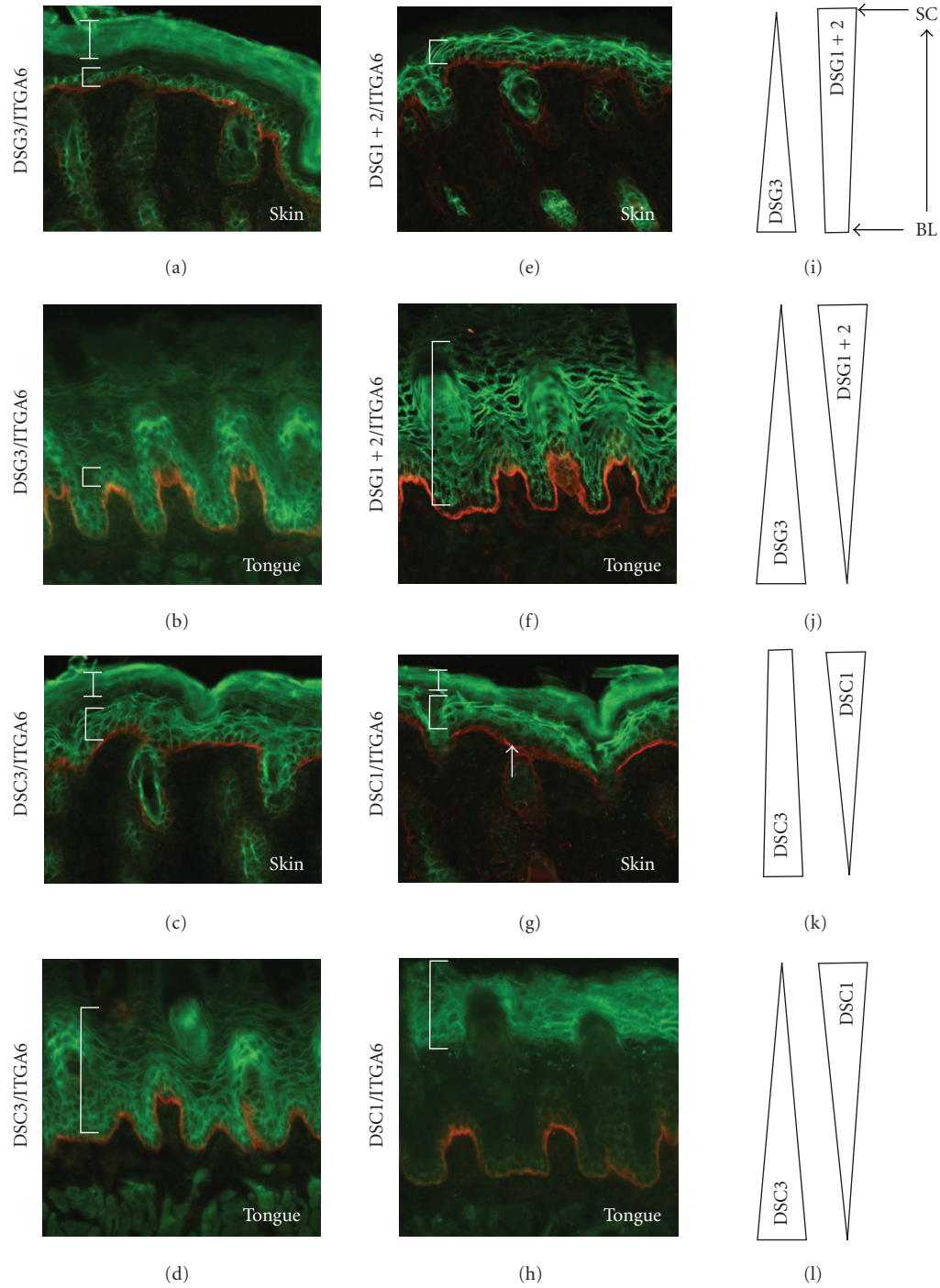

Figure 1: Expression of the desmosomal cadherins desmoglein 1, 2, and 3 (DSG1–3) and desmocollin 1 and 3 (DSC1, 3) as well as α6-integrin (ITGA6) in the interfollicular epidermis and the tongue epithelium of mice: Immunofluorescence staining of newborn epidermis ((a), (e), (c), (g)) and adult tongue ((b), (d), (f), (h)). The cadherins are shown in green while the integrin is shown in red. ((i), (j), (k), (l)) Schematic representation of the distribution of each desmosomal cadherin in the epidermis ((i), (k)) and tongue epithelium ((j and l)). Note that DSC2 antibodies which recognize the mouse isoform are currently not available (see text for details). Immunofluorescence signals from the stratum corneum are due to nonspecific binding of secondary antibodies (*white bar*). Cell layers expressing the relevant proteins are marked with brackets. The white arrow in (g) points towards the basal layer, which does not synthesize DSC1. Note that the expression of DSG1 + 2 in the tongue is low in the basal layers. (i, j, k, l) Distribution of the desmosomal cadherins in stratified epithelia (BL, basal layer; SC, stratum corneum). High expression levels are symbolized by a broad base and low expression levels are symbolized by narrow tips.

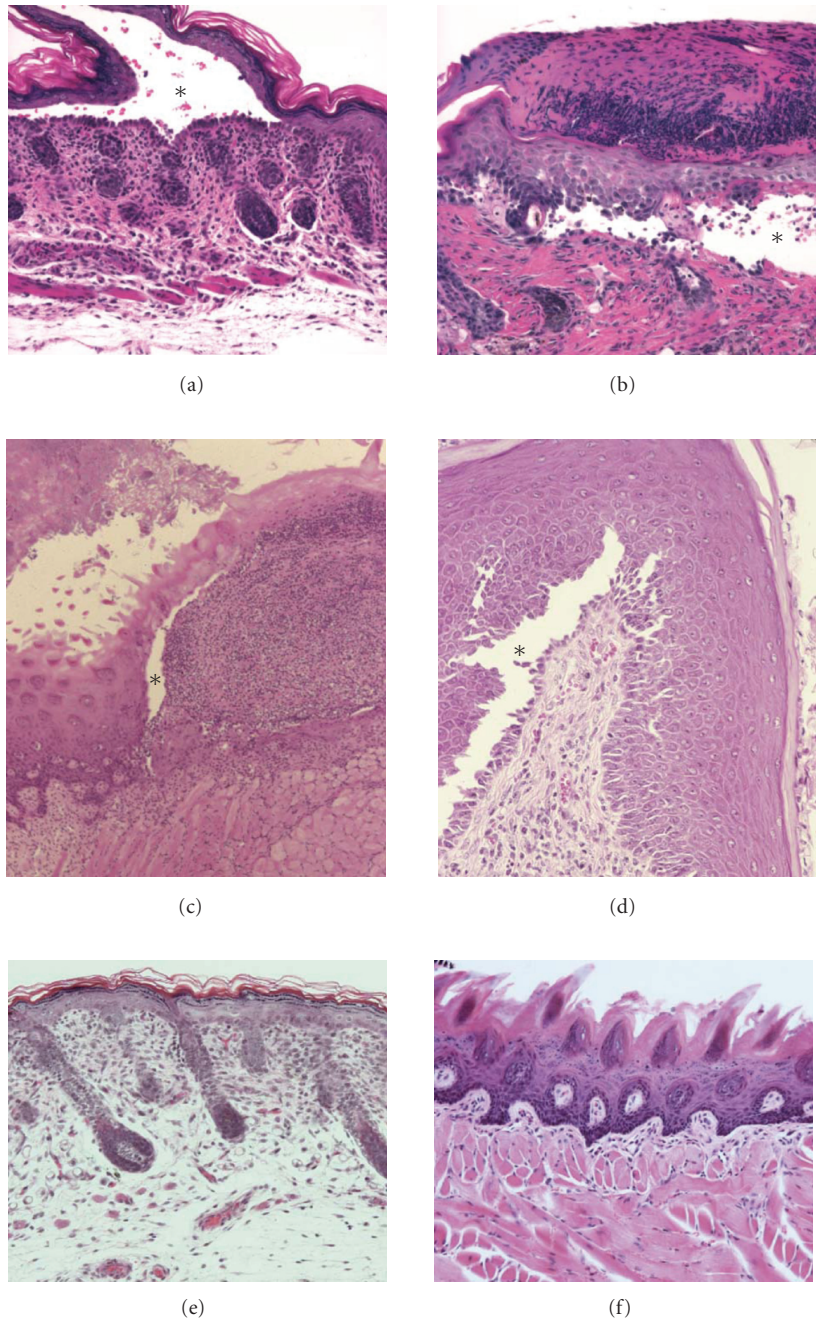

Figure 2: Acantholysis in the interfollicular epidermis and mucous membranes of *Dsc3* and *Dsg3* null epithelia. (a) Acantholysis between the basal and first suprabasal layer in the back skin epidermis of newborn conditional *Dsc3* null mice ($Dsc3^{fl/fl}$/K14-Cre). (b) Severe skin lesions of a 140 day-old $Dsc3^{fl/fl}$/K14-Cre mouse showing blistering in a healing wound. Note that these mice enter a cycle in which acantholysis triggers epithelial tongue formation and secondary blistering in the epithelium that covers the original wound. (c) Tongue section from a 25-day-old *Dsg3* null mouse showing acantholysis and massive inflammation in the epithelium. (d) Vagina of a 6-month-old *Dsg3* null mouse showing acantholysis in the deep epithelium (between basal and suprabasal layer). (e) Back skin of an adult wild type mouse. (f) Tongue histology of an adult wild type mouse. Stars indicate blister cavities.

heterophilic) interactions between desmosomal cadherins in vivo; that is, other models of compensation are possible. Given that homophilic interactions between desmosomal cadherins might occur in vivo, it is also possible that DSG3-DSG3 interactions alone might be sufficient to maintain cell adhesion in the *Dsc3* null mucosa.

The data summarized above suggest that DSG3 and DSC3 have comparable functions in desmosomes. However, they have different roles in cell adhesion of specific tissues, due to the presence of different compensatory proteins in different tissues. Thus, whereas DSG3 appears to be essential for maintaining cell-cell adhesion of internal stratified

epithelia, DSC3 plays a similar role in the epidermis of the skin.

4. Null Mutations in Desmosomal Cadherins Cause Hair Loss

Interestingly, both desmosomal cadherins are required to anchor hair follicles in the skin, as demonstrated by the cyclic hair loss observed both in *Dsg3* null and *Dsc3* null mice (Figure 3). The hair loss in both mutants was initiated around the time of weaning and progressed from the top of the head to the tail. This hair loss was never observed in wild type litter mates, that is, this phenotype was strictly linked to the null mutations in the two desmosomal cadherin genes. Further, loss of telogen hair, as demonstrated by tape hair-stripping experiment (Figures 5(c) and 5(g)), was observed only in the mutant animals and not in wild type littermates. Histology of the bald areas of the skin revealed intraepithelial blistering affecting the two keratinocyte cell layers surrounding the telogen club hair (Figures 3(b) and 3(f)). Acantholysis led to a loss of the hair shaft followed by formation of empty dermal cysts (Figures 3(d) and 3(h)). Nevertheless, the hair in these animals grew back and was lost again in the next telogen phase. This cycle repeated itself several times. Eventually, some of the older mice remained bald, possibly due to hair follicle stem cell depletion.

The patterned loss of hair is due to synchronization of the hair cycle in mice; around the time of weaning hair follicles progress from the first postnatal anagen (the active phase of hair growth cycle) to telogen (resting phase of the hair growth cycle). Further, the wave of hair follicle cycling proceeds from head to tail of the mouse. In rare instances, hair loss has been reported in PF and PV patients [5, 14], suggesting that the mouse models discussed here mimic a human disease phenotype. However, due to the synchronization of the mouse hair cycle (which does not occur in humans), this effect is much more severe in desmoglein and desmocollin null mice than in humans with the autoimmune disease.

5. Dissecting the Roles of Desmosomal Cadherins in Cell Adhesion, Signaling, and Skin Disorders

The *Dsg3* null mice do not develop an autoimmune disease; that is, they do not provide us with a tool to dissect autoantibody-mediated pathology. Nevertheless, these mice enabled us for the first time to link the loss of DSG3 function to PV histopathology. In other words, we were able to functionally identify DSG3 as a likely key target in a subset of pemphigus patients. Subsequent to the development of the *Dsg3*-null mouse model, Amagai and colleagues utilized this mouse line to develop an autoimmune model for PV in mice [15], demonstrating the usefulness of genetically engineered mouse models for understanding the molecular pathology of human diseases. Nevertheless, the mechanism by which PV autoantibodies induce loss of DSG3 function and cause PV is still a controversial issue. The hypotheses range from direct inhibition of desmoglein function to indirect loss of cell-cell adhesion. For instance, PV antibodies appear to trigger aberrant intracellular signaling in several pathways. Several reports have provided a link between pemphigus and abnormal signaling mediated by p38 MAPK pathway and by plakoglobin ([16, 17], see [3]). Based on studies that used cultured keratinocytes, it appears that pemphigus autoantibodies trigger phosphorylation of p38 MAPK which in turn induces acantholysis by affecting downstream effectors such as RhoA or HSP27 [16, 18]. Interestingly, inhibitors of p38 MAPK activation could prevent skin blister formation in newborn mice injected with PV autoantibodies (passive antibody transfer model for PV), which suggests that signaling mechanisms involving p38 MAPK are central to acantholysis in pemphigus. Results from another group implicated depletion of the cellular plakoglobin pool leading to increased expression of c-Myc to be critical for PV pathogenesis [17, 19]. Although the role of c-Myc signaling in acantholysis is not clearly understood, the fact that c-Myc inhibitors abrogated skin blistering induced by PV autoantibodies illustrates that this mechanism could be important in PV pathogenesis. It, therefore, appears that the mechanisms driving the loss of cell adhesion by impaired desmosomal function are more complex than previously believed.

Although it has been well established that DSG3 plays a key role in the development of PV, less is known about a potential role of desmocollins in autoimmune diseases. DSC1 has been linked to IgA pemphigus (see [3]), whereas a role for DSC3 in autoimmune diseases has not yet been established. The phenotypic consequences of a *Dsc3* null mutation in stratified epithelia of mice suggest that loss of function of this protein, either due to mutations or due to autoantibodies, would result in PV-like lesions. Recent reports suggest that this might indeed be the case. Bolling and colleagues reported the case of a patient with PV-like symptoms who had developed DSC3- but not DSG3-autoantibodies [20]. This report did not establish, however, whether the DSC3-specific antibodies from this patient were pathogenic. Further, Ayub and colleagues recently reported a *DSC3* mutation in a family in Afghanistan that was associated with hair loss and recurrent skin blistering [21], that is, a phenotype strikingly similar to the phenotype of our *Dsc3* mutant mice. Nevertheless, this report did not provide histological data demonstrating intraepidermal blistering; that is, further evidence is required to convincingly link impaired DSC3 function to intraepidermal blistering in humans.

Taken together, our mouse study demonstrated that loss of *Dsc3* function can lead to PV-like lesions while the human studies cited above suggest that this finding is relevant for at least a subgroup of patients with PV-like disease. Further studies will be necessary to determine the extent to which DSC3 plays a role in inherited and acquired desmosomal diseases.

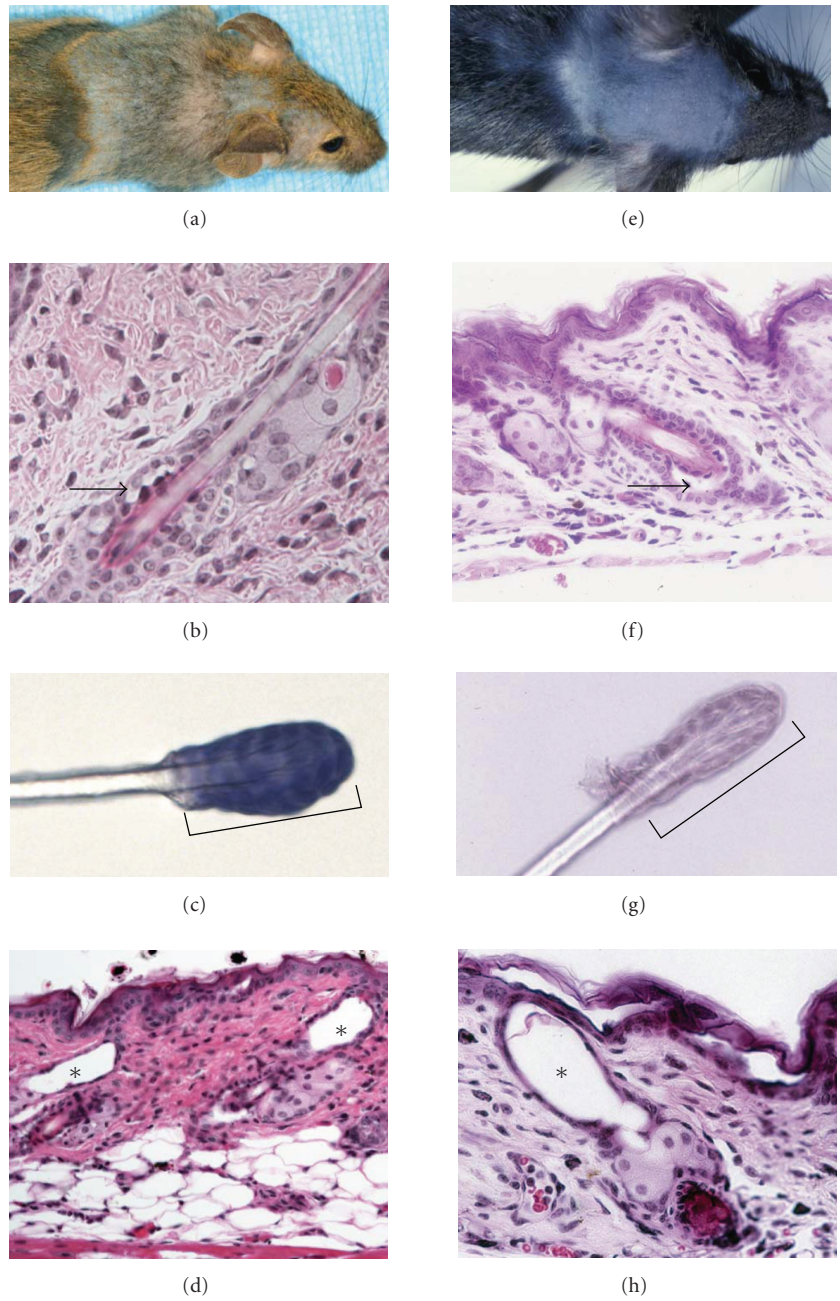

Figure 3: Hair loss phenotype of conditional *Dsc3* null ((a), (b), (c), (d)) and conventional *Dsg3* null mice ((e), (f), (g), (h)). Both mouse lines show cyclic hair loss beginning around the time of weaning when the hair follicles on the back of the head enter telogen, the resting phase of the hair growth cycle. (a) Hair loss of a 56-day-old conditional *Dsc3* null mouse and (b) a 53-day-old *Dsg3* null mouse. (b and f) Acantholysis between the two cell layers surrounding the telogen hair club leads to loss of the hair shaft. Arrows indicate separation of the two cell layers. *Note that early lesions are shown, that is, before the hair shaft is actually lost*. (c and g) Telogen hairs with a single epithelial cell layer surrounding the club hair (brackets, nuclei of the epithelial sheet surrounding the clubs are stained in dark (c) and light (g) blue, resp.). In both cases, hair loss leads to the development of dermal cysts ((d and h); stars). Note that the hair loss in both mouse lines appears to follow the same mechanism, that is, loss of cell-cell adhesion between the two epithelial cell layers which anchor the telogen hair in the skin.

6. Conclusions

The last decade has seen a surge of information regarding the roles of individual desmosomal genes in normal development and diseases based on the analysis of transgenic and knockout mice. The ability to switch genes on or off in specific cell types and tissues at predetermined time points has made the mouse model a premier tool for discovering gene functions and for the elucidation of disease mechanisms. Moreover, the core genetic and physiologic pathways that control epithelial cell differentiation (including appendage development) are highly conserved

between humans and mice. Although the ease of genetic manipulations and the resemblance to human physiology make mice an attractive choice to study skin disorders, there are potential pitfalls, such as the possibility of species-specific differences in the histology of the skin. Further, it is also not possible to exclude the existence of species-specific gene functions, requiring confirmation of experimental results in a human test system. Nevertheless, the mouse will remain a valuable tool for the analysis of human diseases and for the advancement in our understanding of basic epithelial cell biology.

Acknowledgments

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References

[1] J. R. Stanley and M. Amagai, "Pemphigus, bullous impetigo, and the staphylococcal scalded-skin syndrome," *The New England Journal of Medicine*, vol. 355, no. 17, pp. 1800–1810, 2006.

[2] M. Amagai, A. R. Ahmed, Y. Kitajima, et al., "Are desmoglein autoantibodies essential for the immunopathogenesis of pemphigus vulgaris, or just "witnesses of disease"?" *Experimental Dermatology*, vol. 15, no. 10, pp. 815–831, 2006.

[3] A. Schmidt and P. J. Koch, "Desmosomes in development and diseases," in *LaFlamme S. Cell Junctions: Adhesion, Development and Disease*, A. Kowalczyk, Ed., pp. 235–249, Wiley-VCH, Weinheim, Germany.

[4] P. J. Koch, M. G. Mahoney, H. Ishikawa, et al., "Targeted disruption of the pemphigus vulgaris antigen (desmoglein 3) gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris," *Journal of Cell Biology*, vol. 137, no. 5, pp. 1091–1102, 1997.

[5] P. J. Koch, M. G. Mahoney, G. Cotsarelis, K. Rothenberger, R. M. Lavker, and J. R. Stanley, "Desmoglein 3 anchors telogen hair in the follicle," *Journal of Cell Science*, vol. 111, no. 17, pp. 2529–2537, 1998.

[6] V. Spindler, W.-M. Heupel, A. Efthymiadis, et al., "Desmocollin 3-mediated binding is crucial for keratinocyte cohesion and is impaired in pemphigus," *The Journal of Biological Chemistry*, vol. 284, no. 44, pp. 30556–30564, 2009.

[7] N. A. Chitaev and S. M. Troyanovsky, "Direct $Ca2^{+}$-dependent heterophilic interaction between desmosomal cadherins, desmoglein and desmocollin, contributes to cell-cell adhesion," *Journal of Cell Biology*, vol. 138, no. 1, pp. 193–201, 1997.

[8] Z. Den, X. Cheng, M. Merched-Sauvage, and P. J. Koch, "Desmocollin 3 is required for pre-implantation development of the mouse embryo," *Journal of Cell Science*, vol. 119, no. 3, pp. 482–489, 2006.

[9] J. Chen, Z. Den, and P. J. Koch, "Loss of desmocollin 3 in mice leads to epidermal blistering," *Journal of Cell Science*, vol. 121, no. 17, pp. 2844–2849, 2008.

[10] P. J. Koch, M. D. Goldschmidt, R. Zimbelmann, R. Troyanovsky, and W. W. Franke, "Complexity and expression patterns of the desmosomal cadherins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 1, pp. 353–357, 1992.

[11] D. G. Theis, P. J. Koch, and W. W. Franke, "Differential synthesis of type 1 and type 2 desmocollin mRNAs in human stratified epithelia," *International Journal of Developmental Biology*, vol. 37, no. 1, pp. 101–110, 1993.

[12] J. E. Lorimer, L. S. Hall, J. P. Clarke, J. E. Collins, T. P. Fleming, and D. R. Garrod, "Cloning, sequence analysis and expression pattern of mouse desmocollin 2 (DSC2), a cadherin-like adhesion molecule," *Molecular Membrane Biology*, vol. 11, no. 4, pp. 229–236, 1994.

[13] I. A. King, B. D. Angst, D. M. Hunt, M. Kruger, J. Arnemann, and R. S. Buxton, "Hierarchical expression of desmosomal cadherins during stratified epithelial morphogenesis in the mouse," *Differentiation*, vol. 62, no. 2, pp. 83–96, 1997.

[14] A. Mlynek, M. Bar, A. Bauer, and M. Meurer, "Juvenile pemphigus foliaceus associated with severe nonscarring alopecia," *The British Journal of Dermatology*, vol. 161, no. 2, pp. 472–474, 2009.

[15] M. Amagai, K. Tsunoda, H. Suzuki, K. Nishifuji, S. Koyasu, and T. Nishikawa, "Use of autoantigen-knockout mice in developing an active autoimmune disease model for pemphigus," *Journal of Clinical Investigation*, vol. 105, no. 5, pp. 625–631, 2000.

[16] P. Berkowitz, P. Hu, Z. Liu, et al., "Desmosome signaling: inhibition of p38MAPK prevents pemphigus vulgaris IgG-induced cytoskeleton reorganization," *The Journal of Biological Chemistry*, vol. 280, no. 25, pp. 23778–23784, 2005.

[17] L. Williamson, N. A. Raess, R. Caldelari, et al., "Pemphigus vulgaris identifies plakoglobin as key suppressor of c-Myc in the skin," *EMBO Journal*, vol. 25, no. 14, pp. 3298–3309, 2006.

[18] J. Waschke, V. Spindler, P. Bruggeman, D. Zillikens, G. Schmidt, and D. Drenckhahn, "Inhibition of Rho A activity causes pemphigus skin blistering," *Journal of Cell Biology*, vol. 175, no. 5, pp. 721–727, 2006.

[19] L. Williamson, T. Hunziker, M. M. Suter, and E. J. Muller, "Nuclear c-Myc: a molecular marker for early stage pemphigus vulgaris," *Journal of Investigative Dermatology*, vol. 127, no. 6, pp. 1549–1555, 2007.

[20] M. C. Bolling, J. R. Mekkes, W. F. M. Goldschmidt, C. J. M. Van Noesel, M. F. Jonkman, and H. H. Pas, "Acquired palmoplantar keratoderma and immunobullous disease associated with antibodies to desmocollin 3," *British Journal of Dermatology*, vol. 157, no. 1, pp. 168–173, 2007.

[21] M. Ayub, S. Basit, M. Jelani, et al., "A homozygous nonsense mutation in the human desmocollin-3 (DSC3) gene underlies hereditary hypotrichosis and recurrent skin vesicles," *American Journal of Human Genetics*, vol. 85, no. 4, pp. 515–520, 2009.

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Research Article

Superficial Dsg2 Expression Limits Epidermal Blister Formation Mediated by Pemphigus Foliaceus Antibodies and Exfoliative Toxins

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Cell-cell adhesion mediated by desmosomes is crucial for maintaining proper epidermal structure and function, as evidenced by several severe and potentially fatal skin disorders involving impairment of desmosomal proteins. Pemphigus foliaceus (PF) and staphylococcal scalded skin syndrome (SSSS) are subcorneal blistering diseases resulting from loss of function of the desmosomal cadherin, desmoglein 1 (Dsg1). To further study the pathomechanism of these diseases and to assess the adhesive properties of Dsg2, we employed a recently established transgenic (Tg) mouse model expressing Dsg2 in the superficial epidermis. Neonatal Tg and wild type (WT) mice were injected with purified ETA or PF Ig. We showed that ectopic expression of Dsg2 reduced the extent of blister formation in response to both ETA and PF Ig. In response to PF Ig, we observed either a dramatic loss or a reorganization of Dsg1-α, Dsg1-β, and, to a lesser extent, Dsg1-γ, in WT mice. The Inv-Dsg2 Tg mice showed enhanced retention of Dsg1 at the cell-cell border. Collectively, our data support the role for Dsg2 in cell adhesion and suggest that ectopic superficial expression of Dsg2 can increase membrane preservation of Dsg1 and limit epidermal blister formation mediated by PF antibodies and exfoliative toxins.

1. Introduction

Desmogleins and desmocollins are the transmembrane adhesive components of intercellular junctions known as desmosomes. Four distinct desmogleins (Dsg 1-4) have been reported with unique expression profiles dependent on the tissue type and differentiation state [1, 2]. Unlike Dsg1 and Dsg3, whose expression is restricted to complex stratified epithelia, Dsg2 and Dsg4 are expressed in a wide range of other cell types. In stratified epithelia, such as the human epidermis, Dsg2 is expressed at low levels and is restricted to the proliferative basal cell layer while Dsg3 expression extends from the basal into the spinous cell layers. Conversely, Dsg1 and Dsg4 expression is driven by cell differentiation. Dsg1 is expressed from the immediate suprabasal layer up, with marked higher abundance in the differentiated granular cell layers. Dsg4 expression is restricted to the highly differentiated upper cell layers.

It is well established that desmosomal cadherins play a significant role in cell adhesion and tissue integrity, as they have been identified as the target proteins in several autoimmune, infectious, and inherited skin/hair fragility diseases [3]. Pemphigus is a devastating and debilitating autoimmune skin disease [4]. This disorder is generally characterized by the production of pathogenic antibodies targeting different components of cell-cell adhesion, in particular, Dsg1 and Dsg3. The autoantibodies commonly cause cell-cell disadhesion (acantholysis), as well as blisters of the skin and mucous membranes. In pemphigus foliaceus (PF), patients develop pathogenic autoantibodies that target Dsg1 and promote cell-cell disadhesion in the superficial epidermis, where Dsg1 is highly expressed, but which lacks Dsg3 and Dsg2. Passive transfers of purified PF Ig into newborn mice produce blisters similar to those observed in PF patients [5, 6]. Interestingly, in the toxin-mediated disease bullous impetigo (and its

generalized form staphylococcal scalded skin syndrome (SSSS)), the bacterium *S. aureus* produces exfoliative toxins (glutamic-specific serine proteases) that cleave Dsg1 between extracellular domains 3 and 4 [7]. Reminiscent of PF, SSSS patients develop superficial skin blisters [8]. Furthermore, newborn mice treated with exfoliative toxins develop skin blisters similar to those observed with the passive transfer of PF Ig. Both pathogenic pemphigus autoantibodies and exfoliative toxins target specific conformational epitopes found within the N-terminal extracellular domains of desmogleins [9]. These domains are believed to play an important role in cadherin-cadherin interaction, thereby alluding to the importance of desmogleins in controlling intercellular adhesion and in maintaining the structural stability and integrity of the epidermis [10–13].

To assess the ability of Dsg2 to enhance cell adhesion and to test the hypothesis that Dsg2 expression in the suprabasal epidermis can limit PF/ETA blister formation by increasing keratinocyte adhesion, we employed a transgenic mouse model expressing Dsg2 in the superficial epidermis under the involucrin promoter (Inv-Dsg2 Tg) [14]. We selected Dsg2 since it is not a pemphigus antigen [15] and does not appear to have the consensus sequence required for cleavage by exfoliative toxins. We subjected the Tg mice and their WT littermates to ETA and PF Ig treatments and assessed the extent of skin blister formation. These experiments allowed us to compare the relative intensity of the ETA- and PF-induced blister formation in the presence or absence of Dsg2 in the superficial epidermis. The results obtained here provide some insights into the pathomechanisms of diseases targeting Dsg1.

2. Results

2.1. Expression of Dsg2 in the Superficial Epidermis of Inv-Dsg2 Tg Mice. As previously described in detail, we generated Tg mice expressing Dsg2 in the superficial epidermis, under the control of the involucrin promoter [14]. Newborn Tg mice appeared normal, with no gross abnormalities of the skin or hair. Examination of the skin by histology revealed minor epidermal hyperplasia in newborn Tg mice compared to WT littermates (Figure 1(a)). We assessed the expression of the Dsg2-Flag transgene in skin from newborn Tg mice by immunostaining; antibodies against Flag and Dsg2 (MP6) (Figure 1(b)) showed expression of Dsg2-Flag in the superficial cell layers. In keeping with the literature, we observed some negligible expression of endogenous Dsg2 in the basal cell layer.

To confirm the immunoblotting results, we extracted total skin protein from WT and Tg skin in RIPA buffer and resolved it with SDS-PAGE. Immunoblotting with the MP6 and Flag antibodies detected bands of approximately 160 kDa in Tg skin lysates (Figure 1(c)). MP6, but not Flag, antibody picked up a weak signal for a similar sized band in the WT skin, which is indicative of low levels of endogenous Dsg2 in the newborn mouse skin. In summary, we generated transgenic mice expressing Dsg2 in the superficial epidermis of newborn mice.

2.2. Dsg2 Protects Skin from ETA-Mediated Blister Formation. It is well established that ETA cleaves Dsg1 and causes epidermal blisters in the upper layers of the epidermis, where Dsg1 is highly expressed [16]. Mice treated with purified ETA develop blisters similar to those seen in patients infected with *S. aureus*. To determine whether Dsg2 could compensate for the loss of Dsg1, we treated neonatal WT and Tg mice with purified ETA. In response to subcutaneous injection of ETA, we observed dramatic gross blisters in WT, but not Tg, mice (Figure 2(a)). However, the results from the histologic analysis were less definitive, since some Tg mice developed extensive blisters while others showed a resistance to blister formation (Figure 2(b), top panels).

Upon further analysis, we observed that the site of blister formation in WT mice after ETA treatment was superficial and occurred in the middle of the granular cell layer (Figure 2(b), lower left panel), where Dsg1 is highly expressed. However, in the Inv-Dsg2 Tg skin treated with ETA, the site of epidermal splitting was often just beneath the granular cell layer (Figure 2(b), lower right panel). These results suggest that coexpression of Dsg2 with Dsg1 in the superficial epidermis may protect from the loss of Dsg1 by ETA although we cannot rule out the possibility that ectopic expression of Dsg2 may also impair the ETA-digestion of Dsg1 molecules. However, the latter scenario is unlikely, as ETA is enzymatically efficient, and we did not observe any significant increase in uncleaved Dsg1 in ETA-treated Tg mice (see below).

The extent of the histologic blistering was graded on a scale from 0, for no blisters, to +4, for extensive blisters. We observed that overexpression of Dsg2 provided enhanced protection against blister formation in response to ETA (Figure 2(c)). To confirm that ETA cleaves mouse Dsg1 when injected into newborn WT and Tg mice, skin biopsies were taken after ETA treatment and the total cellular proteins were extracted in Laemmli buffer. Western blotting showed that ETA cleaves Dsg1, resulting in a smaller fragment approximately 113 kDa in both WT and Inv-Dsg2 Tg mouse skin (arrow, Figure 2(d)) [8, 16]. We previously showed that antibody 27B2 recognizes an epitope present in the cytoplasmic domain of all mouse Dsg1 (α, β, and γ) isoforms [17]. In that report, we also demonstrated that ETA cleaves only Dsg1-α and -β. Thus, the remaining full-length Dsg1 fragment left after ETA-treatment observed here is most likely Dsg1-γ, but may also show inefficient Dsg1-α or -β cleavage. Interestingly, we observed a slight increase in Dsg1 in the ETA treated Tg skin (Figure 2(d)). This increase in Dsg1 was reflected in a more intense 113 kDa band in response to ETA cleavage. Finally, we showed here that ETA did not cleave Dsg2.

To assess whether ectopic expression of Dsg2 in the Tg skin had an effect on Dsg1 in response to ETA, we performed immunofluorescence staining for Dsg1. We determined the expression pattern of Dsg1-α, -β, and -γ using antibodies specific against each Dsg1 isoform (Figure 3). These antibodies were generated against either synthetic peptides or recombinant proteins localized within the extracellular domain of Dsg1 and would thus detect the full-length, membrane-spanning product after ETA digestion

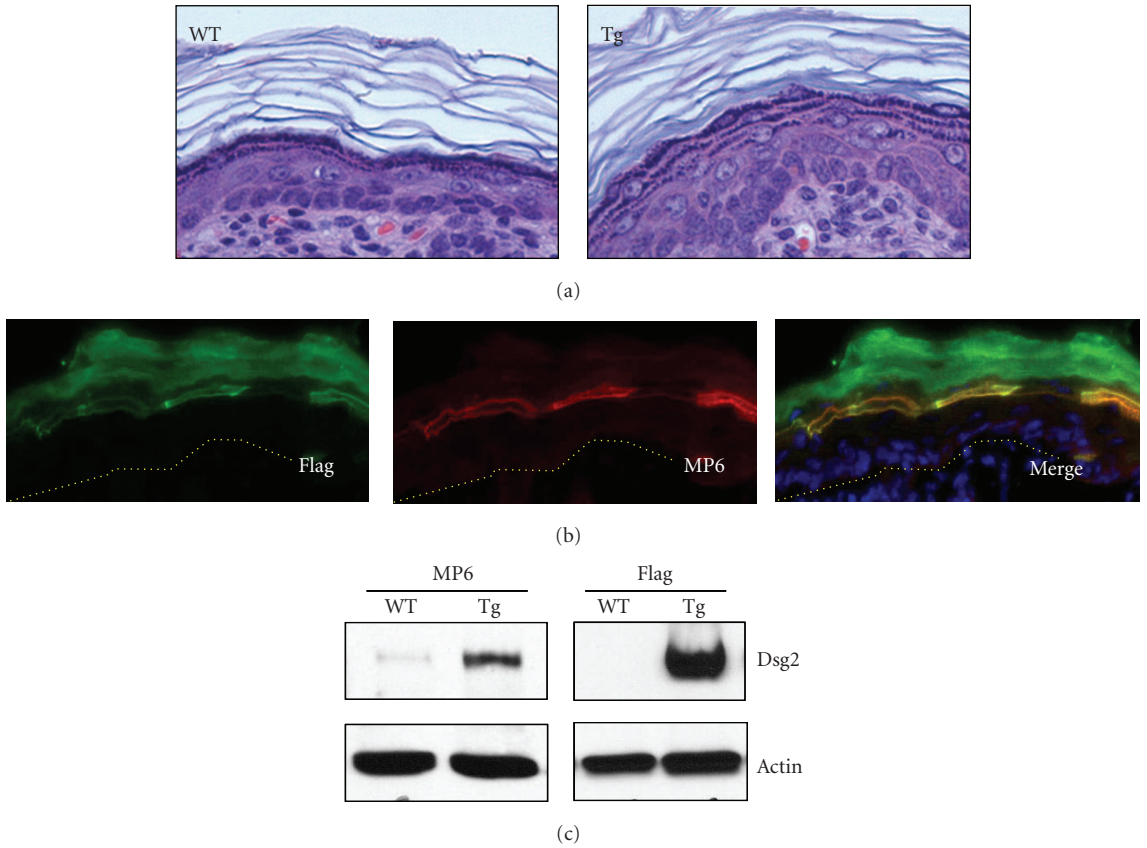

Figure 1: Suprabasal expression of Dsg2-Flag in newborn Inv-Dsg2 transgenic mice. (a) Histology showing slight epidermal hyperplasia in a newborn Inv-Dsg2 Tg overexpressing Dsg2 in the superficial epidermis under the involucrin promoter, but not in WT mice. (b) Immunostaining of newborn Tg skin with Flag (green) and Dsg2-specific MP6 (red) antibodies showing expression of Dsg2-Flag in the differentiated cell layers of the transgenic epidermis. (c) Immunoblot of WT and Tg skin with MP6 and Flag antibodies, showing Dsg2-Flag in the Tg but not WT skin. Actin was used for equal loading.

[17]. Despite the extensive cleavage of Dsg1 by ETA, as shown by Western blotting above, we did not observe a dramatic change in the Dsg1 pattern in either WT or Tg skin. Dsg1 remained intact at the cell-cell border. To further confirm these results, we immunostained ETA-treated WT and Tg skin with antibody 27B2, which was raised against the intracellular domain of human Dsg1 and recognizes all three mouse Dsg1s. Staining with 27B2 showed cell-cell border staining of Dsg1s even at the blister sites (Figure 3). Collectively, our results demonstrate that ectopic expression of Dsg2 could partially compensate for the loss of Dsg1-mediated adhesion in response to ETA digestion.

2.3. Dsg2 Protects against PF Ig-Mediated Blister Formation. Next, we wanted to assess whether ectopic expression of Dsg2 could protect against skin blister formation induced by PF pathogenic antibodies. We purified Ig from the sera of PF patients with the active disease and performed passive transfer of the PF Ig into newborn WT and Tg mice. We, and others, have shown that mice injected with normal human Ig do not develop skin blister formation [6]. In accordance with the literature, we observed extensive gross blisters in WT mice 18 hours after injection with PF Ig [6]. However, the Inv-Dsg2 Tg mice injected with PF Ig developed significantly less extensive blisters (Figure 4(a)). The severity of skin blistering was similar between gross observation and histological analysis (Figure 4(b)). Again similar to the blistering observed with ETA in Figure 2, we observed a slight downward shift in the site of blister formation in Tg, as compared to WT, skin (Figure 4(b), lower panels). The extent of histological blistering was then graded, and the results showed that Tg mice were less susceptible to blister formation in response to PF Ig (Figure 4(c)). Thus, ectopic expression of Dsg2 in the superficial epidermis rendered the Tg mice more resistant to PF Ig-induced skin blisters.

To test the effects of PF Ig on epidermal Dsg1, skin biopsies were collected after PF Ig treatment, and the total cellular proteins were extracted in Laemmli buffer. Western blotting showed that (1) up-regulation of Dsg2 did not alter the expression level of Dsg1 in newborn mouse skin, and (2) incubation with PF Ig reduced the level of Dsg1 (Figure 4(d)). Thus, our results demonstrate that, at the Western blot level, PF Ig depletes Dsg1 and that superficial expression of Dsg2 in Tg mice did not appear to modulate the level of Dsg1 in response to PF Ig.

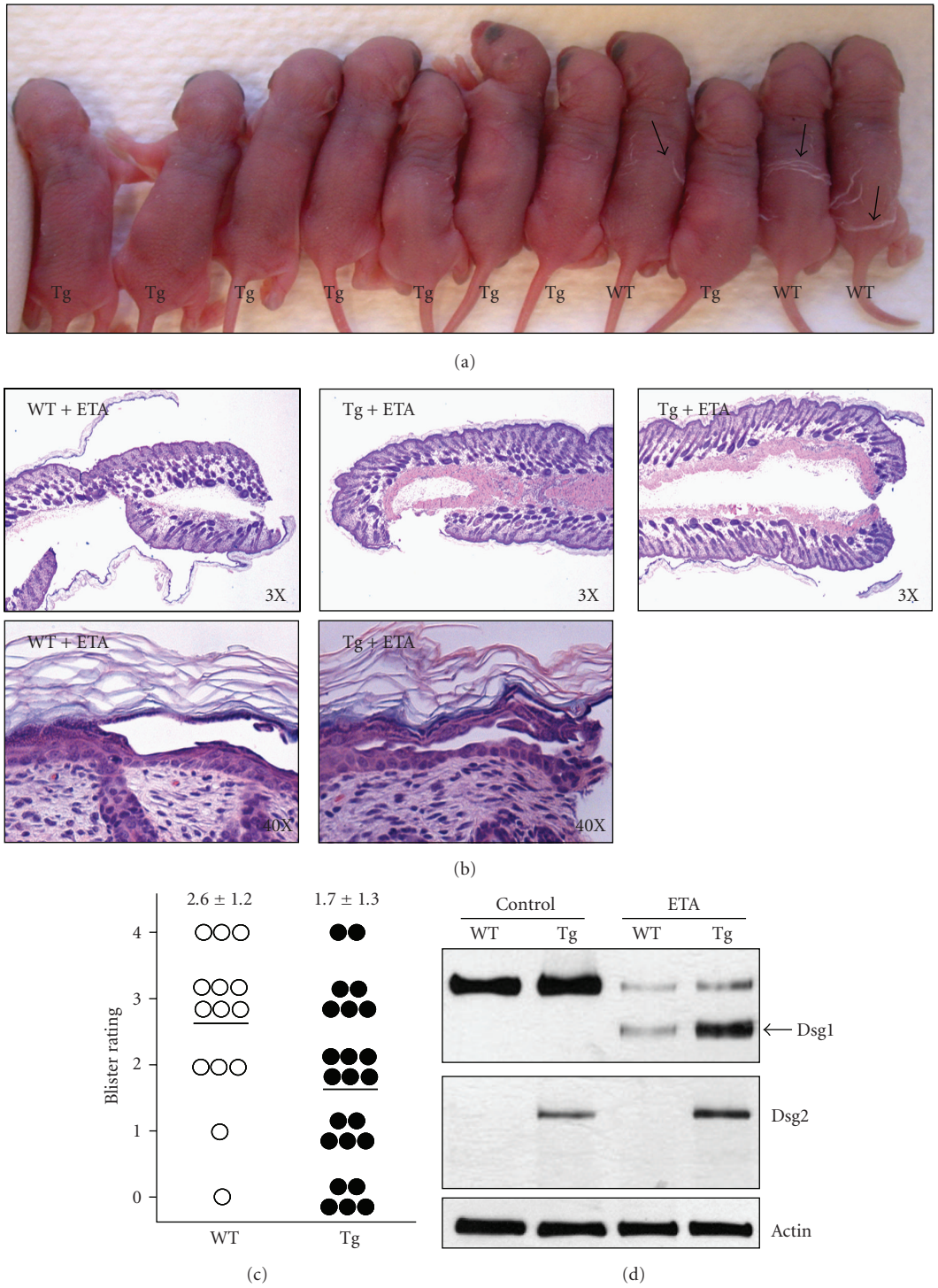

FIGURE 2: Superficial expression of Dsg2 offers protection from ETA-induced blister formation. (a) Newborn WT and Inv-Dsg2 Tg mice were injected subcutaneously with 0.5 μg ETA in PBS. Visible blisters were observed in WT but not Tg mice, 6–8 hours after ETA treatment. (b) Mice were sacrificed, and their skin was processed for histological analysis, revealing slightly more extensive blisters in the WT mice ($n = 14$), as compared to Tg mice ($n = 23$), in response to ETA. Top panels show 3X magnification, and lower panels show 40X magnification of the site of blister formation. (c) The extent of blistering was graded based on the following semiquantitative scale: 0: no blisters, 1+: minor blisters at the edge; 2+: localized blisters <50%; 3+: extensive blisters >50%; and 4+: very extensive blisters >75%. Each dot represents one mouse. The average blister scores were 2.6 ± 1.2 for WT and 1.7 ± 1.3 for Tg. These values were statistically significant, $P \leq .038260921$ (2-tailed unequal variance) or $\leq .01913046$ (1-tailed unequal variance). (d) The back skin biopsies were homogenized in Laemmli buffer, proteins were resolved with SDS-PAGE and immunoblotted for Dsg1 (27B2), Dsg2-Flag (Flag), and Actin (for equal loading). Western blotting demonstrates that ETA cleaved Dsg1 (arrowhead), but not Dsg2. *Note.* antibody 27B2 was raised against the cytoplasmic epitope of human Dsg1, and recognizes mouse Dsg1-a, -β, and -γ. Thus, the full-length signal is most likely ETA-resistant Dsg1-γ.

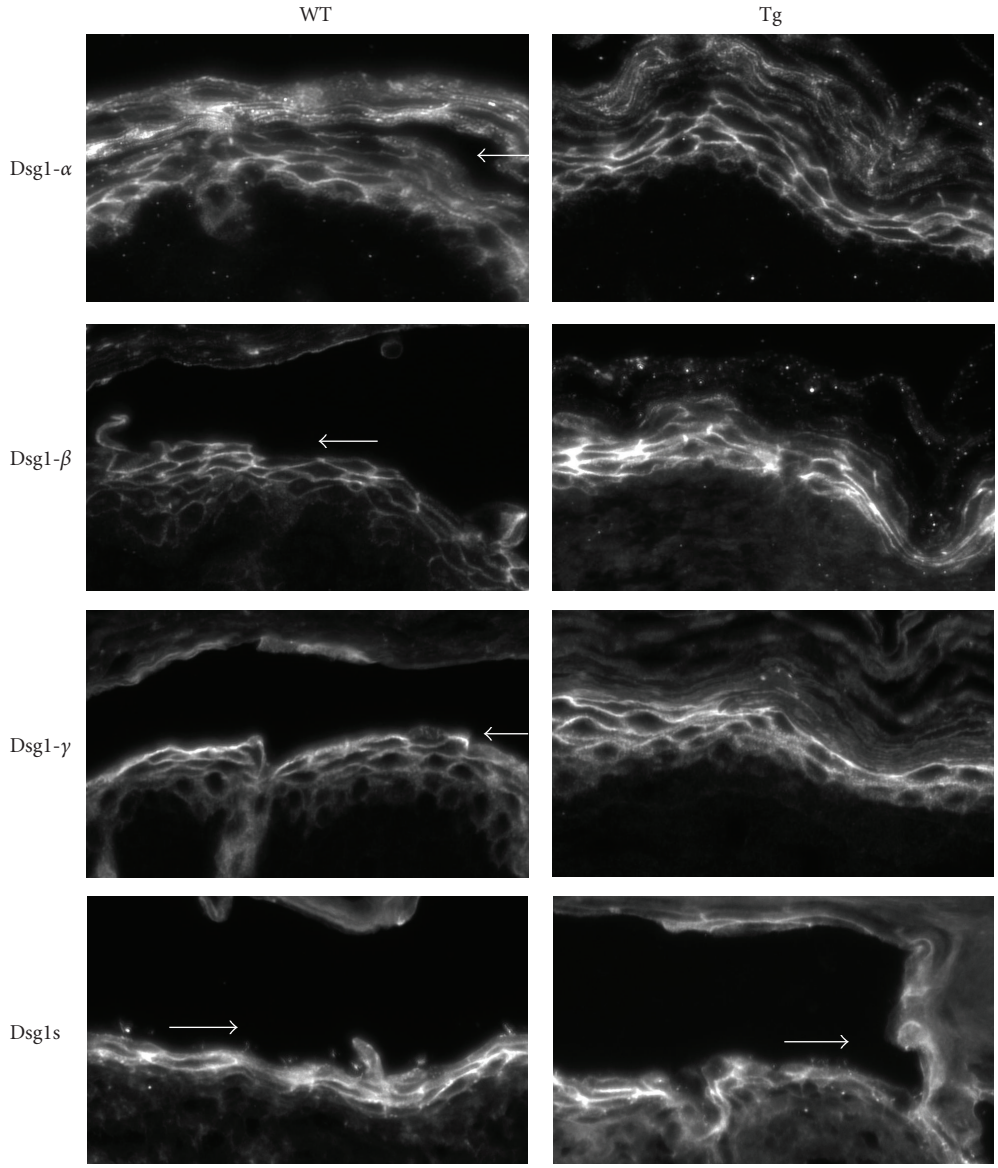

Figure 3: Dsg1 is maintained at the cell-cell border in ETA-treated epidermis. Formalin-fixed paraffin-embedded skin sections from newborn WT and Tg mice treated with ETA, as described in Figure 2, were immunostained with antibodies AP61, AP498, Ab15, and 27B2. Antibodies AP61, AP498, and Ab15 were raised against the extracellular domain of Dsg1-α, Dsg1-β, and Dsg1-γ, respectively [17]. Immunofluorescence shows undisturbed cell-cell border staining of all Dsg1 isoforms in both WT and Tg skins treated with ETA. DAPI (blue) was used as a nuclear stain. Similar results were observed in skin treated with ETA for 18 hours. Arrows demarcate site of blister cleavage.

Next, we wanted to evaluate whether or not superficial expression of Dsg2 had an effect on Dsg1 fate and localization in response to PF Ig. We first confirmed, by direct immunostaining, the presence of human antibodies in the skin of mice treated with PF Ig (Figure 5(a), left panels). The human Ig was detected in the epidermis of both WT and Tg skin. In WT skin, staining for human antibodies was diffuse and was disrupted by some cytoplasmic staining. Interestingly, in the Tg epidermis, the staining for human antibodies was clearly at the cell-cell borders, suggesting, perhaps, the presence of more intact desmosomes (Figure 5(a), lower left panel). We also immunostained the same tissues for Dsg2-Flag to demonstrate that Tg, but not WT, mice expressed the Dsg2-Flag transgene (Figure 5(a), middle panels). Double labeling (Figure 5(a), right panels) showed colocalization (yellow) of Dsg2 (green) and human Ig (red) at the cell-cell border in the superficial epidermis (lower right panel).

Next, we assessed the expression and localization of Dsg1 (α, β, and γ) by indirect immunofluorescence (Figure 5(b)). In WT animals, PF Ig treatment induced extensive redistribution of Dsg1-α and Dsg1-β from a uniform cell-cell border pattern to a disrupted granular pattern, which is indicative of potential dissolution of the desmosomes (Figure 5(b), left panels). The dissolution occurred throughout the epidermis

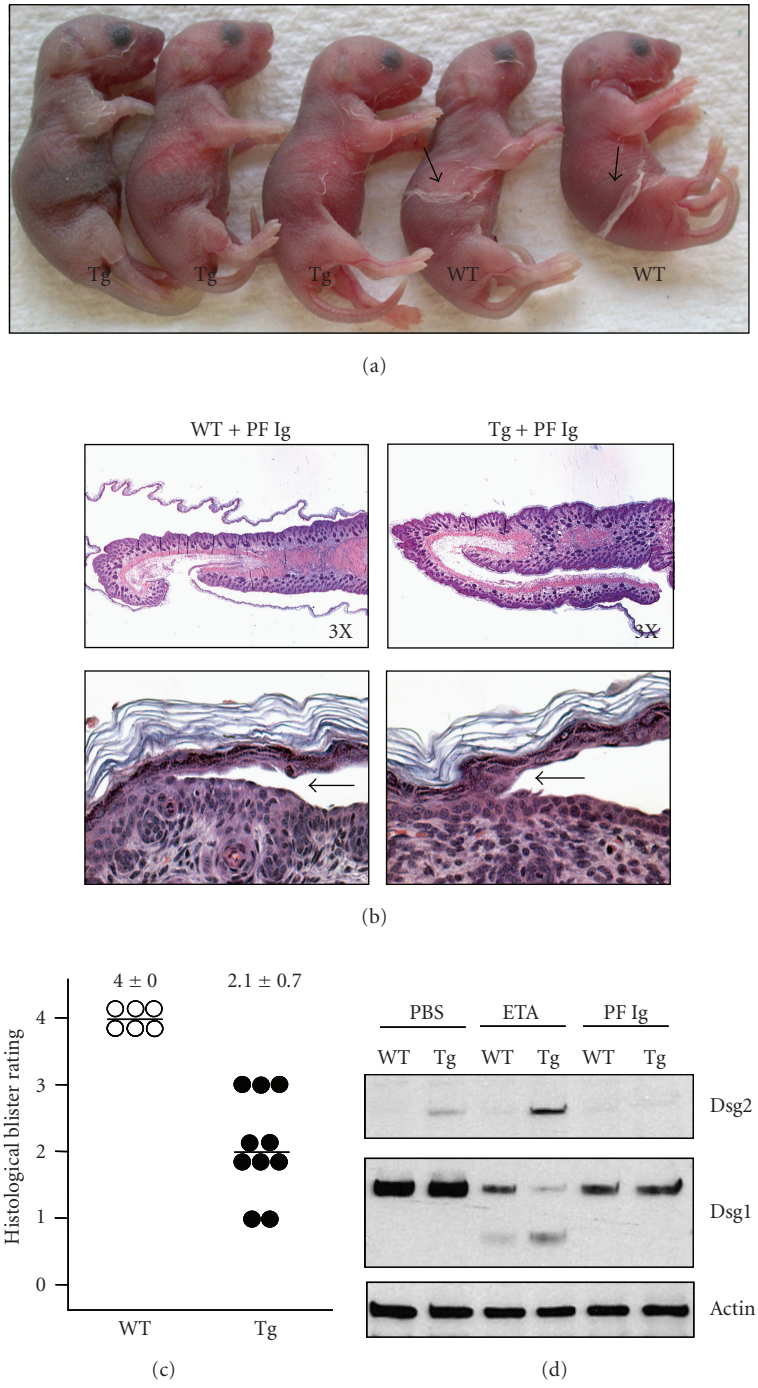

Figure 4: Dsg2 offers protection against PF Ig-induced acantholysis. (a) Newborn WT and Inv-Dsg2 Tg mice were injected subcutaneously with 10 mg of purified PF Ig for 18 hours. Gross blisters were more pronounced in WT mice, as compared to Tg mice. (b) Mice were sacrificed, and their skin was processed for histology, revealing more dramatic blisters in the WT ($n = 6$) than in Tg mice ($n = 10$). Top panels show 3X magnification, and lower panels show 40X magnification of the site of blister formation (arrows). (c) The extent of blistering was graded as described in Figure 2. Each dot represents one animal. The average blister scores were 4.0 ± 0.0 for WT mice and 2.1 ± 0.7 for Tg mice. These values were statistically significant, $P \leq .000022$ (2-tailed unequal variance) or $\leq .000012$ (1-tailed unequal variance). (d) Back skin biopsies of WT and Tg mice treated with PF Ig were homogenized in Laemmli buffer, the proteins were resolved with SDS-PAGE and immunoblotted for Dsg1 (27B2), Dsg2, and Actin. Western blotting demonstrates that superficial Dsg2 slightly enhances retention of Dsg1 in response to PF Ig.

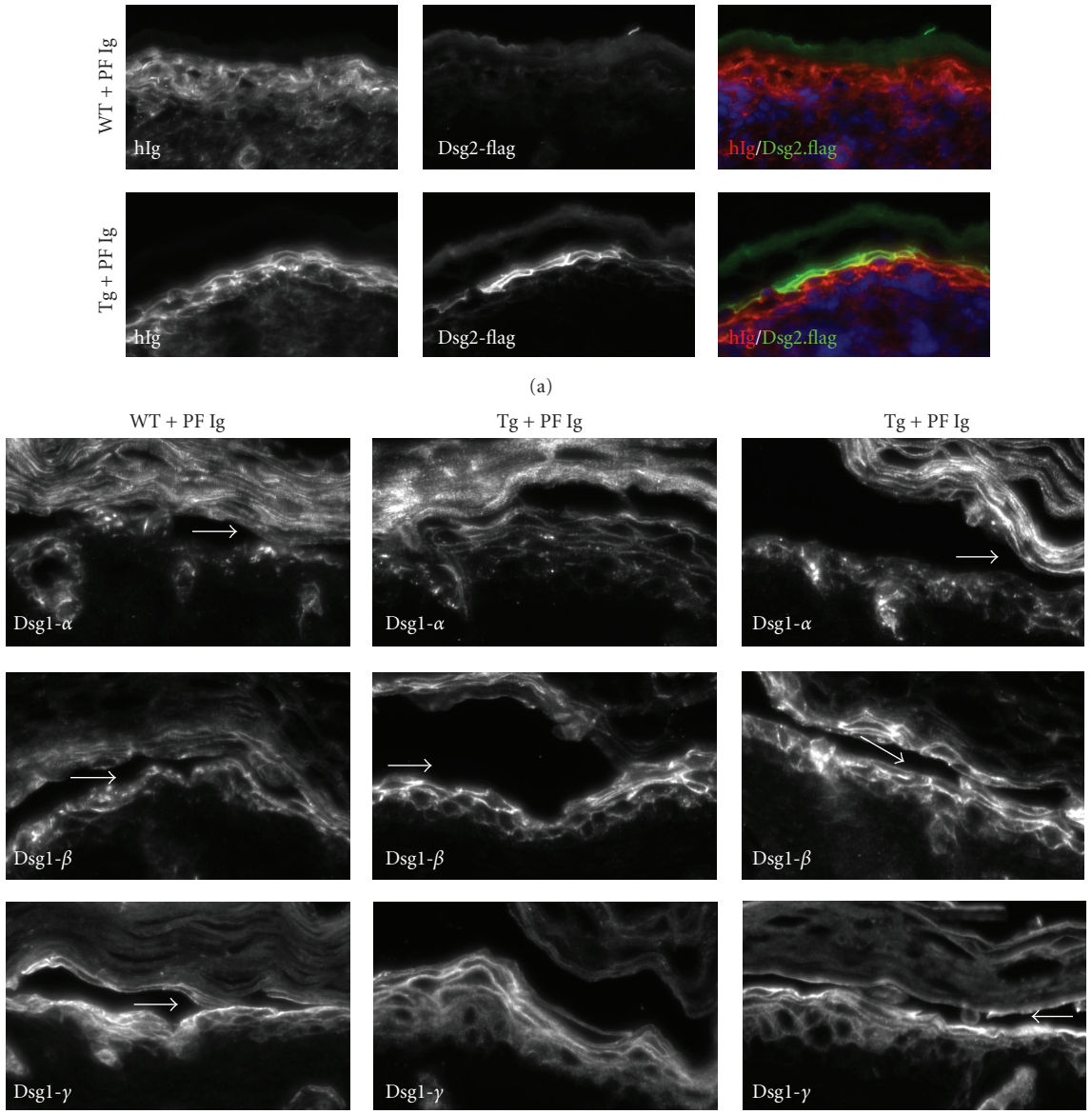

Figure 5: Superficial expression of Dsg2 reduces disruption of Dsg1 in response to PF Ig. Newborn WT and Dsg2 Tg mice were injected subcutaneously with PF Ig, and, 18 hours later, back skin samples were frozen in OCT or were fixed in formalin and embedded in paraffin for immunostaining. (a) Direct immunostaining for human Ig (hIg, left panels), showing localization of human antibodies to the epidermis after PF Ig passive transfer. The same tissue was immunostained with Flag antibodies, demonstrating the presence of Dsg2-Flag in the Tg, but not WT, skin (middle panels). Double labeling for the human Ig (red) and the Flag tag (green) is shown (right panels). Staining of human Ig was considerably more intact at the cell-cell border in the Tg skin, particularly in the superficial epidermis, where Dsg2 was expressed. Thus, superficial Dsg2 retains PF Ig at the cell-cell borders. (b) Lesional and nonlesional back skin sections were then immunostained with antibodies AP61, AP498, and Ab15, which were raised against the extracellular domain of Dsg1-α, Dsg1-β, and Dsg1-γ, respectively. DAPI (blue) was used as a nuclear stain. Arrows demarcate site of blister cleavage (lesional skin). In WT mice, treatment with PF Ig dramatically disrupted cell-cell border staining of Dsg1-α and Dsg1-β but not Dsg1-γ (left panels). Superficial expression of Dsg2 reduced the extent of Dsg1 perturbation (internalization and degradation). There was no significantly observable difference between lesional and non-lesional epidermis.

but was more extensive in the less differentiated cell layers. Intriguingly, PF Ig had a minor effect on Dsg1-γ, showing a slight reduction in the deep epidermis as compared to untreated control skin. In Tg mice treated with PF Ig, the presence of Dsg2 in the superficial epidermis helped retain Dsg1 at the cell-cell border (Figure 5(b), middle and right panels). The level of Dsg1 dissolution was not dramatically different between unaffected and lesional skin. In conclusion, superficial expression of Dsg2 appears to maintain the organization of Dsg1 in response to PF Ig treatment.

3. Discussion

In this paper, we showed that superficial expression of Dsg2 in Tg mice offers protection against skin blister formation in response to the bacterial toxin ETA and pathogenic PF antibodies. These results suggest that Dsg2 can enhance mechanical adhesion, likely contributing to this protective effect. In the case of ETA, this enhancement could counter the loss of adhesive function due to the removal of sequences in the ectodomain of Dsg1 known to be important for desmoglein-dependent adhesion [12, 13]. In the case of PF Ig, increased adhesion may compensate for possible steric hindrance of desmoglein trans-interaction induced by antibody-antigen binding [6, 18, 19].

Despite considerable progress in pemphigus research, there is still a controversy over the mechanism of pemphigus Ig-induced acantholysis—is it a disease of steric hindrance, cell signaling, or both? Steric hindrance of desmoglein trans-interaction induced by antibody-antigen binding was initially proposed based on evidence that one desmoglein could compensate for the loss of another [6, 18, 19]. The most compelling data supporting this hypothesis comes from a study showing that ectopic expression Dsg3 in the superficial epidermis of Tg mice [19] protects from blister formation induced by PF antibodies [20]. Furthermore, toxins such as ETA can induce PF-like skin blisters by proteolysis of Dsg1 in the absence of antibody binding [16]. In this paper, we demonstrate that ectopic expression of Dsg2 in the superficial epidermis could limit both PF Ig- and ETA-induced skin blister formation, suggesting that steric hindrance plays a role in the mechanism of pemphigus.

However, desmoglein compensation in response to pathogenic antibodies may work through mechanisms other than steric hindrance [3, 21, 22]. Indeed, in the case of PF, anti-Dsg1 antibodies can induce blister formation by triggering intracellular signaling pathways, without disrupting Dsg1-Dsg1 interactions [23]. Intracellular signaling may then promote alterations in cortical actin remodeling and may induce other changes that lead to the depletion of desmogleins from the desmosome and lead to consequent blistering. It is possible that Dsg2 counters the impact of PF Ig on these pathways, resulting in the retention of Dsg1 at the cell surface and maintenance of desmosome structure and function.

Also, suprabasal expression of Dsg2 in Tg mice offers greater protection against PF Ig, as compared to ETA (Figure 4). The difference in the response to ETA and PF Ig in transgenic mice may be due to differences in the mechanism by which ETA and PF Ig induce blistering, the mechanism by which suprabasal Dsg2 limits the blistering, or both. For instance, whereas PF antibodies disrupt and reduce cell-cell border staining of Dsg1 (Figure 5), ETA cleavage does not appear to alter the plasma membrane localization of Dsg1 despite inducing extensive skin blisters (Figure 3). It is possible that retention of this truncated Dsg1 molecule at the cell surface may attenuate Dsg2's protective effects. In addition, either alterations in the subcellular distribution of armadillo proteins or the differential impact on other signaling pathways could also account for the observed differences in Dsg2's protective effect between ETA- or PF-treated Tg mice. It has been reported that pemphigus antibodies trigger a rapid turnover and reduction of the nuclear pool of plakoglobin, thereby abolishing plakoglobin's role as a transcriptional repressor of the proto-oncogene c-Myc [24]. We recently demonstrated that Dsg2 enhances c-Myc expression [14]. In that study, we demonstrated that Dsg2 plays a role in cell signaling activation, and many signaling pathways directly involved in epithelial cell growth and survival are activated in Dsg2 Tg mice.

In summary, we propose that desmogleins mediate cellular homeostasis through cell-cell adhesion and activation of signaling pathways; changes in desmosome structure and integrity by pemphigus antibodies or ETA can disrupt this balance. Compensating with another desmoglein can offset this deregulation, thus restoring homeostasis, possibly through a combination of cell-cell adhesion and the regulation of cell signaling. What remains to be addressed in future studies is (1) whether ectopic expression of Dsg2 alters the sensitivity of Dsg1 to degradation by ETA or loss of function upon antibody binding, and (2) whether the loss of Dsg1 function in pemphigus is caused by altered signaling and/or steric hindrance.

Currently, there are no FDA-approved prescription drugs specifically for the treatment of pemphigus. The combination of corticosteroids and other nonsteroidal immunosuppressive or anti-inflammatory drugs offers the most effective means to lower mortality while reducing long-term morbidity due to chronic systemic exposure to steroids [25]. Several ongoing clinical trials targeting cell-signaling molecules such as p38MAPK and TNF-*a* appear promising [26, 27]. As mentioned above, we recently demonstrated that Dsg2 activates multiple growth and survival pathways, including PI 3-kinase/AKT, MEKMAPK, STAT3, and NF-κB. In this paper, we show that Dsg2 can limit epidermal blister formation mediated by PF antibodies. Thus, finding a drug/agent that could increase Dsg2 levels in the skin or, preferably, activate the signaling pathways downstream of Dsg2 offers a potential therapeutic treatment for this life-threatening blistering disease.

4. Materials and Methods

4.1. Histology, Immunohistochemical Staining, and Immunoblotting. For histology, skin tissues were fixed at room temperature overnight in a 10% formalin solution. Tissues were then processed for paraffin embedding, sectioned (5 μm), mounted on glass slides, and stained with Hematoxylin and Eosin.

For immunohistochemistry, mouse skin biopsies were fixed in either OCT or 10% formalin (Sigma) and were embedded in paraffin. Tissues were sectioned (5 μm) as previously described in detail [6, 17]. To detect normal human Ig, the Flag tag, or Dsg1, OCT frozen sections were fixed in methanol (-20°C) for 15 minutes, permeabilized with 1% TX-100 in PBS for 5 minutes, and blocked in IF blocking buffer (5% normal goat serum, 1% BSA, and 0.02% TX-100 in PBS) for 1 hour at RT. Tissues

were incubated with primary antibodies (anti-Flag (1 : 1000, Sigma) and 27B2 (1 : 10)) overnight at 4°C and secondary antibodies for 1 hour at room temperature. Antibodies were incubated in IF blocking buffer. For polyclonal antibodies against mouse Dsg1 isoforms, paraffin embedded tissues were deparaffinized in 100% xylene (5 minutes; 3 times), 100% ethanol (5 minutes; 2 times), 95% ethanol (5 minutes; 2 times), 75% ethanol (2 minutes), 50% ethanol (2 minutes), and H_2O (2 minutes). Antigens were retrieved in an antigen-retrieving medium (Signet, Dedham, MA) by the microwave method and digestion with trypsin (Sigma). Primary and secondary antibodies were suspended in IF blocking buffer. Nuclei were stained with DAPI (Sigma), and slides were mounted for analysis via fluorescence microscopy. Polyclonal antibodies against mouse Dsg1 include AP61 (Dsg1-α; 1 : 100), AP498 (Dsg1-β, 1 : 100), and Ab15 (Dsg1-γ, 1 : 1000). Alexa Fluor-conjugated secondary antibodies (488 and 594 nm) were from Invitrogen (Eugene, OR) and were used at 1 : 200. Images were acquired using a Hamamatsu monochromatic digital camera and Phase 3 Imaging Systems software (Glen Mills, PA, USA; C4742–95).

In preparation for immunoblotting, mouse back skin was pulverized in liquid nitrogen in RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)). Protein concentration was determined (Pierce BCA kit, Pierce Biotech, Rockford, IL), and immunoblotting was performed, as described previously [17], with 20 μg of protein in each lane resolved with 7% SDS-PAGE (Bio-Rad Laboratories, Hercules, CA). Signals were detected with chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ). Antibodies used were 27B2 (1 : 100), Flag (1 : 1000), and Actin (1 : 10 000).

4.2. PF Ig Purification. Preparation of serum Ig was done as previously described [28]. Briefly, human sera (10–20 ml) were dialyzed overnight at 4°C against 20 mM KH_2P0_4 (pH 8.0), and 0.02% sodium azide and was purified with a DEAE Affi-Gel Blue column (Bio-Rad Labs, Hercules, CA). Serum Ig was concentrated using Centriprep10 (Amicon Millipore, Billerica, MA) and was dialyzed against PBS at 4°C. Protein concentration was determined, and the sera were stored at −80°C.

4.3. Passive Transfer of Pemphigus Ig and Injection of ETA into Neonatal Mice. Newborn mice (~2 g) were injected subcutaneously with purified Ig (10 mg in 100 μl) between the shoulder blades with a 1 cc insulin syringe (Becton Dickinson) as previously described [6]. For ETA treatment, newborn control and Tg mice were injected subcutaneously in the back of the neck with either ETA (0.5 μg in 50 μl PBS, Toxin Technology, Sarasota, FL) or PBS alone as previously described [8]. Animals were photographed and sacrificed, and their back skin was processed in 10% PBS-buffered formalin (Sigma) for histology and in OCT for immunohistochemistry or was frozen in liquid nitrogen for DNA and protein extraction.

Abbreviations

ETA: Exfoliative toxin A
Dsg: Desmoglein
PF: Pemphigus foliaceus
PV: Pemphigus vulgaris
Tg: Transgenic
WT: Wild-type.

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References

[1] X. Cheng and P. J. Koch, "In vivo function of desmosomes," *Journal of Dermatology*, vol. 31, no. 3, pp. 171–187, 2004.

[2] M. G. Mahoney, Y. Hu, D. Brennan, H. Bazzi, A. M. Christiano, and J. K. Wahl III, "Delineation of diversified desmoglein distribution in stratified squamous epithelia: implications in diseases," *Experimental Dermatology*, vol. 15, no. 2, pp. 101–109, 2006.

[3] A. S. Payne, Y. Hanakawa, M. Amagai, and J. R. Stanley, "Desmosomes and disease: pemphigus and bullous impetigo," *Current Opinion in Cell Biology*, vol. 16, no. 5, pp. 536–543, 2004.

[4] J. R. Stanley and M. Amagai, "Pemphigus, bullous impetigo, and the staphylococcal scalded-skin syndrome," *New England Journal of Medicine*, vol. 355, no. 17, pp. 1800–1810, 2006.

[5] M. Amagai, K. Tsunoda, D. Zillikens, T. Nagai, and T. Nishikawa, "The clinical phenotype of pemphigus is defined by the anti-desmoglein autoantibody profile," *Journal of the American Academy of Dermatology*, vol. 40, no. 2, pp. 167–170, 1999.

[6] M. G. Mahoney, Z. Wang, K. Rothenberger, P. J. Koch, M. Amagai, and J. R. Stanley, "Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris," *Journal of Clinical Investigation*, vol. 103, no. 4, pp. 461–468, 1999.

[7] Y. Hanakawa and J. R. Stanley, "Mechanisms of blister formation by staphylococcal toxins," *Journal of Biochemistry*, vol. 136, no. 6, pp. 747–750, 2004.

[8] M. Amagai, N. Matsuyoshi, Z. H. Wang, C. Andl, and J. R. Stanley, "Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1," *Nature Medicine*, vol. 6, no. 11, pp. 1275–1277, 2000.

[9] M. Yokouchi, M. A. Saleh, K. Kuroda, et al., "Pathogenic epitopes of autoantibodies in pemphigus reside in the amino-terminal adhesive region of desmogleins which are unmasked by proteolytic processing of prosequence," *Journal of Investigative Dermatology*, vol. 129, no. 9, pp. 2156–2166, 2009.

[10] N. Li, V. Aoki, G. Hans-Filho, E. A. Rivitti, and L. A. Diaz, "The role of intramolecular epitope spreading in the pathogenesis of endemic pemphigus foliaceus (fogo selvagem)," *The Journal of Experimental Medicine*, vol. 197, pp. 1501–1510, 2003.

[11] M. Sekiguchi, Y. Futei, Y. Fujii, T. Iwasaki, T. Nishikawa, and M. Amagai, "Dominant autoimmune epitopes recognized by pemphigus antibodies map to the N-terminal adhesive region of desmogleins," *Journal of Immunology*, vol. 167, no. 9, pp. 5439–5448, 2001.

[12] N. A. Chitaev and S. M. Troyanovsky, "Direct Ca^{2+} dependent heterophilic interaction between desmosomal cadherins, desmoglein and desmocollin, contributes to cell-cell adhesion," *Journal of Cell Biology*, vol. 138, no. 1, pp. 193–201, 1997.

[13] V. Spindler, W.-M. Heupel, A. Efthymiadis, et al., "Desmocollin 3-mediated binding is crucial for keratinocyte cohesion and is impaired in pemphigus," *Journal of Biological Chemistry*, vol. 284, no. 44, pp. 30556–30564, 2009.

[14] D. Brennan, Y. Hu, S. Joubeh, et al., "Suprabasal Dsg2 expression in transgenic mouse skin confers a hyperproliferative and apoptosis-resistant phenotype to keratinocytes," *Journal of Cell Science*, vol. 120, no. 5, pp. 758–771, 2007.

[15] T. Ota, M. Amagai, M. Watanabe, and T. Nishikawa, "No involvement of IgG autoantibodies against extracellular domains of desmoglein 2 in paraneoplastic pemphigus or inflammatory bowel diseases," *Journal of Dermatological Science*, vol. 32, no. 2, pp. 137–141, 2003.

[16] Y. Hanakawa, N. M. Schechter, C. Lin, K. Nishifuji, M. Amagai, and J. R. Stanley, "Enzymatic and molecular characteristics of the efficiency and specificity of exfoliative toxin cleavage of desmoglein 1," *Journal of Biological Chemistry*, vol. 279, no. 7, pp. 5268–5277, 2003.

[17] D. Brennan, Y. Hu, A. Kljuic, et al., "Differential structural properties and expression patterns suggest functional significance for multiple mouse desmoglein 1 isoforms," *Differentiation*, vol. 72, no. 8, pp. 434–449, 2004.

[18] Y. Shirakata, M. Amagai, Y. Hanakawa, T. Nishikawa, and K. Hashimoto, "Lack of mucosal involvement in pemphigus foliaceus may be due to low expression of desmoglein 1," *Journal of Investigative Dermatology*, vol. 110, no. 1, pp. 76–78, 1998.

[19] H. Wu, Z. H. Wang, A. Yan, et al., "Protection against pemphigus foliaceus by desmoglein 3 in neonates," *New England Journal of Medicine*, vol. 343, no. 1, pp. 31–35, 2000.

[20] Y. Hanakawa, N. Matsuyoshi, and J. R. Stanley, "Expression of desmoglein 1 compensates for genetic loss of desmoglein 3 in keratinocyte adhesion," *Journal of Investigative Dermatology*, vol. 119, no. 1, pp. 27–31, 2002.

[21] M. Amagai, A. R. Ahmed, Y. Kitajima, et al., "Are desmoglein autoantibodies essential for the immunopathogenesis of pemphigus vulgaris, or just "witnesses of disease"?" *Experimental Dermatology*, vol. 15, pp. 815–831, 2006.

[22] E. Schmidt and J. Waschke, "Apoptosis in pemphigus," *Autoimmunity Reviews*, vol. 8, no. 7, pp. 533–537, 2009.

[23] J. Waschke, P. Bruggeman, W. Baumgartner, D. Zillikens, and D. Drenckhahn, "Pemphigus foliaceus IgG causes dissociation of desmoglein 1-containing junctions without blocking desmoglein 1 transinteraction," *Journal of Clinical Investigation*, vol. 115, no. 11, pp. 3157–3165, 2005.

[24] L. Williamson, N. A. Raess, R. Caldelari, et al., "Pemphigus vulgaris identifies plakoglobin as key suppressor of c-Myc in the skin," *EMBO Journal*, vol. 25, no. 14, pp. 3298–3309, 2006.

[25] N. Ishii, Y. Maeyama, T. Karashima, et al., "A clinical study of patients with pemphigus vulgaris and pemphigus foliaceous: an 11-year retrospective study (1996–2006)," *Clinical and Experimental Dermatology*, vol. 33, no. 5, pp. 641–643, 2008.

[26] S. E. Dick and V. P. Werth, "Pemphigus: a treatment update," *Autoimmunity*, vol. 39, no. 7, pp. 591–599, 2006.

[27] L. A. O'Neill, "Targeting signal transduction as a strategy to treat inflammatory diseases," *Nature Reviews Drug Discovery*, vol. 5, no. 7, pp. 549–563, 2006.

[28] M. G. Mahoney, Z. H. Wang, and J. R. Stanley, "Pemphigus vulgaris and pemphigus foliaceus antibodies are pathogenic in plasminogen activator knockout mice," *Journal of Investigative Dermatology*, vol. 113, no. 1, pp. 22–25, 1999.

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Review Article

Apoptotic Pathways in Pemphigus

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Pemphigus is a group of human autoimmune blistering diseases of the skin in which autoantibodies to desmosome cadherins induce loss of cell-cell adhesion (acantholysis). In addition to steric hindrance and activation of intracellular signaling, apoptosis has been suggested to contribute to the mechanism by which pathogenic IgG induces acantholysis. We review the current literature examining the role of apoptosis in pemphigus. Current data suggest that apoptosis is not required for blister induction, but that activation of proapoptotic proteins, including caspase cysteine proteinases, may sensitize cells to the acantholytic effects of pemphigus IgG.

1. Introduction

Pemphigus is a group of autoimmune blistering diseases characterized by loss of keratinocyte cell adhesion that leads to clinical blister formation. Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are the two main variants. In these disorders, autoantibodies to desmosome cadherins induce blister formation. In PF, pathogenic IgG target the desmosome cadherin desmoglein-1 (dsg-1) [1], inducing loss of adhesion in the subcorneal region of epidermal epithelia; whereas, in PV, antibodies to desmoglein-3 (dsg-3) in mucosal PV [2] and to dsg-3 and dsg-1 in mucocutaneous PV [3–5] induce loss of adhesion in the suprabasal layer.

2. Signal Transduction and Acantholysis

When injected into neonatal mice, IgG fractions from pemphigus patients induce blister formation reproducing the histology, immunohistology, and clinical appearance of the human disease [6, 7]. This seminal observation provided the evidence that the autoantibodies themselves were pathogenic, inducing blister formation in the target organ, the skin. A variety of mechanisms have been proposed to explain how pemphigus autoantibodies disrupt keratinocyte cell-cell adhesion. The observation that pemphigus IgG were directed against the desmosomal cadherins dsg-1 and dsg-3 led to the suggestion that the IgG inhibited trans interactions of desmosome adhesion proteins across the adhesive interface, a mechanism know as the steric hindrance hypothesis. Alternative hypotheses have been suggested, including antibody induction of intracellular signaling that leads to loss of adhesion, and antibody induction of keratinocyte apoptosis.

Changes in cell-cell adhesion are often accompanied by significant alterations in the biology of the cell. When cells become adherent to adjacent cells, they often cease to migrate and proliferate, a concept referred to as contact inhibition and loss of which often characterizes the malignant phenotype of cancer cells. In contrast, when cells lose adhesion to their neighbors, they can undergo apoptosis, or proliferate, and, or migrate. Which of these later fates occur is often context dependent. For example, loss of adhesion to and migration from the wound margin is typical of the biology of a keratinocyte migrating into a healing wound. Therefore, it is not surprising that alterations in cell adhesion complexes initiate, inhibit, and/or regulate signaling processes.

Kitajima's group was the first to describe the activation of signaling pathways in cells exposed to pemphigus IgG.

They first reported increased intracellular calcium levels in keratinocytes treated with sera from PV patients [8]. In subsequent studies, using either PV sera or fractions enriched for pathogenic IgG, they demonstrated activation of additional signaling molecules including protein kinase C [9] and phospholipase C [10]. Other signaling events have been observed in pemphigus treated keratinocytes and may contribute to loss of or to biologic transitions induced by altered adhesion [11–18].

3. Apoptosis

Programmed cell death is a mechanism used by multicellular organisms to remove unwanted cells. For example, the shaping of tissues during development is a coordinated process of proliferation, migration, and cell death. In fully developed organisms, tissues that continuously proliferate, including the skin, must balance cell proliferation and death to prevent unchecked growth. In pathologic states, including actinic damage that may lead to neoplastic transformation, apoptosis is employed to remove from the host tissue these potentially damaging cells. Apoptosis is characterized by a number of well recognized cytological and biochemical features including, (i) nuclear condensation, (ii) activation of caspase cysteine proteinases, (iii) DNA fragmentation at regular intervals resulting in DNA laddering, (iv) cell shrinkage, and (v) membrane blebbing (reviewed in [19]).

A number of reports indicate that apoptosis occurs as a consequence of exposure of human keratinocytes to pemphigus sera or purified IgG fractions, observed in both in vitro tissue culture systems, in vivo utilizing passive transfer mouse models, or in human patient skin biopsies. Induction of apoptosis or of proapoptotic proteins by pemphigus IgG may be (i) part of the mechanism by which sera and IgG induce acantholysis or (ii) a consequence of loss of adhesion and a result of acantholysis. Apoptosis has been proposed to have a role in the mechanism by which pemphigus IgG induce acantholysis [20, 21]. Initial reports examined histological sections of skin biopsies and TUNEL positive keratinocytes, indicative of DNA fragmentation characteristic of cells undergoing apoptosis, in both lesional PF [22] and PV [20] and in perilesional pemphigus skin [23]. Markers for apoptotic cells, apart from TUNEL include components of the proapoptotic pathway such as caspases, PARP, or Fas. These proapoptotic markers have also been used to investigate the presence of apoptotic cells in PV patient skin biopsies.

4. Immune Cells and Fas/FasL

Although there is a fairly broad consensus that apoptosis can be observed in pemphigus IgG induced disease processes, there remains an ongoing discussion about whether apoptosis is a (i) cause, (ii) result, or (iii) "byproduct" of acantholysis. For example, modeling anoikis, it would be straightforward to think of apoptosis as consequence of acantholysis. Anoikis is a special form of apoptosis of epithelial cells triggered when cells become detached from the substratum. Disruption of integrin-extracellular matrix binding is a main trigger for anoikis [24]. In contrast, the main adhesive forces between cells in the suprabasal layers of the epidermis are adherens junctions and desmosomes; therefore, anoikis seems to be unlikely to be important in pemphigus. Furthermore, it has been reported that PV IgG, but not normal human IgG, cause cell death as measured by trypan blue exclusion in suspension keratinocytes [25]. The ability of pemphigus IgG to induce cell death in nonadherent keratinocytes would seemingly exclude the loss of cell attachment as a trigger for pemphigus IgG induced cell death.

As early as 1994, Sayama and coworkers, and later other groups, reported upregulation of soluble Fas ligand in either PV patient sera and, or skin, suggesting that apoptosis in the target organ skin is triggered via the Fas/FasL system [20, 23, 26, 27]. Importantly, anti-FasL blocking antibodies only partially inhibited this proapoptotic effect of pemphigus sera on cultured keratinocytes suggesting that other mechanisms may contribute to induction of apoptosis. Rodrigues and colleagues also reported observing TUNEL positive keratinocytes in perilesional biopsies in patients with Fogo selvagem, the endemic form of PF [28]. Interestingly, these authors also noted the presence of the inflammatory cytokines TNF-α, IFN-γ, and IL-1 in the lesion exudate which led them to suggest that inflammatory mediators could contribute to the induction of apoptosis in pemphigus. Similarly, Pacheco-Tovar and colleagues suggested that CD8+FasL+ lymphocytes in pemphigus skin may have a role in inducing apoptosis [26]. In contrast, other groups have suggested that pemphigus associated apoptosis is independent of changes in Fas-ligand levels. For example, Reich and colleagues did not see increased levels of FasL in PNP patient sera, despite observing increased apoptotic cells in PNP skin biopsies [29].

Genetic polymorphisms in genes encoding apoptotic factors have been identified in autoimmune disorders. Köhler and Petzl-Erler reasoned that if apoptosis has a role in pemphigus, then genetic polymorphisms in apoptotic factors might similarly explain the susceptibility of certain individuals to this disease; however, they were unable to find an association between specific polymorphisms in the genes encoding either the p53 or BAX in a Brazilian focus of endemic PF [30].

Taken together, pemphigus lesions have been described with or without the infiltration of immune cells suggesting that inflammation is not necessarily an early event nor required for pemphigus disease induction. Nevertheless, the latter observation could explain the contradictory finding of the presence or absence of FasL in pemphigus lesions by different labs.

5. The Temporal Association of Acantholysis and Apoptosis

Apoptosis leads to cell death; whereas, in pemphigus, the engagement of cell surface antigens by pathogenic leads to loss of adhesion, a point discussed by Grando and colleagues in a recent commentary [31]. The timing of these events and their relation to one another, that is the loss of adhesion and activation of proapoptotic proteins, has been subject to

debate and investigation. Grando uses the term apoptolysis to suggest a relationship between the activation of apoptotic proteins including caspases, and acantholysis emphasizing that caspase activation and acantholysis can proceed in the absence of cell death. Namely, that caspases could serve cell regulatory functions in addition to apoptosis. In support of this hypothesis is data demonstrating that many desmosome components as well as intermediate filaments can serve as caspase substrates [32–35]. Thus, caspases may have a role in regulating desmosome protein turnover under normal as well as pathophysiologic states.

Differing reports on the role of apoptosis in pemphigus IgG mediated acantholysis may reflect the different systems and experimental conditions used. For example, different keratinocyte culture systems, including primary human keratinocytes, squamous cell carcinoma cell lines, and the immortalized HaCat keratinocyte cell line, have been used to examine in vitro the role of apoptosis in pemphigus acantholysis. Similarly, cell cultures that have been passaged multiple times, believed to reproduce some of the biochemical and physiologic alterations associated with aging, appear more susceptible to the apoptosis inducing activity of pemphigus IgG [36]. Utilizing cell culture systems, extended incubations are often used to examine the ability of pemphigus sera or pemphigus IgG to induce keratinocyte apoptosis. Incubations with pemphigus sera and/or IgG from 8 to 72 hours have been used in experiments in which activation of proapoptotic markers including TUNEL positivity have been observed at typically later time points [37]. Moreover, monolayer cultures cannot fully replicate the in vivo situation, as cells in culture are proliferating; whereas, the cells affected and involved in pemphigus disease processes are suprabasal keratinocytes that have ceased to proliferate, but are differentiating. Differences in proliferation and differentiation could lead to altered susceptibility to apoptotic stimuli. Skin biopsies are a snapshot in time of the disease progression and reported differences in the biopsies may be attributed to different stages of disease progression. While very early disease lesions may be negative for apoptotic markers, late lesions may show apoptosis as well as immune cell infiltrates.

6. The In Vivo Effect of Caspase Inhibitors

Li and colleagues explored the ability of caspase inhibitors to block acantholysis in vivo using the PF passive transfer mouse model [38]. They reported that pretreatment of neonatal mice with 0.034–6.8 μg/g body weight of the caspase inhibitors Ac-DEVD-cmk or Boc-D-fmk prevented PF IgG induced blister formation in the mice at 20 hours. Interestingly, in time course studies they observed increased amounts of the proapoptotic factor Bax initially and subsequent decreases in the antiapoptotic factor Bcl-xL at later time points, an observation they suggest supports a role for induction of apoptosis via the mitochondrial pathway. They interpret these results to implicate caspase activity in the mechanism of acantholysis. This report stands in contrast to an in vitro study by Schmidt and colleagues suggesting that caspase inhibitors do not block acantholysis [39]. Using the immortalized HaCat keratinocyte cell line as well as normal human epidermal keratinocyte cultures as in vitro model systems, they observed PV IgG induced keratinocyte dissociation and cytokeratin retraction without observable markers of apoptosis such as nuclear condensation, TUNEL staining, and caspase-3 activation. The caspase inhibitor z-VAD-fmk did not block PV IgG mediated acantholysis in cultured keratinocytes. In addition to using pharmacologic inhibitors of apoptosis, genetic approaches also failed to demonstrate a role for apoptosis in PV IgG mediated acantholysis. In these experiments, overexpression in HaCat cells of the Fas-associated death domain-like interleukin-1-β-converting (FLICE)-like inhibitory proteins $FLIP_L$ and $FLIP_S$, inhibitors of caspase-8 mediated activation and apoptosis, blocked Fas induced apoptosis, but failed to block PV IgG mediated acantholysis. An additional important finding in the above mentioned report is that not all lesions from the same patients, particularly very early ones, showed markers of apoptosis, an observation suggesting that apoptosis is not essential for acantholysis.

7. Pemphigus, P38MAPK, Acantholysis, and Apoptosis

In a series of experiments initially designed to look at signaling pathways downstream of changes in desmosome mediated cell-cell adhesion, our research group identified activation of p38 mitogen activated protein kinase (MAPK) as a necessary event for pemphigus IgG induced acantholysis [40]. Phosphorylation is a common, reversible modification that regulates protein structure, function, and activity. Because changes in protein phosphorylation are common in signal transduction cascades and can be readily detected and quantified with radioactive phosphate, we designed a strategy to screen for signaling downstream of pemphigus IgG induced changes in desmosome mediated adhesion. Primary human keratinocytes were loaded with $^{32}P\text{-}H_3PO_4$ and then exposed to purified PV IgG for 30 minutes. Protein extracts were prepared from labeled cells and separated by two-dimensional gel electrophoresis. Radioactive proteins were detected by autoradiography and quantified by phosphoimage analysis. Using this approach, we identified several radioactive spots whose signal was consistently increased in samples treated with PV IgG, but not in normal human IgG nor buffer treated controls. Through this screen we first identified the small heat shock protein (HSP) 27 and p38MAPK as components of a putative signaling cascade downstream of PV IgG induced changes in desmosome adhesion. Because p38MAPK was known to be upstream of HSP27, it was not surprising that keratinocytes pretreated with the p38MAPK inhibitors SB202190 or SB203580, but not the inactive analog SB202474, failed to induce HSP27 phosphorylation when treated with PV IgG. What was surprising was that the p38MAPK inhibitors also blocked PV IgG induced actin reorganization and keratin intermediate filament retraction. This observation suggested that activation of p38MAPK might not be a signal activated by loss of adhesion, but could in fact be part of the mechanism by which PV IgG induced loss of adhesion. These observations

were later confirmed using passive transfer mouse models of both PV [41] and PF [42]. Phosphorylation of both HSP27 and p38MAPK were observed in skin biopsies from PV and PF IgG treated neonatal mice. Significantly, in mice pretreated with p38MAPK inhibitors, p38MAPK and HSP27 phosphorylation and blister formation were blocked. Activation of p38MAPK in keratinocytes treated with PV IgG has now been independently confirmed by several labs actively investigating the mechanism of acantholysis [12, 16, 43].

Our earlier studies had demonstrated a role for p38MAPK in the mechanism by which pemphigus IgG induce acantholysis. Based on (i) the known roles of p38MAPK and HSP27 in regulating both the actin and intermediate filament cytoskeletons [44–50] and (ii) the ability of p38MAPK inhibitors to block PV IgG induced actin reorganization and keratin intermediate filament collapse in keratinocyte tissue cultures [40], we had proposed that p38MAPK was acting upstream of and regulating both actin and keratin intermediate filament cytoskeletal changes induced by pemphigus IgG. Recent work in our lab has also demonstrated a role for p38MAPK in regulating pemphigus IgG induced desmoglein endocytosis [51].

Because of the well-characterized involvement of p38MAPK in apoptosis [52–54], we initiated a series of experiments to investigate the potential relationship of PV IgG induced activation of p38MAPK to apoptosis [55]. In this series of experiments, both tissue culture and animal model systems were employed. Primary human keratinocyte cultures allowed us to very accurately follow the time course of biochemical changes induced by pemphigus IgG; whereas, utilization of the pemphigus passive transfer mouse model allowed us to determine if the events observed in vitro were reflective of the in vivo state. Primary human keratinocytes were exposed to PV IgG and examined biochemically and by confocal immunofluorescent microscopy at various times after addition of PV IgG to the cultures. Interestingly, two sequential peaks of p38MAPK activation were observed, the first beginning within minutes after addition of PV IgG, peaking at 30 minutes, and then dropping down to baseline by 4 hours. A second extended peak of p38MAPK activation was observed to begin at 6 hours and continued out to 10 hours. Analogous to the observations in tissue culture, a similar biphasic activation of p38MAPK in the skin was observed in the passive transfer PF mouse model. An initial peak of p38MAPK activation was seen at 2 to 4 hours after subcutaneous administration of purified PF IgG to neonatal C57BL/6J mice which dropped down to baseline levels at 6 hours. A second peak of p38MAPK was observed in murine skin at 8 hours post PF IgG injection and was sustained out to at least 21 hours post injection. Along with p38MAPK activity, markers of apoptosis, including TUNEL staining, PARP cleavage, and caspase-3 cleavage were also examined at sequential time points after treatment with pemphigus IgG. In both primary human keratinocyte cultures and the passive transfer mouse model, markers of apoptosis were late events occurring coincident or subsequent to the second peak of p38MAPK activation. In the passive transfer mouse model, acantholysis was readily apparent at 21 hours after treatment with PF IgG; however, neither cleaved PARP nor cleaved caspase-3 could be detected at either the 21- or 24-hour-time points, well after the second peak of p38MAPK activity had begun. It was not until 30 hours post PF IgG injection that these markers of apoptosis (cleaved PARP and cleaved caspase-3) were detected by immunoblot of skin extracts. Similarly, only in skin biopsies from mice treated with PF IgG for times exceeding 21 hours were increases in TUNEL positive keratinocytes detected.

To investigate the relationship of the first and second peak of p38MAPK activity to acantholysis, and apoptosis, inhibitor experiments were performed in both primary human keratinocyte culture and passive transfer mouse models. By either pretreating cultures or mice with p38MAPK inhibitors prior to pemphigus IgG or at a time after the first, but prior to the second peak of p38MAPK activity, either both peaks or the second peak of p38MAPK activity could be selectively inhibited. Utilizing this approach, inhibition of the first, but not second, peak of p38MAPK activity inhibited blister formation in vivo and cytokeratin retraction in vitro [55]. We interpreted these results to indicate that the first, but not second peak, of p38MAPK activity observed after exposure to pemphigus IgG was part of the mechanism of acantholysis. Importantly, although it failed to inhibit cytokeratin retraction and acantholysis, selective inhibition of the later second peak of p38MAPK activity inhibited caspase-3 activation in vivo. Thus, the time course studies revealed that apoptosis occurs at or after the second peak of p38MAPK activation and that inhibition of this later peak of p38MAPK activity blocked activation of the proapoptotic proteinase caspase-3, but not acantholysis. Collectively, these observations suggest that the earlier peak of p38MAPK activation is part of the mechanism leading to acantholysis; whereas, the later peak of p38MAPK and apoptosis are subsequent to and likely not essential for acantholysis (Figure 1).

Interestingly, pemphigus IgG induced cytokeratin retraction may trigger apoptosis as suggested by a recent report from the Omary lab implicating a role for keratin intermediate filaments in the induction of apoptotic pathways. Mutations in the hepatocyte keratin K8 were associated with altered mitochondrial morphology and increased susceptibility to proapoptotic stimuli [56]. Null K8 or K8 mutants show decreased mitochondrial size, a shift from diffuse to cortical distribution and clumping, and increased mitochondrial release of cytochrome c from mutants. Therefore, it may be the collapse of the intermediate filament network in pemphigus that contributes to activation of apoptosis in these cells.

8. Making Sense of It All

From studies to date, it is clear that activation of proapoptotic proteins, including caspases, occurs in pemphigus. Although studies from our lab and from Waschke's group indicate that apoptosis is not essential for acantholysis to proceed, Li and coworkers have shown that caspase inhibitors can block acantholysis in the PF passive transfer mouse model. The challenge of resolving these seemingly

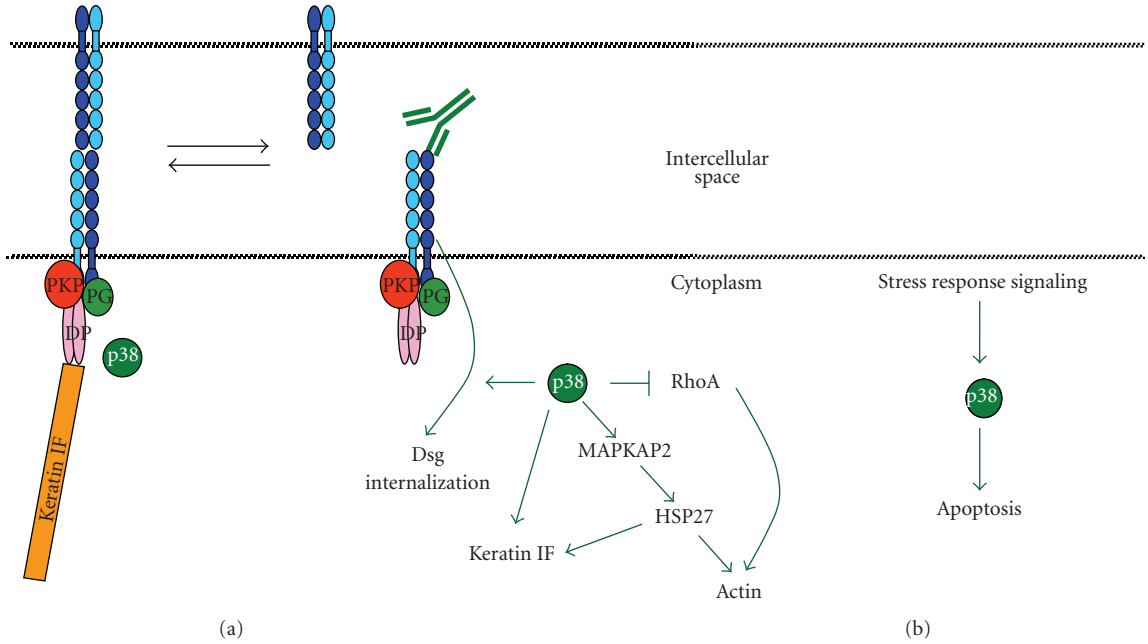

Figure 1: Pemphigus, p38MAPK, acantholysis and apoptosis. Two sequential peaks of p38MAPK activation are observed when keratinocytes are exposed to either PV or PF IgG. (a) Pemphigus IgG binds to dsg and biases the equilibrium of desmosome assembly/disassembly towards disassembly which is linked by an, as yet, undefined mechanism towards activation of p38MAPK. Subsequent p38 dependent alterations in the cell state include RhoA inactivation, dsg endocytosis, HSP27 phosphorylation, keratin intermediate filament retraction, actin, and loss of cell-cell adhesion (acantholysis). (b) A second late peak of p38 activity is observed that is likely a stress response signal induced by loss of cell-cell adhesion and leads to activation of proapoptotic pathways including caspase-3 activation.

mechanistically divergent observations is not as difficult as it would first appear.

Although caspases are cysteine proteinases implicated in apoptosis, they may have additional nonapoptotic biologic functions [57] including regulating desmosome assembly/disassembly. This is particularly relevant because data is accumulating that desmosomes are dynamic complexes in which the adhesive structure is maintained by the equilibrium between desmosome assembly and disassembly. In pemphigus, pathogenic antidsg antibodies promote dsg internalization [14, 15, 51, 58–61] and bias the equilibrium towards disassembly. Similarly, caspases have been shown to cleave desmosome proteins and therefore may be important to the physiologic cycling/turnover of dsg in keratinocytes. The desmosome proteins dsg-3, dsg-1, plakoglobin, and desmoplakin [32, 33], as well as intermediate filaments [34, 35], have all been shown to undergo caspase-dependent cleavage. Furthermore, caspases may also regulate matrix metalloproteinase (MMP) dependent cleavage of desmogleins [33]. In A431 epithelial cells induced to undergo apoptosis by UV exposure, UV-induced MMP cleavage of dsg-1 ectodomain could be inhibited by both MMP specific inhibitors as well as by the caspase inhibitor ZVAD-fmk [33]. Staphylococcal Scalded Skin Syndrome (SSSS) demonstrates that proteolytic cleavage of desmogleins can cause acantholysis. In SSSS, the Staphylococci secrete exfoliative toxin, a serine proteinase that cleaves the ectodomain of dsg-1; thereby, disrupting desmosome mediated adhesion in the subcorneal layers of epidermal epithelia and yielding a phenotype mimicking the subcorneal blisters of PF [62, 63]. Thus, caspase-dependent proteolysis has the potential to augment the acantholytic effects of pemphigus IgG. Analogously, blocking caspase dependent proteolysis of desmosome proteins and intermediate filaments may stabilize keratinocyte cell-cell adhesion thereby increasing their resistance to pemphigus IgG induced acantholysis.

Pemphigus induced acantholysis is a disease specific model system that has facilitated the investigation of desmosome dynamics, adhesion, and the mechanisms by which alterations in desmosome structure impact intracellular signaling and regulatory pathways. Although activation of proapoptotic pathways appears to be a late event and may not be essential for blistering in pemphigus, activation of components of apoptotic signaling, including caspase family member proteinases, could augment the blistering response as downstream effects of p38MAPK activation. Adhesion is a dynamic process that is linked to other biologic events including cell migration, proliferation, differentiation, and death. Changes in desmosome adhesion impact these processes and is likely to involve multiple components and signaling pathways. Elucidating these components and pathways will provide fertile ground for future investigations.

Abbreviations

dsg-1: Desmoglein 1
dsg-3: Desmoglein 3
HSP: Heat shock protein

MMP: Matrix metallo-proteinase
p38MAPK: p38 mitogen activated protein kinase
PF: Pemphigus foliaceus
PNP: Paraneoplastic pemphigus
PV: Pemphigus vulgaris
SSSS: Staphylococcal Scalded Skin Syndrome.

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References

[1] R. W. Eyre and J. R. Stanley, "Human autoantibodies against a desmosomal protein complex with a calcium-sensitive epitope are characteristic of pemphigus foliaceus patients," *Journal of Experimental Medicine*, vol. 165, no. 6, pp. 1719–1724, 1987.

[2] M. Amagai, V. Klaus-Kovtun, and J. R. Stanley, "Autoantibodies against a novel epithelial cadherin in Pemphigus vulgaris, a disease of cell adhesion," *Cell*, vol. 67, no. 5, pp. 869–877, 1991.

[3] X. Ding, V. Aoki, J. M. Mascaro, A. Lopez-Swiderski, L. A. Diaz, and J. A. Fairley, "Mucosal and mucocutaneous (generalized) pemphigus vulgaris show distinct autoantibody profiles," *Journal of Investigative Dermatology*, vol. 109, no. 4, pp. 592–596, 1997.

[4] M. Amagai, K. Tsunoda, D. Zillikens, T. Nagai, and T. Nishikawa, "The clinical phenotype of pemphigus is defined by the anti-desmoglein autoantibody profile," *Journal of the American Academy of Dermatology*, vol. 40, no. 2, part 1, pp. 167–170, 1999.

[5] S. Miyagawa, M. Amagai, T. Iida, Y. Yamamoto, T. Nishikawa, and T. Shirai, "Late development of antidesmoglein 1 antibodies in pemphigus vulgaris: correlation with disease progression," *British Journal of Dermatology*, vol. 141, no. 6, pp. 1084–1087, 1999.

[6] G. J. Anhalt, R. S. Labib, and J. J. Voorhees, "Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease," *The New England Journal of Medicine*, vol. 306, no. 20, pp. 1189–1196, 1982.

[7] J. T. Roscoe, L. Diaz, and S. A. P. Sampaio, "Brazilian pemphogus foliaceus autoantibodies are pathogenic to BALB/c mice by passive transfer," *Journal of Investigative Dermatology*, vol. 85, no. 6, pp. 538–541, 1985.

[8] M. Seishima, C. Esaki, K. Osada, S. Mori, T. Hashimoto, and Y. Kitajima, "Pemphigus IgG, but not bullous pemphigoid IgG, causes a transient increase in intracellular calcium and inositol 1,4,5-trisphosphate in DJM-1 cells, a squamous cell carcinoma line," *Journal of Investigative Dermatology*, vol. 104, no. 1, pp. 33–37, 1995.

[9] K. Osada, M. Seishima, and Y. Kitajima, "Pemphigus IgG activates and translocates protein kinase C from the cytosol to the particulate/cytoskeleton fractions in human keratinocytes," *Journal of Investigative Dermatology*, vol. 108, no. 4, pp. 482–487, 1997.

[10] M. Seishima, Y. Iwasaki-Bessho, Y. Itoh, Y. Nozawa, M. Amagai, and Y. Kitajima, "Phosphatidylcholine-specific phospholipase C, but not phospholipase D, is involved in pemphigus IgG-induced signal transduction," *Archives of Dermatological Research*, vol. 291, no. 11, pp. 606–613, 1999.

[11] Y. Aoyama, M. K. Owada, and Y. Kitajima, "A pathogenic autoantibody, pemphigus vulgaris-IgG, induces phosphorylation of desmoglein 3, and its dissociation from plakoglobin in cultured keratinocytes," *European Journal of Immunology*, vol. 29, no. 7, pp. 2233–2240, 1999.

[12] J. Waschke, V. Spindler, P. Bruggeman, D. Zillikens, G. Schmidt, and D. Drenckhahn, "Inhibition of Rho A activity causes pemphigus skin blistering," *Journal of Cell Biology*, vol. 175, no. 5, pp. 721–727, 2006.

[13] R. Caldelari, A. De Bruin, D. Baumann, et al., "A central role for the armadillo protein plakoglobin in the autoimmune disease pemphigus vulgaris," *Journal of Cell Biology*, vol. 153, no. 4, pp. 823–834, 2001.

[14] C. C. Calkins, S. V. Setzer, J. M. Jennings, et al., "Desmoglein endocytosis and desmosome disassembly are coordinated responses to pemphigus autoantibodies," *Journal of Biological Chemistry*, vol. 281, no. 11, pp. 7623–7634, 2006.

[15] E. Delva, J. M. Jennings, C. C. Calkins, M. D. Kottke, V. Faundez, and A. P. Kowalczyk, "Pemphigus vulgaris IgG-induced desmoglein-3 endocytosis and desmosomal disassembly are mediated by a clathrin- and dynamin-independent mechanism," *Journal of Biological Chemistry*, vol. 283, no. 26, pp. 18303–18313, 2008.

[16] A. I. Chernyavsky, J. Arredondo, Y. Kitajima, M. Sato-Nagai, and S. A. Grando, "Desmoglein versus non-desmoglein signaling in pemphigus acantholysis: characterization of novel signaling pathways downstream of pemphigus vulgaris antigens," *Journal of Biological Chemistry*, vol. 282, no. 18, pp. 13804–13812, 2007.

[17] L. Williamson, N. A. Raess, R. Caldelari, et al., "Pemphigus vulgaris identifies plakoglobin as key suppressor of c-Myc in the skin," *EMBO Journal*, vol. 25, no. 14, pp. 3298–3309, 2006.

[18] A. Lanza, N. Cirillo, R. Rossiello, et al., "Evidence of key role of Cdk2 overexpression in pemphigus vulgaris," *Journal of Biological Chemistry*, vol. 283, no. 13, pp. 8736–8745, 2008.

[19] M. O. Hengartner, "The biochemistry of apoptosis," *Nature*, vol. 407, no. 6805, pp. 770–776, 2000.

[20] X. Wang, F. Brégégère, M. Frušić-Zlotkin, M. Feinmesser, B. Michel, and Y. Milner, "Possible apoptotic mechanism in epidermal cell acantholysis induced by pemphigus vulgaris autoimmunoglobulins," *Apoptosis*, vol. 9, no. 2, pp. 131–143, 2004.

[21] R. Wolf, H. Matz, E. Ruocco, and V. Ruocco, "The putative role of apoptosis in the induction of pemphigus," *Medical Hypotheses*, vol. 64, no. 1, pp. 44–45, 2005.

[22] I. Zuccolotto, A. M. Roselino, L. N. Z. Ramalho, and S. Zucoloto, "Apoptosis and p63 expression in the pathogenesis of bullous lesions of endemic pemphigus foliaceus," *Archives of Dermatological Research*, vol. 295, no. 7, pp. 284–286, 2003.

[23] M. Puviani, A. Marconi, E. Cozzani, and C. Pincelli, "Fas ligand in pemphigus sera induces keratinocyte apoptosis through the activation of caspase-8," *Journal of Investigative Dermatology*, vol. 120, no. 1, pp. 164–167, 2003.

[24] P. Chiarugi and E. Giannoni, "Anoikis: a necessary death program for anchorage-dependent cells," *Biochemical Pharmacology*, vol. 76, no. 11, pp. 1352–1364, 2008.

[25] J. R. Schiltz, B. Michel, and R. Papay, "Pemphigus antibody interaction with human epidermal cells in culture," *Journal of Clinical Investigation*, vol. 62, no. 4, pp. 778–788, 1978.

[26] M. G. Pacheco-Tovar, E. Avalos-Díaz, E. Vega-Memije, et al., "The final destiny of acantholytic cells in pemphigus is Fas mediated," *Journal of the European Academy of Dermatology and Venereology*, vol. 23, no. 6, pp. 697–701, 2009.

[27] K. Sayama, S. Yonehara, Y. Watanabe, and Y. Miki, "Expression of Fas antigen on keratinocytes in vivo and induction of apoptosis in cultured keratinocytes," *Journal of Investigative Dermatology*, vol. 103, no. 3, pp. 330–334, 1994.

[28] D. B. R. Rodrigues, S. A. L. Pereira, M. A. D. Reis, et al., "In situ detection of inflammatory cytokines and apoptosis in pemphigus foliaceus patients," *Archives of Pathology and Laboratory Medicine*, vol. 133, no. 1, pp. 97–100, 2009.

[29] K. Reich, U. Brinck, M. Letschert, et al., "Graft-versus-host disease-like immunophenotype and apoptotic keratinocyte death in paraneoplastic pemphigus," *British Journal of Dermatology*, vol. 141, no. 4, pp. 739–746, 1999.

[30] K. F. Köhler and M. L. Petzl-Erler, "No evidence for association of the TP53 12139 and the BAX-248 polymorphisms with endemic pemphigus foliaceus (fogo selvagem)," *International Journal of Immunogenetics*, vol. 33, no. 2, pp. 141–144, 2006.

[31] S. A. Grando, J.-C. Bystryn, A. I. Chernyavsky, et al., "Apoptolysis: a novel mechanism of skin blistering in pemphigus vulgaris linking the apoptotic pathways to basal cell shrinkage and suprabasal acantholysis," *Experimental Dermatology*, vol. 18, no. 9, pp. 764–770, 2009.

[32] J. Weiske, T. Schöneberg, W. Schröder, M. Hatzfeld, R. Tauber, and O. Huber, "The fate of desmosomal proteins in apoptotic cells," *Journal of Biological Chemistry*, vol. 276, no. 44, pp. 41175–41181, 2001.

[33] R. L. Dusek, S. Getsios, F. Chen, et al., "The differentiation-dependent desmosomal cadherin desmoglein 1 is a novel caspase-3 target that regulates apoptosis in keratinocytes," *Journal of Biological Chemistry*, vol. 281, no. 6, pp. 3614–3624, 2006.

[34] C. Caulín, G. S. Salvesen, and R. G. Oshima, "Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis," *Journal of Cell Biology*, vol. 138, no. 6, pp. 1379–1394, 1997.

[35] N.-O. Ku, J. Liao, and M. B. Omary, "Apoptosis generates stable fragments of human type I keratins," *Journal of Biological Chemistry*, vol. 272, no. 52, pp. 33197–33203, 1997.

[36] X. Wang, F. Brégégère, Y. Soroka, M. Frusic-Zlotkin, and Y. Milner, "Replicative senescence enhances apoptosis induced by pemphigus autoimmune antibodies in human keratinocytes," *FEBS Letters*, vol. 567, no. 2 –3, pp. 281–286, 2004.

[37] A. Baroni, E. Buommino, I. Paoletti, M. Orlando, E. Ruocco, and V. Ruocco, "Pemphigus serum and captopril induce heat shock protein 70 and inducible nitric oxide synthase overexpression, triggering apoptosis in human keratinocytes," *British Journal of Dermatology*, vol. 150, no. 6, pp. 1070–1080, 2004.

[38] N. Li, M. Zhao, J. Wang, Z. Liu, and L. A. Diaz, "Involvement of the apoptotic mechanism in pemphigus foliaceus autoimmune injury of the skin," *Journal of Immunology*, vol. 182, no. 1, pp. 711–717, 2009.

[39] E. Schmidt, J. Gutberlet, D. Siegmund, D. Berg, H. Wajant, and J. Waschke, "Apoptosis is not required for acantholysis in pemphigus vulgaris," *American Journal of Physiology*, vol. 296, no. 1, pp. C162–C172, 2009.

[40] P. Berkowitz, P. Hu, Z. Liu, et al., "Desmosome signaling: inhibition of p38MAPK prevents pemphigus vulgaris IgG-induced cytoskeleton reorganization," *Journal of Biological Chemistry*, vol. 280, no. 25, pp. 23778–23784, 2005.

[41] P. Berkowitz, P. Hu, S. Warren, Z. Liu, L. A. Diaz, and D. S. Rubenstein, "p38MAPK inhibition prevents disease in pemphigus vulgaris mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 34, pp. 12855–12860, 2006.

[42] P. Berkowitz, M. Chua, Z. Liu, L. A. Diaz, and D. S. Rubenstein, "Autoantibodies in the autoimmune disease pemphigus foliaceus induce blistering via p38 mitogen-activated protein kinase-dependent signaling in the skin," *American Journal of Pathology*, vol. 173, no. 6, pp. 1628–1636, 2008.

[43] Y. Kawasaki, Y. Aoyama, K. Tsunoda, M. Amagai, and Y. Kitajima, "Pathogenic monoclonal antibody against desmoglein 3 augments desmoglein 3 and p38 MAPK phosphorylation in human squamous carcinoma cell line," *Autoimmunity*, vol. 39, no. 7, pp. 587–590, 2006.

[44] J. N. Lavoie, E. Hickey, L. A. Weber, and J. Landry, "Modulation of actin-microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27," *Journal of Biological Chemistry*, vol. 268, no. 32, pp. 24210–24214, 1993.

[45] J. N. Lavoie, H. Lambert, E. Hickey, L. A. Weber, and J. Landry, "Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27," *Molecular & Cellular Biology*, vol. 15, no. 1, pp. 505–516, 1995.

[46] J. Guay, H. Lambert, G. Gingras-Breton, J. N. Lavoie, J. Huot, and J. Landry, "Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27," *Journal of Cell Science*, vol. 110, no. 3, pp. 357–368, 1997.

[47] M. D. der Perng, L. Cairns, P. Van Den IJssel, A. Prescott, A. M. Hutcheson, and R. A. Quinlan, "Intermediate filament interactions can be altered by HSP27 and alphaB-crystallin," *Journal of Cell Science*, vol. 112, no. 13, pp. 2099–2112, 1999.

[48] L. Feng, X. Zhou, J. Liao, and M. B. Omary, "Pervanadate-mediated tyrosine phosphorylation of keratins 8 and 19 via a p38 mitogen-activated protein kinase-dependent pathway," *Journal of Cell Science*, vol. 112, no. 13, pp. 2081–2090, 1999.

[49] O. V. Evgrafov, I. Mersiyanova, J. Irobi, et al., "Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy," *Nature Genetics*, vol. 36, no. 6, pp. 602–606, 2004.

[50] S. R. Woll, R. Windoffer, and R. E. Leube, "p38 MAPK-dependent shaping of the keratin cytoskeleton in cultured cells," *Journal of Cell Biology*, vol. 177, no. 5, pp. 795–807, 2007.

[51] P. S. Jolly, P. Berkowitz, M. Bektas, et al., "p38MAPK signaling and desmoglein-3 internalization are linked events in pemphigus acantholysis," *Journal of Biological Chemistry*, vol. 285, pp. 8936–8941, 2010.

[52] Z. Xia, M. Dickens, J. Raingeaud, R. J. Davis, and M. E. Greenberg, "Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis," *Science*, vol. 270, no. 5240, pp. 1326–1331, 1995.

[53] P. Juo, C. J. Kuo, S. E. Reynolds, et al., "Fas activation of the p38 mitogen-activated protein kinase signalling pathway requires ICE/CED-3 family protease," *Molecular and Cellular Biology*, vol. 17, no. 1, pp. 24–35, 1997.

[54] P. A. Henkart, "Ice family proteases: mediators of all apoptotic cell death?" *Immunity*, vol. 4, no. 3, pp. 195–201, 1996.

[55] H. E. Lee, P. Berkowitz, P. S. Jolly, L. A. Diaz, M. P. Chua, and D. S. Rubenstein, "Biphasic activation of p38MAPK suggests that apoptosis is a downstream event in pemphigus acantholysis," *Journal of Biological Chemistry*, vol. 284, no. 18, pp. 12524–12532, 2009.

[56] G.-Z. Tao, K. S. Looi, D. M. Toivola, et al., "Keratins modulate the shape and function of hepatocyte mitochondria: a mechanism for protection from apoptosis," *Journal of Cell Science*, vol. 122, no. 21, pp. 3851–3855, 2009.

[57] C. Schwerk and K. Schulze-Osthoff, "Non-apoptotic functions of caspases in cellular proliferation and differentiation," *Biochemical Pharmacology*, vol. 66, no. 8, pp. 1453–1458, 2003.

[58] S. Futamura, C. Martins, E. A. Rivitti, R. S. Labib, L. A. Diaz, and G. J. Anhalt, "Ultrastructural studies of acantholysis induced in vivo by passive transfer of IgG from endemic pemphigus foliaceus (fogo selvagem)," *Journal of Investigative Dermatology*, vol. 93, no. 4, pp. 480–485, 1989.

[59] Y. Aoyama and Y. Kitajima, "Pemphigus vulgaris-IgG causes a rapid depletion of desmoglein 3 (Dsg3) from the Triton X-100 soluble pools, leading to the formation of Dsg3- depleted desmosomes in a human squamous carcinoma cell line, DJM-1 cells," *Journal of Investigative Dermatology*, vol. 112, no. 1, pp. 67–71, 1999.

[60] N. Cirillo, F. Gombos, and A. Lanza, "Changes in desmoglein 1 expression and subcellular localization in cultured keratinocytes subjected to anti-desmoglein 1 pemphigus autoimmunity," *Journal of Cellular Physiology*, vol. 210, no. 2, pp. 411–416, 2007.

[61] X. Mao, E. J. Choi, and A. S. Payne, "Disruption of desmosome assembly by monovalent human pemphigus vulgaris monoclonal antibodies," *Journal of Investigative Dermatology*, vol. 129, no. 4, pp. 908–918, 2009.

[62] M. Amagai, N. Matsuyoshi, Z. H. Wang, C. Andl, and J. R. Stanley, "Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1," *Nature Medicine*, vol. 6, no. 11, pp. 1275–1277, 2000.

[63] Y. Hanakawa, N. M. Schechter, C. Lin, et al., "Molecular mechanisms of blister formation in bullous impetigo and staphylococcal scalded skin syndrome," *Journal of Clinical Investigation*, vol. 110, no. 1, pp. 53–60, 2002.

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Research Article

Targeted Immunotherapy with Rituximab Leads to a Transient Alteration of the IgG Autoantibody Profile in Pemphigus Vulgaris

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In pemphigus vulgaris (PV), IgG autoantibodies against the ectodomain of desmoglein 3 (Dsg3) have been shown to be directly responsible for the loss of keratinocyteadhesion. The aim of the present study was to study the effect of the B cell depleting anti-CD20 monoclonal antibody, rituximab, on the profile of pathogenic IgG against distinct regions of the Dsg3 ectodomain in 22 PV patients who were followed up clinically and serologically by Dsg3 ELISA over 12-24 months. Prior to rituximab, all the 22 PV patients showed IgG against Dsg3 (Dsc3EC1-5). Specifically, 14/22 showed IgG reactivity against the Dsg3EC1 subdomain, 5/22 patients against Dsg3EC2, 7/22 against Dsg3EC3, 11/22 against Dsg3EC4, and 2/22 against Dsg3EC5. Within 6 months after rituximab, all the patients showed significant clinical improvement and reduced IgG against Dsg3 (5/22) and the various subdomains, that is, Dsg3EC1 (7/22), Dsg3EC2 (3/22), Dsg3EC3 (2/22), sg3EC4 (2/22), and Dsg3EC5 (0/22). During the entire observation period, 6/22 PV patients experienced a clinical relapse which was associated with the reappearance of IgG against previously recognized Dsg3 subdomains, particularly against the Dsg3EC1. Thus, in PV, rituximab only temporarily depletes pathogenic B cell responses against distinct subdomains of Dsg3 which reappear upon clinical relapse.

1. Introduction

Pemphigus vulgaris (PV) is a life-threatening autoimmune blistering disease caused by IgG autoantibodies (auto-Ab) against the extracellular domain (ECD) of desmoglein 3 (Dsg3) and Dsg1, desmosomal adhesion molecules present on epidermal keratinocytes [1]. Auto-Ab production in PV is polyclonal, and in active PV, most auto-Abs are of the IgG_4 subclass [2, 3]. It has been postulated that auto-Ab against Dsg3 and Dsg1 primarily target the NH_2-terminal portion of the Dsg3 ectodomain, that is, the Dsg3EC1 subdomain [4–8]. The EC1 domains of Dsg3 and Dsg1, respectively, according to morphologic studies in desmosomes, are increasingly recognized as the part of the desmosomal cadherin ectodomain which is involved in Dsg transinteraction [9]. Moreover, it seems that IgG auto-Ab reactivity against the Dsg3EC1 domain correlates with active disease in PV although the titers of the auto-Ab do not show a strict correlation with disease activity [10, 11]. Recently, we found a significant correlation between IgG reactivity against Dsg3EC1 and the extent of clinical involvement in PV [8]. IgG against the Dsg3EC1 was seen preferentially in PV patients with mucocutaneous involvement but not in patients with either mucosal or skin involvement only [8].

IgG auto-Ab from pemphigus sera cause loss of adhesion of human skin in vivo and in vitro [12, 13]. Because auto-Ab in pemphigus are directed against desmosomal adhesion molecules, it was suggested that these autoantibodies might directly interfere with Dsg transinteraction binding sites [14–17]. Previous studies showed that IgG against the NH_2-terminus of the Dsg3 ectodomain is pathogenic [18]. Affinity-purified IgG of sera from PV patients injected into neonatal mice which were reactive with the EC1 and EC2 of Dsg3, respectively, induced suprabasilar acantholysis, while

IgG reactive with the EC3-5 of Dsg3 did not [10]. AK23, a mouse monoclonal Ab against Dsg3 which targets the predicted binding motif of the Dsg3EC1, has been shown to be pathogenic in vivo whereas IgG targeting other regions of the Dsg3 ectodomain was not [5, 19]. AK23 directly interferes with homophilic adhesion of two Dsg3 proteins [20] which supports the hypothesis that auto-Ab from PV patients directly inhibit Dsg3-mediated epidermal cell adhesion [21].

Apart from major advances in our understanding of the immune pathogenesis of pemphigus, therapeutic options in cases of recalcitrant pemphigus are rather limited. The standard immunosuppressive treatment of pemphigus consists of systemic glucocorticoids and adjuvant immunosuppressive drugs which induce partial or complete clinical remission in the majority of the patients. In the remaining refractory cases, the B-cell depleting anti-CD20 monoclonal Ab, rituximab, has been recently introduced as a highly effective rescue medication. Rituximab is a chimeric human/mouse IgG1 monoclonal ab and is directed against CD20, a pan B cell glycoprotein on B lymphocytes from the preB cell to the preplasma-cell stage. Among several mechanisms involved in B cell killing, rituximab exerts B cell cytolytic activity mainly through ab-dependent cell-mediated cytotoxicity. A plethora of case series and two prospective clinical trials strongly suggest that rituximab is highly effective in recalcitrant pemphigus [22].

In the present study, the impact of rituximab treatment on the profile of Dsg3-specific auto-Ab was studied in a cohort of 22 well-characterized patients with PV. During a 12 to 24 months' observation period, IgG reactivity against distinct regions of the Dsg3 ectodomain was correlated with clinical parameters such as involvement of body surface area and mucosal surfaces. Our findings suggest that rituximab treatment only temporarily depletes distinct IgG reactivity against the Dsg3 ectodomain and that the reappearance of such auto-ab is associated with clinical relapses. In particular, IgG reactivity against the NH_2-terminal EC1 subregion of the Dsg3 ectodomain was preferentially detected in PV patients who experienced a clinical relapse.

2. Patients and Methods

2.1. PV Patients. Serum samples were obtained from 22 adult patients with PV who were seen at the Dermatology Departments at the Universities of Cologne, Marburg, and Rouen. Patients gave written consent to participate in this study which was adherent to the Declaration of Helsinki Guidelines and which was approved by the local Ethics Committees. The clinical diagnosis of PV was confirmed by (1) histopathology (suprabasal acantholytic blisters), (2) direct immunofluorescence microscopy (epidermal intercellular IgG and/or C3 deposits in perilesional skin), (3) detection of serum IgG auto-ab by indirect immunofluorescence microscopy (intercellular IgG binding to epithelial cells of monkey esophagus), and (4) by ELISA with recombinant Dsg3 and Dsg1. All the 22 PV patients (mean age: 51.3 ± 15.7 years; 11 females and 11 males) mainly presented with mucocutaneous involvement and elevated anti-Dsg IgG titers (Table 1) despite ongoing (> 3 months) immunosuppressive treatment with systemic corticosteroids (initially 1 mg/kg/d; tapered logarithmically upon clinical response) and the immunosuppressive agents, azathioprine (100–150 mg/day) or mycophenolate mofetil (1-2 g/day), respectively (Table 1).

The clinical extent and severity of PV was classified according to the number of blisters or erosions as either mucosal or cutaneous involvement (the later was quantitated by the body surface area; BSA). All the PV patients were treated i.v. with rituximab at 375 mg/m^2 on days 0, 7, 14, and 21 and were kept on an immunosuppressive treatment regimen consisting of prednisolone and azathioprine or mycophenolate mofetil, respectively.

2.2. ELISA with Dsg3 Recombinants. Recombinants representing the entire Dsg3 ectodomain (Dsg3EC1-5) and distinct subregions of the Dsg3 ectodomain, that is, Dsg3EC1-5 (aa 1-566), Dsg3EC1 (aa 1-161), Dsg3EC2 (aa 87-227), Dsg3EC3 (aa 184-349), Dsg3EC4 (aa 313-451) and Dsg3EC5 (aa 424-566) were produced in an eukaryotic baculovirus expression system and were purified using Nickel-NTA as previously described [8, 23]. By ELISA, the patients' sera were tested for IgG reactivity against the Dsg3 ectodomain (Dsg3EC1-5) as well as IgG reactivity against subdomains of the extracellular portion of Dsg3 as recently described [8, 23]. In short, the recombinant Dsg proteins were immobilized on microtiter plates and were incubated with the PV patients' sera at a dilution of 1 : 50. IgG binding was visualized as optical density (OD) at 405 nm. Samples were run at least in duplicate and OD's were expressed as mean values. The threshold for IgG binding of the Dsg3 recombinants in the ELISA yielded an OD value of 0.376 as determined by a ROC curve generated for Dsg3EC1-5 [23]. OD values above the cutoff point defined positive IgG reactivity, while IgG reactivity below the cutoff point was considered to be negative.

2.3. Scoring of Disease Activity in Pemphigus. Scores for mucosal involvement were set as follows: 0, if the patient had no mucosal involvement, 1 point for each additional location of blisters (i.e., oral, dysphagia, genital, conjunctival involvement) and additionally, 1 point for >3 erosions with a diameter >2 mm. Skin involvement was assessed by body surface area and was expressed as percentage of affected area.

3. Results

3.1. Clinical Response to Rituximab of the Studied PV Patients. All the 22 PV patients showed a marked clinical response to rituximab treatment which was expressed as dramatically reduced mucosal (baseline: 4.60 ± 1.96; 1.59 ± 1.80 after 6 months) and BSA (baseline: 11.05 ± 13.14; 0.53 ± 1.01 after 6 months) scores within six months after treatment (Figure 1(a)). Among the studied patients, 18/22 (81.8%) had skin involvement and 18/22 (81.8%) mucosal involvement. Except for patient PV6 whose clinical symptoms were unknown, all the PV patients had mucosal

Table 1: Clinical and immunological characteristics of the pemphigus vulgaris (PV) patients on adjuvant treatment with rituximab.

Patients	Treatment[1]	Sex	Age	Clinical phenotype of PV[2]	Severity[3]		IgG autoantibody profile[4]	
					Mucosa	BSA (%)	Anti-Dsg1	Anti-Dsg3
PV1	Pred., MMF, Dapson	m	53	mucocutaneous	2	3	103	549
PV2	Pred., MMF, MTX	m	67	mucocutaneous	4	1	10	484
PV3	Pred., MMF, Azathioprin	f	69	mucosal	7	0	neg	810
PV4	Pred.	f	69	no mucosal involvement	0	4	163	2368
PV5	Pred. MMF, Azathioprin	f	37	mucocutaneous	3	5	183	94
PV6	n.d.	m	48	n.d.	n.d.	n.d.	979	906
PV7	Pred., MMF	m	50	mucocutaneous	8	1	82	201
PV8	Pred., MMF, Dapson	f	23	mucocutaneous	2	12	357	145
PV10	Pred., MMF	f	57	mucosal	6	0	3	124
PV11	Pred., MMF, MTX	f	28	mucosal	5	0	6	229
PV12	Pred., MMF	f	54	mucocutaneous	7	5	199	175
PV13	n.d.	m	62	mucocutaneous	7	10	78	220
PV14	Pred.	m	79	mucocutaneous	6	10	170	200
PV15	Pred.	m	36	mucocutaneous	4	60	155	201
PV16	n.d.	m	64	mucocutaneous	6	20	163	58
PV17	Pred.	f	51	mucocutaneous	5	10	181	209
PV18	Pred.	f	70	mucocutaneous	4	10	neg	143
PV19	n.d.	m	59	mucocutaneous	4	10	98	156
PV20	Pred.	m	38	mucocutaneous	4	20	36	138
PV21	Pred.	f	41	mucocutaneous	5	10	18	119
PV22	Pred.	m	24	mucocutaneous	4	20	138	135
PV23	Pred.	f	50	Mucocutaneous	5	10	176	163

[1]Systemic prednisolone (Pred) was administered throughout the observation period and was logarithmically tapered upon clinical reponse to treatment; azathioprine (AZA) or mycophenolate mofetil (MMF), respectively, was also administered throughout the observation period. n.d., not determined.
[2]Before treatment with rituximab.
[3]Determined by the extent of cutaneous involvement as body surface area (BSA) and the extent of mucosal involvement.
[4]Determined by enzyme-linked immunosorbent assay (ELISA) with recombinant desmoglein (Dsg) 1 and 3; optical densities are expressed as PIV (protein index value).
[5]PIV cutoff is 20.

involvement. Clinical improvement to rituximab was also monitored as a marked reduction of systemic treatment with prednisolone from 0.63 ± 0.60 mg/kg body weight (baseline) to 0.20 ± 0.17 mg/kg body weight (6 months after rituximab treatment) (Figure 1(a)). Noteworthy, 6/22 PV patients experienced a relapse 12 to 24 months after rituximab treatment.

3.2. IgG Titers against Distinct Dsg3 Subdomains and Disease Activity of the PV Patients Are Reduced upon Treatment with Rituximab. IgG auto-ab of the 22 PV patients against the Dsg3 ectodomain and its subdomains was analysed by ELISA during the course of the disease (Figure 1(b)). Prior to treatment with rituximab, all PV patients showed IgG reactivity against at least some part of the entire Dsg3 ectodomain (Dsg3EC1-5), 14/22 showed IgG reactivity against the Dsg3EC1 subdomain, 5/22 patients against Dsg3EC2, 7/22 against Dsg3EC3, 11/22 against Dsg3EC4, and 2/22 against Dsg3EC5. Within 6 months after treatment, there was a marked reduction of IgG reactivity against the Dsg3 ectodomain as well as against distinct subdomains (Figure 1(b)). Because of the availability of patients sera at all time points, the persistence or disappearance of IgG against distinct Dsg3 regions upon treatment with rituximab was studied in more detail in 16 PV patients.

Among these patients, the number of patients with IgG reactivity against the recombinant proteins of Dsg3 of less than 80% of the initial level was studied 6 and 12 months after rituximab (Figure 2). Initially, 16 PV patients showed IgG reactivity against Dsg3EC1-5, 11 against Dsg3EC1, 4 against Dsg3EC2, 5 against Dsg3EC3, 6 against Dsg3EC4, and 2 against Dsg3EC5. Of the 16 patients, a total of 11 showed reduced (by more than 80%) IgG reactivity against the Dsg3 ectodomain (Dsg3EC1-5): Six months after treatment with rituximab, IgG reactivity against Dsg3EC1 was more than 80% reduced in 4/11 patients, against Dsg3EC2 in 1/4 PV patients, against Dsg3EC3 in 3/5 patients against Dsg3EC4 in 4/6, and against Dsg3EC5 in 2/5 patients. Twelve months after treatment with rituximab, IgG reactivity of less than 80% of the initial value was noticed in 11/16 (Dsg3EC1-5), 4/11 (Dsg3EC1), 1/4 (Dsg3EC2), 2/5 (Dsg3EC3), 3/6 (Dsg3EC4) and 2/2 (Dsg3EC5) PV patients. Specifically, the impact of rituximab treatment on IgG reactivity against distinct Dsg3 subdomains is given in Table 2 (0 and 6 months).

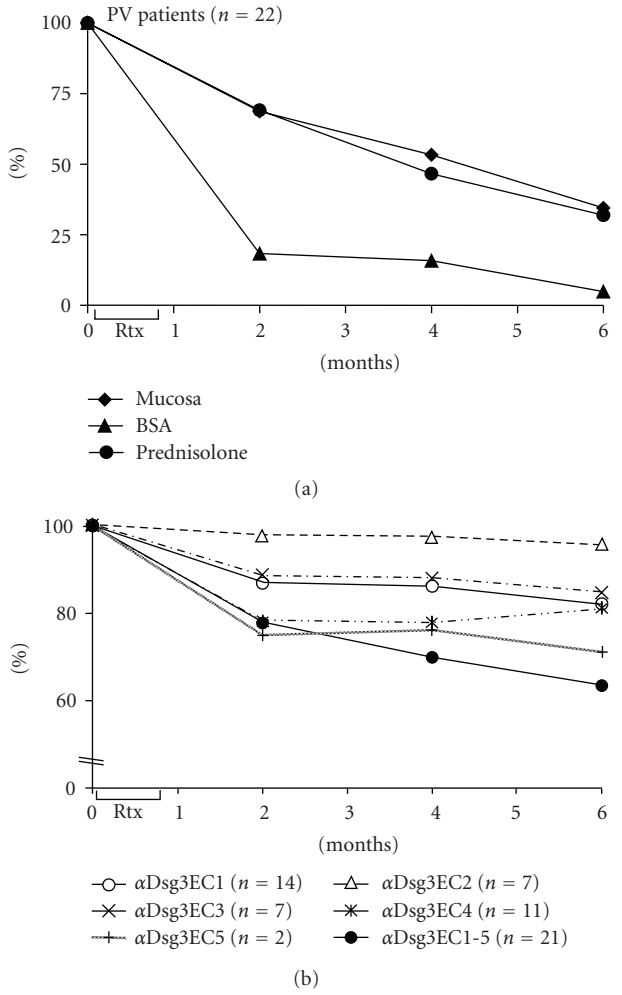

Figure 1: Rituximab (Rtx) treatment of pemphigus vulgaris (PV) patients leads to clinical improvement of skin and mucosal lesions and is accompanied by decreased IgG titers against distinct regions of desmoglein 3 (Dsg3) ectodomain. Treatment with Rtx of 22 PV patients led to a significant improvement of mucosal and skin lesions and is expressed as reduced mucosal and body surface area (BSA) scores within 6 months after treatment (Figure 1(a)). In the same way, the dose of prednisolone was reduced (Figure 1(a)). By enzyme-linked immunosorbent assay, there was clearly a reduction of IgG reactivity against the Dsg3 ectodomain (Dsg3EC1-5) and defined regions of the Dsg3 ectodomain (Figure 1(b)). The number of studied patients or sera, respectively, is given in parenthesis. Rtx (time when rituximab was administered).

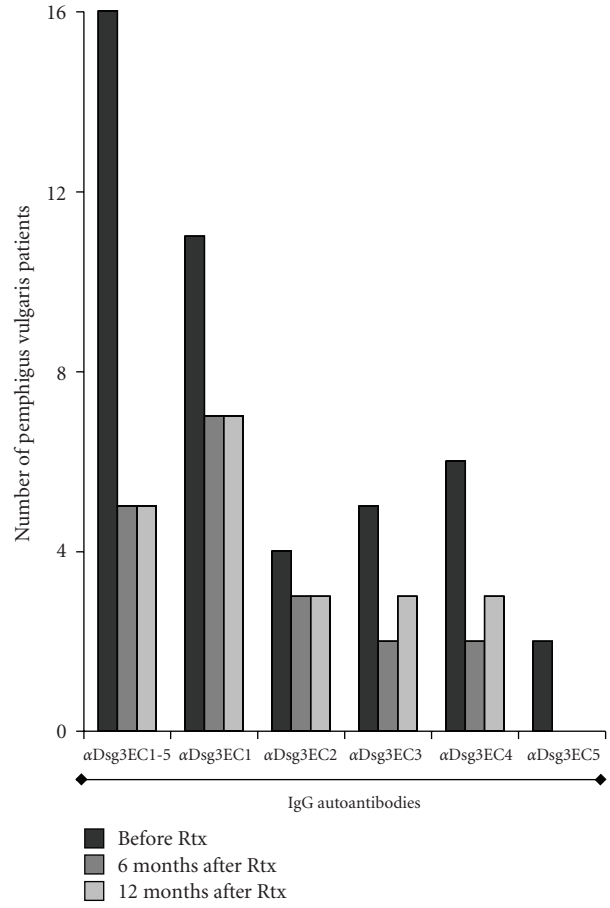

Figure 2: IgG reactivity against desmoglein 3 (Dsg3) subdomains in pemphigus vulgaris (PV) patients on rituximab (Rtx). Illustrated is the number of PV patients showing IgG against Dsg3 subdomains before and 6 and 12 months after treatment with Rtx, respectively.

3.3. Reappearance of IgG against Distinct Regions of the Dsg3 Ectodomain in PV Patients with Clinical Relapses after Rituximab Treatment. Six of the 22 PV patients treated with rituximab showed a clinical relapse 12 months ($n = 3$; PV3, PV5, PV6) or 18 months ($n = 3$; PV13, PV19, PV23), respectively, after rituximab treatment (Table 3). Clinical relapses were defined as newly arisen mucosal or cutaneous lesions which persisted for more than 7 days. All the relapsed PV patients showed increased IgG auto-ab against at least some part of the entire Dsg3 ectodomain (Figure 3) but with individual patterns of IgG recognition of Dsg3 subdomains (Table 3). In the individual PV patients, clinical relapses were associated with the reappearance of distinct auto-ab profiles (Figure 3). Four of the 6 relapsed PV patients had IgG against the Dsg3EC1 subdomain prior to rituximab treatment, that is, patients PV3, PV6, PV13, and PV19 (Figure 3). In these PV patients, IgG auto-ab against Dsg3EC1 decreased (PV6, PV13) or remained constant (P3, P19) upon rituximab treatment. Noteworthy, in all these patients, IgG against the Dsg3EC1 persisted until or reappeared at the time of clinical relapse. PV patient PV23 did not initially show IgG against Dsg3EC1 but at the time of relapse 18 months after rituximab treatment.

Only PV patient PV5 had a different auto-ab profile and showed IgG against Dsg3EC3 and Dsg3EC4 prior to rituximab. At the time of clinical relapse 12 months later, he showed IgG against Dsg3EC4 which exceeded pretreatment levels (Figure 3). In patient PV5, IgG against Dsg3EC1 was not detected at the time of clinical relapse.

Noteworthy, some of these patients showed IgG reactivity against distinct Dsg3 subdomains in addition to IgG against Dsg3EC1 at the time of clinical relapse. Patient PV3 showed IgG against Dsg3EC1, Dsg3EC2, and Dsg3EC4 when

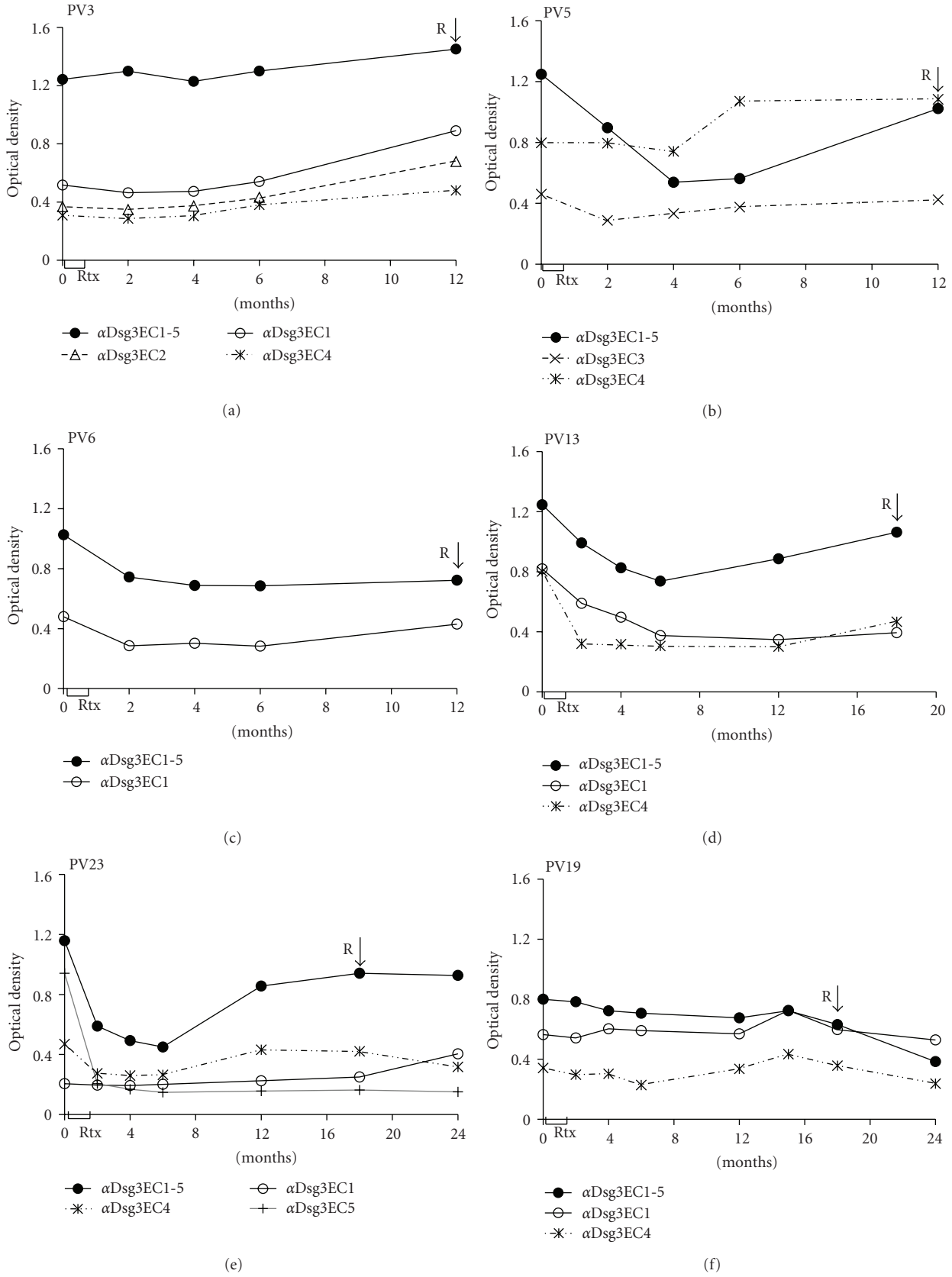

Figure 3: Association of clinical relapses of rituximab (Rtx)-treated pemphigus vulgaris (PV) patients and the reappearance of IgG against distinct regions of the Dsg3 ectodomain. Three of the 6 PV patients (PV3, PV6, PV23) showed an increase of IgG reactive with the Dsg3EC1 subdomain upon clinical relapse after Rtx therapy. Patient PV13 showed increased serum levels of IgG against the Dsg3EC4 subdomain while patients PV5 and PV19 did not show altered IgG levels against any of the studied Dsg3 subdomains upon clinical relapse.

Table 2: Profile of desmoglein 3 (Dsg3)-specific IgG autoantibodies of the pemphigus vulgaris (PV) patients before and 6 months after treatment with rituximab (Rtx).

	Dsg3EC1		Dsg3EC2		Dsg3EC3		Dsg3EC4		Dsg3EC5	
Time point	0	6	0	6	0	6	0	6	0	6
PV1	+	+	+	+	+	+	−	−	−	−
PV2	+	+	+	+	−	−	−	−	−	−
PV3	+	+	−	+	−	−	−	+	−	−
PV4	+	−	−	−	+	+	+	−	−	−
PV5	−	−	−	−	+	−	+	+	−	−
PV6	+	−	−	−	−	−	−	−	−	−
PV7	+	+	+	+	−	−	−	−	−	−
PV8	−	−	−	−	+	−	−	−	−	−
PV10	−	−	−	+	−	−	+	−	−	−
PV11	+	+	−	−	−	−	−	−	−	−
PV12	+	+	+	−	+	+	+	+	−	−
PV13	+	−	−	−	−	−	+	−	−	−
PV14	+	−	+	−	−	−	+	−	+	−
PV15	−	−	−	−	+	−	−	−	−	−
PV16	−	−	−	−	−	−	+	+	−	−
PV17	+	+	−	−	−	−	−	−	−	−
PV18	−	−	−	−	−	−	+	−	−	−
PV19	+	+	−	−	−	−	−	−	−	−
PV20	+	+	−	−	−	−	−	−	−	−
PV21	+	+	−	−	+	+	+	−	−	−
PV22	−	−	−	−	−	−	+	−	−	−
PV23	−	−	−	−	−	−	+	+	+	−

Table 3: Synopsis of the IgG autoantibody profile of the pemphigus vulgaris (PV) patients prior to rituximab (preRtx) treatment and at the time of clinical relapse (CR).

	IgG against extracellular subdomains of Dsg3														
	Dsg3EC1			Dsg3EC2			Dsg3EC3			Dsg3EC4			Dsg3EC5		
Time point	preRtx	6 months	CR	preRtx	6 months	CR	preRtx	6 months	CR	preRtx	6 months	CR	preRtx	6 months	CR
PV3 (CR, months 12)	+	+	+	−	+	+	−	−	−	−	+	+	−	−	−
PV5 (CR, months 12)	−	−	−	−	−	+	+	−	+	+	+	+	−	−	−
PV6 (CR, months 12)	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
PV13 (CR, months 18)	+	−	+	−	−	−	−	−	−	+	−	+	−	−	−
PV19 (CR, months 18)	+	+	+	−	−	−	−	−	−	−	−	+	−	−	−
PV23 (CR, months 18)	−	−	+	−	−	−	−	−	−	+	+	+	+	−	−

he developed new cutaneous lesions and patient PV5 had IgG against Dsg3EC1, Dsg3EC2, Dsg3EC3, and Dsg3EC4 when he relapsed. Finally, PV patient PV23 showed IgG against Dsg3EC1 and Dsg3EC4 upon clinical relapse. None of the 6 PV patients showed IgG against the COOH-terminal Dsg3EC5 subdomain at the time of clinical relapse (Figure 3).

4. Discussion

The present study strongly suggests that rituximab, a monoclonal antibody against CD20 on B cells, does not permanently deplete pathogenic B cell responses against Dsg3 in patients with PV, a potentially lethal autoimmune bullous disorder of skin and mucosa. Here, a total of 22 patients with refractory PV were treated with rituximab which had been recently identified as a potent treatment in refractory PV [24, 25]. Specifically, these patients did not adequately respond to standard immunosuppressive treatment consisting of high dose systemic corticosteroids combined with the immunosuppressive adjuvants, azathioprine, or mycophenolate mofetil, respectively.

In the cohort of studied patients, all of the 22 PV patients initially showed an excellent clinical response to rituximab

treatment. However, within 12–18 months after rituximab treatment, 6/22 PV patients experienced a clinical relapse which was associated with the persistence or reappearance of IgG auto-ab against distinct extracellular subdomains of Dsg3, the major autoantigen of PV (Figure 3). The majority, that is, 5/6 of the relapsed PV patients, showed the reappearance of IgG against the NH_2-terminal subdomain Dsg3EC1 which has been previously identified as the site where most of the pathogenic PV auto-Ab bind [11, 19, 26, 27]. In addition, several of the relapsed patients showed IgG reactivity against additional subdomains of Dsg3 most of which had initially disappeared upon treatment with rituximab. Of note, IgG reactivity against the COOH-terminal EC5 subdomain of Dsg3 was not observed in relapsed patients. This region which is located close to the keratinocyte cell membrane is presumably not a target for pathogenic auto-ab in PV [11]. Thus, IgG reactivity against distinct regions of the Dsg3 subdomain which was abolished by rituximab treatment reappeared in these patients at the time of clinical relapse strongly suggesting that the repertoire of Dsg3-specific B cells was only temporarily deleted by rituximab treatment. The frequent detection of Dsg3EC1-specific IgG in clinically relapsed PV patients supports the concept that pathogenic PV auto-ab preferentially target the NH2-terminal EC1 domain of Dsg3. The relative pathogenic role of IgG against Dsg3 subdomains other than Dsg3EC1 awaits further analysis.

The majority, that is, 16/22 PV patients, were successfully controlled by treatment with rituximab and prednisolone over 12 and 24 months, respectively. The other 6 patients showed a clinical relapse during the observation period. Our findings with rituximab are in line with previous reports demonstrating an excellent clinical response associated with a strong reduction of Dsg1- and Dsg3-specific IgG levels on adjuvant treatment with rituximab [2, 25].

Of note, clinical improvement of mucosal and skin lesions of the rituximab-treated PV patients was significantly associated with a decrease of IgG auto-ab titers. However, we did not find any significant correlation between IgG against distinct subdomains of Dsg3 and skin or mucosal involvement. However, our findings suggest that IgG against the Dsg3EC1 domain is associated with active PV as shown earlier by our group and others [5, 8, 11, 23]. The Dsg3 recombinants of the present studies may not contain all the conformational epitopes that are relevant in the immune pathogenesis of pemphigus. However, as proven in two recent reports, these findings with these Dsg3 recombinants are valid and show differential reactivity against distinct portions of the Dsg3 ectodomain in different pemphigus patients and different diseases stages [8, 23].

Recently, we found a significant correlation between IgG reactivity against the Dsg3EC1 and the extent of clinical involvement in PV [8]. IgG against the Dsg3EC1 was seen preferentially in PV patients with muco-cutaneous involvement but not in patients with either mucosal or skin involvement only [8]. IgG auto-ab from pemphigus sera are sufficient to cause blistering in human skin in vivo and in vitro [12, 13] by direct interference with Dsg transinteraction binding sites [14–17].

Previous studies showed that IgG against the NH_2-terminus of the Dsg3 ectodomain is pathogenic [18]. Affinity-purified IgG from sera of PV patients injected into neonatal mice which were reactive with the EC1 and EC2 of Dsg3 induced suprabasilar acantholysis, while IgG reactive with the EC3-5 of Dsg3 did not [10]. AK23, a mouse monoclonal ab against Dsg3 which targets the predicted binding motif of the Dsg3EC1, has been shown to be pathogenic in vivo whereas IgG that targets other parts of the Dsg3 ectodomain was not [5, 19]. Additionally, AK23, is able to directly interfere with homophilic Dsg3 binding [20] which supports the hypothesis that auto-ab from PV patients directly inhibit Dsg3-mediated epidermal cell adhesion [21].

Sekiguchi et al. suggested that, both pathogenic and nonpathogenic auto-ab exist in PV [5]. This heterogeneity among anti-Dsg3 antibody due to specific epitope recognition was previously demonstrated [19]. In addition to pathogenic IgG auto-ab against Dsg3EC1, IgG against additional Dsg3 subdomains, that is, DsgEC2, DsgEC3, and Dsg3 EC4, may not be pathogenic per se but may act synergistically in inducing the pathology of intraepidermal loss of adhesion [8]. This contention is supported by a recent study showing that nonpathogenic monoclonal IgG ab that recognize regions of the Dsg3 ectodomain other than the NH_2-terminus induce desmosomal loss of adhesion when injected together into mice [28].

Further studies are necessary to investigate the dynamics of pathogenic IgG against the Dsg3EC1 and auto-ab against other Dsg3 subdomains in the immune pathogenesis of PV. At present, it remains to be elucidated why rituximab only temporarily depletes pathogenic auto-ab in PV. Previous observations from our laboratory strongly suggest the presence of short-lived, Dsg3-reactive plasma cells since rituximab rapidly reduced Dsg3-specific IgG auto-ab but not IgG against recall antigens such as tetanus toxoid, Epstein Barr virus or influenza virus [29, 30]. Based on the present and previous observations, detection and monitoring of IgG against the Dsg3EC1 may be a more sensitive marker of disease activity in patients with PV than monitoring IgG against the entire Dsg3 ectodomain.

Abbreviations

Autoantibody:	auto-ab
Dsg:	desmoglein
Dsg3EC1-5:	baculoprotein representing the entire ectodomain (aa1-566) of desmoglein 3
Dsg3EC1:	baculoprotein containing aa1-161 of desmoglein 3
Dsg3EC2:	baculoprotein containing aa87-227 of desmoglein 3
Dsg3EC3:	baculoprotein containing aa184-349 of desmoglein 3
Dsg3EC4:	baculoprotein containing aa313-451 of desmoglein 3
Dsg3EC5:	baculoprotein containing aa424-566 of desmoglein 3
PV:	pemphigus vulgaris.

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References

[1] M. Amagai, P. J. Koch, T. Nishikawa, and J. R. Stanley, "Pemphigus vulgaris antigen (desmoglein 3) is localized in the lower epidermis, the site of blister formation in patients," *Journal of Investigative Dermatology*, vol. 106, no. 2, pp. 351–355, 1996.

[2] A. R. Ahmed, Z. Spigelman, L. A. Cavacini, and M. R. Posner, "Treatment of pemphigus vulgaris with rituximab and intravenous immune globulin," *The New England Journal of Medicine*, vol. 355, no. 17, pp. 1772–1779, 2006.

[3] A. Nagel, A. Lang, D. Engel, et al., "Clinical activity of vulgaris relates to IgE autoantibodies against desmoglein 3," *Clinical Immunology*, vol. 134, no. 3, pp. 320–330, 2010.

[4] Y. Futei, M. Amagai, M. Sekiguchi, K. Nishifuji, Y. Fujii, and T. Nishikawa, "Use of domain-swapped molecules for conformational epitope mapping of desmoglein 3 in pemphigus vulgaris," *Journal of Investigative Dermatology*, vol. 115, no. 5, pp. 829–834, 2000.

[5] M. Sekiguchi, Y. Futei, Y. Fujii, T. Iwasaki, T. Nishikawa, and M. Amagai, "Dominant autoimmune epitopes recognized by pemphigus antibodies map to the N-terminal adhesive region of desmogleins," *Journal of Immunology*, vol. 167, no. 9, pp. 5439–5448, 2001.

[6] M. K. Hacker-Foegen, M. Janson, M. Amagai, J. A. Fairley, and M.-S. Lin, "Pathogenicity and epitope characteristics of anti-desmoglein-1 from pemphigus foliaceus patients expressing only IgG1 autoantibodies," *Journal of Investigative Dermatology*, vol. 121, no. 6, pp. 1373–1378, 2003.

[7] K. Ishii, C. Lin, D. L. Siegel, and J. R. Stanley, "Isolation of pathogenic monoclonal anti-desmoglein 1 human antibodies by phage display of pemphigus foliaceus autoantibodies," *Journal of Investigative Dermatology*, vol. 128, no. 4, pp. 939–948, 2008.

[8] R. Müller, V. Svoboda, E. Wenzel, H.-H. Müller, and M. Hertl, "IgG against extracellular subdomains of desmoglein 3 relates to clinical phenotype of pemphigus vulgaris," *Experimental Dermatology*, vol. 17, no. 1, pp. 35–43, 2008.

[9] A. Al-Amoudi, D. C. Díez, M. J. Betts, and A. S. Frangakis, "The molecular architecture of cadherins in native epidermal desmosomes," *Nature*, vol. 450, no. 7171, pp. 832–837, 2007.

[10] M. Amagai, S. Karpati, R. Prussick, V. Klaus-Kovtun, and J. R. Stanley, "Autoantibodies against the amino-terminal cadherin-like binding domain of pemphigus vulgaris antigen are pathogenic," *Journal of Clinical Investigation*, vol. 90, no. 3, pp. 919–926, 1992.

[11] V. K. Salato, M. K. Hacker-Foegen, Z. Lazarova, J. A. Fairley, and M.-S. Lin, "Role of intramolecular epitope spreading in pemphigus vulgaris," *Clinical Immunology*, vol. 116, no. 1, pp. 54–64, 2005.

[12] J. R. Schiltz and B. Michel, "Production of epidermal acantholysis in normal human skin in vitro by the IgG fraction from pemphigus serum," *Journal of Investigative Dermatology*, vol. 67, no. 2, pp. 254–260, 1976.

[13] G. J. Anhalt, R. S. Labib, and J. J. Voorhees, "Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease," *The New England Journal of Medicine*, vol. 306, no. 20, pp. 1189–1196, 1982.

[14] L. Koulu, A. Kusumi, and M. S. Steinberg, "Human autoantibodies against a desmosomal core protein in pemphigus foliaceus," *Journal of Experimental Medicine*, vol. 160, no. 5, pp. 1509–1518, 1984.

[15] J. C. R. Jones, K. M. Yokoo, and R. D. Goldman, "A cell surface desmosome-associated component: identification of a tissue-specific cell adhesion molecule," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 83, no. 19, pp. 7282–7286, 1986.

[16] M. Amagai, V. Klaus-Kovtun, and J. R. Stanley, "Autoantibodies against a novel epithelial cadherin in Pemphigus vulgaris, a disease of cell adhesion," *Cell*, vol. 67, no. 5, pp. 869–877, 1991.

[17] P. Sharma, X. Mao, and A. S. Payne, "Beyond steric hindrance: the role of adhesion signaling pathways in the pathogenesis of pemphigus," *Journal of Dermatological Science*, vol. 48, no. 1, pp. 1–14, 2007.

[18] K. Ishii, M. Amagai, R. P. Hall, et al., "Characterization of autoantibodies in pemphigus using antigen-specific enzyme-linked immunosorbent assays with baculovirus-expressed recombinant desmogleins," *Journal of Immunology*, vol. 159, no. 4, pp. 2010–2017, 1997.

[19] K. Tsunoda, T. Ota, M. Aoki, et al., "Induction of pemphigus phenotype by a mouse monoclonal antibody against the amino-terminal adhesive interface of desmoglein 31," *Journal of Immunology*, vol. 170, no. 4, pp. 2170–2178, 2003.

[20] W. M. Heupel, W. Baumgartner, B. Laymann, D. Drenckhahn, and N. Golenhofen, "Different Ca^{2+} affinities and functional implications of the two synaptic adhesion molecules cadherin-11 and N-cadherin," *Molecular and Cellular Neuroscience*, vol. 37, no. 3, pp. 548–558, 2008.

[21] J. R. Stanley and M. Amagai, "Pemphigus, bullous impetigo, and the staphylococcal scalded-skin syndrome," *The New England Journal of Medicine*, vol. 355, no. 17, pp. 1800–1810, 2006.

[22] M. Hertl, D. Zillikens, L. Borradori, et al., "Recommendations for the use of rituximab (anti-CD20 antibody) in the treatment of autoimmune bullous skin diseases," *Journal of the German Society of Dermatology*, vol. 6, no. 5, pp. 366–374, 2008.

[23] R. Müller, V. Svoboda, E. Wenzel, et al., "IgG reactivity against non-conformational NH2-terminal epitopes of the desmoglein 3 ectodomain relates to clinical activity and phenotype of pemphigus vulgaris," *Experimental Dermatology*, vol. 15, no. 8, pp. 606–614, 2006.

[24] M. Hertl, R. Eming, and L. Borradori, "Rituximab (anti-CD20 monoclonal antibody)—ultimate or first choice in pemphigus?" *Dermatology*, vol. 214, no. 4, pp. 275–277, 2007.

[25] P. Joly, H. Mouquet, J.-C. Roujeau, et al., "A single cycle of rituximab for the treatment of severe pemphigus," *The New England Journal of Medicine*, vol. 357, no. 6, pp. 545–552, 2007.

[26] K. Ishii, R. Harada, I. Matsuo, Y. Shirakata, K. Hashimoto, and M. Amagai, "In vitro keratinocyte dissociation assay for evaluation of the pathogenicity of anti-desmoglein 3 IgG autoantibodies in pemphigus vulgaris," *Journal of Investigative Dermatology*, vol. 124, no. 5, pp. 939–946, 2005.

[27] M. Yokouchi, M. A. Saleh, K. Kuroda, et al., "Pathogenic epitopes of autoantibodies in pemphigus reside in the amino-terminal adhesive region of desmogleins which are unmasked by proteolytic processing of prosequence," *Journal of Investigative Dermatology*, vol. 129, no. 9, pp. 2156–2166, 2009.

[28] H. Kawasaki, K. Tsunoda, T. Hata, K. Ishii, T. Yamada, and M. Amagai, "Synergistic pathogenic effects of combined mouse monoclonal anti-desmoglein 3 IgG antibodies on pemphigus vulgaris blister formation," *Journal of Investigative Dermatology*, vol. 126, no. 12, pp. 2621–2630, 2006.

[29] A. Nagel, M. Hertl, and R. Eming, "B-cell-directed therapy for inflammatory skin diseases," *Journal of Investigative Dermatology*, vol. 129, no. 2, pp. 289–301, 2009.

[30] A. Nagel, E. Podstawa, M. Eickmann, H.-H. Müller, M. Hertl, and R. Eming, "Rituximab mediates a strong elevation of B-cell-activating factor associated with increased pathogen-specific igG but not autoantibodies in pemphigus vulgaris," *Journal of Investigative Dermatology*, vol. 129, no. 9, pp. 2202–2210, 2009.

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Review Article

A Hypothesis Concerning a Potential Involvement of Ceramide in Apoptosis and Acantholysis Induced by Pemphigus Autoantibodies

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Autoimmune diseases affect more than 50 million Americans, resulting in significant healthcare costs. Most autoimmune diseases occur sporadically; however, endemic pemphigus foliaceus (EPF) is an autoimmune skin disease localized to specific geographic loci. EPF, and the related diseases pemphigus vulgaris (PV) and pemphigus foliaceus (PF), are characterized by skin lesions and autoantibodies to molecules found on epidermal keratinocytes. A variant of EPF in patients from El Bagre, Colombia, South America, has recently been reported to be distinct from previously described loci in Brazil and Tunisia epidemiologically and immunologically. As in PF and EPF, El Bagre EPF patients exhibit autoantibodies towards desmoglein-1, a cell adhesion molecule critical for maintaining epidermal integrity. An association of El Bagre EPF with sun exposure has been detected, and ultraviolet irradiation also exacerbates symptoms in PV, PF and EPF. Our hypothesis is that: (1) the autoantibodies generate pathology through an alteration in ceramide metabolism in targeted keratinocytes, resulting in apoptosis and/or cell death and acantholysis, but only when the cell's ability to metabolize ceramide is exceeded, and (2) apoptosis in response to this altered ceramide metabolism is initiated and/or exacerbated by other agents that increase ceramide levels, such as cytokines, ultraviolet irradiation, and senescence.

1. Introduction

More than 50 million Americans suffer from autoimmune diseases. Because of the chronic nature of this group of diseases, their treatment results in a tremendous cost to healthcare as well as to serious reductions in the quality of life of affected individuals. Pemphigus refers to a class of rare autoimmune skin diseases characterized by epithelial blistering and acantholysis. Pemphigus vulgaris (PV) blisters occur in mucosal tissues and the skin whereas the lesions of pemphigus foliaceus (PF) exhibit a localization to the suprabasal epidermis. The main pathogenic PV autoantibody recognizes desmoglein—(Dsg) 3, a desmosomal cadherin expressed in mucosae and the epidermis. PF antibodies are predominantly directed towards Dsg-1, another cell adhesion molecule critical for the maintenance of epidermal integrity. Most autoimmune diseases, including PV and PF, occur sporadically and are widely scattered geographically. However, endemic pemphigus foliaceus (EPF) represents an autoimmune disorder that is limited to a well-defined geographic area (reviewed in [1, 2]), such as Brazil or, as reviewed below, El Bagre, Columbia in South America [3]. This El Bagre EPF is also characterized by acantholytic skin lesions and by autoantibodies to Dsg-1 [3–5]. However, the mechanism by which the autoantibodies found in the sera of patients with El Bagre EPF, as well as with PV, PF, and EPF, result in the blistering skin lesions typical of pemphigus is largely unknown.

2. El Bagre Endemic Pemphigus Foliaceus (EPF)

A novel variant of EPF in patients from an area around E1 Bagre, Colombia, South America was recently identified

by Abreu-Velez and colleagues [3]. This focus of EPF is distinct from previously described EPF foci in Brazil and Tunisia both epidemiologically and immunologically. Thus, patients with El Bagre EPF are typically men aged from forty to sixty (with a few postmenopausal women), and symptoms often resemble those of paraneoplastic pemphigus, but without the accompanying malignancy [3]. The autoantibody profile of these El Bagre EPF patients is distinct from that of patients with the Brazilian form of EPF, also known as fogo selvagem (see below). As with other EPF foci as well as PF, the El Bagre disease is characterized by skin lesions with hyperkeratosis, acanthosis, and acantholysis. Immunofluorescence studies indicate that the sera of these patients possess autoantibodies that recognize an antigen(s) on the keratinocyte cell surface producing typical intercellular staining in the epidermis [4]. Approximately two-thirds of the E1 Bagre EPF patients exhibit a form localized to the skin; however, one-third develop a more severe form characterized by systemic symptoms resembling lupus. There also appears to be a genetic component to the development of this disease, since certain ethnic groups show a predisposition to acquire E1 Bagre EPF. On the other hand, the involvement of environmental factors in the development of El Bagre EPF is suggested by the restricted geography (this EPF is limited to individuals living in the area surrounding E1 Bagre) as well as the fact that some patients have converted from the systemic to the localized form of the disease after moving from the area [3]. Additional evidence of an environmental parameter is the finding of a strong association between the amount of sun exposure and the development of the disease [3]. However, the precipitating factor(s) that triggers the disease is unknown.

3. Immunologic Features of El Bagre EPF

Indirect immunofluorescence (with El Bagre EPF sera) on human skin sections revealed intercellular staining in all patients, with some EPF sera also showing reactivity with the basement membrane zone [4]. Approximately 10% of the control individuals from within, but not from outside, the endemic area also demonstrated intercellular immunoreactivity. Immunoprecipitation and immunoblotting analyses indicated that the antigens recognized by the EPF autoantibodies comigrated (by gel electrophoresis) with Dsg-1, desmoplakin, envoplakin, and periplakin; and studies using baculovirus-expressed desmosome-associated proteins showed that these patients possess antibodies recognizing Dsg-1, envoplakin, and periplakin [6]. Dsg-1 represents an important antigen for the autoantibodies of patients with fogo selvagem [7] as well; however, this study [6] demonstrated a distinct immunoreactivity profile in El Bagre EPF versus fogo selvagem. These results again point to the idea that El Bagre EPF represents a novel variant of the disease. Nevertheless, the mechanism by which recognition of Dsg-1, and perhaps other antigens, contributes to the observed apoptosis [8, 9] and development of lesions in EPF is not known [1].

4. Pemphigus and Apoptosis, Apoptolysis and Oncosis

Although it is appreciated that there is significant apoptosis in pemphigus, there has been controversy concerning whether or not apoptosis precedes and/or is required for acantholysis (reviewed in [10]). Recent evidence suggests that while other processes, such as oncosis [11], cell shrinkage, and a process that has been termed apoptolysis (reviewed in [12]), play a role in the development of skin lesions, apoptotic cell death also clearly contributes to acantholysis. Indeed, many investigators have reported the ability of inhibitors of apoptosis-activated caspases to inhibit acantholysis both *in vitro* and *in vivo*, in a passive transfer mouse model of pemphigus ([11] and reviewed in [12]). On the other hand, there seems to be some differences in the mechanism of cell death induced by autoantibodies from different patients. Thus, Grando and colleagues [11] found two subsets of PV autoantibodies: for one subset, cell death seemed to be primarily initiated by caspase-dependent processes (although calpain was also involved), and in the other, calpain initiated cell death (although caspases also contributed).

Data in the literature indicate that pemphigus sera are able to trigger lipid metabolism in treated keratinocytes. Based on an in-depth understanding of ceramide and sphingolipid metabolism and the role of ceramide as a mediator of apoptosis in multiple cell systems (reviewed in [13, 14]) as well as data in the literature, we hypothesize that pemphigus autoantibodies contribute to lesional pathology by altering sphingomyelin metabolism to result in elevated ceramide levels that trigger cell death via apoptosis (Figure 1). Furthermore, we hypothesize that this process is exacerbated in keratinocytes exposed to other agents that affect ceramide metabolism, such as cytokines and ultraviolet (UV) irradiation (see below). This hypothesis represents a novel interpretation of the data and could potentially provide a mechanism by which autoantibodies lead to cell death and apoptosis, oncosis and apoptolysis.

5. Ceramide As an Antiproliferative, Proapoptotic Signal

Ceramides are well-known structural components of the skin and help to form the water-permeability barrier of the epidermis (reviewed in [15]). However, ceramide can also function as a lipid second messenger. Approximately two decades ago, investigators began reporting on the ability of certain cytokines and other signals to activate sphingomyelinase, an enzyme that hydrolyzes sphingomyelin to form ceramide and phosphorylcholine (reviewed in [16]). In addition, using synthetic ceramides to mimic the effects of sphingomyelinase-activating agents, researchers demonstrated the ability of this lipid to function as a signaling molecule, mediating a number of important effects. Other studies have shown that ceramide can be generated by a *de novo* pathway initiated by serine palmitoyltransferase as well (Figure 1), for instance, in response to chemotherapeutic

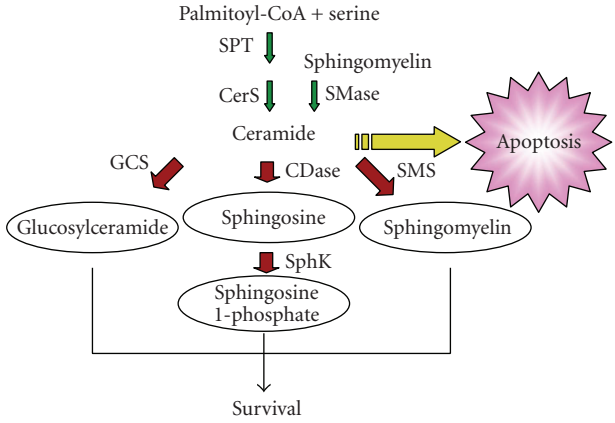

Figure 1: Ceramide Metabolism. Ceramide is produced *de novo* via serine palmitoyltransferase (SPT) and ceramide synthase (CerS), or from sphingomyelin via sphingomyelinase (SMase), and induces apoptosis. In turn, ceramide is metabolized via multiple mechanisms, including glucosylceramide synthase (GCS), ceramidase (CDase) sphingosine kinase (SphK), and sphingomyelin synthase (SMS), with this metabolism of ceramide allowing cell survival.

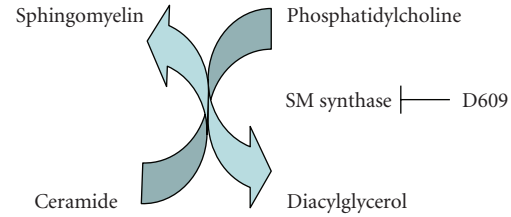

Figure 2: Sphingomyelin synthase. The reaction catalyzed by sphingomyelin synthase (SM synthase) results in a reduction in ceramide and an increase in diacylglycerol and sphingomyelin levels. The reported phosphatidylcholine-specific phospholipase C inhibitor D609 also inhibits sphingomyelin synthase activity.

agents (reviewed in [13]). Most often, these increases in ceramide are associated with growth arrest, differentiation, senescence, and apoptosis, as has been shown in numerous cell types (reviewed in [17]), including keratinocytes [18, 19]. In particular, ceramide produced in response to a number of cell stresses has been shown to trigger apoptosis (reviewed in [17]).

Interestingly, hyperproliferative cancer cells are often able to evade the apoptosis triggered by agents that increase ceramide by utilizing multiple metabolic enzymes to decrease ceramide below critical levels (reviewed in [20]). As an example, in multidrug-resistant cells the enzyme glucosylceramide synthase (GCS), which glycosylates the ceramide induced by chemotherapeutic agents to produce glucosylceramide (Figure 1 and reviewed in [11]), is elevated. Overexpression of GCS converts drug-sensitive cells to resistant ones, whereas decreasing GCS levels with antisense constructs changes resistant cells to a sensitive phenotype [21, 22]. This same enzyme also appears to protect keratinocytes against ceramide-induced stress/apoptosis by converting ceramide to glucosylceramide [23]. Similarly, virally transformed cells (SV40-transformed human lung fibroblasts) exhibit increased sphingomyelin synthase activity [24]. Sphingomyelin synthase transfers the choline headgroup from phosphatidylcholine to ceramide to generate sphingomyelin and diacylglycerol (Figure 2), thereby decreasing ceramide levels. Ceramide can also be metabolized by ceramidase, which hydrolyzes ceramide to sphingosine and is activated, for instance, by growth factors (e.g., [25]). In general, sphingosine is antiproliferative and/or proapoptotic, that is, induces similar effects to ceramide (reviewed in [26]). On the other hand, sphingosine can be phosphorylated to yield sphingosine 1-phosphate (S1P) by sphingosine kinase (of which there are two identified isoforms, sphingosine kinase-1 and -2). S1P can act as a first messenger by binding to a family of GTP-binding protein-coupled receptors, the S1P receptors, but may also signal intracellularly (reviewed in [27]). In most cases, S1P mediates proliferative cell responses and/or cell survival [27], with the combined activities of ceramidase and sphingosine kinase possibly accounting for the observation that in some cell types certain ceramide-elevating agents increase proliferation. Thus, any agent that increases ceramide levels might be expected to activate one or more ceramide metabolic pathways as the cell strives to reduce ceramide and protect itself from the apoptotic effects of this lipid signal. Indeed, 1,25-dihydroxyvitamin D_3-stimulated sphingomyelinase activation, which would be expected to increase ceramide production, appears not to induce apoptosis because of the concominant generation of S1P [28], which in human keratinocytes S1P is reported to be prodifferentiative (e.g., [29]). In contrast, if ceramide levels rise beyond a critical threshold, that is, above the levels that can be metabolized by the various ceramide metabolic pathways apoptosis might be anticipated to result.

6. Pemphigus and Ceramide

Seishima et al. [30] investigated the effect of PV autoantibodies on signaling processes in DJM-1 cells, a squamous cell carcinoma cell line. These authors found that addition of immunoglobulins from pemphigus patients increased the levels of diacylglycerol derived from phosphatidylcholine. This increase was not the result of activation of phosphatidylcholine-hydrolyzing phospholipase D, resulted in the release of phosphorylcholine and could be inhibited by D609. Furthermore, preadsorption of the immunoglobulins with Dsg-1 and -3 prevented the signaling effect [30]. Stanley and colleagues have suggested that autoantibodies to either Dsg-1 or -3 can produce blisters in pemphigus diseases, with the location of the lesions dependent on the distribution of these two molecules in various epithelia [31]. Thus, antibodies directed at either of these desmosomal components should induce similar effects, and anti-Dsg antibodies present in PV, PF, or EPF, including El Bagre EPF, would be expected to trigger the same signaling events as those elicited by the autoantibodies in PV sera. Seishima et al. suggested that the pemphigus autoantibodies activate a phosphatidylcholine-specific phospholipase C [30]; however, no such enzyme has as yet been purified and/or cloned in

mammals. In addition, it has been shown that D609 inhibits sphingomyelin synthase [24, 32] in addition to (or instead of) the reported phosphatidylcholine-specific phospholipase C. As mentioned previously, sphingomyelin synthase transfers the choline headgroup from phosphatidylcholine to ceramide to form diacylglycerol and sphingomyelin (see above and Figure 2). Because phosphatidylcholine is "consumed" and diacylglycerol is generated by this enzyme, it is difficult to distinguish between the activity of sphingomyelin synthase and that of a hypothetical phosphatidylcholine-specific phospholipase C without monitoring sphingolipid metabolism. Thus, the idea that the pemphigus autoantibodies activate a sphingomyelinase to generate ceramide (and release phosphorylcholine), and this ceramide is metabolized by sphingomyelin synthase (to decrease phosphatidylcholine and increase diacylglycerol) is consistent with the observed findings.

The possible involvement of ceramide in the pathology of the skin lesions is also consistent with the observed association of El Bagre EPF disease with certain environmental factors such as sun exposure [3]. Sun exposure has also been reported as an exacerbating factor in PV, PF, and fogo selvagem (e.g., [33, 34]) (see below). UV irradiation is known to raise ceramide levels in keratinocytes [19, 35], as do many cell stresses in other cell types (reviewed in [36]), suggesting that the autoantibodies in and of themselves may not be pathogenic without some additional perturbation of the cells. Indeed, relatives of the El Bagre EPF patients have been observed to possess immunoreactivity to EPF antigens, as measured by immunoprecipitation, immunoblotting [5, 6], and an enzyme-linked immunosorbent assay (ELISA) [37], yet these individuals show no evidence of disease [3, 37]. A similar phenomenon has been observed in fogo selvagem (Brazilian EPF) [38], and, in addition, fogo selvagem autoantibodies are also known to bind to oral mucosa but do not induce lesion formation [39]. On the other hand, cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interferon gamma (IFNγ), are all known to elevate ceramide levels ([40] and reviewed in [41, 42]) and are present in the lesions of autoimmune skin diseases [43–45]. Thus, environmental factors that elevate the quantities of these agents may contribute to the cell perturbations that result in pathology of the autoantibodies. Indeed, Puviani et al. [8] have demonstrated that pemphigus sera contain high levels of Fas ligand (FasL). FasL is also a well-known activator of ceramide production through sphingomyelinase activation (reviewed in [41]) and may stress the keratinocytes sufficiently that they may be unable to metabolize the ceramide below apoptotic levels. If ceramide cannot be reduced below certain levels, the keratinocyte would be triggered to undergo apoptosis, as has been seen in anti-FasL-treated cultured keratinocytes [46] and in acantholytic lesions in pemphigus (reviewed in [10]). Finally, Wang et al. [47] have demonstrated that replicative senescence enhances the apoptotic effect of pemphigus autoantibodies. Ceramide levels are known to be elevated in senescence [48, 49], providing another link between ceramide and apoptotic cell death in pemphigus. Thus, we hypothesize that the autoantibodies, and perhaps FasL [8, 50], and/or other cytokines, present in pemphigus sera increase ceramide levels and in conjunction with other cell stresses, such as sun exposure and age, raise ceramide sufficiently to trigger apoptosis.

7. Possible Mechanisms of Ceramide Action

The question remains: how does ceramide act as a signal to induce apoptosis and other cellular responses? As this lipid second messenger is further studied, it has become apparent that it affects multiple enzymes and proteins, both directly and indirectly, to exert its effects. As an example, ceramide and ceramide-stimulating stresses are known to activate the mitogen-activated protein kinase, p38 (e.g., [51–53]). Activation of p38 has also been observed in keratinocytes upon treatment with pemphigus sera [12], providing another possible link between pemphigus and ceramide. Ceramide has also been shown to activate both a protein phosphatase and a protein kinase, the so-called ceramide-activated protein kinase identified as kinase suppressor of ras (ksr) (reviewed in [13]). In addition, ceramide, or its phosphorylated metabolite ceramide 1-phosphate, appears to modulate the activity of enzymes involved in alternative splicing to yield differential splicing of certain gene products such as caspase-9 (a proapoptotic enzyme) and Bcl-xL (an antiapoptotic mediator) [54]. The result is greater levels of the proapoptotic splice forms of these proteins (Bcl-xS and caspase-9L) [54]. Ceramide also affects multiple proteins involved in the apoptotic pathway, including Akt (an antiapoptotic, prosurvival protein that is inhibited by ceramide action) and cathepsin D, a lysosomal protease involved in apoptosis, among others (reviewed in [55]). In addition, ceramide inhibits mitochondrial function [14], thereby enhancing mitochondrial pathway-mediated apoptosis (reviewed in [14]). Thus, ceramide functions through multiple mechanisms as a proapoptotic cell signal in many cell types including keratinocytes. In addition, ceramide and its metabolites also likely play a role in inflammation (reviewed in [56]).

Although ceramide seems to function generally as an antiproliferative and/or antisurvival (proapoptotic) signaling molecule, there is emerging evidence that the cellular localization of the generated ceramide may affect the ultimate cell response (reviewed in [17, 57]). For instance, overexpression of a bacterial SMase in mitochondria, but not the cytoplasm, Golgi apparatus, endoplasmic reticulum, plasma membrane, or nucleus, triggers apoptosis in MCF7 breast cancer cells [58]. In addition to effects of compartmentalization, different ceramide species, possessing diverse fatty acids from 16 to 24 carbons in length, appear to have different signaling roles. There are five ceramide synthase isoforms, CerS1-5, which have distinct specificities for the formation of various ceramide species with different carbon lengths (reviewed in [17]). Knock down of CerS6, which produces 16 carbon-containing ceramide (C16-ceramide), induces endoplasmic reticulum stress-induced apoptosis, and overexpression of this enzyme protects against ER stress and promotes squamous cell carcinoma tumor growth *in vivo* [59]. On the other

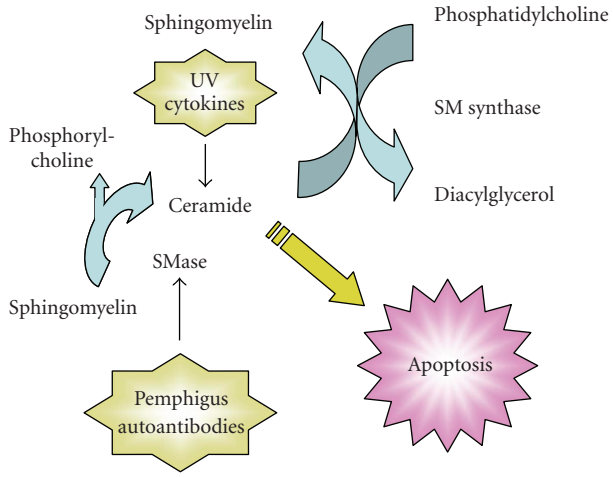

Figure 3: Hypothesized Involvement of Sphingolipid Metabolism in the Keratinocyte Response to Pemphigus Autoantibodies. Our hypothesis is that pemphigus autoantibodies binding to Dsgs results in the activation of sphingomyelinase (SMase) to produce ceramide and phosphorylcholine. In an attempt to survive, keratinocytes activate sphingomyelin synthase (SM synthase). The result is an increase in phosphorylcholine and diacylglycerol levels and a reduction in phosphatidylcholine. Stimuli that overwhelm the ability of the cell to metabolize ceramide, such as cytokines or ultraviolet (UV) light (or senescence), which increase ceramide levels, are proposed to allow manifestation of pathologic skin lesions. Note that UV light increases ceramide in at least two ways: first, by activation of both SMase-mediated [35] and *de novo* ceramide production [19] and second, by decreasing ceramidase activity/expression [60]. Ceramidases are key in the response of keratinocytes to UV light because (1) they metabolize ceramide and decrease its levels and (2) they generate sphingosine, which can be converted to S1P (which allows cell survival) by the action of sphingosine kinase. Indeed, knocking down sphingosine kinase sensitizes keratinocytes to UV-induced apoptosis [60]. In addition, ceramide and its metabolites can activate cytosolic phospholipase A2 and cyclooxygenase-2, suggesting that eicosanoids might be elevated and potentially contribute to the pemphigus disease process (reviewed in [56]).

hand, overexpression of CerS1 (forming C-18 ceramide) inhibits tumor formation, indicating that not only the site of ceramide production but also the species generated is important.

8. Hypothesis

We propose the idea that pemphigus autoantibodies, upon binding to Dsgs, trigger sphingomyelin hydrolysis accompanied by a corresponding stimulation of sphingomyelin synthase activity (Figure 3), as the cell attempts to reduce proapoptotic ceramide amounts. If the cell is efficient enough, no change in ceramide or sphingomyelin levels may occur, although a reduction in phosphatidylcholine levels and an increase in phosphorylcholine release and diacylglycerol quantities would be expected. In this case, acantholysis and other hallmarks of pemphigus would not be observed despite the ability of the antisera to bind Dsgs. Indeed, this need for interaction between the binding of autoantibodies and additional changes in ceramide metabolism may be one explanation for the low levels of anti-Dsg antibodies without concomitant disease seen in some individuals in the El Bagre area [37] and fogo selvagem [61], as well as non-pathogenic antibodies observed in some pemphigus patients (reviewed in [62]). We also hypothesize that coincubation of keratinocytes with pemphigus sera and cytokines, such as FasL, or the combination of pemphigus sera and irradiation with UV light, will result in enhanced levels of ceramide relative to any of the agents alone. Since these combinations would be expected to overwhelm the ability of the cell to metabolize the pemphigus sera-induced production of ceramide, we would also anticipate that cytokines and UV light will act synergistically with pemphigus autoantibodies to stimulate markers of apoptosis and cell death. Consistent with this idea, inhibition of the action of some cytokines, in particular FasL and TNF-α, can inhibit acantholysis in experimental models of pemphigus (reviewed in [12]), and a beneficial action of new TNF-α blocking biological agents in patients has also been reported [63, 64]. In addition, ultraviolet irradiation is known to worsen pemphigus [11]. Similarly, there is a clear association between sunlight exposure and El Bagre EPF [3]. The idea that UV induces ceramide production that is at least partially compensated for by an increase in ceramide clearance is supported by the findings of Uchida and colleagues, who reported (in abstract form) that both knock down of ceramidase levels [60] and incubation with N-acyl-ethanolamine (NAE) compounds that inhibit acidic and neutral ceramidases sensitize keratinocytes to UV-induced apoptosis [65]. These authors also showed that inhibition of the NAE-hydrolyzing enzymes, NAE amidohydrolase, fatty acid amidohydrolase, and NAE-hydrolyzing acid amidase, further exacerbates the effects of UV irradiation. NAE compounds occur naturally and increase with cell stresses including UV exposure and xenotoxics; thus, enhanced production of NAEs, as well as decreased ceramidase activity/expression in response to UV, could significantly impair ceramide clearance [65]. It should be noted that the epidermis has been shown to express all five known ceramidase isoforms, with some localized to the basal layer and others to the differentiating compartments [66]. In addition, alkaline (aCER1) and acid (AC) ceramidases are upregulated during elevated extracellular calcium-induced keratinocyte differentiation and appear to mediate the differentiative effects of calcium [67]. Thus, ceramidases are clearly vital to epidermal physiology and perhaps also pathophysiology, likely by both decreasing ceramide levels and providing sphingosine for production of S1P [60].

On the other hand, it is possible that pemphigus sera do not activate sphingomyelinase but rather stimulate the activity of sphingomyelin synthase through the ligation of cell adhesion molecules. It is widely recognized that cell adhesion can elicit signal transduction processes (reviewed in [68]). Perhaps antibody binding of Dsgs (or possibly other cell surface proteins recognized by pemphigus autoantibodies) results in stimulation of signaling and activation of sphingomyelin synthase. This result would then predict that antibody-mediated loss of the Dsg (or other cell

surface proteins) would inhibit this signal, again leading to apoptosis, either through elevations in ceramide levels or via reductions in protein kinase C-activating diacylglycerol. Diacylglycerol appears to be a prosurvival, antiapoptotic signal in most cell types (reviewed in [13]), presumably in part through its ability to activate sphingosine kinase and stimulate the production of S1P from ceramide [69]. Pemphigus sera have also been reported to stimulate phosphoinositide hydrolysis [70], which would result in the generation of diacylglycerol. Alternatively, it is possible that antibody-mediated loss of Dsg function (rather than of the protein itself) may mediate the changes in cell signaling processes that lead to blister formation in pemphigus. However, this possibility is argued against by recent evidence suggesting that Dsg-1 is proapoptotic, such that RNA interference to decrease Dsg-1 protects keratinocytes against ultraviolet irradiation-mediated apoptosis [71]. In any case, these ideas should be testable by experiments to examine the effect of pemphigus sera on the levels of ceramide and other sphingolipids in keratinocytes, and research into the role of sphingolipid metabolism in the keratinocyte acantholytic response to pemphigus autoantibodies seems warranted.

References

[1] G. J. Anhalt and L. A. Diaz, "Research advances in pemphigus," *Journal of the American Medical Association*, vol. 285, no. 5, pp. 652–654, 2001.

[2] D. A. Culton, Y. Qian, N. Li, et al., "Advances in pemphigus and its endemic pemphigus foliaceus (fogo selvagem) phenotype: a paradigm of human autoimmunity," *Journal of Autoimmunity*, vol. 31, no. 4, pp. 311–324, 2008.

[3] A. M. Abreu-Velez, T. Hashimoto, W. B. Bollag, et al., "A unique form of endemic pemphigus in northern Colombia," *Journal of the American Academy of Dermatology*, vol. 49, no. 4, pp. 599–608, 2003.

[4] A. M. Abreu-Velez, E. H. Beutner, F. Motoya, W. B. Bollag, and T. Hashimoto, "Analyses of autoantigens in a new form of endemic pemphigus foliaceus in Colombia," *Journal of the American Academy of Dermatology*, vol. 49, no. 4, pp. 609–614, 2003.

[5] A. M. Abreu-Velez, P. J. Patino, F. Montoya, and W. B. Bollag, "The tryptic cleavage product of the mature form of the bovine desmoglein 1 ectodomain is one of the antigen moieties immunoprecipitated by all sera from symptomatic patients affected by a new variant of endemic pemphigus," *European Journal of Dermatology*, vol. 13, no. 4, pp. 359–366, 2003.

[6] Y. Hisamatsu, A. M. Abreu-Velez, M. Amagai, M. M. Ogawa, T. Kanzaki, and T. Hashimoto, "Comparative study of autoantigen profile between Colombian and Brazilian types of endemic pemphigus foliaceus by various biochemical and molecular biological techniques," *Journal of Dermatological Science*, vol. 32, no. 1, pp. 33–41, 2003.

[7] M. Olague-Alcala, G. J. Giudice, and L. A. Diaz, "Pemphigus foliaceus sera recognize an N-terminal fragment of bovine desmoglein 1," *Journal of Investigative Dermatology*, vol. 102, no. 6, pp. 882–885, 1994.

[8] M. Puviani, A. Marconi, E. Cozzani, and C. Pincelli, "Fas ligand in pemphigus sera induces keratinocyte apoptosis through the activation of caspase-8," *Journal of Investigative Dermatology*, vol. 120, no. 1, pp. 164–167, 2003.

[9] R. Gniadecki, G. B. E. Jemec, B. M. Thomsen, and M. Hansen, "Relationship between keratinocyte adhesion and death: anoikis in acantholytic diseases," *Archives of Dermatological Research*, vol. 290, no. 10, pp. 528–532, 1998.

[10] E. Schmidt and J. Waschke, "Apoptosis in pemphigus," *Autoimmunity Reviews*, vol. 8, no. 7, pp. 533–537, 2009.

[11] J. Arredondo, A. I. Chernyavsky, A. Karaouni, and S. A. Grando, "Novel mechanisms of target cell death and survival and of therapeutic action of IVIg in pemphigus," *American Journal of Pathology*, vol. 167, no. 6, pp. 1531–1544, 2005.

[12] S. A. Grando, J. C. Bystryn, A. I. Chernyavsky, et al., "Apoptolysis: a novel mechanism of skin blistering in pemphigus vulgaris linking the apoptotic pathways to basal cell shrinkage and suprabasal acantholysis," *Experimental Dermatology*, vol. 18, no. 9, pp. 764–770, 2009.

[13] J. P. Jaffrezou and G. Laurent, "Ceramide: a new target in anticancer research?" *Bulletin du Cancer*, vol. 91, no. 5, pp. E133–E161, 2004.

[14] L. J. Siskind, "Mitochondrial ceramide and the induction of apoptosis," *Journal of Bioenergetics and Biomembranes*, vol. 37, no. 3, pp. 143–153, 2005.

[15] K. C. Madison, "Barrier function of the skin: "la raison d'etre" of the epidermis," *Journal of Investigative Dermatology*, vol. 121, no. 2, pp. 231–241, 2003.

[16] F. G. Tafesse, P. Ternes, and J. C. M. Holthuis, "The multigenic sphingomyelin synthase family," *The Journal of Biological Chemistry*, vol. 281, no. 40, pp. 29421–29425, 2006.

[17] S. A. Saddoughi, P. Song, and B. Ogretmen, "Roles of bioactive sphingolipids in cancer biology and therapeutics," *Sub-Cellular Biochemistry*, vol. 49, pp. 413–440, 2008.

[18] M. Bektas, C. E. Orfanos, and C. C. Geilen, "Different vitamin D analogues induce sphingomyelin hydrolysis and apoptosis in the human keratinocyte cell line HaCaT," *Cellular and Molecular Biology*, vol. 46, no. 1, pp. 111–119, 2000.

[19] Y. Uchida, A. Di Nardo, V. Collins, P. M. Elias, and W. M. Holleran, "De novo ceramide synthesis participates in the ultraviolet B irradiation-induced apoptosis in undifferentiated cultured human keratinocytes," *Journal of Investigative Dermatology*, vol. 120, no. 4, pp. 662–669, 2003.

[20] W. B. Bollag, "Paradoxical effects of sphingosine-1-phosphate," *Journal of Investigative Dermatology*, vol. 120, no. 4, pp. xiii–xiv, 2003.

[21] Y. Y. Liu, T. Y. Han, A. E. Giuliano, and M. C. Cabot, "Expression of glucosylceramide synthase, converting ceramide to glucosylceramide, confers adriamycin resistance in human breast cancer cells," *The Journal of Biological Chemistry*, vol. 274, no. 2, pp. 1140–1146, 1999.

[22] Y. Y. Liu, T. Y. Han, A. E. Giuliano, N. Hansen, and M. C. Cabot, "Uncoupling ceramide glycosylation by transfection of glucosylceramide synthase antisense reverses adriamycin resistance," *The Journal of Biological Chemistry*, vol. 275, no. 10, pp. 7138–7143, 2000.

[23] Y. Uchida, S. Murata, M. Schmuth, et al., "Glucosylceramide synthesis and synthase expression protect against ceramide-induced stress," *Journal of Lipid Research*, vol. 43, no. 8, pp. 1293–1302, 2002.

[24] C. Luberto and Y. A. Hannun, "Sphingomyelin synthase, a potential regulator of intracellular levels of ceramide and diacylglycerol during SV40 transformation: does sphingomyelin synthase account for the putative phosphatidylcholine-specific phospholipase C?" *The Journal of Biological Chemistry*, vol. 273, no. 23, pp. 14550–14559, 1998.

[25] E. Coroneos, M. Martinez, S. McKenna, and M. Kester, "Differential regulation of sphingomyelinase and ceramidase activities by growth factors and cytokines. Implications for cellular proliferation and differentiation," *The Journal of Biological Chemistry*, vol. 270, no. 40, pp. 23305–23309, 1995.

[26] C. Mao and L. M. Obeid, "Ceramidases: regulators of cellular responses mediated by ceramide, sphingosine, and sphingosine-1-phosphate," *Biochimica et Biophysica Acta*, vol. 1781, no. 9, pp. 424–434, 2008.

[27] M. Maceyka, S. Milstien, and S. Spiegel, "Sphingosine-1-phosphate: the Swiss army knife of sphingolipid signaling," *Journal of Lipid Research*, vol. 50, supplement, pp. S272–S276, 2009.

[28] M. Manggau, D. S. Kim, L. Ruwisch, et al., "1α, 25-dihydroxyvitamin D3 protects human keratinocytes from apoptosis by the formation of sphingosine-1-phosphate," *Journal of Investigative Dermatology*, vol. 117, no. 5, pp. 1241–1249, 2001.

[29] R. Vogler, B. Sauer, D. S. Kim, M. Schäfer-Korting, and B. Kleuser, "Sphingosine-1-phosphate and its potentially paradoxical effects on critical parameters of cutaneous wound healing," *Journal of Investigative Dermatology*, vol. 120, no. 4, pp. 693–700, 2003.

[30] M. Seishima, Y. Iwasaki-Bessho, Y. Itoh, Y. Nozawa, M. Amagai, and Y. Kitajima, "Phosphatidylcholine-specific phospholipase C, but not phospholipase D, is involved in pemphigus IgG-induced signal transduction," *Archives of Dermatological Research*, vol. 291, no. 11, pp. 606–613, 1999.

[31] M. G. Mahoney, Z. Wang, K. Rothenberger, P. J. Koch, M. Amagai, and J. R. Stanley, "Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris," *Journal of Clinical Investigation*, vol. 103, no. 4, pp. 461–468, 1999.

[32] A. Meng, C. Luberto, P. Meier, et al., "Sphingomyelin synthase as a potential target for D609-induced apoptosis in U937 human monocytic leukemia cells," *Experimental Cell Research*, vol. 292, no. 2, pp. 385–392, 2004.

[33] Y. Kano, M. Shimosegawa, Y. Mizukawa, and T. Shiohara, "Pemphigus foliaceus induced by exposure to sunlight: report of a case and analysis of photochallenge-induced lesions," *Dermatology*, vol. 201, no. 2, pp. 132–138, 2000.

[34] V. M. S. Reis, R. P. Toledo, A. Lopez, L. A. Diaz, and J. E. C. Martins, "UVB-induced acantholysis in endemic pemphigus foliaceus (fogo selvagem) and pemphigus vulgaris," *Journal of the American Academy of Dermatology*, vol. 42, no. 4, pp. 571–576, 2000.

[35] C. Magnoni, E. Euclidi, L. Benassi, et al., "Ultraviolet B radiation induces activation of neutral and acidic sphingomyelinases and ceramide generation in cultured normal human keratinocytes," *Toxicology in Vitro*, vol. 16, no. 4, pp. 349–355, 2002.

[36] Y. A. Hannun and L. M. Obeid, "The ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind," *The Journal of Biological Chemistry*, vol. 277, no. 29, pp. 25847–25850, 2002.

[37] A. M. Abreu-Velez, M. M. Yepes, P. J. Patino, W. B. Bollag, and F. Montoya, "A sensitive and restricted enzyme-linked immunosorbent assay for detecting a heterogeneous antibody population in serum from people suffering from a new variant of endemic pemphigus," *Archives of Dermatological Research*, vol. 295, no. 10, pp. 434–441, 2004.

[38] S. J. P. Warren, M. S. Lin, G. J. Giudice, R. G. Hoffmann, et al., "The prevalence of antibodies against desmoglein 1 in endemic pemphigus foliaceus in Brazil," *The New England Journal of Medicine*, vol. 343, no. 1, pp. 23–30, 2000.

[39] E. A. Rivitti, J. A. Sanches, L. M. Miyauchi, S. A. P. Sampaio, V. Aoki, and L. A. Diaz, "Pemphigus foliaceus autoantibodies bind both epidermis and squamous mucosal epithelium, but tissue injury is detected only in the epidermis. The Cooperative Group on Fogo Selvagem Research," *Journal of the American Academy of Dermatology*, vol. 31, no. 6, pp. 954–958, 1994.

[40] C. C. Geilen, M. Bektas, T. Wieder, V. Kodelja, S. Goerdt, and C. E. Orfanos, "1α,25-dihydroxyvitamin D3 induces sphingomyelin hydrolysis in HaCaT cells via tumor necrosis factor," *The Journal of Biological Chemistry*, vol. 272, no. 14, pp. 8997–9001, 1997.

[41] I. Petak and J. A. Houghton, "Shared pathways: death receptors and cytotoxic drugs in cancer therapy," *Pathology and Oncology Research*, vol. 7, no. 2, pp. 95–106, 2001.

[42] Y. A. Hannun, "The sphingomyelin cycle and the second messenger function of ceramide," *The Journal of Biological Chemistry*, vol. 269, no. 5, pp. 3125–3128, 1994.

[43] S. A. Grando, B. T. Glukhenky, G. N. Drannik, E. V. Epshtein, A. P. Kostromin, and T. A. Korostash, "Mediators of inflammation in blister fluids from patients with pemphigus vulgaris and bullous pemphigoid," *Archives of Dermatology*, vol. 125, no. 7, pp. 925–930, 1989.

[44] A. Baroni, B. Perfetto, E. Ruocco, R. Greco, D. Criscuolo, and V. Ruocco, "Cytokine pattern in blister fluid and sera of patients with pemphigus," *Dermatology*, vol. 205, no. 2, pp. 116–121, 2002.

[45] C. Feliciani, P. Toto, P. Amerio, et al., "In vitro and in vivo expression of interleukin-1α and tumor necrosis factor-α mRNA in pemphigus vulgaris: interleukin-1α and tumor necrosis factor-α are involved in acantholysis," *Journal of Investigative Dermatology*, vol. 114, no. 1, pp. 71–77, 2000.

[46] K. Sayama, S. Yonehara, Y. Watanabe, and Y. Miki, "Expression of Fas antigen on keratinocytes in vivo and induction of apoptosis in cultured keratinocytes," *Journal of Investigative Dermatology*, vol. 103, no. 3, pp. 330–334, 1994.

[47] X. Wang, F. Bregegere, Y. Soroka, M. Frusic-Zlotkin, and Y. Milner, "Replicative senescence enhances apoptosis induced by pemphigus autoimmune antibodies in human keratinocytes," *FEBS Letters*, vol. 567, no. 2-3, pp. 281–286, 2004.

[48] M. E. Venable, J. Y. Lee, M. J. Smyth, A. Bielawska, and L. M. Obeid, "Role of ceramide in cellular senescence," *The Journal of Biological Chemistry*, vol. 270, no. 51, pp. 30701–30708, 1995.

[49] M. E. Venable, G. C. Blobe, and L. M. Obeid, "Identification of a defect in the phospholipase D/diacylglycerol pathway in cellular senescence," *The Journal of Biological Chemistry*, vol. 269, no. 42, pp. 26040–26044, 1994.

[50] D. B. R. Rodrigues, S. A. L. Pereira, M. A. dos Reis, et al., "In situ detection of inflammatory cytokines and apoptosis in pemphigus foliaceus patients," *Archives of Pathology and Laboratory Medicine*, vol. 133, no. 1, pp. 97–100, 2009.

[51] M. Verheij, R. Bose, X. H. Lin, et al., "Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis," *Nature*, vol. 380, no. 6569, pp. 75–79, 1996.

[52] C. L. Chen, C. F. Lin, W. T. Chang, et al., "Ceramide induces p38 MAPK and JNK activation through a mechanism involving a thioredoxin-interacting protein-mediated pathway," *Blood*, vol. 111, no. 8, pp. 4365–4374, 2008.

[53] H. J. Kim, J. E. Oh, S. W. Kim, Y. J. Chun, and M. Y. Kim, "Ceramide induces p38 MAPK-dependent apoptosis and Bax translocation via inhibition of Akt in HL-60 cells," *Cancer Letters*, vol. 260, no. 1-2, pp. 88–95, 2008.

[54] C. E. Chalfant, K. Rathman, R. L. Pinkerman, et al., "De novo ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adenocarcinoma cells. Dependence on protein phosphatase-1," *The Journal of Biological Chemistry*, vol. 277, no. 15, pp. 12587–12595, 2002.

[55] B. J. Pettus, C. E. Chalfant, and Y. A. Hannun, "Ceramide in apoptosis: an overview and current perspectives," *Biochimica et Biophysica Acta*, vol. 1585, no. 2-3, pp. 114–125, 2002.

[56] G. F. Nixon, "Sphingolipids in inflammation: pathological implications and potential therapeutic targets," *British Journal of Pharmacology*, vol. 158, no. 4, pp. 982–993, 2009.

[57] B. Ogretmen and Y. A. Hannun, "Biologically active sphingolipids in cancer pathogenesis and treatment," *Nature Reviews Cancer*, vol. 4, no. 8, pp. 604–616, 2004.

[58] H. Birbes, S. El Bawab, Y. A. Hannun, and L. M. Obeid, "Selective hydrolysis of a mitochondrial pool of sphingomyelin induces apoptosis," *FASEB Journal*, vol. 15, no. 14, pp. 2669–2679, 2001.

[59] C. E. Senkal, S. Ponnusamy, J. Bielawski, Y. A. Hannun, and B. Ogretmen, "Antiapoptotic roles of ceramide-synthase-6-generated C16- ceramide via selective regulation of the ATF6/CHOP arm of ER-stress-response pathways," *FASEB Journal*, vol. 24, no. 1, pp. 296–308, 2010.

[60] E. Houben, P. M. Elias, W. M. Holleran, and Y. Uchida, "Opposing roles of ceramide hydrolysis and recycling in UVB-induced apoptosis of keratinocytes," *Journal of Investigative Dermatology*, vol. 126, p. S65, 2006.

[61] J. Hilario-Vargas, D. A. Dasher, N. Li, et al., "Prevalence of anti-desmoglein-3 antibodies in endemic regions of fogo selvagem in Brazil," *Journal of Investigative Dermatology*, vol. 126, no. 9, pp. 2044–2048, 2006.

[62] M. Amagai, "Non-pathogenic anti-desmoglein 3 IgG autoantibodies in fogo selvagem," *Journal of Investigative Dermatology*, vol. 126, no. 9, pp. 1931–1932, 2006.

[63] A. Jacobi, G. Schuler, and M. Hertl, "Rapid control of therapy-refractory pemphigus vulgaris by treatment with the tumour necrosis factor-α inhibitor infliximab," *British Journal of Dermatology*, vol. 153, no. 2, pp. 448–449, 2005.

[64] J. Pardo, P. Mercader, L. Mahiques, J. L. Sanchez-Carazo, V. Oliver, and J. M. Fortea, "Infliximab in the management of severe pemphigus vulgaris," *British Journal of Dermatology*, vol. 153, no. 1, pp. 222–223, 2005.

[65] Y. Wang, S. Douangpanya, P. M. Elias, W. M. Holleran, and Y. Uchida, "A novel regulatory system for ceramidase activities that protects human keratinocytes against UVB-initiated ceramide-induced apoptosis," *Journal of Investigative Dermatology*, vol. 127, p. S75, 2007.

[66] E. Houben, W. M. Holleran, T. Yaginuma, et al., "Differentiation-associated expression of ceramidase isoforms in cultured keratinocytes and epidermis," *Journal of Lipid Research*, vol. 47, no. 5, pp. 1063–1070, 2006.

[67] W. Sun, R. Xu, W. Hu, et al., "Upregulation of the human alkaline ceramidase 1 and acid ceramidase mediates calcium-induced differentiation of epidermal keratinocytes," *Journal of Investigative Dermatology*, vol. 128, no. 2, pp. 389–397, 2008.

[68] K. J. Green and C. L. Simpson, "Desmosomes: new perspectives on a classic," *Journal of Investigative Dermatology*, vol. 127, no. 11, pp. 2499–2515, 2007.

[69] K. R. Johnson, K. P. Becker, M. M. Facchinetti, Y. A. Hannun, and L. M. Obeid, "PKC-dependent activation of sphingosine kinase 1 and translocation to the plasma membrane: extracellular release of sphingosine-1-phosphate induced by phorbol 12-myristate 13-acetate (PMA)," *The Journal of Biological Chemistry*, vol. 277, no. 38, pp. 35257–35262, 2002.

[70] M. Seishima, C. Esaki, K. Osada, S. Mori, T. Hashimoto, and Y. Kitajima, "Pemphigus IgG, but not bullous pemphigoid IgG, causes a transient increase in intracellular calcium and inositol 1,4,5-trisphosphate in DJM-1 cells, a squamous cell carcinoma line," *Journal of Investigative Dermatology*, vol. 104, no. 1, pp. 33–37, 1995.

[71] R. L. Dusek, S. Getsios, F. Chen, et al., "The differentiation-dependent desmosomal cadherin desmoglein 1 is a novel caspase-3 target that regulates apoptosis in keratinocytes," *The Journal of Biological Chemistry*, vol. 281, no. 6, pp. 3614–3624, 2006.