Published in final edited form as:
Cell Commun Adhes. 2014 February ; 21(1): 55–63. doi:10.3109/15419061.2013.876015.

Integrating Animal Models and In Vitro Tissue Models to Elucidate the Role of Desmosomal Proteins in Diseases

Maranke I. Koster[1,2], **Jason Dinella**[1,2], **Jiangli Chen**[1], **Charlene O' Shea**[1], and **Peter J. Koch**[1,2,3]

[1]Department of Dermatology, University of Colorado School of Medicine and Charles C Gates Center for Regenerative Medicine and Stem Cell Biology, University of Colorado School of Medicine, Aurora, CO, USA

[2]Graduate Program in Cell Biology, Stem Cells and Development, University of Colorado School of Medicine, Aurora, CO, USA

[3]Department of Cell and Developmental Biology, University of Colorado School of Medicine, Aurora, CO, USA

Abstract

Desmosomes are intercellular junctions that provide tissues with structural stability. These junctions might also act as signaling centers that transmit environmental clues to the cell, thereby affecting cell differentiation, migration, and proliferation. The importance of desmosomes is underscored by devastating skin and heart diseases caused by mutations in desmosomal genes. Recent observations suggest that abnormal desmosomal protein expression might indirectly contribute to skin disorders previously not linked to these proteins. For example, it has been postulated that reduced desmosomal protein expression occurs in patients affected by Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC), a skin fragility disorder caused by mutations in the transcription factor *TP63*. Currently, it is not clear how these changes in desmosomal gene expression contribute to AEC. We will discuss new approaches that combine *in vitro* and *in vivo* models to elucidate the role of desmosomal gene deregulation in human skin diseases such as AEC.

Keywords

Desmosomes; Animal Models; Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC); Ectodermal Dysplasias; Skin Fragility Syndromes; Induced Pluripotent Stem Cells (iPSC); iPSC-Derived Keratinocytes; TP63 (human p63); TRP63 (mouse p63)

Address correspondence to Peter J. Koch, PhD, Department of Dermatology, Charles C. Gates Center for Regenerative Medicine and Stem Cell Biology, 12800 East 19th Avenue, University of Colorado School of Medicine, Aurora, CO 80045, USA. Tel: +1(303) 724-0051. Fax: +1(303) 724-3051. Peter.Koch@ucdenver.edu.

Declaration of interest: The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

DESMOSOMES—CONTEXT AND HISTORICAL PERSPECTIVE

Desmosomes are multiprotein complexes (junctions) that are assembled at the plasma membrane (Figure 1). These junctions serve a dual function: they mediate cell–cell attachment and they provide anchorage points for cytoplasmic intermediate filaments (IF). As such, desmosomes and the IF cytoskeleton form a three-dimensional network of structural proteins that confers resistance to tissues and organs that are exposed to significant mechanical stress forces, such as the skin and the heart (Petrof et al., 2012; Schmidt & Koch, 2007; Cheng et al., 2005; Ganeshan et al., 2010).

Desmosomes consist of a core of type-1 transmembrane glycoproteins (desmogleins [DSG] and desmocollins [DSC]) that connect neighboring cells by participating in hetero- and/or homophilic interactions in the intercellular space (Chitaev et al., 1996; Nie et al., 2011). On the cytoplasmic side of the plasma membrane, DSGs and DSCs interact with plaque proteins (desmoplakin [DSP], plakophilins [PKP], and plakoglobin [JUP]) (Figure 1). These plaque proteins in turn connect DSGs and DSCs to the IF cytoskeleton. Based on their predicted amino acid sequences, DSGs and DSCs are classified as members of the cadherin subfamily, a group of calcium-dependent cell adhesion proteins (Koch et al., 1990; Koch et al., 1991a, 1991b; Buxton et al., 1993). Further, DSGs and DSCs each comprise a group of multiple related proteins (DSG1-4 and DSC1-3 in humans [Buxton et al., 1993; Mahoney et al., 2006]), each of which exhibit tissue-and cell type-specific expression patterns. For example, all DSG and DSC proteins are expressed in the skin, whereas other tissues and cell types express subsets of desmosomal cadherins [e.g. (Koch et al., 1992; Theis et al., 1993)].

Abnormalities in desmosome structure or function underlie a subset of human disorders, mainly affecting the skin and the heart (Petrof et al., 2012). The first diseases unequivocally linked to impaired desmosome function were the autoimmune disorders pemphigus vulgaris and pemphigus foliaceus (Amagai & Stanley, 2012). In these diseases, auto-antibodies are generated against DSGs, leading to blistering lesions in mucous membranes and the skin. Further, auto-antibodies against DSC3 have been linked to atypical pemphigus (Rafei et al., 2011; Hatano et al., 2012). In addition to autoimmune responses targeting desmosomal proteins, mutations in seven desmosomal genes have now been linked to disorders affecting the structure and function of skin and skin appendages (Petrof et al., 2012). Further, a severe form of heart disease, arrhythmogenic right ventricular cardiomyopathy (ARVC), has been linked to mutations in desmosomal genes [*JUP*, *DSC2*, *DSG2*, *DSP*, and *PKP2* (Petrof et al., 2012; Li Mura et al., 2013)].

INSIGHTS INTO THE ROLE OF DESMOSOMAL PROTEINS FROM IN VIVO STUDIES

To gain further insight into the role of individual desmosomal proteins, several mouse lines with null mutations in desmosomal genes have been developed since the early 1990s (e.g., Ganeshan et al., 2010; Cheng et al., 2005; Cheng & Koch, 2004). As describing the individual role of each protein analyzed would exceed the scope of this manuscript, we will highlight a few major conclusions derived from these animal studies. Interestingly, although loss of some desmosomal proteins in mice led to viable mice that could be analyzed, loss of

others was found to be incompatible with life. Embryonic lethality was noted, for example, in mice with null mutations in *Jup*, *Dsp*, and *Dsc3* (Ruiz et al., 1996; Bierkamp et al., 1996; Gallicano et al., 2001, 1998; Den et al., 2006). To overcome the embryonic lethality, tissue-specific null mutations were introduced into desmosomal genes required for embryogenesis (e.g., Chen et al., 2008; Vasioukhin et al., 2001; Li et al., 2011). One key finding obtained by analyzing these mice was that many desmosomal proteins are indeed required to maintain tissue adhesion, as demonstrated, for example, by the blistering skin and hair loss phenotypes of *Dsg3* and *Dsc3* null mice (Koch et al., 1997; Chen et al., 2008). In addition, loss of some desmosomal proteins, such as DSP, led to defects in keratinocyte differentiation (Vasioukhin et al., 2001). Finally, as expected, severe heart defects were observed when desmosomal proteins expressed in the heart were inactivated in genetically engineered mice (Li et al., 2011).

In many cases, the phenotypes of animals with impaired desmosome function provided the rationale to investigate the role of desmosomal proteins in human skin and heart diseases.

However, we are far from fully understanding the role of desmosomal proteins in human disease; although it is clear that complete loss of desmosomal proteins will lead to severe phenotypes, it is not clear whether more subtle changes in desmosomal gene expression contribute to human diseases. For example, increased as well as decreased expression of desmosomal genes has been observed in human cancers (e.g. Chen et al., 2011 and references therein). Developing appropriate animal models will be essential in these cases to establish a causal link between these expression changes and cancer development and progression. In addition, desmosomal abnormalities may be present in skin fragility syndromes with primary defects in upstream regulators of desmosomal genes. Few upstream regulators of desmosomal genes have been identified so far, but their future identification is anticipated to facilitate a more comprehensive understanding of different types of skin fragility disorders (e.g. Tokonzaba et al., 2013 and references therein).

In the following section, we will discuss new disease models which will be essential to understand the role of desmosomal proteins in skin fragility. The specific example we will focus on is Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC), an ectodermal dysplasia caused by mutations in the transcription factor-encoding gene *TP63* (McGrath et al., 2001). As outlined below, it has recently been discovered that desmosomal proteins are deregulated in the skin of AEC patients. However, the contribution of these desmosomal defects to the skin fragility observed in affected individuals is currently not known. We will use this disorder as an example to highlight the challenges faced in modeling diseases and the advantages of utilizing a combination of animal and human cell-based models to understand disease mechanisms.

AEC

AEC is an ectodermal dysplasia characterized by the presence of severe skin erosions, often located to the scalp (Figure 2). In addition to skin erosions, clinical features of AEC include abnormalities in appendages such as hair, nail, teeth, sweat glands, and limbs as well as the presence of cleft lip and/or palate. The severe skin erosions place AEC patients at high risk

for local and systemic infections, and represent the main cause for the morbidity and mortality associated with AEC (Julapalli et al., 2009; Siegfried et al., 2005; Vanderhooft et al., 1993). AEC is caused by dominant mutations in *TP63*, a gene that encodes multiple transcription factors that are critical for the development and homeostasis of the skin and skin appendages (Yang et al., 1998; Koster, 2010). Although all *TP63* isoforms can be detected in the epidermis at the transcript level, it is well-established that the ΔNp63α isoform is the predominantly expressed isoform at the protein level, at least under homeostatic conditions (Parsa et al., 1999; Truong et al., 2006; Yang et al., 1998; Testoni & Mantovani, 2006). Interestingly, AEC-causing *TP63* mutations cluster in exons encoding the SAM domain, a domain that is only present in isoforms that contain the α C-terminus, such as ΔNp63α (McGrath et al., 2001; Rinne et al., 2007). Within the epidermis, ΔNp63α expression levels are highest in the basal layer, and its expression declines rapidly during keratinocyte differentiation through phosphorylation- and ubiquitin-dependent protein degradation (Bellomaria et al., 2010; Browne et al., 2011; Di Costanzo et al., 2009; Yang et al., 1998). Although the expression pattern is well-described, transcriptional networks controlled by ΔNp63α in normal or diseased skin are still relatively poorly characterized. A review of known pathways controlled by ΔNp63α has been provided elsewhere and is beyond the scope of the current manuscript (Koster, 2010).

DEREGULATION OF DESMOSOMAL PROTEINS AND GENES IN AEC

Despite the severity of skin erosions in AEC patients, few studies have addressed the cellular and molecular abnormalities leading to the apparent skin fragility. This is caused, in part, by the lack of availability of skin samples for this rare disorder. We obtained skin biopsies from AEC patients, thus allowing us to interrogate abnormal expression of genes and proteins associated with skin integrity (Fete et al., 2009; Koster et al., 2009; Beaudry et al., 2009). Consistent with reports using smaller sample sets, our analysis of AEC patient skin revealed the presence of suprabasal proliferation, impaired terminal differentiation, and abnormal deposition of basement membrane components (Koster et al., 2009; Marinari et al., 2009; Clements et al., 2012; Browne et al., 2011; McGrath et al., 2001).

In addition to these abnormalities, the skin fragility exhibited by AEC patients suggested that desmosomal protein expression or localization may be aberrant in AEC patient skin. In support of this hypothesis, several desmosomal genes, including *DSP*, *DSC3*, *DSG1*, and *PERP*, were found to be under direct transcriptional control of ΔNp63α in keratinocytes (Ferone et al., 2013; Ihrie et al., 2005). Further, expression of some desmosomal genes was reported to be compromised in AEC patients (Beaudry et al., 2009; Ferone et al., 2013), and structural desmosomal abnormalities were observed in one AEC patient (Payne et al., 2005). However, a systematic analysis of desmosomal abnormalities in AEC patient skin has not been reported. Preliminary data from our group demonstrate that expression of DSP and DSC3 is deregulated in a subset of AEC patient skin samples (Figure 3). These findings are of particular interest since genetic ablation of *Dsc3* and *Dsp* in mouse skin has been associated with loss of cell adhesion (Vasioukhin et al., 2001; Chen et al., 2008). Further, *Dsp* null mutations result in keratinocyte differentiation defects in the mouse (Vasioukhin et al., 2001), a feature also observed in AEC patient skin (Koster et al., 2009; Clements et al., 2012; Marinari et al., 2009).

MODELING AEC IN MICE

Our data, together with published work, clearly demonstrate that desmosomal abnormalities occur in AEC patients. However, these data do not provide insight into the cause or consequence of these abnormalities. To further understand the role of desmosomal protein deregulation in AEC patient skin, it is critical to generate models that replicate the disease. Several mouse models aimed at understanding the role of mutant ΔNp63α proteins expressed in AEC patients (ΔNp63α-AEC) have been generated. The first, generated by our group, is based on the premise that ΔNp63α-AEC proteins function, at least in part, as dominant-negative molecules towards wild-type ΔNp63α (ΔNp63α-wt) protein (Koster et al., 2009; Marinari et al., 2009; Lopardo et al., 2008; Browne et al., 2011). To mimic this dominant-negative effect in a mouse model, we designed genetically engineered mice that allow for the downregulation of ΔNp63 proteins through the inducible expression of a ΔNp63-specific siRNA in the epidermis (Koster et al., 2007). Upon epidermal-specific ΔNp63 downregulation, these mice developed skin erosions within a few days. These erosions mimicked erosions observed in AEC patients on a cellular and molecular level, thus highlighting the value of this model for further understanding AEC (Koster et al., 2009). However, despite the usefulness of this model, it does not take into account that mechanisms other than a dominant-negative effect may contribute to the skin fragility in AEC patients. For example, it has been postulated that ΔNp63α-AEC proteins may harbor gain-of-function effects towards specific genes, although evidence for this hypothesis is currently lacking (Chung et al., 2011; Brunner et al., 2002). To overcome limitations of this siRNA-based mouse model, another group generated mice in which an AEC-causing mutation was engineered into the endogenous *Trp63* gene locus (Ferone et al., 2012). In principle, this model should allow for a more detailed understanding of the role of ΔNp63α-AEC proteins in the epidermis. However, although several features of AEC were replicated in these mice, most notably the presence of a cleft palate, the skin phenotype did not mimic that of AEC patients. In fact, whereas AEC patient skin is hyperplastic and exhibits suprabasal proliferation (Koster et al., 2009; Marinari et al., 2009; Clements et al., 2012; Browne et al., 2011; McGrath et al., 2001), the epidermis of the mice is hypoplastic and exhibits reduced proliferation (Ferone et al., 2012).

NEW APPROACHES OF MODELING COMPLEX DISEASES IN VITRO

As outlined above, mouse models have been extremely informative in modeling aspects of AEC. However, significant discrepancies in the skin phenotype of mice expressing a *TP63* - AEC mutation and AEC patient skin indicate that the mouse model does not truly mimic all aspects of the disease. One possible explanation is a difference in the biology of mouse and human skin. To overcome this issue, *in vitro* approaches using human cell-based systems were designed. The Khavari group ectopically expressed ΔNp63α-AEC proteins in 3D skin equivalents generated from discarded human tissue (Zarnegar et al., 2012; Sen et al., 2012). Interestingly, deregulation of several desmosomal genes was observed in keratinocytes that ectopically expressed the ΔNp63α - AEC protein. Ectopic expression of mutant genes is a potentially powerful approach to assess the function of mutated transcription factors. Disadvantages of this system, however, include the use of viral vectors to express genes which randomly integrate into the keratinocyte genome, mutant gene expression levels that

do not reflect the balance of wild-type and mutant transcripts found in patient cells, and the inability to mimic the effects of a possible change in the ratio of different splice variants of *TP63* (e.g., ΔNp63α and ΔNp63β) in patient keratinocytes. Lastly, the use of genetically heterogeneous sources of keratinocytes in this type of study is of concern. In fact, genetic background effects are well-known to influence the severity of disease phenotypes as demonstrated by the clinical variability observed in related patients carrying the same *TP63* mutation (Bertola et al., 2004; Clements et al., 2010; Dianzani et al., 2003). Further, discarded patient skin is often derived from different body sites. It remains to be determined whether keratinocytes obtained from foreskin and breast tissue, for example, show identical transcriptome changes in response to the expression of mutant TP63. Thus, using patient cells from defined anatomical locations represent an ideal approach to study the disease mechanisms.

What would be the biological requirements for an ideal human cell-based *in vitro* model to study the patho-physiology of AEC? The system would (i) harbor disease-causing *TP63* mutations and (ii) mimic the AEC skin phenotype at the cellular and the molecular levels. Further, to generate reproducible results in a genetically defined system, the model has to be renewable. Induced pluripotent stem cell (iPSC) technology has provided an ideal tool for the generation of tissue models as it allows for the generation of unlimited supplies of human iPSC that can be differentiated into keratinocytes and 3D epidermal equivalents [(Itoh et al., 2011; Tolar et al., 2011); Figure 4]. To generate iPSC, a set of transcription factors (reprogramming factors) is introduced into somatic cells (e.g., skin fibroblasts isolated from a skin biopsy (Park et al., 2008b; Park et al., 2008a; Okita et al., 2007; Takahashi et al., 2007)). These reprogramming factors dedifferentiate somatic cells to a state similar to that of embryonic stem (ES) cells. These cells can then be differentiated into keratinocytes. Because iPSC can be amplified indefinitely, this technology enables investigators to produce unlimited quantities of patient-derived keratinocytes, thereby circumventing the problem of the limited lifespan and amplification potential of cultured primary human keratinocytes *in vitro* (Green et al., 1977; Chapman et al., 2010).

iPSC-based approaches also allow for the correction of point mutations in the *TP63* gene of AEC patients. The development of genome editing tools such as zinc finger nucleases, TALEN and CRISPR/CAS (Hockemeyer et al., 2009; Hockemeyer et al., 2011; Mali et al., 2013; Cong et al., 2013) enables us to correct these mutations and generate pairs of conisogenic iPSC-derived keratinocytes that differ only with respect to the presence or absence of a *TP63* -AEC mutation. The identical genetic background of these pairs of cells facilitates disease pathway analysis using advanced transcriptome and proteome tools. Because this approach eliminates genetic background variation effects, it is conceptually superior to using “normal controls” from donors without *TP63* mutations.

A potentially unlimited source of patient iPSC-derived keratinocytes enables us to generate *in vitro* skin equivalents designed to mimic the histo-pathology of AEC. Further, xenotransplantation approaches (Lichti et al., 2008), that is, the generation of human epidermis with iPSC-derived keratinocytes on immunosuppressed mice, will enable researchers to evaluate AEC epidermis in an *in vivo* environment. Of particular interest with respect to desmosomal proteins is the question of whether these systems will be able to

reproduce the aberrant expression patterns of key targets in AEC, such as DSP and DSC3. Next, it will be possible to define the signaling pathways that lead to the deregulation of desmosomal genes in this context using advanced transcriptome and proteome analyses. Finally, using conventional overexpression and knockdown experiments, this *in vitro* skin equivalent system should be ideally suited to mechanistically define the contribution of individual desmosomal proteins to the skin fragility observed in AEC patient skin.

SUMMARY AND CONCLUSIONS

Although it is undisputed that null mutations in many desmosomal genes can cause severe diseases in humans, it is not clear in which way more subtle changes in desmosomal gene expression or protein synthesis contribute to skin disorders such as AEC. The case of AEC is of particular interest. Several desmosomal genes are targets of the transcription factor TP63. However, only a few desmosomal genes are downregulated in nonlesional skin of AEC patients, in particular DSC3 and DSP. It will be of interest to determine whether reduced expression of these genes contributes to the tissue fragility and keratinocyte differentiation defects observed in the skin of AEC patients. Given that mouse models have limitations in their ability to mimic the AEC skin phenotype, we propose that new human cell-based tissue models of this disease are needed. Using stem cell (iPSC) technology, we now have the tools to generate patient-derived keratinocytes for cell biological, transcriptome, and proteome analysis. This technology also enables us to generate conisogenic pairs of cells from patients that differ only with respect to the presence and absence, respectively, of disease causing mutations such as those causing AEC. These genetically defined cells will be invaluable for identifying disease pathways. Further, the approach described here will be applicable to any monogenetic skin disorder. In the future, this technology will also be essential for generating patient-derived and gene-corrected replacement tissue for transplantation, for example, in cases of severe skin fragility disorders.

Acknowledgments

We would like to thank the University of Colorado School of Medicine Histology Core (www.medschool.ucdenver.edu/histology) and iPSC Core (www.medschool.ucdenver.edu/iPS) for technical support. We would like to thank the National Foundation for Ectodermal Dysplasias (NFED) for providing patient photographs and assistance in obtaining patient skin biopsies.

PJK and MIK were supported by a grant from the NFED and by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) under Award Numbers R00 AR054696 (MIK), R01 AR061506 (MIK), and R01 AR053892 (PJK). JD is supported by a pre-doctoral fellowship from the Colorado Clinical & Translational Science Institute (TR001081).

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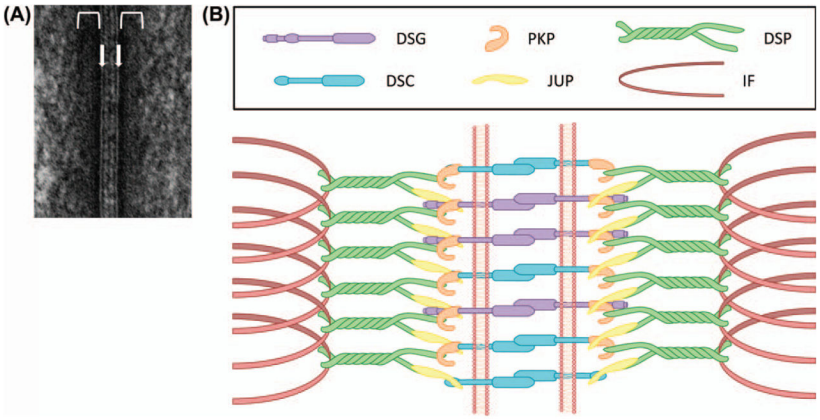

Figure 1.

Desmosomal structure and composition. (A) Electron micrograph of a desmosome from mouse epidermis. The arrows demarcate the positions of the plasma membranes of neighboring cells. The brackets indicate the location of the cytoplasmic plaques. (B) Schematic representation of the proteins that assemble into desmosomes. Note that the transmembrane components (DSG and DSC) connect on the cytoplasmic surface of the desmosome with the plaque proteins (JUP, DSP and PKP) which in turn link to the intermediate filament (IF) cytoskeleton.

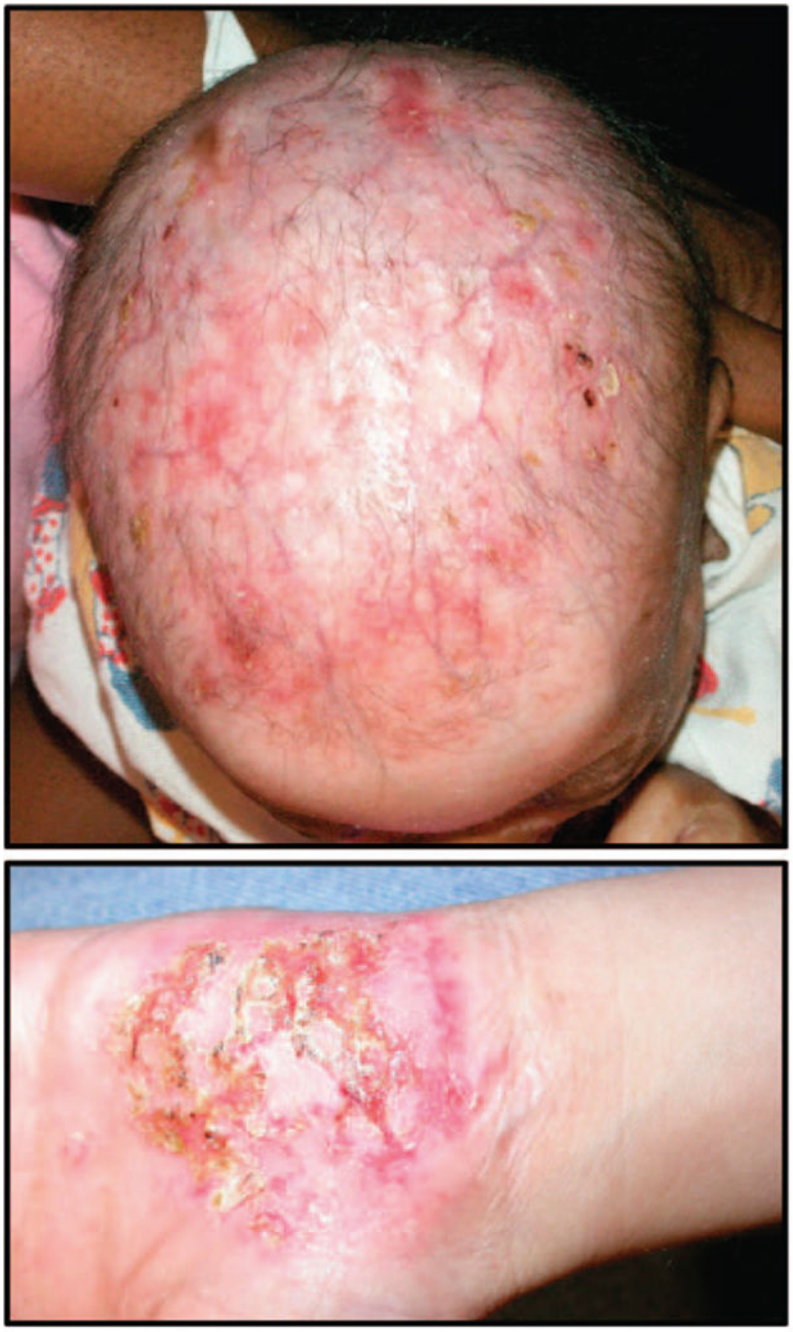

Figure 2.

Clinical presentation of AEC patients. Scalp and palmar erosions on two patients affected by AEC. Patient images provided by the National Foundation for Ectodermal Dysplasias (NFED).

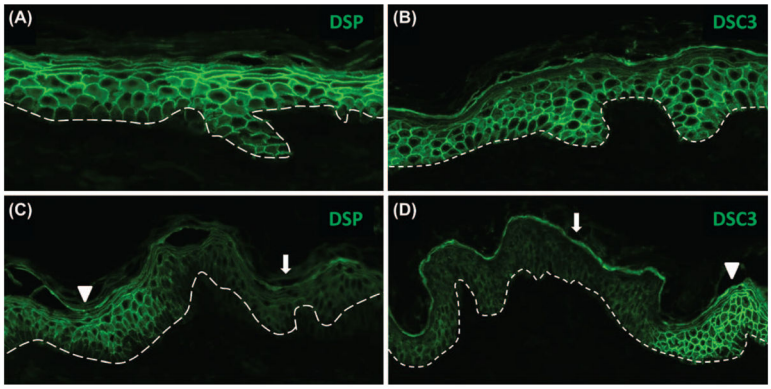

Figure 3.

Focal downregulation of DSP and DSC3 expression in non-lesional skin of an AEC patient. (A,B) Normal human skin and (C, D) skin of an AEC patient. The dashed line demarcates the epidermal–dermal junction. The arrowheads in panels (C) and (D) point to areas of normal DSP and DSC3 staining, respectively, while the arrows indicate area in which expression of either DSC3 or DSP is significantly downregulated.

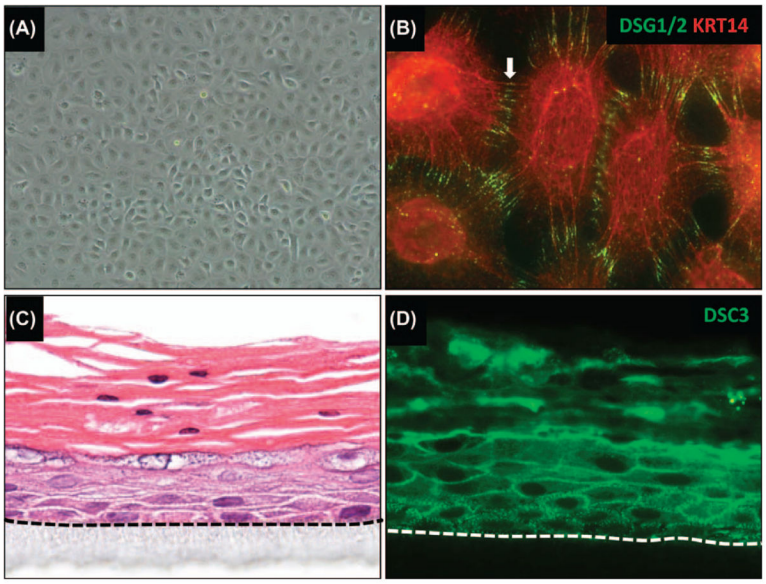

Figure 4.

Generating *in vitro* tissue models using iPSC-derived keratinocytes. (A) iPSC-derived keratinocytes were generated following a protocol similar to a previously published procedure (Itoh et al., 2011). (B) iPSC-derived keratinocytes express keratin 14 (KRT14) and desmoglein 1/2 (DSG1/2). Note that these cells also express other epithelial markers, such as keratin 5, TP63, and α6β4 integrin (data not shown). (C) *in vitro* skin equivalent generated from human iPSC-derived keratinocytes on an artificial surface. Note that the epithelium stratified with a notable granular and cornified layer. Parakeratosis (retention of nuclei in the stratum corneum) occurs occasionally in *in vitro* skin equivalents. (D) Staining of the skin equivalent shown in (C) with an antibody for desmocollin 3 (DSC3). Note that these iPSC-derived skin equivalents also express other epithelial markers such as KRT5/14, KRT1/10, and loricrin (data not shown). The dotted line demarcates the junction between stratified epithelium and the artificial matrix on which the cells are growing.