Available online at www.sciencedirect.com
ScienceDirect
Matrix Biology 27 (2008) 163–170
MATRIX BIOLOGY
www.elsevier.com/locate/matbio

The basement membrane microenvironment directs the normalization and survival of bioengineered human skin equivalents

Nadav Segal [a], Frank Andriani [a], Lawrence Pfeiffer [a], Padmaja Kamath [a], Ning Lin [a], Kapettu Satyamurthy [b], Christophe Egles [c], Jonathan A. Garlick [c,*]

[a] *Department of Oral Biology and Pathology, School of Dental Medicine, SUNY at Stony Brook, Stony Brook, New York, United States*
[b] *Manipal Academy of Higher Education, Department of Biotechnology, Manipal, India*
[c] *Division of Cancer Biology and Tissue Engineering, Tufts University School of Dental Medicine, Boston, Massachusetts, United States*

Received 3 January 2007; received in revised form 10 August 2007; accepted 6 September 2007

Abstract

Epithelial–mesenchymal interactions promote the morphogenesis and homeostasis of human skin. However, the role of the basement membrane (BM) during this process is not well-understood. To directly study how BM proteins influence epidermal differentiation, survival and growth, we developed novel 3D human skin equivalents (HSEs). These tissues were generated by growing keratinocytes at an air–liquid interface on polycarbonate membranes coated with individual matrix proteins (Type I Collagen, Type IV Collagen or fibronectin) that were placed on contracted Type I Collagen gels populated with dermal fibroblasts. We found that only keratinocytes grown on membranes coated with the BM protein Type IV Collagen showed optimal tissue architecture that was similar to control tissues grown on de-epidermalized dermis (AlloDerm) that contained intact BM. In contrast, tissues grown on proteins not found in BM, such as fibronectin and Type I Collagen, demonstrated aberrant tissue architecture that was linked to a significant elevation in apoptosis and lower levels of proliferation of basal keratinocytes. While all tissues demonstrated a normalized, linear pattern of deposition of laminin 5, tissues grown on Type IV Collagen showed elevated expression of α6 integrin, Type IV Collagen and Type VII Collagen, suggesting induction of BM organization. Keratinocyte differentiation (Keratin 1 and filaggrin) was not dependent on the presence of BM proteins. Thus, Type IV Collagen acts as a critical microenvironmental factor in the BM that is needed to sustain keratinocyte growth and survival and to optimize epithelial architecture.

Keywords: Basement membrane; Human skin equivalent; Type IV Collagen; Microenvironment; Keratinocytes

1. Introduction

The maturation and function of human skin is dependent on the regulation of keratinocyte growth, terminal differentiation and survival. It is known that the underlying connective tissue can modulate these events through two mechanisms. First, fibroblast-derived diffusible factors can act in a paracrine fashion to regulate the behavior of basal keratinocytes (Fusenig, 1994; Szabowski et al., 2000). Secondly, interactions between basement membrane (BM) proteins and adjacent basal keratinocytes at their stem cell niche are known to direct keratinocyte fate decisions (Jones et al., 1995). Thus, epithelial–mesenchymal interactions act in concert to promote tissue morphogenesis and to maintain epithelial homeostasis (Fusenig, 1994).

Since the BM plays an important role in keratinocyte differentiation, survival and growth it is important to understand how its development is linked to tissue phenotype. When keratinocytes are grown in 2D, monolayer culture, cells are hyperproliferative, undergo a minimal degree of stratification and do not achieve normalized, 3D tissue architecture. This limitation was partially overcome by the development of bioengineered 3D cultures that mimic human skin, known as human skin equivalents (HSEs), in which epidermal cells are grown at an air–liquid interface on a connective tissue substrate

* Corresponding author. Division of Cancer Biology and Tissue Engineering, South Cove Bldg — Room 116, 136 Harrison Avenue, Tufts University School of Dental Medicine, Boston, MA 02111, United States. Tel.: +1 617 636 2478; fax: +1 617 636 2915.
E-mail address: Jonathan.Garlick@tufts.edu (J.A. Garlick).

0945-053X/$ - see front matter © 2007 Elsevier B.V./International Society of Matrix Biology. All rights reserved.
doi:10.1016/j.matbio.2007.09.002

Table 1
Summary of phenotypic properties of 3D, human skin equivalents grown on a variety of extracellular matrix or basement membrane protein interfaces

	Alloderm	Collagen I	Collagen IV	Fibronectin
Tissue stratification	*****	**	*****	***
Tissue architecture	Normal, well-polarized basal cells	Aberrant flat basal cell layer	Normal, well-polarized basal cells	Loss of basal cell polarity
Laminin 5	Linear	Linear	Linear	Linear
Collagen VII	Linear	Faint	Linear, patchy	Linear, patchy
Collagen IV	Linear	Patchy	Linear	Absent
α±6 integrin	Linear	Patchy	Linear	Faint
Filaggrin	+++	++	+++	+++
K1	+++	++	+++	++
Brdu (LI)	25%	14%	25%	17%
% Tunel-positive basal cells	0	7%	0	17%

***** — full stratification, *** — moderate stratification, ** — little stratification.
+++ — strong staining, ++ — moderate staining.
Phenotypic properties of epithelial tissues grown on varied interfaces.

harboring viable fibroblasts (Andriani et al., 2003; Berking and Herlyn, 2001). A variety of connective tissue substrates have been used to construct these HSEs to generate stratified epithelia that display many morphologic and functional characteristics of native skin. However, while HSEs express BM components (Fusenig, 1994; Marinkovich et al., 1993), only limited success has been realized in demonstrating morphologically-identifiable BM in these cultures (Zieske et al., 1994; Andriani et al., 2003). Since intact BM is known to be a critical signal for the control of normal epidermal growth and differentiation, it is important to fabricate tissues that generate structured BM.

No previous studies have investigated how purified BM or ECM components direct the tissue phenotype of HSEs. To address this, we have developed novel HSEs in which keratinoctyes were grown on purified ECM or BM components coating polycarbonate membranes that were placed on a contracted collagen gel harboring viable fibroblasts. By growing keratinocytes at an air–liquid interface on these substrates, we have fabricated human 3D tissues that can be studied and analyzed in the presence or absence of particular BM or ECM proteins. In this report, we describe the directive influence of BM components on the normalint tissue organization. These findings demonstrated that keratinocytes require the presence of BM proteins at the dermal–epidermal tissue interface to optimize their architecture and phenotype during the development and organization.

2. Results

2.1. Basement membrane components support optimal epidermal morphogenesis

NHK were seeded onto polycarbonate membranes coated with either the BM component Type IV Collagen or with ECM components not found in the BM (Type I Collagen and fibronectin). Coated membranes were placed on contracted Type I Collagen gels containing fibroblasts and were cultured at an air–liquid interface for 7 d to allow tissue development and maturation (Fig. 1). As control tissues for the presence of intact BM, NHK were seeded onto a de-epidermalized, human cadaver dermis (AlloDerm™) that was also layered onto the Type I Collagen gel. 3D tissues grown directly on AlloDerm have previously been shown to enable rapid tissue normalization and BM organization and thus served as a positive control for optimal tissue organization (Andriani et al., 2003). The phenotypic properties of HSEs grown on these different interfaces are summarized in Table 1. Keratinocytes seeded onto AlloDerm generated an epithelium with in vivo-like tissue organization characterized by the presence of all morphologic strata and by polarized, cuboidal basal cells which were nested in undulating rete pegs (Fig. 2D). Similarly, keratinocytes

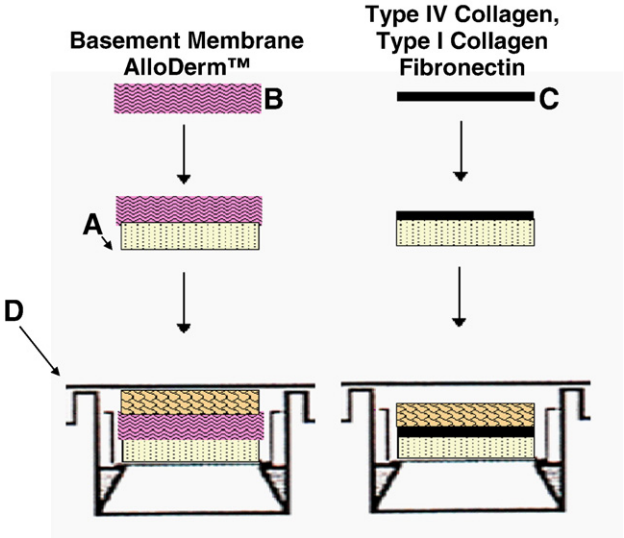

Fig. 1. Human skin equivalent models developed to study the role of basement membrane components or extracellular matrix proteins on epidermal organization, growth and survival. Connective tissue was constructed by generating a contracted Type I Collagen gel containing dermal fibroblasts (A). Culture in the presence of intact basement membrane was achieved by seeding keratinocytes on a de-epidermalized dermal substrate (AlloDerm™) (B) which was layered on the collagen gel to allow its repopulation with fibroblasts. Alternatively, keratinocytes were seeded on either Type I Collagen, Type IV Collagen or fibronectin by layering a polycarbonate membrane (C) coated with these proteins on the contracted Type I Collagen gel. All cultures were grown submerged in media for 4 d and at an air–liquid interface for an additional 7 d (D).

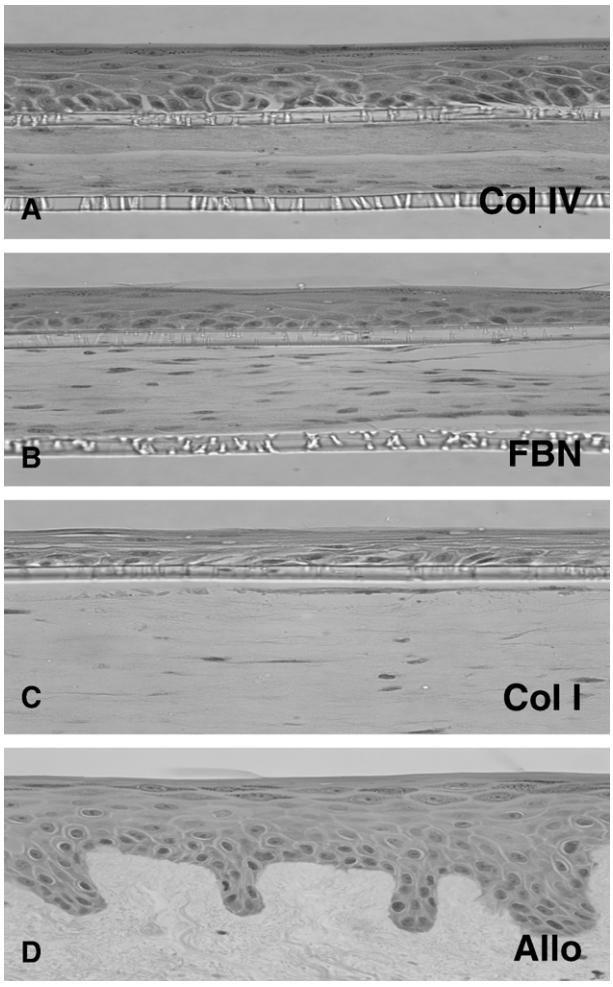

Fig. 2. Type IV Collagen and basement membrane proteins normalize epidermal morphogenesis. Keratinocytes were seeded onto coated poycarbonate membranes and grown as 3D tissues. Type IV collagen-coated (A) and AlloDerm (D) interfaces generated thick epithelial tissues showing all morphologic strata and well-polarized basal keratinocytes. Cultures grown on fibronectin- (B) and Type I Collagen (C) demonstrated aberrant morphology.

seeded on polycarbonate membranes coated with Type IV Collagen generated an epithelial tissue with polarized basal cells, a well-formed spinous layer and the presence of a thin stratum granulosum and stratum corneum (Fig. 2A). In contrast, cultures grown on a polycarbonate membrane coated with ECM components not found in BM demonstrated a greater degree of tissue disorganization and altered tissue development. For example, 3D tissues grown on fibronectin (Fig. 2B) demonstrated a poorly-developed epithelium in which basal cells appeared flattened without the polarized, cuboidal morphology seen for tissues grown on Type IV Collagen and AlloDerm. Even greater alterations in morphology were seen when tissues were grown on Type I Collagen-coated membranes (Fig. 2C). These tissues demonstrated a thin epithelium characterized by non-polarized, flattened basal cells and a poorly-formed spinous layer. Thus, epidermal morphogenesis was enhanced by the presence of the BM component Type IV Collagen at the stromal interface.

2.2. Cell proliferation and survival are supported by the presence of Type IV Collagen but not by fibronectin and Type I Collagen

In order to establish the tissue phenotype that was linked to the improved morphologic appearance of epithelia grown on the Type IV Collagen interface, cell proliferation and survival were directly analyzed in 3D tissues. To assess cell growth, the proliferation rate of basal keratinocytes was determined following BrdU labeling of 3D tissues grown on various substrates. Numbers of BrdU-positive basal cells were counted and calculated as the percentage of the total number of basal cells (LI = labeling index) (Fig. 3). Tissues grown on BM-containing AlloDerm and on Type IV Collagen demonstrated highest levels of cell proliferation (LI=25%), showing that Type IV Collagen was sufficient to support growth at a level similar to tissues grown on intact BM. In contrast, tissues grown on fibronectin and Type I Collagen showed significantly lower levels of proliferation (17% and 14% respectively), showing that optimal cell growth required the presence of BM proteins.

In situ TUNEL assay was then performed to determine apoptosis of keratinocytes when tissues were grown on different interfaces. When the percentage of TUNEL-positive basal cells was calculated, it was found that tissues grown on fibronectin (17%) and Type I Collagen (7%) displayed TUNEL-positive cells in the basal layer of the epithelium (Fig. 3). TUNEL-positive cells were seen primarily in the basal layer when grown on Type I Collagen (Fig. 4B) while TUNEL-positive cells were seen in both the basal and suprabasal layers of tissues grown on fibronectin (Fig. 4A). In contrast, no TUNEL-positive basal or suprabasal cells were seen when tissues were grown on the Type IV Collagen interface (Fig. 4C). In these tissues, TUNEL-positive cells were only seen in the most superficial layer of the epithelium, as would be expected in a normal epithelium (Fig. 4C, arrow). Thus, the presence of a BM protein, such as Type IV Collagen, sustained basal keratinocyte survival, demonstrating that BM components promoted optimization of tissue

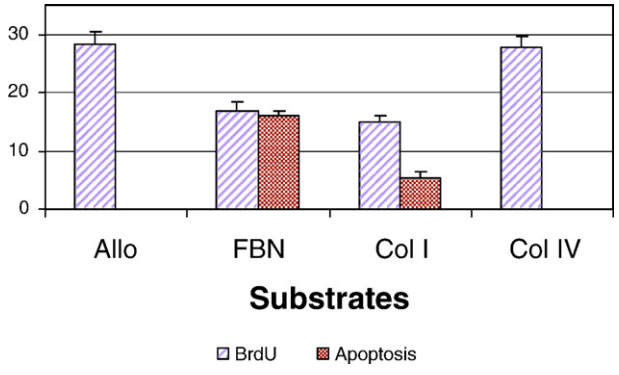

Fig. 3. Survival and proliferation of basal keratinocytes are promoted by Type IV Collagen and basement membrane proteins. Tissues were stained by TUNEL assay to determine the percentage of apoptotic basal keratinocytes and using a BrdU antibody to measure the percentage of proliferating cells in the basal layer. Proliferating and apoptotic cells were calculated as the percentage of TUNEL- and BrdU-positive basal cells by counting a total of 1000 nuclei in the basal cell layer present in 8 serial sections found 100 μm apart.

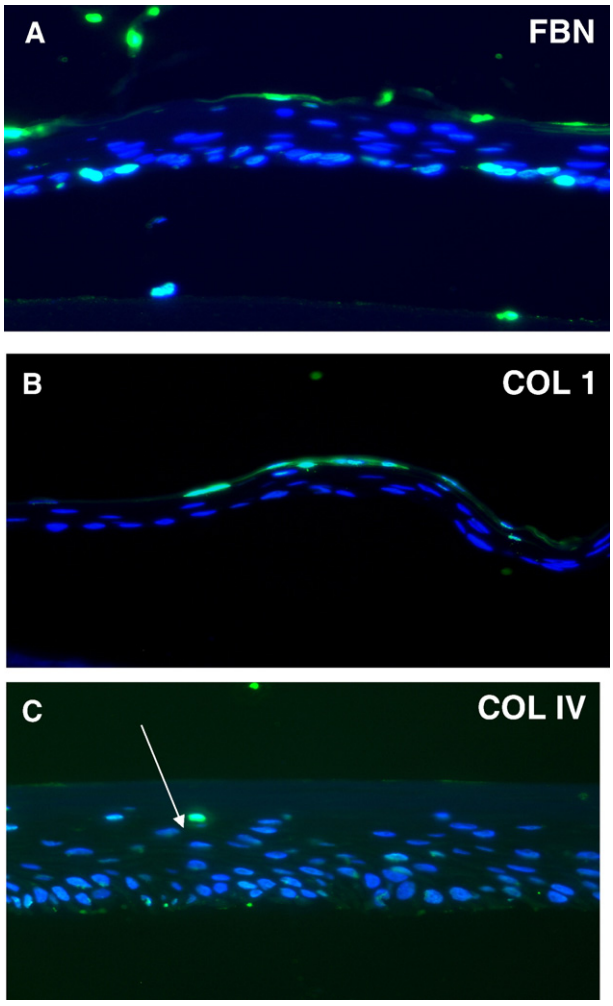

Fig. 4. Type IV Collagen and BM proteins prevent induction of basal cell apoptosis. In situ TUNEL assay determined large numbers of TUNEL-positive cells were seen on fibronectin- (A) and Type I Collagen-coated membranes (B) (blue-green nuclei showing an overlap of DAPI (blue) and FITC (green) signals). Tissues grown on the Type IV Collagen substrates showed no TUNEL-positive basal cells and apoptotic nuclei were restricted to the most superficial epithelial layer (C arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

organization by preventing aberrant apoptosis of basal keratinocytes while augmenting keratinocyte growth.

2.3. Maturation of the BM zone is linked to the presence of Type IV Collagen

We next determined if optimal tissue normalization and enhanced cell growth and survival seen in the presence of either Type IV Collagen or AlloDerm were linked to the early stages of BM organization and development. To accomplish this, tissues were analyzed for the presence of laminin 5, Type VII Collagen, Type IV Collagen and α6 integrin, as markers of BM organization. As laminin 5 and α6 integrin are produced strictly by keratinocytes (Marinkovich et al., 1993) and are known to be linked to the earliest stages of BM development (Fleischmajer et al., 1998), their distribution would serve as an indicator of how various epithelial–stromal interfaces could enable initiation of BM organization. When keratinocytes were seeded onto the AlloDerm substrate, deposition of laminin 5 (Fig. 5G) and α6 integrin (Fig. 5H), as well as Type IV Collagen and Type VII Collagen (Fig. 6) were found to be linear and strictly polarized along the BM interface. In light of our previous findings that the linear, co-localization of these proteins was linked to BM assembly (Andriani et al., 2003), this distribution is an indication of organization of structured BM in these tissues. Cells grown directly on Type I Collagen (Fig. 5E), fibronectin (Fig. 5C) and Type IV Collagen (Fig. 5B) all demonstrated a linear pattern of laminin 5 deposition.

In contrast, tissues grown on fibronectin showed a faint staining for α6 integrin (Fig. 5D) and the Type I Collagen substrate showed a patchy localization of this protein (Fig. 5F). In contrast, localization of this integrin subunit was considerably elevated as staining was seen in a pericellular pattern in which staining extended into the immediate suprabasal layer of tissues grown on Type IV Collagen (Fig. 5B). This suggested that while α6 integrin expression was elevated on Type IV collagen, it had not yet fully co-localized with the linear pattern seen for laminin 5. This ectopic expression may be linked to the somewhat elevated proliferation seen in these tissues. Tissues grown on Type I Collagen showed patchy deposition of Type IV Collagen (Table 1) and only faint, perinuclear staining for Type VII Collagen (Fig. 6C), while growth on fibronectin showed no deposition of Type IV Collagen (Table 1) and patchy, linear localization of Type VII Collagen (Fig. 6A). Type VII Collagen staining of tissues grown on Type IV Collagen-coated membranes also showed a linear, patchy localization (Fig. 6B) while a linear distribution was seen on control, AlloDerm tissues (Fig. 6D). This suggests that Type VII Collagen, a late marker of BM organization thought to be primarily derived from sub-epithelial fibroblasts, may not play a major role in the initial development of the BM. Taken together, the altered deposition of epithelial-derived BM components when tissues were grown on a Type I Collagen- or fibronectin-coated interface suggested a delayed development of the initial stages of BM formation. In contrast, the more normalized deposition of these proteins in tissues grown in the presence of Type IV Collagen suggested a more complete organization of initial BM formation that was linked to the capacity of these tissues to sustain their growth and survival.

2.4. Expression of epidermal differentiation (K1) was independent of the BM interface

The in vivo-like architecture of 3D tissues grown on de-epidermalized dermis was reflected in the normalized distribution of differentiation markers seen upon immunofluorescent staining. The early marker of keratinocyte differentiation, Keratin 1 (K1) was expressed in suprabasal keratinocytes in a strata-specific pattern (Fig. 6) while filaggrin was restricted to the superficial layers of the epithelium (Table 1). Tissues grown on all coated membranes demonstrated a similar strata-specific expression of K1 that was limited to the suprabasal layers (Fig. 6E,F,G). Thus, expression of markers of keratinocyte differentiation were found

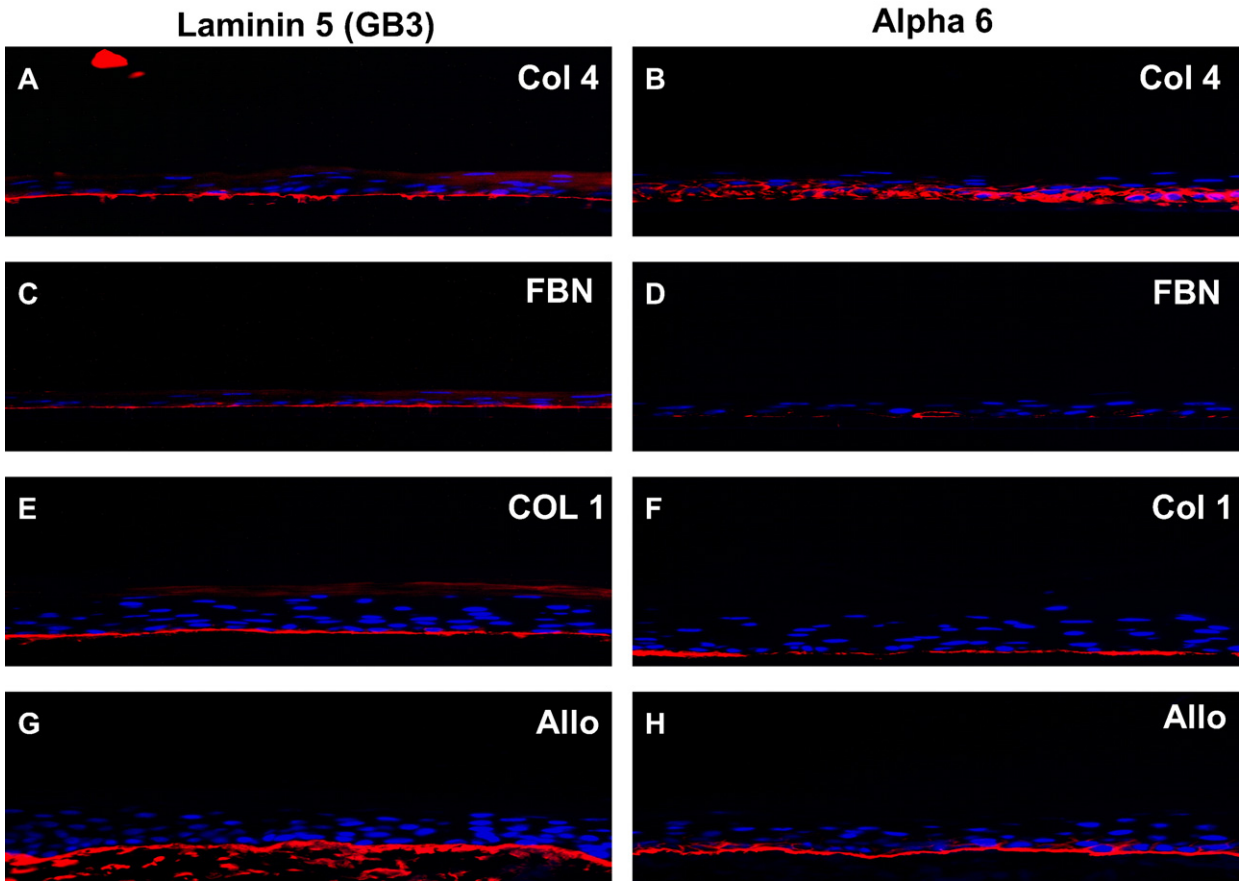

Fig. 5. Laminin 5 and α6 integrin deposition are more normalized on basement membrane proteins. Tissues were stained for immunohistochemical localization of laminin-5 (A, C, E, G) and α6 integrin (B, D, F, H). Tissues were grown on Type I Collagen- (F) and fibronectin- (D), Type IV collagen- (B) and AlloDerm- (H) coated inserts. Background staining is seen in the upper dermis of AlloDerm for laminin 5 stain (G).

to be independent of the nature of proteins present at the BM interface.

3. Discussion

The epidermal basement membrane is known to establish adhesion between the epithelium and dermis and to regulate the growth, differentiation, survival and morphogenesis of human skin (Alonso and Fuchs, 2003). However, it remains unclear how BM assembly is linked to these tissue phenotypes. To address this, we have engineered novel 3D human skin equivalent (HSE) tissues in which keratinocytes were grown in the presence of either BM proteins (Type IV Collagen) or extracellular matrix (ECM) proteins not found in the BM (fibronectin, Type I Collagen). For the first time, this approach has enabled growth of HSEs on individual BM or ECM components at the dermal–epidermal interface in order to study how these proteins can direct tissue phenotype and organization. We found that 3D tissues grown in the presence of Type IV Collagen or pre-existing BM proteins showed normalized tissue architecture, improved organization of BM components, supported keratinocyte growth and prevented apoptosis when compared to tissues grown on proteins not found in the BM. These results indicated that BM proteins play a critical role in the generation of a well-structured epidermis and in the regulation of normalized epithelial phenotype in a 3D model of human skin.

While the synthesis of BM components by keratinocytes and fibroblasts has most commonly been studied in 2D, monolayer cultures, keratinocytes do not express their in vivo phenotype in these cultures. Since epithelial BMs are composed of an intricate network of proteins at the epithelial–stromal interface (Yurchenko and O'Rear, 1994; Christiano and Uitto, 1996), BM assembly needs to be studied in biological systems in which a high degree of tissue complexity can be achieved, such as seen in human skin equivalents (HSEs) (Andriani et al., 2003; Berking and Herlyn, 2001). However, HSEs have shown limited success forming structured BM (Black et al., 2005; Yang et al., 2006) due to MMP-mediated degradation of BM components after their synthesis and secretion (Amano et al., 2001; Amano et al., 2005). These studies have shown that Type IV Collagen can suppress this MMP-9 expression and stabilize the BM interface during the early stages of BM assembly. We have similarly shown that the presence of Type IV Collagen can support the early stage of BM maturation and lead to normalization of tissue organization and optimization of cell growth and survival. Thus, it appears that the balance between degradation and stabilization of newly synthesized BM components is essential for BM assembly and maturation (Amano et al., 2001).

It has been shown that pre-existing BM proteins serve as a template for the deposition and integration of newly synthesized proteins at the dermal–epidermal junction (Vailly et al., 1995).

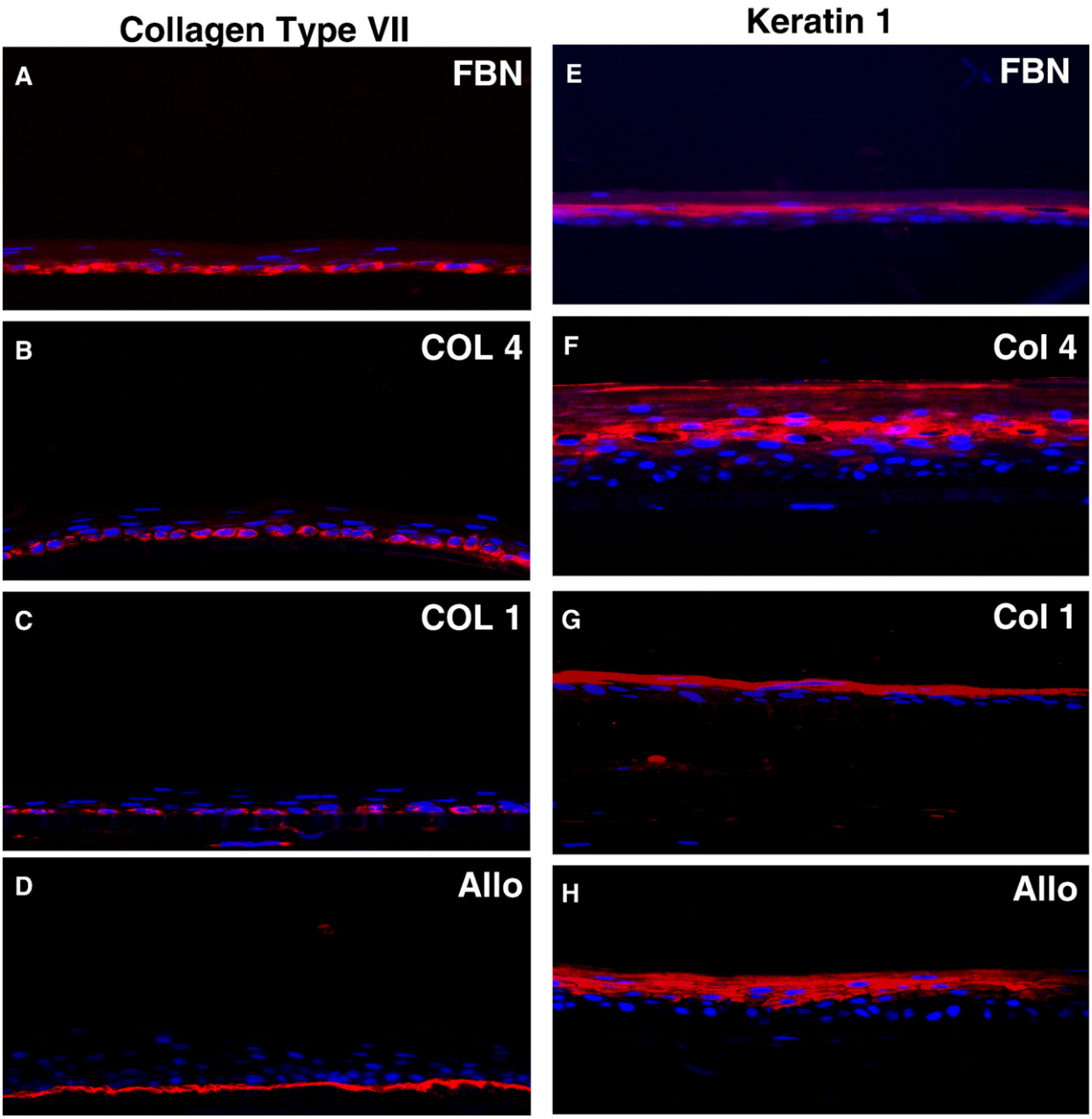

Fig. 6. Expression of Collagen Type VII and Keratin 1 are independent of ECM or BM protein substrate. The distribution of the basement membrane component Type VII Collagen and the tissues grown on keratinocyte differentiation keratin 1 (K1) marker was determined by immunohistochemical stain. Type VII Collagen was seen in a linear but patchy distribution for fibronectin (A) and Type IV Collagen (B), was faint for Type I Collagen (C) and linear when grown on AlloDerm (D). K1 expression was appropriately limited to suprabasal cells cultures grown either on fibronectin (E), Type IV collagen (E), and Type I collagen (G) in a pattern similar to AlloDerm controls (H).

HSEs constructed by seeding keratinocytes on an interface containing pre-existing BM components, such as de-epithelialized skin (Andriani et al., 2003) or amniotic membrane (Yang et al., 2006) promote rapid assembly of well-structured BM. This is thought to occur due to interactions between Type IV Collagen and β1 integrins (Fleischmajer et al., 1998) that have been shown to provide an early scaffold for BM organization. Our finding that the presence of Type IV Collagen is linked to the assembly of BM supports these previous findings and extends the significance of these events by linking them to the concurrent normalization of epithelial growth, morphogenesis and survival during the early stages of tissue organization. Interestingly, differences in tissue phenotype were evident during these earliest stages of tissue formation demonstrating that the BM interface is a dominant determinant in initially establishing 3D tissue phenotype. Thus, the tissue models described are very useful for studying the early development and organization of epithelial tissue phenotype.

Factors directing the de novo establishment of BM upon restoration of epithelial integrity are important to understand in the context of reepithelialization occurring during wound repair (Singer

and Clark, 1999). In this light, cutaneous wound healing has been described as a model for cell-matrix interactions (Martin, 1997; Woodley et al., 1985) and as a process that is mimicked by HSEs as they organize their nascent BM (Andriani et al., 2003). In vitro and in vivo models of wound healing have demonstrated that Type IV Collagen was important in the initiation of newly synthesized BM, and its secretion triggered a switch, as wound epithelium transitioned from a migratory to a stationary phenotype (Larjava et al., 1993; Woodley et al., 1985). Our findings therefore demonstrated that the switch from contact with stromal components that are present during the migratory phase of healing (Type I Collagen and Fibronectin) to BM components such as Type IV Collagen is a requisite step for initiation of BM assembly and have implications for the reestablishment of BM integrity following wounding.

The BM is known to be a critical signal for the control of normal epidermal growth (Fleischmajer et al., 1998; Marinkovich et al., 1993) and establishes basal cell polarity associated with hemidesmosomes (Dowling et al., 1996). Our findings support these observations, as only tissues grown on substrates such as Type IV Collagen, were permissive for improved BM organization and improved epithelial tissue architecture. Furthermore, it appears that survival of basal keratinocytes is substrate dependent, as only BM components were able to prevent apoptosis of basal cells. The fact that growth of tissues on Type I Collagen and fibronectin led to apoptosis of significant numbers of keratinocytes supports previous findings that disruption of cell-matrix interactions induces apoptosis (Frisch and Francis, 1994) and anoikis (Frisch and Ruoslahti, 1997). Since these previous studies were performed in 2D, monolayer culture systems in which apoptosis was induced by forced suspension, our findings extend these observations to a tissue environment in which cell–matrix interactions are in their appropriate context. Since the goal of HSE culture is to fabricate stratified epithelia that demonstrate in vivo-like epidermal morphology, growth and survival (Andriani et al., 2003; Berking and Herlyn, 2001) it is critical to generate 3D tissues that develop in the context of an organizing BM.

Our findings show that development of a well-organized stratified squamous epithelium is dependant on interactions with the underlying connective tissue substrate. This supports previous studies showing that self-renewal and regulation of basal keratinocyte fate is modulated by proteins in the BM (Alonso and Fuchs, 2003). However, further study is needed to demonstrate how specific proteins in the BM microenvironment provide signals in the stem cell niche that control these regulatory functions. Further elucidation of particular signaling inputs by organizing BM components on the growth, survival and development of human skin will be advanced by the use of novel tissue models that can allow investigation of BM formation in a 3D tissue microenvironment.

4. Experimental procedures

4.1. Culture of 3D human skin equivalents

Normal human keratinocytes (NHK) derived from foreskin were first grown in 2D, monolayer culture on irradiated 3T3 fibroblasts. Foreskin fibroblasts were grown in media containing Dulbecco's Modified Eagle's Medium (DMEM) and 10% fetal calf serum. HSEs were grown by adding fibroblasts to neutralized Type I Collagen (Organogenesis, Canton, MA) to a final concentration of 2.5×10^4 cells/ml. Three ml of this mixture was added to each 35 mm well of a 6 well plate (Organogenesis, Canton, MA) and incubated for 6 d in media containing DMEM and 10% fetal calf serum, until the collagen matrix showed no further shrinkage. Polycarbonate membranes coated with the purified, murine BM protein Type IV Collagen or ECM components not found in BM (Fibronectin, Type I Collagen) (Becton Dickinson, Billerica, MA), were layered onto the collagen gels and 5×10^5 keratinocytes were seeded on them (Fig. 1). As controls for tissues with well-structured BM (Andriani et al., 2003), keratinocytes were grown in the presence of pre-existing BM components on a de-epidermalized dermis derived from human skin (Alloderm™, LifeCell Corp. Branchburg, New Jersey), that was layered onto the contracted collagen gel. Cultures were maintained submerged in low calcium epidermal growth media (EGM) for 2 d, submerged for 2 d in normal calcium EGM and raised to the air–liquid interface for an additional 7 d by feeding from below. Human foreskins were procured and used for the described experiments in accordance with all institutional approvals from Stony Brook University's and Tufts University's Human Subjects Committees.

4.2. Immunofluorescence

Specimens were frozen in embedding media (Triangle Biomedical, Durham, NC) in liquid nitrogen vapors after being placed in 2 M sucrose for 2 h at 4 °C. Tissues were serial sectioned at 6 μm and mounted onto gelatin–chrome alum-coated slides. Sections were incubated with monoclonal antibodies to Keratin 1 (AE2, ICN, Cosa Mesa, CA), Filaggrin (BTI Inc., Stoughton, MA) Type IV Collagen (Sigma, St. Louis, MO), Type VII Collagen (Sigma, St. Louis, MO), α6 integrin subunit (Chemicon International Inc., Temecula, CA), bromodeoxyuridine (Boehringer Mannheim, Indianapolis, IN) and laminin 5 (GB3, gift of Dr. G. Meneguzzi) and were detected with Alexa 488™-conjugated rabbit anti-mouse IgG (Molecular Probes, Eugene, OR). Fluorescence was visualized using a Nikon OptiPhot microscope. For routine light microscopy, tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and 4 um sections were stained with hematoxylin and eosin.

4.3. Analysis of apoptosis and proliferation

Frozen sections were analyzed for the presence of apoptotic cells by in situ TUNEL assay. This was performed using ISOL (In Situ Oligo Ligation) end-labeling and immunofluorescent detection of apoptotic cells (Boehringer Mannheim, Indianapolis, IN). The percentage of apoptotic basal cells was calculated by counting 1000 nuclei in the basal and layers that were present in eight serial sections found 100 μm apart in the specimen. For proliferation assays, bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO) was added to 3D cultures 8 h prior to harvesting at a final concentration of 10 μM. Proliferation of was measured as the percentage of BrdU positive nuclei in the basal layer (LI = Labeling Index) after counting stained nuclei in five serial sections.

Acknowledgements

We would like to thank Dr. Lina Nguyen, Dr. Zehra Pradhan, Dr. Taly Argov and Marc Mendelsohn for their technical assistance and Laura Bertolotti and Jennifer Landmann for their assistance in the preparation of this manuscript. This work was supported by the U.S. Army Medical Research and Materiel Command under Grant DAMD17-01-1-0688.

References

Alonso, L., Fuchs, E., 2003. Stem cells of the skin epithelium. Proc. Natl. Acad. Sci. U. S. A. 100 (Suppl 1), 11830–11835.

Amano, S., Akutsum, N., Matsunaga, Y., Nishiyama, T., Champliaud, M.F., Burgeson, R.E., Adachi, E., 2001. Importance of balance between extracellular matrix synthesis and degradation in basement membrane formation. Exp. Cell Res. 271, 249–262.

Amano, S., Ogura, Y., Akutsu, N., Matsunaga, Y., Kadoya, K., Adachi, E., Nishiyama, T., 2005. Protective effect of matrix metalloproteinase inhibitors against epidermal basement membrane damage: skin equivalents partially mimic photoageing process. Br. J. Dermatol. 153 (Suppl 2), 37–46.

Andriani, F., Margulis, A., Lin, N., Griffey, S., Garlick, J.A., 2003. Analysis of microenvironmental factors contributing to basement membrane assembly and normalized epidermal phenotype. J. Invest. Dermatol. 120, 923–931.

Berking, C., Herlyn, M., 2001. Human skin reconstruct models: a new application for studies of melanocyte and melanoma biology. Histol. Histopathol. 16, 669–674.

Black, A.F., Bouez, C., Perrier, E., Schlotmann, K., Chapuis, F., Damour, O., 2005. Optimization and characterization of an engineered human skin equivalent. Tissue Eng. 11, 723–733.

Christiano, A.M., Uitto, J., 1996. Molecular complexity of the cutaneous basement membrane zone. Revelations from the paradigms of epidermolysis bullosa. Exp. Dermatol. 5, 1–11.

Dowling, J., Yu, Q.C., Fuchs, E., 1996. Beta4 integrin is required for hemidesmosome formation, cell adhesion and cell survival. J. Cell Biol. 134, 559–572.

Fleischmajer, R., Utani, A., MacDonald, E.D., Perlish, J.S., Pan, T.C., Chu, M.L., Nomizu, M., Ninomiya, Y., Yamada, Y., 1998. Initiation of skin basement membrane formation at the epidermo–dermal interface involves assembly of laminins through binding to cell membrane receptors. J. Cell Sci. 111 (Pt 14), 1929–1940.

Frisch, S.M., Francis, H., 1994. Disruption of epithelial cell–matrix interactions induces apoptosis. J Cell Biol. 124, 619–626.

Frisch, S.M., Ruoslahti, E., 1997. Integrins and anoikis. Curr. Opin. Cell Biol. 9, 701–706.

Fusenig, N.E., 1994. Epithelial–mesenchymal interactions regulate keratinocyte growth and differentiation in vitro. In: Leigh, I., Lane, B., Watt, F. (Eds.), The Keratinocyte Handbook. Cambridge University Press, Cambridge, pp. 71–97.

Jones, P.H., Harper, S., Watt, F.M., 1995. Stem cell patterning and fate in human epidermis. Cell 80, 83–93.

Larjava, H., Salo, T., Haapasalmi, K., Kramer, R.H., Heino, J., 1993. Expression of integrins and basement membrane components by wound keratinocytes. J. Clin. Invest. 92, 1425–1435.

Marinkovich, M.P., Keene, D.R., Rimberg, C.S., Burgeson, R.E., 1993. Cellular origin of the dermal–epidermal basement membrane. Dev. Dyn. 197, 255–267.

Martin, P., 1997. Wound healing- aiming for perfect skin regeneration. Science 276, 75–81.

Singer, A.J., Clark, R.A., 1999. Cutaneous wound healing. N. Engl. J. Med. 341, 738–746.

Szabowski, A., Maas-Szabowski, N., Andrecht, S., Kolbus, A., Schorpp-Kistner, M., Fusenig, N.E., Angel, P., 2000. c-Jun and JunB antagonistically control cytokine-regulated mesenchymal–epidermal interaction in skin. Cell 103, 745–755.

Vailly, J., Pulkkinen, L., Miquel, C., Christiano, A.M., Gerecke, D., Burgeson, R.E., Uitto, J., Ortonne, J.P., Meneguzzi, G., 1995. Identification of a homozygous one-basepair deletion in exon 14 of the lamb3 gene in a patient with Herlitz junctional epidermolysis bullosa and prenatal diagnosis in a family at risk for recurrence. J. Invest. Dermatol. 104, 462–466.

Woodley, D.T., O'Keefe, E.J., Prunieras, M., 1985. Cutaneous wound healing: a model for cell–matrix interactions. J. Am. Acad. Dermatol. 12, 420–433.

Yang, L., Shirakata, Y., Shudou, M., Dai, X., Tokumaru, S., Hirakawa, S., Sayama, K., Hamuro, J., Hashimoto, K., 2006. New skin-equivalent model from de-epithelialized amnion membrane. Cell Tissue Res. 326, 69–77.

Yurchenco, P.D., O'Rear, J.J., 1994. Basement membrane assembly. Methods Enzymol. 245, 489–518.

Zieske, J.D., Mason, V.S., Wasson, M.E., Meunier, S.F., Nolte, C.J., Fukai, N., Olsen, B.R., Parenteau, N.L., 1994. Basement membrane assembly and differentiation of cultured corneal cells: importance of culture environment and endothelial cell interaction. Exp. Cell Res. 214, 621–633.