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Basement Membrane Reconstruction in Human Skin Equivalents Is Regulated by Fibroblasts and/or Exogenously Activated Keratinocytes

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This study was undertaken to examine the role fibroblasts play in the formation of the basement membrane (BM) in human skin equivalents. For this purpose, keratinocytes were seeded on top of fibroblast-free or fibroblast-populated collagen matrix or de-epidermized dermis and cultured in the absence of serum and exogenous growth factors. The expression of various BM components was analyzed on the protein and mRNA level. Irrespective of the presence or absence of fibroblasts, keratin 14, hemidesmosomal proteins plectin, BP230 and BP180, and integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_4$ were expressed but laminin 1 was absent. Only in the presence of fibroblasts or of various growth factors, laminin 5 and laminin 10/11, nidogen, uncein, type IV and type VII collagen were decorating the dermal/epidermal junction. These findings indicate that the attachment of basal keratinocytes to the dermal matrix is most likely mediated by integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$, and not by laminins that bind to integrin $\alpha_6\beta_4$ and that the epithelial–mesenchymal cross-talk plays an important role in synthesis and deposition of various BM components.

Key words: basement membrane/fibroblasts/growth factors/human skin equivalent/keratinocytes/laminins
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The basement membrane (BM) is a specialized structure separating the epidermis from the underlying dermis (Marinkovich *et al*, 1992; Moll and Moll, 1998).

Detailed information on the mechanisms controlling the BM formation is difficult to obtain by *in vivo* studies. *In vitro* studies performed with conventional submerged keratinocyte cultures have markedly contributed to the present knowledge on keratinocyte biology. These cultures are, however, not suitable for investigations aiming to establish the interactive role of epidermal and mesenchymal cells on BM formation, as the spatial tissue organization in these cultures is missing. The *in vivo* situation can be closely approached with organotypic skin cultures. In these cultures, generated by culturing of keratinocytes seeded onto the fibroblast-populated matrix and cultured at the air–liquid (A/L) interface, a reconstructed tissue is formed with features closely mimicking the native counterpart (Bell *et al*, 1981; Boyce *et al*, 1988; Parenteau *et al*, 1991; Ponec *et al*, 1997; Stark *et al*, 1999; El Ghalbzouri *et al*, 2002a, b, 2004). Marinkovich *et al* (1993) have provided important information on BM formation in the presence of growth factors in these cultures. They also showed that type VII and type IV collagen are produced both by keratinocytes and fibroblasts. Recent studies from our laboratory revealed that next to the use of serum-free medium, the number and the functional state of fibroblasts incorporated into the matrix also strongly affects the normalization of the epidermal differentiation program (El Ghalbzouri *et al*, 2002a, b). The information on the role fibroblasts play in regulation of synthesis and deposition of proteins at the dermal–epidermal junction (DEJ) is limited. To gain such information, studies with human skin equivalents (HSE) generated in the presence or absence of fibroblasts is an attractive approach. Previous studies mainly used fibroblast-populated dermal matrices (Breitkreutz *et al*, 1997; Fleischmajer *et al*, 1998; Smola *et al*, 1998) or media supplemented with growth factors (Breitkreutz *et al*, 1997; Smola *et al*, 1998) and studies with organotypic keratinocyte monocultures are scarce (Smola *et al*, 1998). In this study we used two in-house skin models in which epidermis was reconstructed using two different matrices, collagen or de-epidermized dermis (DED). Both skin models were established in serum-free media in the absence of growth factors. As the majority of the studies performed until now used media supplemented with growth factors, the effects of exogenously added growth factors were examined as well. The results presented in this study indicate that plectin, BP230, BP180, and integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_4$ are constitutively produced by keratinocytes, and most importantly that fibroblasts or exogenously added growth factors may contribute for deposition of the BM proteins laminin 5, laminin 10/11, Ladinin-1, uncein, and type IV and type VII collagen.

Abbreviations: ADAM, *a d*isintegrin *a*nd *m*etalloproteinase; BM, basement membrane; DED, de-epidermized dermis; DEJ, dermal–epidermal junction; TACE, TNF-α converting enzyme

Results

Fibroblast-induced modulation of BM and hemidesmosomal proteins To examine the role of fibroblasts on

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hemidesmosomal (HD) and BM formation, HSE were generated either on fibroblast-free (organotypic keratinocyte monocultures) or fibroblast-populated dermal matrices (organotypic keratinocyte-fibroblast co-cultures). As shown in Fig 1, the presence of fibroblasts was crucial for the expression of a great variety of BM proteins in HSE generated on collagen matrices. In the absence of fibroblasts, collagen IV, VII, nidogen (Fig 1*a*), uncein (Fig 1*b*), and laminin 5 and laminin 10/11 (Fig 1*c*) were not detected. The absence of laminin 5 was established with four different antibodies: BM165 (directed against the laminin α_3-chain, also recognizes laminin 6 ($\alpha_3\beta_1\gamma_1$) and laminin 7 ($\alpha_3\beta_2\gamma_1$) which covalently associates with laminin 5 (Marinkovich *et al*, 1992), D4B5 (directed against laminin γ_2 chain), P3E4, and 110+ (data not shown) (Fig 1*c*). In contrast to laminin 5 and laminin 10/11, laminin 1 was not detected both in the keratinocytes monocultures and keratinocyte-fibroblast cocultures (Fig 1*c*). The expression of integrin subunits α_6 and β_4 (Fig 1*b*), both the intracellular (IC) and the extracellular (EC) portions of the BP180, LAD-1 (Fig 1*d*), K14, and the HD proteins BP230 and plectin (Fig 1*e*) was not dependent on the presence of fibroblasts. The expression of various BM components was affected by fibroblasts. In the absence of fibroblasts, K14 expression was confined to the basal cell layer, whereas in their presence the first three suprabasal cell layers were positively stained (Fig 1*e*). In the presence of fibroblasts the expression of BP180 was more pronounced at the DEJ but that of BP180IC not (Fig 1*d*). The increased staining of BP180EC in the presence of fibroblasts can be attributed to the increase of shedded BP180EC that cross-reacts with MoAb NCC-Lu226. In the absence of fibroblasts, the deposition of LAD-1 at the DEJ was marginal (Fig 1*d*) and virtually absent when DED was used as a dermal matrix (Fig 2*a*). In the presence of fibroblasts, LAD-1 decorated the DEJ. As with the collagen matrix, also with DED in the absence of fibroblasts BP180EC (Fig 2*a*); the integrin subunits α_6 and β_4 (Fig 2*b*) decorated the DEJ and uncein (Fig 2*a*) was absent. In contrast to organotypic keratinocyte monocultures generated on collagen, laminin 5 was clearly continuously decorating the DEJ when DED was used (Fig 2*b*). This can be ascribed to the preservation of various BM components during the isolation of this matrix (Prunieras *et al*, 1983; Ponec *et al*, 1988; Andriani *et al*, 2003).

Fibroblasts favor the synthesis of laminin 5 The results obtained with immunohistochemistry were clearly indicative for the important role fibroblasts play in the deposition of various BM proteins. To examine whether the expression of these proteins was regulated at translational or post-translational level, RNA was isolated from keratinocytes and fibroblasts were grown either in organotypic mono- or co-cultures. Laminin 5 consists of three different chains: α_3, β_3, and γ_2. Using RT-PCR, we found that laminin 5 α_3-chain mRNA was not expressed in organotypic keratinocyte monocultures (Fig 3). In contrast to this finding, laminin 5 α_3-chain mRNA expression was clearly detected in fibroblast mono- and keratinocyte-fibroblast co-cultures. At mRNA level, expression of the γ_2-chain was detected in both keratinocytes and fibroblasts; irrespective of the fact that cells were grown in mono- or co-cultures. A comparable expression was found for the β_3-chain, except for organotypic fibroblast-monocultures, where no β_3-mRNA was detected.

The results shown in Fig 3 further indicate that, independently of culture conditions used, nidogen mRNA is expressed in fibroblasts but it is regulated in keratinocytes by fibroblasts. No nidogen mRNA expression was observed in keratinocytes grown in monocultures. In contrast to this, when grown in co-culture with fibroblasts, a weak expression was detected. To assure that this finding is not resulting from the presence of fibroblasts co-isolated with keratinocytes, an experimental setup has been chosen, in which a direct contact between the epidermal and dermal compartments was prevented by placing a filter in between these layers. Also in this case, detectable amounts of nidogen mRNA was found.

To assess whether the differences in the deposition of LAD-1, observed on immunohistochemical level between organotypic mono- and co-cultures, can be ascribed to differences in TACE (TNF-α converting enzyme) expression, one of the enzymes responsible for the cleavage of the BP-180 ectodomain leading to the formation of 120 kDa soluble BP180EC (Franzke *et al*, 2002) has been evaluated as well. We observed that TACE was expressed irrespective of the cells were grown in mono- or co-cultures.

Epidermal growth factor, keratinocyte growth factor, and GM-CSF induce laminin 5 and laminin 10/11 deposition in organotypic keratinocyte monocultures To examine whether the laminin 5 and laminin 10/11 deposition in keratinocyte monocultures can be induced by various growth factors, the culture media were supplemented with EGF (1 and 5 ng per mL), KGF (5 ng per mL) or GM-CSF (0.01 and 1 ng per mL). As shown in Fig 4*A*, the number of viable epidermal cell-layers increased and laminin 5 was expressed along the DEJ. When 0.01 ng per mL of GM-CSF was, however, added to the medium, epidermal improvement was achieved but no expression of the α_3-chain laminin 5 was noticed (Fig 4*Ae*). When a higher GM-CSF concentration was used (0.1 ng per mL), laminin 5 was deposited along the DEJ (Fig 4*Af*). Similar results were also obtained when specimens were stained for laminin 10/11 (data not show).

The observations made with immunohistochemistry were similar to the results obtained on mRNA level (Fig 4*B*). Laminin 5 (α_3-chain) mRNA expression was not observed in growth factor-free organotypic keratinocyte monocultures and in cultures stimulated with 0.01 ng per mL GM-CSF (Fig 4*B*). Stimulation with EGF, KGF or higher GM-CSF concentrations resulted in the induction of the α_3-chain mRNA expression.

Integrin $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ facilitate epidermal–dermal attachment Although laminin 1, laminin 5, laminin 10/11, and collagen type IV and type VII were not synthesized, keratinocytes were able to proliferate and differentiate in organotypic keratinocyte monocultures (Fig 5). This indicates that the attachment of basal keratinocytes to the underlying dermal compartment is not mediated by these proteins but most probably by other proteins (e.g., collagen type I, fibronectin) that bind to $\alpha_1\beta_1$, $\alpha_2\beta_1$, and/or $\alpha_3\beta_1$ integrins. That is, staining with integrin-subunits α_1, α_2, α_3,

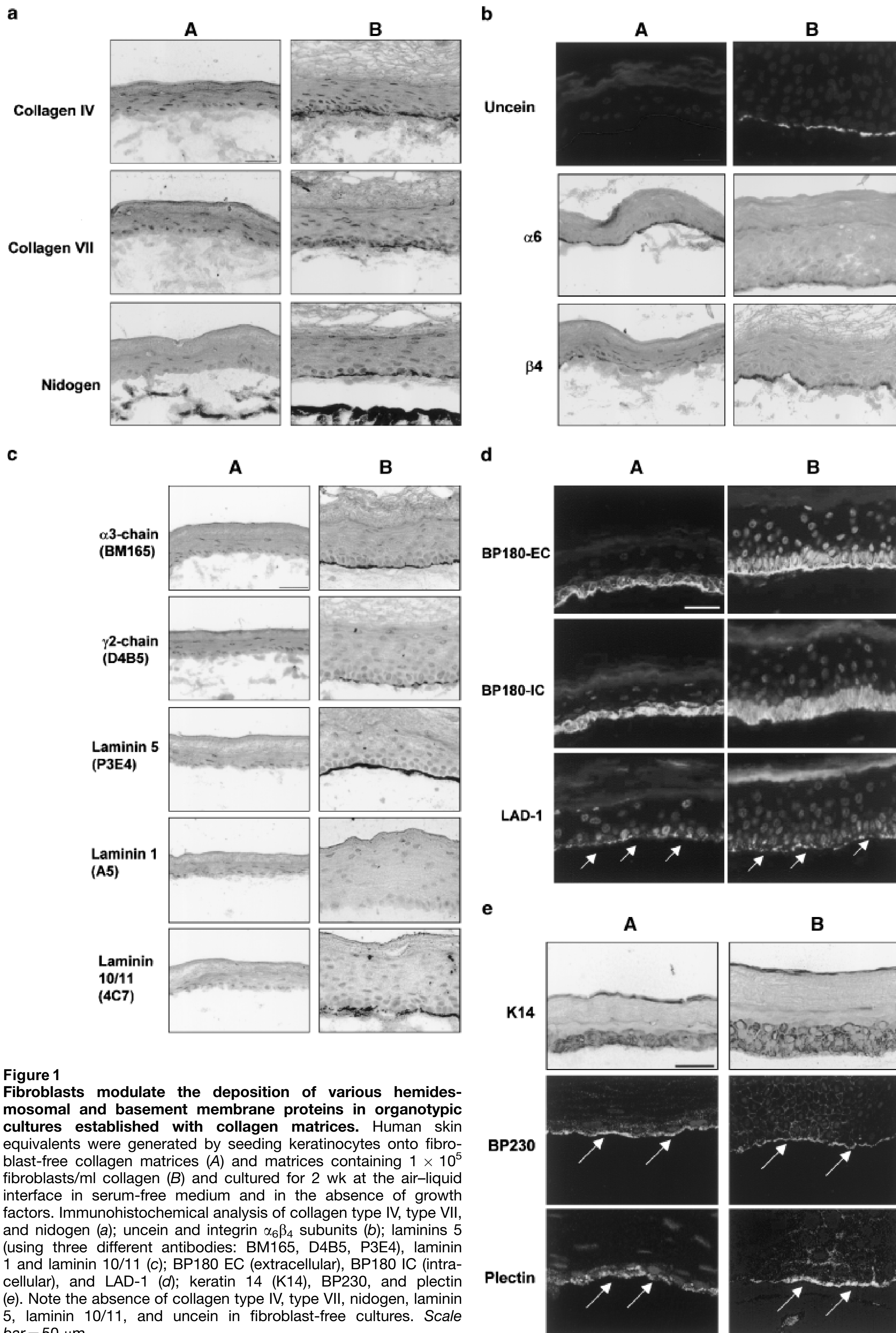

Figure 1
Fibroblasts modulate the deposition of various hemidesmosomal and basement membrane proteins in organotypic cultures established with collagen matrices. Human skin equivalents were generated by seeding keratinocytes onto fibroblast-free collagen matrices (*A*) and matrices containing 1×10^5 fibroblasts/ml collagen (*B*) and cultured for 2 wk at the air–liquid interface in serum-free medium and in the absence of growth factors. Immunohistochemical analysis of collagen type IV, type VII, and nidogen (*a*); uncein and integrin $\alpha_6\beta_4$ subunits (*b*); laminins 5 (using three different antibodies: BM165, D4B5, P3E4), laminin 1 and laminin 10/11 (*c*); BP180 EC (extracellular), BP180 IC (intracellular), and LAD-1 (*d*); keratin 14 (K14), BP230, and plectin (*e*). Note the absence of collagen type IV, type VII, nidogen, laminin 5, laminin 10/11, and uncein in fibroblast-free cultures. *Scale bar* = 50 μm.

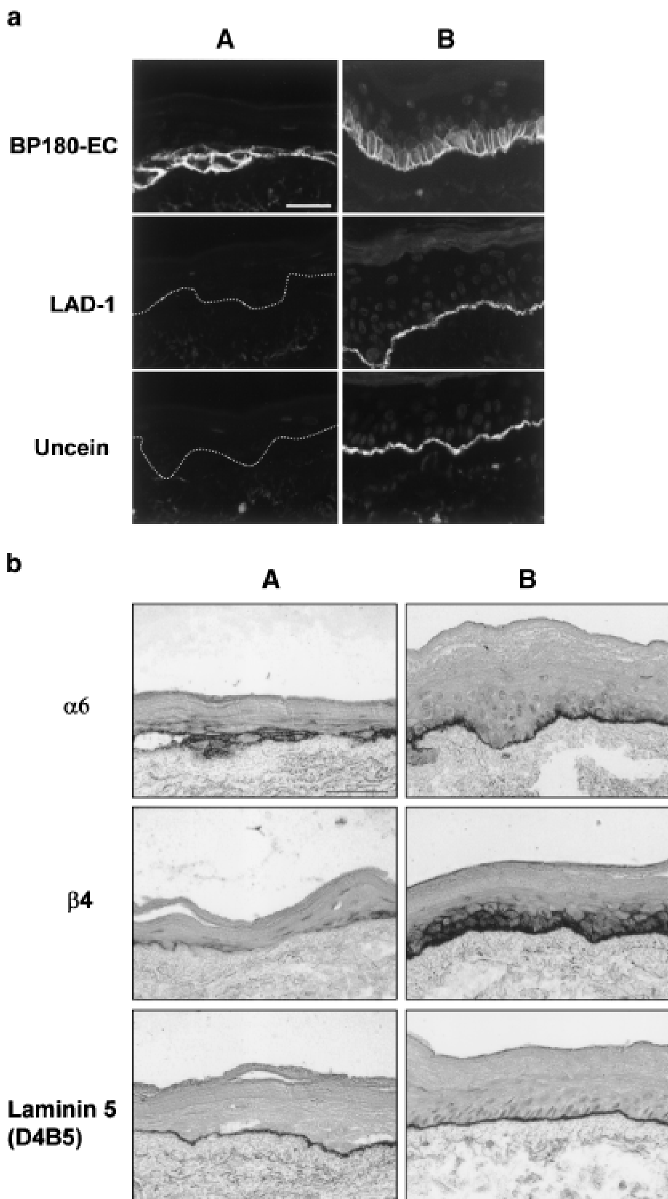

Figure 2
Fibroblasts modulate the deposition of various hemidesmosomal and basement membrane proteins in organotypic cultures established with de-epidermized dermis (DED). Human skin equivalents were generated by seeding keratinocytes onto fibroblast-free DED matrices (*A*) and matrices containing 1×10^5 fibroblasts/cm^2 DED (*B*) and cultured for 2 wk at the air–liquid interface in serum-free medium and the absence of growth factors. Immunohistochemical analysis of BP180 EC (extracellular), LAD-1 and uncein (*a*); integrin $\alpha_6\beta_4$ subunits and laminin 5 (D4B5) (*b*). Note the absence of LAD-1 and uncein but not of laminin 5 and BP180 EC in fibroblast-free cultures. *Scale bar* = 50 μm.

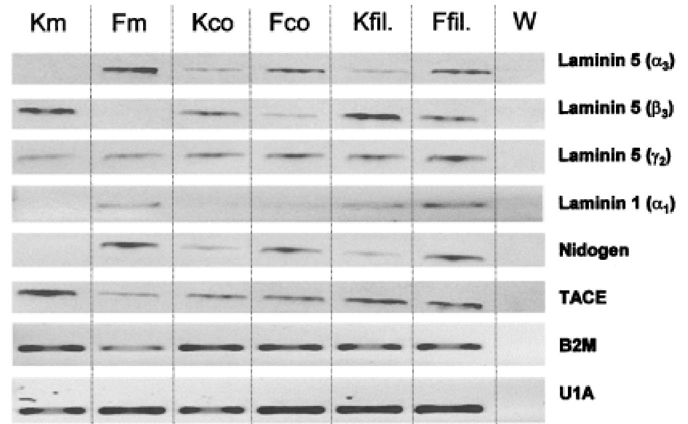

Figure 3
No detectable amounts of the laminin α_3-chain and nidogen mRNA are present in keratinocyte monocultures. Semiquantitative RT-PCR analysis of laminin 5 α_3, β_3, and γ_2 chains, nidogen, and TACE (TNF-α converting enzyme) mRNA expression in keratinocytes and fibroblasts grown either in organotypic monocultures (Km or Fm, respectively), co-cultures (Kco and Fco, respectively) or co-cultures in which keratinocytes were separated from the underlying fibroblast-populated collagen matrix by a filter (Kfil. and Ffil, respectively); lane 1—keratinocytes from monoculture (Km), lane 2—fibroblasts from monoculture (Fm), lane 3—keratinocytes from co-culture (Kco), lane 4—fibroblasts from co-culture (Fco), lane 5—keratinocytes from filter-separated co-cultures (Kfil), lane 6—fibroblasts from filter-separated co-cultures (Ffil), and lane 7—negative control (no cDNA added) (W: water). Small nuclear ribonucleoprotein particle U1A and β_2-microgobulin were used as quantity controls.

and β_1 revealed the deposition of these proteins along the DEJ in organotypic keratinocyte–fibroblast co-cultures and in keratinocyte monocultures. The expression of integrin subunits α_1, α_2, and α_3 was mostly confined to the DEJ, whereas the β_1 subunit stained the a few suprabasal cell layers. In native skin these integrin subunits were expressed at the DEJ and in the suprabasal cell layers (data not shown).

Discussion

In this study, we used a three-dimensional HSE to determine the role fibroblasts play in BM formation. To exclude the potential effect of various endogenous factors, the skin was reconstructed in serum-free media and in the absence of exogenous growth factors. Under these conditions, fibroblasts markedly modulate synthesis of cutaneous BM components. Only in the presence of fibroblasts, uncein, type IV and type VII collagen, and laminin 5 and laminin 10/11 were continuously decorating the BM zone.

The major discrepancy between the results of the present and earlier studies concerns the differences in laminin 5 deposition in keratinocyte monocultures (Zhang and Kramer, 1996; Qin and Kurpakus, 1998; Smola *et al*, 1998; Goldfinger *et al*, 1999; Nguyen *et al*, 2000, Decline and Rousselle, 2001, Andriani *et al*, 2003). Laminin 5 deposition found in these studies can most probably be attributed to the presence of exogenous EGF or serum, as we could establish that both the EGF and serum facilitates the synthesis of laminin 5. Also, other growth factors often used as media supplements for *in vitro* reconstruction of the skin (KGF and GM-CSF) showed similar induction of laminin 5 deposition. In addition, the RT-PCR analyses revealed the presence of β_3 and γ_2 laminin mRNA but the absence of α_3-chain mRNA expression in keratinocytes grown in monocultures. In contrast to keratinocytes, α_3 laminin mRNA was detected in fibroblasts irrespective of whether they were grown as monoculture or co-cultured with keratinocytes. These findings clearly indicate that fibroblasts may contribute to the laminin 5 deposition in human skin reconstructed in the absence of exogenous growth factors.

Maturation of laminin 5 has been reported to require processing of its α_3 and γ_2 chains (Amano *et al*, 2000). This process seems to be favored by the presence of fibroblasts (Elkhal *et al*, 2004). In a previous study, Veitch *et al* (2003) demonstrated that both the mammalian Tolloid-like 2

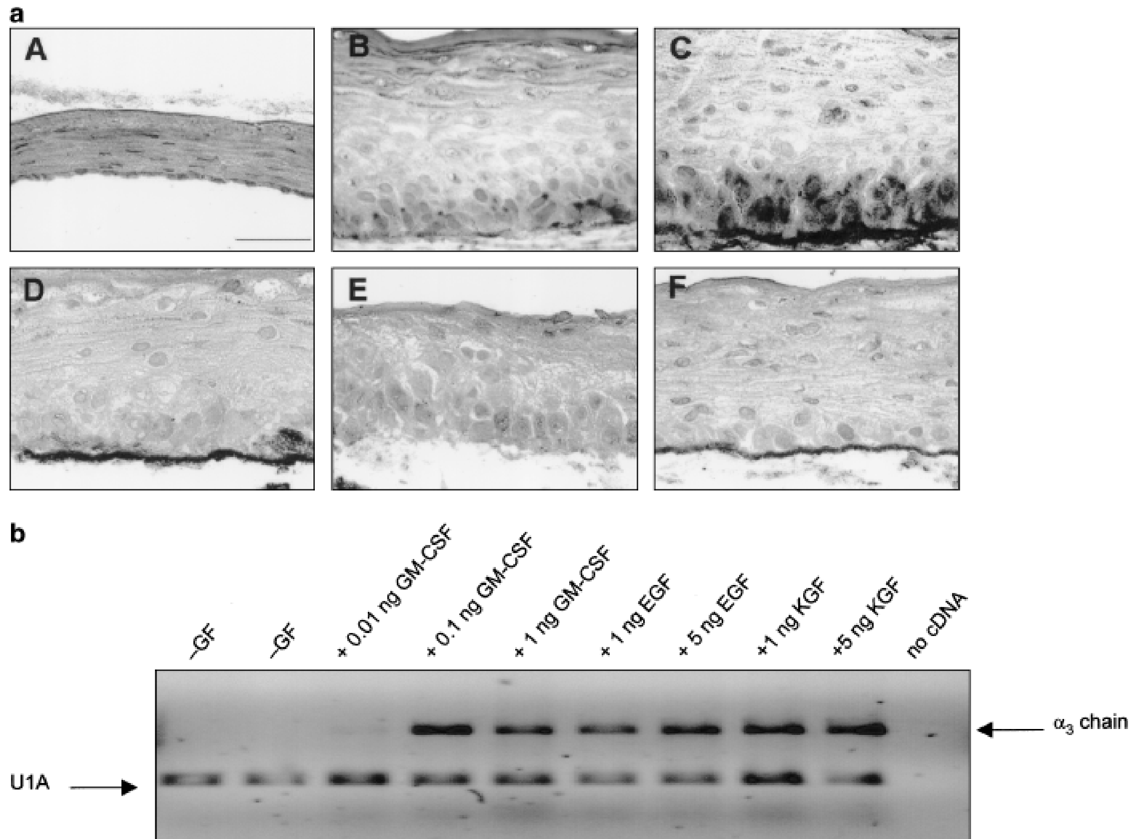

Figure 4
Laminin 5 expression is modulated by growth factors. Human skin equivalents were generated by seeding keratinocytes onto fibroblast-free collagen matrices and cultured for 2 wk at the air–liquid interface in the absence or presence of growth factors. *a*: (*A*), no growth factor; (*B*) 1 ng per mL EGF; (*C*) 5 ng per mL EGF; (*D*) 5 ng per mL KGF; (*E*) 0.01 ng per mL GM-CSF; and (*F*) 1 ng per mL GM-CSF. Immunohistochemical analysis of laminin 5 with BM165 antibody. Note the absence of laminin 5 in cultures stimulated with 0.01 ng per mL of GM-CSF but its deposition at the DEJ in the presence of 1 ng per mL of GM-CSF. *Scale bar* = 50 μm. *b*: GM-CSF, EGF, and KGF induce the laminin α_3-chain mRNA expression. Representative laminin 5 α_3-chain expression in cDNA isolated from keratinocytes grown in monocultures in the absence or presence of growth factors: lanes 1 and 2—no growth factors added, lane 3—GM-CSF 0.01 ng per mL, lane 4—GM-CSF 0.1 ng per mL, lane 5—GM-CSF 1 ng per mL, lane 6—EGF 1 ng per mL, lane 7—EGF 5 ng per mL, lane 8—KGF 1 ng per mL, lane 9—KGF 5 ng per mL, and lane 10—negative control (no cDNA added). Small ribonucleoprotein particle U1A was used as quantity control.

(mTLL-2) and bone morphogenetic protein 1 (BMP-1), two related astactin like enzymes, are involved in specific processing of laminin 5 γ_2 and α_3 chains. In addition, these authors showed that mammalian tolloid is produced by keratinocytes and BMP-1 is mainly produced by fibroblasts. Both enzymes may contribute to the additional laminin 5 processing seen in co-cultures.

In a recent study performed with submerged keratinocyte cultures, the processing of laminin 5 was observed only when keratinocytes were co-cultured with fibroblasts (Tunggal *et al*, 2000). In contrast to these reports, Marinkovich and his group have clearly demonstrated the processing of laminin 5 in keratinocyte monocultures, indicating that fibroblasts are not necessary for this process (Marinkovich *et al*, 1992; Fleischmajer *et al*, 1998; Amano *et al*, 2000). Growth factors, cytokines, and other secreted proteins released during the keratinocyte-fibroblast interaction may contribute to the laminin 5 deposition in organotypic keratinocyte monocultures, as laminin 5 was found to decorate the DEJ in keratinocyte monocultures grown in conditioned media derived from keratinocyte-fibroblast co-cultures (Marinkovich *et al*, 1993; El Ghalbzouri *et al*, 2004b). Similar observations have been made for nidogen deposition. Its expression was reported to be strictly confined to fibroblasts (Fleischmajer *et al*, 1995). Our results contradict these earlier observations, as its expression at the mRNA level could be clearly detected in keratinocytes harvested from keratinocyte-fibroblast co-cultures. These findings indicate that in the absence of exogenous growth factors the epithelial–mesenchymal cross-talk plays an important role in establishing the profile of released factors regulating proliferation and differentiation of keratinocytes and BM formation.

One may speculate that laminin 1, laminin 10, or laminin 11 are the surrogates for laminin 5 in this process. In this study laminin 1 was, however, found to decorate the DEJ in HSE generated with fibroblast-populated matrices in the absence of growth factors only at late time points; its expression was only detected after 3 wk of culture (data not shown). Similar results have been made by Fleischmajer *et al* (2000), who reported the absence of laminin 1 and integrin $\alpha_6\beta_4$ but the presence of laminin 5 and the integrin α_2 and α_3 subunits in 7 d organotypic keratinocyte-fibroblasts co-cultures. In addition, Smola *et al* (1998) reported deposition of laminin 1 only in the presence of fibroblasts after 14 d of air exposure. Both studies were performed with

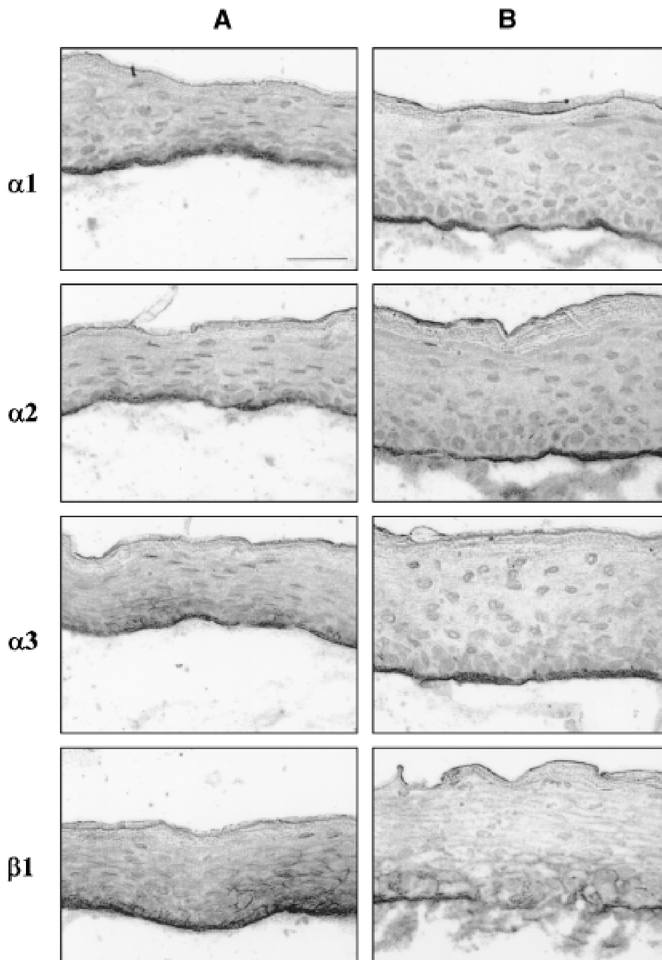

Figure 5
Integrin subunits α_1, α_2, α_3, and β_1 are expressed in keratinocyte monocultures. Human skin equivalents were generated by seeding keratinocytes onto fibroblast-free collagen matrices (*A*) and matrices containing 1×10^5 fibroblasts per mL collagen (*B*) and cultured for 2 wk at the air–liquid interface in serum-free medium and the absence of growth factors. Immunohistochemical analysis of integrin subunits α_1, α_2, α_3, and β_1. *Scale bar* = 50 μm.

culture medium supplemented with fetal calf serum. In the latter study, EGF was added to the medium and might be responsible for the early expression of laminin 1.

Laminin 10 and laminin 11 have been recently reported as components of the BM (Paulsson *et al*, 1987; Miner *et al*, 1997; Kikkawa *et al*, 1998; Aumailley and Roussselle, 1999). Pouliot *et al* (2002) provided strong evidence that laminin 10/11 could function as an alternative adhesive ligand for epidermal keratinocytes with a functional role in promoting proliferation and migration in submerged keratinocyte monocultures grown in the presence of EGF. Also, in this study, laminin 10/11 decorated the DEJ when keratinocyte monocultures were established in the presence of EGF, KGF, or GM-CSF. In the absence of growth factors and serum no laminin 10/11 deposition was observed.

Due to the absence of laminin 1, laminin 5, and laminin 10/11, other integrins than $\alpha_3\beta_1$ and $\alpha_6\beta_4$ could be involved in the attachment, proliferation, and migration of keratinocytes on the cell-free collagen matrix consisting predominantly of type I collagen. The potential adhesion molecules are integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ that have been reported to bind to type I collagen (Kirchhofer *et al*, 1990; Kern *et al*, 1994; Rich *et al*, 1999; Jokinen *et al*, 2004). Indeed, their expression was noticed along the DEJ of organotypic keratinocyte monocultures, indicating the important role of these integrins for skin reconstruction *in vitro*.

Another remarkable finding was the presence of the shed EC of BP180, which comprises the LAD-1 antigen (Hirako *et al*, 1998). Although this process was strongly modulated by fibroblasts, the shed ectodomain was present under all experimental conditions. The cleavage of EC domains of various integral membrane proteins is mediated by the so-called ADAM (*a disintegrin and metalloprotease*) (Turner and Hooper, 1999). Three ADAM metalloproteases, ADAM-9, ADAM-10, and TACE, have been reported to act as "sheddases" through a process called ectodomain shedding. They are synthesized as pro-enzymes and one can speculate that fibroblasts are involved in their activation through, e.g., the modulation of synthesis of metalloproteinases and their inhibitors (Amano *et al*, 2000, Han *et al*, 2001; Zigrino *et al*, 2001). The absence or very low expression of LAD-1 in keratinocyte monocultures is suggestive that either ADAM-9 or ADAM-10 are involved in the cleavage of the BP180 ectodomain, as TACE mRNA was detected in keratinocytes isolated from both monocultures and co-cultures.

The results of this study clearly show that laminins are expressed by keratinocytes and mediate their attachment to the dermal compartment only when fibroblasts or exogenous growth factors are present. In addition to laminins, also the expression of uncein, type IV and type VII collagen, and the shedding of the BP180 ectodomain (LAD-1) seems to be regulated by fibroblasts. The attachment of basal keratinocytes to the dermal matrix is most likely initiated by the integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$, as these molecules were deposited at the DEJ in organotypic keratinocyte monocultures established in the absence of serum and exogenous growth factors.

In conclusion, this study illustrates the importance of epithelial–mesenchymal cell interplay in the regulation of synthesis of BM components. Organotypic cultures representing functional skin equivalents provide an attractive model to elucidate the role different cell types play in the regulation of epidermal morphogenesis and BM formation.

Material and Methods

Cell culture Cultures of normal human keratinocytes (NHK) and fibroblasts were established from human mammary skin, as described earlier (Ponec *et al*, 1997). For experiments, passage two (keratinocytes) or two to five (fibroblasts) were used.

Dermal equivalents

Collagen Hydrated collagen gels were prepared using 4 mg per mL collagen solution isolated from rat-tails, as described in detail elsewhere (El Ghalbzouri *et al*, 2002a).

DED DED was prepared, as described earlier (Ponec *et al*, 1988) and populated with fibroblasts using centrifugal seeding obtaining a final fibroblast density of 1×10^5 cells per cm^2 (El Ghalbzouri *et al*, 2002b).

Reconstruction of epidermis For generation of reconstructed epidermis, secondary keratinocyte cultures were seeded onto fibroblast-free or fibroblast-populated collagen matrices or DED. The cultures were incubated overnight in keratinocyte medium

containing 5% serum, followed by culture in medium supplemented with 1% serum. For collagen matrices, the lifting to the A/L interface was conducted after overnight incubation and serum-free medium was used 1 d after air exposure. DED cultures were lifted to the A/L interface after 3 d. The cultures were grown for 14 d at the A/L interface in serum-free medium. In some experiments the cultures were supplemented with EGF (1 or 5 ng per mL), KGF (1 or 5 ng per mL), or GM-CSF (0.01 or 0.1 ng per mL) (Sigma-Aldrich, Zwijndrecht, the Netherlands). In some experiments, keratinocytes were seeded onto Transwell filters (Corning, Life Sciences, VWR International B.V., Amsterdam, the Netherlands) and cultured for 2 d under submerged conditions. Thereafter, the filters were transferred onto fibroblast–collagen matrices and cultured for 14 d at the A/L interface.

Immunohistochemistry and Immunofluoresence microscopy Harvested cultures were washed in PBS, one part was snap-frozen in liquid nitrogen and another part was fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. After deparaffinization, sections (5 μm) were used for morphological or immunohistochemical analysis of K14, laminin 5, integrin β_4 subunit, and type IV and type VII collagen. Immunohistochemical analysis of other HD and BM proteins was performed using 5 μm frozen sections. The primary antibodies used in this study are listed in Table I. After incubation with primary antibodies, sections were stained with avidin–biotin–peroxidase complex system (streptABcomplex/HRP, DAKO, Glostrup, Denmark), as described earlier (El Ghalbzouri *et al*, 2002a, b). All sections were counterstained with hematoxylin.

For immunofluorescence, the harvested cultures were processed as previously described (Jonkman *et al*, 1992). Digital fluorescence microscopy was performed according to Bruins (Bruins *et al*, 1995), allowing detection of low levels of fluorescence.

Table I. Primary antibodies used for immunohistochemical and immunofluoresence staining of on frozen tissue sections

Antibody designation	Source[a]
K14 (RCK107)	Monisan, Uden, the Netherlands
Laminin 1 (A5)	Abcam, Cambridge, UK
Laminin 5 (P3E4)	Chemicon, Temecula, California
Laminin 5 and 6 (MB165)	Dr M. Aumailley, Cologne, Germany
Laminin 5 (110+)	Dr R. Timpl, Martinsried, Germany
Laminin 10/11 (4C7)	DAKO, Carpinteria, California
Type IV collagen (PHM12)	Chemicon, Temecula, California
Type VII collagen (LH7.2)	Dr I. M. Leigh, London, England
Integrin α_1 (TS2/7)	TCS Cellworks, Buckingham, England
Integrin α_2 (P1E6)	Telios Pharmaceuticals, San Diego, California
Integrin α_3 (P1B5)	Telios Pharmaceuticals, San Diego, California
Integrin α_6 (JEB5)	Dr A. Sonnenberg, Amsterdam, the Netherlands
Integrin β_1 (P4C10)	Life Technologies B.V., Breda, the Netherlands
Integrin β_4 (3E1)	Biomol, Hamburg, Germany
BP180EC (NCC-Lu-226)	Dr S. Hirohashi, Tokyo, Japan
BP180IC (1A8c)	Dr K. Owaribe, Nagoya, Japan
LAD-1 (123)	Dr M. P. Marinkovich, Stanford, California
Uncein (19-DEJ-1)	Dr J. D. Fine, Chapel Hill, North Carolina
BP230 (5E10D)	Dr T. Hashimoto, Fukuoka, Japan
Plectin (P1/P2)	Dr H. Herrmann, Bonn, Germany

[a]Antibodies not purchased from indicated sources were personal gifts from the investigator named.

RT-PCR Total cellular RNA was extracted from homogenized samples using RNeasy RNA isolation kit and Proteinase K solution (Qiagen, Hilden, Germany). cDNA synthesis was performed on 2 μg total RNA after treatment with RQ1 DNase I (Promega, Madison, Wisconsin) using Superscript II reverse transcriptase (Invitrogen, Breda, the Netherlands) and an oligo(dT)$_{12–18}$ primer (Invitrogen).

PCR amplification of cDNA was performed with 6 primer pairs covering a part of the coding region of the laminin 1 α_1, laminin 5 α_3, β_3 and γ_2 subunit, TACE, and nidogen gene (Table II). As controls, the maintenance genes, small nuclear ribonucleoprotein particle U1A, and β_2-microgobulin were used. Each PCR was carried out in a 25 μL reaction volume with the final concentrations of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 25 μM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.5 units Platinum *Taq* DNA Polymerase (Invitrogen), and 0.4 μM of each primer. PCR was performed using a Touchdown PCR-program: denaturing for 30 s at 94°C; annealing for 60 s at 70°C declining 1°C per cycle; extension for 60 s at 72°C; for 15 cycles; denaturing for 30 s at 94°C; annealing for 60 s at 55°C; and extension for 60 s at 72°C. PCR products were separated by electrophoresis on a 12.5% acrylamide gel and stained using PlusOne DNA Silver Staining Kit (Amersham Pharmacia, Uppsala, Sweden).

Table II. Primer combinations

Molecule	Primers	(Mw) bp
Laminin 5 α_3		
Sense Antisense	*5′-GGA AGA CTG GAA GCT TGT GC-3′* *5′-CCT GGG TCT TGG GAA GTG GT-3′*	663
Laminin 5 β_3		
Sense Antisense	*5′-CCA AGC CTG AGA CCT ACT GC-3′* *5′-GGA ATC TCC TGT CCA GGT CC-3′*	194
Laminin 5 γ_2		
Sense Antisense	*5′-AGG CTG TCC AAC GAA ATG GG-3′* *5′-GGA GCT GTG ATC CGT AGA CCA-3′*	240
Laminin 1 α_1		
Sense Antisense	*5′-AAG TGT GAA GAA TGT GAG GAT-3′* *5′-CAC TGA GGA CCA AAG ACA TT-3′*	316
TACE (TNF-α converting enzyme)		
Sense Antisense	*5′-ACA GAT ACA TGG GCA GAG GG-3′* *5′-TGT GGA GAC TTG AGA ATG CG-3′*	159
Nidogen		
Sense Antisense	*5′-TGG GGA AGG TTT ATT ATC GAG-3′* *5′-GAG AAT GTC GTA TGG AAC TGC-3′*	270
U1A		
Sense Antisense	*5′-CAG TAT GCC AAG ACC GAC TCA GA-3′* *5′-GGC CCG GCA TGT GCT GCA TAA-3′*	225
β_2-microglobulin		
Sense Antisense	*5′-CCA GCA GAG AAT GGA AAG TC-3′* *5′-GAT GCT GCT TAC ATG TCT CG-3′*	370

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