

Interactions between Fibroblasts and Keratinocytes in Morphogenesis of Dermal Epidermal Junction in a Model of Reconstructed Skin

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De novo dermal epidermal junction morphogenesis was studied in a skin model including dermal fibroblasts and epidermal keratinocytes. Sequential gene expression, protein deposition, and localization of basement membrane zone components were studied during 15 days. The morphogenesis of dermal epidermal junction is characterized by an implementation of the different components and then a subsequent plateau phase occurring at day 11. Three groups of genes were identified depending on cellular origin and expression profile: 1/genes of fibroblastic origin (*col I alpha1*, *col III alpha1*, *nidogen*, and *fibrillin 1*); 2/genes expressed in fibroblasts and keratinocytes with symmetrical expression pattern between both cell types (*col IV alpha1*, *col VII alpha1*, and *tenascin C*); 3/*laminin beta3* only expressed in keratinocytes. Use of modified organotypic models excluding one cell type revealed a tight interplay between fibroblasts and keratinocytes for synthesis and localization of the components of dermal epidermal junction. Keratinocytes downregulated mRNA and proteins of fibroblastic origin, upregulated col VII in fibroblasts and were absolutely required for dermal-epidermal junction localization of fibroblastic proteins. Fibroblasts downregulated mRNA of keratinocytes and were needed for extracellular secretion and correct localization of type VII collagen and laminin 5.

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INTRODUCTION

Dermal epidermal junction (DEJ) is a complex macromolecular structure which establishes the boundary between two major skin compartments, epidermis and dermis. Apart from its role in cohesion, DEJ also constitutes an exchange zone between epidermal keratinocytes and dermal connective tissue. The major components of DEJ are laminins, collagen types IV and VII, perlecan, and nidogen. DEJ also includes extracellular matrix (ECM) proteins such as tenascin, types I and III collagens and fibrillin-1.

Several *in vivo* and *in vitro* models have been used to assess formation of the DEJ. *In vitro*, monocultures allowed to investigate cell ability to synthesize specific DEJ proteins. *In vivo* studies provided valuable insights into the formation and regeneration of basement membrane (BM) zone. However, the kinetic of expression, deposition and regulation of DEJ proteins, their cellular origin and interactions between keratinocytes and fibroblasts are complex parameters often

difficult to analyze in these systems. Three-dimensional organotypic co-cultures have therefore been considered as suitable models to investigate DEJ morphogenesis. DEJ proteins are deposited in a time-dependent process (Stanley *et al.*, 1982; Compton *et al.*, 1989; Regauer *et al.*, 1990). Assembly of laminins through binding to cell membrane receptors, as well as a scaffold formation of type IV collagen constitute the early phase of the DEJ formation (Fleischmajer *et al.*, 1997, 1998). Both the keratinocytes and fibroblasts contribute to protein deposition to the DEJ (Stanley *et al.*, 1982; O'Keefe *et al.*, 1987; König and Bruckner-Tuderman, 1991; Marinkovich *et al.*, 1992, 1993; Yamane *et al.*, 1996; Fleischmajer *et al.*, 1997). Some components have a specific cellular origin, such as laminin-5, only synthesized by keratinocytes, while others are produced by both cellular compartments. Interaction and cooperation between epithelial and mesenchymal skin cells in DEJ formation were underlined (Marinkovich *et al.*, 1993; Smola *et al.*, 1998). These authors showed a cooperative expression profile of DEJ components in both keratinocytes and fibroblasts, with different kinetics and reciprocal regulatory patterns. However, the real contribution of epithelial and mesenchymal cells in BM assembly has only been partially addressed and coordinated cooperation between both cell types remained unclear.

Furthermore, components of the ECM linked to DEJ, such as types I and III procollagens, fibrillin, and tenascin C, have

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Abbreviations: BM, basement membrane; DEJ, dermal-epidermal junction; ECM, extracellular matrix; NHK, normal human keratinocytes; RS, reconstructed skin

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never been studied in parallel with DEJ proteins. Finally, most of DEJ proteins, such as nidogen, laminins, types IV and VII collagens have never been studied simultaneously.

The aim of this study was to analyse DEJ morphogenesis in a model of human reconstructed skin (RS) *in vitro*, composed of a fully differentiated epidermis built on a fibroblasts populated dermal equivalent (Asselineau *et al.*, 1985, 1989). In such model, there is no pre-existing BM, thus allowing the analysis of *de novo* DEJ morphogenesis. In order to assess the respective role of keratinocytes and fibroblasts, both present in the initial skin model, two modified models were used comprising only one cell type. Using these three systems, the study of the cellular origin of the different markers, their tight deposition and regulation, the respective role of both cell types and their interactions during DEJ morphogenesis was performed. Gene expression quantification and deposition of the proteins associated to DEJ-like types IV and VII collagens, laminin-5, nidogen, types III and type I procollagens, fibrillin-1, and tenascin C were analyzed at different time points. The complementary approaches allowed to show that the DEJ and ECM components did not share the same kinetic of synthesis and origin, fibroblasts and keratinocytes playing a coordinated role, with tight reciprocal interactions, in the synthesis and localization of DEJ components.

RESULTS

De novo DEJ morphogenesis was checked in the complete RS model during the emersion period. In agreement with previous reports (Marinkovich *et al.*, 1993; Fleischmajer *et al.*, 1998; Smola *et al.*, 1998) BM proteins and sub-epidermal ECM proteins were progressively visualized until day 8 (see Figures 4–6). The ultrastructural organization of the BM zone was confirmed by transmission electron microscopy (Figure 1).

Cellular origin and time course regulation of mRNA level of DEJ markers

Using the complete skin model (comprising fibroblasts and keratinocytes), mRNA levels of DEJ and ECM constituents were studied to determine their cellular origin, their kinetic of

expression, and their regulation during the formation of skin BM. mRNA levels were quantified separately in dermal fibroblasts and in epidermal keratinocytes, at day 0, 2, 4, 8, 11, and 15 of emersion phase, using real-time PCR.

Cellular origin of mRNA. Three groups of genes could be obtained depending on their cellular expression.

Genes expressed by fibroblasts (Figure 2a): *Col I α 1*, *col III α 1*, *nidogen*, and *fibrillin-1* genes were mostly expressed by dermal fibroblasts and had a similar profile of expression. Between days 0 and 4, a linear increase in mRNA level was observed, followed by a decrease phase from days 4 to 8 and a stabilization until day 15. In keratinocytes, *col I α 1*, *col III α 1*, and *fibrillin-1* mRNA levels were close to the 0 value. *Nidogen* mRNA was detected at low but significant levels.

Genes expressed by fibroblasts and keratinocytes (Figure 2b): *Col IV α 2*, *Col VII α 1*, and *tenascin C* were expressed by both cell types.

In fibroblasts, for all the subcited markers, mRNA levels increased between days 0 and 4 and reached a maximum at that time point. Then, *col IV α 2* and *col VII α 1* mRNA decreased until day 11 and remained stable until day 15. For *tenascin C*, gene expression was quite stable between days 4 and 11, and then decreased at day 15.

In keratinocytes, mRNA levels of *col IV α 2*, *col VII α 1*, and *tenascin C* decreased between days 0 and 8. After that level of expression was maintained until day 15 for *col IV α 2* and *tenascin C*, whereas *col VII α 1* mRNA levels increased between days 8 and 11 and then remained stable.

Gene expressed by keratinocytes (Figure 2c): Expression of *laminin β 3* gene (specific for the laminin-5 isoform) was restricted to epidermal keratinocytes. *Laminin β 3* mRNA level was maximal at the 1st day of emersion phase and progressively decreased 6.5-fold. from days 0 to 15.

Morphology of the different organotypic skin models

To better understand the respective role of fibroblasts and keratinocytes as well as their interactions, two simplified models were obtained from the complete skin model, allowing to suppress one cell type, as described in Materials and Methods and Figure 3.

Morphological analysis of the three models (Figure 3) revealed that the complete RS model displayed a fully differentiated epidermis laying on a living dermal equivalent. The model lacking keratinocytes was limited to a living dermal equivalent. The aspect of fibroblasts was not modified by the absence of keratinocytes, but their number was 1.25-fold decreased compared to complete model ($P < 0.1$). Finally, the model lacking fibroblasts displayed a normal global morphology of the epidermis, with formation of horny layers. However, the epidermis was thinner compared to complete skin model, but contained a higher number of granular layers.

Influence of mesenchymal and epidermal cells on deposition of DEJ and ECM components

To assess the respective participation and influence of each cell type with regard to deposition of DEJ and ECM proteins,

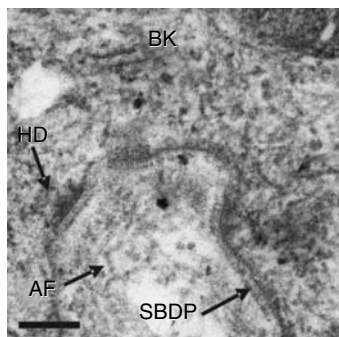


Figure 1. Transmission electron microscopy of complete RS at day 8 of emersion period. BM zone could be visualized with hemidesmosomes (HD) in basal keratinocytes (BK), sub-basal dense plaque (SBDP) and anchoring fibrils (AF). Bar = 0.22 μ m.

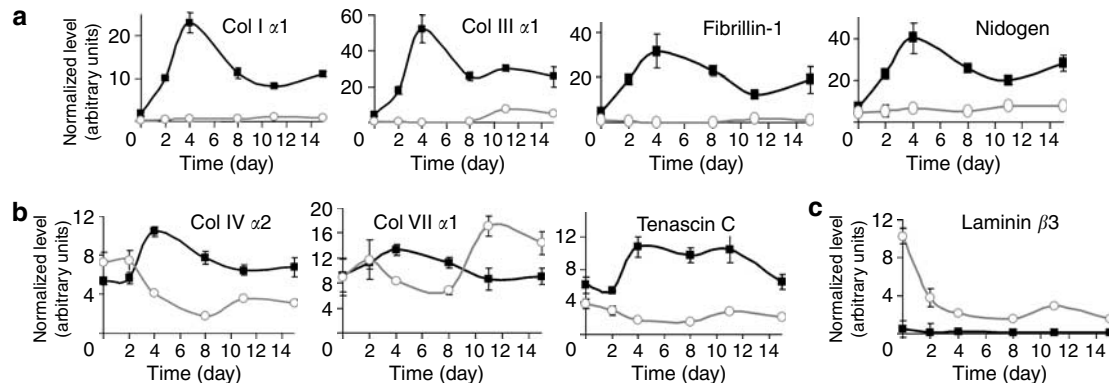


Figure 2. mRNA levels of DEJ components in complete RS. (a): mRNA components of fibroblastic origin; (b): mRNA components originated from keratinocytes and fibroblasts; (c): mRNA of laminin $\beta 3$. mRNA levels were quantified in fibroblasts (\blacksquare) and in keratinocytes (\circ) separately using quantitative reverse transcriptase-polymerase chain reaction, at different time points during the 15 days of emersion culture phase. Each point shows the mean value of normalized mRNA quantity ($n=3$). Data are expressed as mean \pm standard error of mean.

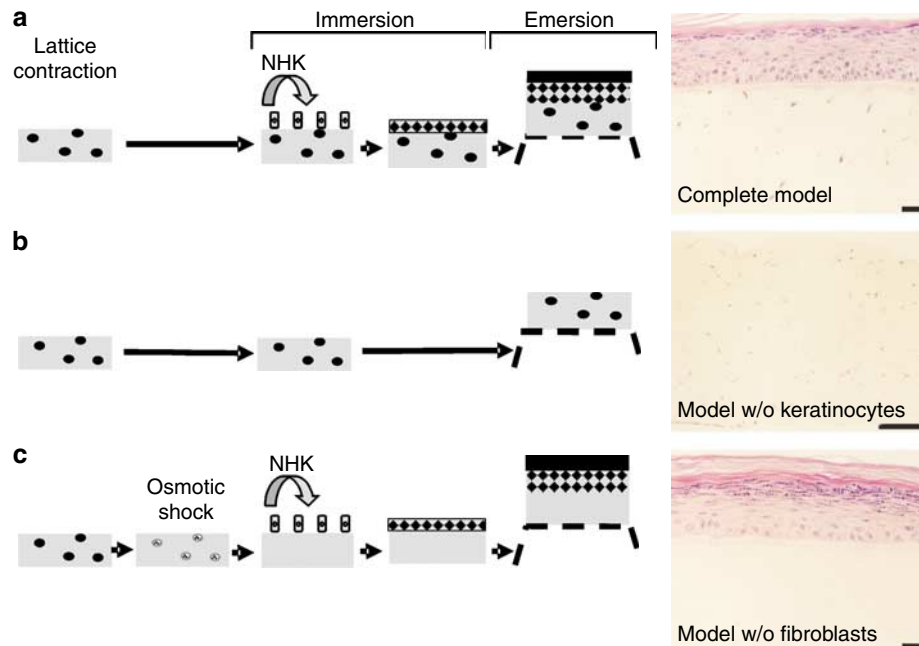


Figure 3. Experimental procedure for generation of the three organotypic models and classical histology at day 8 of the emersion phase. (a): Complete model of RS. Human dermal fibroblasts (\bullet) are embedded in a collagen gel (lattice, \square). After contraction, keratinocytes (Ⓜ) were seeded, cultured for 7 days in submerged conditions, and raised at the air-liquid interface to form a fully differentiated epidermis (\blacksquare) for 8–15 days. (b): Model lacking keratinocytes. All the culture periods were carried out except seeding of keratinocytes. (c): Model lacking fibroblasts. After contraction of the lattice, fibroblasts were killed by osmotic shock. Keratinocytes were then seeded on the dead lattice and cultured as described for (a). Bar = 25 μm .

the expression and distribution of several proteins were studied at day 8 in the models described above.

Markers of fibroblastic origin (Figure 4): Procollagens I and III, fibrillin-1, and nidogen. In the complete skin model, these proteins were mostly detected in the subepidermal zone. In the model devoid of fibroblasts, types I and III procollagens and fibrillin-1 were not detected, while, nidogen was barely detectable at the DEJ and in basal keratinocytes. These results are in concordance with gene

expression data (see Figure 2a). In the model lacking keratinocytes, types I and III procollagens, nidogen, and fibrillin-1 were detected in the dermal equivalent in agreement with mRNA data. Surprising results were found with regard to the intensity and distribution of stainings. In the model lacking keratinocytes, types I and III procollagens stainings were stronger compared to complete skin model, suggesting that the presence of keratinocytes might inhibit the synthesis of these proteins. Furthermore, types I and III procollagens, nidogen, and fibrillin-1 were randomly

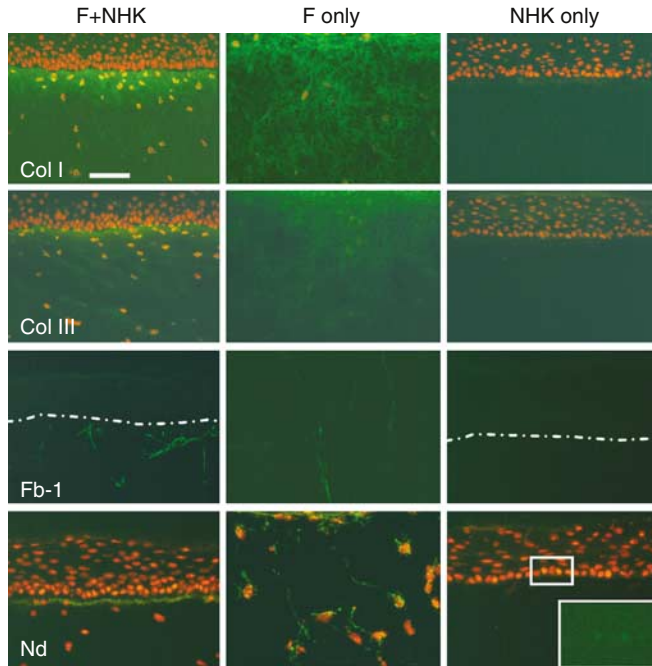


Figure 4. Immunostainings of DEJ proteins of fibroblastic origin, in the three organotypic models. Immunostainings of type I procollagen (col I), type III procollagen (col III), fibrillin-1 (Fb-1), and nidogen (Nd) were performed at day 8 of the emersion phase, in the complete model of RS (F + NHK), in model lacking keratinocytes (F only) and in model lacking fibroblasts (NHK only). Note a preferential subepidermal localization in the complete model, a dermal distribution in the model lacking keratinocytes, and the absence of staining in the model lacking fibroblasts, except a weak staining for nidogen in epidermal basal keratinocytes (inset, bar = 100 μ m). Nuclei were counterstained with propidium iodide (red). Dotted line indicates DEJ. Bar = 50 μ m.

distributed in the whole dermal equivalent and not restricted to the dermal epidermal zone as found in the complete RS model, indicating that keratinocytes are essential for this specific localization.

Markers having both fibroblast and keratinocyte origins (Figure 5): types IV and VII collagens, tenascin-C. In complete RS model, these proteins were mostly detected at the DEJ with a linear staining. Type VII collagen was also detected with a punctiform staining, within basal keratinocytes. Tenascin C was also found in the whole dermal equivalent as well as within dermal fibroblasts. In the model lacking keratinocytes, type IV collagen and tenascin C were localized in the cytoplasm and around dermal fibroblasts. Again, these results indicate that epidermal cells are required for specific DEJ localization of these markers. In this model, type VII collagen immunostaining was negative. However, *col VII α 1* mRNA was significantly detected in fibroblasts from complete skin model (see Figure 2b).

In the model devoid of fibroblasts, type IV collagen and tenascin-C were detected at the DEJ. Type VII collagen stainings was restricted to the cytoplasm of basal keratinocytes, indicating that fibroblasts are essential for their DEJ localization. The intensity of stainings for types IV and VII

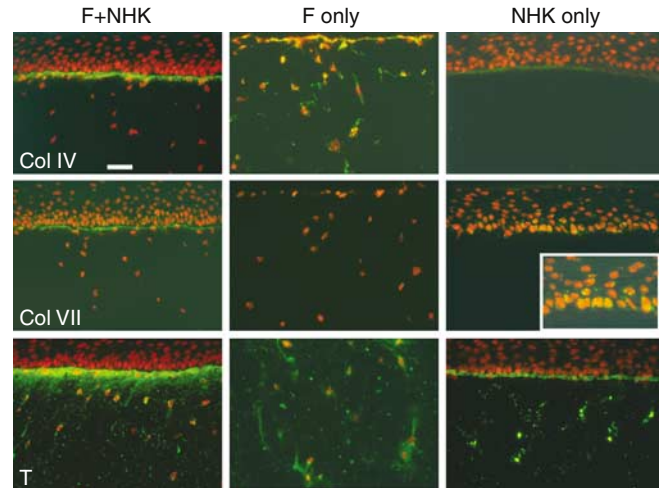


Figure 5. Immunostainings of DEJ proteins having both keratinocyte and fibroblast origin, in the three organotypic models. Immunostainings of type IV collagen (Col IV), type VII collagen (Col VII) and tenascin C (T) were realized at day 8 of the emersion phase, in the complete model of RS (F + NHK), in model lacking keratinocytes (F only) and in model lacking fibroblasts (NHK only). Note the better distribution at the DEJ and the higher staining intensity in the complete model compared to the models lacking one cell type. Note that staining for collagen VII is restricted within basal keratinocytes in the model lacking fibroblasts (inset, bar = 100 μ m). Bar = 50 μ m.

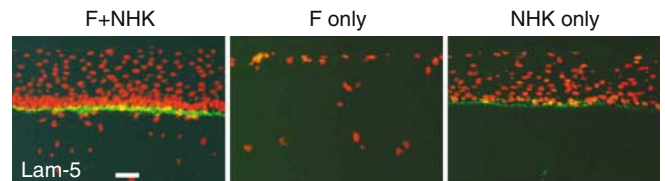


Figure 6. Immunostaining of laminin-5 in the three organotypic models. Immunostainings were performed at day 8 of the emersion phase in the complete model of RS (F + NHK), in model lacking keratinocytes (F only) and in model lacking fibroblasts (NHK only). Bar = 50 μ m.

collagens and tenascin-C was weaker in one-cell type models compared to complete skin model suggesting addition of fibroblasts and keratinocytes protein synthesis.

Marker having a keratinocyte origin (Figure 6): laminin-5. While laminin-5 immunostaining was visualized as a thin layer at the DEJ in the complete RS model, it was not detected in the model lacking keratinocytes. In contrast, in the model lacking fibroblasts, laminin-5 was found at the DEJ and also within basal keratinocytes. The intensity of staining was weaker when only keratinocytes were present.

These results show two types of interaction between fibroblasts and keratinocytes, (i) regarding protein localization at the DEJ zone, epidermal keratinocytes being essential for nidogen, type I, III, and IV collagens and tenascin-C and fibroblasts being necessary for nidogen, type VII collagen and laminin-5 and (ii) regarding the amount of secreted protein.

Influence of skin compartments on gene expression of DEJ and ECM markers

To precise the interactions of both cell types in term of synthesis capacity, analysis of mRNA levels were performed in keratinocytes and fibroblasts obtained from the three organotypic models at day 8 of the emersion phase (Figure 7).

Influence of epidermal compartment on mRNA levels in fibroblasts (Figure 7). mRNA levels of DEJ and ECM markers were quantified in dermal fibroblasts from complete skin model and from model lacking keratinocytes. *Col 1 α 1*, *col III α 1*, *fibrillin-1*, and *nidogen* mRNA levels were significantly higher in dermal fibroblasts cultured in the absence of keratinocytes, with 3.2-, 8.5-, 3.5-, 4.6-, and 4.1-fold induction, respectively. This result strongly suggests that the presence of epidermal keratinocytes decreased gene expression of the subcited markers in dermal fibroblasts at day 8. These data may explain lower protein stainings in the complete skin model compared to the model lacking keratinocytes (Figure 4). In contrast, *col VII α 1* mRNA levels in dermal fibroblasts were significantly reduced (2.8-fold) in the absence of epidermal keratinocytes compared to complete reconstructed human skin. This result also agrees with data at protein level (Figure 5). The levels of *tenascin C* and *col IV α 2* mRNA were found identical in both organotypic models.

Influence of dermal fibroblasts on mRNA levels in epidermal keratinocytes (Figure 7). mRNA levels for DEJ and ECM markers were quantified in keratinocytes obtained from the complete skin model and the model lacking fibroblasts. For markers expressed in keratinocytes such as *nidogen*, *tenascin C*, *col VII α 1*, *col IV α 2*, and *laminin β 3*, the absence of fibroblasts in organotypic model led to a significant increase in mRNA levels (up to 3.7-fold for *nidogen*). These results suggest that fibroblasts may regulate negatively gene expression of these markers in epidermal keratinocytes.

DISCUSSION

The aim of this study was to assess simultaneously several parameters in human DEJ morphogenesis. The sequential analysis permitted us to get insight into kinetic events during DEJ morphogenesis at gene expression, protein deposition, and distribution levels.

Our data bring new clues in time course appearance of DEJ components. The RS model devoid of pre-existing BM was therefore suitable to study kinetic events during DEJ formation leading to its achievement. Two major phases were identified. During a first period, implementation of the different DEJ components was observed as well as important modulation in mRNA levels until days 8–11. During a second phase, protein levels are stabilized reaching a sort of plateau phase, together with stabilization of mRNA level.

Interesting results were found regarding the respective role of mesenchymal and epidermal cells, in the synthesis of DEJ components. The complete RS and the single cell type modified models allowed to assess the respective contribution of keratinocytes and fibroblasts. Using this approach, genes could be gathered in three groups according to their time-dependent expression pattern.

The first group included components of fibroblastic origin which exhibited remarkably similar expression profiles such as types I and III procollagens, fibrillin 1 and nidogen. mRNA for *Col 1 α 1*, *Col III α 1*, and *fibrillin-1* were only found in fibroblasts. This result was confirmed by complete absence of corresponding proteins in the model devoid of fibroblasts, indicating their strict dermal fibroblastic origin. These results are in agreement with previous *in vitro* or *in vivo* studies, except for *fibrillin-1* which had previously been found to be synthesized by monolayered cultured keratinocytes (Haynes *et al.*, 1997; Dzamba *et al.*, 2001). In our experiments and in agreement with previous studies, nidogen was found to be mostly synthesized and secreted by fibroblasts as shown by Q-PCR and immunostainings data (Smola *et al.*, 1998). However, in contrast to the study of Fleischmajer *et al.*, we confirmed very recent data showing that epidermal keratinocytes also represented a slight source of nidogen (Fleischmajer *et al.*, 1995; el Ghalbzouri *et al.*, 2002, 2005). Thanks to the high sensibility of Q-PCR method, low but significant levels of *nidogen* mRNA were detected in epidermal keratinocytes. A mesenchymal contamination of epidermis samples was firmly ruled out since no *col 1 α 1*, *col III α 1*, or *fibrillin-1* mRNA were detected in these samples. Moreover, using the three-dimensional model lacking fibroblasts, we could detect low amounts of nidogen protein.

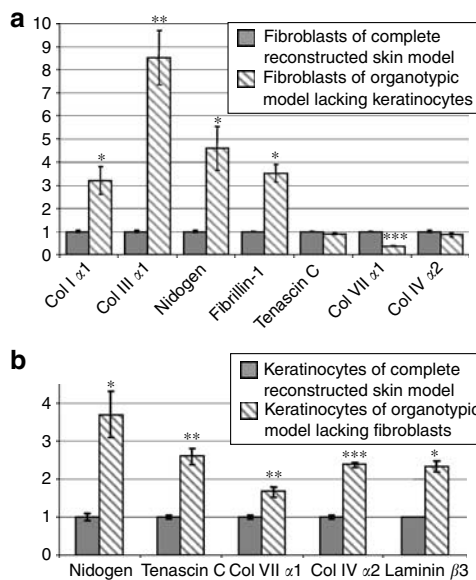


Figure 7. Comparison of mRNA levels of DEJ components at day 8 of the emersion phase. (a): mRNA levels in fibroblasts isolated from complete RS model and model lacking keratinocytes. (b): mRNA levels in keratinocytes isolated from complete RS model and model lacking fibroblasts. Detectable mRNA was quantified by real-time PCR. mRNA quantity in fibroblasts (a) or keratinocytes (b) of complete skin model was taken as control and adjusted to the 1 value. Each histogram bar represents the mean value of the normalized and adjusted mRNA quantity ($n=4$). Data are expressed as mean \pm SEM. * $P<0.05$; ** $P<0.01$; *** $P<0.0001$.

The second group of genes included components synthesized by both fibroblasts and keratinocytes such as tenascin C, type IV, and VII collagens. For type IV collagen and tenascin C, our study reinforced previous data (Rettig *et al.*, 1994; Fleischmajer *et al.*, 1997, 1998; Latijnhouwers *et al.*, 1997; Smola *et al.*, 1998) but also showed that the amount of corresponding proteins was lower in the single cell type models compared to complete skin. This indicated an additive participation of keratinocytes and fibroblasts, and supported recent data suggesting a dual cell types involvement (Lee and Cho, 2005). Type VII collagen represents a particular case. Keratinocytes were currently considered as the main source of type VII collagen (Burgeson, 1993; Chen *et al.*, 2002). However, in our study, type VII collagen mRNA was also detected in fibroblasts, although the protein could not be found when keratinocytes were missing. This supported the fact that keratinocytes could synthesize type VII collagen independently of the presence of fibroblasts. In contrast, fibroblasts seemed to require epidermal cells to produce collagen VII since the presence of keratinocytes led to increased *col VII α1* mRNA levels in fibroblasts. It suggested that fibroblasts may be able to synthesize type VII collagen in the presence of keratinocytes. This hypothesis could be tested in a RS including keratinocytes deficient in type VII collagen and normal fibroblasts. The last category was represented by laminin-5, which is restrictively produced by keratinocytes, (Rousselle *et al.*, 1991; Fleischmajer *et al.*, 1998; Smola *et al.*, 1998; Amano *et al.*, 2001).

Other major interactions between keratinocytes and fibroblasts occurred regarding the influence on gene expression

and protein synthesis of DEJ components. A tightly regulated epithelial-mesenchymal interplay in time is evidenced by results obtained for genes expressed at similar levels in keratinocytes and fibroblasts (*col VII α1*, *col IV α2*, *tenascin*). Their expression profiles in epidermis and in dermis from days 0 to 8 was symmetrical to a horizontal axis, indicating a reciprocal time-dependent regulation of keratinocytes and fibroblasts in terms of gene expression, already described for *col IV α2* (Smola *et al.*, 1998).

Secondly, keratinocytes downregulated mRNA level of *col I α1*, *col III α1*, *nidogen*, and *fibrillin-1* in fibroblasts, leading to lower amounts of types I and III procollagens in complete model. This supported results described for *nidogen*, and suggested a negative feedback loop (Fleischmajer *et al.*, 1995). In parallel, fibroblasts downregulated mRNA levels of *col IV α2*, *tenascin C*, *laminin β3*, *nidogen*, and *col VII α1* in keratinocytes. These cell type interacting effects could be mediated by secreted soluble factors which could diffuse through BM (Kondo *et al.*, 1997). Several cytokines and growth factors have been shown to modulate expression of various collagen genes but also nidogen, fibrillin-1 or laminin-5 (Kurata and Hata, 1991; König and Bruckner-Tuderman, 1992; Mauviel *et al.*, 1994; Korang *et al.*, 1995; Talts *et al.*, 1995; Neubauer *et al.*, 1999). Integrins, such as $\alpha2\beta1$ may also be involved in these processes (Riikonen *et al.*, 1995).

Moreover, our study indicates that the presence of both keratinocytes and fibroblasts is crucial for an optimal localization of DEJ components (Figure 8c). Keratinocytes were absolutely required for the preferential localization at

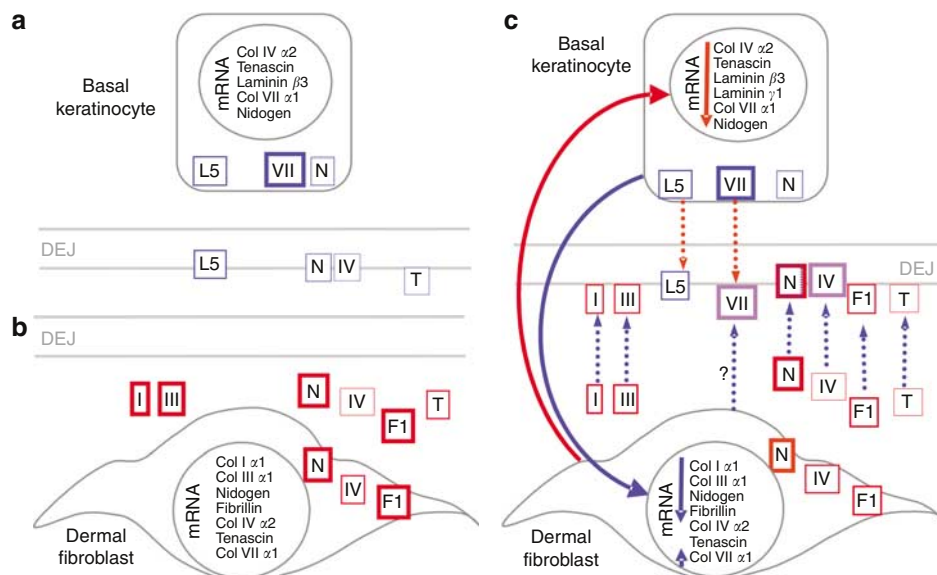


Figure 8. Diagram representation of respective role and influences of fibroblasts and keratinocytes during DEJ morphogenesis. (a): Synthesis and deposition of BM components by keratinocytes in the absence of fibroblasts. (b): Synthesis and deposition of BM components by fibroblasts in the absence of keratinocytes. (c): Interactions between keratinocytes and fibroblasts. $\cdots\blacktriangleright$ Influence of cell types interactions on proteins localization. In blue, influence of keratinocyte, in red influence of fibroblast. \rightarrow Influences of cell type interactions on gene expression. In blue, by keratinocyte on mRNA levels in fibroblasts, in red by fibroblasts on mRNA levels in keratinocytes. \uparrow mRNA increase. \downarrow mRNA decrease. X: proteins synthesized by keratinocyte (blue rectangle), by fibroblast (red rectangle), by both (purple rectangle). Width of rectangle indicates level of synthesized protein. IV: type IV collagen; L5: laminin-5; N: nidogen; VII: type VII collagen; I: type I procollagen; III: type III procollagen; T: tenascin C; F1: fibrillin-1.

DEJ of all proteins synthesized by fibroblasts. On the other hand, the presence of fibroblasts was required for correct deposition at the DEJ of type VII collagen and laminin-5. Indeed, in the absence of mesenchymal cells, proteins were completely or partially retained within keratinocytes (Figure 8a).

Several proteins produced by keratinocytes such as integrins, type VII collagen, laminin-5, or other anchoring proteins may be implicated in correct DEJ components localization. This hypothesis is supported by different aspects. First, structural organization of the BM zone is thought to result from a self assembly process, knowing that several components exhibit mutual physical interactions *in vitro* (Aumailley *et al.*, 1989). Second, in three-dimensional *in vitro* skin models, the presence of pre-existing BM proteins provides an important permissive clue for the rapid formation and maturation of complete BM (Ralston *et al.*, 1999; Andriani *et al.*, 2003). Third, laminin-5 has the potential to accelerate the formation of BM in cultured skin equivalents and *in vivo* (Tsunenaga *et al.*, 1998; Nishiyama *et al.*, 2000). Other actors could be implicated in correct DEJ component localization, as recently revealed for PRELP protein (Bengtsson *et al.*, 2002).

In conclusion, cellular origin and sequential expression of DEJ components together with participation and interactions between epidermal keratinocytes and fibroblasts are key aspects of DEJ morphogenesis process.

MATERIALS AND METHODS

Keratinocyte and fibroblast cultures

Epidermal normal human keratinocytes (NHK) were isolated from breast skin obtained from plastic surgery procedures with volunteer informed consent. Cells were cultured as described by Rheinwald and Green (1975) on a feeder layer of Swiss 3T3 fibroblasts. Human dermal fibroblasts were isolated after spreading from mammary skin explants.

Organotypic skin models

Complete model. Complete model of RS *in vitro* comprising dermal fibroblasts embedded in collagen gel and epidermal keratinocytes was performed as described (Asselineau *et al.*, 1985). Magnesium L-ascorbyl-2-phosphate (AsPM, Showa Denko K.K., Tokyo, Japan, 2.8×10^{-4} M) was added during the last medium change of immersion phase, and then at each medium change during the emerged culture period. Experiments were performed in the dark. The culture was kept 8–15 days at the air-liquid interface.

Model without keratinocyte. The procedure was exactly the same than that for complete model except that medium without keratinocyte was seeded on the dermal equivalent.

Model without fibroblast. After the contraction of the dermal equivalent, the culture medium was replaced by sterile distilled water, changed three times during the day, and left overnight at 37°C. This procedure induced the lysis of fibroblasts by an osmotic shock (Coulomb *et al.*, 1989). After seeding human epidermal keratinocytes on this acellular dermal equivalent, culture conditions followed rigorously the standard protocol.

Transmission electron microscopy

Samples were fixed for 4 hours in 2.5% glutaraldehyde, then washed in cacodylate buffer, and postfixed with 1% osmium tetroxide. Ultra-thin sections were counterstained with uranyl acetate and lead citrate and observed with a LEO electron microscope.

Classical histology and immunostainings

They were performed as described by Bernerd and Asselineau (1997).

Mouse monoclonal antibodies were against: type IV collagen (CIV22, Dako, Denmark, 1:10); type VII collagen (LH7.2, Chemicon Inter, Temecula, CA, 1:200); tenascin C (TN2, Novocastra Lab, Newcastle Upon Tyne, UK, 1:50). Rabbit polyclonal antibody was against type III procollagen (Chemicon Inter, 1:100). Rat monoclonal antibody was against type I procollagen (M-58, Chemicon Inter, 1:100); laminin-5 (GB3, gift from Dr G. Meneguzzi, INSERM U385, Nice, France, 1:1). FITC-conjugate rabbit anti-mouse immunoglobulins (1:80) or FITC-conjugate swine anti-rabbit immunoglobulins (1:40) or FITC-conjugate rabbit anti-rat immunoglobulins (1:200), Dako, Denmark, were used as second antibodies. Nuclear counterstaining using propidium iodide was carried out routinely.

Total RNA extraction

For complete model, RS was rinsed in phosphate-buffered saline Dulbecco's without calcium and magnesium (Gibco BRL, Cergy Pontoise, France). Epidermis was peeled off from dermal equivalent using fine forceps. Immediately after collection, epidermis and dermal equivalent were immersed separately in lysis buffer (Rneasy mini-kit, Qiagen, Courtaboeuf, France). Epidermis disruption was performed by vortexing. Disruption of dermal equivalent was performed using a 5 mm stainless-steel bead and a Mixer Mill MM300 (Retsch, Germany) for 2 minutes at 25 Hz.

For the model without keratinocytes, dermal equivalent was rinsed in phosphate-buffered saline Dulbecco's without calcium and magnesium (Gibco BRL) and disrupted as described above.

For the model without fibroblasts, the procedure was only carried out for epidermis as described.

Tissue lysates were homogenized using QIASredder columns (Qiagen) and total RNA was obtained according to the manufacturer's instructions using Rneasy mini-kit (Qiagen). Dnase I treatment (27 U, 15 minutes) of total RNA was performed directly on the spin columns to eliminate genomic contamination of RNA samples.

Quantitative reverse transcriptase-PCR

1 µg of total RNA was used for first strand cDNA synthesis using an Advantage RT-for-PCR kit (Clontech, Saint Quentin en Yvelines, France), according to the manufacturer's instructions. Quantitative PCR was performed using the LightCycler and the LightCycler-FastStart DNA Master Sybr Green kit (Roche Diagnostics, Meylan, France). The amplification program consisted of 1 cycle of 95°C with 10 minutes hold (hot start), followed by a primer-specific number of amplification cycles of 95°C with 15-seconds hold, specified annealing temperature with 10-seconds hold, 72°C with an amplicon-depending time and primer-specific acquisition temperature with 2-second hold. Specific parameters of primer sets are given in primer list (Supplementary material). Specificity of amplification was checked by melting curve analysis. A relative standard curve constructed with serial dilutions of cDNA was performed for each

gene and at each run. *Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)*, *ribosomal protein L13a (RPL13A)* and *beta-2-microglobulin (B2M)* mRNA were quantified with the LightCycler in each sample and used for normalization using geNorm application (Vandesompele et al., 2002; Savli et al., 2003).

Statistical analysis

Means of mRNA quantity were compared using two-tailed Student's *t*-test ($P < 0.05$).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Primer list: primers and conditions for quantitative reverse transcriptase-polymerase chain reaction.

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