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Development of human skin equivalents mimicking skin aging : contrast between papillary and reticular fibroblasts as a lead

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Chapter 7

Differential effect of extracellular matrix derived from papillary and reticular fibroblasts on epidermal development *in vitro*

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Abstract

Papillary and reticular fibroblasts have different effects on keratinocyte proliferation and differentiation. The aim of this study was to investigate whether these effects are caused by differential secretion of soluble factors or by differential generation of extracellular matrix from papillary and reticular fibroblasts.

To study the effect of soluble factors, keratinocyte monolayer cultures were grown in papillary or reticular fibroblast-conditioned medium. To study the effect of extracellular matrix, keratinocytes were grown on papillary or reticular-derived matrix.

Conditioned medium from papillary or reticular fibroblasts did not differentially affect keratinocyte viability or epidermal development. However, keratinocyte viability was increased when grown on matrix derived from papillary, compared with reticular, fibroblasts. In addition, the longevity of the epidermis was increased when cultured on papillary fibroblast-derived matrix skin equivalents compared with reticular-derived matrix skin equivalents.

The findings indicate that the matrix secreted by papillary and reticular fibroblasts is the main causal factor to account for the differences in keratinocyte growth and viability observed in our study. Differences in response to soluble factors between both populations were less significant. Matrix components specific to the papillary dermis may account for the preferential growth of keratinocytes on papillary dermis.

Introduction

The dermis can be divided into two morphologically different layers: the papillary and reticular dermis (1). Fibroblasts isolated from these layers appear and behave differently in monolayer culture (2-6). In addition, reticular and papillary fibroblasts have been shown to behave differently in human skin equivalents (HSEs) (7-9). Results from these studies indicate that papillary fibroblasts are better at supporting keratinocyte proliferation and differentiation. This seems logical, since the papillary fibroblasts are located closer to the epidermis *in vivo*.

Currently, it is not clear what causes the different effects of papillary and reticular fibroblasts on the epidermis. Both fibroblast subtypes release different amounts of soluble factors; for example, the secretion of keratinocyte growth factor is increased in reticular fibroblasts (8, 10). It is possible that the spectrum of soluble factors secreted by papillary fibroblasts are more effective in stimulating keratinocyte proliferation and differentiation than that of reticular fibroblasts. Another possibility is that the papillary-generated dermis is a better environment and provides a richer basis for keratinocytes. The papillary microenvironment consists of (soluble) factors secreted by fibroblasts and the extracellular matrix (ECM) generated by fibroblasts. The ECM generated by papillary fibroblasts is different from the ECM generated by reticular fibroblasts, both *in vivo* and *in vitro* in HSEs (5, 7). Most strikingly, ECM generated by papillary fibroblasts is less dense than ECM generated by reticular fibroblasts. Several constituents of the matrix are differentially expressed between reticular and papillary fibroblasts, perhaps most notably proteoglycan decorin (5, 11).

In the present study, two types of HSEs were used: epidermal equivalents (the Leiden epidermal model; LEM) and fibroblast derived matrix (FDM) equivalents. LEMs consist only of an epidermal part, but, in contrast to keratinocyte monolayer cultures, differentiate and form a complete epidermis, including the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum. FDM equivalents are full-thickness HSEs that consist of both a dermal and an epidermal compartment. For the generation of FDM, fibroblasts are stimulated to produce ECM. As such, keratinocytes will grow on the ECM secreted by the fibroblasts in the dermal part of the HSE (12).

The aim of this study was to elucidate the cause of the different effects of papillary and reticular fibroblasts on keratinocyte proliferation and differentiation; are these caused by differential secretion of soluble factors, differential ECM composition, or both? Therefore, we investigated keratinocyte viability and development in response to conditioned medium of papillary and reticular fibroblasts. This was performed in both keratinocyte monolayer cultures and LEMs. Furthermore, we investigated the growth of keratinocyte monolayer cultures on ECM secreted by both fibroblast subpopulations and investigated

the development and longevity of the epidermis in FDM HSEs generated with papillary or reticular fibroblasts.

Materials and Methods

Isolation and cell culture

Isolation of reticular and papillary fibroblasts was performed as described earlier (13). First, skin obtained from plastic surgery was cleaned thoroughly. Then the skin was dermatomed at 300 μm to obtain the epidermis and papillary dermis. For the reticular dermis, the deep skin was removed by dermatome and scalpel, and the upper part was discarded. This deep dermis was then used for isolation. Fibroblasts were isolated by treatment with collagenase (Invitrogen, Breda, The Netherlands) and dispase (Roche Diagnostics, Almere, The Netherlands), mixed in a 3:1 ratio for 2 hours at 37°C.

Fibroblasts were cultured in DMEM medium (Gibco/Invitrogen, Breda, The Netherlands) containing 5% foetal calf serum (FCS, HyClone, Thermo Scientific, Etten-Leur) and 1% penicillin-streptomycin (Invitrogen). They were kept at 37°C at 5% CO₂. Both reticular and papillary fibroblasts were isolated from all donors; consequently, all analyses were performed on a pairwise basis. When reaching confluency, fibroblasts were passaged at a 1:3 ratio. Fibroblasts used for experiments were at passage 3-6. All experiments were performed with tissue from at least three different, middle-aged, female donors.

Normal human epidermal keratinocytes were isolated from skin obtained from plastic surgery. First, the entire skin was treated with dispase II to separate the dermis from the epidermis. Subsequently, the epidermis was incubated in trypsin to isolate the keratinocytes. After filtering with a cell strainer (70- μm pore size), the keratinocytes were seeded and cultured at 37°C in 7.3% CO₂. Keratinocyte medium consisted of DMEM and Ham's F12 medium (3:1), supplemented with 5% FCS, 0.5 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin, 100 U/ml penicillin (Invitrogen), and 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen).

All constituents of media were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), unless otherwise mentioned.

Patient consent was not acquired because the use of surplus material obtained in accordance with the Dutch Law on Medical Treatment Agreement does not require patient consent.

Conditioned medium

To generate fibroblast-conditioned medium for monolayer keratinocytes, papillary or reticular fibroblasts were cultured seven days in fibroblast medium, as described above.

The medium was then collected and frozen until use. Conditioned medium was then combined with Ham's F12 (3:1) and supplemented with 1.25% FCS, 0.5 μ M hydrocortisone, 1 μ M isoproterenol, 0.1 μ M insulin, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

To generate fibroblast-conditioned medium for LEMs, papillary or reticular fibroblasts were cultured for seven days in serum-free HSE medium. HSE medium consisted of DMEM and Ham's F12 (3:1), supplemented with 0.5 μ M hydrocortisone, 1 μ M isoproterenol, 0.1 μ M insulin, 100 U/ml penicillin and 100 μ g/ml streptomycin, 53 μ M selenious acid, 10 mM L-serine, 10 μ M L-carnitine, 1 μ M dL- α -tocopherol-acetate, and 250 μ M ascorbic acid phosphate.

Leiden epidermal models

LEMes were generated as described earlier (14). In short, 200,000 keratinocytes were seeded in 12-well filter inserts (0.4- μ m pore size Transwell inserts; Corning Incorporated, Schiphol-Rijk, The Netherlands). The cells were cultured for four days in Dermalife K medium (CellSystems, Troisdorf, Germany). Then, the keratinocytes were air-exposed and cultured in conditioned medium, as described above, with an additional supplement. The supplement consisted of 24 μ M bovine serum albumin, 25 μ M palmitic acid, 30 μ M linoleic acid, and 7 μ M arachidonic acid, and was added to the medium immediately before addition to the LEMes. After ten days of air-exposed culture, the LEMes were harvested for analysis.

Cell viability assay

Cell viability was measured by a WST-1 assay (Roche). The WST-1 reagent was added at 1:10 to the cell culture medium. After incubation for 2 hours, absorption was measured at 465 nm and referenced at 650 nm.

Generation of ECM substrates

For the generation of ECM substrates, to test with keratinocytes, 50,000 papillary or reticular fibroblasts were seeded, per well, in 12-well plates. These were then cultured for seven days in standard fibroblast medium with 50 μ M ascorbic acid phosphate to stimulate ECM generation. The fibroblasts were then killed by exposing them to UVB (TL-12 UV source, Philips, Eindhoven, The Netherlands) at 450 mJ/cm². Keratinocytes were seeded only if no fibroblast viability was detected, visually and by WST-1 assay, 72 hours after UV irradiation.

After removal of fibroblasts, 35,000 keratinocytes were seeded onto each ECM substrate and left to attach for 15 minutes. After rinsing, the keratinocytes were cultured in

standard keratinocyte medium, as described above for 10 days. Keratinocyte viability was measured by WST-1.

Generation of human skin equivalents

Human fibroblast-derived matrix (FDM) equivalents were generated as described earlier (12). Briefly, 200,000 fibroblasts were seeded into six-well filter inserts (0.4- μ m pore size Transwell inserts, diameter: 24 mm, Corning) and cultured submerged for three weeks using CNT-05 medium (CELLnTEC, Huissen, The Netherlands), supplemented with 50 μ M ascorbic acid.

After generation of the FDM dermal equivalents, 500,000 keratinocytes were seeded on top using a stainless-steel ring (diameter: 10 mm). This approach allowed us quantify keratinocyte outgrowth. Cultures were incubated overnight in keratinocyte medium, as described above. After, the HSEs were cultured for two days in keratinocyte medium with 1% FCS, supplemented with 53 μ M selenious acid, 10 mM L-serine, 10 μ M L-carnitine, 1 μ M dL- α -tocopherol-acetate, 250 μ M ascorbic acid phosphate, 24 μ M bovine serum albumin, and a lipid supplement containing 25 μ M palmitic acid, 15 μ M linoleic acid, and 7 μ M arachidonic acid (Sigma-Aldrich). After another two days, the cultures were air-exposed and cultured in supplemented keratinocyte medium, as described above, except that FCS was omitted and the concentration of linoleic acid was increased to 30 μ M. Medium was refreshed twice a week. After 21, 49 and 70 days of air-exposed culture, HSEs were harvested for analysis.

Immediately before the equivalents were processed for further analysis, macroscopic pictures were taken. These were used to measure the growth area of the keratinocytes using imaging software. The percentage was calculated by dividing the surface area of the epidermis by the total surface area of the HSE.

Western blot

For each sample, 5 (for β -actin detection) or 10 μ g (for collagen type VI α 2 and tenascin c detection) of protein was added to loading buffer, heated to 90°C for 5 minutes, and loaded on a Mini protean TGX gel (Bio-Rad, Veenendaal, The Netherlands). Proteins were blotted on a PVDF membrane (Bio-Rad). Blocking was performed with 10% dried milk (Campina, The Netherlands) in PBS-T (0.1% Tween). Primary antibodies were incubated overnight at 4°C. Afterwards, membranes were incubated with stabilized HRP-conjugated anti-rabbit (Thermo Scientific/Pierce; dilution: 1:1500). For detection of the bands, the Supersignal West Femto ECL system (Thermo Scientific / Pierce, Etten-Leur, The Netherlands) was applied to the membrane. Bands were visualized using Bio-Rad Chemidoc MP. The intensities of the bands were measured using the ImageJ software.

The relative intensity was calculated as the intensity of collagen type VI $\alpha 2$ or tenascin c/intensity of β -actin.

Immunohistochemistry

HSEs and *ex vivo* skin tissue were processed and snap-frozen in liquid nitrogen or fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Sections were cut (at 5 μ m) and rehydrated in xylene and ethanol. For cryosections, 5- μ m sections were cut and fixed with acetone. Following incubation with the primary antibody, sections were stained using the avidin-biotin-peroxidase system (GE Healthcare, Hoevelaken, The Netherlands), as described by the manufacturer's instructions. Staining was visualized with AEC (3-amino-9-ethylcarbazole) and sections were counterstained with haematoxylin. For fluorescent stainings, sections were labelled with the primary antibody, as described above. Secondary antibodies were conjugated to Cy3 and counterstaining was performed with DAPI.

Global morphological analysis was performed on 5- μ m thick paraffin sections stained with haematoxylin and eosin (H&E).

Masson's trichrome staining was performed with the Masson's Trichrome Stain Kit (25088, Polysciences, Warrington, USA), per the manufacturer's instructions.

Antibodies

The antibodies used in this study were: β -actin (4967, Cell Signaling, Leiden, The Netherlands) at 1:2,000 for western blots, calponin (CALP, Abcam, Cambridge, UK) at 1:50, keratin 16 (LL025, Serotec, Düsseldorf, Germany) at 1:50, Ki67 (MIB1, DAKO, Heverlee, Belgium) at 1:100, laminin 332 (BM165, kind gift from Dr. A. Aumailly) at 1:75, nidogen-1 (AF2570, R&D Systems, Minneapolis, USA) at 1:300, collagen type VI $\alpha 2$ (ProteinTech, Manchester, UK) at 1:250 for IHC and 1:200 for western blots, tenascin c (EPR4219, GeneTex, Irvine, CA, US) at 1:1,000, and integrin $\beta 1$ (4B7R, Santa Cruz Biotechnology, Heidelberg, Germany) at 1:150.

Statistics

For analysis of numerical data, a t-test or ANOVA was performed on all data. If the ANOVA was significant, the TukeyHSD post-hoc test was used to determine the differences between the individual groups. Results were considered significant if the p-value was below 0.05.

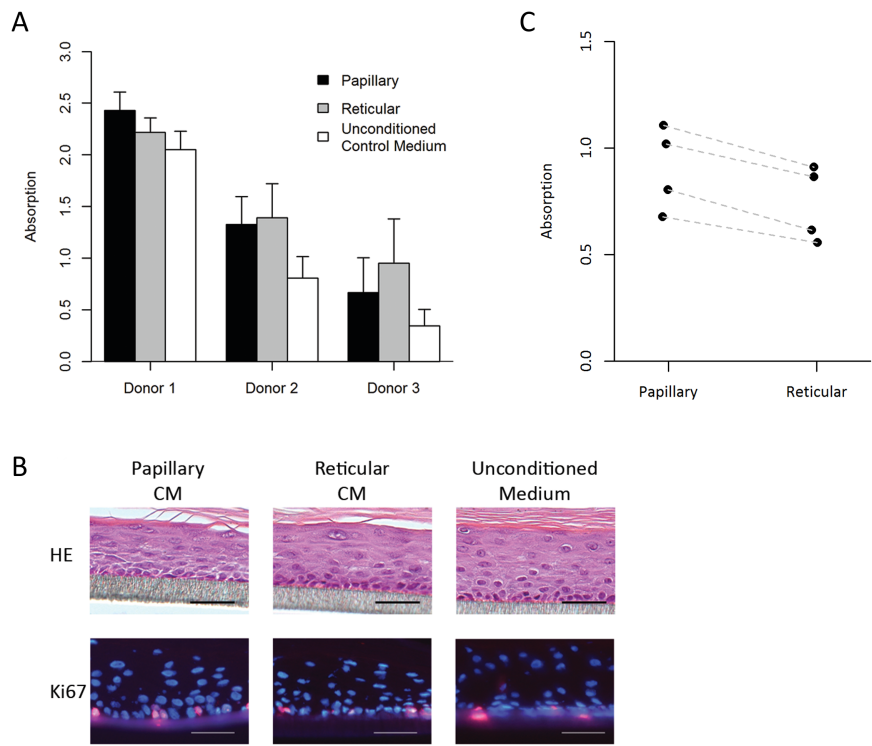


Figure 1: A) WST-1 viability measurements of monolayer keratinocytes cultured in conditioned media of papillary or reticular fibroblasts, or in unconditioned normal keratinocyte medium. Based on an ANOVA with TukeyHSD post-hoc test, there were no significant differences between reticular and papillary conditioned media, but keratinocytes had a significantly higher viability in conditioned medium compared to unconditioned medium. Error bars represent the SD, based on three biological replicates per donor and condition. B) Leiden epidermal equivalents generated with papillary or reticular conditioned medium (CM), or with unconditioned control medium. The equivalents showed no differences with regards to morphology (H&E) or immunohistochemical analysis of Ki67 staining. Scale bars: 50 μ m. C) WST-1 viability assay of keratinocytes grown on ECM secreted by papillary or reticular fibroblasts. Keratinocytes showed less viability when grown on matrix secreted by reticular fibroblasts. Keratinocytes and fibroblasts from two donors were used; samples from the same donors are connected by a dashed line. Based on an ANOVA analysis with TukeyHSD post-hoc test, the difference between reticular and papillary fibroblasts was significant.

Results

Conditioned medium from papillary and reticular fibroblasts have similar effects on keratinocyte growth

To investigate whether the growth of keratinocytes is affected by soluble factors from papillary and reticular fibroblasts, monolayer cultures of keratinocytes were cultured in conditioned medium from both fibroblasts subpopulations. After seven days of culture, the viability of the keratinocytes was measured by WST analysis. No differences were detected in the viability of the keratinocytes cultured in papillary or reticular fibroblast-conditioned medium, but there was considerable variability between different donors (figure 1). The use of conditioned medium did lead to increased keratinocyte viability, when compared with unconditioned medium.

Next, LEMs were generated and cultured in conditioned medium from papillary and reticular fibroblasts. The equivalents were assessed based on their morphology and proliferation marker Ki67. Again, no differences were found between the effects of reticular and papillary fibroblast-conditioned media. The LEMs had a similar morphology compared with LEMs cultured in unconditioned control medium and Ki67 was not differentially expressed in basal keratinocytes, compared within donors (figure 1).

Keratinocytes preferentially grow on papillary fibroblast-generated ECM

To test whether the ECM causes the differential effects between papillary and reticular fibroblasts on keratinocytes in HSEs, keratinocytes were grown on papillary and reticular ECM substrates. To generate the matrix, fibroblasts were stimulated to generate matrix with ascorbic acid (similar to the generation of the FDM dermis). Subsequently, the fibroblasts were killed by a lethal dose of UVB before seeding the keratinocytes. Keratinocytes that were seeded on a papillary ECM substrate had a significantly higher viability than keratinocytes seeded onto a reticular ECM substrate, as measured by WST-1 (figure 1).

Keratinocytes lose proliferative capacity earlier on reticular FDM than on papillary FDM

FDM HSEs were generated with papillary and reticular fibroblasts. The HSEs were harvested 3, 7 and 10 weeks after keratinocyte seeding in order to study whether keratinocyte proliferative capability was maintained longer on papillary or reticular HSEs.

To quantify the outgrowth of the keratinocytes, the relative area of the epidermis covering the FDM was measured. There was considerable variability between keratinocyte donors. However, the growth area was larger on papillary FDM than on reticular FDM in all experiments performed (figure 2).

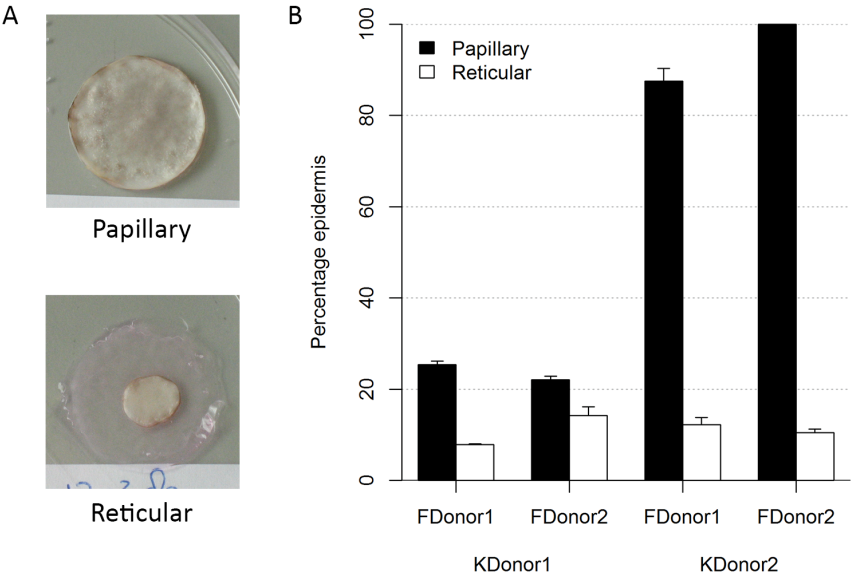


Figure 2: Area measurements of the epidermis of FDM equivalents after 10 weeks, following keratinocyte seeding. A) Macroscopic images used for quantifying the growth area, corresponding to the second keratinocyte donor in the plot. B) Plot of the percentage of the FDM surface covered by epidermis for different keratinocyte (KDonor) and fibroblast donors (FDonor). There was considerable variability between keratinocyte donors, but on papillary FDMs, the keratinocytes covered a larger surface than on reticular equivalents. The error bars represent the SD of the measurement, based on two independent measurements per equivalent.

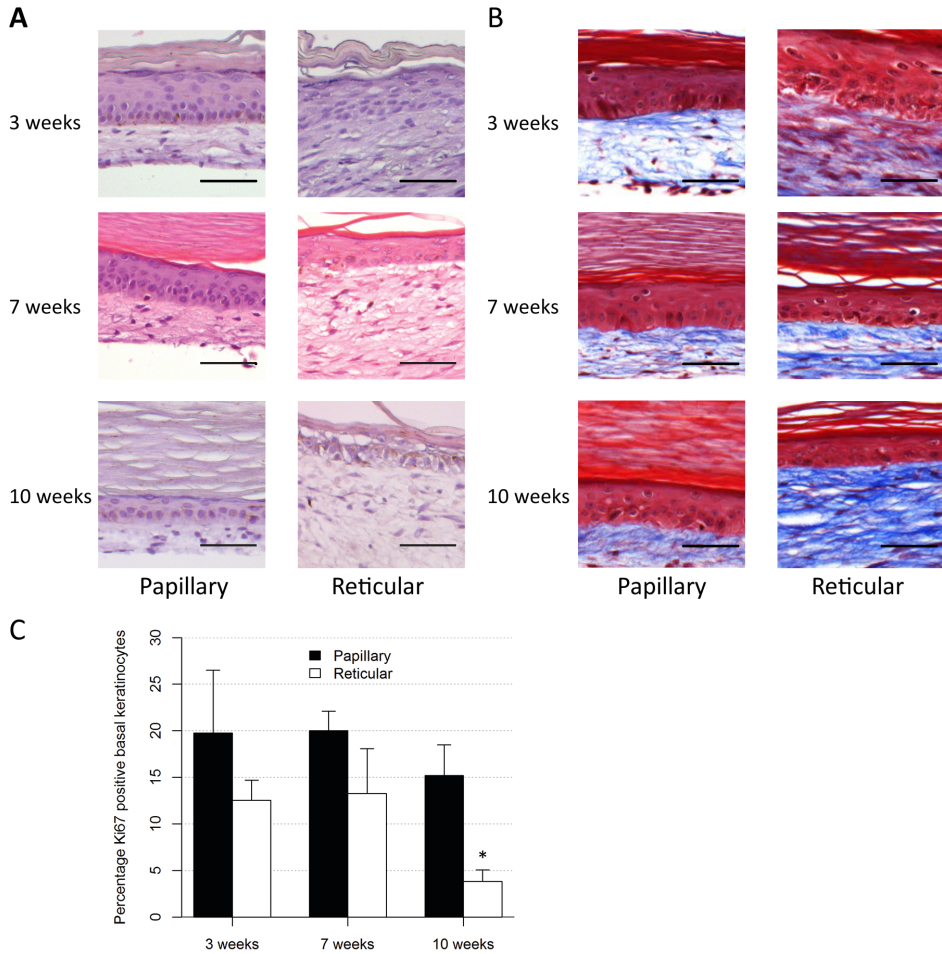


Figure 3: A) H&E staining of FDM equivalents generated with papillary or reticular fibroblasts, cultured for 3, 7, or 10 weeks after keratinocyte seeding. The epidermis was more developed on papillary HSEs. After long-term culture, the epidermis of reticular HSEs consisted of fewer viable cell layers than the epidermis of papillary HSEs. B) Trichrome staining of FDM equivalents generated with papillary or reticular fibroblasts, cultured for 3, 7, or 10 weeks after keratinocyte seeding. The papillary dermis showed, in general, less intense staining compared to the reticular dermis, indicating a less dense dermal matrix. C) Percentage of Ki67-positive basal keratinocytes. The number of Ki67-positive keratinocytes was higher in papillary HSEs compared to reticular HSEs. In addition, after 10 weeks of culture, the percentage of proliferating keratinocytes dropped. After performing an ANOVA and TukeyHSD post-hoc test, the differences between papillary and reticular HSEs, as well as between 10 weeks and the other time points, were significant. Error bars represent the SD of the different HSEs (three donors). Scale bars: 50 μ m.

After three weeks, the differences between papillary and reticular HSEs were similar, as described earlier (7). The epidermis of papillary HSEs showed better development than the epidermis of reticular HSEs. This was evident from the number and organization of keratinocytes. After three weeks of culture, papillary HSEs had 7-8 viable epidermal cell layers, while reticular HSEs had 5-7 viable epidermal cell layers. After seven weeks, the viable epidermis became progressively thinner and the stratum corneum became progressively thicker in both papillary and reticular HSEs; papillary HSEs had 5-7 viable cell layers and reticular HSEs had 3-4 viable cell layers. After 10 weeks, the viable epidermis in reticular HSEs had reduced to just 1-2 viable cell layers, while papillary HSEs still had 3-4 viable cell layers (figure 3A). In addition, papillary fibroblasts generated a loose matrix while reticular fibroblasts generated a dense matrix, as shown by the Masson trichrome staining. Papillary dermis cultured for 3, 7, or 10 weeks after keratinocyte seeding demonstrated, in general, a less blue intense staining compared to the reticular dermis, indicating a less dense dermal matrix (figure 3B). Epidermal proliferation was measured by counting the number of Ki67-positive basal keratinocytes. In general, the proliferation was significantly higher in papillary HSEs compared with reticular HSEs. After 10 weeks, the number of Ki67-positive basal keratinocytes dropped. This drop was more pronounced in reticular HSEs, which had about 5% Ki67-positive basal keratinocytes, compared with papillary HSEs, which still had about 15% Ki67-positive basal keratinocytes (figure 3C).

In order to validate the long-term cultures, we evaluated several fibroblast and epidermal biomarkers by immunohistochemistry. Only the HSEs harvested three and 10 weeks post keratinocyte seeding were analysed. Reticular marker CNN1 was not expressed in papillary HSEs, but was expressed in several fibroblasts of reticular HSEs at both three and 10 weeks. Integrin $\beta 1$, which is known to be important for keratinocyte adhesion and a potential marker for keratinocyte stem cells (15), was expressed more strongly in the membrane of basal keratinocytes after three weeks in papillary HSEs, compared with reticular HSEs. After 10 weeks, the difference disappeared and the expression levels were similar in papillary and reticular HSEs. Epidermal activation-associated marker keratin 16 showed very low expression in all papillary HSEs, low expression in reticular HSEs cultured for three weeks, and high expression in reticular HSEs cultured for 10 weeks. Basement membrane protein laminin 332 and nidogen were expressed, as expected, in the basement membrane zone in all conditions (figure 4).

Collagen type VI α 2 is predominantly expressed in the papillary dermis *ex vivo* and in HSEs generated with papillary fibroblasts

Because the extracellular matrix appears responsible for the different effects of papillary and reticular fibroblasts on the epidermis, we investigated which ECM molecule(s) are

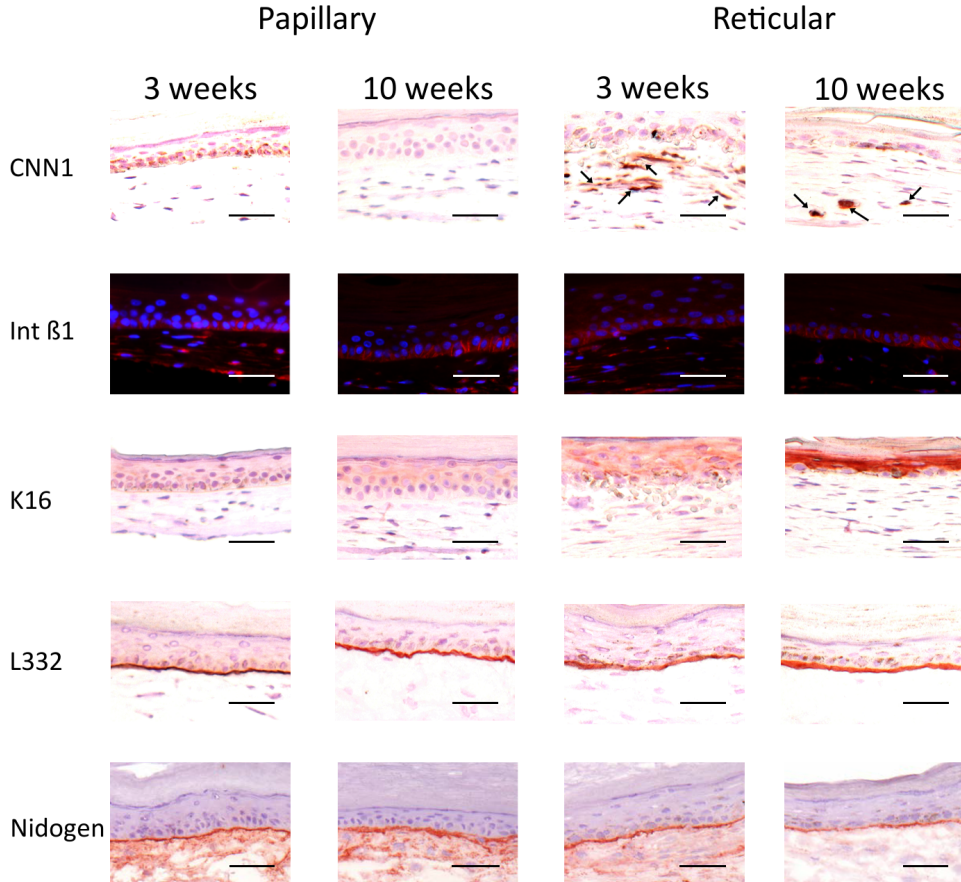


Figure 4: Immunohistochemical analysis of integrin β 1 (Int β 1), keratin 16 (K16), calponin 1 (CNN1), laminin 332 (L332), and nidogen in HSEs. Reticular marker CNN1 was not expressed in papillary HSEs, but was expressed in several fibroblasts of reticular HSEs at both three and 10 weeks (arrows). Integrin β 1 was strongly expressed in the membrane of basal keratinocytes after three weeks in both papillary and reticular HSEs. After 10 weeks, the expression in reticular HSEs was reduced in the basal layer, while in papillary HSEs, the basal layer still showed strong expression. Epidermal activation-associated marker keratin 16 showed very low expression in all papillary HSEs, low expression in reticular HSEs cultured for three weeks, and high expression in reticular HSEs cultured for 10 weeks. Basement membrane proteins, laminin 332 and nidogen, were expressed in the basement membrane zone in all conditions. Scale bars: 50 μ m.

differentially expressed between both cell types. Specifically, we selected several candidate matrix genes from an earlier gene expression study (13) that were more highly expressed in papillary fibroblasts compared to reticular fibroblasts. Of these genes, tenascin C and collagen type VI $\alpha 2$ showed the greatest difference in expression *in vitro*. These two genes were validated at the protein level by western blotting using samples from monolayer cultures of papillary and reticular fibroblasts and by immunohistochemistry on *ex vivo*- and FDM HSE sections. As shown by western blot, the expression of both tenascin C and collagen type VI $\alpha 2$ was significantly higher in samples from papillary fibroblasts than from reticular fibroblasts (figure 5).

In *ex vivo* tissue, tenascin C and collagen type VI $\alpha 2$ were only expressed in the papillary dermis (figure 6A, B). In sections from older individuals, some epidermal collagen type VI $\alpha 2$ staining could be detected. In FDM HSEs, tenascin C was expressed in the dermis of equivalents generated with both papillary fibroblasts and with reticular fibroblasts (data not shown). However, collagen type VI $\alpha 2$ was expressed in the dermis of papillary HSEs, most strongly underneath the epidermis. In reticular HSEs, collagen type VI $\alpha 2$ was expressed at a very low level to not at all in the dermis, but did show a strong level of expression in the layers above the stratum basale in the epidermis. It was not expressed in the epidermis of papillary HSEs (figure 6C).

Discussion

Papillary and reticular fibroblasts have different effects on keratinocytes. The main aim of this study was to investigate whether the main cause of these differences is based on a difference in soluble secretion or ECM generation by the fibroblast populations. The differences between the effects of soluble factors from papillary and reticular fibroblasts on the *in vitro* growth and viability of keratinocytes were minimal. It appears that the ECM secreted by papillary and reticular fibroblasts is the causal factor of the differential effects of the fibroblast subpopulations on keratinocytes.

One potential problem with conditioned medium experiments is the fact that the unconditioned medium already contains nutrients and growth factors. Interestingly, in monolayer experiments, there was a considerable difference between unconditioned and conditioned medium, but not in LEMs. A possible explanation for this is the period of growth before conditioned medium is added. LEMs were first cultured in unconditioned medium for four days, and then ten days in conditioned medium. Monolayer cultures were cultured in conditioned medium right from the start of the experiment. Perhaps the extra growth factors in conditioned media have little influence on the viability and morphology of LEMs if added after four days of culture.

To verify and isolate the role of the ECM, experiments were performed in wells contain-

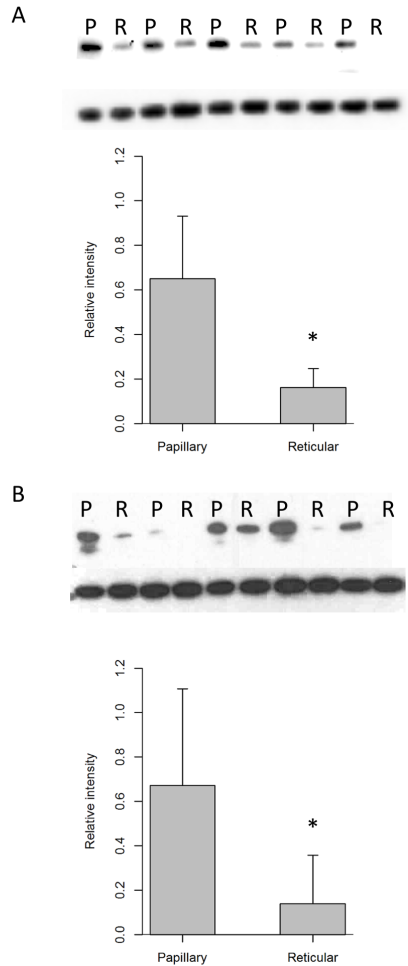


Figure 5: Western blot analysis of (A) collagen type VI $\alpha 2$ and (B) tenascin C expression in papillary and reticular fibroblasts from five donors. Samples from papillary fibroblasts are marked with P and from reticular fibroblasts with R. Immunoblotting for β -actin was used as a control. The mean relative band intensity, calculated as the intensity of the protein band of interest divided by that of the respective β -actin band, was significantly higher in papillary samples compared to reticular samples for both collagen type VI $\alpha 2$ and tenascin C (t-test). Error bars represent the SD of the intensities.

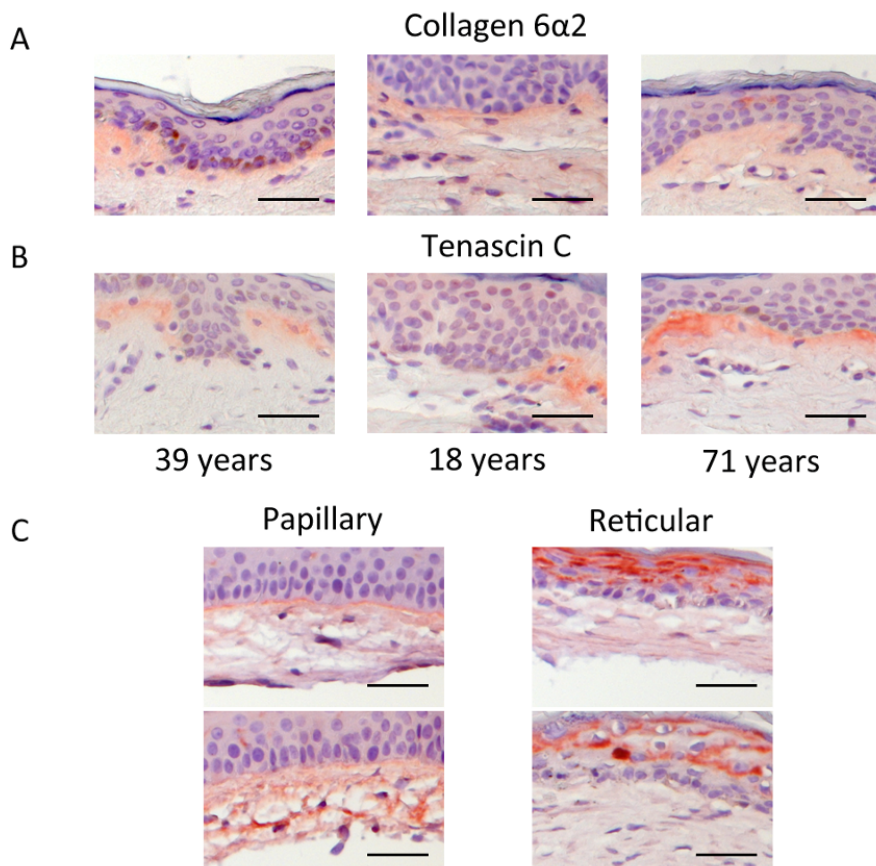


Figure 6: A) Immunohistochemical analysis of tenascin C expression in *ex vivo* sections from three donors. Expression was identified only directly underneath the epidermis. B) Immunohistochemical analysis of collagen type VI $\alpha 2$ expression in *ex vivo* sections from three donors. Expression was detected in the upper layers of the dermis. C) Immunohistochemical analysis of collagen type VI $\alpha 2$ in FDM equivalents generated with papillary or reticular fibroblasts. In papillary HSEs, collagen type VI $\alpha 2$ was expressed in ECM in the dermis, while in reticular HSEs, it was expressed in the epidermis. Scale bars: 50 μm .

ing ECM secreted by papillary or reticular fibroblasts, with the fibroblasts subsequently killed. Complete removal of fibroblasts proved essential, since any surviving, remaining fibroblasts distorted the results. These experiments indicated that ECM is an important factor in the differential effects of papillary and reticular fibroblasts on keratinocyte viability and growth. A main advantage of the type of HSEs used in this study is that keratinocytes grow on ECM secreted by the fibroblasts themselves. Others have shown differences between papillary and reticular fibroblasts on the epidermis in HSEs with a more artificial, uniform dermal part (collagen type I gel) (9). However, in our hands, there are no differences between reticular and papillary collagen gel-based HSEs (data not shown), but there are in FDM-type HSEs.

Since the ECM appears to be an important factor for the interaction between keratinocytes and fibroblasts, the question remains as to which components, present in the papillary ECM, cause the keratinocytes to grow preferentially on matrix secreted by papillary fibroblasts, compared with reticular fibroblasts. Based on earlier gene expression data, several genes were selected and validated that had a higher expression level in papillary fibroblasts, compared with reticular fibroblasts. Of these, collagen type VI $\alpha 2$ was expressed only in the papillary dermis, directly underneath the epidermis *in vivo*. In HSEs, collagen type VI $\alpha 2$ was predominantly expressed in the dermal matrix of HSEs generated with papillary fibroblasts. The exact role of collagen type VI ($\alpha 2$) in the dermis is not known. It appears to play a role in the attachment of cells through integrins (16, 17).

Interestingly, in reticular HSEs, collagen type VI $\alpha 2$ was only very sporadically expressed in the dermal matrix, but was highly expressed in the epidermis. A possible explanation could be that when an epidermis is cultured on ECM generated by reticular fibroblasts, it exhibits poor morphology and cell structure. One can assume that the interactions with this epidermis result in an altered and incomplete BM and thereby result in small leakages of the BM.

Collagen type VI can be expressed in the epidermis *in vivo* (18). In the *ex vivo* sections analysed in the present study, collagen type VI $\alpha 2$ was also occasionally detected in the epidermis. The expression of epidermal collagen type VI $\alpha 2$ was very high in reticular HSEs. Epidermal expression of collagen type VI $\alpha 2$ can be a backup mechanism in case the dermal matrix does not contain enough of collagen type VI.

Recently, it was shown that the presence of papillary fibroblasts is important for the final stages of wound healing (19). The speed of wound healing, especially wound closure, reduces with age (20). This hints at a deficiency, either functionally or numerically, of papillary fibroblasts in aged skin. As such, it could be interesting to investigate components of the papillary matrix for their role in wound healing and as a potential treatment for wound healing problems, such as non-closing wounds or scar formation. It has already been shown that papillary matrix-specific glycoprotein decorin can reduce

scar formation (21, 22).

In conclusion, we have demonstrated that keratinocytes grow differentially on ECM secreted by papillary and reticular fibroblasts. The differences in soluble factors secreted by both fibroblast populations seem to be less important for epidermal growth and viability.

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Conflict of interest

GS and CM are employees of CHANEL Parfum Beauté. AEG declares the receipt of a grant from CHANEL. The other authors declare no conflict of interest.

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