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Making More Matrix: Enhancing the Deposition of Dermal–Epidermal Junction Components *In Vitro* and Accelerating Organotypic Skin Culture Development, Using Macromolecular Crowding

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Skin is one of the most accessible tissues for experimental biomedical sciences, and cultured skin cells represent one of the longest-running clinical applications of stem cell therapy. However, culture-generated skin mimetic multicellular structures are still limited in their application by the time taken to develop these constructs *in vitro* and by their incomplete differentiation. The development of a functional dermal–epidermal junction (DEJ) is one of the most sought after aspects of cultured skin, and one of the hardest to recreate *in vitro*. At the DEJ, dermal fibroblasts and epidermal keratinocytes interact to form an interlinked basement membrane of extracellular matrix (ECM), which forms as a concerted action of both keratinocytes and fibroblasts. Successful formation of this basement membrane is essential for take and stability of cultured skin autografts. We studied interactive matrix production by monocultures and cocultures of primary human keratinocytes and fibroblasts in an attempt to improve the efficiency of basement membrane production in culture using mixed macromolecular crowding (mMMC); resulting ECM were enriched with the deposition of collagens I, IV, fibronectin, and laminin 332 (laminin 5) and also in collagen VII, the anchoring fibril component. Our *in vitro* data point to fibroblasts, rather than keratinocytes, as the major cellular contributors of the DEJ. Not only did we find more collagen VII production and deposition by fibroblasts in comparison to keratinocytes, but also observed that decellularized fibroblast ECM stimulated the production and deposition of collagen VII by keratinocytes, over and above that of keratinocyte monocultures. In confrontation cultures, keratinocytes and fibroblasts showed spontaneous segregation and demarcation of cell boundaries by DEJ protein deposition. Finally, mMMC was used in a classical organotypic coculture protocol with keratinocytes seeded over fibroblast-containing collagen gels. Applied during the submerged phase, mMMC was sufficient to accelerate the emergence of collagen VII along the *de novo* DEJ, together with stronger transglutaminase activity in the neoepidermis. Our findings corroborate the role of fibroblasts as important players in producing collagen VII and inducing collagen VII deposition in the DEJ, and that macromolecular crowding leads to organotypic epidermal differentiation in tissue culture in a significantly condensed time frame.

Introduction

The extracellular matrix (ECM) that surrounds and separates tissue structures in organ systems provides a tissue-specific niche and mediates signals that trigger or suppress proliferation, differentiation, or senescence.[1] The dermal–epidermal junction (DEJ)[2,3] of the skin is a complex carpet of specialized ECM that provides anchorage for the waterproof epidermis[4–6] to the mechanical buffering dermis below.[7] Ultrastructurally, the basal keratinocyte layer of the epidermis is anchored to the type IV collagen-rich extracellular lamina densa through interactions with collagen XVII and laminin 332. The lamina densa is in turn fastened to the papillary dermis by anchoring fibrils, composed of collagen type VII that interlace with collagen I/III/V heterotypic fibrils of the dermis. This complex assembly of proteins is essential to maintain healthy skin tissue, and defects in any component results in tissue

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fragility disorders; for example, mutations in the collagen VII gene *COL7A1* result in dystrophic epidermolysis bullosa.

Current tissue culture techniques attempt to replicate this cell-specific matrix to facilitate optimal cell growth and function *in vitro*. However, one of the bottlenecks in tissue engineering is the slow processing of collagen *in vitro,* and thus poor ECM formation. Whereas fibronectin deposition is readily demonstrated in tissue culture, the deposition of collagen, the primary biological component essential for the basic structural formation of all tissues and organs, is enzymatically rate limited.[8,9] Although it takes several weeks to produce tissue sheets which contain sufficient ECM *in vitro*,[10] our group has shown that with macromolecular crowding, we are able to significantly shorten that waiting time while enhancing collagen production by fibroblasts.[11]

Collagen type VII plays a crucial role in the integrity of the DEJ. It is synthesized by both keratinocytes and fibroblasts in culture,[12–14] although the relative contribution of each cell type in DEJ formation has not been fully described. In addition, supramolecular assembly and deposition of collagen VII into a pericellular matrix has never been achieved experimentally *in vitro*. Drawing from studies that applied mixed macromolecular crowding (mMMC) to cell monolayers, we developed a three-dimensional (3D) coculture system which allows fibroblasts and keratinocytes to interact in a more physiologically realistic way, which is closer to intact human skin. This is shown to facilitate generation of a skin equivalent construct with an enriched basement membrane in a condensed time frame. This technology may be of significant value, both for the development of better skin-mimetic cultures for *in vitro* screening and toxicity assays, as well as for generating cultured autografts with higher efficiency for clinical applications.

Materials and Methods

Isolation of primary fibroblasts and keratinocytes from human skin

Human dermal fibroblasts and human primary keratinocytes were isolated from normal human female skin obtained from surgical waste skin remnants (abdominoplasty), with full local ethical approval. Fibroblasts and keratinocytes from at least three different donors were studied each time. Skin was cleaned with ethanol and soaked in sterile phosphate buffered saline (PBS); excess fat was trimmed off using sterile surgical scissors. The skin was then incubated in 2× antibiotic–antimycotic solution [(100×) stabilized: penicillin, streptomycin, amphotericin B] (#A5955; Sigma Aldrich) for 15 min at room temperature, followed by two washes in PBS. The skin was then cut into 1 × 1 cm cubes and immersed in 2.4 U/mL Dispase solution (Roche) for 16 h at 4°C. The epidermis was then separated from the dermis by peeling it away with sterile forceps, and then incubated in 0.125% trypsin for 15 min at 37°C before filtering through a 100 μm nylon cell strainer (BD Falcon, BD Biosciences) to remove tissue fragments. The resulting cell suspension was pelleted and resuspended in CNT-57 (Cell-N-Tec) or Keratinocyte Serum-Free Media (KSFM; Invitrogen). After separation from the epidermis, the dermis was cut into smaller cubes and placed in a T-75 flask to generate explant cultures; fibroblasts began to migrate out from the explants after a few days.

Cell culture and macromolecular crowding

Fibroblasts were cultured in a fibroblast medium (FM) made up of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics; 100 U/mL penicillin (GIBCO-Invitrogen) and 100 U/mL streptomycin (GIBCO-Invitrogen). Culture medium was changed every 2 days. Human primary keratinocytes were cultured in CNT-57 or KSFM. In addition, a keratinocyte cell line was used, NEB1.K14wt-GFP,[15] cultured in RM+ media containing DMEM (Invitrogen), Ham's F12 (Invitrogen) supplemented with 10% FBS (Invitrogen), antibiotics (100 U/mL penicillin (GIBCO-Invitrogen) and 100 U/mL streptomycin (GIBCO-Invitrogen), 0.4 μg/mL hydrocortisone, 5 μg/mL insulin, 1.8×10^{-4} M adenine, 10 ng/mL epidermal growth factor, 5 μg/mL transferrin, and 2×10^{-11} M triiodothyronine. All cell cultures were maintained in a 37°C humidified incubator with 5% CO_2.

A Ficoll cocktail consisting of 37.5 mg/mL of Ficoll 70 and 25 mg/mL of Ficoll 400 (Fc 70/400; SciMed Asia) was used for mMMC as described earlier.[11,16] Briefly, cells were seeded onto the culture dish and allowed to attach overnight. The next day, the culture medium was replaced with medium containing the macromolecular crowders together with ascorbic acid and cultured for 6 days with medium changes every 2 days.

To generate cocultures, equal numbers of keratinocytes and fibroblasts were seeded together in a single well of a 24-well plate. To differentiate between keratinocytes and fibroblasts, NEB1.K14wt-GFP keratinocytes were used, as they could be distinguished from the fibroblasts by their green fluorescent keratin filament network.

Preparation of decellularized ECM

Cells were grown in either noncrowded or crowded conditions before removing the cells to generate decellularized matrix. Cells were seeded onto 24-well plates and left to attach for 24 h. Culture medium was then removed and new medium, with or without crowders, was added and cell cultures were maintained for 6 days, with media change every 2 days. Cell monolayers were decellularized using 0.5% sodium deoxycholate (Prodotti Chimici E Alimentari). Briefly, cell layers were washed twice with PBS, and 230 μL of sodium deoxycholate was added to the cell monolayers and left for 10 min on ice to lyse the cells. Lysates were then aspirated and the process was repeated two more times. After removing all cells, the remaining ECM preparations obtained were washed twice with distilled water and air-dried for 15 min in a laminar flow hood. ECM preparations are referred to in the text as follows: *f-mat* = fibroblast-derived ECM, *k-mat* = keratinocyte-derived ECM, and ECMs derived from cocultures of fibroblasts and keratinocytes = *co-mat*. Fibroblasts were seeded on top of *k-mat* and left to adhere overnight. Similarly, keratinocytes were seeded on *f-mat* and left to adhere overnight.

Immunocytochemistry and histochemistry

Cell layers and decellularized ECMs were fixed with either methanol or 3% paraformaldehyde. Nonspecific binding of antibodies was blocked by incubating with 3% bovine

serum albumin or 10% goat serum for 30 min. Primary antibody was then added for 90 min at room temperature. Primary antibodies include rabbit anti-collagen I (Abcam; dilution 1:100), mouse anti-collagen IV (NovoCastra; PHM-12 clone, dilution 1:100), mouse anti-collagen VII (clone LH7.2), and mouse anti-fibronectin (Abcam; dilution 1:100). Secondary antibodies (AlexaFluor 594 goat anti-rabbit, AlexaFluor 594 goat anti-mouse, Alexa Fluor 488 chicken anti-rabbit, and AlexaFluor 488 goat anti-mouse; Molecular Probes) were then added for 30 min at room temperature. Nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI). Coverslips were mounted onto a glass slide using the Hydromount mounting media (National Diagnostics). Samples were left to air dry for 24 h before visualization using an inverted Olympus fluorescent microscope or a Zeiss Confocal microscope. The transglutaminase assay measuring transglutaminase activity (TGA) was conducted as previously described.[17]

Histology

Organotypic cultures were cut into pieces and fixed separately, one snap frozen and one in 10% neutral buffer formalin, and processed into wax blocks for tissue sectioning. For frozen tissue, blocking and subsequent primary and secondary antibody incubation could be performed immediately. For formalin-fixed paraffin-embedded sections, the sections were subjected to a routine histological protocol for hematoxylin and eosin (H&E) staining.

Immunoblotting

Cell layers (seeded on six-well plates) were washed twice in PBS, then scraped into 100 μL lysis buffer and then centrifuged at 15,330 *g* for 30 min at 4°C. The supernatant was collected, mixed with sample buffer, and heated for 10 min at 95°C. After spinning down to collect the condensation water, a final volume of 20 μL was loaded onto precast 3–8% NuPAGE gels (Invitrogen).

The concentration of proteins in the samples were determined by the Pierce BCA Protein Assay Kit (ThermoScientific) and equal amounts of protein were loaded onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (Invitrogen) as confirmed by the actin blots. Separated proteins were transferred to nitrocellulose membranes (Biorad) and blocked with 5% milk powder for 1 h at room temperature. Primary antibody used was mouse anti-collagen VII (clone LH 7.2, 1:1000). Secondary antibody used was goat anti-mouse-HRP. After washing with PBS-Tween, membranes were incubated with the ECL detection reagents (GE Healthcare) and chemiluminescence detected on light-sensitive film (GE Healthcare).

Interference reflection microscopy combined with fluorescence microscopy

Image acquisition was performed by using a Confocal Laser Scanning Microscope (LSM510; Zeiss) with an EC Plan-Neofluar 40x/1.30 Oil objective. The pinhole was set up at 74 μm. The filters used were LP 505 for the interference reflection microscopy (IRM) channel and BP 575-615 IR for the fluorescence red channel. The beam splitters used were NT 80/20 for the IRM channel and HFT 405/488/561, NFT 565, plate for the fluorescence red channel. The lasers used were DPSS 561-10 (wavelength 561 nm) at 1.1% power for the fluorescence red channel and HeNe633 (wavelength 633 nm) at 5.0% power for the IRM channel.

Three-dimensional organotypic cocultures

Collagen gel preparation. Keeping all solutions on ice, 10 mL of collagen solution (rat tail collagen type I (BD Biosciences), suspended in acetic acid) was mixed with 1 mL DMEM followed by 0.5 mL 1 M NaOH for neutralization. 0.5 mL of DMEM containing 1,200,000 fibroblasts was added. 2 mL of the collagen–fibroblast suspension was loaded into a cell culture insert (BD Biosciences) in each well of a six-well plate and the plate transferred to 37°C to allow the collagen to gel for 1 h.

After the collagen gel had solidified, FM was added to the fibroblast-containing gel in the cell culture insert. After 24 h, FM was replaced with FM containing the Fc 70/400 Crowder cocktail and ascorbic acid. On the third day, the medium was aspirated and keratinocytes were seeded on top of the collagen gel. After 3 h, the keratinocytes have attached to the gel, after which CNT-57 or KSFM was added in the cell culture insert. The following day, the medium was replaced with CNT-57 or KSFM containing the Fc 70/400 Crowder cocktail and ascorbic acid. After 1 week of submerged culture, the keratinocytes have formed a confluent layer on top of the gel. Cell culture inserts are then raised to the air–liquid interface using deep well plates (BD Biosciences) to facilitate keratinocyte stratification and differentiation. After 3–5 weeks, the mature organotypic cultures are harvested for analysis.

Electron microscopy

Organotypic skin cocultures were fixed in 2.5% glutaraldehyde (in PBS) for 72 h. Samples were then washed in PBS and cut into small pieces (1 mm^3). Postfixation was carried out in 1% osmium tetroxide, pH 7.4, for 1 h at room temperature. Samples were then washed in distilled water and dehydrated through an ascending ethanol series. Samples were infiltrated, embedded in araldite resin and left to polymerize at 60°C for 24 h. Ultrathin sections were viewed using a Transmission Electron Microscope, JEOL JEM-1010 (100 kV).

Results

Macromolecular crowding enhances the deposition of ECM components synthesized by fibroblasts, keratinocytes, and a fibroblast–keratinocyte coculture in vitro

Upon the addition to the culture medium of a Ficoll 70/400 macromolecular crowding mixture, collagen type I, IV, and fibronectin production by fibroblasts was dramatically enhanced in 6 days as compared with uncrowded controls. Decellularization of the mMMC-treated fibroblast cell layer yielded an enriched matrix on the tissue-culture polystyrene surface (TCPS) (Fig. 1), demonstrating a significantly increased amount of matrix (containing collagen type I, IV, and fibronectin) deposited by the fibroblasts, as compared with control cultures in which minimal matrix was detected.

As keratinocytes do not synthesize collagen I, mMMC only enhances intrinsic deposition of various amounts of fibronectin and in particular DEJ components such as collagen type IV (Fig. 1).[18] The highest level of collagen type I,

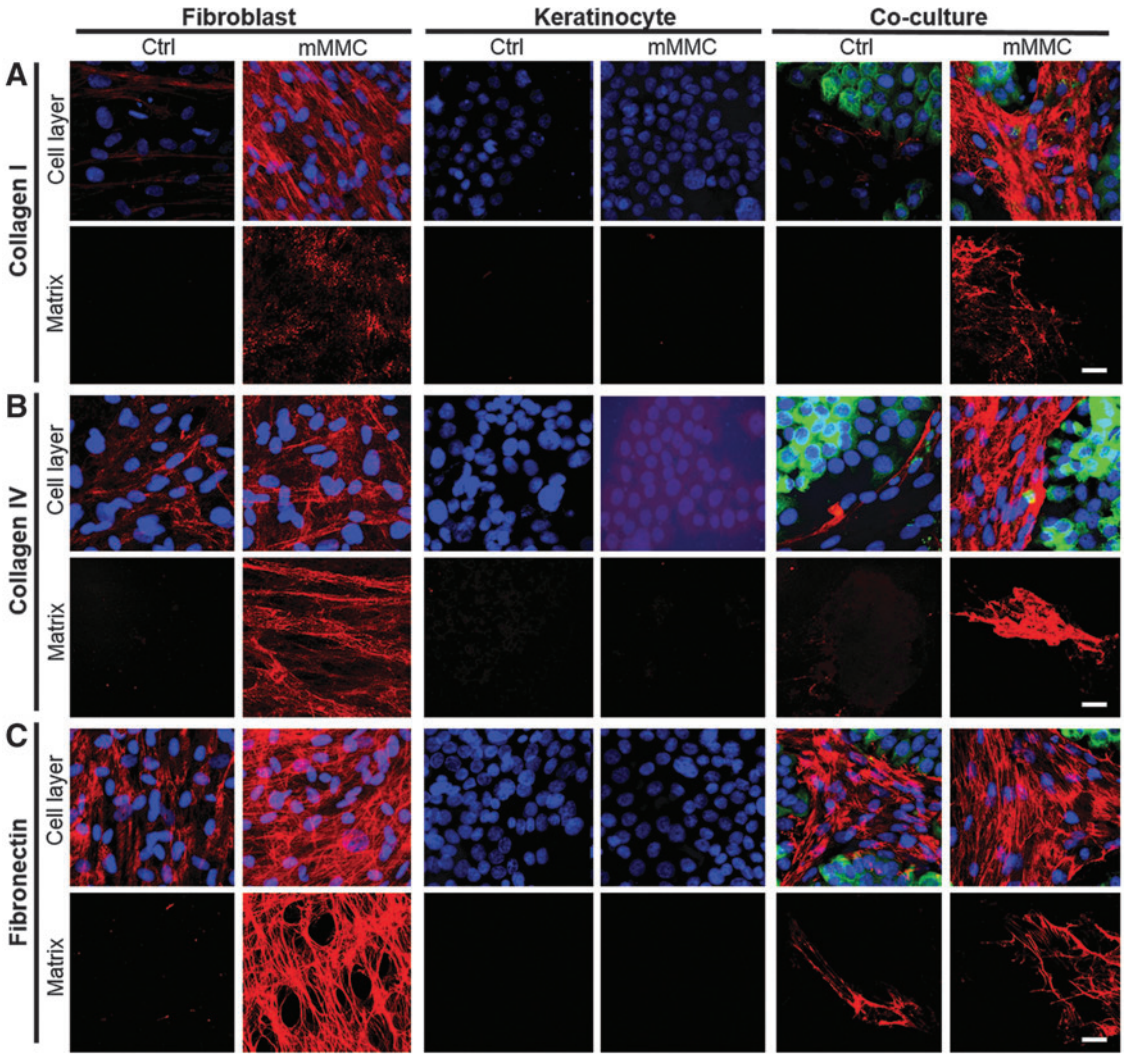

FIG. 1. Mixed macromolecular crowding (mMMC) enhances deposition of dermal–epidermal junction components *in vitro*. **(A)** Collagen I deposition is enhanced by mMMC (cell layer and matrix) in fibroblasts only. Crowding of cocultures produce the most collagen I and show that keratinocytes stimulated collagen I production by fibroblasts. **(B)** Collagen IV deposition by fibroblasts is enhanced by crowding. This is seen even more clearly in crowded cocultures. Of note, keratinocytes stained for collagen IV show mostly cell-associated or intracellular collagen IV, but not a pericellular matrix. In cocultures, both cell types segregate with collagen IV being predominantly associated with fibroblasts sparing keratinocyte islands. **(C)** Fibronectin deposition was only seen with fibroblasts, and therein strongly enhanced by crowding (cell layer and matrix). In cocultures, a reticular mesh of fibronectin was associated with fibroblasts only, sparing islands of keratinocytes. Scale bars = 20 μm.

IV, and fibronectin deposition was observed in confrontation cultures of fibroblasts and keratinocytes, cultured under mMMC suggesting a stimulatory role of keratinocytes on fibroblasts. Although fibroblasts and keratinocytes were initially seeded as a heterogeneously mixed cell suspension, the two cell types demixed and segregated into distinct populations. Keratinocyte colonies could be distinguished from fibroblasts by the green fluorescence of the GFP-coupled keratin-14 (K14) filaments. Comparing the three cell seeding situations and the resulting ECM after decellularization, it became obvious that although mMMC-treated fibroblasts produce a rich ECM, they deposited significantly more in the coculture with keratinocytes. In our hands, collagen VII was synthesized predominantly by fibroblasts and to a lesser extent by keratinocytes. While there was a small amount of collagen type VII produced by both cell types *in vitro*, mMMC dramatically enhances this production and deposition (Fig. 2A). In particular, strong collagen type VII staining was noted in fibroblast cultures crowded with Fc 70/400. In contrast, a coculture of fibroblasts and keratinocytes under the same conditions, but without mMMC treatment, failed to yield significant amounts of collagen type VII. Whole cell lysates also show an increased amount of collagen type VII production after mMMC exposure when compared with uncrowded controls (Fig. 2B, C) suggesting a rich pericellular matrix.

Fibroblast-derived matrices induce keratinocytes to deposit more collagen type VII in vitro

Upon seeding onto a fibroblast-derived matrix (*f-mat*), keratinocytes were able to synthesize a larger amount of

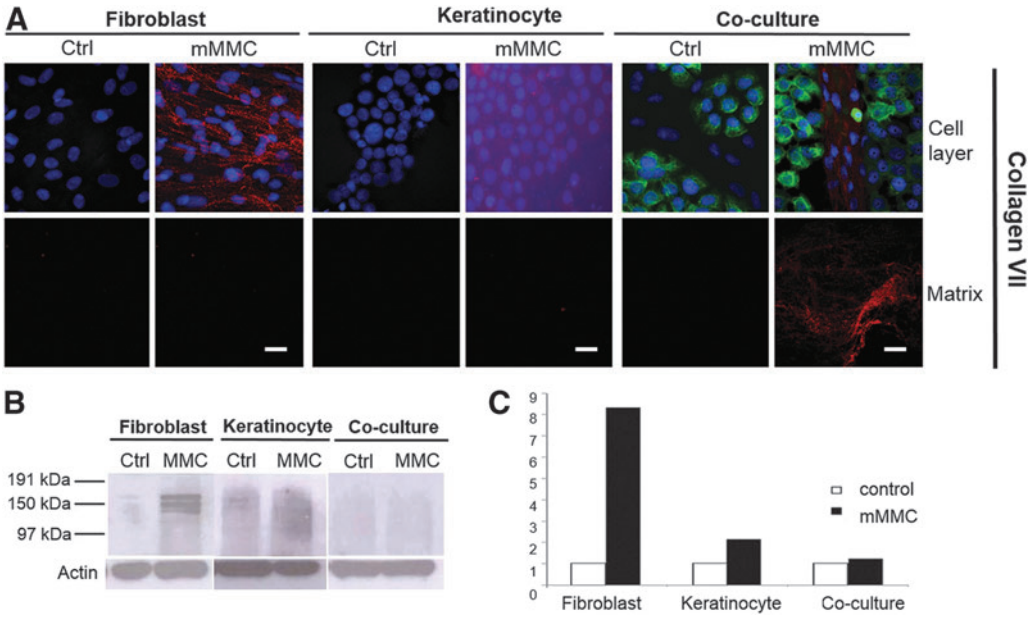

FIG. 2. mMMC facilitates the deposition of anchoring fibril building collagen VII. **(A)** A reticular deposition pattern of collagen VII deposition is evident with fibroblasts only under mMMC. In cocultures, extracellular collagen VII is strongly associated with fibroblast colonies in between keratinocyte islands. Keratinocytes show pericellular and intracellular collagen VII more strongly expressed in the presence of mMMC. After cell lysis, collagen VII footprints are seen in a fine granular layer in mMMC-treated fibroblast cultures, but a discernible fibrillar deposition is retrieved from cocultures. **(B)** Immunoblot analysis of lysed cell layers shows that both crowded fibroblasts and keratinocyte cultures contain significantly more collagen VII compared with uncrowded controls. The retrieved collagen VII is mainly pericellular derived. **(C)** Densitometric analysis of **B** shows that mMMC increases the amount of cell-associated collagen VII by a factor of 8 in fibroblasts and a factor of 2 in keratinocytes. Scale bars = 20 μm.

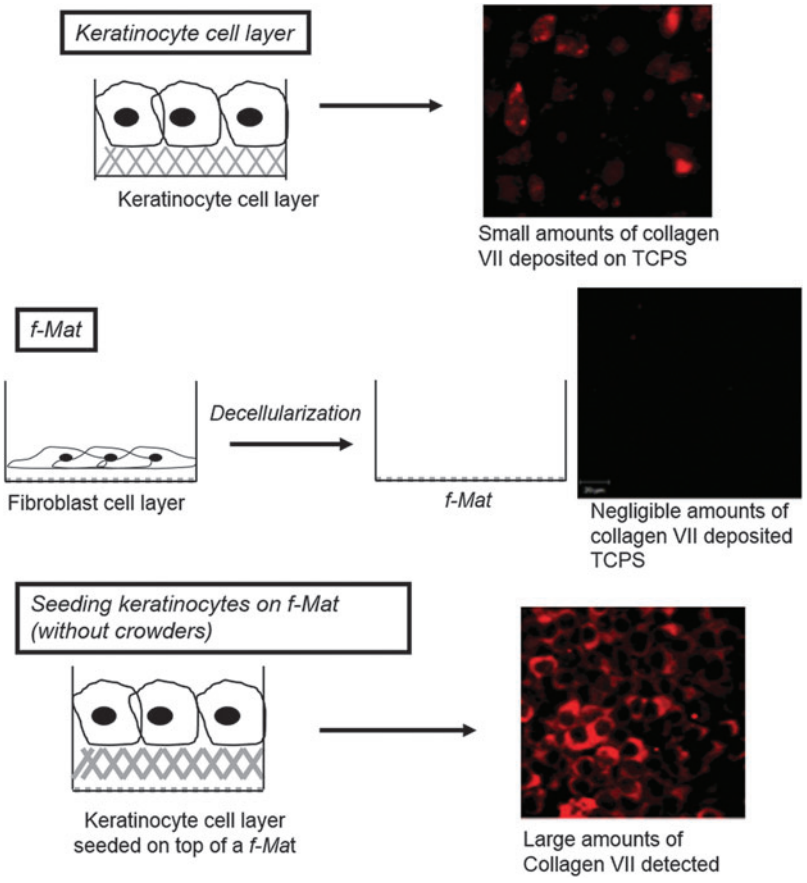

FIG. 3. Keratinocytes adhere most effectively to *f-Mat* and produce the most amount of collagen VII. Keratinocytes seeded on uncoated tissue culture polystyrene surface dishes produce little amounts of extracellular matrix (ECM) (*red* = collagen VII). Decellularized uncrowded fibroblast cell layers, *f-Mat*, produce negligible amounts of ECM. Keratinocytes seeded on top of *f-Mat* produce significantly more ECM.

collagen type VII than keratinocytes cultured only on TCPS. This points to an effect of the *f-mat* on the keratinocytes and the capacity of this matrix to stimulate ECM production in cells with which it comes into contact (Fig. 3). Colony-forming assays showed that keratinocytes were able to adhere and form colonies on *f-mat* and *k-mat* (data not shown). Keratinocytes were also induced to produce significant amounts of collagen type IV when they were seeded on a previously Ficoll 70/400-crowded *f-mat* (data not shown).

Interference reflection microscopy demonstrates larger amount of ECM deposited under mMMC

Interference reflection microscopy was applied to visualize the whole ECM deposited by and in contact with the glass coverslip. All the matrix components which were in contact with the glass coverslip generated a dark print by IRM (black area). In this study, fibroblasts were cultured under crowded and noncrowded conditions and subsequently these cell layers were removed to reveal the underlying matrix (fibroblast footprint). Upon antibody staining with collagen IV, uncrowded cultures showed only patches of collagen IV. In crowded fibroblast cultures, the amount of collagen IV increases significantly (Fig. 4; collagen IV antibody). With IRM, we were able to detect the whole extent of the ECM and comparing between crowded and control fibroblast footprints, it is evident that crowding causes fibroblasts to deposit more matrix extracellularly as is seen when comparing the IRM-crowded and IRM-control images.

We also studied the deposition of fibronectin and noted that the ECM is composed largely of this component (Fig. 4; IRM-fibronectin antibody merge). mMMC increases this production and deposition by fibroblasts as seen in both antibody and IRM images.

Macromolecular crowding enhances the deposition of collagen type VII in the DEJ of organotypic cocultures in vitro

The ECM deposition enhanced by macromolecular crowding was evaluated in organotypic cultures by H&E staining

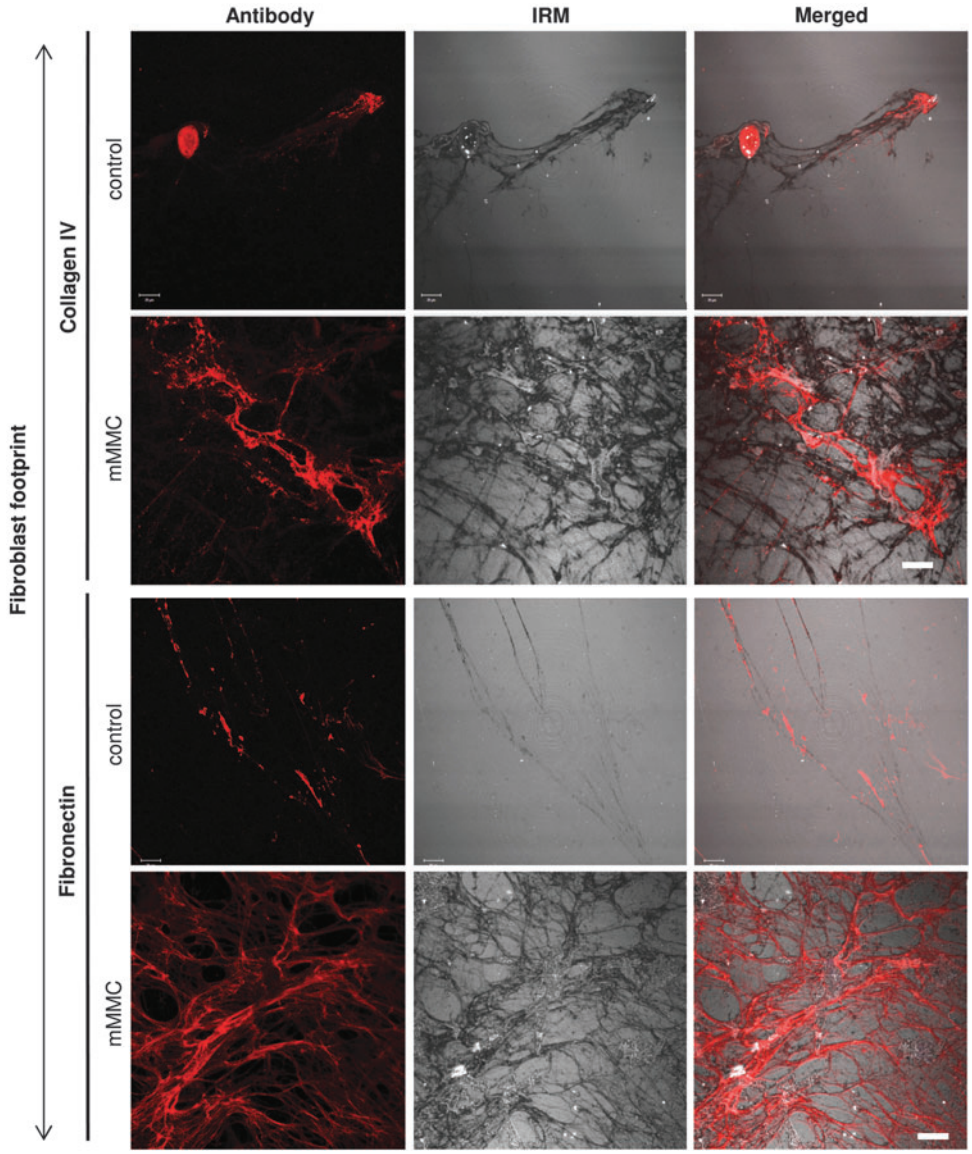

FIG. 4. Fibroblast footprints contain more total ECM as visualized by interference reflection microscopy (IRM). On analysis of individual ECM components (collagen IV and fibronectin), culture under mMMC enhanced the extracellular deposition. To visualize the total ECM deposited, IRM was used to quantify all ECM as antibody staining had their limitations. IRM clearly showed the total matrix quantity and pattern under mMMC as compared with control conditions.

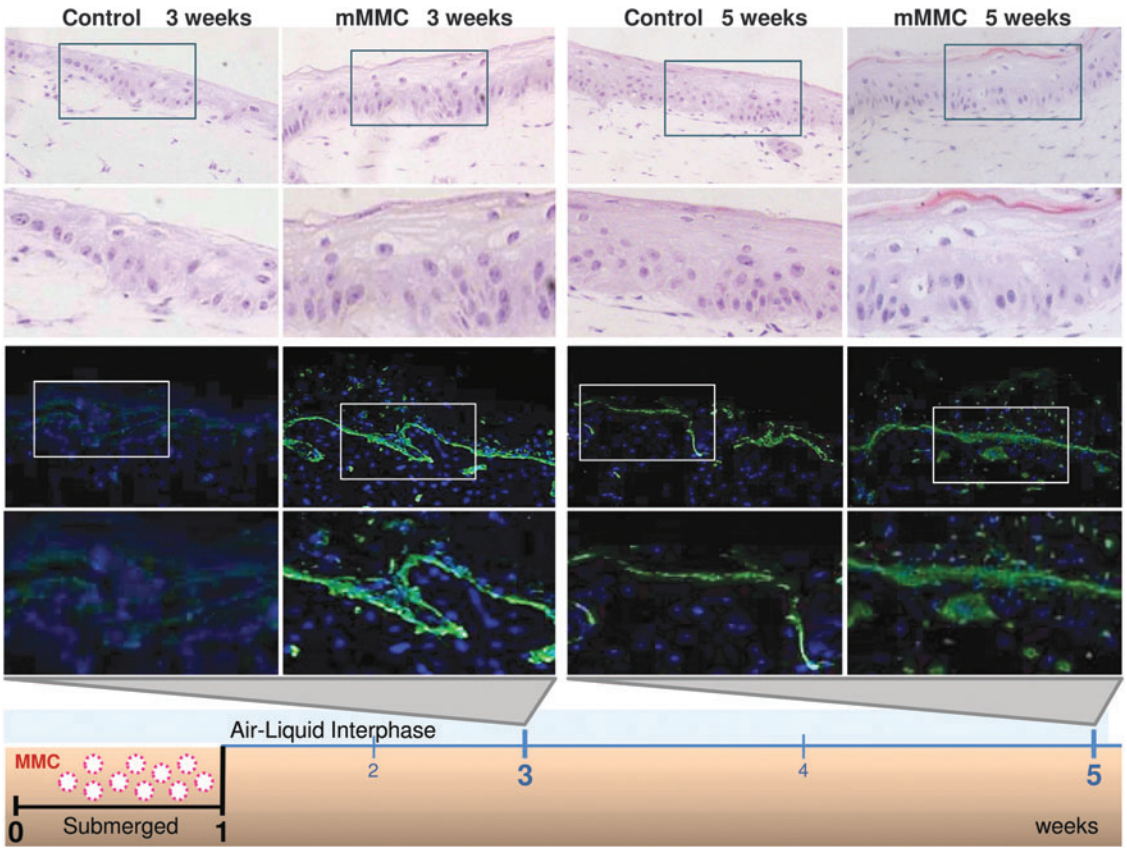

FIG. 5. mMMC during the submerged phase enhances maturation of the dermal–epidermal junction (DEJ) in skin equivalents. Fibroblast-containing collagen gels were seeded with keratinocytes on top and kept submerged for 1 week, then lifted to air–liquid interface. In the classical protocol, collagen VII was absent after a total of 3 weeks in culture, but appeared in skin equivalents after 5 weeks. In contrast, under mMMC, collagen VII was already strongly evident after 3 weeks and even more strongly stained after 5 weeks compared with standard cultures. Hematoxylin and eosin staining confirmed that with this rapid protocol, stratification and maturity of the skin equivalent were maintained and accelerated.

as well as by antibody staining of specific proteins. As a starting scaffold to host dermal fibroblasts, rat tail collagen type I was used as a dermal substitute. Fibrin gel was also used as a dermal scaffold to allow for quantitation of *de novo* synthesized and deposited collagen type I (data not shown). In addition to observing the 3D pattern of ECM enhancement, the organotypic cocultures also allow to study the stratification status of the epidermis. We evaluated mMMC as a potential accelerator of DEJ maturation and epidermal stratification that was applied during the first week, the immersion phase. Two weeks after the constructs had been lifted to the air–liquid interface (meaning a total age of 3 weeks), the *de novo* DEJ showed under mMMC, strong collagen VII which was negligible in noncrowded controls (Fig. 5). Only 5-week noncrowded controls started showing some presence of collagen VII expression along the neo-DEJ, albeit sporadic and still reduced compared to the 5-week-old skin equivalent cultured with mMMC.

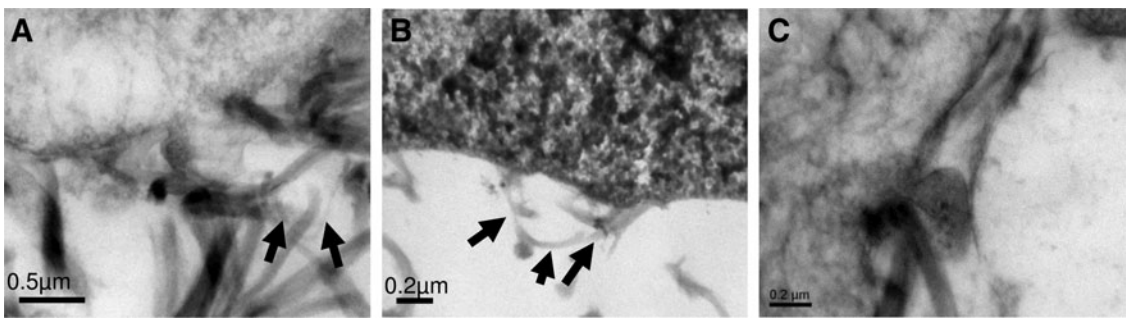

FIG. 6. Evidence of *de novo* formation of anchoring fibrils in skin equivalents generated under mMMC. Ultrastructural studies of the nascent dermal–epidermal junction of organotypic cocultures after a 3 week culture protocol with mMMC **(A, B)** suggests structures akin to anchoring fibrils (*arrows*) that are absent in noncrowded skin equivalents **(C)** after 3 weeks of culture.

Interestingly, we observed anchoring fibrils in the neo-DEJ of 3-week-old skin equivalents generated with mMMC (Fig. 6A, B; arrows), but not in control cultures, pointing to the probability that the increased amount of collagen VII secreted under crowded conditions has the capability to form anchoring fibrils *in vitro*.

Also, epidermal maturation was improved by mMMC. After 3 weeks we noted a mature pluristratified epidermis under mMMC, which was less evident in noncrowded controls (Fig. 5). After 5 weeks of culture, both crowded and noncrowded skin equivalents showed a stratified epidermis. Organotypic culture maturity was further assessed by proliferation and stratification markers, including Ki67, keratin 10, and transglutaminase activity (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). Keratin 10 expression was present in the epidermal layers of all organotypic cultures showing stratification of skin equivalents under the various conditions. While transglutaminase activity was present in the epidermal layers of all organotypic cultures, it was observed that in 3-week-old mMMC organotypic cultures, transglutaminase activity was more evenly expressed in the whole epidermal layer. Ki67 positive cells were observed to be most abundant in 3-week-old mMMC organotypic cultures as compared with 3-week-old controls. Five-week-old mMMC organotypic cultures showed negligible amounts of Ki67 positive cells suggesting an extremely low proliferative capacity of the epidermal layer.

Discussion

We show in this study that two different cell types, fibroblasts and keratinocytes cooperate to build the DEJ *in vitro* at the two-dimensional and 3D levels. This cellular cooperation is based on cell–matrix reciprocity effects. To dissect these effects, we applied a novel technology in tissue engineering, macromolecular crowding, a system based on the introduction of carbohydrate-based macromolecules in a culture system. The resulting excluded volume effects drive enzymatic processes that control ECM processing and also supramolecular assembly.[11] We were particularly intrigued to observe that it was possible under mMMC to effect deposition of collagen VII in fibroblast cultures to the extent that after removal of these cells, a footprint remained. This is the first report on the successful *in vitro* deposition of collagen VII, the major component of anchoring fibrils and essential guarantors of dermo-epidermal cohesion. In confrontation cultures of fibroblasts and keratinocytes, we observed a segregation of cell types and a demarcation of the boundaries between both cell types characterized by the deposition of collagens IV and VII; again the deposition of collagen VII is a novelty that allowed us to investigate the effects of fibroblast matrices (*f-mats*) on keratinocytes and vice versa. mMMC technology helped to show that dermal fibroblasts are the main producers and depositors of collagen type VII. This ties in with earlier work[12] and strongly supports strategies suggested earlier[19] to move the focus of attention for gene therapeutic intervention of dystrophic epidermolysis bullosa (a heritable collagen VII defect leading to severe skin blistering) from keratinocytes toward dermal fibroblasts.

MMC initially augments the production of ECM as it mimics more closely the *in vivo* environment. Once ECM is deposited, a solid-phase microenvironment has arisen in which the producing cells find themselves embedded. This ECM contains biochemical cues that fuel dynamic cell–matrix reciprocity.[16] After decellularization, crucial information is retained in these matrices and thus is still available to cells that are freshly seeded onto these matrices. In this study, a vast difference became apparent between ECM that had been generated in standard culture or under mMMC. Utilizing the cell-derived matrices (*f-Mat*, *k-Mat*, *co-Mat)* for bioengineering applications is advantageous over commercial coatings, as they are less denatured, fully cell-type specific, fully processed, secreted, and deposited onto the culture surface containing a full portfolio of ligands (such as proteoglycans and growth factors).[16] Thus, the mMMC-generated ECMs, at this point the *Mats* (*f-Mat, k-Mat, co-Mat*) represent highly complex mixtures of ECM molecules produced and processed by the cell, as opposed to an artificial layer of a single basement membrane component commonly used as coating. This would predict a much more physiological *in vivo* extracellular environment. Interestingly, keratinocytes seeded on *f-Mat* were induced to produce and deposit collagen type VII, which they do not normally do in such amounts when cultured on TCPS alone, pointing to the effect of matrix reciprocity.

Taking these observations to a tissue equivalent level, we introduced MMC to 3D organotypic cultures and found a marked improvement of DEJ protein expression and epidermal stratification. Current organotypic culture protocols involve at least 2 weeks at the air–liquid interface with a total culture time of 4–5 weeks. Using mMMC in a modified organotypic culture protocol, we were able to reduce the time needed to generate a skin equivalent from 5 to 3 weeks, involving 1 week of submerged culture in the presence of MMC followed by a 2 week air–liquid interface. This generated an organotypic skin equivalent with a pluristratified epidermis as well as an enhanced basement membrane, in particular, the deposition of collagen type VII in the DEJ is enriched, as compared with the uncrowded control, which shows a spotty and discontinuous expression of collagen type VII in the DEJ. Ultrastructural investigations suggested an early stage of anchoring fibril appearance in mMMC-treated organotypic cultures.

As macromolecular crowding works as an amplifier of enzymatic activity, the role of matrix metalloproteinases, drivers of remodeling,[20] should also be predictably enhanced under mMMC. We observed a faster dissolution of fibroblast-seeded collagen gels under crowded conditions (data not shown) which ties in with observations of dissolving chondrocyte pellets under MMC.[21] Recent findings confirm that MMP2 activity is increased and more closely associated with the pericellular matrix under MMC.[16] While from the physiological point of view this will open highly interesting avenues to study tissue remodeling under MMC, biotechnological considerations would suggest to use MMC in selected time windows to preferably harness the anabolic phase of tissue growth. In this study, we found that the first week in submerged conditions was optimal to accelerate the maturation of organotypic skin equivalents. The findings have major implications for the preparation of autografts for

skin defects or organotypic skin constructs for pharmacological studies. One of the biggest problems in autologous keratinocyte grafting is the time it takes to grow keratinocyte sheets or living skin equivalents. In the first case, the stabilization of the DEJ after grafting involves the cooperative formation of a neoepidermis and neodermis and it takes 120 days, at least *in vivo*, until the formation of sizeable anchoring fibrils are evident.[22] As long as the DEJ is not well formed, the grafts are fragile. Transplanting preconstructed skin equivalents with a prestabilized DEJ would be a desirable alternative to pure keratinocyte autografting. Culturing autologous cocultures of fibroblasts and keratinocytes as skin equivalents, for example in a fibrin hydrogel, has long been the gold standard in skin grafting protocols.[23] The current time required from harvesting of the cells to maturation of the skin equivalent can range from 4 to 8 weeks. Therefore, our method of culturing fibroblasts and keratinocytes in a mixed macromolecular crowded hydrogel to generate an organotypic skin equivalent in a shorter time frame while still maintaining a pluristratified epidermis and promoting mature DEJ formation holds great potential in tissue engineering and wound healing.

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Authors' Contributions

P.B., C.B., E.B.L. and M.R. designed the experiments. P.B. and C.B. carried out experiments. All authors contributed to data analysis, wrote the article, and provided feedback.

Disclosure Statement

No competing financial interests exist.

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