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Sub-Populations of Dermal Skin Fibroblasts Secrete Distinct Extracellular Matrix: Implications for Using Skin Substitutes in the Clinic

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Running title: Bioinspired extracellular matrices for skin engineering

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Bulleted Statements:

What is already known about this topic?

- There are several types of skin fibroblasts within the dermis which can be defined by their spatial location: papillary fibroblasts (Pfi) of the papillary dermis, reticular fibroblasts (Rfi) of the reticular dermis and dermal papilla fibroblasts (DPfi) of the hair follicle dermis.
- Extracellular matrix (ECM) composition is distinct with regard composition and architecture within the papillary, reticular and hair follicle dermis in vivo.
- When skin is injured, dermal replacement substitutes used for tissue repair do not reflect the heterogeneity observed within the skin dermis.

What does this study add?

- Self-assembled ECMs from different sub-populations of skin fibroblasts can be generated *in vitro*.
- Cell assembled ECMs made in vitro from Pfi, Rfi and DPfi reflect dermal heterogeneity seen in vivo, and are morphologically, functionally, and compositionally distinct from one another.

- Inspiration should be taken from cell assembled ECMs from distinct fibroblast sub-populations, to improve the design of therapeutic biomaterials in skin engineering applications.

SUMMARY

Background: While several commercial dermo-epidermal scaffolds can promote wound healing of the skin, the achievement of complete skin regeneration still represents a major challenge.

Objective: We postulated that three dimensional structures derived through the physiological cell secretion of extracellular matrix (ECM) may be a bioinspired scaffold for skin tissue engineering. In the present study we performed a biological characterization of self-assembled ECMs from three different sub-populations of fibroblasts found in human skin; papillary fibroblasts (Pfi), reticular fibroblasts (Rfi), and dermal papilla fibroblasts (DPfi).

Methods: Fibroblast sub-populations were cultured with ascorbic acid to promote cell assembled matrix production for 10 days. Subsequently, cells were removed and the remaining matrices were characterized. Additionally, in another experiment, keratinocytes were seeded on the top of cell depleted ECMs to generate epidermal only skin constructs.

Results: We found that the ECM self-assembled by Pfi exhibited randomly oriented fibers associated with highest interfibrillar space, reflecting ECM characteristics which are physiologically present within the papillary dermis. Mass spectrometry followed by validation with immunofluorescence analysis showed that THROMBOSPONDIN 1 (THBS1) is preferentially expressed within the DPfi derived matrix. Moreover we observed that epidermal constructs grown on DPfi or Pfi matrices exhibited normal basement membrane formation, while Rfi matrices were unable to support membrane formation.

Conclusion: We argue that inspiration can be taken from these different ECMs, to improve the design of therapeutic biomaterials in skin engineering applications.

Introduction:

Skin is a multi-layered structure comprised of an underlying supporting dermis and functional epithelium at the skin surface. This is oversimplified, as within each layer there are multiple cells with different functions which contribute to tissue homeostasis. Within the epidermis, keratinocytes are the most abundant cell type; these differentiate and stratify towards the skin surface equipping skin with its barrier function [1]. Within the skin dermis the most abundant cell type are fibroblasts, whose primary role is to secrete components of the extracellular matrix (ECM) for structural support [2]. Largely unconsidered, there are several types of skin fibroblasts within the dermis which can be defined by their spatial location [3], and exist as morphologically and functionally-heterogeneous sub-populations [4-6]. For example, the fibroblast population within the dermal layer adjacent to the basement membrane and epidermis known as the papillary dermis are called papillary fibroblasts (Pfi). These are distinct from those residing within the lower reticular dermis, which are termed reticular fibroblasts (Rfi) [4,6]. There are also fibroblasts with a fibrogenic tendency which are thought to be responsible for scar deposition after wounding [5]. However these are distributed in a speckled pattern throughout the reticular and papillary dermis, and are therefore harder to define by spatial location [5]. If we label cells according to their location, rather than their behaviour, then Pfi reside adjacent to the basement membrane where they direct growth and differentiation of epidermal keratinocytes [7]. Comparatively, the deeper Rfi produce the bulk of the dermal ECM and are responsible for the first wave of dermal repair following a full thickness wound [6]. In hairy skin there are also fibroblasts associated with the hair follicle, located in the dermal papilla and the connective tissue sheath [3,8-12]. Dermal papilla fibroblasts (DPfi) have specialized signalling properties required for hair follicle

morphogenesis and coordination of hair growth [13]. In the hair follicle a specialised basement membrane, termed a glassy membrane, separates the dermal papilla from the surrounding epithelial matrix [14]. In addition, the human dermal papilla is rich in interstitial collagens such as type I and type III, in addition to fibrillar matrix proteins such as fibronectin (FN1) glycoproteins such as THBS1 [14-17], and proteoglycans such as versican [18]. Various studies have also demonstrated that ECMs produced by Pfi and Rfi within the interfollicular dermis are distinct with regard to their composition and architecture [4,12,19,20]. Pfi in the upper dermis secrete ECM which is constituted of thin, poorly-organized collagen fiber bundles, while thick well-organized collagen bundles are characteristic within the lower dermis which is produced by resident Rfi [4]. The papillary dermis also has a higher ratio of collagen type-III to I, higher levels of the dermatan sulfate proteoglycan decorin, yet lower levels of the chondroitin sulfate proteoglycan versican compared to the reticular dermis [4,20-22]. Not only do differences between fibroblast subtypes control how they behave and interact with surrounding cell types, but the ECM secreted by fibroblasts in distinct sub-anatomic locations has a key role in the regulation of these interactions. When fibroblasts are isolated from the skin and grown in culture they retain distinct transcriptional signatures despite being removed from environmental cues [23]. Concomitantly with this, Pfi have been shown to synthesise more decorin than Rfi in culture, reflecting the expression pattern of decorin in the skin in vivo [20].

It is important to understand fibroblast behavior within the skin, especially in the context of wound healing if we want to successfully modulate this process. After injury in the skin, there are coordinated processes that lead to reepithelialisation and establishment of a new functional barrier; however, it is the repair and ECM deposition within the skin dermis which results in scar formation. Specifically, fibroblasts synthesise highly aligned and bulky ECM fibers after injury, and a consequence of this is that complete skin regeneration cannot be achieved [24,25].

After injury, or in the case of a chronic skin wound, there are a number of biological products available on the market which promote healing which have been developed combining primary skin cells together with biomaterials [26-29]. These skin substitutes are biologically active through the release of growth factors and cytokines which aid the recruitment and adhesion of host cells [30-32]. There are a small number of bioengineered skin products that replace both the epidermal and dermal layers of the skin; one such example is Apligraf®, which is composed of neonate-derived fibroblasts cultured in a bovine collagen matrix, over which neonate-derived keratinocytes are seeded to produce a stratified epidermis. In recent years, a group of biological scaffolds comprised of ECMs which are used solely as a dermal replacement have gained increasing interest [33-36]. One of these is Integra®, a dermal regeneration template [37] composed of a layer of bovine tendon collagen type I matrix and shark chondroitine-6-sulfate juxtaposed against a silicone layer that acts as a temporary pseudo-epidermis [38]. Despite the success of Integra, there are some limitations. Two operations are necessary, while there are risks of infection under the silicone layer and a risk that the silicone will become detached [39]. An alternate to this is MatriDerm®, which is an engineered dermal template specially developed to provide a one-step grafting procedure. Matriderm is a scaffold consisting of a native bovine type I, III and V collagen fiber template incorporating elastin hydrolysate that is converted into native host collagen within weeks following application [40]. In addition to engineered scaffolds, decellularized skin dermis from cadaveric donors is now becoming more popular as a scaffold for use after injury where there is extensive skin loss [41]. The common feature of all these decellularized dermal scaffolds is that the native collagen fibers guide fibroblasts and possibly other cells toward dermal regeneration, while the presence of elastin in the collagen matrix diminishes the formation of granulation tissue in the early phase of wound healing [42]. As a result, a high-quality neodermis with randomly organised collagen bundles can be regenerated. These decellularized dermal scaffolds are commonly taken from reticular dermis rather than papillary dermis. Reticular dermis is much thicker than papillary dermis, and therefore provides a greater area for cell infiltration. In addition, its relatively low cellularity in

comparison to the papillary dermis means it is easy to decellularise. Lastly, the reticular dermis shows a strong mechanical resistance when compared to papillary dermis making it easy to handle [43]. Despite these advantages, decellularized reticular dermis does not have a basal membrane and this feature can make re-epithelization of grafts by host keratinocytes cells more difficult [44].

As highlighted above, one problem with dermal replacement scaffolds is they do not reflect the heterogeneity observed within the skin dermis, where different fibroblasts sub-types including the ones described in this manuscript have divergent functions [1]. Furthermore, with decellularized scaffolds there can be issues associated with inflammation, and these foreign body reactions have lead scientists to investigate new strategies based on in vitro organogenesis approaches [45]. A therapeutic strategy such as this requires cells to first be obtained from their native tissue, then kept in long term culture with appropriate growth factors to produce a tissue substitute rich in ECM [46-49]. In this respect, ECM produced by cells via a self-assembly approach has been shown to act as a key cell adhesion site and a mechanically strong scaffold free support for tissue engineering [50-52]. Lastly, cell derived ECM of mesenchymal cells from anatomically distinct sites, such as the bone marrow and adipose tissue, can be created in vitro and reflect the distinct ECM found in these different sites [53].

Based on the above observations, in this study we decided to investigate cell derived ECMs as potential scaffolds for use in skin engineering. We had two main objectives in our study; the first was to generate and biologically characterise ECMs produced by fibroblast sub-populations found both within the hair follicle dermis, and the papillary and reticular dermis of human skin. We refer to these fibroblasts using names based on their sub-anatomical origin. Our second aim was to evaluate how ECMs from fibroblasts in different dermal locations, which are from areas juxtaposed to basement membrane or devoid of basement membrane, can interact with and instruct basement membrane formation in epithelial only skin constructs.

Materials and Methods:

Human tissue samples and cell culture

Scalp skin tissues were taken from healthy donors using IC-REC approved consent forms and used for the isolation of epidermal keratinocytes, and dermal sub-populations. Specifically, Pfi and Rfi were obtained from the upper and lower regions of interfollicular dermal tissue. To isolate cells, the adipose tissue was first cut off the scalp tissue using a scalpel. The tissue was then laid flat within a petri dish containing PBS, and using a stereo dissection microscope to view the tissue a scalpel was used to transect the dermis 100-200µm below the epidermis. The piece of tissue containing epidermis and papillary dermis was used to isolate Pfi, while the deeper dermis was used for isolation of Rfi. These pieces of superficial (papillary) and deep (reticular) tissue were minced, then placed in culture in different 35mm dishes in Dulbecco's Modified Eagle's Medium (DMEM) containing GlutaMAX (Gibco, Invitrogen) supplemented with 20% Fetal Bovine Serum (FBS) (Gibco, Invitrogen), 1% antibiotics-antimycotics (Gibco, Invitrogen). Tissue were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air for 10 days, during which time Pfi and Rfi migrated from their respective dermal explants. After migration of fibroblasts outward from the explants, cells were amplified in culture in DMEM GlutaMAX, 10% FBS and 1% antibiotics at 37°C in a humidified atmosphere of 5% CO_2/95% air, and passaged until p3, at which point they were used to establish cell derived ECMs.

Intact dermal papillae were also isolated from the same piece of scalp skin using a microdissection approach to obtain DPfi cultures from the same donors as the Pfi and Rfi [54]. Follicles were transected just above the level of the dermal papilla to isolate end bulbs, which were inverted using 27G needles to remove the matrix and expose the dermal papilla. Papilla were then separated from the follicle by cutting through their stalk. For culture, 8 papillae were transferred to 35mm dishes containing 20% FBS in DMEM GlutaMAX, with 1% antibiotics-antimycotics. Cells migrated from the papillae, and when the dish was

approaching confluence, DPfi were passaged at a 1:2 ratio using 0.5% trypsin-EDTA (Gibco, Invitrogen) for detachment. After the initial 2 weeks of culture, cells were grown in DMEM GlutaMAX, 10% FBS and 1% antibiotics. After passaging, DPfi were cultured in the same manner as Pfi and Rfi. Matched sets of cells, from 3 different male donors were used for ECM generation and subsequent analysis.

To assess proliferation characteristics of fibroblast sub-types, cells were seeded at 6000 cells/cm^2. After 24 and 168 hours, 100 µl of alamarBlue® reagent (Invitrogen) was added directly to cells in 1ml culture medium. The cells were incubated for 3 hours at 37ºC, protected from direct sunlight. After 3 hours, 100 µl aliquots were taken in duplicate and their absorbance was measured at 570 nm, using 600 nm as a reference wavelength. The fluorescent intensity is proportional to cell number.

For keratinocyte isolation, skin samples were washed briefly in phosphate buffered saline (PBS) containing 2% antibiotics-antimycotics, then incubated in Dispase (Stem Cell Technologies) overnight at 4°C. The epidermis and dermis were then separated from each other with fine forceps. Next, the epidermal layer was minced with scissors, and incubated in 0.5% trypsin-EDTA at 37°C for 30 minutes. Digested tissue was filtered through a 70µm cell strainer, then cells were pelleted and resuspended in Epilife medium with 1% antibiotics and EDGS (Gibco, Invitrogen). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air and used for epithelial only skin construct generation at p2.

Self-assembly approach for matrix generation

To generate ECMs from dermal fibroblast sub-populations we followed previously described protocols [55]. Sterile 13mm glass coverslips in a 24 well-plate were coated with 0.2% sterile gelatin (gelatin type B, Sigma Aldrich) for 60 min at 37°C. They were washed 3 times with PBS, cross-linked with 1% sterile glutaraldehyde (Sigma Aldrich) for 30 min at room temperature (RT) and again washed 3 times with PBS. Crosslinking was quenched with 1M sterile glycine in PBS for 20 min at RT, followed by 3 more washes in PBS. Coverslips were

then incubated in growth medium (DMEM GlutaMAX, 10% FBS, 1% antibiotics) for 30 min at 37°C. Finally coverslips were washed 3 more times with PBS, then used immediately. The three different sub-populations of fibroblasts (DPfi, Pfi, Rfi) were plated onto coverslips in 24 well plates. A total of 65,000 cells (34,000 cells/cm^2) were seeded into each well in DMEM GlutaMAX, 10% FBS, 1% antibiotics and cultured overnight at 37°C, 5% CO2/95% air to achieve a confluent lawn of fibroblasts. The next day, growth medium supplemented with 50 µg/ml ascorbic acid (Sigma Aldrich) was added to each culture to promote self-assembly of ECM from each cell type. Medium was exchanged for fresh medium every two days for a total of ten days. After ten days of culture in ascorbic acid supplemented medium, cells were removed. First, medium was aspirated and cells were washed once with PBS before pre-warmed extraction buffer (20mM NH4OH, 0.5% Triton X-100 in PBS) was added and left for 4 min to allow cell lysis. Half of the buffer was carefully removed and PBS was added. The same step was repeated until no intact cells were visible. The DNA residue was digested with 10 µg/ml DNase I (Roche) in PBS for 30 min at 37°C, 5% CO2, followed by two washes with PBS. Matrices were either used straight away or stored at 4°C in PBS with 1% antibiotics for up to 4 weeks.

Immunofluorescence staining and analysis of cell assembled ECMs

We performed immunofluorescence of specific proteins in the self-assembled matrices using antibodies against individual ECM components. Matrices were fixed using 4% paraformaldehyde (PFA) for 20 minutes, followed by wash and blocking steps with 5% goat serum (Vector Laboratories) in PBS for 30 minutes. Primary antibodies (Table 1) were diluted in PBS and placed on matrices overnight at 4°C. Secondary antibodies (Table 1) were used for 1 hour at room temperature. Finally they were washed 3 times in PBS and mounted on glass slides with Vectashield mounting medium (Vector Laboratories). After staining, ECMs were imaged using a Zeiss LSM-510 inverted confocal microscope. Three random Z-stack images of matrices were acquired for each FN1 stained sample, which were used for the evaluation of alignment and fiber measurements. Moreover the thickness of the

ECMs was determined by taking Z-stack images on the confocal microscope and subtracting the distance between the top and the bottom of assembled FN1 matrix. The images were subsequently processed with Image J and Fiji programs. Each Z-stack set of images was converted to a single image by using the maximum projection function. The plugin Dimensionality was applied to calculate the orientation distribution of fibers, while the diameter of fibers and interfibrillar spaces were assessed with the plugin BoneJ [56]. All graphs were generated using GraphPad Prism version 6.01. Data are shown as the mean while error bars (±) are the standard deviation of the mean. A one-way ANOVA followed by Bonferroni's correction was performed for experiments, with $p < 0.05$ considered significant.

Generation of epidermal only skin constructs

For this experiment we used fibroblasts and keratinocytes as isolated in section 2.1. To start we placed several cell inserts (Millicell-24 Cell Culture Insert Plate, polycarbonate, 0.4µm) into a 60mm cell culture dish, and coated using the same method for coverslip coating as described in section 2.2. We seeded each fibroblast sub-populations at a density of 30,000 cells/insert in DMEM GlutaMAX, 10% FBS, 1% antibiotics and allowed the fibroblasts to produce ECMs for 10 days in ascorbic acid supplemented medium on the inside of each insert. After removing fibroblasts from these matrices, we seeded 250,000 keratinocytes inside each insert on the top of the cell depleted ECMs in Cnt Prime Medium (CellnTec). After 2 days, we removed the Cnt Prime Medium from the inserts and added 3D Barrier Medium (CellnTec) both inside and outside the inserts. The next day all 3D Barrier Medium was removed, and replaced with fresh 3D Barrier Medium on the outside of the inserts only. This enables establishment of an air liquid interface which is required for epithelial stratification. Finally we kept the cells for 14 days in culture at the air liquid interface, prior to analysis as described in 2.5.

Skin constructs viability, Immunofluorescence staining and Transmission electron microscopy

We used an alamar blue assay to assess cell viability of epidermal skin constructs after 14 days of culture. Resazurin, the active ingredient of alamarBlue® reagent, is reduced upon entering cells to resorufin, a compound that is red in color and highly fluorescent. Viable cells continuously convert resazurin to resorufin, increasing the overall fluorescence and color of the media surrounding cells. Alamar blue dye at a concentration of 10% in PBS was added to each skin construct and incubated for 4 h at 37 °C in 5% CO2/95% air. Subsequently, 100 µl of the medium was transferred to a fresh 96 well plate and absorbance was read at 570 nm. Data are expressed as an absorbance value unit.

Alternatively, after the growth period of 14 days at the air-liquid interface, samples were snap-frozen in OCT mounting medium and stored at -80°C until processing. Frozen samples were sectioned into 7µm slides using a cryostat. For staining, these were fixed with either 4% PFA in PBS for 10 min at RT, or 100% methanol for 7 minutes at -20C. After fixation, the samples were rinsed 3x with PBS and pre-treated for 30 min with PBS containing 5% goat serum, followed by incubation overnight at 4°C with primary antibodies (Table 1). The sections were thoroughly rinsed with PBS and then incubated with secondary antibodies for 1hr at RT. Coverslips were mounted using Vectashield containing DAPI (Vector Laboratories), which subsequently labels nuclei. Confocal microscopy (Zeiss LSM-510 inverted) was used to visualize and capture immunostained cells. As a positive control, human skin was stained using the same antibodies.

For transmission electron microscopy, 14 day old constructs were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2-7.4, washed in 0.1 M cacodylate buffer at pH 7.2, postfixed in 1% OsO4 in 0.1 M cacodylate buffer at pH 7.2, dehydrated in graded ethanol and embedded in Araldite (Serva). Ultrathin sections were counterstained with

uranyl acetate and lead citrate and examined under a Zeiss EM109 transmission electron microscope to evaluate basement membrane formation within the constructs.

ECM samples directly analyzed by mass spectrometry

The protocol we used for mass spectrometry was described previously by Plieger et al. [57]. After cell elimination, ECM proteins were directly proteolyzed in each well using 2.5µg trypsin (Gibco, Invitrogen) in 250 µL of 30mM Tris (pH 8.0) at 37°C overnight. Predigested matrix proteins were scraped off from the wells using a cell scraper and collected into 1.5mL microfuge tubes. The samples were then reduced by the addition of DTT (final concentration 10mM) and incubated for 30 min at 56°C. After reduction, Iodoacetadime (IAM, final concentration 55mM) was added to keep the disulfide bonds separate and unfolded, and samples were incubated for 30 min at RT in the dark. Finally, the samples were further digested by trypsin at 37°C overnight before terminating the digest with the addition of 10% trifluoroacetic acid (final concentration 0.5%).

Samples were analysed by LC–MS using a nanoAcquity UPLC™ system (Waters MS Technologies, Manchester, UK). 1 µL (1-3 µg protein digest) of sample was injected onto each trapping column (Waters, C18, 180µm×20mm) using partial loop injection, for 1 min at a flow rate of 15µL/min with 0.1% (v/v) formic acid. Samples were resolved on an analytical column (Waters, nanoACQUITY UPLC™ M-class HSS T3 75µm×150mm 1.8µm column) using a gradient of 97% A (0.1% (v/v) formic acid) 3% B (99.9% acetonitrile 0.1% (v/v) formic acid) to 60% A 40% B over 36 min at a flow rate of 300nL/min. The nanoAcquity UPLC™ was coupled to a Synapt™ G2 mass spectrometer (Waters) and data was acquired using a MS^E program with 1s scan times and a collision energy ramp of 15–40eV for elevated energy scans. The mass spectrometer was calibrated before use and throughout the analytical run at 1min intervals using the NanoLockSpray™ source with glufibrinopeptide.

To quantitatively analyse the mass spectrometry results peptide identification was performed using the ProteinLynx Global SERVER™ (PLGS) v3.0.3 (Waters). The data was processed using a low energy threshold of 250. A fixed carbamidomethyl modification for cysteine was specified. The search thresholds used were: minimum fragment ion matches per peptide 3; minimum fragment ion matches per protein 7; minimum peptides per protein 1 and a false positive value of 4. The maximum protein mass identified was 50kDa. Data was searched against the most recent UniProtKB/Swiss-Prot human database entry [58]. For normalisation, each sample had a known Trypsin concentration, which was used as an internal standard. All protein hits were identified with a confidence of above 95%. The PLGS protein score was based on the probability that the observed match between the experimental data and the PLGS database was not a random event, thus minimising potential random hits. Furthermore, if two or more proteins shared an identical peptide but the peptide in question was regulated differently, then the peptide was not included in the analysis. Peptide intensity was determined as the sum of the peptide ions for all unique peptides for each protein. A total peptide score was taken as the integrated total of all normalised peptide intensities, for all identified ECM proteins [59]. Analysis was performed on matrices derived using cells from two different patients.

Results:

Fibroblast subpopulations produced self-assembled ECMS with different morphology and architectural structure

We first generated cell assembled matrices from each of the three fibroblast sub-populations; DPfi, Pfi and Rfi. These were seeded to achieve full confluency straight away and switched to matrix assembly media at 24 hours to ensure differential proliferation profiles had no effect on matrix production (Figure 1A). Using confocal microscopy images of FN1 (Figure 1B-D) coupled with image analysis algorithms we found that the architecture of the self-assembled ECMs varied between each of the three fibroblast sub-populations

used. Specifically, Pfi generate matrices with significantly thicker fibers than DPfi and Rfi, which are associated with highest amount of interfibrillar space between each fiber (Figure 1E, F). The Pfi derived ECM fibers were also significantly anisotropic compared to both Rfi and DPfi derived matrices (Figure 1G). This bears resemblance to the papillary dermis, which is disorganised in comparison to the reticular dermis [4]. Lastly, when we assessed matrix thickness, the DPfi deposited significantly more matrix than the Pfi (Figure 1H).

Fibroblast sub-populations differentially support basement membrane formation

After determining that ECMs could be successfully generated from each fibroblast subtype, we decided to employ a protocol to generate epithelial only skin constructs, replacing the collagen coating which is usually used with ECM assembled from each cell type. Using alamarBlue to assess keratinocyte viability, we found no significant statistical differences between epithelial only constructs grown on different self-assembled ECMs compared to the control (Figure 2A). Next, we specifically wanted to assess basement membrane formation, since our fibroblast sub-populations were derived from sub-anatomical locations subjacent to, and devoid of basement membrane. We used antibodies against COLLAGEN IV (COL4) and COLLAGEN VII (COL7) to assess basement membrane characteristics. COL4, which is deposited by both fibroblasts and keratinocytes [60], was observed only in keratinocytes grown on Pfi ECM and DPfi ECM (Figure 2B), but not in keratinocytes supported by Rfi ECM, or control constructs (without matrix). COL7, whose production in keratinocytes is stimulated by fibroblasts [61], was also found in keratinocytes in both DPfi and Pfi matrix supported constructs. However, COL7 was not expressed in the control group, or in the Rfi ECM supported constructs (Figure 2C). In addition to immunofluorescence, we used transmission electron microscopy to assess basement membrane formation in our epithelial only skin constructs (Figure 2D). Basement membranes were detected in the control group despite not identifying COL4 or COL7 by immunofluorescence. We also detected basement membrane in constructs supported by Pfi and DPfi matrices, but not Rfi derived matrices. The skin constructs grown on Pfi matrices had a very clear and continuous basal lamina

structure compared to the control. In contrast, the skin constructs supported by DPfi derived ECM had a very thick basement membrane; however this was not continuous across the entire construct.

Mass spectrometry revealed different matrix compositions

Given the different fiber morphologies in our self-assembled ECMS from different fibroblast sub-populations, and a divergent ability to support establishment of a basement membrane, we wanted to determine whether there were also differences in matrix composition. To do this, we performed mass spectrometry analysis of digested matrices devoid of cells.

Our analysis identified a number of different proteins with the maximum protein mass of 50KDa. In total, 12 ECM proteins were identified which passed the 95% accuracy threshold within the DPfi, Pfi and Rfi cell assembled ECMs (Table 2). Based on the total PLGS score, DPfi produced significantly more ECM compared to either the Rfi or Pfi over a 10 day time frame. All identified proteins were interstitial ECM rather than basement membrane ECM. Specifically, FN1, which was used for image analysis was produced equally by all three different fibroblasts subpopulations. Surprisingly, COLLAGEN 1 (COL1), which is generally thought to be expressed throughout the dermis, was not found in either of the Pfi ECM samples, even if we considered peptides that didn't pass the accuracy cutoff threshold.

Interstitial ECM is usually classified into fibrous ECMs (collagens and elastin), proteoglycans, or 'other'. Intriguingly our analysis identified collagens and proteins which fell into the 'other' category, but no proteoglycans (Table 2). Of the proteins in the 'other category, nearly all were adhesive glycoproteins; these are linker proteins which connect cells with fibrous ECM. Of the collagens, the mass spectrometry revealed that while alpha 1 chain COLLAGEN VI (COL6A1) and alpha 3 chain COLLAGEN V1 (COL6A3) were present in all ECMs derived from all cell types, alpha 2 chain COLLAGEN 1 (COL1A2) was only in the DPfi and Rfi matrices, while alpha 2 chain COLLAGEN VI (COL6A2) and alpha 1 chain COLLAGEN 1 (COL1A1) were only in the DPfi ECM. Of the glycoproteins, TENASCIN

(TNC) and FIBULIN 2 (FBLN2) were present in all ECMs, while THBS1 was only present at detectable levels in the DPfi ECM. VITRONECTIN (VTN) was present in the both the DPfi and Pfi ECM, while conversely FIBRILIN 1 (FBN1) and EMILIN 1 (EMIL1) were only found in the Rfi ECM (Figure 3A). As noted above, we did not find any proteoglycans when the accuracy threshold was set to 95%. We did however identify one proteoglycan, BIGLYCAN (BGN), in the DPfi matrix at low levels when the accuracy threshold was lowered to 50%. We cannot be sure if others proteoglycans were expressed within our matrices as no other proteoglycans were detected with our technique. It is highly possible that the selected ECM extraction method selectively obtained only fibrous ECM, and adhesive glycoproteins anchored to these.

In order to verify that proteins detected in our mass spectrometry analysis were expressed within our cell derived ECMS, we stained each cell assembled ECM with antibodies against COL1 (all chains), COL6 (all chains), TNC and THBS1. Despite not finding COL1A1 or COL1A2 in our Pfi ECM by mass spectrometry we found COL1 in all samples (Figure 3B). The COL6 antibody we used also detects all alpha chains, and it appeared to be slightly more, but not significantly abundant in the Pfi ECM, which correlates with observations from an earlier study [55] (Figure 3C). Likewise, TNC was present at similar levels across all cell derived ECMs (Figure 3D). THBS1 was predominantly expressed in DPfi derived matrix (Figure 3E), which corroborates our mass spectrometry results. We also used image analysis to quantify expression levels, and confirm these observations (Figure 3F-I). These results highlight that cells in vitro deposit matrices similar to the ECM they produce in vivo. For example, THBS1 expression is higher within the dermal papilla than the interfollicular skin dermis [16].

Discussion

In the last two decades the role of ECM in cell biology has become more evident. Indeed, ECM is known to regulate cell behaviour, and it plays an essential role during organ development, function and repair [62,63]. On this basis, ECM as a molecular scaffold is fundamental for tissue homeostasis and alterations in a specific ECM component can lead to disruption of this process [64]. Physical properties such as topography and porosity of ECM can influence various anchorage-related biological functions such as cell division and migration [65].

Given the importance of ECM in cell biology, ECM scaffolds are becoming commonplace for tissue repair [45]. However, within the skin at least, dermal scaffolds used to promote regeneration are insufficient. They are composed of only a simple ECM, or decellularized reticular dermis, and they do not take into account the complexity of heterogeneity within whole skin dermis [1]. These limitations highlight the need for bioinspired scaffolds for skin repair.

ECM scaffolds derived from cultured cells offer several advantages compared with decellularized tissues; there are lower risks of pathogen transmission and undesirable inflammatory and immunological reactions [34]. In this study we showed that ECMs derived from three distinct fibroblast sub-types located within the skin dermis were both morphologically, functionally, and compositionally distinct from one another. In particular, the observation that Pfi generate matrices with significantly thicker fibers than DPfi and Rfi is different to ECM organization in vivo, where the papillary dermis contains thinner fibers than the reticular dermis[4]. However, with regards to organization we observed parallels in our cell assembled ECMs, to ECM in different sub-anatomical locations from which the fibroblast sub-types were initially isolated. Specifically, the Pfi assembled matrix had significantly anisotropic fibers, which is analogous to the papillary dermis organisation. The composition of cell assembled ECMs in vitro is also similar to the ECM in the spatial locations from where

the cells were initially derived, indicating that fibroblasts in vitro have a memory of their sub-anatomical origin (Figure 4). In particular, THBS1 was preferentially deposited by DPfi, while the in vivo dermal papilla is rich in THBS1. In addition it is worth mentioning the absence of elastin, and proteoglycans, which are usually key components of dermal ECMs [66]. While we did detect BGN in our DPfi matrices, this was only at low levels when the accuracy threshold was lowered to 50%. However, previous studies of cell-assembled ECM (by GT and JTC) from dermal fibroblasts have identified proteoglycans including versican and decorin using antibodies for detection [55]. We therefore believe the lack of proteoglycans is an artifact of matrix processing for mass spectrometry. For the lack of elastin in our matrices we have an alternative explanation. In development, elastin is deposited relatively late, onto a scaffold of FBN1 microfibrils which forms at an earlier time point [67]. It may be the case that elastin was just not deposited in the 10 days timeframe over which ECMs were generated. In support of this, matrisome analysis of cell assembled matrices derived from neonatal dermal fibroblasts indicates that they also lack elastin [53]. Despite this, fibrillins were observed in both our Rfi assembled matrices and the neonatal dermal fibroblasts matrices, which act as a guide for elastin fiber deposition.

It is well known that the skin dermis and its interaction and crosstalk with epithelial cells is important for the production of the basement membrane components [17]. In our study of epithelial only constructs, COL4 and COL7 were only found in keratinocytes of Pfi and DPfi ECM supported constructs, but not keratinocytes on Rfi ECM or control constructs. This observation is supported by previous studies, where authors have observed that decellularized fibroblast ECM stimulated the production and deposition of COL7 by keratinocytes [61]. Refocusing on constructs supported by Rfi matrices, here we found no evidence of basement membrane establishment with either antibody staining or transmission electron microscopy. This is despite basement membrane formation occurring within our control samples and suggests that there may be an inhibitor of membrane formation within the Rfi matrices. While we removed cells from our matrices, growth factors may have

remained attached to the ECMs onto which keratinocytes were seeded. We therefore cannot conclude that this observation is due specifically to the different ECMs, and it is highly likely that the growth factors constituents produced by the different fibroblast sub-types play an important role directing the establishment of a basement membrane [68]. Other studies have shown that when Pfi and Rfi are grown in co-cultures with keratinocytes, they form cysts [4]. In Rfi-keratinocyte cysts COL7 is absent, however, Pfi-keratinocyte cysts express all basement membrane components evaluated [4]. Based on our observation and the aforementioned study, we postulate that basement membrane formation cannot be supported by ECM produced by Rfi. This again reflects the dermal locations of the cells in vivo, where supporting establishment of a basement membrane is not a usual Rfi cell function. In contrast, DPfi support establishment of a very thick basement membrane, reflecting their location subjacent to a thickened basement membrane termed a glassy membrane in vivo [14]. It is postulated that the expression of high number of basement membrane ECMs in the dermal papilla, as appose to the interfollicular dermis means they have a key role in directing follicular physiology [15].

In conclusion, the differences we observed in ECM composition, fiber morphology, and behavior between the different cell derived ECMs reflects differences that are physiologically present between the papillary, reticular, and hair follicle dermis within the skin. We believe that inspiration can be taken from these physiologically different cell-derived ECMs to improve the design of reliable biomimetic materials with improved therapeutic potential for skin tissue engineering. Self-assembled scaffolds may be useful for clinical application because they mimic natural ECM structurally and compositionally, yet they do not elicit an immunogenic response. The self-assembly process can also be used to establish ECMs of different thicknesses and strengths [49]. Decellularised papillary dermis is not usually used as a tissue engineered product as it is very thin compared to the reticular dermis. Moreover, dermal papilla, which are less than 100μm in diameter have never, to our knowledge, been decellularised and used in tissue engineering; they are simply too small. Using a self-

assembly approach, matrices derived from both DPfi or Pfi cultured in vitro could be superior to ex vivo Rfi derived dermis, or engineered dermal templates, as an effective product for skin engineering.

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Figure Legends

Figure 1: Morphological characterization of cell assembled ECMs. (A) Graph of cell number 1 day and 7 days after seeding cells at equivalent densities highlights fibroblast sub-type specific differences. Representative immunofluorescence images of FN1 stained ECMs generated by DPfi (B), Pfi (C) or Rfi (D) after 10 days in culture. Corresponding graphs determined from analysis of 9 representative images show quantification of fiber diameter for each cell assembled ECM (E), interfibrillar space (F), Gaussian standard deviation of fiber orientation (G) and total ECM thickness (H). Data are represented as the mean + standard deviation. (N=3, n=3). **p<0.01, ****p<0.0001. Scale bars in A-C: 50µm.

Figure 2: Epithelial only skin constructs grown on cell assembled ECMs. (A) Viability assay of cultured keratinocytes on different fibroblast derived matrices (mean ± standard deviation). Immunofluorescence images of COL4 (B) and COL7 (C) in entire skin and epithelial only constructs cultured on coating matrix, or DPfi, Pfi and Rfi derived ECM. (D) Ultrastructural analysis of the basement membrane (arrows) in the epithelial only skin constructs. (n=3) Scale bars for B-C: 50µm. Scale bars for D: 400nm.

Figure 3: ECMs composition. (A) Venn diagram showing distribution of 12 ECM components in matrices assembled from DPfi, Pfi and Rfi. Representative immunofluorescence images of COL1 (B), COL6 (C), TNC (D) and THBS1 (E) in ECMs generated by DPfi, Pfi or Rfi cells after 10 days in ascorbic acid supplemented medium. Quantification of immunofluorescence is plotted in graphs as the mean + standard deviation (F-I) (N=3, n=3). *p<0.05, **p<0.01. Scale bars B-E: 50µm.

Figure 4: Results summary. Human skin in vivo has distinct features established by the fibroblast sub-types within the dermis which create and deposit ECM. For example, the DP is rich in THBS1 while the Rfi deposited matrix contains aligned ECM fibers. Fibroblasts isolated from the skin maintain distinct identities reflecting their origin, and subsequently deposit ECM in vitro which reflects the location from which they were initially isolated.

Tables

Antigen	Source	Catalog number	Species (raised in)	Dilution used
FIBRONECTIN	Sigma-Aldrich	F3648-5mL	Rabbit	1:500
COLLAGEN 1	Abcam	ab90395	Mouse	1:300
TENASCIN C	Abcam	ab6393	Mouse	1:300
COLLAGEN 6	Abcam	ab6588	Rabbit	1:500
THROMBOSPONDIN	Abcam	ab1823	Mouse	1:50
COLLAGEN 7	Abcam	ab93350	Rabbit	1:500
COLLAGEN 4	Abcam	ab6583	Mouse	1:50
Anti mouse-ALEXA fluor 488	Molecular Probes	A-11001	Goat	1:200
Anti rabbit-ALEXA fluor 546	Molecular Probes	A-11010	Goat	1:200

Table 1: Primary and secondary antibodies used in this study

Protein Symbol	Protein ID	DPfi ECM			Pfi ECM			Rfi ECM			ECM type
		PLGS Score	Coverage (%)	Peptide Intensity Sum	PLGS Score	Coverage (%)	Peptide Intensity Sum	PLGS Score	Coverage (%)	Peptide Intensity Sum	
FN1	P02751	13937	49	9138740	4245	44	4349942	4511	47	6198948	Adhesive glycoprotein
COL1A1	P02452	241	25	319566	--	--	--	--	--	--	Fibrous
COL1A2	P08123	1018	15	398538	--	--	--	89	14	26986	Fibrous
COL6A1	P12109	5787	42	1399723	208	18	79824	259	26	142226	Fibrous
COL6A2	P12110	2448	28	721444	--	--	--	--	--	--	Fibrous
COL6A3	P12111	2561	34	3888636	226	23	263054	268	25	683339	Fibrous
TNC	P24821	1931	30	1579325	549	27	647935	681	30	885657	Adhesive glycoprotein
THBS1	P07996	1014	31	575557	--	--	--	129	21	106958	Adhesive glycoprotein
FBLN2	P98095	431	23	171414	129	17	61035	238	24	217243	Ca2+ binding glycoprotein
VTN	P04004	403	16	44674	95	18	12991	--	--	--	Adhesive glycoprotein
FBN1	P35555	--	--	--	--	--	--	118	17	136846	Adhesive glycoprotein
EMIL1	Q9Y6C2	--	--	--	--	--	--	95	9	21431	Adhesive glycoprotein
Total Peptide Intensity		**18237614**			**5414781**			**8392648**			

Table 2: Mass Spectrometry Data of ECM matrices for each fibroblast subtype.
Listed in the table are the protein symbol and accession number (ID) from Swiss-prot for all the ECM proteins detected. PLGS Score: A statistical measure of peptide assignment accuracy. Coverage: Percentage of protein sequence covered by identified peptides for each protein. Peptide intensity sum: Sum of the peptide ion intensities for each protein, normalized against a trypsin internal control. Total Peptide Intensity: Sum of the peptide ions for all peptides identified, for each ECM protein. Values are the average of matrices from N=2.