



Trouillas Marina (Orcid ID: 0000-0001-9196-6588)

## **Influence of fibrin matrices and their released factors on epidermal substitute phenotype and engraftment**

**Short running title:** Fibrin matrices for epidermal substitute engraftment

Maia M Alexaline, Brice Magne, Amparo Zuleta Rodríguez, Muriel Nivet, Daniel Bacqueville, Jean-Jacques Lataillade, Marina Trouillas

Maia M. Alexaline, PhD

Inserm U1197 - IRBA/CTSA, 1 rue du lieutenant Raoul Batany, 92141 Clamart, France

Celogos, 15 rue Béranger, Paris, France

Email: maia.alexaline@gmail.com

Footnote for author's present address if different to where the work was carried out: CEMAG Care, 55 rue de Turbigo, Paris, France

Brice Magne

Inserm U1197 - IRBA/CTSA, 1 rue du lieutenant Raoul Batany, 92141 Clamart, France

Scarcell therapeutics, Paris, France

Email: b.fmagne@gmail.com

Amparo Zuleta Rodríguez

Inserm U1197 - IRBA/CTSA, 1 rue du lieutenant Raoul Batany, 92141 Clamart, France

Email: amparo.zuleta@gmail.com

Footnote for author's present address if different to where the work was carried out: CELLUS Medicina Regenerativa S.A. Av. Kennedy 5757, Las Condes, Santiago, Chile

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Muriel Nivet

Inserm U1197 - IRBA/CTSA, 1 rue du lieutenant Raoul Batany, 92141 Clamart, France

Email: [muriel\\_lizou@yahoo.fr](mailto:muriel_lizou@yahoo.fr)

Daniel Bacqueville

Service Pharmacologie Division 2 et Pharmacocinétique cutanée, Département Pharmacologie,  
Centre R&D Pierre Fabre Dermo-Cosmétique, Toulouse, France

Email: [daniel.bacqueville@pierre-fabre.com](mailto:daniel.bacqueville@pierre-fabre.com)

Jean-Jacques Lataillade

Inserm U1197 - IRBA/CTSA, 1 rue du lieutenant Raoul Batany, 92141 Clamart, France

Email: [jjlataillade@gmail.com](mailto:jjlataillade@gmail.com)

Marina Trouillas

Inserm U1197 - IRBA/CTSA, 1 rue du lieutenant Raoul Batany, 92141 Clamart, France

Email: [trouillasmarina@gmail.com](mailto:trouillasmarina@gmail.com)

**Corresponding author:**

Marina Trouillas, PhD

Unité mixte Inserm U1197 - Institut de Recherche Biomédicale des Armées (IRBA)

Antenne Centre de Transfusion Sanguine des Armées

1 rue du Lieutenant Raoul BATANY

BP 410

92141 CLAMART cedex

Email : [trouillasmarina@gmail.com](mailto:trouillasmarina@gmail.com)

Tel : + 33.1.41.46.72.09

Fax : + 33.1.41.46.72.81

## SUMMARY

Cultured Epithelial Autografts (CEAs) represent a life-saving surgical technique for full-thickness skin burns covering more than 60% total body surface area. However, CEAs present numerous drawbacks leading to heavy cosmetic and functional *sequelae*. In our previous study, we showed that human plasma-based fibrin matrices (hPBM) could improve the reparative potential of CEAs. Therefore, in the present work, we sought to investigate the role of hPBM compared to Fibrin from Purified Fibrinogen (FPF) or plastic support on epidermal substitute formation and engraftment. The use of hPBM for epidermal substitute culture improved keratinocyte migration, proliferation and epidermal substitute organization to a better extent than FPF *in vitro*. Both fibrin matrices favored greater dermal-epidermal junction protein deposition and prevented their degradation. Keratinocyte differentiation was also decreased using both fibrin matrices. Basement membrane protein deposition was mainly influenced by matrix while growth factors released from fibrin especially by hPBM were shown to enhance *in vitro* keratinocyte migration, proliferation and epidermal substitute organization. Ultimately, epidermal substitutes grown on hPBM displayed better engraftment rates than those cultured on FPF or on plastic support in a NOD-SCID model of acute wound with the formation of a functional dermal-epidermal junction. Together, these results show the positive impact of fibrin matrices and their released growth factor on epidermal substitute phenotype and grafting efficiency. Fibrin matrices, and especially hPBM, may therefore be of interest to favor the treatment of full-thickness burn patients.

## KEYWORDS

Cultured epidermal autograft, severe burn, fibrin matrices, growth factors

## INTRODUCTION

Despite a decrease of burn incidence and mortality, severe burns are still a major public health issue correlated with high morbidity (Smolle *et al.*, 2017). Severe burns are characterized by large and deep damages to the skin, suppressing the external barrier essential for survival. Therefore, the restoration of the skin and more specifically of the outer compartment (the epidermis) is crucial. In the most severe burn cases, regenerative stem cells residing in the basal layer of the epidermis and in the dermis are fully destroyed, therefore limiting the reepithelialization to keratinocyte migration from the wound edges (Singer *et al.*, 1999).

To date, the standard medical treatment for severe skin burns after debridement is the autologous Split-Thickness Skin Graft (STSG) coming from an uninjured body area of the patient himself (Papini, 2004). When burns spread over 50 to 60 % of the total body surface area, the lack of healthy skin tissues leads medical staffs to consider alternative treatments to STSG. In those cases, skin allograft cannot be an option for permanent epidermal restoration, as allogenic epidermis is ultimately rejected (Burd *et al.*, 2005). Meshed STSG, meek grafting, micrografting and epidermal blister grafting are neither satisfactory since they result in poor cosmetic and functional outcomes (Stone Li *et al.*, 2018; Ter Horst *et al.*, 2018). Epidermal substitutes of cultured keratinocytes, such as Cultured Epithelial Autograft (CEA) have been used as an alternative treatment since the 1980's (O'Connor *et al.*, 1981). Other techniques to deliver keratinocytes have been further developed such as topical sprays. These techniques are successful to cover large body surfaces and increase patient survival. However, CEA are known to be fragile, induce contracture, produce hyperkeratosis, trigger blistering (possibly due to dermal-epidermal junction (DEJ) immaturity), and be complex and expensive to prepare in laboratory (Chua *et al.*, 2016; Hynds *et al.*, 2018; Ter Horst *et al.*, 2018).

Over the past 30 years, several strategies have been developed to enhance the therapeutic efficacy of epidermal substitutes for the treatment of severe burns (Hynds *et al.*, 2018). For example, specific culture medium supplementations can be added to enhance keratinocyte growth and stemness (Cerqueira *et al.*, 2013; Green *et al.*, 1979). Other strategies consist in employing matrices as supports for keratinocytes growth. Scaffolds of Fibrin obtained from Purified Fibrinogen (FPF) are good candidates due to their unique mechanical properties, their ability to maintain keratinocyte clonality and their successful use in the clinic (Chua *et al.*, 2018; Ronfard *et al.*, 2000). But one way to combine both approaches is the use of clotted human plasma that results in the formation of a Plasma-based Fibrin Matrix (hPBM): a fibrin

scaffold containing all plasma factors without platelets. hPBM have been more recently used as skin scaffolds *in vitro*, *in vivo* (Alexaline *et al.*, 2015; Llamas *et al.*, 2004; Monfort *et al.*, 2013) and on patients (Gomez *et al.*, 2011; Llamas *et al.*, 2006; Mohamed Haflah *et al.*, 2018) showing interesting properties in skin bioengineering. Indeed, we have previously shown that human plasma-based epidermal substitutes (hPBES) have a better regenerative potential than conventional CEAs, with less clonal conversion (Alexaline *et al.*, 2015). FPF and hPBM present several advantages as keratinocyte supports. Both scaffolds allow the production of epidermal sheets less fragile than those produced with traditional methods, easier to handle and able to retain their original size after detachment from culture flasks (Ronfard *et al.*, 2000). Moreover, both FPF and hPBM are biodegradable materials that are removed physiologically after grafting by the fibrinolytic system (Janmey *et al.*, 2009). Additionally, the detachment procedure of these two matrices (Alexaline *et al.*, 2015; Meana *et al.*, 1998) do not necessitate enzymatic treatment (Osada *et al.*, 2016), thus allowing the preservation of DEJ proteins. At last, rete ridges-like structures were observed one month after grafting epidermal substitutes grown on FPF, but not CEA (Ronfard *et al.*, 2000). Thus, the presence of DEJ protein at the grafting time seems to be crucial to enhance epidermal engraftment. Both fibrin scaffolds have shown interesting properties to support skin substitutes. Yet, the different impacts of hPBM or of FPF on keratinocyte phenotype *in vitro* and on the grafting process *in vivo* have never been studied.

In this study, we investigated the influence of hPBM compared to FPF or no matrix on the quality of epidermal substitute formation and its grafting efficiency. We found that fibrin matrices enhanced cellular organization and DEJ protein deposition, while keeping a low level of epidermal cell differentiation. hPBM and FPF influenced stemness, proliferation and *in vivo* engraftment. Eventually, we investigated the role played by fibrin released factors in these differences.

## **MATERIALS AND METHODS**

### ***Cell culture***

Human keratinocytes and human dermal fibroblasts were isolated from skin obtained from female patients undergoing breast reduction surgeries after informed consent. As described previously (Alexaline *et al.*, 2015), skin pieces were incubated overnight at 4°C in 1.8 UI/ml Dispase II (Roche) and 0.0625 % trypsin (Biochrom). Epidermis pieces were separated from

dermis using forceps, and further digested at 37°C in 0.05 % trypsin/EDTA (Gibco) for 30 min. Keratinocytes were immediately frozen and stored. The dermal tissue was digested in 2.4 UI/ml Dispase II and 2.4mg/ml Collagenase II (Gibco), and dermal fibroblasts were plated at 4000 cells/cm<sup>2</sup> in DMEM (Gibco,) supplemented with 5% platelet lysate, 10 µg/ml ciprofloxacin (Bayer Pharma), and 2 UI/ml CHOAY heparin (Sanofi). At 80% of confluence, fibroblasts were either replated after using 0.05 % trypsin/EDTA or frozen. Cryopreserved human dermal fibroblasts were thawed, received a dose of 60 Grays of  $\gamma$  rays to prevent further proliferation and were seeded at 20,000 cells/cm<sup>2</sup>. 4 to 12 h later after this step, primary keratinocytes were thawed and plated at a density of 2,400 cells/cm<sup>2</sup> on growth-arrested fibroblasts in a medium previously described (Alexaline *et al.*, 2015). Before reaching 70 % of keratinocyte confluence, remaining fibroblasts were removed from the flasks by flushing, and keratinocytes were detached from plastic with 0.017 % and 0.05 % trypsin/EDTA, respectively.

### ***Epidermal substitute preparation and fibrin matrices released factor analysis***

hPBM was prepared with a pool of fresh frozen human plasma, issued from 10 volunteers, which were biologically qualified in accordance with French legislation. A mix solution of 39.8 % of pooled plasma (initial fibrinogen concentration at 2.5 mg/mL), 4.66mg/mL NaCl (Fresenius), 0,8 mg/ml Calcium Chloride (Laboratoire Renaudin), and 0.39 mg/mL Exacyl (Sanofi), were poured on appropriate culture plates (0.3 mL/cm<sup>2</sup>) and left to polymerize at 37°C for minimum 3 h. FPF was prepared with the kit Tisseel (Baxter). Solution of 3 UI/mL thrombin and 426 µg/mL Exacyl (prepared in saline solution 1.1 % NaCl and 1 mM CaCl<sub>2</sub>) and fibrinogen (diluted in saline solution to a final concentration of 18 mg/mL with 593 UIK/ml aprotinin) were mixed on a proportion 1:1, poured on appropriate culture plates (0.3 mL/cm<sup>2</sup> for *in vitro* study and 0.1 mL/cm<sup>2</sup> for *in vivo* study) and left to polymerize at 37°C for minimum 3 h. Passage 1 keratinocytes were seeded at 2,400 cells/cm<sup>2</sup> on previously seeded growth-arrested fibroblasts (20,000 cellules/cm<sup>2</sup>) on hPBM, FPF or on plastic from culture plates (No Matrix). After 14 days of culture, epidermal substitutes were analyzed by immunohistochemistry (IHC), by western blot, by transmission electron microscopy (TEM) or treated as a skin biopsy to isolate keratinocyte and perform migration or and long-term proliferation assay. For specific experiments, hPBM and FPF were manually detached from culture plate while epidermal substitutes with no matrix were detached using 0.8 UI/mL dispase at 4°C overnight. These detached epidermal substitutes were either analyzed through IHC, or grafted *in vivo* after less than 10 hours of transport.

Fibrin matrices (hPBM or FPF) without cells were incubated in keratinocyte culture medium which was renewed at 24 h, day 3, 7, 10 and 14. Supernatants were then collected at each medium renewal and stored at -80°C. Factors released from hPBM and FPF in the culture medium at 24 h were analyzed by quantibody array analysis (TEBU) according to the supplier's instructions. Transforming Growth Factor beta-1 (TGF-β1, R&D Systems) were analyzed in supernatant at day 1, 3, 7, 10 and 14 by ELISA (Quantikine® Kits, R&D Systems). Then, culture of keratinocyte with No matrix was achieved during 14 days in a medium supplemented with TGF-β1 (R&D Systems) at the concentrations determined by ELISA at day 1, 3, 7, 10 and 14 (respectively 99.31, 65.63, 26.62, 15.73 and finally 0 pg/ml).

### ***Histology and Immunohistochemistry (IHC)***

*In vitro* and *in vivo* tissue samples were rinsed, fixed with buffered 4% formalin (LaboNord) for 1 day and then dehydrated with a graded series of ethanol treatment prior paraffin (Thermo Scientific) embedding. Paraffin sections of 5µm thickness were dried, deparaffinized, and stained with Hematoxylin, Phloxin, and Safranin (HPS) (All from Dako). Epidermal layer quantification was performed on HPS staining from two observers (10 quantifications per field). For IHC, paraffin section of 5 µm thickness were fixed on polylysine slides (Thermo Scientific). Sections were dried overnight at 37°C and deparaffinized. Antigen retrievals were performed in target retrieval solution 1x pH 6 (for Keratin 19, Filaggrin, Integrin β1, Ki67) or pH 9 (for Keratin 10, Perlecan) (Dako) or enzymatically in a solution containing pronase 1 mg/ml (Sigma) and CaCl<sub>2</sub> 0.1 mg/ml (Renaudin) for 10 min at 37°C (for Collagen IV and Laminin 332) or in a solution containing 80 mg/ml pepsin and 0.2 M HCl (for Collagen VII). 3 % H<sub>2</sub>O<sub>2</sub> (Dako, Denmark) was used to block endogenous peroxidases. Sections were then incubated at room temperature for 30 minutes with primary antibodies: Keratin 10 (1/50, M7002, Dako), Keratin 19 (1/600, ab9221, Abcam), Collagen IV (1/25, M0785, Dako), Collagen VII (1/50, ab6312, Abcam), Filaggrin (1/200, ab218397, Abcam), Integrin β1 (1/100, ab3167, Abcam), Ki67 (1/2, IS626, Dako), Laminin 332 (1/600, ab78286, Abcam), Perlecan (1/250, ab23418, Abcam). Following steps were performed using LSAB™2 Kit (Dako) with Dako autostainer instrument. Images were analyzed with Fiji or Image J software. Percentage of basal keratinocyte expressing Keratin 19 or Ki67 were quantified as the ratio between the number of stained cells and total cells on the basal layer. Percentage of keratinocyte expressing Keratin10 was quantified as the ratio between positive staining and total epidermal surface and the result was normalized by the average number of cell layers. For *in vivo* experiment, as previously described (Alexaline *et al.*, 2015), human reepithelialization percentage was

calculated as the ratio between the length of the Integrin  $\beta 1$  positive stained region (human specific) and the total length of the wound (zone deprived of epidermal appendages). For each epidermal substitute, animals were assigned to a group corresponding to 0 to 10%, 10% to 50% or 50% to 100% of human reepithelialization. Results are presented as a percentage of mice in each classification group.

### ***Animal model of dorsal acute wound***

All procedures were carried out under a protocol approved by the Ethical Committee of “Paris Sud n°26” in accordance with French regulations for animal experiments (01481.03, 2016). NOD/SCID mice were anesthetized via intraperitoneal injection of 6 mg/kg xylazine (Bayer Pharma) and 80 mg/kg ketamine (Virbac). Epidermal Substitutes were grafted on a single full-thickness wound of 1 x 1 cm<sup>2</sup> on the back of each mouse and were protected by a silicon device (Interchim). Mice were left with the silicon protection until day 14 post-grafting, and were sacrificed with an overdose of anesthetics after sedation according to the French Institutional Animal Guidelines. Wounds were excised and fixed in formalin for histology preparation. 18 mice were used per conditions and in each condition 3 donors of keratinocytes were evaluated.

### ***Long term growth and CFE Assay***

Keratinocytes extracted from epidermal substitutes with hPBM, FPF or no matrix were serially passaged. Cells were plated at 2,400 cells/cm<sup>2</sup> in 25 cm<sup>2</sup> culture flasks on growth-arrested murine 3T3-J2 feeder layer (60,000 cells/cm<sup>2</sup>). Before reaching 70% of confluency, keratinocytes were trypsinized, counted, and replated at the same density until passage 10. For growth factor evaluation on colony-forming efficiency (CFE) assay, keratinocytes were plated at low densities (200 cells) in 60 cm<sup>2</sup> petri dishes on growth-arrested murine 3T3 feeder layer (60,000 cells/cm<sup>2</sup>). Keratinocytes were grown during 12 days in culture medium alone or complemented with growth factors: Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) at 23.8 pg/mL (Peprotech), Granulocyte-Stimulating Growth Factor (G-CSF) at 58.97 pg/mL (R&D Systems), Hepatocyte Growth Factor (HGF) at 4.5 pg/ml (Peprotech), Insulin-like Growth Factor-1 (IGF-1) at 5750 pg/mL (Peprotech), InterLeukin-1 $\alpha$  (IL-1 $\alpha$ ) at 2.25 pg/ml (Peprotech), IL-6 at 6.1 pg/ml (R&D Systems), IL-10 at 2.8 ng/mL (Peprotech), Matrix MetalloProteinase-9 (MMP-9) at 215 pg/mL (Life Technologies), PDGF-BB at 23.6 pg/ml (R&D Systems), RANTES at 624 pg/mL (Life Technologies) and Tissue Inhibitor of

MetalloProteinases-1 (TIMP-1) at 2448.1 pg/mL (Life Technologies). Colony surface area was measured using Image J freeware.

### ***Migration Assay***

24-well plates containing 2 well silicone inserts cell-free gap (Ibidi) were seeded at 250,000 or 450,000 cells/cm<sup>2</sup> with irradiated keratinocyte obtained after primary culture trypsinization or isolated from epidermal substitute respectively in keratinocyte culture medium. After 24 h, Ibidi silicone insert were removed and cells were washed three times with PBS 1X. New medium were added supplemented or not with culture insert (PET membrane, EMD Millipore) with pore size of 0.4 μm, containing 100 μL of hPBM or FPF. Pictures of the gap were taken at 20, 24 and 48 h and analyzed by Image J software. Migration percentage were calculated as followed:  $((A_{T0}-A_{Tx})/A_{T0}) \times 100$ , A<sub>T0</sub>: Area of the gap at 0h, A<sub>Tx</sub>: Area of the gap at 20, 24 or 48 h.

### ***Western Blot***

Epidermal substitutes were rinsed with PBS 1X, cut in small pieces and lysed in a buffer containing PBS 1X, 1 % NP 40, 0.1 % of SDS, 0.5 % of deoxycholic acid and 1X of protease inhibitor cocktail (all from Sigma). Bio-rad Protein Assay were used to measure the protein concentrations of cell lysates. Equal amount of protein samples were loaded, separated on SDS-PAGE 10% (Bio-Rad) and then electro-transferred on PVDF membrane (Immobilon-P Transfer Membrane, Millipore). The membranes were blocked in PBS 1X, 2 % Tween 20 and 5 % skimmed milk for 1 h and were incubated overnight at 4°C with primary antibodies β-actin (1/1000, ab8227, Abcam), Laminin 332 (1/800, MAB1956, Merck Millipore 2) or Perlecan (1/250, 13-4400, Invitrogen). Membranes were then incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (IgG) (Santa-Cruz). Chemiluminescence reagents (Bio-Rad) were used to reveal antibody-binding and signals intensity were detected with Chemidoc instrument and analyzed with Image Lab software.

### ***Transmission Electron Microscopy (TEM)***

The tissues were fixed in 2 % glutaraldehyde in 0.1 M Sorensen phosphate buffer (pH 7.4) at 4°C for at least 4 h, and washed overnight in 0.2 M phosphate buffer (Centre de Microscopie Electronique Appliquée à la Biologie, CMEAB, Toulouse, France). They were post-fixed in 1 % osmium tetroxide in 250 mM saccharose and 0.05 M phosphate buffer for 1 h in the dark at

room temperature. The samples were then dehydrated in a series of graded ethanol solutions, up to 70 % ethanol. From then, samples were embedded in Embed 812 resin (*Electron Microscopy Sciences*) using a Leica EM AMW automated microwave tissue processor for electron microscopy. Finally, the tissues were sliced into 70-nm thick sections (Ultracut Reichert Jung) and mounted on 100-mesh collodion-coated copper grids prior to staining with 3 % uranyl acetate in 50 % ethanol and Reynold's lead citrate. Examinations were carried out on a Hitachi HT7700 transmission electron microscope at an accelerating voltage of 80 kV.

### ***Statistics***

Statistical analysis was achieved using R software (3.5 version) with the use of RVAideMemoire, lme4 packages. Prism 6 Graphpad software was used to realize graphical representations. Depending on experiments, paired t-tests, one sample t-tests, Anova and pairwise comparisons using paired t tests, Permutation Analysis of Variance Table and Pairwise comparisons using permutation paired t tests or Kruskal-Wallis and Pairwise comparisons using Wilcoxon rank sum test were used. When multiple comparisons were used inside a single experiment, p-values were corrected using the FDR method.

## **RESULTS**

### ***hPBM allows the engineering of epidermal substitutes with proliferative and well-organized undifferentiated keratinocytes.***

To better understand the enhancement of epidermal regeneration using hPBM observed in our previous study (Alexaline *et al.*, 2015), we first compared cellular organization, proliferation, stemness and differentiation of epidermal substitutes cultured for 14 days on hPBM, FPF, or without any matrix (no matrix). Keratinocytes cultivated on hPBM formed a well-organized basal layer of cuboidal cells similar to the basal layer of healthy skin (Figure 1A and Supplemental Data 1A). In comparison, epidermal substitutes with no matrix were composed of spindle shaped keratinocytes. The mean number of keratinocyte layers formed on hPBM was significantly higher than on FPF (1.56-fold,  $p < 0.05$ ) or on no matrix (1.71-fold,  $p < 0.05$ ) (Figure 1B). This increase in cell layers could be related to the higher number of proliferating basal keratinocytes (expressing Ki67 proliferation marker) on hPBM compared to FPF (2.08-fold,  $p < 0.05$ ) or no matrix (1.8-fold,  $p < 0.05$ ) (Figure 1A and 1C). Percentage of Keratin 19 positive basal cells was significantly higher on FPF compared to no matrix (2.69-fold,  $p < 0.01$ )

or hPBM (3.17 fold,  $p < 0.01$ ) (Figure 1A and D). Culture on a fibrin matrix (hPBM or FPF) significantly reduced the percentage of the supra-basal differentiation marker Keratin 10 compared to no matrix (8.25 and 14.28-fold respectively, Figure 1A and E). Similar results were obtained for the terminal differentiation marker Filaggrin (Data not shown). TEM analysis confirmed these results and showed features of incomplete differentiation (Supplemental Data 1B), desmosomes between keratinocytes (Supplemental Data 1C) and stratum granulosum (Supplemental Data 1D). Overall, fibrin matrices (hPBM or FPF) thus improve cellular organization and decrease differentiation of epidermal substitutes. The use of FPF specifically increases the expression of epidermal stemness markers, while hPBM stimulates the expression of cell proliferation markers.

### ***Fibrin matrices enhance basement membrane protein deposition and conservation***

We next investigated by IHC, by TEM and by western blot whether fibrin matrices affect basement membrane protein deposition during epidermal substitute culture. Therefore, we first analyzed by western blot Laminin 332 and Perlecan expressions during the culture of epidermal substitutes. Cultures on hPBM or FPF significantly expressed more Perlecan (84.51 and 120.21-fold respectively,  $p < 0.05$ , Figures 2A and B) and Laminin 332 (8.56 and 8.21-fold respectively,  $p < 0.05$ , Figures 2A and C) compared to no matrix. Then, we assessed by IHC Perlecan, Laminin 332 and Collagen IV expressions in epidermal substitutes detached from their culture plastic flasks by mechanical or enzymatic treatment for epidermal substitutes with fibrin matrices or with no matrix respectively. We observed that these proteins remained deposited all along the junction separating basal keratinocytes from their matrices in hPBM and FPF (Figure 2D). TEM observations of epidermal substitutes grown on hPBM further showed immature DEJ features, including forming hemidesmosomes (Supplemental data 2). However, we could not see these proteins in epidermal substitutes cultured on no matrix (Figure 2D), suggesting that their detachment caused the loss of DEJ proteins like Laminin. Together, these results therefore suggest that fibrin matrices do not only allow the conservation of DEJ proteins after epidermal substitute detachment, but also enhance DEJ protein deposition early in keratinocyte culture.

### ***Keratinocyte long-term growth and migration are improved by the use of hPBM***

We deepened our investigations to characterize fibrin matrix influence, by studying keratinocyte properties (i.e. long-term growth and migration) after extraction from epidermal substitutes. We first explored whether Keratin 19 expression could be linked to long-term

growth and clonal potential of keratinocytes extracted from our cultured epidermal substitutes. Our experiments indicated a higher number of keratinocytes directly after extraction from epidermal substitutes grown on hPBM compared to FPF (Figure 3A). When keratinocytes extracted from epidermal substitutes were subjected to long-term culture (from passage 1 to 10), the slope of hPBM keratinocyte cumulative numbers was slightly higher than with FPF after the 7<sup>th</sup> passage (Figure 3A). We next evaluated the migration potential of extracted keratinocytes in an *in vitro* wound closure assay. Our results showed that keratinocytes extracted from hPBM migrate faster than those extracted from no matrix or FPF at 20 and 24 h ( $p < 0.01$  and  $p < 0.05$  respectively, Figure 3B). In summary, our results suggest that hPBM has a long-lasting influence on keratinocyte properties, as it stimulates their growth and migration even after their detachment from it.

***The use of hPBM as a culture support for epidermal substitutes allows better engraftment in a NOD-SCID model of dorsal acute wound.***

We then compared the *in vivo* grafting ability of epidermal substitutes grown on hPBM, FPF and no matrix in a NOD-SCID model of acute wound injury. After 2 weeks, epidermal substitutes cultured on hPBM induced a better human wound reepithelialization than those grown on FPF or no matrix ( $p < 0.01$ , Figure 4A). The *in vivo* grafting of epidermal substitutes grown on fibrin matrices (hPBM or FPF) induced the formation of a regenerated epidermis with a basal layer containing columnar keratinocytes expressing Integrin  $\beta 1$ , Ki67 and Keratin 19 (Figure 4B, Supplemental data 3). While, Collagen type VII was only slightly deposited along the basement membrane, other DEJ proteins such as Collagen type IV, Laminin 332 and Perlecan were continuously deposited 14 days after grafting (Figure 4B and Supplemental data 3). These proteins were well organized into mature hemi-desmosomes as shown by TEM (Figure 4C). All suprabasal layers were stained with Keratin 10 while uttermost layers expressed Filaggrin as in a healthy epidermis (Figure 4B, Supplemental data 3). Stratification of all epidermal layers with terminal differentiation and cornification was also confirmed by TEM (Figure 4D). The stratum granulosum and spinosum displayed typical features such as keratohyalin granules (Figure 4E) and numerous desmosomes respectively (Figure 4F). Thus, two weeks after wound grafting, epidermal substitutes grown on hPBM induce better reepithelialization compared to other substitutes, and promote the formation of a neo-epidermis close to healthy skin in terms of basal layer organization, DEJ assembly and stratification.

### ***hPBM matrix releases more factors than FPF.***

To better understand the mechanisms of action of the two fibrin matrices on epidermal growth and grafting, we then decided to investigate the factors released by hPBM and FPF in culture medium (Figure 5). Some factors were only released by hPBM such as IFN $\gamma$  (2.18 pg/mL), HGF (3.73 pg/mL), GM-CSF (23.78 pg/mL), PDGF-AB (43.54 pg/mL) and IGF-1 (9087.6 pg/mL). hPBM released other factors to a greater concentration than FPF, including PDGF-BB (23.82 vs. 0.56 pg/mL), VEGF (45.76 vs. 3.34 pg/mL), G-CSF (53.68 vs. 13.32 pg/mL), TGF- $\beta$ 1 (99.31 vs. 3.9 pg/mL), MMP-9 (218.87 vs. 112.04 pg/mL), RANTES (623.39 vs. 31.3 pg/mL) and TIMP-1 (2492.48 vs. 418.29 pg/mL). Besides, IL-6, IL-1 $\alpha$ , bFGF and MMP-1 were released both from hPBM and FPF at a similar level. We then established the release kinetic of TGF- $\beta$ 1 and other factors from the two fibrin matrices in the culture medium following established timing of medium renewal for epidermal substitute culture. We observed an initial burst of factor release followed by a progressive decrease along medium changes. TGF- $\beta$ 1 was more released in the medium from hPBM than from FPF (data not shown). Thus we showed that both fibrin matrices release factors during the culture process, with more factors brought by hPBM.

### ***hPBM-released factors enhance keratinocyte morphogenesis, proliferation, migration and basement membrane deposition.***

Based on our previous results, we hypothesized that the differences observed between hPBM, FPF and no matrix on epidermal substitute development and engraftment could be due to the release of specific factors. Therefore, we evaluated the effect of a medium supplemented with total released factors from hPBM or FPF on keratinocytes cultured with no matrix. We first observed that the addition of total released factors from hPBM improved epidermal substitute basal cell organization (Figure 6A). The percentage of Ki67 positive basal cells was also enhanced in epidermal substitutes grown with no matrix and hPBM-released factors compared to control ( $p < 0.01$ , Figure 6B). We then investigated the role of hPBM- and FPF-released factors on keratinocyte migration *in vitro*. hPBM-released factors enhanced keratinocyte wound closure at 24 and 48 h compared to control ( $p < 0.05$ ) and FPF-released factors (not significant) (Figure 6C). At last, as both hPBM and FPF favored DEJ protein deposition, we evaluated the role of fibrin matrix-released factors on Laminin 332 and Perlecan deposition during epidermal substitute culture. Our results showed that both proteins were slightly more expressed in the presence of hPBM and FPF-released growth factors (Figure 6D, E and F),

despite expressing levels did not reach those observed when cultured on fibrin matrices (data not shown). Overall, we showed that both hPBM and FPF released factors in culture medium slightly enhance DEJ protein expression. hPBM released factors enhance basal keratinocyte morphology, proliferation, and migration.

We next investigated whether some hPBM released factors could influence keratinocyte clonogenicity. Thus, we evaluated separately the effect of several factors released from hPBM at their level after one day immersed in the medium (Figure 6G). Our experiments showed that G-CSF significantly enhanced the mean area of keratinocyte colonies ( $p < 0.05$ ), while GM-CSF, HGF, IL-1A, MMP9, PDGF-BB and TIMP1 only slightly enhanced it (not significant, Figure 6G). As TGF- $\beta$ 1 was released at higher concentration by hPBM (Figure 5), and since it is a well-known regulator of DEJ synthesis, we then evaluated the effect of a medium supplemented with TGF- $\beta$ 1 on DEJ expression in epidermal substitutes grown on no matrix. Our results showed a small increase of Laminin 332 expression in the presence of TGF- $\beta$ 1 (not significant, Figure 6H). These data thus indicate that G-CSF and TGF- $\beta$ 1 could play a role in keratinocyte clonogenicity and DEJ protein deposition respectively but are not sufficient to explain the differences observed between hPBM and FPF.

## DISCUSSION

During the last 30 years, the development of skin substitutes has considerably improved with the arising of scaffolds in tissue engineering. FPF matrices have been first introduced, followed, more recently, by hPBM for the culture of epidermal substitutes. In this work, we sought to understand the mechanisms underlying the favorable properties of hPBES, an epidermal substitute cultured on hPBM, and developed in our laboratory (Alexaline *et al.*, 2015). For the first time, this study also aimed to underline the best scaffold among hPBM, FPF and no matrix to support epidermal substitute growth and *in vivo* engraftment. Herein, we showed that both fibrin matrices (hPBM and FPF) supported the formation of organized and undifferentiated epidermal substitutes, presenting well-preserved DEJ protein networks. Notably, hPBM scaffolds stimulated pro-migratory and pro-proliferative properties in keratinocytes and improved epidermal substitutes quality leading to better *in vivo* engraftment, compared to FPF and no matrix. Mechanistically, we showed that hPBM-released factors were at least partly responsible for the improvement of keratinocyte proliferation, morphology, migration and stemness during culture.

Fibrin matrices have been used for the culture of several cell types, especially keratinocytes and fibroblasts, and reported to enhance cell proliferation (Sese *et al.*, 2011). Moreover, it has been shown that the presence of basement membrane proteins such as Laminin 332 (Yamada *et al.*, 2018), Collagen IV (Segal *et al.*, 2008), or Perlecan (Nakamura *et al.*, 2011) would be favorable to the proliferation of keratinocyte. In our study, we showed that the use of fibrin matrices (hPBM and FPF) enhanced the deposition and the preservation of basement membrane proteins, and favored basal keratinocyte proliferation, compared to cultures with no matrix (Figure 1). These results thus suggest that the preservation of DEJ proteins may help maintain the proliferative properties of keratinocytes. A higher level of keratinocyte proliferation is required so that a neo-epidermis rapidly forms after grafting, with a number of cell layers close to that of normal skin. In fact, we showed in our study that basal keratinocytes cultivated on hPBM were more proliferative compared to FPF due to the nature of hPBM-released factors (Figure 1 and 6). Among those factors, IGF-1, GM-CSF, PDGF-AB and HGF are known to stimulate keratinocyte proliferation (Shirakata, 2010). Another study also showed that some of these factors like PDGF-AB could favor the formation of a thicker epidermis once combined with a biodegradable matrix in a burn wound model (Mittermayr *et al.*, 2016). Therefore, fibrin matrices containing growth factors are likely to enhance keratinocyte proliferation, which is important for reepithelialization and wound healing.

During wound healing, reepithelialization is a crucial step to close the wound and needs to be achieved rapidly. At this step, keratinocytes migrate from the edges of the wound to close the gap. In case of severe burns, keratinocytes migrate from SSG or CEA to reepithelialize the gap between the graft and the wound edges. Therefore, it is crucial that keratinocytes from epidermal substitutes keep their migratory potential. In our study, we demonstrated in a wound closure assay that hPBM-released factors induced more keratinocyte migration *in vitro* compared to control (Figure 6C). In fact, some factors only released by hPBM are known to induce migration such as HGF, PDGF-AB and IGF-1 (Peplow *et al.*, 2013). Nam *et al.* showed for example that HFG used at 6 ng/ml was able to induce keratinocyte migration (Nam *et al.*, 2010). However, this group also showed that a combination of HGF and TGF- $\beta$ 1 could result in a better migration profile of keratinocyte. Therefore, a combination of several growth factors even at low levels might be responsible for the observed enhancement of migration in our study. Several groups have also demonstrated that basement membrane proteins such as Laminin 332 (Tjin *et al.*, 2014), Collagen IV (Bush *et al.*, 2007) or Perlecan (Nakamura *et al.*, 2015) favor keratinocyte adhesion and are implicated in migration processes (Frank *et al.*,

2004). In our study, we demonstrated that keratinocytes extracted from epidermal substitutes cultivated on hPBM could (thus exposed to hPBM released factors and basement membrane proteins) retain their higher migratory potential (Figure 3B) and had a better adhesion level (data not shown). This therefore indicates that keratinocytes extracted from epidermal substitutes grown on hPBM possess a higher migratory potential, probably due to the fact that these substitutes contain pro-migratory factors and DEJ proteins.

The aim of using an autologous epidermal substitute is to maintain long-term epidermal homeostasis. However, this is only possible when epidermal stem cells are preserved within the graft. Indeed, if progenitor cells appear to participate in the normal renewal of the epidermis, it is the stem cell population of keratinocytes that ensures the regeneration of the epidermis in the long term (Mascre *et al.*, 2012). In our study we therefore evaluated the impact of fibrin scaffolds on keratinocyte stemness. We found that FPF scaffolds were better than hPBM to induce the expression of the epidermal stemness marker Keratin 19 in basal keratinocytes (Figure 1A and C). TGF- $\beta$ 1 is known to decrease Keratin 19 expression in epithelial cells or carcinoma (Sato *et al.*, 2010) and could thus explain the low level of Keratin 19 observed in hPBES. However, this result was uncorrelated to functional assays, as keratinocytes extracted from epidermal substitutes grown on hPBM displayed a better long-term growth potential than those extracted from FPF scaffold *in vitro* (Figure 3A). Our mechanistic study showed that some hPBM released factors, like HGF, had a slight positive impact on keratinocyte clonogenicity (Figure 6G), while TGF- $\beta$ 1 tended to decrease it (data not shown). The final clonogenic potential of keratinocytes extracted from epidermal substitutes grown on hPBM is relatively good compared to those used clinically (Alexaline *et al.*, 2015), possibly due to a synergy of several growth factors released by hPBM. In our study, both fibrin matrices induced a decrease in the keratinocyte differentiation level (Figure 1A and E). This finding correlates with the detachment of differentiated keratinocytes from fibrin *in vitro* observed by Geer and Andreadis (Geer *et al.*, 2003). Thus, fibrin matrices seem to select undifferentiated keratinocytes in the first phase of culture by adhesion to fibrin, similarly to the selection of high Integrin  $\beta$ 1-expressing keratinocytes obtained with collagen IV substrates (Fortunel *et al.*, 2011). Moreover, we observed that both fibrin matrices, and more particularly hPBM, favored the formation of a well-organized basal keratinocyte layer in cultured epidermis (Figure 1A). This appeared to be due to the hPBM-released factors that were shown to improve the basal organization of epidermal substitutes cultured on no matrix (Figure 6A). This enhancement could involve the Insulin/IGF-1 signaling pathway that controls p63 activity and

epidermal morphogenesis (Gunschmann *et al.*). The presence of DEJ components, such as Perlecan, Collagen IV or *rete ridges*, contributes to epidermal morphogenesis and homeostasis (Nie *et al.*, 2013). As a matter of fact, exogenous supplies of Collagen IV has been shown to enhance Keratin 19 expression in epidermal substitutes (Yang *et al.*, 2016). Therefore, the unique properties of stemness, organization and low differentiation displayed by epidermal substitutes grown on hPBM may be due to fibrin and plasma factors.

In our study, we showed that the use of both fibrin matrices allowed the deposition and the preservation of Laminin 332, Collagen IV and Perlecan, whereas none of these proteins were present on epidermal substitutes with no matrix after disperse detachment at the time of grafting (Figure 2D). The enzymatic step is not solely causing the absence of these proteins as only Laminin 332 and Perlecan were expressed at a low level in epidermal substitutes before enzymatic treatment (Figure 2A, (Matsumura *et al.*, 2013)). Moreover, hPBM- and FPF-released factors slightly enhanced Laminin 332 and Perlecan in epidermal substitutes with no matrix (Figure 6D, E and F). PDGF-AB, IGF-1 and TGF- $\beta$ 1 have been shown to positively influence basement membrane protein expression (Amano *et al.*, 2004; Eming *et al.*, 1998). In our study, the addition of TGF- $\beta$ 1, at the release level observed in hPBM, slightly enhanced Laminin 332 expression in epidermal substitutes with no matrix. However, this positive effect on basement membrane production was not sufficient to reach the expression observed with hPBM or FPF scaffolds. Other studies have shown that the conservation of basement membrane before grafting was essential for a good graft take of epidermal substitutes (Osada *et al.*, 2016). In our study, although the basement membrane of epidermal substitutes grown on hPBM was not fully organized before grafting (Supplemental data 2), it may have contributed to a favorable effect on engraftment and DEJ development later on *in vivo* (Figure 4C). Indeed, two weeks after grafting, the regenerated epidermis showed a well-organized DEJ with numerous hemidesmosomes (Figure 4C). Thus fibrin scaffolds are important to make keratinocytes start the formation of a mature DEJ and favor engraftment.

A good engraftment rate is the key for a successful wound healing in patients. CEA grafting with no dermal preparation often leads to disastrous clinical failure (Herzog *et al.*, 1988). In our study, we developed an excisional wound NOD/SCID mouse model in which the grafting of epidermal substitutes was devoid of a dermal preparation, so to simulate a situation in which the quality of the wound bed is unsatisfactory. In this specific context, we showed that fibrin matrices allowed a better engraftment of epidermal substitutes in comparison to those grown on plastic dishes two weeks after grafting (Figure 4). These good engraftment rates could be

explained by an improvement of the DEJ formation, a higher keratinocyte proliferation and migration potential, but might also be the result of a better neoangiogenesis induced by the presence of fibronectin and fibrin degradation byproducts in hPBM and FPF scaffolds (Bootle-Wilbraham *et al.*, 2001). Ultimately, the presence of fibrin matrices in epidermal substitutes may act as a temporary papillary dermis, releasing growth-factors and providing ECM proteins, needed to support an *in vitro* keratinocyte pro-reparative phenotype, a good engraftment rate, and the formation of an healthy neo-epidermis *in vivo*. At present, these promising results would need further investigations to bring fibrin matrices, and more particularly hPBM, from the bench to the bed side.

## CONCLUSION

In our study, we compared a well-known type of scaffolds used for skin bioengineering that is fibrin from purified fibrinogen (FPF), to a more recently introduced fibrin matrix obtained from clotted human plasma (hPBM). We have shown that hPBM combines the advantages of a fibrin polymer and a plasma factor-releasing scaffold for skin tissue engineering. Hence, hPBM appears to be a relevant matrix for improving clinical epidermal substitutes with superior properties than FPF or no matrix, and a fabrication that can be easily translated to a clinical grade process. Essentially, epidermal substitutes grown on hPBM could be transformed or combined to other cell types so as to obtain a skin substitute including all layers from hypodermis to epidermis and skin appendages.

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## CONFLICT OF INTEREST STATEMENT

The authors indicate no potential conflicts of interest.

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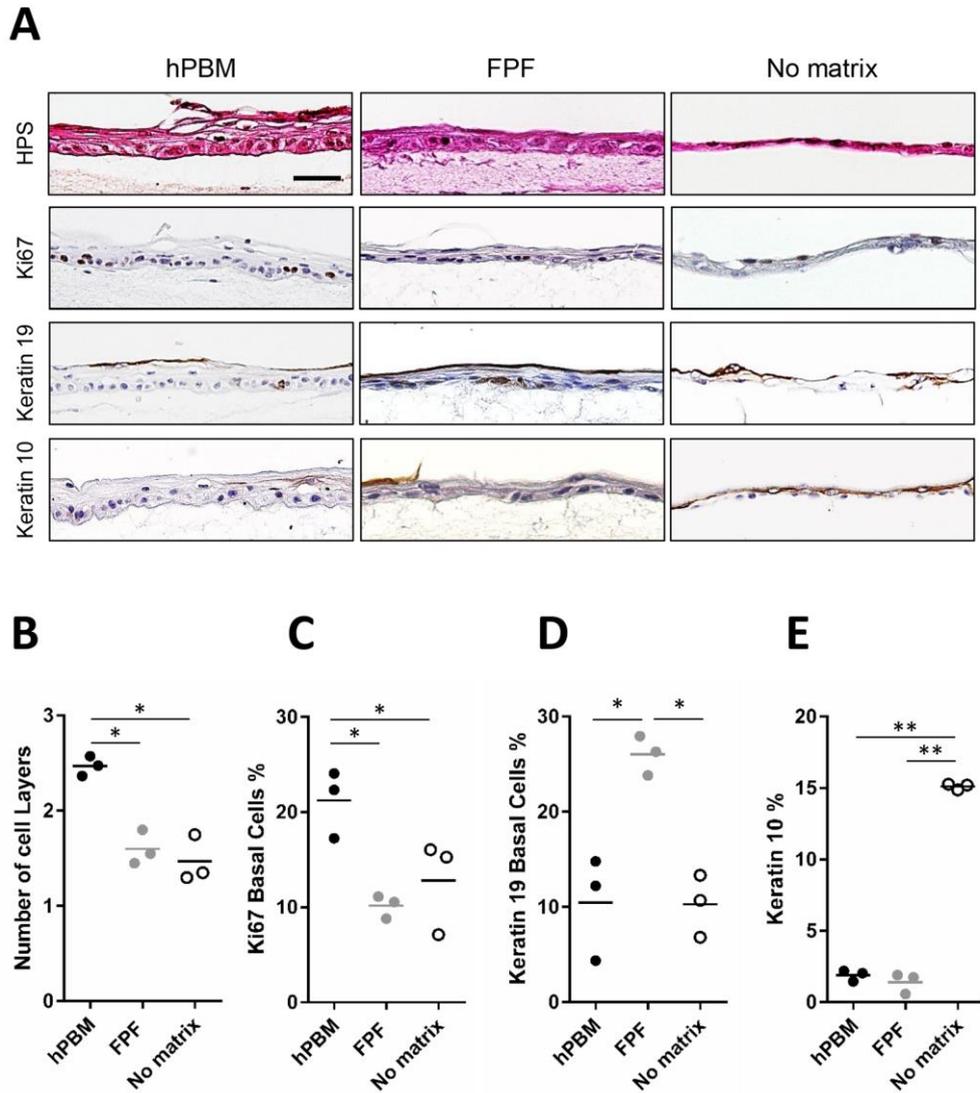
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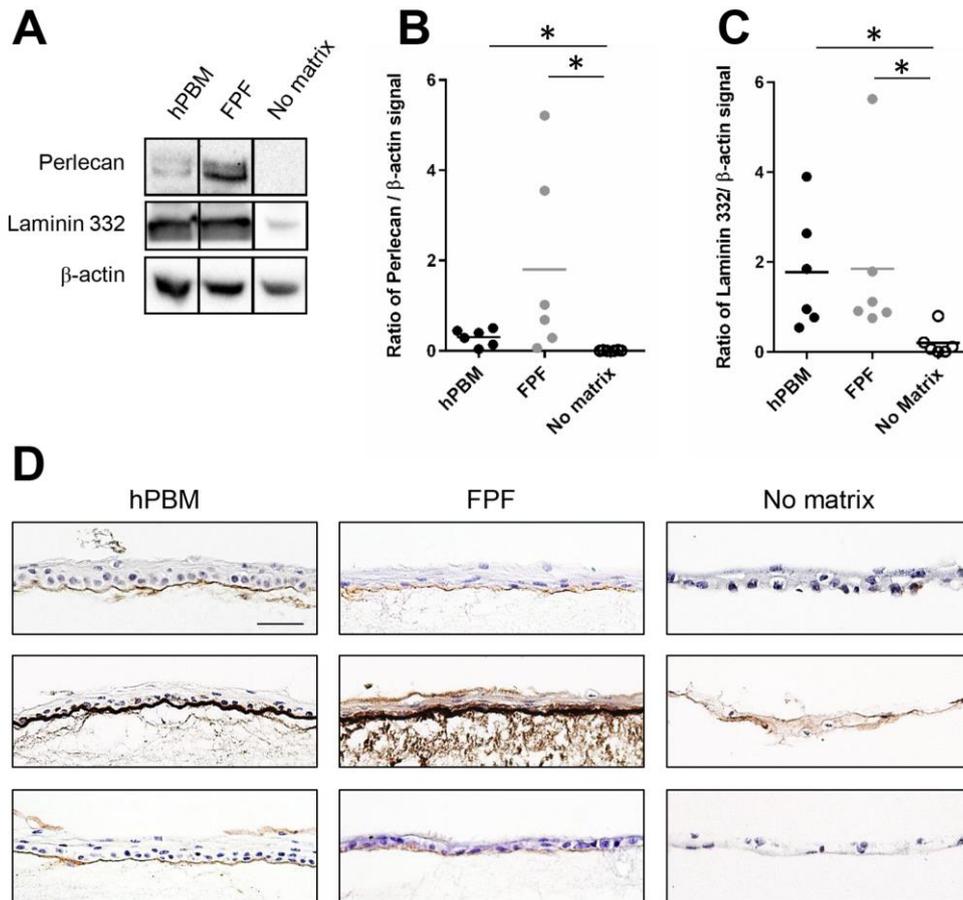
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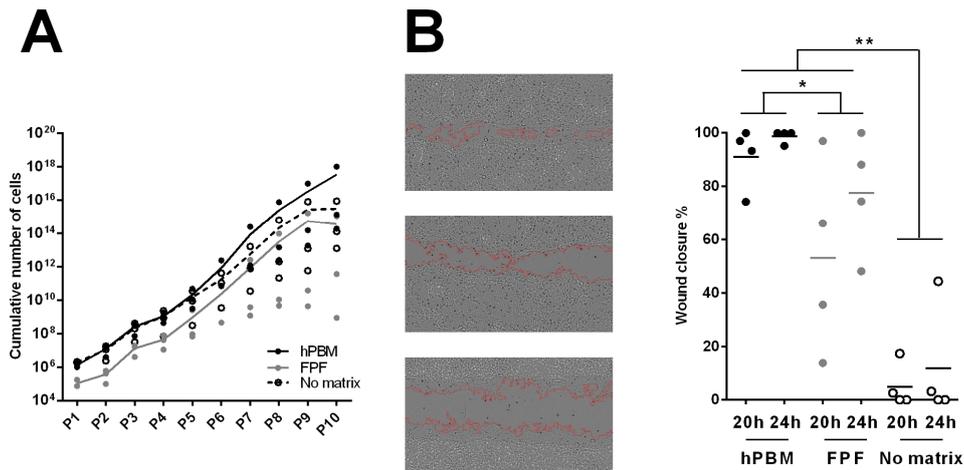
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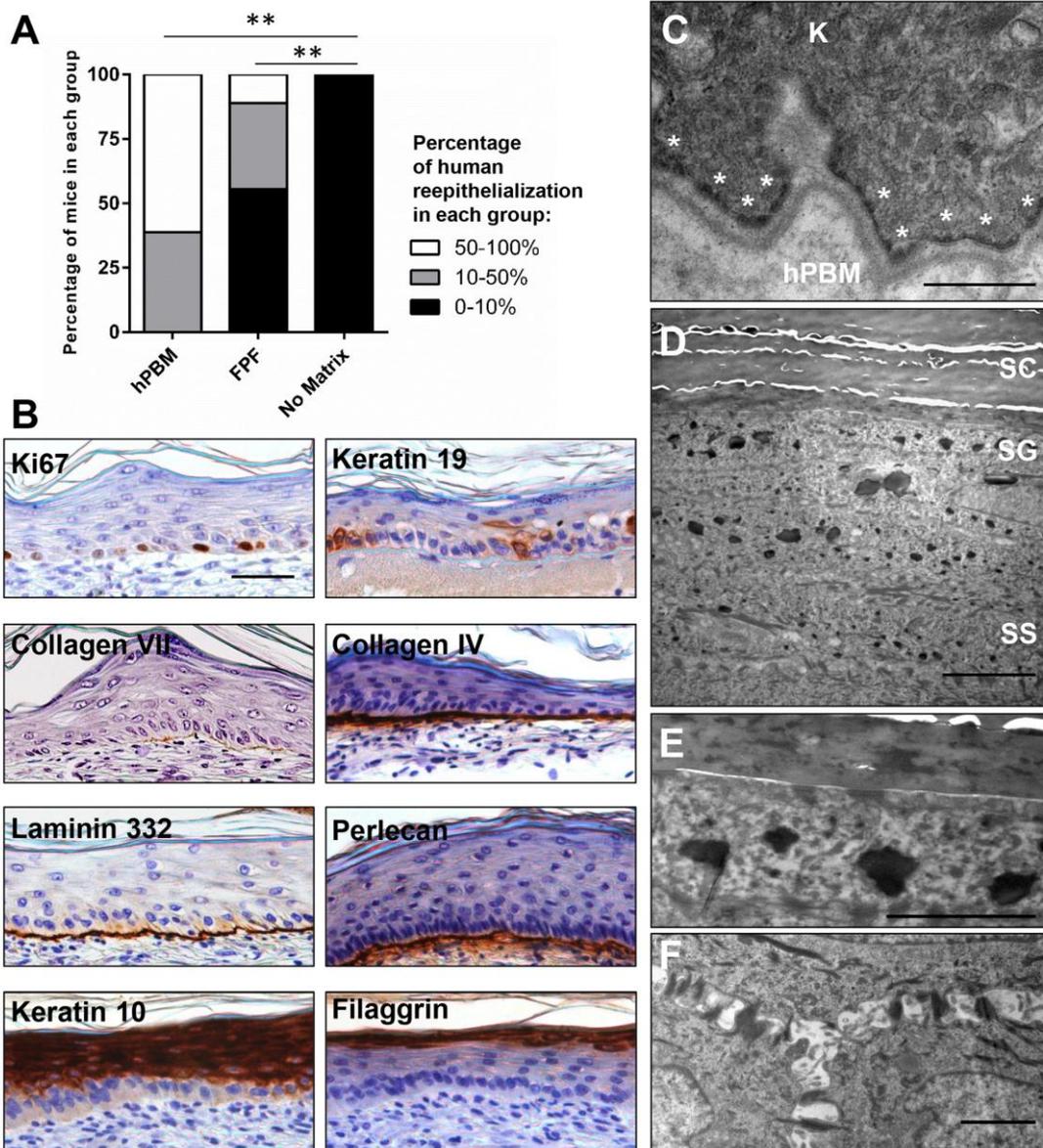
**Figure 1: Fibrin matrices influence keratinocyte organization, proliferation and differentiation.** (A) Representative photos of HPS and IHC staining of Ki67, Keratin 19 and Keratin 10 (scale bar: 50  $\mu$ m) of epidermal substitute after 14 days *in vitro* culture. Staining of the upper layer with the Keratin 19 antibody is nonspecific. (B) Quantification of cell layer number, and quantifications measured on IHC staining for (C) percentage of basal keratinocytes expressing Ki67, (D) percentage of basal keratinocytes expressing Keratin 19 and (E) percentage of keratinocytes expressing Keratin 10. Mean was represented on the dot plot. ANOVA and Pairwise comparisons using paired t tests with FDR correction were used to compare two distinct experimental groups (\* $<0.05$ , \*\*  $p<0.01$ ,  $n=3$  donors of keratinocytes).



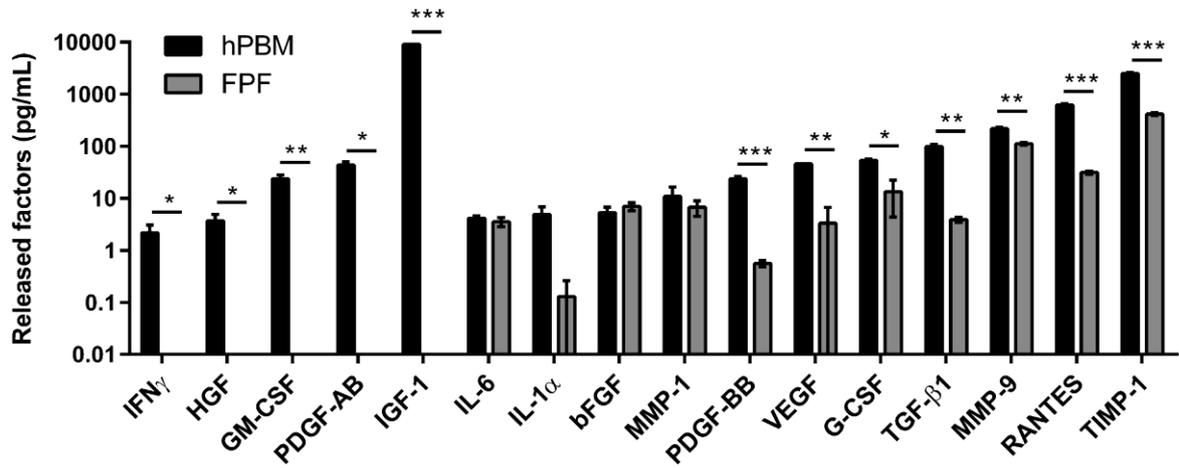
**Figure 2: Fibrin matrices allow basement membrane protein deposition and conservation.** (A) Western blot analyses of Laminin 332, Perlecan and  $\beta$ -actin expression in epidermal substitutes with hPBM, FPF or No matrix, after 14 days of culture without detachment procedure. Quantification of (B) Laminin 332 or (C) Perlecan band intensity, presented as the ratio of signals of interest on  $\beta$ -actin signals. Mean was represented on the dot plot (n=6 donors of keratinocytes), Kruskal-Wallis and Pairwise comparisons using Wilcoxon rank sum test were used to compare two conditions (\*  $p < 0.05$ ). (D) Representative IHC stainings of Laminin 332, Collagen IV and Perlecan on epidermal substitute after manual (hPBM or FPF) or enzymatic (No matrix) detachment (n=3) (Scale bar: 50  $\mu$ m).



**Figure 3: Culture on hPBM matrix does not affect long term amplification and favors migration potential.** (A) Cumulative number of cells, extracted from epidermal substitutes on hPBM, FPF or no matrix, at each passage. (B) Representative pictures (at 20H) and quantification of percentage of *in vitro* wound closure by keratinocyte extracted from epidermal substitutes cultivated on hPBM, FPF or no matrix, at 20 and 24 h. Mean was represented on the dot plot (n=4 donors of keratinocytes), Permutation Analysis of Variance Table was used to compare experimental groups (\* p<0.05, \*\* p<0.01).

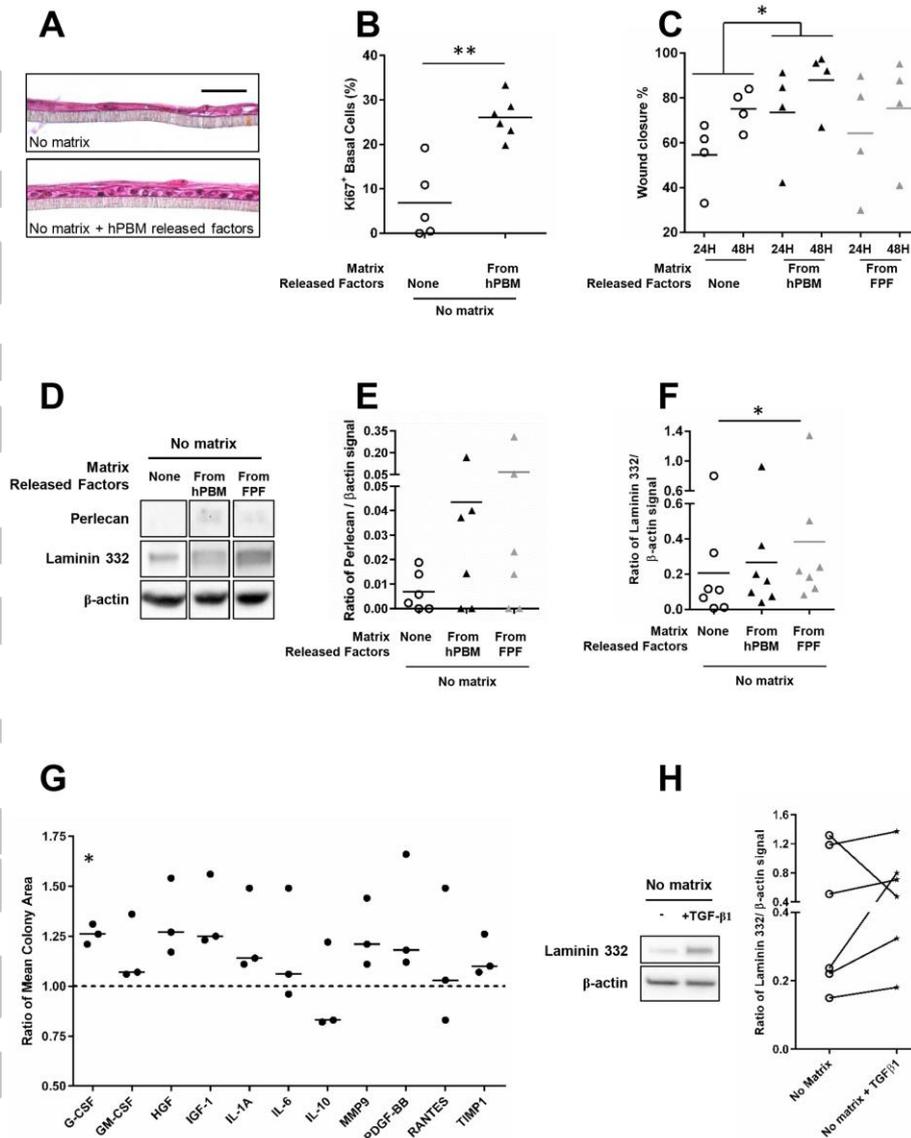


**Figure 4: Epidermal substitutes on hPBM engraft well in an acute wound and regenerate a neo-epidermis close to healthy skin.** (A) Reepithelialization scores are expressed as a percentage of mice present in each reepithelialization groups (0-10%, 10-50%, 50-100%). (n= 18). Kruskal-Wallis and Pairwise comparisons using Wilcoxon rank sum test were used to compare two conditions (\*\*p<0.01). (B) Representative pictures of regenerated epidermis stained with Ki67, Keratin 19, Collagen VII, Collagen IV, Laminin 332, Perlecan, Keratin 10 and Filaggrin by IHC (scale bar: 50  $\mu$ m) and (C, D, E, F) TEM pictures of regenerated epidermis from hPBES after 14 days of *in vivo* engraftment. Pictures were marked as following: asterisks for hemidesmosomes, K for keratinocytes and hPBM for matrix, SS for stratum spinosum, SG for stratum granulosum, SC stratum corneum and arrows for keratohyalin granules. Scale bar: 500nm (C), 5 $\mu$ m (D), 2  $\mu$ m (E and F).



**Figure 5: HPBM and FPF released different factors.** Concentration of factors released by FPF or hPBM in medium at day 1. Mean +/- sem was presented on the graph (n=2 to 4). Statistics: paired t-tests, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001).

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**Figure 6: Fibrin matrices released factors enhance DEJ deposition while hPBM released factors specifically enhance proliferation and migration.** Representative photos of HPS (A) and (B) percentage of basal keratinocyte expressing Ki67 in epidermal substitutes with no matrix cultivated 14 days in control medium, or in medium with total hPBM released factors (scale bar: 50  $\mu$ m). Mean was represented on the dot plot (n=4 donors of keratinocytes). Kruskal-Wallis and Pairwise comparisons using Wilcoxon rank sum test were used to compare different conditions (\*\*p<0.01). (C) Percentage of wound closure *in vitro* by keratinocytes in presence or not of matrix released factors (hPBM or FPF) at 24 and 48 h. Mean was represented on the dot plot (n=4 donors of keratinocytes). Permutation Analysis of Variance Table and Pairwise comparisons using permutation paired t tests were used to compare different conditions. (D) Western blot analyses of Perlecan, Laminin 332, and  $\beta$ -actin expression in epidermal substitutes with No matrix, cultivated 14 days in control medium, or in medium with total matrices (hPBM or FPF) released factors. Quantification of (E) Perlecan or (F) Laminin 332 band intensity, displayed as the ratio of signals of interest on  $\beta$ -actin signals. (G) Ratio of mean colony area of keratinocytes in supplemented mediums with cytokines at levels of hPBM released factors over keratinocytes in normal culture medium. Mean was represented on the dot plot (n=3 donors of keratinocytes). One sample t-

tests was used to compare experimental groups to the control baseline, (\* $p < 0.05$ ). (H) Western blot analyses of Laminin 332, and  $\beta$ -actin expression in epidermal substitute with no matrix, cultivated 14 days in control medium, or in medium supplemented with TGF- $\beta$ 1 (at hPBM released level). Quantification of Laminin 332 band intensity, displayed as the ratio of signals of interest on  $\beta$ -actin signals. 6 donors of keratinocytes were presented on the dot plot. Kruskal-Wallis and Pairwise comparisons using Wilcoxon rank sum test were used to compare different conditions.

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