

# Epithelial and Stromal Developmental Patterns in a Novel Substitute of the Human Skin Generated with Fibrin-Agarose Biomaterials

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## Key Words

Human skin substitute • Tissue engineering • Fibrin-agarose biomaterials

## Abstract

Development of human skin substitutes by tissue engineering may offer new therapeutic alternatives to the use of autologous tissue grafts. For that reason, it is necessary to investigate and develop new biocompatible biomaterials that support the generation of a proper human skin construct. In this study, we generated a novel model of bioengineered human skin substitute using human cells obtained from skin biopsies and fibrin-agarose biomaterials and we evaluated this model both at the ex vivo and the in vivo levels. Once the dermal fibroblasts and the epithelial keratinocytes were isolated and expanded in culture, we used fibrin-agarose scaffolds for the development of a full-thickness human skin construct, which was evaluated after 1, 2, 3 and 4 weeks of development ex vivo. The skin substitutes were then grafted onto immune-deficient nude mice and analyzed at days 10, 20, 30 and 40 postimplantation using transmission electron

microscopy, histochemistry and immunofluorescence. The results demonstrated that the fibrin-agarose artificial skin had adequate biocompatibility and proper biomechanical properties. A proper development of both the bioengineered dermis and epidermis was found after 30 days in vivo, although the tissues kept ex vivo and those implanted in the animal model for 10 or 20 days showed lower levels of differentiation. In summary, our model of fibrin-agarose skin equivalent was able to reproduce the structure and histological architecture of the native human skin, especially after long-term in vivo implantation, suggesting that these tissues could reproduce the native skin.

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## Abbreviations used in this paper

CKs	cytokeratins
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
PBS	phosphate-buffered saline

## Introduction

As the largest organ in the body, the skin is the primary protective barrier between an individual and his environment. Skin consists of three structurally and embryologically distinct tissue layers: the superficial epidermis, the inner connective dermis and the subcutaneous tissue [Hoath and Leahy, 2003]. Histologically, the epidermis mainly consists of a cell population of keratinocytes that express specific cytokeratins (CKs) that are responsible for many of the functions of the epithelium [Jacques et al., 2005; Proksch et al., 2008].

Numerous diseases and conditions may affect the human skin, including tumors, ulcers, infections, burns and trauma, among others. Currently, the treatment of the different skin pathologies requires surgical reconstruction or even substitution of the damaged tissues, and plastic surgeons are often confronted with a shortage of skin to replace the excised tissues. In fact, the surgical treatment of extensive affections of the skin depends on the use of skin flaps or grafting from nonaffected donor areas, which are not always available, especially when the skin damage is over a large area [Shevchenko et al., 2010]. In addition, the management of many patients may require more than one surgical procedure, which is often associated with high morbidity at the donor and recipient sites, and surgeons need to avoid making the patient's condition acutely worse by removing too much epidermis from healthy areas of the body [MacNeil, 2007]. For all these reasons, the search for alternative sources of skin tissues is needed.

Tissue engineering is a novel scientific discipline that combines the principles of engineering and biological sciences [Vacanti, 2006]. The main focus of tissue engineering is the development of artificial biological substitutes for a specific kind of human tissue. Different models of artificial substitutes of the human skin have been developed in the laboratory using tissue engineering protocols [Larouche et al., 2009; Shevchenko et al., 2010; Lazic and Falanga, 2011]. Most of the available human skin substitutes use artificial stromas based on type I collagen [Harrison et al., 2006; Falanga et al., 2007; Priya et al., 2008; Huang et al., 2010], collagen-chitosan/fibrin glue [Han et al., 2010], collagen-GAG sponges [Boyce et al., 1993; Damour et al., 1994], gelatin [Lee et al., 2003], acellular dermis [Gibbs et al., 2006], fibrin [Meana et al., 1998; Llames et al., 2006], synthetic scaffolds [Blackwood et al., 2008], or self-assembly technique [Auger et al., 2002]. However, most of these artificial skin models are subject to several drawbacks and problems, including the heterologous or even xenologous origin of the structures used

for the artificial skin, the contraction of the collagen scaffolds [Harrison et al., 2006] and the low consistency and difficult surgical handling of the autologous fibrin-based scaffolds [Ronfard et al., 2000]. Recently, we developed a novel stromal scaffold based on a mixture of agarose and human fibrin, that was efficiently used for construction in the laboratory of artificial human corneas and oral mucosa by tissue engineering, displaying good physical properties that allowed the surgical implantation and management of the bioengineered tissues [Alaminos et al., 2007; Gonzalez-Andrades et al., 2009; Garzon et al., 2009b]. However, the potential usefulness of this type of bioengineered stroma substitute for the development of human artificial skin has not been determined to date.

In addition, our model of fibrin-agarose bioengineered human skin could have many nonclinical research applications, especially 3-dimensional models. In fact, bioengineered skin is currently delivering value in aspects of skin biology research as diverse as reducing animal experimentation, investigation of cell-to-cell and cell-to-extracellular-matrix interactions, skin barrier penetration, wound healing, angiogenesis, regulation of pigmentation, skin contraction and the investigation of skin diseases such as melanoma invasion, psoriasis and skin-blistering disorders [MacNeil, 2007].

In this work, we have developed a novel model of human bioengineered skin substitute using fibrin-agarose stroma scaffolds. We have evaluated the structure and function of the skin model both *ex vivo* and *in vivo* using immune-deficient mice in comparison with other skin models previously described.

## Methods

### *Establishment of Primary Cultures of Human Skin Fibroblasts and Keratinocytes*

Small biopsies of normal human skin were obtained from healthy donors subjected to minor surgery at the Department of Plastic Surgery of the Virgen de las Nieves University Hospital Granada, Spain. All biopsies corresponded to total-thickness skin. Immediately after extraction, all tissues were kept at 4°C in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with antibiotics and antimycotics (100 U/ml of penicillin G, 100 mg/ml of streptomycin and 0.25 mg/ml of amphotericin B; Sigma-Aldrich) and processed in the following 24 h. All patients gave their written consent to participate in the study. The work was approved by the local research committee.

Skin biopsies were washed twice in phosphate-buffered saline (PBS) and incubated overnight at 4°C in dispase II (5 mg/ml in PBS; Gibco) to enzymatically detach the epithelium from the connective tissue. Subsequently, the detached epithelium was mechanically fragmented into small pieces and incubated in trypsin-

EDTA at 37°C for 10 min. After this, the trypsin was neutralized with DMEM supplemented with 10% fetal bovine serum (FBS) and all detached cells were harvested by centrifugation and cultured in culture flasks using a 3:1 mixture of DMEM and Ham's F12 culture media supplemented with 10% FBS, 1% antibiotics, 24 µg/ml adenine, 0.4 µg/ml hydrocortisone, 5 µg/ml insulin, 10 ng/ml epidermal growth factor, 1.3 ng/ml triiodothyronine and 8 ng/ml of cholera toxin (all from Sigma-Aldrich). This culture medium was called keratinocyte medium. No feeder cells were used in this experiment.

To obtain primary cultures of human skin fibroblasts, the de-epithelized skin was digested in a mixture of DMEM and 2 mg/ml of *Clostridium histolyticum* collagenase I (Gibco BRL Life Technologies, Karlsruhe, Germany) at 37°C during 6 h. Detached fibroblasts were collected by centrifugation and expanded in culture flasks containing DMEM supplemented with antibiotics (100 U/ml of penicillin G, 10 mg/ml of streptomycin and 0.025 mg/ml of amphotericin B) and 10% of FBS. This culture medium was called MF.

In all cases, cells were incubated at 37°C in 5% CO<sub>2</sub> using standard culture conditions. The medium was changed every 3 days, and subcultivation of the cells was carried out using a trypsin/EDTA (0.5/0.2 g/l) solution at 37°C for 10 min. Keratinocytes and fibroblasts used for tissue engineering of the tissue models always corresponded to the first 4 cell subcultures.

#### *Generation of Human Skin Substitutes by Tissue Engineering*

Biological orthotypical substitutes of the human skin were developed in the laboratory using 24-mm Transwell® porous inserts (Costar, Corning Inc., Corning, N.Y., USA) as previously described [Garzon et al., 2009a, b; Gonzalez-Andrades et al., 2009]. Briefly, a stromal substitute was first generated using a mixture of human fibrin obtained from frozen plasma (kindly provided by Dr. Fernández-Montoya, Human Tissue Bank of Granada, Spain) and 0.1% type VII agarose in PBS. To produce a 5-ml dermal substitute, 3.8 ml of human plasma were added to 375 µl of DMEM with 150,000 cultured skin fibroblasts. To prevent gel fibrinolysis, the mixture was supplemented with 75 µl of tranexamic acid (Amchafibrin®, Fides Ecopharma, Spain). Then 500 µl of 1% CaCl<sub>2</sub> were added to induce fibrin polymerization, followed by the immediate addition of 250 µl of melted concentrated agarose (2% in PBS) at 40°C. The final concentration of agarose in the stromal substitute was 0.1%. Finally, the mixture was rapidly aliquoted on porous inserts and allowed to solidify at 37°C for 2 h. Twenty-four hours after the stroma had solidified, 500,000 keratinocytes from the primary cultures were seeded on top of the artificial stroma, and the skin constructs were cultured for 15 days submerged in culture medium. As controls, acellular tissues were generated using the same method but without cells. Finally, all bioengineered skin substitutes were submitted to an air-liquid culture technique for 15 days to induce the proper differentiation of the multilayered epithelium (4 weeks in total). Tissues were kept in culture using keratinocyte medium at 37°C with 5% CO<sub>2</sub>. Samples were taken for analysis after 1, 2, 3 and 4 weeks of development ex vivo.

#### *In vivo Evaluation of Bioengineered Skin Substitutes*

To evaluate the in vivo behavior of the skin substitutes generated by tissue engineering, we implanted the artificial tissues in 20 6-week-old Fox 1<sup>nu/nu</sup> immune-deficient athymic mice (Hartman, USA).

All animals were anesthetized by intraperitoneal injection of a mixture of acepromazine (Calmo-Neosan® 0.001 mg/g of weight of the animal) and ketamine (Imalgene 1000® 0.15 mg/g of weight of the animal) after subcutaneous administration of atropine. All procedures were performed in a biological safety cabinet and animals were housed in filter-topped cages in a laminar flow isolator. Skin substitutes were grafted onto the back of the animals without the use of silicone chamber, in direct contact with air. Briefly, we first created a full-thickness skin wound (including the panniculus carnosus) of approximately 4 cm<sup>2</sup> above the shoulder of the mouse, in the interscapular region of the animal. The artificial human skin substitutes developed by tissue engineering were then implanted onto the wound bed of the host mice using 4-0 PDS sutures. All skin constructs were sutured on both the skin (8 stitches) and the wound bed (4 stitches). Finally, a porous nylon membrane was sutured onto the surrounding mouse skin to protect the implanted tissues from any mechanical damage.

Mice were euthanized for histological analysis by lethal administration of anesthetics (acepromazine and ketamine) at 10, 20, 30 and 40 days after the implantation of the bioengineered skin.

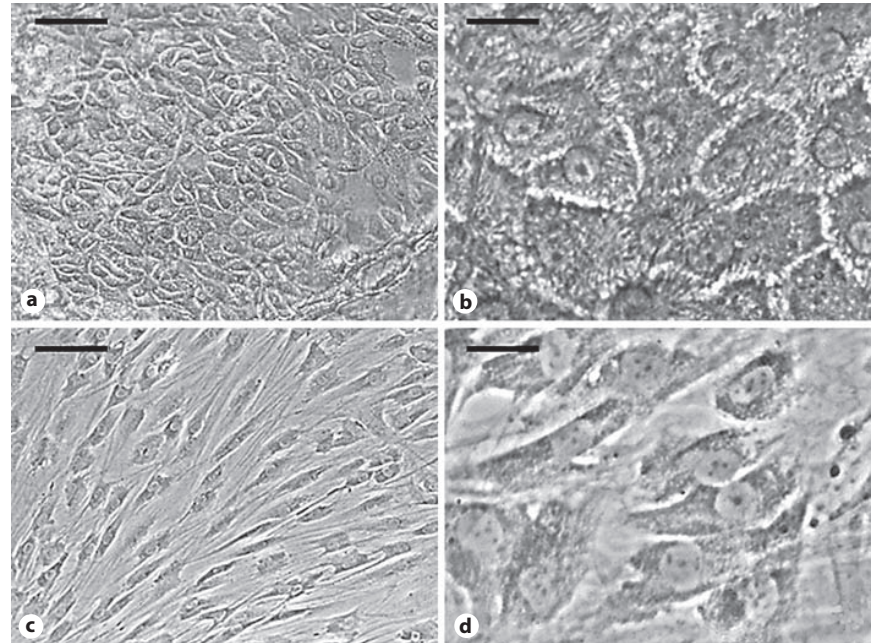
#### *Histological Analyses*

For light microscopy analysis, samples corresponding to normal human control skin and bioengineered skin substitutes (both ex vivo and in vivo) were fixed in 4% formaldehyde, dehydrated and embedded in paraffin, and 4-µm-thick sections were stained with hematoxylin and eosin. For the study of tissues by transmission electron microscopy, the samples were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C for 4–6 h, and then washed twice in 0.1 M phosphate buffer (pH 7.2) at 4°C. The tissues were then embedded in Spurr resin for 24 h at 4°C, ultrathin 100-nm-thick sections were obtained using an Ultracut Reichert and the tissues were stained with 2% uracil acetate for 10 min and in lead citrate for 5 min. The observation was carried out with a transmission electron microscope, Zeiss EM902 (Oberkochen, Germany). For scanning electron microscopy, samples were fixed in 3% glutaraldehyde as described above, washed, dehydrated in increasing concentrations of acetone (30, 50, 70, 95 and 100%), and dried by the critical point method. Once dried, the tissues were covered with gold and palladium atoms and analyzed in a scanning electron microscope FEI Quanta 200 using the high vacuum mode.

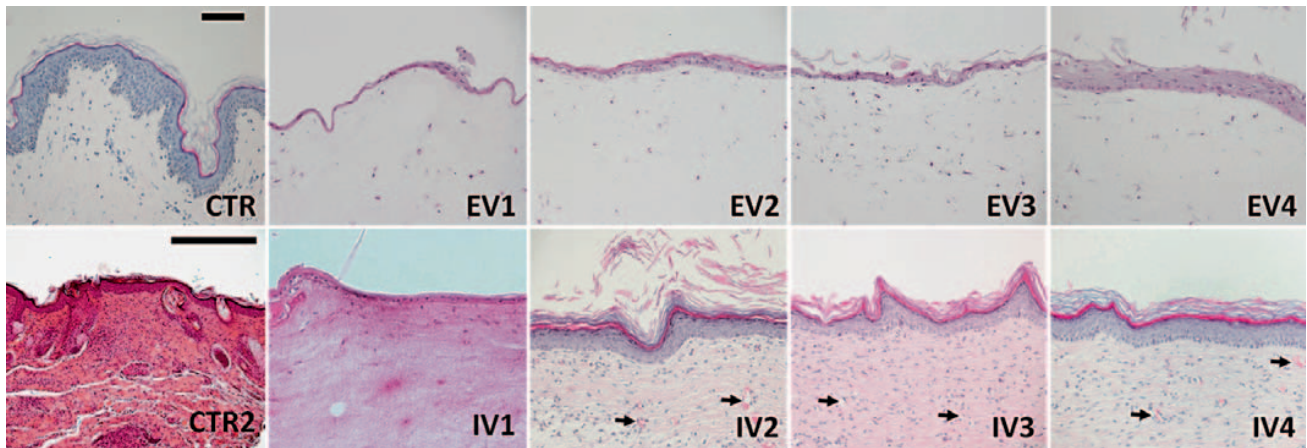
The histochemical analysis of the dermal extracellular matrix was performed with the following histochemical methods: Picrosirius stain for detection of collagen fibers (including polarized light microscopy), Alcian blue (pH 4) for the identification of acid mucopolysaccharides and glycosaminoglycans, the Gomori method for reticular fibers, and the Orcein method for elastic fibers.

To determine the protein expression of several epithelial markers in the bioengineered epidermis, immunofluorescence was carried out using CK 5, CK 1, CK 10, filaggrin and involucrin primary antibodies using 4-µm-thick sections of the different tissues embedded in paraffin. In all cases, tissue sections were deparaffinized in xylene, rehydrated in alcohol series and incubated in the different primary antibodies used here (anti-CK 5, prediluted, Master Diagnostica; anti-CK 1, dilution 1:500, Sigma-Aldrich; anti-CK 10, prediluted, Master Diagnostica; antiinvolucrin, dilution 1:500, Sigma-Aldrich; antifilaggrin, dilution 1:50, ABCam).





**Fig. 1.** Primary cell cultures of human skin keratinocytes (**a, b**) and human dermal fibroblasts (**c, d**) at confluence in monolayer culture (phase contrast microscopy images). Scale bar: 100  $\mu\text{m}$  (**a, c**) and 20  $\mu\text{m}$  (**b, d**).



**Fig. 2.** Light microscopy histological analysis of the fibrin-agarose human skin substitutes generated in this study stained with hematoxylin and eosin. CTR is human native normal skin used as control; EV1–4 are samples kept ex vivo for 1, 2, 3 and 4 weeks, respectively, with EV3 and EV4 subjected to an air-liquid culture technique. CTR2 is control fibrin-agarose acellular scaffold

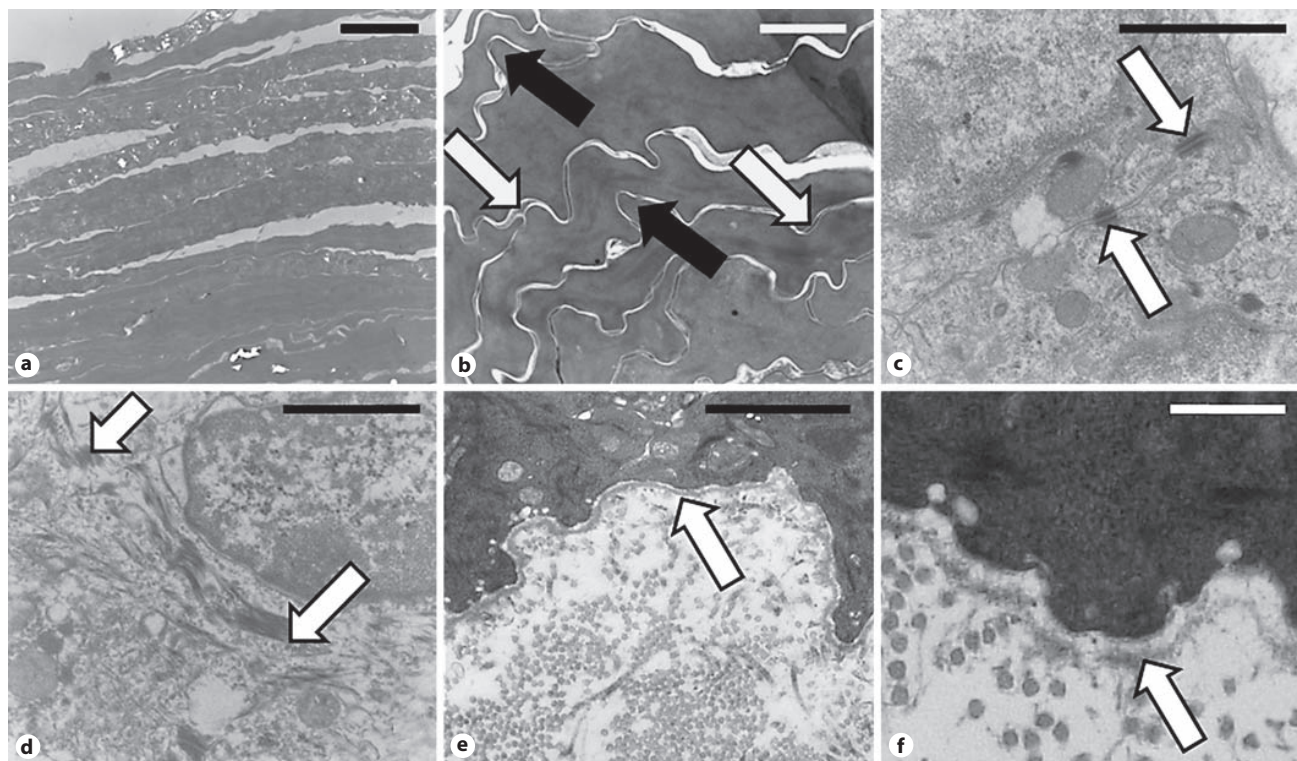
implanted on nude mice for 4 weeks, where the implanted fibrin-agarose scaffold area has been significantly reduced; IV1–4 are samples implanted in vivo in nude mice for 1, 2, 3 and 4 weeks, respectively. The arrows show the newly formed blood vessels in the artificial dermis from IV2 to IV4 figures. Scale bar: 100  $\mu\text{m}$ .

Thereafter, the samples were washed in PBS and a secondary anti-mouse or anti-rabbit antibody, labeled with a fluorescent pigment (FITC or Cy3), was applied for 60 min and washed with PBS. Finally, nuclei were counterstained with DAPI, samples were covered with glass coverslips and analyzed in a Nikon Eclipse 90i fluorescent microscope.

## Results

### *Ex vivo Development of an Artificial Skin Substitute by Tissue Engineering*

In this work, we first established primary cultures of human skin keratinocytes (fig. 1a, b) and fibroblasts (fig. 1c, d). In culture, cells showed early attachment to the



**Fig. 3.** Transmission electron microscope analysis of the epithelial layer of the fibrin-agarose human skin substitutes implanted in vivo in athymic mice. **a–d** Analysis of the epidermal keratinocytes. **a** Stratified epithelium showing several cell layers. **b** Interdigitations between several epithelial cells (black arrows) and

squasmosomes (white arrows). **c** Desmosomes (arrows). **d** Keratohyalin granules (arrows) in the cytoplasm of a keratinocyte. **e, f** Analysis of the basal membrane (arrows) at the base of the bioengineered epidermis. Scale bar: 3  $\mu\text{m}$  (**a**), 1  $\mu\text{m}$  (**b–d**), 500 nm (**e**) and 100 nm (**f**).

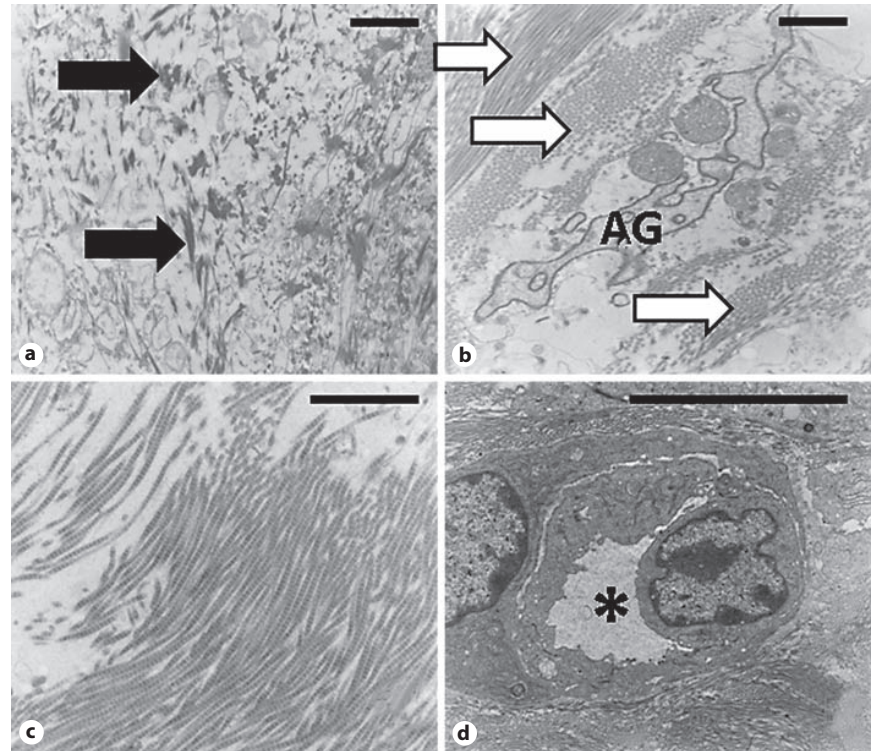
surface of the culture flasks, with a rapid growth rate, reaching cell confluence after 7 days (fibroblasts) and 21 days (keratinocytes). The use of human fibrin and agarose scaffolds allowed us to efficiently develop a full-thickness artificial skin substitute ex vivo. The histological analysis of these skin substitutes revealed the presence of an epithelium on top of the fibrin-agarose dermal substitute (fig. 2), with the presence of a single cell layer of keratinocytes at the first week of development in submerged culture (fig. 2, panel EV1) and 2–3 layers after 2 weeks (fig. 2, EV2). When the epithelium was exposed to direct contact with air, 2–3 epithelial cell layers could be identified at week 3 (fig. 2, EV3), with the most superficial layer showing evident signs of desquamation and a multistratified epidermis with 8–10 layers after 4 weeks in culture (fig. 2, EV4). The analysis of the bioengineered human dermis showed a proper development of the fibroblasts in the fibrin-agarose biomaterial. No blood vessels, hair follicles or chorial papillae were found in the bioengineered stroma.

#### *In vivo Evaluation of the Human Bioengineered Skin Model on Nude Mice*

In vivo evaluation of the human skin substitutes developed by tissue engineering showed that the bioengineered tissues integrated properly into the wound bed of the recipient mice. The surgical procedure was well tolerated by all animals, and no intra- or postoperative mortality was observed. As shown in figure 2, histological analysis of the tissue substitutes grafted on nude mice using light microscopy revealed the presence of a well-developed, properly integrated full-thickness skin in all mice. On the one hand, all the bioengineered cellular skin samples displayed a stratified epithelium with well-differentiated basal, spinosum, granulosum and corneum strata, especially 20 days after implantation (fig. 2, IV2). However, the typical dermoepidermal interface specializations of the normal skin (i.e. the rete ridges of the epithelium and the chorial papillae of the dermis, hair follicles and glands) were not found in any of the samples.



**Fig. 4.** Transmission electron microscope analysis of the dermal layer of the fibrin-agarose human skin substitutes implanted in vivo in athymic mice. **a** Presence of fibrin rests (black arrows) 10 days after being implanted in mice. **b** Presence of agarose rests (AG) 20 days after being implanted in mice, with the presence of collagen fibers in different orientations (white arrows). **c** Well-organized and oriented collagen fibers in samples grafted for 30 days in the animal model. **d** Newly formed blood vessel in the artificial dermis implanted in vivo for 40 days. Scale bar: 1  $\mu\text{m}$  (**a–c**), and 10  $\mu\text{m}$  (**d**). \* = Lumen of the vessel.



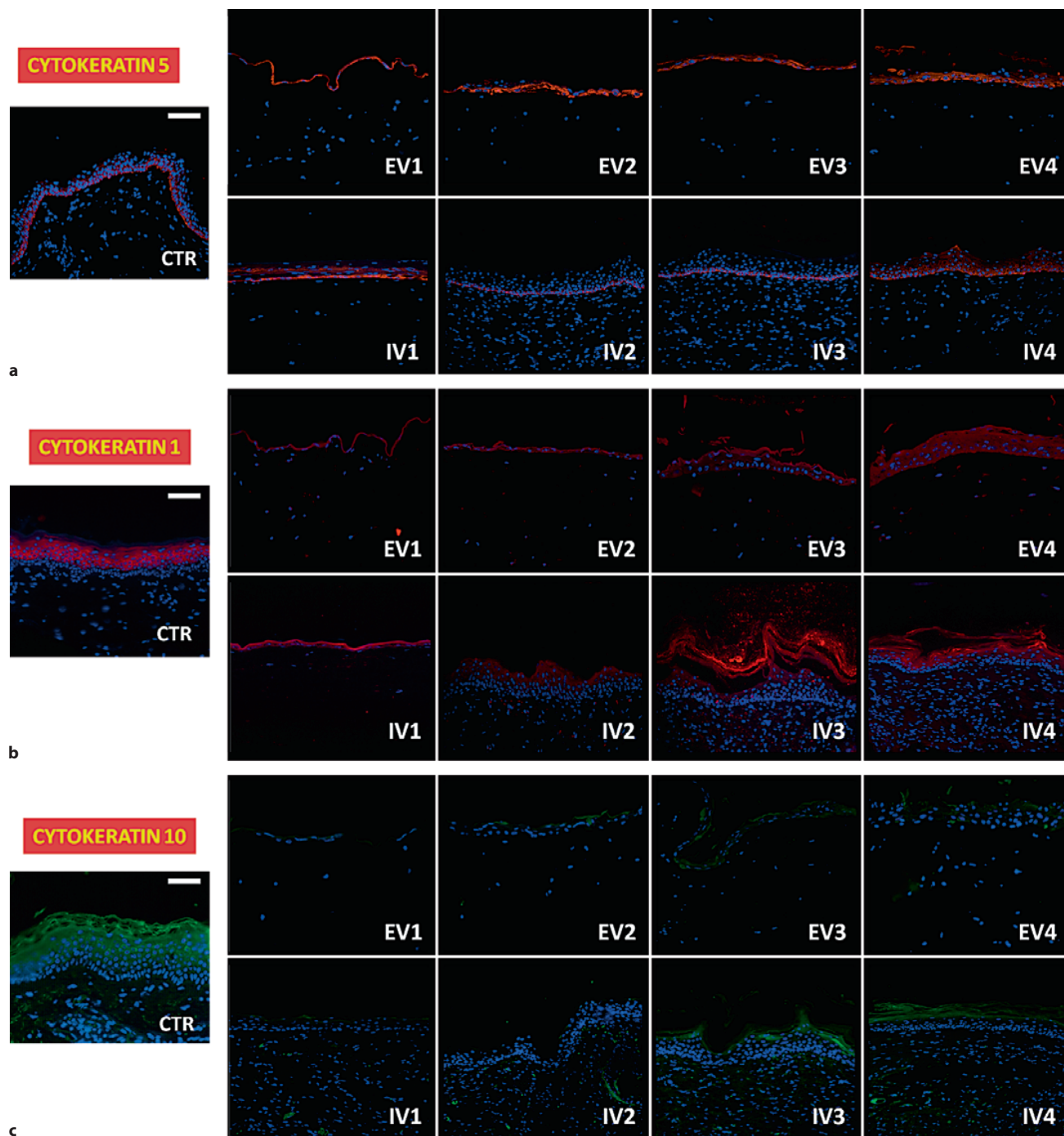
Interestingly, control mice in which an acellular fibrin-agarose scaffold was implanted resulted in the formation of a tissue with 2–3 epithelial cell layers that did not properly differentiate into the different strata of the human epidermis, being very similar to the pseudodystrophic native skin of these animals.

In addition, the analysis of the samples using transmission electron microscopy (fig. 3) showed that cells tended to stratify in vivo, with the formation of a well-differentiated cornified stratum corneum on top (fig. 3a) with cells attached by interdigitations and squamosomes (fig. 3b), cells at the stratum granulosum with high amounts of keratin and keratohyalin in their cytoplasm (fig. 3d) and cells at the stratum spinosum attached by desmosomic intercellular junctions (fig. 3c). Furthermore, our electron microscopy analysis revealed the presence of a basement membrane from day 10 after grafting (fig. 3e, f). On the other hand, the structure and ultrastructure of the bioengineered human dermis (fig. 4) revealed the presence of numerous fibroblasts and blood vessels from the 10th day after implantation, with the fibrin-agarose scaffold being remodeled very early. In fact, some remains of the fibrin fibers and agarose were identi-

fied at day 10 by both light (fig. 2, IV1) and electron microscopy (fig. 4a, b), but these biomaterials were completely substituted by collagen starting on day 20 after implantation in the athymic mice. Blood vessels could be identified from day 10 after implantation (fig. 2, IV2, IV3, IV4 and fig. 4d).

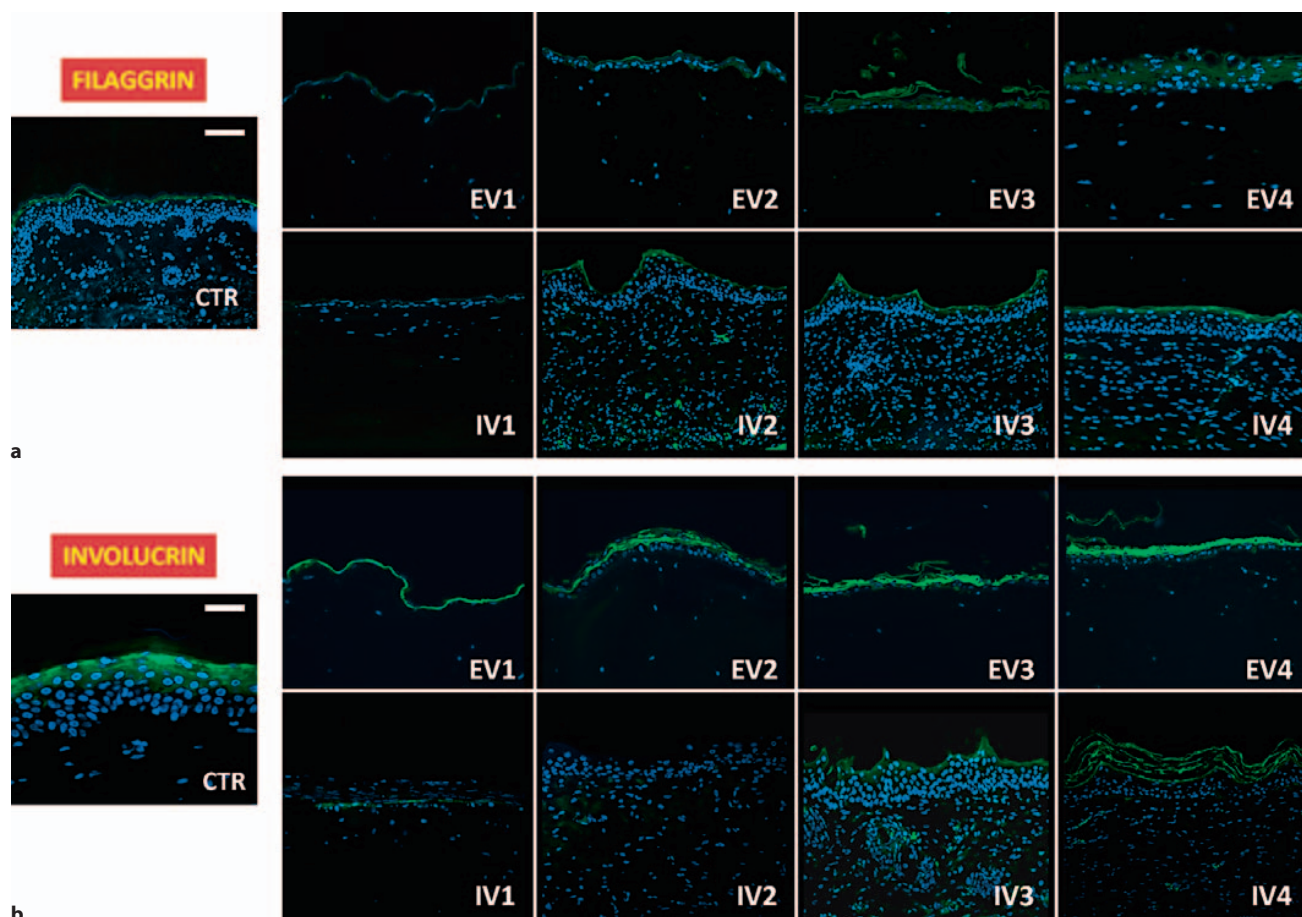
#### *Immunofluorescence Analysis of the Bioengineered Human Skin Epidermis*

The analysis of the epithelial layer of the bioengineered human skin using anti-CK 5 primary antibodies showed that the expression of CK 5 was intense in all ex vivo samples, and the CK 5 expression pattern was restricted to the basal strata of all in vivo samples (fig. 5a) and after 40 days in vivo, the CK 5 expression pattern was comparable to the native human skin control (fig. 5, CTR, IV4). The expression of the cornification marker CK 10 was minimal in samples maintained ex vivo and became more intense after 30 and 40 days in vivo (fig. 5c, IV3 and IV4), although CK 1 tended to show higher expression in all ex vivo and in vivo samples (fig. 5b). Strikingly, the expression pattern of CK 1 and CK 10 was mainly restricted to the most superficial cell layers of the bioengineered sam-



**Fig. 5.** Immunofluorescence analysis of the fibrin-agarose human skin substitutes generated in this work using anti-CK 5 (**a**), anti-CK 1 (**b**) and anti-CK 10 (**c**) antibodies. CTR is human native normal skin used as control; EV1–4 are samples kept ex vivo

for 1, 2, 3 and 4 weeks, respectively, with EV3 and EV4 subjected to an air-liquid culture technique; IV1–4 are samples implanted in vivo in nude mice for 1, 2, 3 and 4 weeks, respectively. Scale bar: 100  $\mu$ m.



**Fig. 6.** Immunofluorescence analysis of the fibrin-agarose human skin substitutes generated in this work using anti-filaggrin and anti-involucrin antibodies. CTR is human native normal skin used as control; EV1–4 are samples kept ex vivo for 1, 2, 3

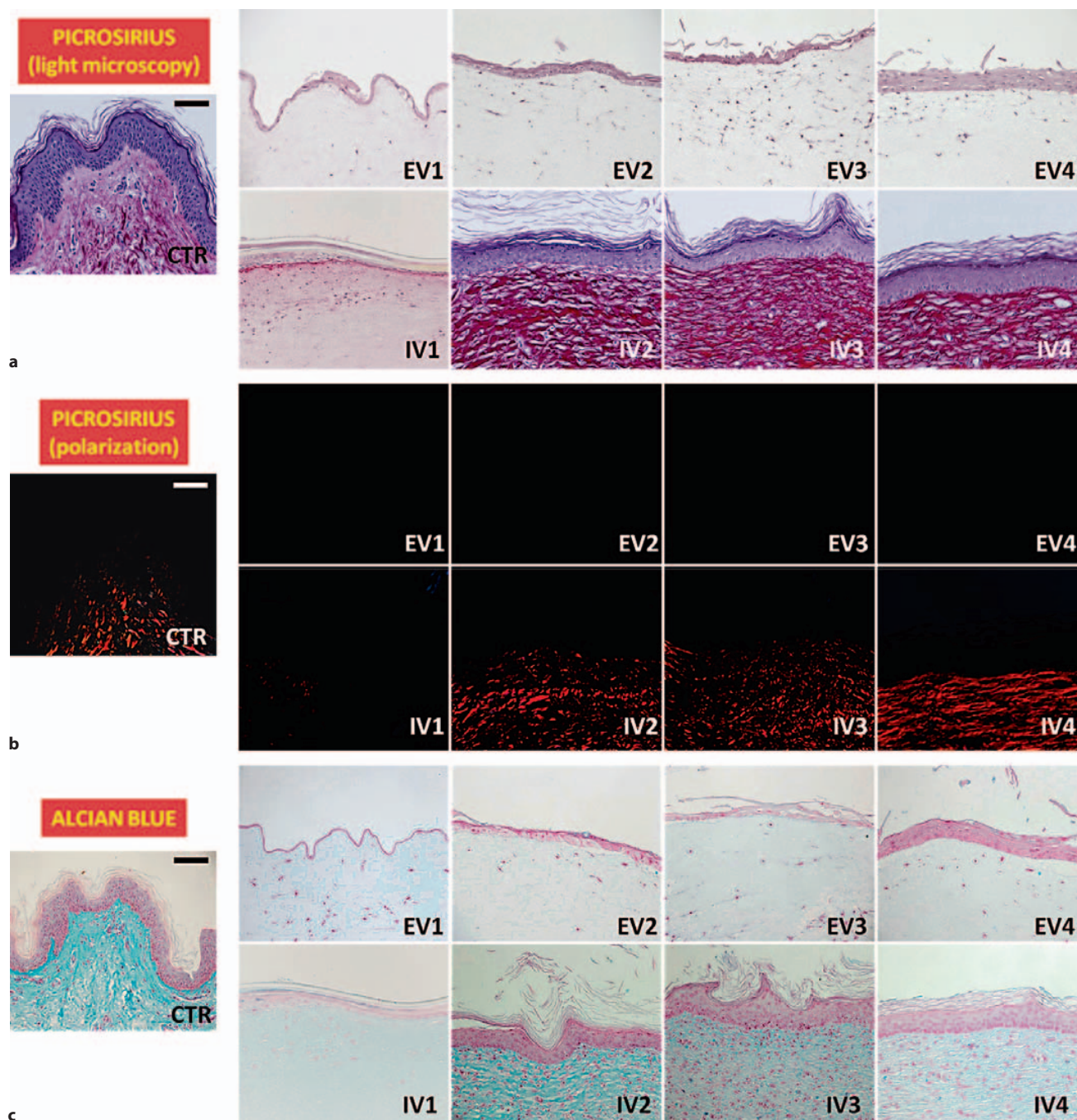
and 4 weeks, respectively, with EV3 and EV4 subjected to an air-liquid culture technique; IV1–4 are samples implanted in vivo in nude mice for 1, 2, 3 and 4 weeks, respectively. Scale bar: 100  $\mu\text{m}$ .

ples. When the markers of epithelial differentiation filaggrin and involucrin were analyzed, we found that the expression of both markers was comparable to the native human skin controls for all samples maintained ex vivo and in vivo, while samples implanted for 10 and 20 days in the animal model showed lower expression than tissues implanted for longer time periods (fig. 6). These results confirm the human nature of the bioengineered epithelium, since some of the antibodies used in this work (especially involucrin) specifically recognize human proteins and do not cross-react with mouse epidermis [Hudson et al., 1992].

#### *Sequential Development and Remodeling of the Bioengineered Human Dermal Extracellular Matrix*

Using the Picrosirius histochemical technique, all samples kept ex vivo showed a complete absence of collagen fibers (fig. 7, top panel, EV). In contrast, all tissues grafted in vivo in the animal model showed intense staining of the collagen fibers at the bioengineered dermis, especially after 20 or more days in vivo (fig. 7a, IV2, IV3 and IV4). As shown in figure 7, the amount of collagen fibers was slightly lower in the 10-day in vivo samples (fig. 7a, IV1) than in the control human skin and in samples grafted for 20, 30 or 40 days in the mice (fig. 7a, IV2, IV3 and IV4). However, when the orientation of the collagen fibers was analyzed using polarization micros-





**Fig. 7.** Histological analysis of the extracellular matrix of the fibrin-agarose human skin substitutes generated in this work. The top and middle panels correspond to tissue constructs stained using Picrosirius histochemistry to demonstrate the presence and spatial organization of collagen fibers and were analyzed using light microscopy (a) and polarized light (b). c Histochemical

staining of mucopolysaccharides and glycosaminoglycans in the dermis of the artificial tissues using Alcian blue. EV1–4 are samples kept ex vivo for 1, 2, 3 and 4 weeks, respectively, with EV3 and EV4 subjected to an air-liquid culture technique; IV1–4 are samples implanted in vivo in nude mice for 1, 2, 3 and 4 weeks, respectively. Scale bar: 100  $\mu$ m.

copy (fig. 7b), we found that the collagen fibers that were previously identified in the 10-day in vivo samples were not properly orientated (fig. 7b, IV1). Instead, they formed a disorganized mesh that showed no signal when polarized light was used. Conversely, bioengineered human skin implanted for 20 or more days in the animal model had a high number of well-orientated collagen fibers.

In addition, our analysis using Alcian blue histochemistry demonstrated the presence of detectable acid proteoglycans in all in vivo implanted skin substitutes, especially after 20, 30 and 40 days (fig. 7c, IV2, IV3 and IV4), with a very weak signal at day 10 (IV1). All ex vivo tissues were negative for this staining technique (EV).

Finally, the histochemical analysis for the presence of reticular fibers showed a complete absence of these fibers in all bioengineered skin samples. Interestingly, some of the control samples corresponding to human native skin showed positive expression of these fibers, whereas others were negative for histochemical reactions (data not shown).

## Discussion

Tissue engineering can be defined as a rapidly growing interdisciplinary field involving physical, engineering and life sciences and seeking to develop clinical therapies for the repair, maintenance, replacement and/or enhancement of biological function [Nerem, 2010]. In this context, skin replacement has been a challenging task for surgeons ever since the introduction of skin grafts by Reverdin in 1871 [Priya et al., 2008], and several biomaterials have been used for this purpose in vitro [Han et al., 2010; Huang et al., 2010; Shevchenko et al., 2010]. However, a fully functional bioengineered human skin substitute has not been developed to date. Although some models of fibrin-based human skin have demonstrated clinical usefulness [Llames et al., 2006], fibrin scaffolds normally have poor biomechanical properties, and surgical management of these tissues is very difficult [Ronfard et al., 2000].

Generation of an orthotypic organ substitute of the human skin is strongly dependent on the availability of adequate sources of human immune-compatible cells and biomaterials. In this study, we used an autologous source of dermal and epidermal cells – human skin biopsies – and a novel, natural and highly compatible biomaterial consisting of a mixture of human fibrin and agarose. Fibrin-agarose biomaterials have been previously used for

generating artificial models of the human cornea in the laboratory [Gonzalez-Andrades et al., 2009] and oral mucosa [Alaminos et al., 2007; Sanchez-Quevedo et al., 2007; Garzon et al., 2009a, b] with adequate biocompatibility and proper biomechanical properties, including consistency, resistance to traction forces and elasticity. However, fibrin-agarose scaffolds had not been previously used for the generation of a skin substitute by tissue engineering. The use of this type of scaffold has several advantages when compared to other biomaterials, including the highest biocompatibility, accessibility, cytocompatibility, absence of shrinkage of the bioengineered mesh and reduced price.

In this study, we demonstrated that our model of fibrin-agarose skin equivalents prepared with human epidermal keratinocytes seeded onto a fibrin-agarose cellular mesh was able to reproduce the structure and histological architecture of the native human skin, especially after in vivo implantation without the use of silicone chamber. The analysis of the epidermis that forms on top of the skin substitute revealed that this epithelial tissue was able to fully differentiate and mature on top of the fibrin-agarose mesh, especially when the artificial skin was implanted in vivo. In fact, the analysis of samples maintained ex vivo showed a regular tissue with an increasing number of cells with time in culture, and initial signs of desquamation when the air-liquid culture technique was used, but this artificial epidermis was very immature and no specific layers could be identified. In contrast, when samples were grafted in vivo, a fully differentiated epidermis was found, with the formation of a basement membrane and epithelial basal, suprabasal, spinous and cornified layers that resembled the native human skin, with the presence of numerous cell-cell junctions and intracellular keratohyalin granules in cells corresponding to the most superficial layers. In addition, the expression of CKs by the bioengineered epithelium of the ex vivo samples was not comparable to the native skin, and the expression of CK 5 was highly positive. However, CK 1 and CK 10 were very low or completely absent from these tissues, suggesting that the bioengineered human skin samples kept ex vivo were very immature, and that the implant in vivo is necessary for the complete development of these tissues. This is in agreement with previous reports from our group demonstrating that human bioengineered oral mucosa substitutes require an in vivo environment for a full differentiation and maturation and that samples kept ex vivo correspond to an early stage of development and do not form well-differentiated layers (basal, spinosum, granu-

losum and cornified strata) [Garzon et al., 2009a, b]. Furthermore, our analysis revealed that maturation of the epithelium requires at least 30–40 days of implantation *in vivo*, and the expression of epithelial markers (CKs, filaggrin and involucrin) is immature and similar to that of the *ex vivo* samples when kept *in vivo* for shorter periods. In fact, the CK, filaggrin and involucrin expression patterns suggest that full maturation of the bioengineered epidermis could require a relatively long time period after *in vivo* implantation. This phenomenon was previously demonstrated by authors using different skin models, although some of these models tended to mature and fully differentiate earlier [Medalie et al., 1996]. The temporal differences could be due to the different structure and chemical composition of the scaffolds used in the different skin models [Shevchenko et al., 2010]. In the same sense, the CK 5 is linked to CK 14 as the primary keratins of the mitotically active basal cells of stratified epidermis [Pekny and Lane, 2007; Bragulla and Homberger, 2009], and the production of this keratin is not correlated with the differentiation of epithelial cells or the stratification of the epithelium [Bragulla and Homberger, 2009]. Therefore, the CK 5 expression pattern suggests that the basal layer of the artificial skin epithelium is normal and could have efficient levels of cell proliferation in the basal strata of the *ex vivo* and *in vivo* samples [Bragulla and Homberger, 2009]. As regards CK 1 and CK 10, both are produced in the suprabasal layers of the epidermis and can therefore be considered as important proteins for postmitotic differentiation in stratified keratinizing and cornifying epithelia [Kartasova et al., 1993; Pekny and Lane, 2007; Bragulla and Homberger, 2009]. In this context, the expression of CK 1 precedes CK 10 expression and CK 1 is therefore first associated with preformed CK 5/CK 14 filaments [Kartasova et al., 1993]. We found that the expression of CK 5 by the artificial epithelium was comparable to the native control epidermis. However, CK 1 and CK 10 expression was partly different to that of the native control epidermis, being more intense in superficial cell layers. These results correlated with previous studies [Kartasova et al., 1993; Pekny and Lane, 2007; Bragulla and Homberger, 2009; Han et al., 2010], and suggest that our model of artificial skin may need some additional time to normalize the CK expression and become fully equivalent to the native skin. Altogether, these results imply that the *ex vivo* epidermis could have a progressive stage of differentiation with mitotic activity of the basal epithelial cells at *ex vivo* and *in vivo* levels demonstrated by CK 5-positive expression, as was the case with the human oral mucosa

substitutes generated using fibrin-agarose scaffolds [Garzon et al., 2009a, b].

Our novel skin substitute showed a progressive differentiation of the bioengineered tissues as revealed by electron microscopy, morphological, histochemical and immunohistochemical studies. All these results suggest that our model of artificial human skin has some similarities to the human native skin.

The analysis of the bioengineered human dermis demonstrated the convenience of the fibrin-agarose biomaterials for the generation of an artificial substitute of the human skin. First, the evaluation of the skin constructs kept *ex vivo* revealed that the fibrin-agarose lattice remained stable after 4 weeks of development, allowing the proper growth of the dermal fibroblasts immersed within. Interestingly, *in vivo* implantation of the artificial skin resulted in a proper differentiation of the bioengineered dermis, with the progressive biodegradation of the fibrin and agarose molecules and the increasing synthesis of collagen fibers, proteoglycans and blood vessels from day 10 *in vivo*. Besides, collagen fibers became properly oriented, as shown by polarization analysis, 20 days after the implant in the animals, implying that the artificial dermis could be fully mature after that time *in vivo*.

In summary, in this study we have demonstrated that our novel model of fibrin-agarose skin substitute could have similarities to native human skin. The biochemical and histological composition of the composite graft described in this paper could resemble that of the native human skin at both the epidermal and dermal layers, showing good biointegration at *in vivo* levels. For all these reasons, we hypothesize that fibrin-agarose human skin substitutes might be useful for pharmacological, biological and mechanical testing. Further long-term preclinical and clinical studies should determine the potential usefulness of these skin substitutes, the possibility of large scale production, the biocompatibility of agarose for transplantation in humans and the long-term persistence of the grafted cells.

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