

Skineering I: engineering porcine dermo-epidermal skin analogues for autologous transplantation in a large animal model

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Abstract

Background Extended full thickness skin defects still represent a considerable therapeutic challenge as ideal strategies for definitive autologous coverage are still not available. Tissue engineering of whole skin represents an equally attractive and ambitious novel approach. We have recently shown that laboratory-grown human skin analogues with near normal skin anatomy can be successfully transplanted on immuno-incompetent rats. The goal of the present study was to engineer autologous porcine skin grafts for transplantation in a large animal model (pig study = intended preclinical study).

Materials and methods Skin biopsies were taken from the pig's abdomen. Epidermal keratinocytes and dermal fibroblasts were isolated and then expanded on culture dishes. Subsequently, highly concentrated collagen hydrogels and collagen/fibrin hydrogels respectively, both containing dermal fibroblasts, were prepared. Fibroblast survival, proliferation, and morphology were monitored using fluorescent labelling and laser scanning confocal microscopy. Finally, keratinocytes were seeded onto this dermal construct and allowed to proliferate. The resulting in vitro generated porcine skin substitutes were analysed by H&E staining and immunofluorescence.

Results Dermal fibroblast proliferation and survival in pure collagen hydrogels was poor. Also, the cells were mainly round-shaped and they did not develop 3D-networks. In collagen/fibrin hydrogels, dermal fibroblast survival was significantly higher. The cells proliferated well, were spindle-shaped, and formed 3D-networks. When these latter dermal constructs were seeded with keratinocytes, a multilayered and partly stratified epidermis readily developed.

Conclusion This study provides compelling evidence that pig cell-derived skin analogues with near normal skin anatomy can be engineered in vitro. These tissue-engineered skin substitutes are needed to develop a large animal model to establish standardized autologous transplantation procedures for those studies that must be conducted before “skineering” can eventually be clinically applied.

Keywords Tissue engineering · Skin culture · Transplantation · Pig model

Introduction

Large full thickness skin defects typically result from burns, massive avulsion injuries, septic skin necroses, or from extended excision of scars or nevi [1–10]. The coverage of such lesions still poses a very significant challenge: the functionally and cosmetically best therapeutic option is transplanting full thickness autologous skin, however, donor sites are limited in a prohibitive way when there is extensive demand [11–14]. On the other end of the therapeutical spectrum stands the almost unlimited supply of cultured autologous epithelial autografts (CEA) (in numerous variations) that, despite enormous basic science

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and clinical research efforts, still requires 2–3 weeks of cultivation time and still yields far from ideal results [15–23].

Clearly, the clinical introduction of nowadays widely used dermal regeneration templates (e.g. IntegraDRT[®] and Matriderm[®]) and the development of more sophisticated laboratory-grown skin substitutes containing keratinocytes, fibroblasts, extracellular matrix components, growth factors, and other cytokines have pushed the frontiers further [8, 16, 17, 24–29].

Yet, these techniques are still plagued with considerable problems including intricate and time consuming laboratory processes, fragility of grafts, staged operative procedures, susceptibility to infection, poor graft take, and, finally and most importantly, not really satisfactory long term results in terms of both functionality and cosmetic appearance of the reconstituted skin [30–32].

We have, therefore, invested over 10 years of laboratory research into the ambitious project of engineering an anatomically near normal skin analogue. Briefly summarized, we have managed to grow a hydrogel-based dermo-epidermal construct featuring a correctly stratified epidermis, a basement membrane, the characteristic structures of the dermo-epidermal junction, and a close to normal, prevascularized dermis [33]. These grafts, cultured from human cells, were then successfully grafted onto immuno-incompetent rats [34–36].

Theoretically, this progress may be seen as the indispensable evidence required before an eventual clinical application in human patients can be envisioned. Practically, however, we are facing the problem that the above mentioned human tissue grafts used in our rat model are way too small and ill-configured (round grafts, 2.5 cm diameter, covering about 4 cm²) when looking at grafts suitable for large scale transplantation in human patients. Ideally, such transplants should be rectangular and large (covering 50–100 cm²).

Consequently, we have embarked on culturing skin substitutes with near normal anatomical architecture, mechanical properties allowing standard surgical handling, and appropriate size matching the prerequisites for large scale autologous transplantation in a large animal model (=immunocompetent setting). This article describes in vitro engineering of such grafts to be later used in pigs.

Materials and methods

In view of the envisioned animal model for large scale autologous transplantation, we have chosen the pig (Schweizer Edelschwein). Biopsies were taken as outlined in detail in the companion paper published in the same issue (Skinengineering II).

Isolation and culture of keratinocytes and fibroblasts

Porcine skin samples (3 × 1 cm) were cut to 2–3 mm² pieces and incubated for 15–18 h at 4°C in 12 U/ml dispase in HBSS containing 5 µg/ml gentamycin. The epidermis was then separated from the dermis using forceps. Epidermal cells were isolated by incubation in 1% trypsin, 5 mM EDTA for 3 min at 37°C. After centrifugation, the cell pellet was resuspended in CT57 keratinocyte medium containing 5 µg/ml gentamycin (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland), then seeded on collagen type I-coated cell culture dishes (BD Falcon, Basel, Switzerland). Medium was changed every 2–3 days.

The dermal tissue was digested in 2 mg/ml collagenase for about 60 min at 37°C. Isolated cells were seeded on 10 cm cell culture dishes containing fibroblast growth medium (DMEM supplemented with 10% FCS, 4 mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, and 5 µg/ml gentamycin) and allowed to attach overnight, before dead cells and erythrocytes were removed by three washes in phosphate-buffered saline (PBS). Collagenase was from Sigma (Buchs, Switzerland), and all other compounds were from Invitrogen (Basel, Switzerland).

Organotypic skin cultures

Organotypic cultures were prepared using a previously established transwell system (4.2 cm² 6-well cell culture inserts with membranes of 3.0 µm pore-size, BD Falcon, Basel, Switzerland). A highly concentrated acidic solution of bovine collagen type I (5 mg/ml, Symatase Biomatériaux, 69630 Chaponost, France) was neutralized on ice by dropwise addition of 0.5 M NaOH and immediately mixed with 5 × 10⁴ human primary dermal fibroblasts (passage 1–2) suspended in fibroblast growth medium. For the fibrin-containing preparations, fibrinogen was added to the collagen before neutralization, thrombin was added after neutralization, and mixed thoroughly. All components were chilled on ice during gel preparation. The mixture was transferred into 6-well culture inserts immediately and allowed to gel for 10 min at room temperature before being transferred into a cell culture incubator.

The fibroblast-containing gels were grown in fibroblast growth medium for 3–7 days before 7.5 × 10⁵ keratinocytes were seeded onto the entire surface of each gel. After initial submersed cultivation in Rheinwald and Green keratinocyte differentiation medium (RGM) [37] for 3–7 days, the medium level was lowered to 1.5 ml to expose the developing epidermis to air (air–liquid interface) for additional 7–10 days. Medium changes were performed every 2–3 days.

Fluorescein diacetate (FdA) vital cell staining

Fluorescein diacetate (FdA) staining was performed as published and proven to be suitable for the determination of cell viability in tissue-engineered skin [38, 39]. In short, cell culture medium was replaced for 2 min with the equal volume of 5 μ M FdA in PBS, freshly prepared from a stock solution of 5 mM FdA in acetone. The FdA was removed by washing twice in PBS before fresh culture medium was applied.

Actin and nuclear staining

Gels were fixed in 4% paraformaldehyde and permeabilised in acetone/methanol (1:1) for 20 min at -20°C , air dried, and washed $3\times$ in PBS. Then they were blocked in PBS containing 2% BSA (Sigma) for 30 min. Cytoskeletal actin was stained with phalloidin-TRITC in blocking buffer for 1 h at room temperature. Slides were washed twice for 5 min in PBS. Thereafter sections were incubated for 5 min in PBS containing 1 mg/ml Hoechst 33342 (Sigma) and then washed twice for 5 min in PBS. Finally, the samples were mounted with Dako fluorescent mounting solution (Dako, Baar, Switzerland).

Fluorescence microscopy

Fluorescence microscopy pictures were photographed using a DXM1200F digital camera connected to a Nikon Eclipse TE2000-U inverted microscope, equipped with Hoechst 33342, FITC, and TRITC filter sets or Nikon SMZ1500 stereo microscope with FITC filters (Nikon AG, Egg, Switzerland), (Software: Nikon ACT-1 vers. 2.70).

For confocal imaging, a Nikon C1 Laser Scanning Microscope upgrade on the Eclipse TE2000-U was used. A helium–neon laser with 543-nm excitation was used for tetramethyl rhodamine iso-thiocyanate (TRITC) and 408-nm excitation was used for Hoechst 33342. With the Plan Apo 40 c/N.A.0.95 objective 50 optical sections were captured. Images were processed with Imaris 6.4.0 (Bitplane, Zurich, Switzerland) and Photoshop 7.0 (Adobe Systems Inc, Munich, Germany).

Results

Cell isolation

Porcine dermal fibroblasts could be isolated efficiently from the dermis according to the protocol and media established for the isolation of human dermal fibroblasts [35]. Starting from a 3×1 cm skin biopsy, we received approximately one million fibroblasts within 2–3 days that

could be further expanded and passaged. This efficiency as well as proliferation rate is comparable to that of human fibroblasts. Importantly, the morphology of porcine fibroblasts was typical and similar to human fibroblasts (Fig. 1a) [40]. Most cells were viable as shown by viability assay using FdA (Fig. 1b).

Trypsinisation of the porcine epidermis resulted in rapid release of keratinocytes that attached within 2 h to bovine collagen type I-coated cell culture dishes. Within 5–8 days the keratinocytes could be expanded to six million cells in a medium optimised for human keratinocyte precursor enrichment. The morphology of confluent keratinocytes resembled the typical cobblestone pattern of epithelial cells with some interspersed large cells (Fig. 1c). By staining with FdA we could show that the majority of cells were vital (Fig. 1d). Therefore our procedure used for human skin cell isolation and cultivation could be applied for porcine cells without any changes [35].

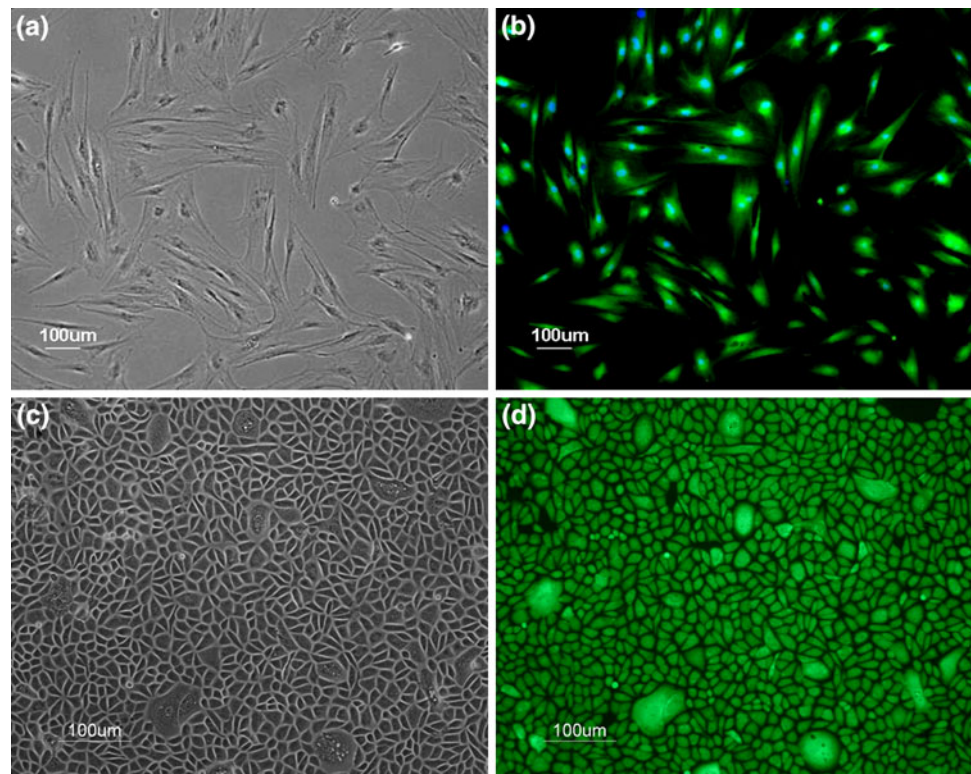
Fibroblast behaviour in collagen

When porcine fibroblasts were cultivated in highly concentrated collagen type I hydrogels (>3 mg/ml), FdA staining revealed that only few vital cells were present 2 days after gel preparation. Furthermore, the majority of cells were round and did not show dendritic or stellate morphology typical for human fibroblasts in collagen gels and in normal dermis [40]. After prolonged cultivation in collagen gels (10 days), some porcine fibroblasts started to proliferate and acquired stellate morphology (data not shown). Porcine fibroblasts seeded in fibrin gels however, were fastly proliferating and showed dendritic or stellate morphology already 1 day after gel preparation (data not shown). We therefore included increasing fibrin concentrations from 0.2 to 1.0 mg/ml in collagen gels to stimulate porcine fibroblasts. Confocal scanning laser microscopy of actin-stained fibroblasts showed a clear effect of fibrin on cell density and morphology. Already 3 days after gel preparation, this effect was detectable and it intensified over the ensuing days. As little as 0.2 mg/ml fibrin in collagen increased the number of vital cells with mainly stellate or dendritic morphology (Fig. 2a, b). This effect was slightly more pronounced with 0.5 and 1.0 mg/ml fibrin (Fig. 2c, d).

Porcine in vitro skin model

Already one day after seeding porcine keratinocytes on the fibroblast-containing gels, a confluent keratinocyte layer was established, indicating that porcine keratinocytes can attach and survive on collagen gels (data not shown). This keratinocyte layer was stable during the initial submerged cultivation phase. During the following air-exposed

Fig. 1 Two-dimensional cell culture of primary porcine fibroblasts and keratinocytes. **a** Phase contrast microscopy shows typical spindle like morphology of fibroblasts in two-dimensional culture. **b** Positive FdA labelling indicates the viability of all adherent fibroblasts. **c** The majority of confluent porcine primary keratinocytes display typical epithelial cobblestone morphology, while few cells are significantly larger and round. **d** FdA staining reveals that both, the large and the small keratinocytes, are vital. [scale bars 50 μm (**a** + **b**); 100 μm (**c** + **d**)]



cultivation phase, a partly stratified epidermis developed (Fig. 3a): a distinct basal layer, 5–7 suprabasal layers, and a well developed stratum corneum (conjunctum and disjunctum) were present. Notably, a stratum granulosum and spinosum could not be distinguished morphologically at this stage. Also, the dermo-epidermal junction was not yet fully established, resulting in an apparently accidental separation of epidermis and dermis in histological sections (cutting artefact). Fibroblasts were present within the dermal component (Fig. 3b). In summary, these features of the engineered porcine skin were typical for in vitro generated skin at early stages that will develop further after transplantation [35].

Discussion

This is the first report on in vitro engineering of a pig cell-derived skin analogue consisting of a multilayered epidermis, a preformed dermo-epidermal junction, and a collagen gel-based dermis-like structure containing fibroblasts and essential extracellular matrix components. In other words, the desirable basic graft profile is basically matched. The following crucial aspects deserve a detailed comment.

First, and most importantly, the graft does exhibit the most essential anatomical hallmarks of skin, although not fully developed. For instance, the epidermis is not yet normally stratified and differentiated. Also, the dermo-

epidermal junction is still weak in that cutting for histology easily leads to disruption between epidermis and dermis which is not the case in normal skin. Similarly, the dermal compartment of the construct is, in reality, to be seen as a competent template for the later establishment of a mature dermis. Although abundant dermal fibroblasts, viewed as the key effector cells of the dermis [41], are present, and although certain crucial ECM components are part of the template, the physiological inventory of components and spatial arrangement of these is not yet accomplished. Moreover, certain morphologic characteristics like rete ridges and dermal vasculature are absent at this stage. Obviously, skin appendages are absent.

This “set of imperfections” is mostly identical to what we found when engineering small human cell-derived skin analogues [34, 35]. Interestingly, after transplantation onto immuno-incompetent rats, we observed maturation and differentiation processes finally leading to a near normal skin anatomy (apart from missing skin appendages) [34, 35]. It is, therefore, reasonable to assume that similar mechanisms will also take place after autologous transplantation of the described grafts in the pig model.

Second, since the planned transplantation of the skin substitutes described here in a pig model is intended to be the last preclinical evaluation, it is imperative that all laboratory procedures (cell isolation, expansion, and organotypic culture system) should be identical or at least very similar to our already established human cell-based

Fig. 2 Laser scanning confocal microscopy of porcine fibroblasts in three-dimensional collagen gels, 7 days after preparation. Actin cytoskeleton staining (*red*) and nuclear staining (*blue*). **a** In highly concentrated collagen gels, only few porcine fibroblasts survive and most cells are round or have few extensions. **b–d** By adding increasing fibrin concentrations, more fibroblasts are present and the prevailing morphology is stellate or dendritic. Fibrin concentrations were **(b)** 0.2 mg/ml, **(c)** 0.5 mg/ml, **(d)** 1.0 mg/ml. (scale bars 50 μ m, grids 20 μ m)

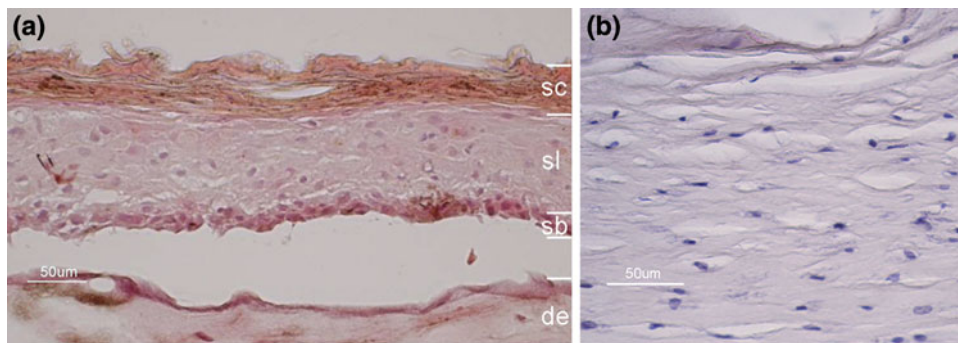
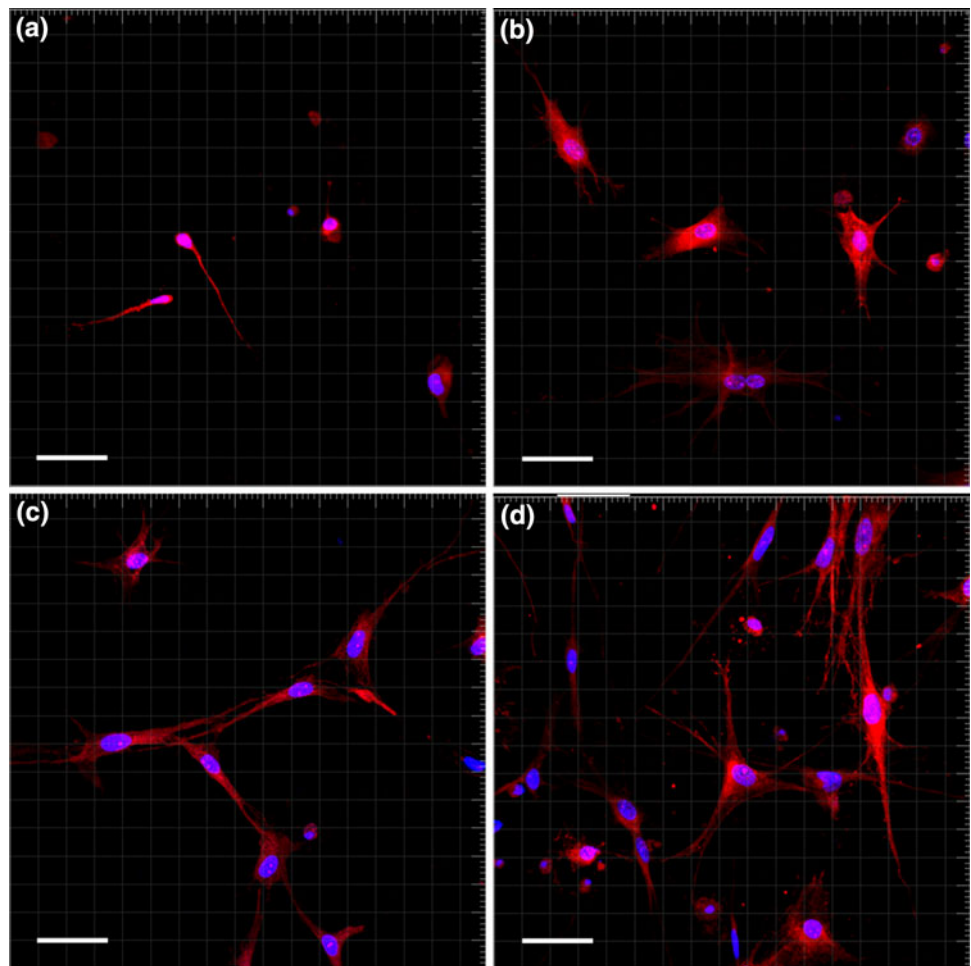


Fig. 3 Histological evaluation of in vitro engineered porcine skin, 20 days in culture, H&E staining. **a** A partly stratified epidermis with a well defined stratum corneum (*sc*), 5–7 suprabasal layers (*sl*), and a dense stratum basale (*sb*) have developed on the dermal equivalent

(*de*). Of note, the junction between epidermis and dermis is still weak and disrupted when preparing sections. **b** Fibroblasts were present within the dermal component (gel). (scale bars 50 μ m)

system [34, 35]. This is in fact the case for most aspects. In particular, we used the identical cell isolation technique as well as media and growth conditions as we did for our human cell-derived experiments [34, 35]. The only initial lack of correspondence was that porcine fibroblasts did not develop as well in highly concentrated collagen gels as human fibroblasts did. By addition of fibrin, however, this

problem could be solved effectively in that porcine fibroblasts were stimulated to behave like human fibroblasts. This observation is interesting in two ways. It raises the questions why porcine fibroblasts do not tolerate high collagen concentrations and whether the already favourable growth conditions for human fibroblasts could be further improved by adding fibrin to the collagen matrix.

In summary, this study shows that pig cell-derived skin analogues closely approaching normal skin anatomy can be engineered in vitro. Evidently, the next experimental step is the development of an appropriate pig model in order to establish a standardized transplantation procedure for further studies.

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