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ORIGINAL ARTICLE

Impact of human mesenchymal cells of different body site origins on the maturation of dermo-epidermal skin substitutes

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Abstract

Aim of the study The use of autologous bio-engineered dermo-epidermal skin substitutes (DESS) yields a pivotal opportunity to cover large skin defects in human patients. These skin grafts consist of both epidermal and dermal compartments necessary for robust and permanent functional wound closure. In this study, we investigated the impact of mesenchymal cells derived from different body site origins on the expression pattern of diverse markers within DESS.

Methods Human keratinocytes were obtained from interfollicular epidermis, and mesenchymal cells were isolated from foreskin, palmar skin, fat tissue, and tonsils. After expansion, epidermal cells were seeded on collagen I hydrogels containing stromal cells. These human DESS were transplanted on the back of immune-incompetent rats. After 3 weeks, transplants were excised and analyzed using immunohistology techniques.

Main results The macroscopic appearance of skin grafts containing tonsil, fat tissue, or palmar derived mesenchymal cells, was similar to substitutes with foreskin derived dermal fibroblasts. All skin grafts had a strong membrane-localized expression of Lingo-1 in the epidermis. Additionally, we observed an intense expression of transglutaminase 5 in upper epidermal cell layers of the skin grafts confirming a proper keratinocyte differentiation. Tropoelastin was localized throughout the dermal compartments and tightly in contact with the dermo-epidermal junction suggesting an advanced maturation of all skin grafts.

Conclusions Our data implicate that stromal cells derived from tonsil, fat tissue, and palmar skin can assume fibroblast functions supporting keratinocyte proliferation and differentiation. These findings indicate that distinct types of mesenchymal cells can be clinically used for skin engineering purposes.

Keywords Skin tissue engineering · Human dermo-epidermal skin substitutes · Mesenchymal cells · Lingo-1 · Tropoelastin

Introduction

One of the main interests in regenerative medicine is finding an appropriate skin substitute to cover deep and extensive skin wounds. Autologous spilt-thickness skin grafts, containing an epidermis and a thin part of the dermis, are the most frequently used grafts, as they are able to cover large wound areas, and their rate of rejection is low [1, 2]. However, the clinical application of large split-thickness skin grafts is limited by shortage of undamaged donor skin. Therefore, the development of autologous tissue-engineered skin substitutes is one of the most promising methods to overcome this limitation. The primary attempt to replace patient's skin loss was the growth of multilayered epithelial autografts containing human keratinocytes (CEA) [1, 3–5]. Although the CEA grafts were an enormous milestone to achieve a faster re-epithelialization of burn wounds, the complete absence of a dermal compartment often limited their clinical application due to poor quality of the resulting epidermis [6]. Currently, an auspicious approach to cover extensive burn wounds is the application of tissue-engineered dermo-epidermal skin substitutes (DESS). These skin equivalents consist of both epidermal and dermal compartments to qualify the critical parameters of clinically applied

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skin transplants. The addition of a dermal compartment, populated with fibroblasts, to skin equivalents resulted in an improved graft take, accelerated wound healing, and better esthetic appearance [7].

In general, fibroblasts are responsible for extracellular matrix production and deposition. When incorporated into skin equivalents, they support keratinocyte proliferation and differentiation, participate in the formation of dermo-epidermal junction and, through the constant mesenchymal–epithelial interaction, maintain homeostasis of overlying epidermis [8].

In this study, we aimed to characterize the influence of stromal cells derived from distinct body sites on maturation of tissue-engineered DESS in vivo. We have previously shown that interfollicular dermal fibroblasts derived from human foreskin samples are a good source of stromal cells for development of tissue-engineered skin substitutes [9–11]. Here, our goal was to test whether fibroblasts from other alternative body sites, such as from palmar skin, tonsils and adipose tissue, can successfully substitute dermal fibroblasts in DESS. We were particularly interested in the expression pattern of tropoelastin and transglutaminase 5 (TG5) in skin equivalents containing fibroblasts from various body sites. Tropoelastin is a soluble precursor of elastin fibers in dermal extracellular matrix, while TG5 is expressed in the epithelium being a marker for terminally differentiated keratinocytes [12, 13]. In addition, we investigated in-depth the expression of leucine-rich repeat Ig domain-containing Nogo-interacting protein 1 (Lingo-1) in tissue-engineered DESS.

Our data demonstrated that stromal cells derived from tonsil, fat tissue, and palmar skin can completely substitute fibroblast functions in tissue-engineered dermo-epidermal skin grafts supporting keratinocyte proliferation, differentiation and maintaining tissue homeostasis. These findings implicate that several alternative fibroblasts sources can be clinically applied for tissue engineering purposes.

Materials and methods

Human skin samples

All experiments were performed according to the Declaration of Helsinki Principles and after permission by the Ethics Commission of the Canton Zurich. Human skin samples were obtained from patients 1–16 years of age after parents or/and patients gave their informed consent. Human subcutaneous adipose tissue samples were obtained from donors aged between 18 and 68 years, male or female, all undergoing a surgical fat liposuction or an excision operation, mostly from abdominal body locations. For histological analysis, tissue samples were embedded in OCT (Sakura Finetek, Switzerland) and kept at − 20 °C.

Isolation and culturing of primary cells

Human epidermal keratinocytes were isolated and expanded from foreskin samples, as described previously [14]. Stromal cells were isolated from human foreskin, palmar skin, or tonsil and propagated as described previously [8, 14]. Adipose-derived stromal cells were isolated from fat tissue after liposuction and cultivated as described previously [15].

Preparation of dermo-epidermal skin analogs

Dermo-epidermal skin substitutes were prepared by mixing collagen type I (Symatese, France) with 1×10^5 stromal cells derived from distinct body locations, and casted into 6-well cell culture inserts (3.0 µm pore-size membranes) (BD Falcon, Switzerland). After 7 days of cultivation in DMEM medium supplemented with 10% fetal calf serum (Invitrogen, Switzerland), 5% Pen/Strep, 5% HEPES, human keratinocytes from interfollicular epidermis were seeded onto the dermal equivalents at a density of totally 5×10^5 cells/gel. Skin substitutes were cultivated in CnT-57 (CellnTec, Switzerland) for additional 1 week and subsequently transplanted on the back of immune-incompetent rats.

Transplantation of cultured skin substitutes

The surgical protocol was approved by the Local Committee for Experimental Animal Research (permission number 76/2011). In brief, dermo-epidermal skin substitutes were transplanted on full thickness skin defects created on the back of immune-incompetent female nu/nu rats, 8–10 weeks old (Charles River, Germany). To prevent wound closure by surrounding rat skin, custom made surgical steel rings (diameter 2.6 cm) were sutured to the skin of rats using non-absorbable polyester sutures (Ethibond®, Ethicon, USA). Silicon foil (Silon-SES, BMS, USA), a polyurethane sponge (Ligasano, Ligamed, Austria) and a tape (Leukoplast, BSN medical, Germany) were used as wound dressing. Dressing changes were performed once a week. The transplants were excised 3 or 13 weeks after transplantation and embedded in OCT compound for further analysis.

Immunohistochemical staining and analysis

Immunofluorescence stainings were performed as described previously. Briefly, Lingo 1 (1:50, Abcam, Switzerland), and transglutaminase 5 (1:50, Santa Cruz, USA) were used to visualize the components of epidermis, while tropoelastin (1:50, EPC, USA) was used to characterize the dermal

compartment in analyzed skin analogs in vivo. To visualize basement membrane laminin 5 (1:50, Santa Cruz, USA) antibody was used. As secondary antibodies FITC or TRITC-conjugated immunoglobulins were applied. Additionally, some of the primary antibodies were pre-labeled with Alexa 488 or 555 labeling kit, according to manufacturer's instructions (Zenon Mouse IgG Labeling Kit, Molecular Probes, Invitrogen). A DXM1200F digital camera connected to a Nikon Eclipse TE2000-U inverted microscope was used to take pictures of immunofluorescence stainings. The device is equipped with Hoechst 33342-, FITC-, and TRITC-filter sets (Nikon AG, Switzerland; Software: Nikon ACT-1 version 2.70). Images were processed with Photoshop 11.0 (Adobe Systems Inc., Germany).

Results

Expression of tropoelastin in DESS containing stromal cells of different body site origins

The dermal maturation of skin substitutes containing mesenchymal cells derived from interfollicular dermis, palmar dermis, fat tissue or tonsil was investigated using an antibody against the human precursor of elastic fibers in extracellular matrix, tropoelastin (Fig. 1a–f). Additionally, to visualize the dermo-epidermal junction, a staining against laminin 5 was performed. Tropoelastin was localized throughout the dermal compartments and appeared in tight contact with dermo-epidermal junctions suggesting an advanced maturation of all skin grafts.

Of note, as compared to other skin substitutes, an extensive and dense network of tropoelastin was found in analogs with adipose-derived stromal cells (ASC) (Fig. 1d). In addition, 13 weeks after transplantation, skin graft with interfollicular fibroblasts (Fig. 1b) demonstrated an increased

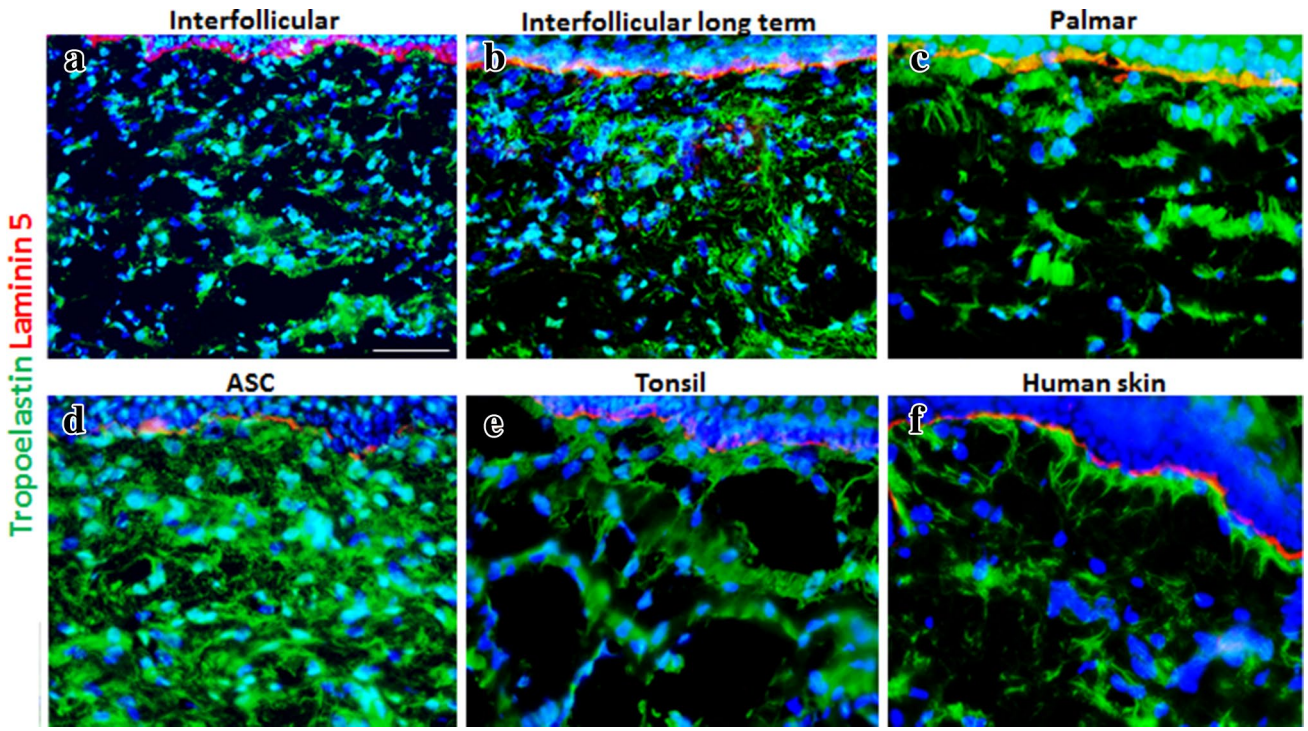

Fig. 1 Expression pattern of the dermal marker tropoelastin in transplanted tissue-engineered skin analogs. Immunofluorescence double staining for tropoelastin (green) and laminin 5 (red) in skin substitutes consisting of interfollicular fibroblasts **a** 3 weeks and **b** 13 weeks after transplantation. Laminin 5 visualizes the dermo-epidermal junction (DEJ) between epidermis and dermis. Tropoelastin can be found throughout the dermis and appears in tight contact with the DEJ. Note a significantly enhanced expression of tropoelastin after 13 weeks in vivo. Immunofluorescence double staining for tropoelastin (green) and laminin 5 (red) in transplanted skin substitutes containing stromal cells derived from **c** palmar skin, **d** adipose tissue, and **e** tonsil. Tropoelastin is distributed throughout the human neodermis and is in tight contact with the DEJ. Note that the expression of tropoelastin is increased in skin grafts with ASC. **f** Tropoelastin (green) and laminin 5 (red) expression in native human skin. Cell nuclei are stained with Hoechst (blue). Scale bar 50 µm. *ASC* adipose-derived stromal cells

expression of tropoelastin as compared to 3 weeks transplants (Fig. 1a). In all skin grafts, the pattern of tropoelastin expression was comparable to normal human foreskin (Fig. 1f).

The influence of stromal cells of different body site origins on the expression of transglutaminase 5 in DESS

To verify in-depth keratinocyte differentiation and the assembly of cornified cell envelope in skin grafts, we performed immunofluorescence analysis using TG5 and laminin 5 antibodies (Fig. 2a–f). In all skin analogs, the TG5 antibody decorated the upper layers of epithelium, suggesting the proper keratinocyte differentiation. Three weeks post-transplantation, TG5 expression in skin analogs with interfollicular (Fig. 2a), palmar (Fig. 2c), fat tissue (Fig. 2d) and tonsil (Fig. 2e) derived stromal cells followed a gradient from the spinous to granular layers. Additionally, this marker was also detectable at very low levels in basal cell layers.

After 13 weeks in vivo, the skin substitutes with interfollicular fibroblasts (Fig. 2b) exhibited an increased expression of TG5 as compared to 3 weeks transplants (Fig. 2a). This abundance of TG5 protein in long-term DESS in vivo was comparable to normal human foreskin (Fig. 2f).

The influence of stromal cells of different body site origins on expression of epidermal marker Lingo-1 in DESS

Skin grafts with stromal cells derived from distinct body site origins were double stained for Lingo-1 and laminin 5 (Fig. 3a–f). The epidermis of all skin analogs showed a multilayered, stratified, and cornified epithelium. Already 3-weeks post-transplantation, all skin grafts demonstrated a strong membrane-localized expression of Lingo-1 in the uppermost layers of epidermis (Fig. 3a–e) showing a similar pattern as observed in native human skin (Fig. 3f). The only identifiable difference was a more pronounced expression of Lingo-1 throughout all suprabasal epidermal layers in the transplants constructed with interfollicular (Fig. 3a) and palmar (Fig. 3c) derived fibroblasts as compared to other DESS.

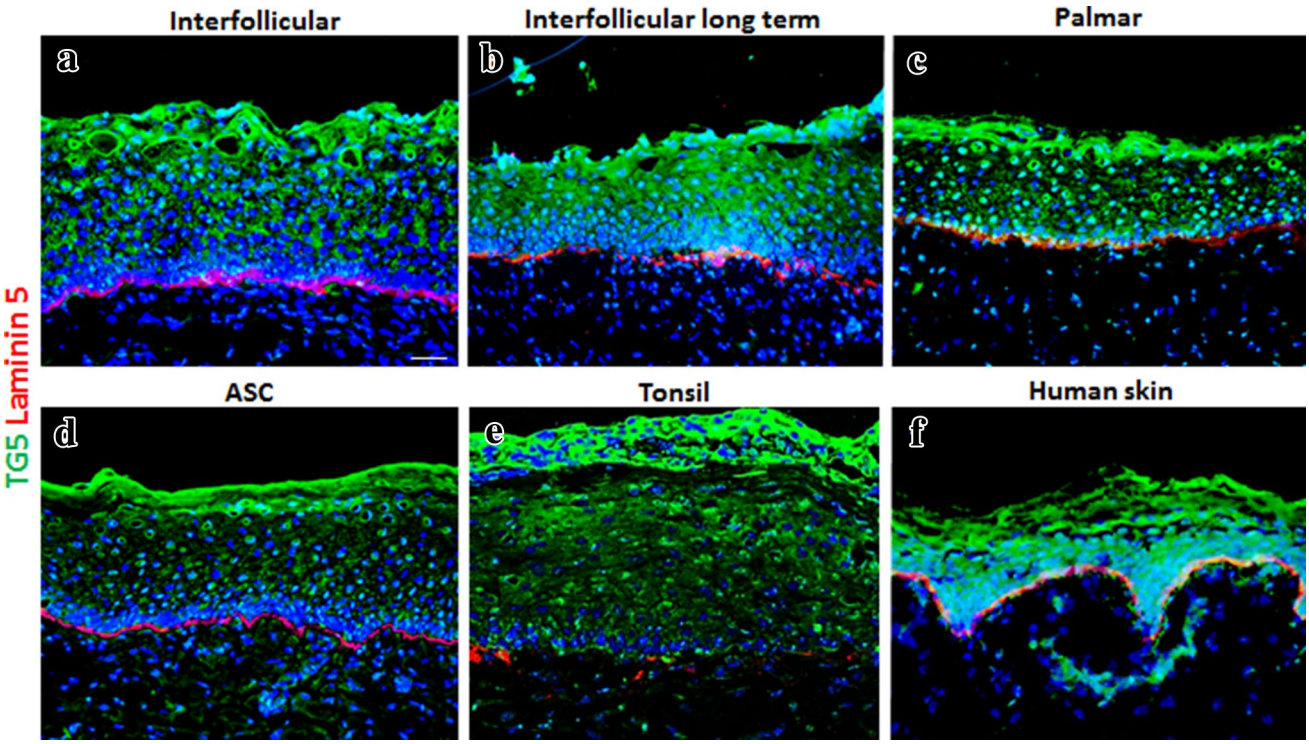

Fig. 2 Expression pattern of epidermal marker transglutaminase 5 (TG5) in transplanted tissue-engineered skin analogs. Immunofluorescence co-staining for TG5 (green) and laminin 5 (red) in transplanted DESS with interfollicular fibroblasts after **a** 3 weeks and **b** 13 weeks in vivo. Laminin 5 stains the dermo-epidermal junction (DEJ). TG5 is expressed in upper differentiated layers of the epithelium. Staining for TG5 (green) and laminin 5 (red) in skin equivalents constructed with **c** palmar skin, **d** adipose tissue, and **e** tonsil-derived mesenchymal cells. TG5 is also present in the upper layers of the epidermis. **f** Expression of TG5 (green) and laminin 5 (red) in native human skin. Cell nuclei are stained with Hoechst (blue). Scale bar 50 µm. *TG5* transglutaminase 5

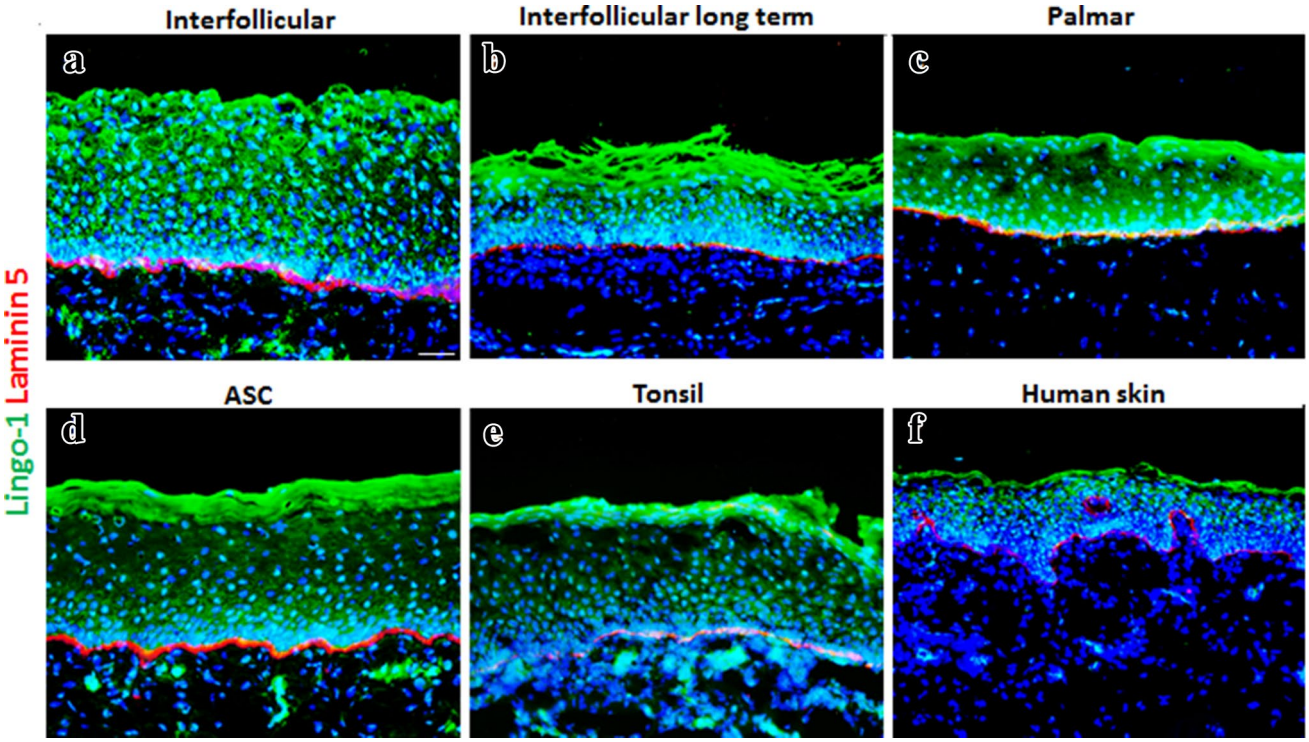

Fig. 3 Expression pattern of Lingo-1 in transplanted human dermo-epidermal skin substitutes. Immunofluorescence double staining for Lingo-1 (green) and laminin 5 (red) in skin equivalents containing interfollicular fibroblasts after **a** 3 weeks and **b** 13 weeks in vivo. Laminin 5 demonstrates dermo-epidermal junction between epidermis and dermis. Lingo-1 shows a strong membrane-localized expression in upper layers of the epithelium. Staining for Lingo-1 (green) and laminin 5 (red) in transplanted skin analogs containing mesenchymal cells derived from **c** palmar skin, **d** adipose tissue, and **e** tonsil. Expression of Lingo-1 is also visible in the upper layers of epidermis. **f** Expression pattern of Lingo-1 (green) and laminin 5 (red) in native human skin. Cell nuclei are stained with Hoechst (blue). Scale bar 50 µm

Discussion

Here, we report on the influence of mesenchymal cells derived from distinct body site origins on the maturation of human tissue-engineered dermo-epidermal skin substitutes in vivo. In particular, we analyzed in-depth the expression of certain markers, such as tropoelastin, TG5, and Lingo-1, in tissue-engineered DESS containing interfollicular, palmar, fat tissue, and tonsil-derived stromal cells. In a global picture, our data suggest that mesenchymal cells from different body locations can assume fibroblasts functions supporting the process of epidermal and dermal maturation. These findings deserve further comments.

In the present study, we observed that tropoelastin, a soluble precursor of elastic fibers in extracellular matrix, is abundantly expressed throughout the dermal compartments of all skin substitutes in a pattern comparable to the native human skin. The association of tropoelastin with other supporting microfibril proteins, such as fibrillin-1 and 2 and fibulin-4 and 5, leads to the formation of mature and insoluble elastic fibers responsible for skin elasticity [13, 16]. Hence, our findings confirm the achievement of already advanced dermal homeostasis in our DESS 3 weeks after transplantation.

Of note, we observed an enhanced expression of tropoelastin in skin grafts containing adipose-derived stromal cells (ASC) as compared to other transplants. Recently, several studies have confirmed that ASC secrete distinct cytokines and growth factors that improve wound healing via paracrine signaling [17, 18]. One of these factors secreted by ASC is Transforming Growth Factor β1 (TGFβ1), known to play an important role in the regulation of extracellular matrix (ECM) components [19]. Therefore, we suggest that the extensive and abundant tropoelastin network in skin grafts with ASC may be due to an increased synthesis of TGFβ1, which in turn leads to the accelerated ECM component production. In addition, we observed an increased expression of tropoelastin in skin grafts with interfollicular dermal fibroblasts after 13 weeks in vivo indicating a more advanced maturation of those human DESS.

In general, TG5, a known marker of terminally differentiated keratinocytes, is an Ca^{2+} crosslinking enzyme that catalyzes the formation of isopeptide bonds between neighboring polypeptides playing the significant role in the assembly

of cornified envelope in skin [20]. In this study, TG5 was detected in the upper layers of the epithelium and followed the expression gradient reaching from spinous to granular layers. This support the notion that all types of transplants exhibited a multilayered stratified epidermis with a thick stratum corneum. This finding suggests that already after 3 weeks in vivo, interfollicular, palmar, adipose tissue, and tonsil-derived mesenchymal cells are able to support keratinocyte differentiation, allowing for the proper formation of cornified cell envelope.

Additionally, for the first time, we investigated in-depth the expression of leucine-rich repeat Ig domain-containing Nogo-interacting protein 1 (Lingo-1) in human DESS containing stromal cells derived from different body site origins in vivo. Lingo-1, a transmembrane glycoprotein, was originally found in neurons and oligodendrocytes in spinal cord and brain [21]. It is described as a curtail component of a cell-surface receptor that negatively regulate various central nervous system (CNS) functions including neuronal differentiation and growth, oligodendrocyte myelination as well as axon branching and regeneration [21–23]. However, the expression of Lingo-1 is not exclusively restricted to CNS. Choi et al. investigated the presence of Lingo-1 in human skin before and after UVB irradiation [24]. The authors demonstrated that Lingo-1 is visible in both keratinocytes and melanocytes in unexposed skin and it is dramatically enhanced upon exposure to UVB light. Here, we have shown that Lingo-1 exhibit a strong membrane-localized expression in the uppermost layers of epithelium of all skin grafts. Interestingly, the expression pattern of Lingo-1 is independent on the type of mesenchymal cells used for the preparation of the human DESS. Nevertheless, the exact role of Lingo-1 in human skin remains unknown and requires further investigation.

It is known that mesenchymal cells derived from distinct body locations influence the expression pattern of certain proteins in epithelium. Biedermann et al. have shown that stromal cells present in human dermis significantly influence the pigmentation process in skin substitutes [25]. Other studies demonstrated that dickkopf 1 (DKK1) is the main factor secreted by palmoplantar fibroblasts that inhibit melanin synthesis leading to hypopigmentation of palms and soles [26, 27]. In addition, it has been shown that fibroblasts influence not only melanocytes but also keratinocytes in their expression pattern of cytokeratins. Only palmoplantar stromal cells induce the expression of Cytokeratin 9 (CK9) in keratinocytes, whereas non-palmoplantar situated fibroblasts cause the expression of Cytokeratin 2e (CK2e) in keratinocytes in human skin and human DESS [25, 28]. Of note, the proteins investigated herein were observed in almost the same pattern. This suggests that at least the occurrence of these proteins are not, or only in minor fashion, hampered by the stromal cells derived from various origins.

In conclusion, this is, to our best knowledge, the first study comprising the influence of mesenchymal cells derived from distinct body site origins on the dermal and epidermal marker expression of tropoelastin, TG5 and Lingo-1 in tissue-engineered DESS in vivo. Apparently, these distinct stromal cells can fully assume the essential fibroblasts functions supporting keratinocytes proliferation and differentiation. From a global picture, these findings indicate that distinct types of mesenchymal cells can be clinically used for human skin engineering purposes.

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Compliance with ethical standards

Conflict of interest ER and MM are co-founding members and shareholders of "Cutiss AG", a company to fund the further development of the tissue-engineered skin substitutes. All other authors declare that they have no conflict of interest.

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