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TISSUE-ENGINEERED BIOLOGICAL DRESSING ACCELERATES SKIN WOUND HEALING IN MICE VIA FORMATION OF PROVISIONAL CONNECTIVE TISSUE

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SKIN EQUIVALENT FOR WOUND HEALING

KEY WORDS: wound healing, tissue engineering, living skin equivalent, wound dressings, collagen-based scaffolds

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ABSTRACT

Despite recent advances in bioengineered therapies, wound healing remains a serious clinical problem. In acute full-thickness wounds, it is desirable to replace both the damaged dermis and epidermis in a single procedure. This approach requires appropriate properties of tissue-engineered dressings to support simultaneous regenerative processes in the dermis and epidermis while they are temporally separated in the natural wound healing process.

In this study, a collagen-based scaffold inhabited by skin cells was employed. Its ability to stimulate the skin repair of full-thickness excisional splinting wounds in a murine model was evaluated in comparison with that of acellular collagen and commercially available gelatin porous sponge Spongostan\textsuperscript{®}.

The study showed that cell-based skin equivalent promoted the immediate filling of the wound bed and provided simultaneous reorganization of the dermal component into highly vascularized granulation-like tissue and rapid epithelialization, thus improving the quality of healing. Inflammation was delayed and less pronounced. In contrast, acellular collagen and especially Spongostan\textsuperscript{®} failed to demonstrate similar results. The porous structure of Spongostan\textsuperscript{®} prevented effective long-term epithelialization and impeded the formation of an adequate connective tissue at the wound bed.
INTRODUCTION

Although treatments for most skin wounds are effective, healing an extensive area of wounds and burns, particularly chronic wounds, remains a major clinical problem. The effective healing of large-area wounds requires additional approaches with the use of skin substitutes to facilitate repair and regeneration (Balasubramani et al., 2001). There is a huge variety of wound dressings aimed to accelerate wound healing available today (Broussard and Powers, 2013; Wood, 2014; Gould, 2016), based on the use of natural or synthetic materials, as well as the presence or absence of cells. Acellular synthetic scaffolds can be produced in the form of hydrogels, sponges, or lattices and exhibit any required mechanical and physical characteristics (such as microstructure, porosity, tensile strength and elasticity) to promote cell-biomaterial interactions (Zhong et al., 2010). Many natural polymers, including collagen, chitosan, fibrin, elastin, gelatin, and hyaluronic acid, have been investigated for wound treatment applications (Bell et al., 1981; Tanaka et al., 2005; Zhong et al., 2010; Collins and Birkinshaw, 2013; Croisier and Jerome, 2013). Collagen has been widely used in skin tissue engineering due to its structural and functional similarity to native extracellular matrix (Zhong et al., 2010). Gelatin, as a partially hydrolyzed version of collagen, has shown promising results in wound healing when used in formulations such as sponges and films (Choi et al., 2001; Tanaka et al., 2005). Different techniques have been developed to design and fabricate two-dimensional cellular dressings, such as cell sheets, epidermal constructs, dermal equivalents, and bilayered skin substitutes (Zhong et al., 2010). The advantages of three-dimensional scaffolds containing cells are doubtless. When placed in the wound bed, the three-dimensional matrix serves as a substrate for seeding cells and as a physical support to guide tissue regeneration and the formation of new tissue. Combining stem cells with biomaterial scaffolds provides a promising strategy to treat acute and chronic cutaneous wounds (Branski et al., 2009; Ozturk and Karagoz, 2015; Heublein et al., 2015).

Living skin equivalent (LSE), a cell-based collagen living tissue dressing, has been used successfully in burns, acute wound healing, chronic skin ulcers, and even non-skin defects (Bell et al., 1981; Falanga et al., 1998; Kirsner, 1998; Waymack et al., 2000; Rogovaya et al., 2015). Tissue-engineered skin containing both dermal and epidermal components may be the best alternative to split-thickness grafts for wound healing treatment to support the simultaneous regenerative processes in the dermis and epidermis (Krejci et al., 1991). It is not fully understood how this skin equivalent initiates wound healing and promotes more rapid and successful wound healing, which suggests that the donor cells release factors that activate the host keratinocytes and stimulate their migration and re-epithelialization (Falanga et al., 2002; Brem et al., 2003; El Ghalbzfouri and Ponec, 2004; Spiekstra et al., 2007).
This study describes the application of LSE in cutaneous wound healing and shows that a cell-based skin equivalent promotes the immediate filling of the wound bed and provides simultaneous reorganization of the dermal component into highly vascularized granulation-like tissue and rapid epithelialization, thus improving the quality of healing. The efficiency of wound healing by LSE was compared when using an acellular collagen gel and a commercially available gelatin sponge Spongostan®.

MATERIALS AND METHODS

All experiments were approved by the local ethical review committee of the Koltsov Institute of Developmental Biology, Russian Academy of Science.

Isolation and culture of primary mouse neonatal cells

Mouse cells were isolated and cultured from neonatal C57Bl/6 mice. Skin pieces from the dorsal and ventral sides of the P1-P3 mouse pups were removed, washed in Hank’s balanced salt solution containing 80 µg/ml gentamicin, and incubated in 0.05% Dispase (Gibco) in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) for 12 h at 4°C. The epidermis was then gently separated from the underlying dermis using forceps, transferred into a 1:1 phosphate buffered saline (PBS):Trypsin (0.25%; Gibco) mixture, and incubated at 37°C for 7–10 min. Trypsin was inhibited with fetal bovine serum (FBS, Gibco), and single keratinocytes were obtained by gentle pipetting. The cell suspension was transferred to another tube and centrifuged at 200 g for 5 min. The cell pellet was resuspended in CnT-07 medium (CELLnTEC).

The dermis remaining after the epidermis separation was cut into 2x2 mm² pieces and digested in 0.25% collagenase over 1-1.5 h at 37°C. Isolated fibroblasts were seeded and expanded in DMEM with 10% FBS (Gibco). Fibroblasts and keratinocytes were cultured at 37°C and 5% CO₂, and the medium was changed every 3 days.

Fabrication of the skin equivalents

Dermal equivalents were prepared with type I collagen extracted from rat tail tendons with 0.1% acetic acid. Eight volumes of ice-cold collagen solution were mixed with one volume of 199 Eagle’s medium followed by neutralization with 0.34 N NaOH (Sigma). One volume of
FBS was added with suspended mouse dermal fibroblasts (passage 5-6) and mixed thoroughly, resulting in a final concentration of $2 \times 10^5$ cells/ml. Collagen was allowed to gel at 37°C in a CO$_2$ incubator for 15 min. Then, the collagen gel was immersed in DMEM containing 10% FBS and GlutaMAX (Gibco). The next day, neonatal mouse keratinocytes (passage 1-2) were seeded and maintained in DMEM/F-12 (1:1) containing 10% FBS, insulin (5 mg/ml, Sigma), isoproterenol ($10^{-6}$ M, Sigma), transferrin (5 µg/ml, Sigma), and epidermal growth factor (10 ng/ml, Sigma) for 10 days. The day before transplantation the medium was changed for the one containing the same components except for FBS.

**Animals and surgical procedure**

Forty C57Bl/6 mice (ten animals per group) were used in the study. Animals were provided with food and water *ad libitum*. On the day of surgery, mice were anesthetized with an intraperitoneal injection of Avertin solution (250 mg/kg), and the skin on their backs was depilated. A wax and rosin mixture (Beauty Image, Cemsa, Spain) was applied to the site of surgery and around, then quickly removed after cooling. The flap was excised down to the panniculus carnosus muscle with a 5-mm-diameter sterile biopsy punch to create a standard wound. To prevent contraction, a donut-shaped silicone splitting ring was applied to the wound. The inner diameter of the splint was 6 mm. Cyanoacrylate adhesive Super Glue™ was spread on one side of a splint, and the splint was carefully placed around the wound (with the glue side down) so that the wound was centered within the splint. The splint was then sutured to the skin with five interrupted sutures (5-0 Softsilk SS-673 – Covidien or 4-0 Silk 3096-6 Volot’). A gelatin sponge was cut by a 5-mm punch and soaked in PBS for 10 min before transplantation. Tissue equivalents or control materials were applied onto wound beds followed by Tegaderm™, which was further covered by self-adhering elastic bandage (Coban, 3M). The adhesive was tested on the mouse skin prior to this experiment, and no skin irritation or allergic reaction was observed.

Mice were sacrificed on day 6 and 13 when skin samples including the wound and 3 mm of the surrounding skin were harvested. The wound was bisected, and the specimens were processed for histological and immunohistological examination.
**Wound closure test**

For the evaluation of the wound closure dynamics, digital photographs of the wounds were analyzed using ImageJ, a Java-based image processing program developed at the National Institutes of Health (USA). The wound closure percentages were measured at different time points (on days 0, 6 and 13) to calculate the residual wound area non-covered by healed skin. Residual wound area (%) = Area on day n/ Area on day 0 x 100.

**Epithelialization rate analysis**

The epithelialization dynamics were evaluated by measuring the epithelial gap in the digital photographs of wounds taken on day 6 and 13. Three sections from at least three mice were examined in each group.

**Histology**

Wound samples with a margin of healthy skin were harvested on days 6 and 13 postoperatively, fixed overnight in 10% formalin (Biovitrum), embedded in paraffin blocks, and cut into 5-µm sections. Hematoxylin and eosin staining was performed according to standard protocols. Mallory’s trichrome staining was performed with Picro Mallory trichrome kit (Bio-Optica) according to manufacturer’s instructions.

**Immunohistochemistry**

Wound samples harvested as above were immersed in OCT Cryomount (HistoLab, Gothenburg, Sweden) and frozen in liquid nitrogen. Cryostat sections were prepared and dried overnight at room temperature. They were fixed for 10 min in 4% paraformaldehyde, washed thoroughly in PBS, incubated for 5 min with 3% hydrogen peroxide in distilled water followed by PBS wash step. Sections were then incubated for 1 h at 37°C or overnight at 4°C with primary antibodies in blocking solution (5% of bovine serum albumin, 0.1% Triton in PBS). Further staining was performed using the ImmPRESS HRP reagents and ImmPACT DAB peroxidase substrate (Vector Labs) according to instructions from the manufacturer. Primary antibodies were rabbit anti-keratin 5 (1:250; Abcam), rabbit anti-Ki67 (1:50; Abcam), rabbit
anti-CD31 (1:50; Abcam), rabbit anti-CD68 (1:300; Abcam). Light microscopy was performed with a Keyence BZ-9000 Instrument.

Image analysis and statistics

All statistical data are represented as the mean ± SEM. All results were replicated in at least three different animals. Keyence Analyzer software was used for digital quantifications of measuring wound epithelialization distances, and cells, vessels and hair follicles counting. An ANOVA model for repeated measures was used. Statistical analysis was carried out with GraphPad Prism software. A probability (p) value <0.05 was considered statistically significant.

RESULTS

Addition of cells to collagen gel accelerates wound healing

Living skin equivalents (LSEs) were fabricated from fibroblast-populated collagen gel followed by seeding of cultured keratinocytes on its surface. Allogeneic cell transplantation was performed in this study. The LSEs were transplanted onto the wound after a 10-day cultivation period. The culture time is necessary for LSE maturation, when the keratinocytes differentiate and the collagen-containing fibroblasts undergo a contraction process shrinking the gel volume by approximately 40%. Our preliminary studies have shown that the mature LSE is more effective in wound healing than the fresh one. Before grafting, the LSE showed a well-differentiated epidermis, positive for keratin 5 (Fig. 1).

A splint excisional wound model was used to evaluate the wound healing. The ability of the LSE to regenerate the wound was compared with a commercial porous gelatin sponge Spongostan®, acellular collagen and a non-treated control. First, the dynamics of wound closure were evaluated, and it was found that the LSE group showed significant improvement in the wound closure rate (Fig. 2). After 6 days, only the acellular collagen and LSE were able to reduce the wound area significantly while the wounds of the non-treated control and gelatin sponge group kept their initial size with no significant difference between the latter two groups. On day 13, only the LSE was able to close the wound almost completely, showing a significantly decreased residual wound area compared to the other groups (Fig. 2).
**LSE prevented inflammatory cells from migration inside the graft and provided a favorable connective tissue environment for regeneration**

Wound healing begins with the appearance of a fibrin clot followed by migration of the immune system cells and fibroblasts, which later on participate in collagen synthesis and generation of the granulation tissue. The granulation tissue is an important indicator for the wound healing prognosis and outcome. Mallory's trichrome stain allows for evaluating connective tissue remodeling in the course of the wound healing process. Mallory-stained sections of wounds revealed the difference in the repair outcome between our groups. On day 6, the collagen and LSE groups had collagen substrate in the wound bed that came from transplants. The porous structure of the gelatin sponge appeared as a fragmented meshwork of blue fibrils in accordance with its content (Fig. 3a). On day 13, the LSE wound beds contained collagen fibers, which was in contrast to the collagen and Spongostan® groups; these groups demonstrated intensive pink staining (Fig. 3a), which may be attributed to fibroglia. This means that collagen synthesis was delayed in these groups. Compared to other groups, a greater deposition of collagen fibers and the reduction of the inflammatory process manifested by the infiltration of wound beds were observed in the LSE group on day 13. Thus, the LSE accelerated granulation tissue formation during wound healing. However, it should be mentioned that intensive matrix remodeling was almost completed in the control group on day 13, while the LSE group demonstrated a certain delay in this process, which may be attributed to the additional collagen gel introduced into the wound. Nevertheless, this did not prevent epithelialization.

To evaluate the inflammation in the wounds, cryosection staining for the macrophage-lineage marker CD68 was performed, and positive cells at the margin and in the center of wounds were counted on day 6. Interestingly, collagen gel attracted the greatest number of inflammatory cells to the margins of the wounds (Fig. 3b), while for the same matrix with the addition of fibroblasts (LSE) this count was comparable to the control wounds. Remarkably, LSE prevented the migration of inflammatory elements inside the graft, and there were significantly less CD68-positive cells in the center of the wound in the LSE group compared to the other groups (Fig. 3b).
Porous substrate failed to maintain long-term persistence of the epithelium while LSE stimulated epithelialization and cell proliferation

Since re-epithelialization of the wound is critical for successful wound healing, the effect of transplants on epithelialization was compared. For this purpose, the gap between keratin 5-positive leading edges was measured as this specific keratin of basal keratinocytes demarcates the leading edge of the migrating epidermal sheets. The decrease of the distance between the leading edges indicates better epithelialization.

Acellular collagen promoted rapid epithelialization; however, the addition of cells in the collagen gel (LSE) accelerated this process even more after 6 days (Fig. 4a,d). The gelatin sponge group showed rapid epithelialization on day 6, when keratinocytes actively migrated into the pores of the sponge, but failed to maintain cell viability and integrity of the epidermis on day 13 (Fig. 4a,d).

Adequate and quick assembly of the basal lamina may accelerate the attachment and migration of keratinocytes. Wound cryosections were analyzed for laminin 5 and type IV collagen expression as typical components of the basal lamina (Fig. 4b,c). The type of grafts does not seem to influence the protein expression. The comparable patterns of laminin 5 and type IV collagen deposition were observed on day 6 and 13 of wound healing upon injury (Fig. 4b,c).

The proliferation of the keratinocytes is a key step of epithelialization, as the coverage of the wound area requires the multiplication of keratinocytes. The migration of newly formed epithelial cells from the wound edge occurs as a result of the enhanced cell division in the basal layer of the epidermis. To investigate the role of LSE in the wound healing processes, histological sections were examined (Fig. 5a), and the amount of Ki-67-positive cells in the epithelium and dermis was evaluated using immunohistochemistry (Fig. 5b,c). On day 6, the gelatin sponge group had 1.5 times more epidermal proliferation (p<0.05) compared with the LSE and control groups (Fig. 5b). At the same time, the dermal proliferation was significantly upregulated in the LSE group, which may indicate effective formation of granulation tissue (Fig. 5c).

However, day 13 brought other results. Compared with the control group, the LSE stimulated epidermal proliferation in the center of the wound 3-fold (p<0.05) (Fig. 5b) and promoted proliferation in the dermis both in the marginal zones and in the center of the wound 3.5 and 2-fold, respectively (p<0.05) (Fig. 5c).
LSE stimulated angiogenesis and hair follicle morphogenesis

The efficacy of the LSE in angiogenesis was analyzed further by evaluating the expression of the endothelial cell marker CD31. A significant increase in the number of blood vessels was detected in the LSE-treated wounds ($p<0.05$) 13 days after graft in contrast to the non-treated control, acellular collagen and gelatin sponge-treated wounds (Fig. 6a,b).

The number of vessels in the marginal wound zones grew more than 4.5 times and almost doubled in the center of the wound compared to the control. There was no significant difference in the number of vessels between the control and the gelatin sponge groups (Fig. 6b).

From analyzing the structure of the regenerated dermis, an obvious increase in the number of hair follicles was observed at the marginal zones of LSE-treated group compared with the acellular collagen, gelatin sponge and non-treated control groups on both days 6 and 13 (Fig. 6c). In the LSE group, newly formed follicles were generated not only at the wound edges but also close to the center of the wound. Compared to the control groups, the hair follicle number of the LSE-treated group grew by 5 times at the wound edges as early as day 6 (Fig. 6c).

DISCUSSION

Wound healing in humans comprises a sequence of complex biological processes, including migration, proliferation, angiogenesis, granulation tissue formation, extracellular matrix deposition and remodeling of the connective tissue, resulting in eventual re-epithelialization of the wound (Chen et al., 2009). Despite general similarities in regenerative processes between humans and mice, wound contraction is very important for mouse wound healing. To imitate the features observed in human wound healing, an excisional wound splinting model was followed, which prevents skin contraction, so the wound healing occurs through the same human granulation and epithelialization processes (Galiano et al., 2004; Wang et al., 2013). To compensate temporarily for the deficiency of the connective tissue in the wound and fill the defect, various matrices, distinguished by cell presence and porosity, were tested. In this study, the healing qualities of LSE were superior to that of the control groups. To create controls similar to the LSE collagen gel but without cells, the gelatin sponge Spongostan® and non-treatment were chosen. Wound treatment with different matrices showed what kind of matrix features favor excisional wound healing. Type I collagen matrices, inhabited by cells or not, favored keratinocyte migration, which is important for re-epithelialization. It was shown before that type I collagen has a stimulatory effect on keratinocyte migration (Fu et al., 2014). The addition of skin cells to collagen gel (LSE) accelerated the re-epithelialization process, as well as
angiogenesis, hair follicle regeneration and granulation tissue formation, suggesting the advantageous effect of the LSE in wound healing. LSE comprises allogeneic epithelial and mesenchymal cells. Allogeneic cells can survive approximately 20 days in vivo (Morimoto et al., 2005) and showed a promoting effect on host cells in different studies (You and Han, 2014). Mesenchymal cells, in particular dermal fibroblasts, are distributed in the volume of the collagen structure and serve as an activator for the regeneration and repair processes of connective tissue. Epithelial keratinocytes activate the regeneration and repair processes of epithelial tissue. Transplantation of such cellular grafts to the wound bed can construct an environment advantageous for recruiting autologous keratinocytes for an accelerated re-epithelialization. The proliferation of epithelial cells was statistically expressed in the LSE group, which apparently contributed to the accelerated re-epithelialization and closure of the wound. Cell proliferation in the dermis was statistically high 6 days after the injury and treatment with LSE, suggesting the promoting effect of the allogeneic donor cells. Dermal cell proliferation reduction on day 13 may argue for the transition to the remodeling phase. It is known that cutaneous tissue damage initiates fibroblast proliferation, resulting in repopulating the damaged area and producing new granulation tissue (Eming et al., 2007a). The granulation tissue finally turns into mature connective tissue by a slow process called tissue remodeling, and fibroblast proliferation decreases. The acellular gel promoted cell proliferation on day 13, suggesting a delay in the wound healing process.

Neovascularization is a crucial step in the wound healing process to support the newly formed granulation tissue and the survival of keratinocytes. In our study, the acellular collagen gel and LSE promoted angiogenesis, suggesting the collagen matrix in the form of the gel did not impede vessel penetration. Adding cells to collagen gel accelerated vascularization on day 13, which shows that LSE cells release pro-angiogenesis paracrine factors. The study found that dermal fibroblast conditioned medium expressed a high level of VEGF in culture. It is well known that VEGF is the most specific growth factor that regulates angiogenesis by stimulating endothelial cell proliferation, migration, and organization into tubules (Fam et al., 2003). It is also known that fibroblasts deposit a complex provisional wound matrix consisting of glycosaminoglycans, proteoglycans, collagen III, thrombospondin, fibronectin, and vitronectin, which promote endothelial tube formation and vessel growth (Eming et al., 2007b). VEGF is expressed at a low level in normal human skin, whereas its expression is highly upregulated in keratinocytes during wound healing (Berse et al., 1992; Brown et al., 1992; Kishimoto et al., 2000). VEGF may be crucial for angiogenesis during the proliferation phase of granular tissue formation during wound repair (Nissen et al., 1998).
LSE-treated wounds exhibited a significantly increased number of hair follicles, suggesting that paracrine factors released by engrafted skin cells in the wound also play an important role in skin appendage regeneration. There is evidence that the transplantation of tissue engineering skin analogues containing cells affect the wound healing process, in particular, it prevents scarring and stimulates formation of hair follicles (Xie et al., 2016). The increased number of hair follicles may indicate the ability of LSE to prevent scar formation, promoting an aesthetic effect, despite the stimulation of the provisional connective tissue in the course of wound healing. It was shown that hair follicle regeneration could prevent tissue scarring by activating embryonic pathways and converting myofibroblasts to adipocytes (Plikus et al., 2017).

Commercial gelatin-based Spongostan® is a resorbable, water-insoluble, porous, and pliable sponge obtained from purified pork skin gelatin. In our study, the gelatin sponge group keratinocytes actively migrated in the pores on day 6, showing rapid re-epithelialization, but on day 13 most of the keratinocytes on the surface of the gelatin sponge was lost, and the gelatin sponge was not able to maintain the viability of the cells. This was probably connected with the degradation of the sponge or/and insufficient blood supply due to porous formulation of the sponge. Rapid epithelialization within first 6 days did not correspond to a visible wound decrease, which probably accounts for the thin epithelial layer and the absence of full-thickness regenerated neodermis. The matrix porosity plays an important role in wound healing. It is known that epithelial cells prefer a smooth surface for adhesion, while other types of cells (osteoblasts, for example) may prefer a rough surface (Kunzler et al., 2007; Nothdurft et al., 2015). The high porosity and the absence of a supportive basal membrane in most commercially available dermal matrices interfere with the correct proliferation and differentiation of keratinocytes in vitro and may delay re-epithelialization in vivo (Paul et al., 2015).

In full-thickness wounds, migration of the epithelium from the wound edges is connected with the filling of the tissue defect with granulation tissue. As the cells do not show any tendency to descend into the wound bed, they can only crawl on a smooth, flat and moist surface. Our research showed the importance of matrix selection. Whereas a gelatin sponge promoted proliferation and rapid epithelialization but not re-epithelialization or granulation tissue formation, its porous surface impeded vascular invasion for proper nutrition of the epidermis. It is well known that successful wound healing requires the accumulation of a competent provisional matrix. The collagen gel proved itself an ideal substrate for re-epithelialization, while the addition of cells further improved the process of skin regeneration.

Collagen gel served as a provisional connective tissue favoring the re-epithelialization process and preventing wound inflammation. The penetration of collagen gel by new blood
vessels and fibroblasts may suggest that the collagen provides a suitable environment. Skin cells incorporated in the collagen gel (LSE) promoted the efficiency of wound healing. LSE favors a more intensive wound matrix reorganization and may disturb epithelial coverage, which does not affect the clinical picture. LSE contributed to wound shrinkage, vessel and hair follicle number increase, and development of skin appendages. All these factors may indicate favorable wound healing. The collagen gel has just showed its advantage in supporting keratinocyte migration, resulting in proper re-epithelization.

The results of this study add new proof to the wound healing promoting effect of tissue-engineered scaffolds. They demonstrate that despite rapid epithelialization wound healing may fail in the case of inappropriate dermal regeneration. Thus, one should use proper components of tissue engineered skin equivalents, avoiding highly porous materials without the stimulation of provisional granulation tissue.

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FIGURES

Fig. 1. The structure of LSE before grafting. Expression of keratin 5 by keratinocyte (green), nuclei of keratinocytes (keratin 5 – positive layer) and fibroblasts (exemplified by arrows) are stained with DAPI. Scale bar, 100 µm.

Fig. 2. The dynamics of the wound area in the groups with transplantation of LSE, collagen gel, gelatin sponge and non-treated control on days 6 and 13 after the transplantation (data are presented as the percentage from initial wound area). The results are presented as the mean ± SEM. Symbol: * p<0.05.

Fig. 3. Wound matrix reorganization analysis and inflammation. Picro Mallory trichrome staining (a) demonstrates collagen content and matrix remodeling and the diagram (b) shows the content of CD86-positive cells in the center and at the edge of wounds counted on cryosections stained using immunohistochemistry and dianaminobenzidine staining. Scale bars in (a), 200 µm. The results in (b) are presented as the mean ± SEM. Symbols: * p<0.05 relative to the control, # p<0.05 relative to collagen gel.

Fig. 4. Epithelialization of the wound bed. Wound sections stained for keratin 5-positive cells: (a) Movement of the epidermal sheets over the wound bed. (b) Expression of type IV collagen on day 6. (c) Expression of laminin 5 (exemplified by arrows) on day 6. (a, b, c) Immunohistochemistry on cryosections with diaminobenzidine staining. Brown color indicates positive staining. Diagram (d) shows the distance between epidermal leading edges in control, gelatin sponge, collagen gel and LSE groups on days 6 and 13 measured as indicated in the materials and methods section, which demonstrates the advantage of the LSE group. Scale bars in (a) are 200 µm and in (b, c) are 100 µm. The results in (d) are presented as the mean ± SEM. Symbol: * p<0.05.

Fig. 5. Epithelialization and cell proliferation in the wounds. Histological analysis of wounds (a) at day 6 shows general view of epidermal sheets movements. Diagrams demonstrate the number of Ki-67-positive cells in the epithelium (b) and dermis (c). The results for the epithelium were normalized per 1 mm length of the epithelium and per 0.1 mm² for the dermis. Scale bars in (a) are 100 µm. The results in (b) and (c) are presented as the mean ± SEM. Symbols: * p<0.05 relative to the control, # p<0.05 relative to collagen gel, ** p<0.05 relative to LSE.

Fig. 6. Angiogenesis and hair follicle morphogenesis on day 13: (a) immunohistochemistry for CD31 (exemplified by arrows), (b) diagram of vessel count, and (c) diagram of hair follicle morphogenesis in the wounds. Scale bars in (a) are 200 µm. The results in (b) and (c) are
presented as the mean ± SEM, Symbols: * p<0.05 relative to the control, # p<0.05 relative to collagen gel, ** p<0.05 relative to LSE.

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