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Expression of human β-defensins HBD-1, HBD-2, and HBD-3 in cultured keratinocytes and skin substitutes

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Abstract

Defensins are effector molecules of the innate host defense system with antimicrobial activity against a variety of pathogens, including microorganisms commonly found in burn units. β-Defensins are variably expressed in the epithelia of skin and other organs. Cultured skin substitutes (CSS) grafted to burn wounds lack a vascular plexus and are therefore more susceptible to microbial contamination than split thickness skin autograft. To investigate whether β-defensins can contribute to host defense in CSS, we examined expression of human β-defensins HBD-1, HBD-2, and HBD-3 in cultured keratinocytes and CSS from uninjured donors and burn patients. HBD-1 was expressed in all keratinocyte strains analyzed. HBD-2 expression in keratinocyte monolayers was highly variable but did not correlate with burn injury. HBD-3 was expressed at variable levels in all but one keratinocyte strain. CSS were prepared from two donors that lacked expression of HBD-2 in keratinocyte monolayers. All three genes were readily detected in CSS from both donors, suggesting up-regulation of HBD-2 and HBD-3. In sections of CSS, HBD-1, HBD-2, and HBD-3 proteins were localized to distinct epidermal regions. We conclude that β-defensins can potentially contribute to innate immunity in CSS, but their levels may be too low to prevent contamination after grafting.

Keywords: Defensins; Cultured skin substitutes; Keratinocyte; Skin

1. Introduction

Cultured skin substitutes (CSS), comprised of cultured keratinocytes, fibroblasts, and biopolymers, are an adjunctive treatment for the healing of burns [1,2]. Such wounds are often contaminated with microorganisms at the time of grafting. Because CSS are avascular they are susceptible to microbial contamination, particularly from the time of grafting until vascularization is achieved [3]. This has been managed clinically through application of irrigation fluids containing multiple antimicrobial drugs during the early healing period [4]. This serves to protect the grafts but may also facilitate the emergence of resistant organisms. The development of a cultured skin model with innate resistance to contamination could prospectively reduce or eliminate the requirement for exogenous antibiotics.

Gene-encoded antimicrobial peptides are effector molecules of the innate host defense system in widely divergent species [5–8]. In vertebrates, a number of antimicrobial peptides, including members of the α-defensin and β-defensin families, are variably expressed in phagocytes and epithelia of multiple organs [8]. These cationic peptides have antimicrobial activity against a wide variety of pathogens, including bacteria that are associated with burn wound infection and sepsis, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* [9–14]. The microbicidal activity of defensins is thought to result from their ability to disrupt microbial membranes [7,8,15]. Because their mechanism of action is distinct from standard antibiotic drugs, and they can be effective against even multiply drug-resistant bacteria [9,14], defensins represent an alternative approach for infection control. α-Defensins are primarily found in neutrophils and intestinal Paneth cells, whereas β-defensins are expressed in epithelia [5]. The human β-defensin (HBD) family has at least six members; the HBD-1 and HBD-2 genes have been extensively studied. HBD-1 is constitutively expressed in epithelia from the colon, small intestine, airway, mammary gland, and skin, as well as the pancreas and kidney [16–20]. HBD-2 is also expressed in the gastrointestinal tract, pancreas, and lung, but in contrast to the constitutive expression of HBD-1, HBD-2 expression is more variable and is inducible in several tissues [10]. For example, analysis of several skin samples from different

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donors and body sites showed variable HBD-2 expression, whereas all samples showed HBD-1 expression [20]. HBD-2 expression in skin is induced by cytokine stimulation, inflammation, or exposure to microorganisms [11]. Interestingly, expression of HBD-2 was found to be reduced in human burn wounds [21]. Human β-defensin-3 (HBD-3), originally isolated from lesional psoriatic scales, is less widely expressed than HBD-1 and HBD-2 [14]. HBD-3 is expressed at high levels in tonsils and skin, as well as cultured keratinocytes from skin and lung epithelial cells, and its expression is induced by cytokines and bacteria [14,22].

Hypothetically, expression of antimicrobial peptides in CSS could decrease microbial load, in the wound bed and at the graft surface, and facilitate graft healing. The purpose of the current study was to examine expression of human β-defensins HBD-1, HBD-2, and HBD-3, in CSS and cultured keratinocytes from burn patients and uninjured donors, to determine if these antimicrobial peptides can potentially contribute to defense against microbial contamination of CSS.

2. Methods

2.1. Cell culture and preparation of cultured skin substitutes

Primary cell cultures were derived from biopsies of human skin (Table 1) as previously described [23,24]. Fibroblasts and keratinocytes from burn patients ($n = 8$) were isolated from non-burned skin biopsies obtained from patients enrolled in an Institutional Review Board (IRB)-approved cultured skin study [1,2,25]. The cells used for the present study were in excess of those needed for grafting CSS to patients. Fibroblasts and keratinocytes from non-injured donors ($n = 7$) were isolated from skin obtained with IRB approval from healthy adult females undergoing cosmetic mammoplasty or abdominoplasty.

Table 1
Skin donor demographics. Donors 1–8, burn injured; donors 9–15, non-injured

Donor number	Age	TBSA (%)	Gender	Race	Body site
1	16	75	Male	C	NR
2	13	66	Male	A	NR
3	14	71	Female	C	NR
4	1	50	Male	A	NR
5	5	71	Male	C	NR
6	10	77	Male	C	NR
7	29	90	Male	C	NR
8	7	71	Female	A	NR
9	27	NA	Female	A	Breast
10	35	NA	Female	A	Breast
11	43	NA	Female	A	Breast
12	23	NA	Female	C	Breast
13	38	NA	Female	A	Breast
14	53	NA	Female	C	Breast
15	40	NA	Female	C	Abdomen

Abbreviations: TBSA, total body surface area burned; C, Caucasian; A, African American; NA, not applicable; NR, not recorded.

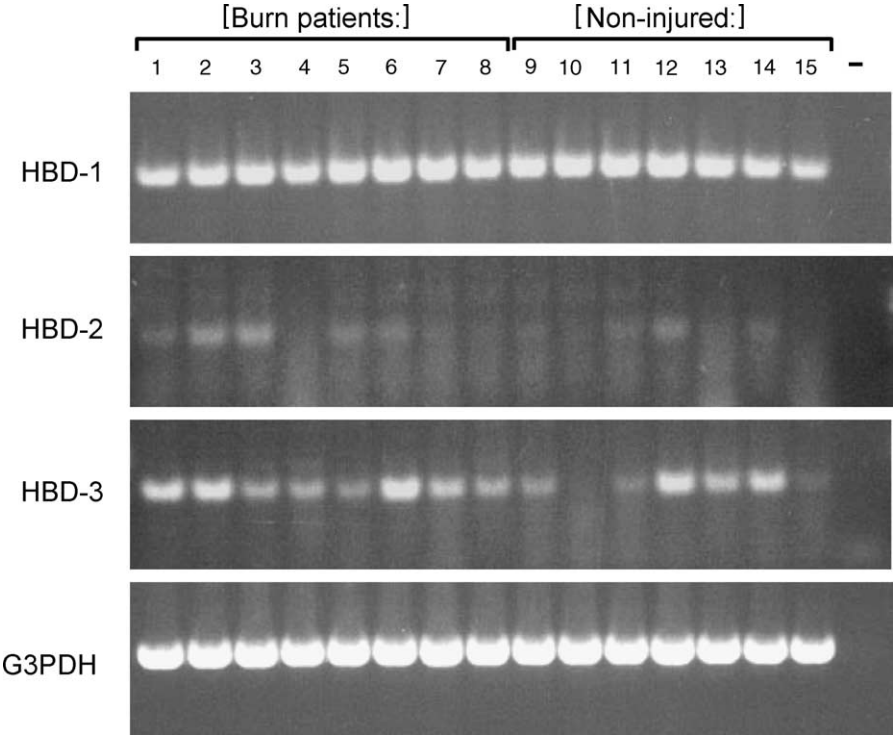

Fig. 1. β-Defensin mRNA expression in keratinocytes cultured from burn patients and non-injured donors. Lane numbers correspond to donor numbers listed in Table 1. G3PDH represents a housekeeping gene, used as a positive control for RNA integrity and concentration. Negative control reactions (−) contained no RNA template.

CSS were prepared as previously described [24,26]. Briefly, dermal substitutes were prepared by inoculating acellular bovine collagen-glycosaminoglycan substrates with fibroblasts (5×10^5 cm^{-2}). One day later, keratinocytes (1×10^6 cm^{-2}) were inoculated onto the dermal substrates. Six CSS, approximately 80 cm^2 starting surface area each, were prepared (Table 1, donor strains 8 and 15; $n = 3$ each). CSS were cultured for 14 days at the air–liquid interface (37 °C, 5% CO_2) with daily medium changes.

2.2. β-Defensin expression analysis

RNA was isolated from keratinocytes and CSS using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). β-Defensin gene expression was analyzed by reverse-transcription polymerase chain reaction (RT-PCR) using the Titan One-Tube RT-PCR Kit (Roche Applied Science, Indianapolis, IN). Gene-specific primers were synthesized by the University of Cincinnati DNA Core Facility (Cincinnati, OH). Primer sequences follow:

HBD-1 left	5′-CCCAGTTCCTGAAATCCTGA-3′
HBD-1 right	5′-CTTCTGGTCACTCCCAGCTC-3′
HBD-2 left	5′-CATGAGGGTCTTGTATCTCCTCTT-3′
HBD-2 right	5′-CAGCTTCTTGGCCTCCTCAT-3′
HBD-3 left	5′-CTTCGGCAGCATTTTCGGCCA-3′
HBD-3 right	5′-AGCCTAGCAGCTATGAGGATC-3′

G3PDH primers (BD Biosciences/Clontech, San Diego, CA) were used for positive control reactions. To control for RNA levels, batches were set up for each RNA sample, with all reagents except primers. Each batch was equally divided into four separate tubes; primers were added, and amplifications were performed simultaneously using a GeneAmp 9600 PCR System (Perkin-Elmer, Boston, MA). RT-PCR was performed using 1 μg total RNA per reaction, following the manufacturer's protocol (35 cycles total) with an annealing temperature of 56 °C. RNA was omitted from negative control reactions. Reaction products were visualized by electrophoresis on 1.5% agarose gels. For each pair of primers, a representative PCR fragment was cloned and sequenced to confirm amplification of the appropriate defensin gene (data not shown).

2.3. Immunohistochemistry

Biopsies of CSS, and native human skin (Table 1, donor 15), were frozen in M-1 Embedding Matrix (Lipshaw, Pittsburgh, PA). Cryostat sections were dehydrated in methanol and fixed in acetone at −20 °C. After air-drying, sections were rehydrated in phosphate-buffered saline. Sections were incubated for 1 h at room temperature with rabbit polyclonal antibodies against HBD-1 (2 μg/ml; Alpha Diagnostics International, San Antonio, TX), HBD-2 (2 μg/ml; Alpha Diagnostics International), or HBD-3 (0.75 μg/ml; Novus Biologicals, Littleton, CO). For negative controls, sections were incubated with non-immune Rabbit IgG (2 μg/ml; R&D Systems, Minneapolis, MN). Antibody detection was performed using the Vectastain® Elite ABC Universal kit and the DAB Peroxidase Substrate kit (Vector Labs, Burlingame, CA). Sections were counterstained using Vector® Hematoxylin QS (Vector Labs).

3. Results

3.1. RNA expression

Expression of HBD-1 was readily detected in all keratinocyte strains analyzed (Fig. 1). There were no differences in HBD-1 expression between keratinocyte strains isolated from burn patients or non-injured donors. Expression of HBD-2 was low compared with HBD-1, and was highly variable between individual strains (Fig. 1). However, the observed differences were not related to burn injury. HBD-3 expression was detected at low to moderate levels

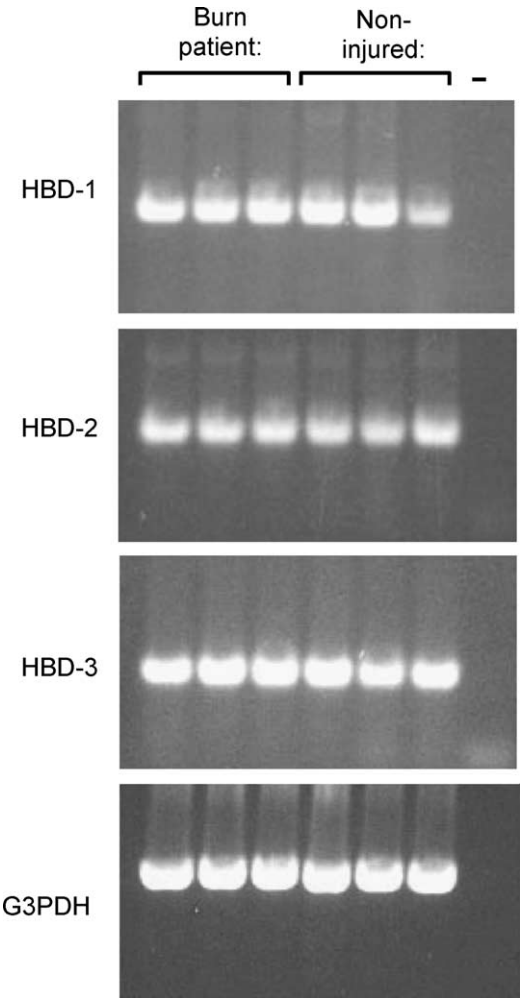

Fig. 2. β-Defensin mRNA expression in cultured skin substitutes. G3PDH was used as a positive control; negative control reactions (–) had no RNA template.

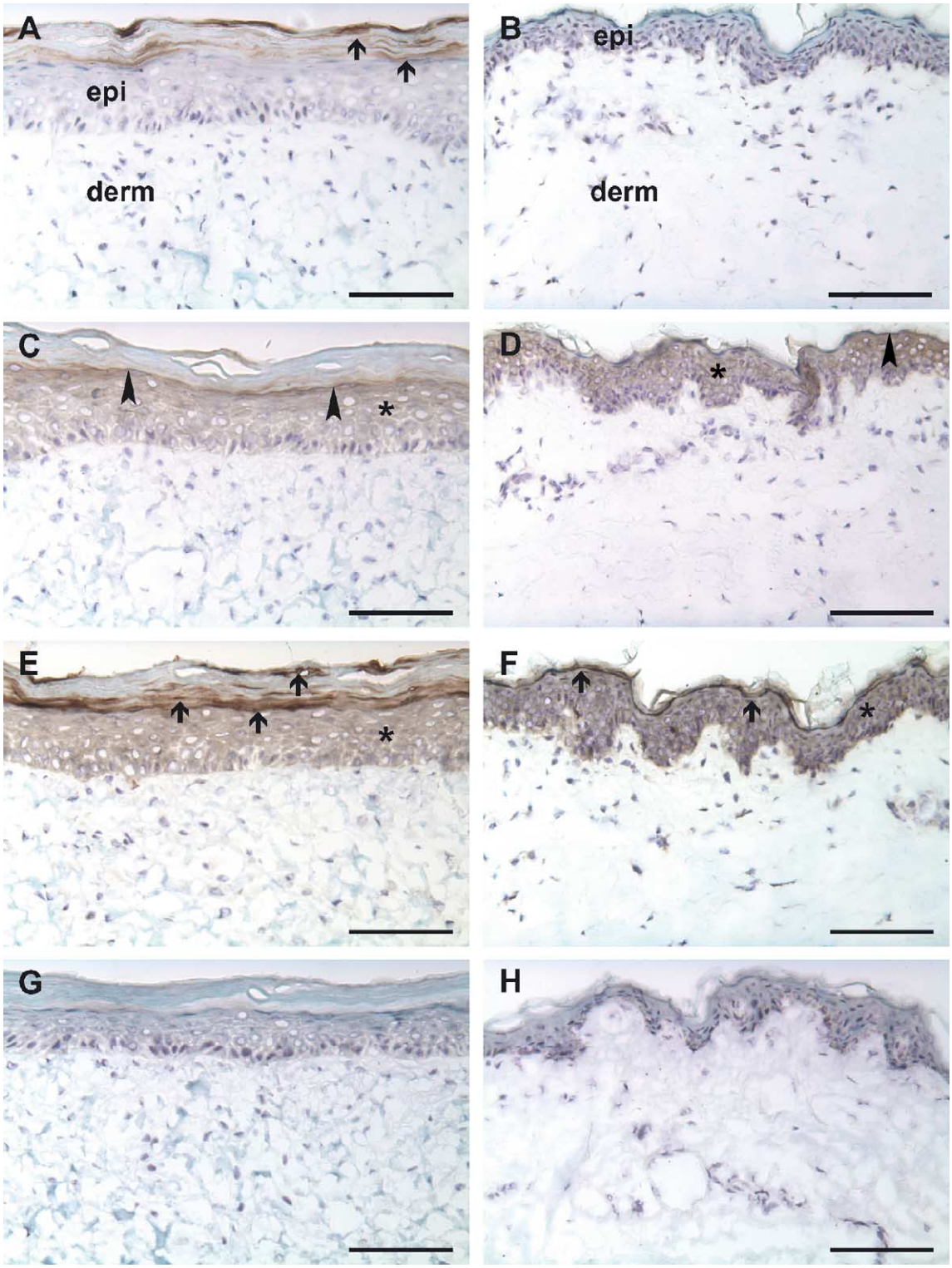

Fig. 3. Immunohistochemical localization of β-defensin proteins. Shown are sections of CSS (left), prepared from non-injured donor skin, and native human skin (right) from the same donor. There was no difference between CSS prepared with cells from a burn patient (not shown) and a healthy donor. Brown color indicates positive staining. (A) HBD-1 protein localized to the cornified layers of the epidermis of CSS (arrows). (B) HBD-1 was not detected in this native human skin sample, which contained very little stratum corneum. (C) HBD-2 protein localized to the suprabasal layers of the cultured epidermis (asterisk) and the region of transition between non-cornified and cornified layers (arrowheads). (D) The pattern of HBD-2 localization in native human skin is similar to the pattern seen in CSS. (E) HBD-3 protein in CSS was found at relatively low levels throughout the epidermis (asterisk) and was regionally concentrated in the cornified layers (arrows). (F) HBD-3 staining in native human skin is similar to the pattern seen in CSS. (G–H) Non-immune rabbit IgG was used as a negative control for the antibody staining procedure in CSS and native human skin, respectively. Abbreviations: epi, epidermis; derm, dermis. Scale bars = 100 μm.

in all but one keratinocyte strain analyzed (Fig. 1), and like HBD-2, the variability did not appear to be significantly related to burn injury.

CSS were prepared using cells from two strains that did not express detectable levels of HBD-2 in keratinocytes: one from a burn patient and the other from a non-injured donor. As seen in keratinocytes in monolayer culture, HBD-1 was expressed in all the cultured skin samples (Fig. 2). However, in contrast to the variable HBD-2 and HBD-3 expression levels seen in keratinocytes, expression was easily detected in all CSS from both strains examined.

3.2. Protein localization

After 2 weeks of in vitro incubation, the epidermal component of the CSS stratified into layers resembling those seen in native human skin. HBD-1 protein was found in the upper, cornified layers of the epidermis of CSS (Fig. 3A). HBD-1 was not detected in the native human skin sample (Fig. 3B), but this may be due to the absence of much of the stratum corneum in this skin biopsy. HBD-2 protein was localized to the suprabasal layers of the epidermis of CSS and was found in the lower cornified layers (Fig. 3C). A similar pattern was observed in native human skin (Fig. 3D). HBD-3 was found throughout the epidermis of CSS and was highest at the lower regions of the cornified layer (Fig. 3E). A similar pattern of localization was seen in native skin (Fig. 3F). No specific staining was seen with a non-immune antibody, used as a negative control (Fig. 3G–H).

4. Discussion

Burn patients are particularly susceptible to infection, due both to the nature of the burn wound and to immunosuppression that can accompany burn injury [27,28]. Other investigators have reported the absence of β-defensin HBD-2 in burn blister fluid and reduced expression in burn wounds, leading to speculation that altered defensin expression could promote the growth of microorganisms [21,29]. The mechanism of reduced defensin expression in burn wounds remains unclear. It is well known that burn injury results in the release of multiple inflammatory mediators [30]. Many of the cytokines that are elevated in burn patients, including interleukin-1 (IL-1), IL-6, interferon gamma, and tumor necrosis factor-α, have been implicated in the regulation of defensin expression in vitro [5,11,14,22,31], so these mediators may theoretically play a role in altered defensin expression in burn patients.

Skin substitutes, which are becoming increasingly important for closure of massive burn wounds, are avascular, and are therefore more susceptible to microbial contamination than split thickness skin autograft. We examined expression of β-defensin genes in CSS, to begin to address their role in contamination defense and/or susceptibility. Hypothetically, reduced expression in cells cultured from burn patients, or in CSS prepared with these cells, could contribute to a reduced capacity to defend against microbial infection. However, the results reported here indicate no reduction of β-defensin expression in keratinocytes cultured from burn patients. We did not analyze expression in the native skin used to establish cultures from these patients, so we cannot determine whether β-defensins were expressed in these skin samples, or if keratinocyte culture affected defensin expression. There were differences in the levels of expression observed between different cell strains, but these may be due to individual variation since there was no significant correlation with burn injury. Others have reported individual variation in β-defensin expression in skin from different body sites [20], but it is not known whether these expression patterns would be maintained if the cells were grown in culture. In the current study, the donor sites for burn patients' skin biopsies were not recorded; however, all but one of the non-injured skin samples were isolated from breast tissue. Thus, the variation in keratinocytes is likely due to genetic differences rather than the site of the biopsy used to prepare the cells. Despite the variation in HBD-2 and HBD-3 expression in keratinocytes in monolayer culture, both genes, as well as HBD-1, were expressed in CSS. This observed up-regulation in CSS compared with monolayer keratinocytes may be due to differences in the culture media; keratinocyte growth medium is optimized for proliferation, whereas skin substitute medium is designed to promote epidermal maturation [32]. Additionally, elevated defensin expression may be due to the increased differentiation of the keratinocytes in this organotypic culture system [11].

Evaluation of β-defensin protein expression showed similar localization patterns in CSS and in native human skin. Localization of β-defensins to the epidermis, and in particular, HBD-1 and HBD-3 in the cornified layers of CSS, is consistent with a role for these proteins in defense against environmental contamination. Therefore, expression of these defensins in CSS may contribute to host defense. However, because of the high incidence of infection in burn patients, the levels of defensins present may be insufficient to combat microbial growth in CSS in clinical settings.

Because antimicrobial peptides are structurally and mechanistically different from conventional antibiotics, they have the potential to be used as novel therapeutic agents for treating infection. The need for development of new therapeutic options is critical, to combat emerging drug-resistant strains of bacteria. Natural and synthetic antimicrobial peptides have been shown to be effective for reducing microbial levels in a number of preclinical studies [13,33–36]. Future studies aimed at increasing the expression of β-defensins in CSS may lead to improved resistance to microbial contamination after grafting, contributing to improved engraftment and reduced utilization of topical antibiotics.

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