Chapter 7

Development, validation and testing of a human tissue engineered hypertrophic scar model

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ABSTRACT

Abnormal hypertrophic scars can form after healing of full-thickness skin wounds. Currently reliable animal and in vitro models to identify and test novel scar reducing therapeutics are scarce. Here we describe the development and validation of a tissue-engineered human hypertrophic scar model based on a reconstructed epidermis on a dermal matrix containing adipose tissue-derived mesenchymal stem cells (ASC). Although obtained from normal, healthy skin, ASC, in contrast to dermal mesenchymal cells, were found to facilitate HTscar formation. Quantifiable HTscar parameters were identified: contraction; thickness of dermis; collagen-1 secretion; epidermal outgrowth; epidermal thickness and also cytokine secretion (IL-6, CXCL8). The model was validated with therapeutics currently used for treating scars (5-fluorouracil, triamcinolon) and a therapeutic known to be unsuccessful in scar reduction (1,25-dihydroxyvitamin-D₃). Furthermore it was shown that atorvastatin, but not retinoic-acid may provide a suitable alternative for scar treatment. Each therapeutic selectively effected a different combination of parameters, suggesting combined therapy may be most beneficial. This animal free hypertrophic scar model may provide an alternative model for mechanistic studies as well as a novel in vitro means to test anti-scar therapeutics thereby reducing the use of animals.
INTRODUCTION

Cutaneous wound healing is a natural complex response to tissue injury and normally results in a scar. The most desirable scar is thin and flat and is mostly seen after superficial injury. This type of scar is called a normotrophic scar (NTscar). However extensive trauma, deep burns and sometimes even standard surgery can result in wound closure with an abnormal scar formation which is red, firm, raised, itchy and painful. This abnormal scar is known as a hypertrophic scar (HTscar). The quality of life of patients with HTscars can be severely affected due to loss of joint mobility, contractures and disfigurements which lead to accompanying psychological problems (like depression and social avoidance).

HTscars occur more often after full-thickness wounding, where no viable dermis is left and adipose tissue is exposed. Therefore the deeper the wound, the greater the possibility of HTscar formation. Superficial wounds generally heal with a NTscar. The pathogenesis of HTscar formation in humans is not well understood and although there are various treatment strategies, it is generally accepted that current strategies are still far from optimal. A major limitation in the progress of scar management is the lack of physiologically relevant human models to explore the pathogenesis of HTscar formation and to test new therapeutics. Nowadays patients, animal models and in vitro cell culture models are used to study skin scar formation. Patient studies are essential, but are limited due to logistical and ethical problems. Common alternatives are animal studies. Despite the large number of studies describing pigs, mice, rabbits, and other animals as models to investigate hypertrophic scarring, the wound healing process in these species presents significant differences when compared with human scarring. Pig skin most closely represents human skin and the red durac pig model has recently been validated since these pigs have been described to develop HTscars similar to human HTscars in a number of ways. However, extensive research with this model is limited due to the lack of pig specific biomarkers such as detected by monoclonal antibodies. Rabbit skin also shows some similarities to human scar formation, however the rabbit ear scar model encounters similar restrictions to the pig model. Mouse models are most extensively used even though mouse skin physiology poorly represents human skin, and mice do not form adverse scars after wounding. Therefore in order to humanize mouse models, studies have been described using CXCR3−/− mice and transplanting human skin onto the backs of nude mice. In addition to difficulties in interpreting results due to differences in skin physiology and in particular scar formation, inflicting large full thickness trauma and burn wounds to animals has substantial ethical consequences worldwide. In vitro cell culture models have been used to gain insight into different aspects of scar pathogenesis. For example adipose tissue-derived mesenchymal cells have been described as having a number of similar characteristics to mesenchymal cells found within HTscar tissue e.g.: both are α-SMA. Also a scratch assay has been described in which
an increase in the single parameter connective tissue growth factor (CTGF) has been proposed for testing scar therapeutics. However, no attempts have been made so far to create a robust and physiologically relevant *in vitro* HTscar model for *in vitro* testing of therapeutics with multiple scar forming parameters. With increasing pressure from the EU (Directive 86/609/EEC) who strongly stimulate the replacement, reduction, and refinement of the use of animals models, there is an urgent need to develop a physiologically relevant *in vitro* human HTscar model, in order to investigate the pathogenesis of HTscar formation. This in turn can facilitate identifying and testing new therapeutics, and thus lead to novel treatment strategies.

Therefore, we have developed and validated a tissue-engineered HTscar model consisting of a reconstructed epidermis on a dermal matrix populated with mesenchymal cells. We compared full-thickness skin equivalents (SE) constructed from mesenchymal stem cells isolated from the deep cutaneous adipose tissue (ASC) with SE constructed from more superficial mesenchymal stromal cells found within the reticular dermis (R-DSC) and papillary (P-DSC) in order to mimic HTscar formation, NTscar formation and Nskin respectively. We hypothesized that ASC in the exposed wound bed might most rapidly regenerate dermal tissue in order to close life threatening deep cutaneous wounds at the cost of HTscar formation whereas more superficial wounds are repaired from DSC within the flanking and underlying dermis generally resulting in NTscar formation.

In order to develop, validate and further test the HTscar model, a number of quantifiable parameters typical for HTscars were identified: 1) contraction since HTscars are highly contractile; 2) thickness of the dermis and 3) collagen-1 secretion since more connective tissue is formed in HTscars than in NTscars; 4) the degree of epithelialization, since it has been described that the extent of HTscar formation corresponds with delayed wound closure; 5) thickness of the regenerating epidermis, since it is known that HTscars have more epidermal cell layers than NTscars. In addition to the scar forming parameters we assessed the secretion of two cytokines, IL-6 and CXCL8, known to contribute to wound healing. The HTscar model was validated with therapeutics generally used in the clinic for scar treatment (5-Fluorouracil and a triamcinolone (kenacort®-A40)) and a therapeutic known to be unsuccessful in scar reduction (1,25-dihydroxy vitamin D3) (Table 1). The HTscar model was further tested with two potential scar reduction therapeutics (All-trans-retinoic acid and atorvastatin calcium salt trihydrate).
MATERIALS AND METHODS

Normal skin and scar tissue
Human adult skin samples were obtained from healthy individuals undergoing abdominal dermolipectomy or breast reduction surgery (n = 9; age: 25-50 years; sex: 8 x female, 1 x male). Scar tissue samples were obtained from patients who underwent plastic surgery for scar excision (HTscar n = 8; age: 25-55 years; sex: 7 x female, 1 x male; location: abdomen, breast and flank; age of scar: >1 year and NTscar n = 7; age: 15-60 years; sex: 6 x female, 1 x male; location: abdomen and breast; age of scar: >1 year). HTscars were defined as raised above skin level (>1 mm) for at least 1 year and NTscar were defined as never raised above skin level. VU University medical center approved all the experiments described in this manuscript. The study was conducted according to Declaration of Helsinki 1975.

Cell isolation and culture of normal healthy skin
Epidermal keratinocytes were isolated from healthy (non scarred) human adult skin and cultured as described earlier. Keratinocytes were cultured until 80% confluency and then stored in the vapor phase of liquid nitrogen for later use.

Papillary dermal, Reticular dermal and Adipose tissue-derived mesenchymal cells were isolated by collagenase type II / dispase II treatment from healthy (non scarred) human adult skin as previously described by Kroeze et al. In short, split thickness skin (0.4 mm) was removed using a dermatome (Acculon II, Braun, Tuttligen, Germany) to separate the papillary dermis from reticular dermis and adipose tissue. The cells in the papillary dermis (upper layer) are further referred to as papillary dermal derived mesenchymal stromal cells (P-DSC). From the remaining reticular dermis all adipose tissue was removed. Cells in the reticular dermis are further referred to as reticular dermal derived mesenchymal stromal cells (R-DSC). Adipose derived mesenchymal stem cells (ASC) were isolated in the same way as P-DSC and R-DSC. All mesenchymal cells were cultured under identical conditions and upon reaching 80% confluency were stored in the vapor phase of liquid nitrogen until required. Notably within a single experiment KC, P-DSC, R-DSC and ASC were all from the same donor. Cells at passage 3 were used to construct SE and DE. Of note P-DSC and R-DSC are the same cell population often referred to as dermal fibroblasts.

Skin equivalents (SE) and dermal equivalents (DE)
In this study we choose the sponge-like collagen-elastin-matrix (matriderm®; Dr. Suwelack Skin & Health Care, Billerbeck, Germany) since it provides an initial scaffold for seeding the cells into but then is very easily remodeled by the cells within the matrix thus enabling potential scar-like phenotypes to be formed. Mesenchymal cells (4.10^5) were
seeded into the collagen-elastin-matrix (2.2 x 2.2 cm) and cultured submerged for three weeks in culture medium containing DMEM (BioWhittaker, Verviers, Belgium)/Ham’s F-12 (Invitrogen, Gibco, Paisley, UK)(3:1), 2% UltraSerG (UG)(BioSepra SA, Cergy-Saint-Christophe, France), 1% penicillin/streptomycin (P/S) (Invitrogen, Gibco, Paisley, UK), 5 μg/ml insulin, 65 μg/ml ascorbic acid and 5 ng/ml epidermal growth factor (EGF). Unless otherwise stated, all culture additives were obtained from Sigma-Aldrich (St. Louis, MO, USA). Medium obtained after the last refreshment before keratinocytes were seeded onto the surface of DE was collected and is referred to as medium of dermal equivalents (DE). After 3 weeks of culturing keratinocytes (5.10^5 cells/culture) were seeded onto the surface of mesenchymal cell-populated matrixes. The cultures were cultured then as follows: 4 days submerged cultured in DMEM/Ham’s F-12 (3:1), 1% UG, 1% P/S, 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin and 1 ng/ml KGF. Hereafter SE were cultured at the air-liquid interface in DMEM/Ham’s F-12 (3:1), 0.2% UG, 1% P/S, 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin, 1.0 × 10^{-3} μM L-carnitine, 1.0 × 10^{-3} μM L-serine, 1 μM DL-α-tocopherol acetate and enriched with a lipid supplement containing 25 μM palmitic acid, 15 μM linoleic acid, 7 μM arachidonic acid and 24 μM bovine serum albumin for another 10 days. SE were harvested after an entire culture period of 5 weeks for histological analysis and culture supernatants were collected for ELISA. The cultures received new culture medium twice a week.

**Application of therapeutics**

SE containing ASC were generated as described above with the addition of therapeutics supplemented in culture medium from the first medium renewal after starting the culture onwards. The constructs were cultured with 10^{-7} M all-trans-retinoic acid (Sigma-Aldrich, St. Louis, MO, USA), 10^{-8} M 5-Fluorouracil (Sigma-Aldrich, St. Louis, MO, USA), 10^{-7} M Atorvastatin calcium salt trihydrate (Sigma-Aldrich, St. Louis, MO, USA) (all dissolved in 0.01% dimethylsulfoxide (DMSO), or 10^{-5} M kenacort®-A40 (Bristol-Myers Squibb B.V., Woerden, The Netherlands) (dissolved in 0.01% benzyl alcohol) or 10^{-8} M 1,25-dihydroxy vitamin D3 (Sigma-Aldrich, St. Louis, MO, USA) (dissolved in 0,0095% ethanol). Corresponding vehicles were used as controls. The concentrations were determined from dose response studies on ASC monolayers and were the concentrations where no inhibition of ASC metabolic activity, which corresponds to proliferation (2 days exposure), was observed by MTT assay (see below).

**Histological and immunohistochemical analysis**

Paraffin embedded sections of normal tissue, scar tissue and SE were used for morphological (haematoxylin and eosin staining) and immunohistochemical analysis (alpha-smooth muscle actin (α-SMA) (clone 1A4; 1:200, Dako, Glostrup, Denmark))23. The dermal thickness of SE was quantified from photos of H&E stainings (Nikon Eclipse 80i Düs-
seldorf, Germany) taken at 200 fold magnification using NIS-Elements AR 2.10 software. The epidermal thickness was quantified by taking the mean of the number of living cell layers at 5 different regions within a single tissue section.

**Measurement of matrix contraction and outgrowth of epidermis**
Matrix contraction and outgrowth of the epidermis were determined by taking photographs of the constructs at the first medium change and then again at the time of harvesting of the cultures. Photographs were taken with a Nikon coolpix 5400 digital camera (Japan). The surface area of the constructs and the outgrowth of the epidermis outside of the original 1 cm diameter seeding area were determined using NIS-Elements AR 2.10 imaging software (Nikon).

**Keratinocyte migration**
Chemotactic migration of keratinocytes towards DE conditioned medium (dose response of 0.3%, 3% and 30%) with the aid of a modified Boyden well chamber technique using a 24-transwell system with 8μm was assessed and quantified as previously described.

**Cell proliferation**
ASC: A MTT assay was used to measure mitochondrial activity, which is representative of viable number of ASC. The assay was performed as described by the supplier (Sigma-Aldrich, St. Louis, MO, USA).

Keratinocytes: Relative proliferation of keratinocytes was determined by quantifying the amount of house keeping enzyme lactate dehydrogenase (LDH) released into the supernatant after 100% cell lysis with 0.1% Triton X-100 as earlier described by Krooze et al.

**Enzyme-linked immunosorbent assay for cytokine production**
All reagents were used in accordance to the manufacturer’s specifications. For collagen I quantification commercially available ELISA antibodies and recombinant proteins obtained from Rockland, Gilbertsville, PA, USA were used. For IL-6 commercially available paired ELISA antibodies and recombinant proteins obtained from R&D System Inc. (Minneapolis, MN, USA) were used. For CXCL8 quantification, a Pelipair reagent set (CLB, Amsterdam, The Netherlands) was used.

**Statistical analysis**
At least three independent experiments were performed with each experiment being from a different donor and having an intra experimental duplicate. Importantly all experiments using KC, P-DSC, R-DSC and ASC were donor matched and performed in parallel. Difference in thickness and contraction of the matrix, outgrowth of the epider-
mis, number of epidermal cell layers and collagen 1 secretion were compared between the different constructs using a repeated measures ANOVA test followed by Bonferroni’s multiple comparison test. Difference in number of epidermal cell layers in native skin and scar tissues were compared using a one-way analysis of variance test, followed by Bonferroni’s multiple comparison test. Differences in biomarkers of ASC model treated with therapeutic (compared to vehicle control) were compared using paired t-test. Differences were considered significant when $p < 0.05$.

RESULTS

Qualitative macroscopic and microscopic comparison of native scars with the in vitro HTscar model

In order to determine which characteristics are typical for a HTscar we first compared HTscar with native human NTscar and Nskin. Macroscopically, HTscar is more raised and red than NTscar and Nskin (Figure 1a). Microscopically, HTscar has a thicker epidermis than NTscar and Nskin. Rete ridges are almost absent in HTscar and occur to a lesser extent in NTscar compared to Nskin (Figure 1a). In order to identify the presence of myofibroblasts, which are thought to be mainly responsible for skin contraction after wounding, an α-SMA staining was performed. In HTscars α-SMA positive staining was not only observed around blood vessels but also in single cells in lower regions of the dermis. In contrast, both in NTscar and Nskin α-SMA staining was mainly restricted to blood vessels (Figure 1a).

Next we determined whether the SE constructed with either ASC, R-DSC or P-DSC showed typical macroscopic and microscopic characteristics of HTscar, NTscar and Nskin, respectively. Macroscopically, the SEASC are more contractile than SERDSC and SEDSC (Figure 1b). Similar to HTscar, microscopical examination of tissue sections showed that SEASC had increased thickness of the epidermis. This was not observed with SERDSC and SEDSC. There was increased α-SMA staining in SE particularly in SEASC, where it is mainly located directly underneath the epidermis. The α-SMA staining was much less and spread throughout the dermis in SERDSC and SEPDSC (Figure 1b).

Clearly SE constructed with ASC populated matrixes represent HTscars both macroscopically and microscopically, and have the potential for use in an in vitro HTscar model. In contrast R-DSC and P-DSC visually represent NTscar and Nskin, respectively. Before the HTscar model can be implemented, quantifiable and relevant parameters typical for HTscar need to be identified. Therefore we next determined whether: thickness of dermis; contraction; collagen 1 secretion; number of epidermal cell layers and outgrowth of epidermis were suitable parameters. In addition we determined whether the secretion of two cytokines, IL-6 and CXCL8, related to wound healing differed in the 3 different models.
Figure 1. Macroscopic and microscopic comparison of healthy skin with scar tissue and SE. (a) Macroscopic overview, histological haematoxylin and eosin (H/E) staining, and immunohistochemical α-SMA staining of human Nskin, NTscar, and HTscar tissue. (b) Macroscopic overview, histological H/E staining, and immunohistochemical α-SMA staining of SE composed with P-DSC, R-DSC, and ASC. Bars macroscopic pictures = 1 cm and bars microscopic stainings = 100 μm.
Identification of dermal parameters in HTscar model

In skin wound healing, the development of HTscar is characterized by an overproduction of extracellular matrix, increased contraction and augmented α-SMA expression compared to NTscar\textsuperscript{15}. For this reason we first compared SE\textsuperscript{ASC} with SE\textsuperscript{R-DSC} and SE\textsuperscript{P-DSC} with regards to thickness of the dermis, contraction and collagen 1 secretion (Figure 2).

The dermal thickness was not significantly different between the three SE (Figure 2a). An increase in contraction, is represented by a decrease in surface area of the SE. The contraction in SE\textsuperscript{ASC} was increased compared to SE\textsuperscript{R-DSC} and SE\textsuperscript{P-DSC} (Figure 2b). SE\textsuperscript{ASC} secreted significantly more collagen 1 compared to SE\textsuperscript{R-DSC} (Figure 2c).

From these results, contraction and collagen 1 secretion were identified as suitable dermal parameters for assessing HTscar formation in vitro using SE.

**Figure 2. Identification of dermal parameters for HTscar formation.** (a) Thickness of dermis of SE (μm); (b) relative matrix contraction of SE (surface area after 5 weeks of culture divided by surface at day 0); (c) collagen 1 secretion into culture supernatants (ng/ml per equivalent per 24 h). Experiments were performed with SE constructed from three different donors each in duplicate. Keratinocytes, P-DSC, R-DSC, and ASC were all from the same donor within a single experiment. Data are presented as the mean (n=3 ±SEM) thickness of dermis, contraction, or secretion of collagen 1. Statistical significant differences were calculated using a repeated measures ANOVA test followed by a Bonferroni’s multiple comparison test. *, p<0.05; **, p<0.01.
Identification of epidermal parameters in HTscar model

It was observed that native HTscar had a thicker epidermis than NTscar and Nskin (Figure 1a). This observation was confirmed by quantification of the number of epidermal cell layers: HTscar showed more epidermal cell layers (7.9 ± 1.6) than NTscars (6.9 ± 1.0) and Nskin (5.8 ± 0.6) (Figure 3a). Next we determined whether this increased epidermal thickness in native epidermis also occurred in the HTscar model. Indeed, SE\textsuperscript{ASC} had increased number of epidermal cell layers (8.00 ± 1.3) compared to SE\textsuperscript{R-DSC} (6.5 ± 0.6) and SE\textsuperscript{P-DSC} (5.3 ± 1.1) (Figure 3b). Notably, all of these findings correlated very closely to native tissue and in particular HTscars had the same number of epidermal cell layers as SE\textsuperscript{ASC}.

Since the probability of HTscar formation is increased in wounds with delayed wound closure\textsuperscript{2}, we next determined whether ASC were responsible for the delayed epidermal outgrowth compared to DSC. Indeed, SE\textsuperscript{ASC} had significant slower outgrowing epidermis compared with SE\textsuperscript{R-DSC} and SE\textsuperscript{P-DSC} (Figure 3c). However since the contraction is also greater in SE\textsuperscript{ASC} compared with SE\textsuperscript{R-DSC} and SE\textsuperscript{P-DSC} it could not be entirely excluded from these findings that contraction confounded this result. To exclude the confounder a chemotactic transwell migration experiment was performed with keratinocytes using conditioned supernatant derived from the three types of DE. The keratinocyte migration was reduced with supernatant derived from DE\textsuperscript{ASC} compared with supernatants derived from DE\textsuperscript{R-DSC} and DE\textsuperscript{P-DSC} (Figure 3d). The parallel proliferation experiment showed that this decrease in migration was not due to changes in keratinocyte proliferation (Figure 3e) indicating that ASC do indeed stimulate less epidermal migration than R-DSC and P-DSC.

From these results, the increase in number of epidermal cell layers and delayed outgrowth of epidermis were identified as suitable epidermal parameters for assessing HTscar formation in vitro using SE.

Cytokine IL-6 and CXCL8 secretion

Most probably, already at the onset of wound healing, scar formation is initiated. Cytokines such as IL-6 and CXCL8 are reported to play a role in inflammation and granulation tissue formation during the wound healing process\textsuperscript{18}. Therefore the secretion of IL-6 and CXCL8 was assessed in culture supernatants derived from SE for their use as potential future novel scar parameters (Figure 4).

The secretion of IL-6 was slightly lower (trend) when ASC were incorporated into SE than when P-DSC were used. The secretion of CXCL8 by the SE was significantly lower when ASC were incorporated into SE than when P-DSC were used.

From these results, decreased IL-6 and CXCL8 secretion were identified as a characteristic of SE\textsuperscript{ASC}. 
Figure 3. Identification of epidermal parameters for HTscar formation. The epidermal thickness shown as the mean number of keratinocyte cell layers within the epidermis of (a) native tissue biopsies and (b) skin equivalents. (c) The area of outgrowth of the epidermis outside of the original 1 cm diameter seeding area of SE (mm²). (d) Keratinocyte migration towards DE conditioned supernatant was assessed with a chemotactic transwell migration experiment. (e) Relative proliferation of keratinocytes exposed to DE conditioned supernatant was determined by LDH assay. Experiments (triplicate) were performed from three different donors each in duplicate. Keratinocytes, P-DSC, R-DSC, and ASC were all from the same donor within a single experiment. Data are presented as the mean ± SEM (n=3). Statistically significant differences were calculated using a repeated measures ANOVA test followed by a Bonferroni’s multiple comparison test, except for the difference in number of epidermal cell layers in native skin and scar tissues, which were compared using one-way analysis of variance test, followed by Bonferroni’s multiple comparison test. *, p<0.05; **, p<0.01.
Validation and testing of the in vitro HTscar model with anti-scarring agents

Clearly SE constructed from ASC populated matrices not only visually represent HTscars, but also enabled quantifiable parameters to be identified, which are representative for HTscars. These were increases in 1) thickness of dermis; 2) contraction; 3) collagen 1 secretion; 4) number of epidermal cell layers and decreases in 5) degree of epithelialization. In addition SE ASC showed reduced IL-6 secretion and reduced CXCL8 secretion.

The HTscar model was next validated by culturing with two standard therapeutics (5-fluorouracil (5FU) and triamcinolon (TC)) which result in partial scar correction in patients, (positive controls) and a therapeutic that is known to be not effective in scar reduction (1,25-dihydroxy vitamin D3 (VitD3)), which functioned as negative control therapeutic (Table 1). Additionally potential novel scar therapeutics (Atorvastatin and All-trans-retinoic acid (RAI)) were tested (Table 1). For all therapeutics, vehicle controls were tested in parallel. No significance was found between control condition (nothing added) and vehicle control conditions for the selected parameters. The results of this validation study are described below and summarized in Table 2, Figure 5 and Figure 6.

5-fluorouracil (SFU): standard care (partially effective therapeutic)

Supplementing SE ASC with SFU led to reduced contraction (Figure 5a and 6a) and reduced number of epidermal cell layers of SE compared to control (6.3 ± 0.8 versus 7.8 ± 0.9) (Figure 5b and 6b). Notably, SE ASC treated with SFU had approximately the same number of epidermal cell layers as NTscars (6.9 ± 1.0) and SE ASC (6.5 ± 0.6) (Figure 3a and b). No differences were found with regards to the other parameters (Figure 6).
Triamcinolon (TC): standard care (partially effective therapeutic)

Supplementing TC reduced collagen 1 secretion of SE$^{ASC}$ (Figure 6a). Also the number of epidermal cell layers of SE$^{ASC}$ decreased after treating with TC compared to control (7.0 ± 1.2 versus 7.8 ± 0.9) (Figure 5b and 6b). No differences were found with regards to the other parameters.

1,25-dihydroxy vitamin D$_3$ (VitD$_3$): clinically non effective therapeutic

Supplementing VitD$_3$ led to less contraction of SE$^{ASC}$ (Figure 5a and 6a). The number of epidermal cell layers of SE$^{ASC}$ increased after treating with VitD$_3$ compared to control (9.1 ± 1.1 versus 7.8 ± 0.9) (Figure 5b and 6b). Notably, SE$^{ASC}$ treated with VitD$_3$ (9.1 ± 1.1) had even more epidermal cell layers than HTscars (7.9 ± 1.6) (Figure 3a). The secretion of
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**IL-6 by SE<sup>ASC</sup>** was even further reduced by adding VitD<sub>3</sub>. (Figure 6c). No differences were found with regards to the other parameters.

**All-trans-retinoic acid (RA): potential novel scar therapeutic**

Supplementing potential novel scar therapeutic RA only partially normalized collagen 1 secretion of the HTscar model (Figure 6a). No differences were found after supplementing SE<sup>ASC</sup> with RA with regards to the other parameters. These results indicate that RA was not an effective anti scar therapeutic in the HTscar model.

**Atorvastatin: potential novel scar therapeutic**

Supplementing SE<sup>ASC</sup> with atorvastatin reduced the thickness of the dermis (Figure 5b and 6a). The number of epidermal cell layers of SE<sup>ASC</sup> decreased after treating with atorvastatin compared to control (6.4 ± 1.0 versus 7.8 ± 0.9) (Figure 5b and 6b). Notably, SE<sup>ASC</sup> treated with atorvastatin had approximately the same number of epidermal cell layers as NTscars (6.9 ± 1.0) and SE<sup>R-DSC</sup> (6.5 ± 0.6) (Figure 3a and b). The secretion of CXCL8 by SE<sup>ASC</sup> was increased by adding atorvastatin (Figure 6c). No differences were found with regards to the other parameters. Atorvastatin was the only therapeutic tested which resulted in partial normalization of three parameters.

### Table 2. HTscar parameters and cytokine secretion

| Scar parameters                   | Therapeutics applied to *in vitro* HTscar model | Standard care | Not effective | Potential novel |
|-----------------------------------|-----------------------------------------------|--------------|--------------|-----------------|
|                                   | SFU TC VitD<sub>3</sub> RA Atorvastatin       |--------------|--------------|-----------------|
| Thickness of dermis               | ↑ = = = = =                                     | ↑ =          | ↑ = =         | ↓* = = =        |
| Contraction                       | ↑ ↑ ↓* =                                       | ↑ =          | ↓* =         | = = =           |
| Collagen 1 secretion              | ↑ ↑ = ↓*                                        | ↑ =          | ↓* =         | ↓* = = =        |
| Epidermal Thickness               | ↑ ↑ ↓* = =                                      | = = =        | = =         | = = =           |
| Outgrowth of epidermis            | ↓ ↓ =                                           | = = =        | = = =     | = = =           |

IL-6 by SE<sup>ASC</sup> was even further reduced by adding VitD<sub>3</sub>. (Figure 6c). No differences were found with regards to the other parameters.

*HTscar compared to NTscar; ASC model compared to model containing R-DSC and P-DSC; = comparable, ↑ increased, ↓ decreased compared to control condition, ? unknown. Statistical significant difference between *in vitro* HTscar model cultured with therapeutics compared to *in vitro* HTscar model cultured with corresponding vehicle controls (*p < 0.05 and **p < 0.01, paired t-test)*
Figure 5. Macroscopic and microscopic assessment of HTscar model cultured with therapeutics. (a) Macroscopic overview (bars = 1 cm) and (b) Histological H/E staining (bars = 100 µm) of SE cultured without (control condition) and with therapeutics (5FU, TC, RA, VitD₃, atorvastatin). For all therapeutics, vehicle controls were tested in parallel. The vehicle control conditions were similar to the control condition (no vehicle added), data not shown.
DISCUSSION

In this study we show that ASC and keratinocytes both isolated from healthy full thickness human skin which is readily obtained as rest material after standard surgical procedures may be used to make an in vitro HTscar model to test anti-scarring therapeutics. The HTscar model had similar characteristics as HTscars and enabled relevant and quantifiable HTscar parameters to be identified and tested. Our first results shown...
in this study indicate that the *in vitro* HTscar model may be used to test new anti-scar therapeutics. Testing with combinations of known therapeutics and novel therapeutics is now required to further indicate the true value of the HTscar model with regards to replacement, reduction, and refinement of the use of animal models.

The first part of this study involved developing the HTscar model and selecting relevant and quantifiable HTscar parameters. We found that SE constructed with ASC visually represents HTscars. In contrast, incorporation of R-DSC and P-DSC, which are cells isolated from the more superficial layers of the skin, led to SE visually representing NTscar and Nskin respectively. This observation is in line with the clinical observation that HTscars occur more often after the closure of full-thickness wounds. Relevant and quantifiable parameters typical for HTscars that were identified in the HTscar model were contraction; collagen 1 secretion; outgrowth of epidermis and epidermal thickness. Additionally 2 cytokines typically involved in wound healing were assessed. The decrease in both IL-6 and CXCL8 secretion was characteristic for the HTscar model only and therefore it would now be interesting to determine whether HTscar *in vivo* also show decreased expression of these cytokines. In literature no consensus was reached whether IL-6 and CXCL8 are up or down regulated during HTscar formation. The confusion may be due to size, location and age of the studied scars samples. Although we did observe an increase in collagen 1 secretion no increased thickness of the dermis was observed in the presented HTscar model compared to SE composed with R-DSC and P-DSC. However, the thickness of the dermis was greater in DE when only ASC were incorporated into the matrix (without keratinocytes on top) than when R-DSC or P-DSC were used (data not shown). At present the reason for this is unknown, however this discrepancy between SE and DE may be related to cultured keratinocytes being very active in secreting proteins which degrade the collagen matrix as it forms.

Our results showed that dermal fibroblasts exhibited less hypertrophic scar characteristics than ASC even though they have been reported to produce TGF beta 1 and many cytokines involved in wound healing and scar formation. This indicates, in line with others, that dermal fibroblasts are involved in normal wound healing whereas ASC may be involved in adverse scar formation. Our finding that the SEASC model secreted less cytokine IL-6 and CXCL8 may be of significance for the pathophysiology of scar formation and our model now provides an excellent means to investigate this further in parallel with *in vivo* patient derived data in the future. Of note, previously we have shown that ASC and dermal fibroblast both display a mesenchymal stem cell phenotype (CD31−, CD34+, CD45−, CD54+, CD90+, CD105+, CD166+) and show similar multi-lineage differentiation potential. These characteristics were more pronounced for ASC. This suggests that, possibly, potent mesenchymal stem cell capacity may correlate to poor scar quality and requires further investigation. Although our results are in line with the clinical observation that HTscars show increased α-SMA compared to
NTscar and Nskin, it was noticed though that α-SMA was strongly expressed directly below the basement membrane in the SEASC HTscar model. This indicates that cultured keratinocytes may secrete a factor which stimulates differentiation into α-SMA positive cells. Interestingly DEASC showed very little α-SMA expression supporting this hypothesis. Since the immunohistochemical staining of α-SMA positive cells is difficult to quantify, this biomarker was not selected as a scar forming parameter.

The second part of this study was to validate the HTscar model with two therapeutics regularly used in the clinic for scar treatment (5FU and TC) and one therapeutic known to be unsuccessful in scar reduction (VitD3). Supplementing the HTscar model with 5FU resulted in partial normalization of the contraction and the epidermal thickness. Interestingly, the other therapeutic, TC, resulted in partial normalization of two different parameters: collagen 1 secretion and epidermal thickness. This finding indicates that combined therapy with 5FU and TC may have a better therapeutic affect than either single therapy. Indeed it has been shown in a clinical study (60 patients) that the combination of 5FU and TC does give a better response rate than either therapeutic alone.

Not all parameters (thickness of dermis, outgrowth of epidermis; IL-6 secretion; and CXCL8 secretion) were favorably influenced by these two therapeutics. This result is in line with clinical results for 5FU and TC since it is known that neither of these therapies can completely restore scar tissue to a normal skin phenotype in all patients (Table 1). Both standard therapeutics normalized only two parameters out of seven, indicating that for a therapeutic to be potentially effective it should also partially normalize at least two parameters.

VitD3 was used as a negative control therapeutic in our study based on clinical evidence. In line with the negative clinical results, we found an increased number of epidermal cell layers after adding VitD3. After adding VitD3 both IL-6 and CXCL8 were even further reduced. However, we also observed a decrease in contraction in SE which may be due to VitD3 inhibiting ASC proliferation resulting in fewer cells in the matrix at time of harvesting. Indeed, FACScan flow cytometry analysis of 3 mm punch biopsies isolated from SE showed 48% less CD90+ cells within the dermis of VitD3 exposed SE compared to control vehicle exposed SE (data not shown). Despite the thusfar reported clinical results properly dosed VitD3 may possibly prove to be beneficial to the patient since a decrease in the number of fibroblasts would result in fewer α-SMA positive cells and less contraction. Therefore further clinical studies are justified.

After testing the positive and negative controls, two therapeutics of unknown capacity to reduce HTscar characteristics (RA and atorvastatin) were tested. RA is an active metabolite of vitamin A and was included into this study since it decreases fibroblast proliferation and collagen production. However our results indicate that RA may only have limited value for scar treatment since it only partially normalized one HTscar parameter (reduction of collagen 1 secretion). Furthermore this favourable effect may
be counteracted by simultaneously decreasing collagen degradation\(^{20}\). On the other hand, atorvastatin shows distinct therapeutic potential since it was the only therapeutic to partially normalize three parameters (thickness of the dermis, epidermal cell layers and CXCL8 secretion. These results are in line with literature describing atorvastatin to prevent cardiac hypertrophy in rabbits and fibrotic adhesions in rats\(^{22,36}\). Of note, this was the only therapeutic to reduce the thickness of the dermis, a major parameter for a HTscar model. Since we showed \textit{in vitro} that both 5FU and TC have partly complementary properties compared to atorvastatin, they may potentially be used as combined therapies with atorvastatin. \textit{Our in vitro} HTscar model will easily permit such pre-clinical investigations in the future.

The HTscar model constructed with ASC not only assesses HTscar reduction but also HTscar prevention since therapeutics were already applied to the culture medium from day 4 before SE were fully developed. This mimics early treatment after surgery. All selected parameters typical for HTscar were affected in SE by at least one of the tested therapeutics with the exception of the outgrowth of the epidermis. This indicates that the model may be able to identify combinations of therapeutics which compliment each other in correcting adverse scar formation.

As with all \textit{in vitro} models, the HTscar model has a number of limitations which should be addressed. The main limitation is that it lacks an immune component since it is well known that infiltrating cells e.g. macrophages, monocytes etc influence wound healing\(^{16}\). Currently the model is being further developed to include these immune cells in co-culture with the HTscar model. Also neuro-endocrine signals\(^{37}\) and an angiogenic component\(^{38}\) are not incorporated in this HTscar model yet. Furthermore the current number of scar forming parameters might be further expanded. Extensive screening for more parameters such as increased TGF\beta1\(^{39}\) or CTGF\(^{14}\) might further improve the model and provide more insight into human HT scar formation. Another limitation is that only therapeutics which can be dissolved in the culture medium have been studied so far. It has yet to be determined whether similar results will be obtained if therapeutics are added topically to the stratum corneum of the SE. If this is the case, the model will also be suitable for testing water insoluble therapeutics in the form of creams and ointments. Also the model will need further adapting if it is to test pressure and silicone dressings, both widely used in HTscar treatment\(^{40}\). The negative control therapeutic VitD\(_3\) gave one false positive result (contraction) and one correctly assessed result (increase in epidermal thickness) in addition to a decreased IL-6 and IL-8 secretion. However, it may be possible that the false positive result is a valid result and that the single clinical study described was performed under sub optimal conditions with regards to VitD\(_3\) concentration. In general though, a single false positive result can be minimized due to the assessment of multiple scar parameters.
In most academic research and during drug discovery studies, many animal experiments are used in the early phases to define and refine research questions and potential future applications. It is possible that these early stages in drug development can be replaced by our human *in vitro* HTscar model system, limiting animal experiments to the final *in vivo* confirmation and risk assessment phases. Generally, these final phases require maximally one-tenth of the total number of animals used (http://www.buzzle.com/articles/animal-testing-statistics.html).

In summary we developed and validated a HTscar model using ASC and keratinocytes isolated from healthy skin and identified relevant and quantifiable parameters typical for HTscars. In line with the clinical experience, 5FU and TC only partially restored HTscar to normal skin phenotype. Each therapeutic selectively affected a different combination of parameters. These findings indicate that the *in vitro* model may be useful for selecting combinations of therapeutics with complementary properties. This will be a future area for investigation. Although the number of therapeutics tested in this initial study is small, our results indicate that this animal free HTscar model may be used to test novel anti-scar therapeutics and thereby may lead to the reduction of the use of animals in HTscar research.

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