Universiteit
Leiden
The Netherlands

Skipping to improve scar management: modulation of TGF-B in hypertrophic scars via exon skipping

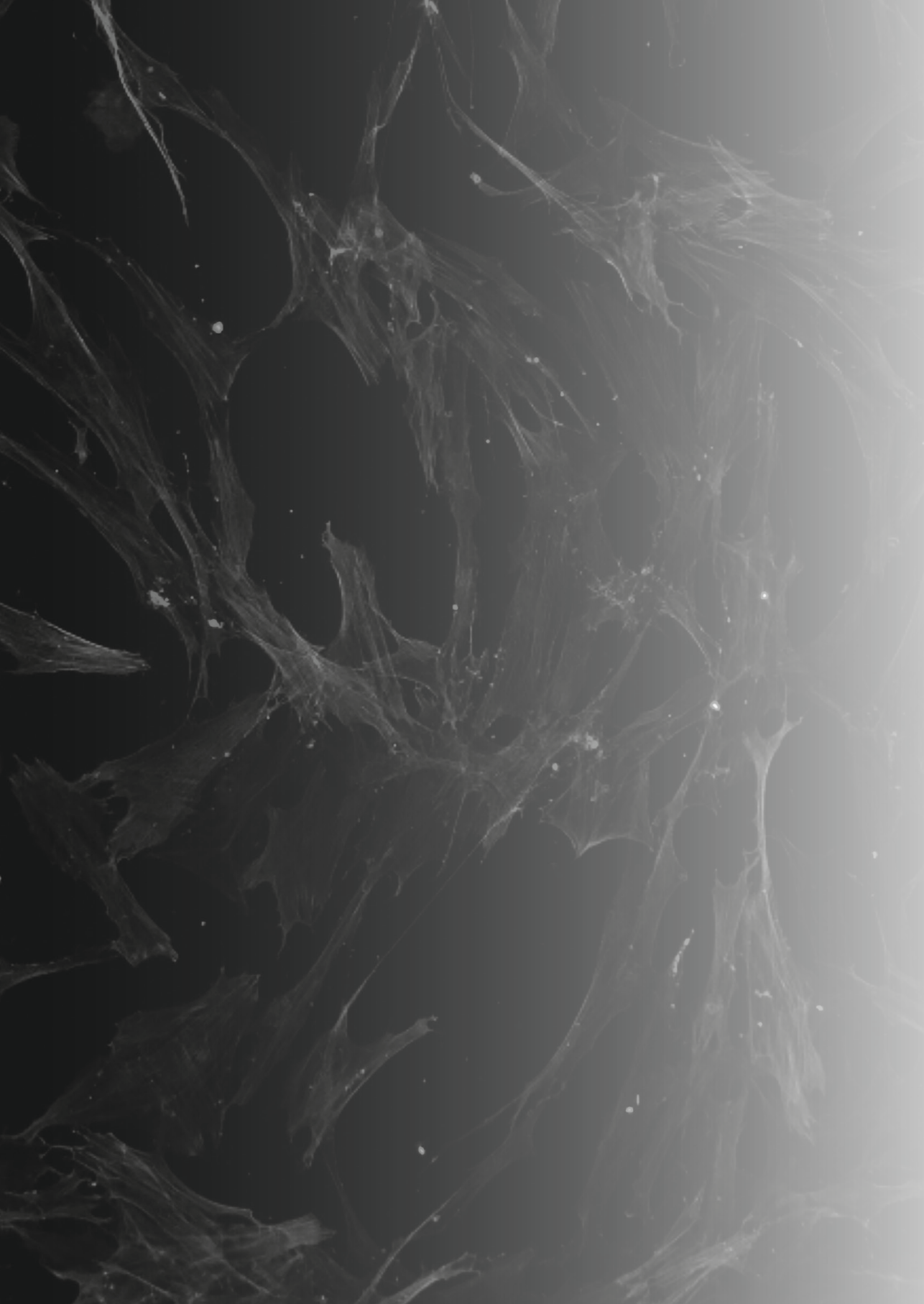
Raktoe, R.S.

Citation

Raktoe, R. S. (2024, November 5). *Skipping to improve scar management: modulation of TGF-B in hypertrophic scars via exon skipping*. Retrieved from https://hdl.handle.net/1887/4107638

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/4107638

Note: To cite this publication please use the final published version (if applicable).



CHAPTER

7

Summary and Perspectives

SUMMARY

The aim of this thesis was to investigate how interfering with TGF-β signaling, through either chemical inhibition or antisense-oligonucleotide (AON)-mediated exon skipping in mRNA of TGF-β receptors (specifically ALK5), impacts human hypertrophic burn scar fibroblasts and tissue. The rationale was that blocking TGF-β signaling could ameliorate hypertrophic scars (HTSs), and exon skipping could be exploited as a novel technique for designing a highly specific alteration in an essential protein, like ALK5. For this purpose, we have first set up fibroblast cultures and fibroblast-derived matrix (FDM) models. In these models we investigated the effects of ALK5 exon skipping on TGF-β signaling, fibroblast differentiation status and collagen remodeling. Since vivo-morpholinos (ViMs), modified AONs, do not require incorporation into a vector to enter the cell (due to their octaguanidinium delivery moieties), they can simply be added to the medium (1). Throughout the studies, we have used the pharmacological ALK5-specific inhibitor SB431542 as a positive control. SB431542 is extensively used *in vitro* and proven to be a potent inhibitor of TGF-β signaling. However, pre-clinical *in vivo* studies have shown that SB431542 is pharmacokinetically unstable (2).

In **chapter 2** of this thesis, we investigated the effects of ALK5 exon skipping on TGF-β signaling in HTS-derived fibroblasts. Once we confirmed the anti-fibrotic effects in HTS fibroblasts, we addressed the fibroblast heterogeneity. Since the HTS fibroblast cultures consisted of papillary fibroblasts (PFs), reticular fibroblasts (RFs) and myofibroblasts (MFs) the question was to what level the different subtypes were affected by exon skipping. Previous studies have reported that PFs from healthy skin possess anti-fibrotic features as opposed to RFs and MFs (3–6). Therefore, the subsequent question was if ALK5 exon skipping was able to target especially the pro-fibrotic cells which could result in a fibroblast culture consisting primarily of the anti-fibrotic PFs. In **chapter 3** we have set up PF, RF and HTS fibroblast cultures and treated them with ALK5ViM to quantify the biomarker expressions of these fibroblast subtypes. The study was concluded with a 3D-contraction assay to asses if ALK5 exon skipping successfully reduced the presence of pro-fibrotic fibroblasts, which are known to possess a contractile apparatus (7). Therefore, collagen lattices (FPLCs) established by and populated with the different fibroblast subtypes, were treated with ALK5ViM. Subsequently, the surface areas of the different FPLCs were measured and αSMA staining was applied. Here we found that ALK5ViM reduced αSMA expression in the FPLCs. We established FDM models that were formed by HTS fibroblast in order to mimic a fibrotic environment. In this model (**chapter 4**), we assessed the effect of ALK5 exon skipping on one of the hallmarks of HTS tissue: parallel alignment of collagen bundles which is linked to increased stiffness of the tissue (8–11). We cultured FDMs for three weeks including weekly treatment with ALK5ViM. After three weeks, collagen architecture was assessed using image processing by Second-Harmonic Generation (SHG) microscopy. In short, SHG microcopy is an imaging technique that makes use of the SHG signal that is transmitted by biological materials such as collagen, allowing live tissue imaging of the FDMs (12). Mechanical characteristics of the FDMs were quantified using micro-indentation. Confirmation of skipping of exon 2 was obtained by qRT-PCR. Here, we found that ALK5 exon skipping reduced collagen density and parallel alignment. In accordance,

micro-indentation demonstrated that stiffness of the FDMs was indeed reduced upon ALK5 exon skipping.

Further, in **chapter 5** we reported on ALK5 inhibition experiments in HTS explants. The aim of this study was to determine if injection of ALK5ViMs was effective on fully developed HTSs, albeit *ex vivo*, and would detectably modulate TGF-β signaling. First, we assessed ViM delivery in the HTS *ex vivo* cultures using a Lissamine (red fluorescent)-tagged ScrViM (about 1/3 of fibroblast turned out positive). Next, the explants were injected with ScrViM, or ALK5ViM followed by a seven day incubation, showing reduced expression of ALK5 downstream targets with ALK5ViM compared to ScrViM. Since phosphorylation of SMAD2 is a crucial step in propagation of TGF-β signaling, we also performed an immunohistochemical staining for pSMAD2. Here, we found a significant reduction in pSMAD2 expression after ALK5ViM treatment.

In **chapter 6**, we developed a three-layered Full thickness model (FTM) by incorporating the underlying adipose tissue. The initial aim was to investigate the molecular mechanisms behind the anti-fibrotic features of fat grafting in HTSs. We established FTMs that were populated by normal fibroblasts (NFs) or HTS fibroblasts, with or without the addition of adipose tissue. In addition, we assessed if the addition of adipose tissue could augment re-epithelialization. However, before proceeding, we first needed to determine the viability of this newly developed three-layered FTM in representing native skin in comparison to the currently used two-layered FTM. This issue evolved into the main focus of this chapter. The proof-of-principle with the three-layered FTM provided a solid foundation of this model to investigate fat grafting in a fibrotic environment and, more generally, as a novel platform for drug testing.

Altogether, the studies presented in the preceding chapters provided more detailed insights in how to reduce the fibrotic wound response and normalize the fibrotic environment in HTS development from different perspectives. Thus, our preclinical studies clearly made way toward the ultimate goal of optimizing HTS management.

PERSPECTIVES

Perspectives on modulation of TGF-β signaling in different (HTS-derived) fibroblast subpopulations

Chapter 2 of this thesis describes the modulatory effects of ALK5 exon skipping on TGF-β signaling in fibroblasts isolated from HTSs. We based our study on that of researchers who have injected *ex vivo* models of Dupuytren's tissue with ALK5ViM and found a clear reduction in fibrotic load (13). This raised the question whether ALK5ViM would work equally well on HTS tissue. However, a crucial difference between Dupuytren's disease (DD) and HTSs is that DD is a fibroproliferative disorder, whereas HTS is not and tends to regress over time (14,15). But TGF-β signaling was also suspected to be crucial in maintaining HTS (16,17). In HTS fibroblasts cultures, we found that exon skipping in the TGF-β receptor ALK5 pre-mRNA was successful in that TGF-β downstream targets were affected. Over the years, numerous compounds have been reported to target TGF-β signaling successfully. Many compounds were found to reduce the fibrotic load and most of them actively inhibiting TGF-β signaling and thereby, for example,

diminished αSMA expression, typically high in HTS. Unfortunately, to this date no drugs are available for HTS treatment (2,18). The ultimate goal would be to prevent HTS formation (or even better, achieve scarless wound healing) and therefore one could consider TGF-β inhibitory treatments applied during wound healing. It should be feasible to conserve proper wound healing while preventing overactivity in TGF-β signaling. Because of its specificity by design, exon skipping would appear ideal in the prevention of HTS development. To do so, it has become clear throughout this thesis that fibroblasts appear to be the target of interest.

Research on dermal fibroblasts has proven that this cell population is highly heterogenous showing spatial and functional differences between the different fibroblast subtypes (19–23). These differences could possibly be utilized to control wound healing and, thereby, reduce the risk of HTS formation, or ultimately achieve scarless wound healing. The three most well-known fibroblast subtypes are the PFs, RFs and MFs. Information on changes in functionality of the subtypes and fibrotic response are scarce. In **chapter 3**, we have compared the responses of PFs, RFs and HTS fibroblasts (referred to as scar fibroblasts (SFs) in this study) to ALK5 inhibition by either ALK5 exons skipping or SB431542 treatment. Furthermore, we investigated whether a reduction in RF and MF would further propel an anti-fibrotic effect. Gene expression analysis on specific fibroblast markers confirmed that after ALK5 inhibition PF marker (CCRL1 and NTN1) expression was higher compared to RF (CNN1, CDH2, TGM2) of MF (ACTA2) markers, indicating that ALK5 exon skipping induced a desirable shift in the subtypes. We expanded this analysis to other marker proteins. We selected TNC (PF marker), TGM2 (RF marker) and αSMA (MF marker), as we have shown specificity of these markers for proper distinction between the subtypes (24,25). Surprisingly, we found that TNC responded like a pro-fibrotic marker in showing downregulation after ALK5 inhibition. Other skin fibrosis-related studies also found TNC to be a fibrotic marker (25–29). TGM2 and αSMA data were inconclusive. Additionally, western blot analyses of untreated PF, RF and SF cultures confirmed that PF cultures exhibit high levels of PDPN (PF marker), and low CDH2 (RF marker) and αSM (high in MF), whereas RF and SF cultures conversely expressed substantially higher levels of CDH2 and αSMA compared to PDPN. These results, especially on TNC, indicate that expression of these markers should be interpreted with great care after treatment with ALK5ViM.

We then continued experimentation with a contraction assay on FPCLs and stain these for αSMA to mark pro-fibrotic fibroblasts. Here, we found that exon skipping in ALK5 was able to counteract the TGF-β-induced contractility. As expected, due to its anti-fibrotic features, PF-FPCLs showed substantially less contraction than RF- and SF-FPCLs. We hypothesized that this could be attributed to decorin which is highly expressed by PFs (30,31). Predominance of PFs introduced by ALK5 exon skipping could thus block, and perhaps wind back, the differentiation into MFs, as presented in **figure 1**. The scheme in **figure 1** is derived from the two-stage model of myofibroblast differentiation described by Tomasek and colleagues, indicated by the red arrows (32). Here we propose that the fibroblasts in the two-stage model start from PFs. **1)** Due to mechanical stress during wound healing, the PFs differentiate into proto-myofibroblasts that could comprise phenotypical RFs. **2)** Under further influence of TGF-β, ED-A fibronectin and mechanical stress proto-myofibroblasts differentiate into MFs. The MF-block induced by ALK5 exon skipping proceeds as follows (indicated in blue): **3)** ALK5 exon skipping reduces

TGF-β downstream signaling. Reducing (the risk of) severe scarring, **4)** In parallel, ALK5 exon skipping primarily targets the pro-fibrotic cells and yields a dominance of anti-fibrotic PFs over RFs and MFs. In turn, PFs exert their anti-fibrotic functions through, for example, decorin and even further reduce scar formation. At the same time, proper wound healing can still be completed. Translating this potential beneficial effect of ALK5 exon skipping on fibroblast heterogeneity, one could develop 'ALK5-tuned' human skin substitutes for burn victims and prevent severe scar formation.

Although we were able to isolate viable PFs and RFs by selection on cell-surface markers, extraction of an MF population for sequential culturing proved impossible. Fluorescence-activated cell sorting (FACS) of αSMA positive MFs is possible, but these cells need to be permeabilized and fixed for fluorescent labeling of αSMA, precluding any possibility of subsequent culturing. Staining for surface markers, on the other hand, leaves cells viable and suitable for further culturing. Prior to the study presented in **chapter 3**, we attempted to isolate the MF fraction from HTS fibroblasts by cell sorting for cell-surface marker amine oxidase copper containing 3 (AOC3; also known as vascular adhesion protein 1 (VAP1)) which is in volved in lymphocyte–endothelial interactions (33). A previous study had shown that colorectal MFs could be purified based on AOC3 in cell sorting because these cells uniformly expressed AOC3. Furthermore, researchers were able to distinguish these $AOC3^+$ MFs from TGF-β-activated skin fibroblasts. However, AOC3 expression on skin-residing MFs was somewhat elusive. Therefore, we performed cell sorting analysis on HTS fibroblasts from three donors by labeling AOC3 with an Alexa-555 fluorophore. In **figure 2A** the output of the analysis is shown. Here, $AOC3^+$ fibroblasts are displayed on the x-axis and the $AOC3^-$ fibroblasts were captured in the FITC channel which is displayed on the y-axis. Next, we defined the $AOC3^-$ and $AOC3^+$ by gating the data (P7 = negative; P6 = positive). However, gating appeared to somewhat difficult, since the $AOC3^+$ fraction was not explicit (one would expect to find a higher cell count on Alexa-555-positive cells). Therefore, we set the P6 gate with limited overlap with $AOC3^-$ fibroblast portion. However, one should take into account the presence of false positive AOC3 fibroblasts. After setting the gates, only a small proportion (around 0.5%) of the cells showed to be $AOC3^+$. Eleven days of separately culturing of the AOC3-negative and positive fibroblasts, revealed that $AOC3^-$ fibroblasts presented a typical spindle-shape like phenotype (**figure 2B**). $AOC3^+$ fibroblasts cultures were composed of polygonal-shaped cells, as seen in RFs and MFs (**figure 2C**). Extracting the MF population from a heterogenous fibroblast population should facilitate dissecting the MF-specific response upon modulation of TGF-β signaling and how this is propagated into their environment, more specifically, the deposition of ECM. Altogether, further elucidation of function and responses of the different fibroblast subtypes could augment more precise targeting of the subtypes in order to treat burn wounds, HTSs and ultimately other fibrotic disorders. In addition, such information could also contribute in optimizing the development of autologous full-skin equivalents to treat burn victims.

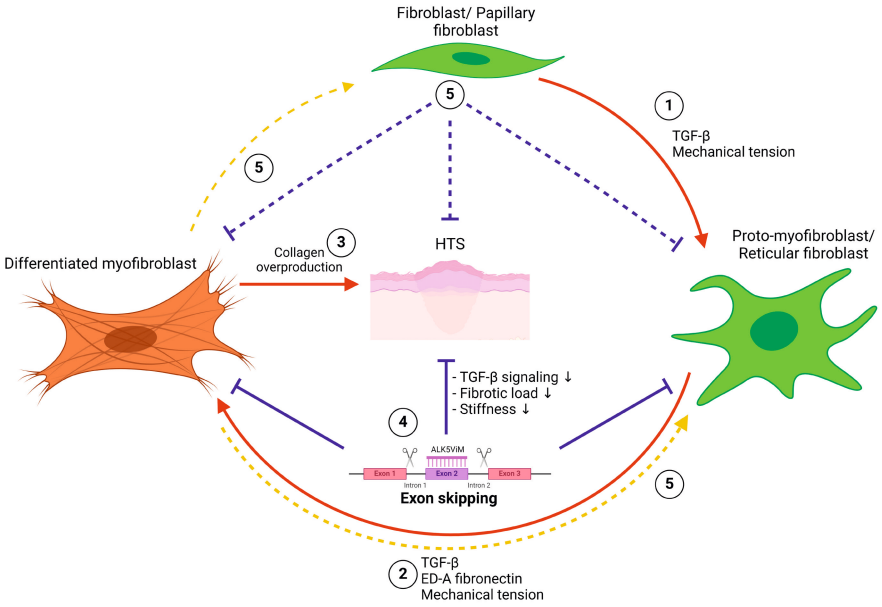


Figure 1: The effects of ALK5 exon skipping on different fibroblast subsets. Based on the two-step model of MF differentiation fibroblasts, including PFs, differentiate into proto-myofibroblasts/RFs under mechanical stress during wound healing **(1)**. As wound healing continues, mechanical tension increases, TGF-β is excreted which in turn induces ED-A fibronectin expression, propagating proto-myofibroblast/RFs differentiation **(2)** towards fully differentiated MFs **(3)**. During HTS development, MFs become less sensitive for apoptotic signals and start to produce excessive amounts of collagen. ALK5 exon skipping is able to reduce the fibrotic load of HTSs and presence of pro-fibrotic RFs and MFs (indicated by the blue flathead arrows) **(4)**. As a result, PFs could assume dominance and continue to exert its anti-fibrotic properties and augmenting inhibitory effects **(5)** of HTS, RFs and MFs (indicated by the blue, dashed flathead arrows) and perhaps induce de-differentiation of MFs (indicated by the yellow arrows). Created with BioRender.com.

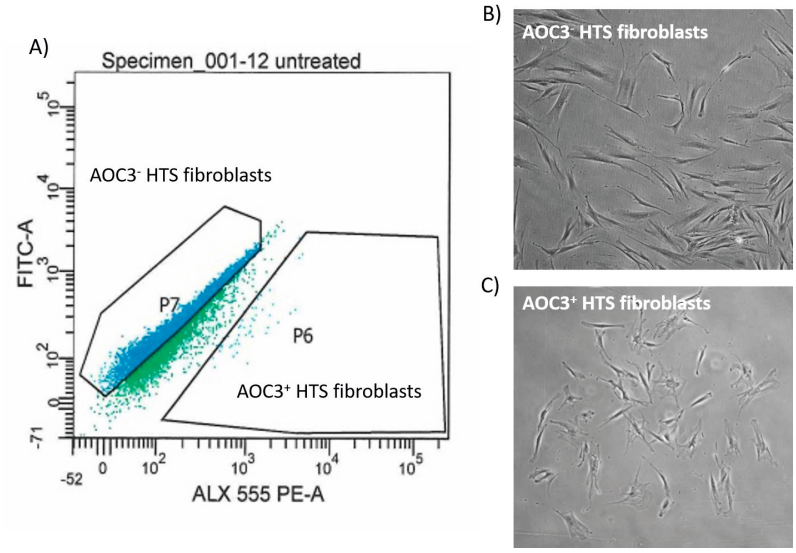


Figure 2: Sorting for AOC3⁺ fibroblasts in an HTS-derived fibroblast culture. The fibroblasts were incubated with a VAP-1 (AOC3) primary antibody, followed by incubation with Alexa-555 secondary antibody. **A)** AOC3⁻ (collected in the FITC channel; P7) and AOC3⁺ (collected in the Alexa-555 channel; P6) fibroblasts were sorted and collected in culture medium (DMEM containing 5% FBS, 1% P/S). The sorted fibroblasts were cultured at 37 ˚C, 5% CO_2 and medium was changed twice a week. **B)** AOC3⁻ fibroblasts after 11 days of culturing. The fibroblasts are typically spindle-shaped. **C)** AOC3⁺ fibroblasts, which are the putative MFs. At day 11, the fibroblasts harbor a stellate and polygonal-shaped like morphology, hence, resembling RFs and MFs. Image magnification: 40X; N = 3.

Effects of ALK5 exon skipping on the fibrotic environment

In **chapters 2** and **3** we focused on the effects of exon skipping on TGF-β signaling and how it affected the different fibroblasts. The next question was whether impairing TGF-β signaling has an any impact on dermal fibrotic tissue. To answer this question in **chapter 4**, we established an FDM that was formed by HTS fibroblasts (HTS-FDM). Therein we determined collagen organization and measured the stiffness of the deposited ECM after ALK5 inhibition. ALK5 exon skipping reduced collagen density, bundle thickness and parallel alignment. Furthermore, indentation measurements showed that stiffness was reduced. Hence, exon skipping clearly affects the fibrotic environment beneficially.

As posed in **chapter 4,** it is warranted to further expand the experiments by establishing FDMs using NFs. ALK5ViM-treatment of this heterogenous population of fibroblasts could shift the nature of their matrix toward either papillary or reticular (6,34). Consequently, such a change in the dermis may well influence development of the overlying epidermis (34,35). ALK5 inhibition substantially reduced αSMA staining, indicating a reduction in MFs, paralleled by a decrease in contractile tension. Or could there be more at play, such as the intriguing possibility of de-differentiation of MFs to RFs or even PFs?

Furthermore, SHG microscopy and indentation measurements were performed. Thus, we were able to correlate the changes in collagen organization with stiffness. In order to tighten this correlation, these measurements should in future be performed on the very same area. For clinical purposes, combining both techniques in a (portable) device could help clinicians in objectively monitoring HTS severity in the course of treatment.

In the next step reported on in **chapter 5**, we resorted to explants of HTS tissue as a model closer to native HTS, and injected these explants with ALK5ViM. In the DD study that was used as the basis for our study, *ex vivo* DD tissue was injected with ALK5ViM, or SB431542 was added to the culture medium for comparison (13). We used a similar experimental setup and started injecting *ex vivo* HTS tissue with a Lissamine (red fluorescent)-tagged ScrViM, in order to determine delivery of the ViM into the fibroblasts. With only 36% lissamine-positive cells, delivery was considerably lower than the virtually complete uptake in monolayers of fibroblasts. Gene expression analyses, where we compared ScrViM versus ALK5ViM treatment, revealed that TGF-β downstream target SERPINE-1 (PAI-1) was downregulated. As mentioned earlier, phosphorylation of SMAD2 is a crucial step in further activation of TGF-β downstream targets. Phosphorylated SMAD2 (pSMAD2) was also significantly reduced after ALK5 inhibition. Altogether, the data obtained in **chapter 5** showed that HTS explants could serve as a 3D culture model to further study the effects of ALK5 inhibition on cellular level and the fibrotic microenvironment. In comparison, the *in vitro* culture models presented in **chapter 2, 3 and 4** simulate development of an HTS environment in which ALK5 exon skipping could serve as a preventive modality. In contrast, the *ex vivo* HTS model in **chapter 5** is a fully developed post-burn HTS. This model is therefore more suitable to investigate ALK5 exon skipping as a therapeutic regimen rather than a preventive treatment option.

Perspectives on the development of full functional skin substitutes

We initially conducted the study in **chapter 6** of this thesis because we were interested in the anti-fibrotic mechanisms behind autologous grafting of fat tissue into HTSs (36–38). Therefore, we added adipose tissue (ADT) underneath the dermal compartment of a FTM to conform to the normal anatomical organization. Before embarking on experiments with this three-layered FTM, we first had to assess its quality and validity in mimicking skin in comparison to our current two-layered FTM. Addition of ADT turned out to improve epidermal morphogenesis, showing more resemblance with NHS than the two-layered FTM. And αSMA was indeed significantly reduced in the ADT-FTMs, in line with an anti-fibrotic effect of fat grafting. Thus, the introduction of ADT proved to be a two-edge sword in both an enhancement in mimicking the skin and a reduction in potentially fibrotic MFs.

The results in **chapter 6** were subsequently primarily focused on a quality assessment of the three-layered FTM which provided a solid foundation for further experimentation on molecular mechanisms involved. An unbiased exploration could start with extensive gene expression analyses of dermal and epidermal compartments in three-layered FTM versus two-layered FTM, or in treated three-layered FTM with HTS fibroblasts versus untreated control three-layered FTM. Next, a verificative analysis on the protein level should be carried out.

In our study we solely used fresh, unprocessed ADT from resected skin. However, in the clinical studies the ADT is obtained by liposuction and prepared further. This adipose material is then injected underneath the scar. This procedure is known as the *Coleman technique* (39,40). In our study, we kept the ADT as much as possible in its 3D confirmation. It is highly desirable to elucidate the effects of the different types of ADT processing on epidermal morphogenesis and anti-fibrotic impact in FTMs. In addition, experimentation is warranted on exclusive incorporation of adipose stromal cells in FTMs to further elucidate the anticipated anti-fibrotic effects of these cells, e.g. by reducing the number of MFs (41,42). In addition, adipose-residing mesenchymal cells should be investigated for their role in (fibrotic) scar development in wound healing studies and in FDMs (43).

The future perspective of FTM is primarily two-fold: 1) The three-layered organotypic skin model should be developed further as a more valid *in vitro* model of human skin (44,45), 2) with a better resemblance to NHS, it should preferentially be used in drug screening. In addition, further research should be conducted on the effects of ADT use on barrier function three-layered skin model. In a previous study, conducted by Mieremet et al., improved barrier function was reported upon addition of the macromolecule chitosan in the dermal compartment of the two-layered skin model (46). Will incorporation of ADT in combination with chitosan have a synergistic effect on barrier function?

With respect to ALK5 exon skipping, this newly developed three-layered FTM would also appear a more suitable 3D *in vitro* model to study further the effects on TGF-β signaling and the influence of ADT on HTS.

To humanize the skin model fully, the dermal part (conventionally grown on rattail collagen) should be fully grown from human fibroblasts, as the FDMs in **chapter 4**, before adding keratinocytes to establish an overlying epidermal layer (44). In **chapter 6** we started experimentation on FDMs with underlying ADT. However, preparing this advanced model is time-consuming in comparison to a conventional FTM. Therefore, further optimization is warranted and desirable. Furthermore, these organotypic skin models are isolated models, without physiological perfusion carrying growth factors, cytokines etc. Therefore, future experiments should include perfusion with essential physiological components and, optionally, drugs, e.g. by growing three-layered FTMs on a chip enabling perfusion (47).

Important in developing FTMs into skin substitutes to treat burn wounds, is clinical approvement. To this end, the dermal compartment should consist of fibroblasts embedded in clinically approved artificial or human-derived dermal matrices (45).

Altogether, this newly developed three-layered organotypic skin model offers a broad spectrum of applications, among which a promising perspective in developing treatments for burn wounds, severe scars and skin fibrosis in general.

Targeting TGF-β signaling and skin regeneration after (burn) injury: towards scarless wound healing

Throughout this thesis it has become evident that ALK5 exon skipping showed great potential in treating HTSs, and possibly, for scar free wound healing. In mammalian adult skin, the common outcome of injury is scar formation, instead of a desirable perfect regeneration of

the skin. The latter is still to be considered the holy grail in curing wounds. In humans, scarless wound healing only occurs in fetal skin, but is lost in the third trimester (48). In recent years, advances have been made in elucidating the molecular processes contributing to scarless wound healing (as seen in fetal skin) and in how to exploit this to optimize (burn) wound treatment (49). It turns out that fibroblast heterogeneity plays a crucial role (50). Lineage tracing and molecular profiling have demonstrated an apparent corresponding functional heterogeneity in wound healing (51). This analysis was based on the most well-characterized subtypes, PFs, RFs and MFs. PFs hold the potential for dermal regeneration and the formation of skin appendages, such as hair follicles (6,52). Hair follicles are absent in severe scars, whereas it has been reported that hair follicles improve wound healing and that follicular stem cells are involved in regeneration of epithelial structures (53).

Recently, the discovery and isolation of the engrailed-1 $(En1)^+$ murine fibroblast (EPFs) lineage has greatly propelled regenerative research. In humans, the En1 equivalent is CD26 (dipeptidyl peptidase 4; Dpp4) (54). Mascharak and colleagues showed that $En1^+$ fibroblasts, residing in the reticular dermis, mediate scarring (55). In addition, researchers found that $En1^-$ (papillary) fibroblasts (ENFs) in the reticular dermis activate En1 expression in the wound environment in which mechanical cues are of utmost importance. This process resembles differentiation of fibroblasts into putative RFs under mechanical stress during wound healing, as proposed in the two-stage model of MF differentiation (32). In short, migrating fibroblasts move into the wound along provisional collagen fibrils. The tractional forces, exerted by the migrating fibroblasts cause the collagen matrix to remodel. The mechanical tension induces stress fibers in the fibroblasts and their eventual differentiation. Consequently, researchers inhibited Yes-associated protein (YAP), since this is a crucial mechanotransducer in fibroblasts (56). YAP and transcriptional coactivator with PDZ-binding motif (TAZ) are key downstream targets of the Hippo pathway, which is involved in regulation of tissue volume (57). Functions of YAP include inhibition of apoptosis (58). Pro-fibrotic features of YAP lie in its activation of connective tissue growth factor (CTGF) which is known to be upregulated in HTS formation (59,60). Moreover, a previous study reported that YAP-TAZ-deficient fibroblasts are less responsive to TGF-β stimulation, produce less ECM and show reduced expression of PAI-1 and αSMA (56). With regard to the mechanotransducing properties, Mascharak and colleagues found that fibroblasts on low-stiffness hydrogels showed no En1 activation. And of note, PFs showed low En1 activation on high-stiffness plates, whereas RFs responded with strong En1 activation. Hence, lessening mechanical tension could reduce scarring. With respect to **chapter 4**, we hypothesize that ALK5ViM reduces En1 activation by lowering the ECM stiffness trough reduced YAP activity, and, thus deactivates pro-fibrotic fibroblasts.

In wound healing studies in mice, Mascharak et al found that YAP inhibition blocked En1 activation and promoted ENF-mediated skin regeneration, including recovery of functional HFs including appendages (55). Moreover, PFs have been characterized as CD26-negative and RFs as CD26-positive (22). Since we have found that pro-fibrotic fibroblasts were affected by ALK5 exon skipping (**chapter 3**), we hypothesize that ALK5 exon skipping could reduce CD26 expression. This calls for further investigation of fibroblast plasticity during healing of burn wounds. For instance, are $CD26^+$ fibroblast (pro-fibrotic) able to differentiate into $CD26^-$

fibroblasts (anti-fibrotic)? These interconversions between fibroblast subtypes are likely to take place during wound healing since prevalence of markers of the different populations shift over time, revealing phenotypical dynamics (51). Further understanding of these mechanisms in fibroblast plasticity could propagate regeneration of the skin after injury.

Another approach for the potential prevention of HTS development is reprogramming of MFs. Previous studies have shown bone morphogenetic protein (BMP)- dependent differentiation of MFs into adipocytes which are involved in wound healing and skin regeneration (42,61,62). Moreover, paracrine secretion by adipocytes substantially reduces expression of αSMA and ECM components by reprogramming of HTS-derived MFs through BMP4 and peroxisome proliferator-activated receptor gamma (PPARγ) (42). Significant reduction in αSMA upon fat grafting was supported in **chapter 6**. This anti-fibrotic effect could very well be attributable to the same mechanism as reported on by Hoerst and colleagues (42).

Other approaches to achieve scarless wound healing, entail the use of stem cells. The different types of stem cells can be divided in the following groups: pluripotent stem cells (including embryonic stem cells and induced pluripotent stem cells), multipotent hematopoietic stem cells and mesenchymal stem cells/ adipose stem cells (ADSCs) (63–69). Especially, ADSCs are of great interest, and with their abundance in ADT easy to harvest (68,69). As mentioned earlier, the anti-fibrotic effects of fat grafting are also attributed to the ADT-residing ADSCs. ADSCs are involved in wound healing and regeneration because they can give rise to a multitude of different cell lineages, thus making them potentially pivotal in developing optimal human skin substitutes to treat burn patients and achieve scarless wound healing (70–72). With respect to MF de-differentiation and potential scarless wound healing, it has been shown that ADSCs differentiate into MFs when treated with TGF-β1 which is reversed by basic fibroblast growth factor (bFGF) treatment. ADSCs differentiate into spindle shape-like fibroblasts with increased TNC expression and enhanced migratory behavior, as seen in PFs (34,73).

Altogether, the above mentioned anti-fibrotic modalities provide potential methods for avoiding HTS formation and, possibly, in achieving scarless wound healing. Based on our data and available literature, we have developed the scheme in **figure 3**, which is an extension of **figure 1**. Here, we propose additional interactions between the treatment modalities and their contribution to minimizing scar formation in wound healing (**figure 3)**.

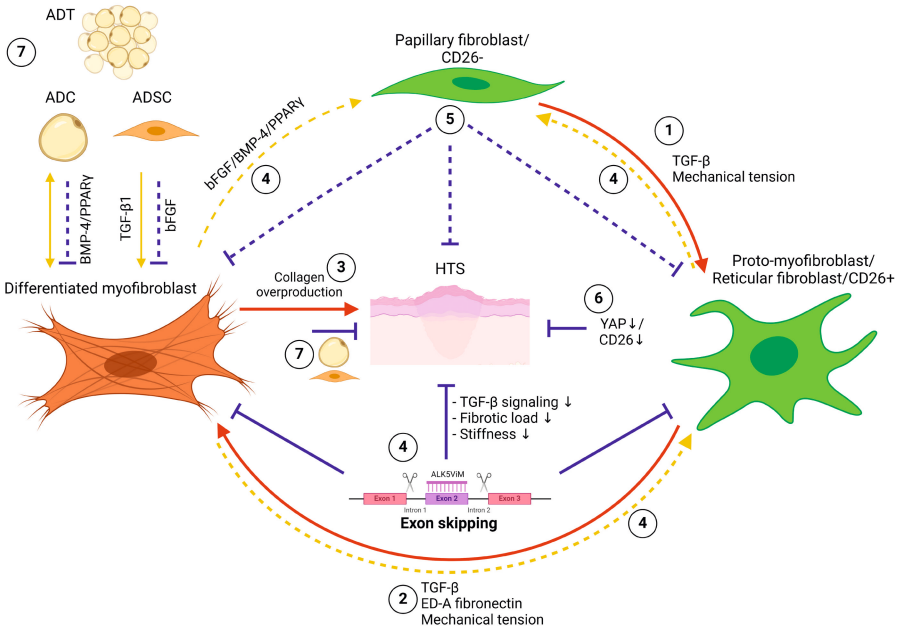


Figure 3: The proposed interaction between different anti-fibrotic treatment options and possible contribution to scarless wound healing. This scheme is an extension of figure 1 that displays the effects of ALK5 exon skipping on PFs, RFs and MFs. **1)** Upon (burn) injury of the skin, fibroblasts migrate to the wound in a provisional collagen matrix, exerting mechanical tension. As a result of this increasing tension, fibroblasts start to differentiate into a more pro-fibroblastic phenotype and start to express TGF-β1. The anti-fibrotic $CD26^-$ PFs differentiate into proto-myofibroblast/$CD26^+$ RFs. **2)** MF differentiation is further propagated by TGF-β1 and ED-A fibronectin together with mechanical tension, caused by the movement of fibroblasts along collagen fibers. **3)** In HTS development, MFs become less sensitive to apoptotic signals resulting in collagen overexpression and overproduction. **4)** ALK5 exon skipping causes downregulation of TGF-β signaling, reduction in fibrotic load and stiffness. As reported in chapter 3, ALK5 exon skipping affects primarily RFs and MFS shifting predominance to anti-fibrotic PFs. In addition, ALK5 exon skipping together with bFGF/BMP4/PPARγ could cause reprogramming of MFs towards PFs. **5)** PFs exert their anti-fibrotic effects directly by reducing the fibrotic load and indirectly by inhibiting RFs and MFs. This could contribute to scarless wound healing. **6)** YAP inhibition and CD26 inhibition cause depletion of $CD26^+$ RFs, thereby reducing HTS development/ possibly enhancing scarless wound healing. **7)** The anti-fibrotic effects of fat grafting, including both ADCs and ASCs, have an inhibitory effect on MF differentiation (blue dashed flathead arrows). Furthermore, reported MF plasticity (yellow arrows) could be utilized to reduce of MFs. Furthermore, paracrine secretion directly affects HTS development (blue flathead arrows). Created with BioRender.com.

Concluding remarks and recommendations

The main goal of this thesis was to contribute to effective management of hypertrophic scars by ALK5 inhibition through exon skipping. Throughout the chapters, our findings showed that transient knockdown of ALK5 exon 2 elicits anti-fibrotic effects and, thereby could offer a novel treatment option for burn patients suffering from HTSs.

An important question is whether exon skipping is either more suited as a curative or as preventive treatment, or whether it is suited for both. In most of the chapters, the models that were set up represented an active fibrotic environment. This was accomplished by initial stimulation with TGF-β1 to augment the fibrotic response and induce myofibroblast differentiation (74). In **chapter 5**, we used HTS explants. In the latter case, ALK5 exon skipping was evidently tested as a curative option rather than a preventive one. As mentioned in **chapter 1**, HTSs tend to regress overtime and becoming less active in terms MF abundance and proliferation. Although we found anti-fibrotic responses in HTS explants after ALK5 exon skipping, our earlier *in vitro* findings suggests that ALK5 exon skipping could be very effective in preventing severe scar formation. By quenching the fibrotic response in deep burn wounds, one could potentially prevent severe scarring, such as HTS or keloid development. The latter is recognized to be a progressive type of scarring (75). Therefore, ALK5 exon skipping could be very effective in immediate post-burn treatment. Hence, ALK5 exon skipping could be used either to treat the scar or prevent a severe fibrotic outgrowth.

HTSs can cause substantial cutaneous dysfunction and disfigurements. ALK5 exon skipping could potentially help to ameliorate these problems. It is of great importance to explore broadening the scope of pertinence of this intervention; for example, in treating contractures that arise after burn injuries. Contractures are characterized by shortening of the collagen fibers, which can cause severe impairment of joint motion and can be accompanied by HTS development. Elaborating further on the applicability of ALK5 exon skipping, one could hypothesize that it could be a potential treatment modality for scleroderma, notoriously difficult to treat. One of the main manifestations in scleroderma is the overproduction of collagens in connective tissue of the skin, resulting in thickening and hardening of the skin. This effect is primarily attributed to myofibroblasts (76). Moreover, the fibrosis in scleroderma can reach beyond the skin, severely affecting quality of life of patients, therefore making ALK5 exon skipping an interesting treatment option in this generalized fibrotic disease. Altogether, since TGF-β signaling is the pivotal pathway in the fibrotic response, it is evident that ALK5 exon skipping could be a potential tool that goes far beyond severe scarring of the skin, e.g. treating cancers by targeting their cancer-associated fibroblasts (CAFs) (77).

However, the fibrotic response is not limited to derailed TGF-β signaling, and should rather be considered as a multiple-facetted process, including, for example, the immune response especially in the early phases of wound healing. Therefore, further investigation of ALK5 exon skipping should include possible administration in combination with other anti-fibrotic agents that target other facets of the fibrotic response, thus attaining an enhanced and potentially synergistic effect.

The basic conception of targeting TGF-β signaling in order to achieve an anti-fibrotic effect is not new. However, the optimal approach is still undecided, as is the issue of minimizing adverse side effects. To this date, no anti-TGF-β treatment has successfully reached the stage of a clinical trial (78). As mentioned in **chapter 1**, the use of AONs harbor great potential to overcome the barriers to a clinical trial. For example, there are numerous AON modifications possible to fine-tune its effects. An encouraging development in antisense technology is its

approval by the US Food and Drug Administration for the treatment of Duchenne muscular dystrophy (79).

Another serious option that should be investigated is the use of CRISPR/Cas9 in targeting TGF-β signaling. This emerging technique has shown a plethora of possibilities with regard to gene editing. However, one crucial difference between CRISPR/Cas9 and antisense technology is that AONs do not alter a gene but target gene expression on transcriptional level, causing a transient effect. It may turn out that a patient would then require periodical treatment. CRISPR/Cas9 would edit the DNA, thus introducing permanent changes in specific genes, which could also be a severe drawback. Depending on the intended impact, gene knockdown or knockout, one could therefore choose either an antisense or a CRISPR/Cas9 treatment, respectively.

In this thesis we have shown that ALK5 exon skipping features effects that could reduce a fibrotic process. It is therefore warranted to investigate this anti-fibrotic potential further in *in vivo* models, assessing toxicity and pharmacokinetics. This should lead up to deploying the antisense therapy in clinical trials. Altogether, ALK5 exon skipping is a promising novel tool that could open up an avenue for effective scar treatment and beyond.

REFERENCES

1. Morcos PA, Li Y, Jiang S. Vivo-Morpholinos: A non-peptide transporter delivers Morpholinos into a wide array of mouse tissues. Biotechniques. 2008;45(6):613–23.
2. Akhurst RJ, Hata A. Targeting the TGFβ signalling pathway in disease. Nat Rev Drug Discov Internet. 2012;11(11):886–886. Available from: http://www.nature.com/doifinder/10.1038/nrd3878
3. Schafer I, Pandy M, Ferguson R, Davis B. Comparative observation of fibroblasts derived from the papillary and reticular dermis of infants and adults: Growth kinetics, packing density at confluence and surface morphology. Vol. 31, Mechanisms of ageing and development. 1985. 275–293 p.
4. Sorrell JM, Baber MA, Caplan AI. Construction of a bilayered dermal equivalent containing human papillary and reticular dermal fibroblasts: use of fluorescent vital dyes. Tissue Eng. 1996;2(1):39–49.
5. Sorrell JM, Caplan AI. Fibroblast heterogeneity: more than skin deep. J Cell Sci. 2004 Feb;117(Pt 5):667–75.
6. Varkey M, Ding J, Tredget EE. Advances in Skin Substitutes-Potential of Tissue Engineered Skin for Facilitating Anti-Fibrotic Healing. J Funct Biomater. 2015 Jul;6(3):547–63.
7. Kendall RT, Feghali-Bostwick CA. Fibroblasts in fibrosis: novel roles and mediators. Front Pharmacol Internet. 2014 May 27;5:123. Available from: https://pubmed.ncbi.nlm.nih.gov/24904424
8. Verhaegen PDHM, Van Zuijlen PPM, Pennings NM, Van Marle J, Niessen FB, Van Der Horst CMAM, et al. Differences in collagen architecture between keloid, hypertrophic scar, normotrophic scar, and normal skin: An objective histopathological analysis. Wound Repair Regen Internet. 2009 Sep 1;17(5):649–56. Available from: https://doi.org/10.1111/j.1524-475X.2009.00533.x
9. Kischer CW. Collagen and dermal patterns in the hypertrophic scar. Anat Rec Internet. 1974 May 1;179(1):137–45. Available from: https://doi.org/10.1002/ar.1091790111
10. Dunn MG, Silver FH, Swann DA. Mechanical Analysis of Hypertrophic Scar Tissue: Structural Basis for Apparent Increased Rigidity. J Invest Dermatol Internet. 1985;84(1):9–13. Available from: http://www.sciencedirect.com/science/article/pii/S0022202X15435705
11. Clark JA, Cheng JCY, Leung KS. Mechanical properties of normal skin and hypertrophic scars. Burns Internet. 1996;22(6):443–6. Available from: http://www.sciencedirect.com/science/article/pii/0305417996000381
12. Chen X, Nadiarynkh O, Plotnikov S, Campagnola PJ. Second harmonic generation microscopy for quantitative analysis of collagen fibrillar structure. Nat Protoc Internet. 2012;7(4):654–69. Available from: https://doi.org/10.1038/nprot.2012.009
13. Karkampouna S, Kruithof BP, Kloen P, Obdeijn MC, van der Laan AM, Tanke HJ, et al. Novel Ex Vivo Culture Method for the Study of Dupuytren's Disease: Effects of TGFβ Type 1 Receptor Modulation by Antisense Oligonucleotides. Mol Ther - Nucleic Acids Internet. 2014;3(June 2013):e142. Available from: http://linkinghub.elsevier.com/retrieve/pii/S2162253116302815
14. Trojian TH, Chu SM. Dupuytren's disease: diagnosis and treatment. Am Fam Physician. 2007 Jul;76(1):86–9.
15. Ehrlich HP, Desmoulière A, Diegelmann RF, Cohen IK, Compton CC, Garner WL, et al. Morphological and immunochemical differences between keloid and hypertrophic scar. Am J Pathol Internet. 1994 Jul;145(1):105–13. Available from: https://pubmed.ncbi.nlm.nih.gov/8030742
16. Lichtman MK, Otero-Vinas M, Falanga V. Transforming growth factor beta (TGF-β) isoforms in wound healing and fibrosis. Wound Repair Regen Internet. 2016 Mar 1;24(2):215–22. Available from: https://doi.org/10.1111/wrr.12398
17. Pohlers D, Brenmoehl J, Löffler I, Müller CK, Leipner C, Schultze-Mosgau S, et al. TGF-beta and fibrosis in different organs - molecular pathway imprints. Biochim Biophys Acta. 2009 Aug;1792(8):746–56.
18. Zhang T, Wang X-F, Wang Z-C, Lou D, Fang Q-Q, Hu Y-Y, et al. Current potential therapeutic strategies targeting the TGF-β/Smad signaling pathway to attenuate keloid and hypertrophic scar formation. Biomed Pharmacother Internet. 2020;129:110287. Available from: https://www.sciencedirect.com/science/article/pii/S0753332220304790

19. Haydont V, Neiveyans V, Fortunel NO, Asselineau D. Transcriptome profiling of human papillary and reticular fibroblasts from adult interfollicular dermis pinpoints the 'tissue skeleton' gene network as a component of skin chrono-ageing. Mech Ageing Dev Internet. 2019;179:60–77. Available from: http://www.sciencedirect.com/science/article/pii/S0047637418301908
20. Haydont V, Neiveyans V, Perez P, Busson E, Lataillade J, Asselineau D, et al. Fibroblasts from the Human Skin Dermo-Hypodermal Junction are Distinct from Dermal Papillary and Reticular Fibroblasts and from Mesenchymal Stem Cells and Exhibit a Specific Molecular Profile Related to Extracellular Matrix Organization and Modeling. Cells. 2020 Feb;9(2).
21. Haydont V, Neiveyans V, Zucchi H, Fortunel NO, Asselineau D. Genome-wide profiling of adult human papillary and reticular fibroblasts identifies ACAN, Col XI α1, and PSG1 as general biomarkers of dermis ageing, and KANK4 as an exemplary effector of papillary fibroblast ageing, related to contractility. Mech Ageing Dev Internet. 2019;177:157–81. Available from: http://www.sciencedirect.com/science/article/pii/S0047637418300423
22. Philippeos C, Telerman SB, Oulès B, Pisco AO, Shaw TJ, Elgueta R, et al. Spatial and Single-Cell Transcriptional Profiling Identifies Functionally Distinct Human Dermal Fibroblast Subpopulations Internet. Vol. 138, Journal of Investigative Dermatology. Society for Investigative Dermatology; 2018. 811–825 p. Available from: https://doi.org/10.1016/j.jid.2018.01.016
23. Korosec A, Frech S, Gesslbauer B, Vierhapper M, Radtke C, Petzelbauer P, et al. Lineage Identity and Location within the Dermis Determine the Function of Papillary and Reticular Fibroblasts in Human Skin. J Invest Dermatol Internet. 2019;139(2):342–51. Available from: https://doi.org/10.1016/j.jid.2018.07.033
24. Janson D, Rietveld M, Mahé C, Saintigny G, El Ghalbzouri A. Differential effect of extracellular matrix derived from papillary and reticular fibroblasts on epidermal development in vitro. Eur J Dermatology. 2017;27(3):237–46.
25. Janson DG, Saintigny G, van Adrichem A, Mahé C, El Ghalbzouri A. Different Gene Expression Patterns in Human Papillary and Reticular Fibroblasts. J Invest Dermatol Internet. 2012;132(11):2565–72. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022202X15355226
26. Schalkwijk J, Steijlen PM, Van Vlijmen-Willems IMJJ, Oosterling B, Mackie EJ, Verstraeten AA. Tenascin expression in human dermis is related to epidermal proliferation. Am J Pathol. 1991;139(5):1143–50.
27. Pearson CA, Pearson D, Shibahara S, Hofsteenge J, Chiquet-ehrismann R. Tenascin : cDNA cloning. 1988;7(10):2977–82.
28. Jinnin M, Ihn H, Asano Y, Yamane K, Trojanowska M, Tamaki K. Tenascin-C upregulation by transforming growth factor-beta in human dermal fibroblasts involves Smad3, Sp1, and Ets1. Oncogene. 2004;23.
29. Midwood KS, Chiquet M, Tucker RP, Orend G. Tenascin-C at a glance. J Cell Sci. 2016;129(23):4321–7.
30. Schönherr E, Beavan LA, Hausser H, Kresse H, Culp LA. Differences in decorin expression by papillary and reticular fibroblasts in vivo and in vitro. Biochem J. 1993 Mar;290 (Pt 3(Pt 3):893–9.
31. Zhang Z, Garron TM, Li X-J, Liu Y, Zhang X, Li Y-Y, et al. Recombinant human decorin inhibits TGF-β1-induced contraction of collagen lattice by hypertrophic scar fibroblasts. Burns. 2009;35(4):527–37.
32. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol Internet. 2002;3(5):349–63. Available from: http://www.nature.com/doifinder/10.1038/nrm809
33. Hsia L, Ashley N, Ouaret D, Wang LM, Wilding J, Bodmer WF. Myofibroblasts are distinguished from activated skin fibroblasts by the expression of AOC3 and other associated markers. Proc Natl Acad Sci Internet. 2016;113(15):E2162–71. Available from: http://www.pnas.org/lookup/doi/10.1073/pnas.1603534113
34. Janson D, Rietveld M, Mahe C, Saintigny G, El Ghalbzouri A. Differential effect of extracellular matrix derived from papillary and reticular fibroblasts on epidermal development in vitro. Eur J Dermatol. 2017 Jun;27(3):237–46.

35. Varkey M, Ding J, Tredget EE. Superficial dermal fibroblasts enhance basement membrane and epidermal barrier formation in tissue-engineered skin: implications for treatment of skin basement membrane disorders. Tissue Eng Part A Internet. 2013/10/17. 2014 Feb;20(3–4):540–52. Available from: https://pubmed.ncbi.nlm.nih.gov/24004160

36. Negenborn VL, Groen J-W, Smit JM, Niessen FB, Mullender MG. The Use of Autologous Fat Grafting for Treatment of Scar Tissue and Scar-Related Conditions: A Systematic Review. Plast Reconstr Surg Internet. 2016;137(1). Available from: https://journals.lww.com/plasreconsurg/Fulltext/2016/01000/The_Use_of_Autologous_Fat_Grafting_for_Treatment.16.aspx

37. Jaspers MEH, Brouwer KM, Van Trier AJM, Groot ML, Middelkoop E, Van Zuijlen PPM. Effectiveness of Autologous Fat Grafting in Adherent Scars: Results Obtained by a Comprehensive Scar Evaluation Protocol. Plast Reconstr Surg. 2017;139(1):212–9.

38. Jaspers MEH, Brouwer KM, van Trier AJM, Middelkoop E, van Zuijlen PPM. Sustainable effectiveness of single-treatment autologous fat grafting in adherent scars. Wound Repair Regen. 2017;25(2):316–9.

39. Coleman SR. Long-term survival of fat transplants: controlled demonstrations. Aesthetic Plast Surg. 1995;19(5):421–5.

40. Coleman SR. Structural fat grafting: more than a permanent filler. Plast Reconstr Surg. 2006 Sep;118(3 Suppl):108S-120S.

41. Spiekman M, van Dongen JA, Willemsen JC, Hoppe DL, van der Lei B, Harmsen MC. The power of fat and its adipose-derived stromal cells: emerging concepts for fibrotic scar treatment. J Tissue Eng Regen Med Internet. 2017 Nov 1;11(11):3220–35. Available from: https://doi.org/10.1002/term.2213

42. Hoerst K, van den Broek L, Sachse C, Klein O, von Fritschen U, Gibbs S, et al. Regenerative potential of adipocytes in hypertrophic scars is mediated by myofibroblast reprogramming. J Mol Med. 2019;97(6):761–75.

43. Van Den Bogaerdt AJ, Van Der Veen VC, Van Zuijlen PPM, Reijnen L, Verkerk M, Bank RA, et al. Collagen cross-linking by adipose-derived mesenchymal stromal cells and scar-derived mesenchymal cells: Are mesenchymal stromal cells involved in scar formation? Wound Repair Regen Internet. 2009 Jul 1;17(4):548–58. Available from: https://doi.org/10.1111/j.1524-475X.2009.00501.x

44. Trottier V, Marceau-Fortier G, Germain L, Vincent C, Fradette J. IFATS collection: Using human adipose-derived stem/stromal cells for the production of new skin substitutes. Stem Cells. 2008 Oct;26(10):2713–23.

45. van Zuijlen P, Gardien K, Jaspers M, Bos E, Baas D, van Trier A, et al. Tissue engineering in burn scar reconstruction. Burn Trauma Internet. 2015;3(2015):1–11. Available from: http://dx.doi.org/10.1186/s41038-015-0017-5

46. Mieremet A, Rietveld M, Absalah S, van Smeden J, Bouwstra JA, El Ghalbzouri A. Improved epidermal barrier formation in human skin models by chitosan modulated dermal matrices. PLoS One Internet. 2017 Mar 23;12(3):e0174478. Available from: https://doi.org/10.1371/journal.pone.0174478

47. Zhang Q, Sito L, Mao M, He J, Zhang YS, Zhao X. Current advances in skin-on-a-chip models for drug testing. Microphysiological Syst. 2018;1:1–1.

48. Larson BJ, Longaker MT, Lorenz HP. Scarless fetal wound healing: a basic science review. Plast Reconstr Surg Internet. 2010 Oct;126(4):1172–80. Available from: https://pubmed.ncbi.nlm.nih.gov/20885241

49. Moore AL, Marshall CD, Barnes LA, Murphy MP, Ransom RC, Longaker MT. Scarless wound healing: Transitioning from fetal research to regenerative healing. WIREs Dev Biol Internet. 2018 Mar 1;7(2):e309. Available from: https://doi.org/10.1002/wdev.309

50. Jiang D, Rinkevich Y. Scars or Regeneration?-Dermal Fibroblasts as Drivers of Diverse Skin Wound Responses. Int J Mol Sci Internet. 2020 Jan 17;21(2):617. Available from: https://pubmed.ncbi.nlm.nih.gov/31963533

51. Griffin MF, desJardins-Park HE, Mascharak S, Borrelli MR, Longaker MT. Understanding the impact of fibroblast heterogeneity on skin fibrosis. Dis Model & Mech Internet. 2020 Jun 1;13(6):dmm044164. Available from: http://dmm.biologists.org/content/13/6/dmm044164.abstract

52. Rippa AL, Kalabusheva EP, Vorotelyak EA. Regeneration of Dermis: Scarring and Cells Involved. Vol. 8, Cells . 2019.
53. Kiani MT, Higgins CA, Almquist BD. The Hair Follicle: An Underutilized Source of Cells and Materials for Regenerative Medicine. ACS Biomater Sci Eng Internet. 2017/03/21. 2018 Apr 9;4(4):1193–207. Available from: https://pubmed.ncbi.nlm.nih.gov/29682604
54. Rinkevich Y, Walmsley GG, Hu MS, Maan ZN, Newman AM, Drukker M, et al. Identification and isolation of a dermal lineage with intrinsic fibrogenic potential. Science (80-) Internet. 2015 Apr 17;348(6232):aaa2151. Available from: http://science.sciencemag.org/content/348/6232/aaa2151.abstract
55. Mascharak S, DesJardins-Park HE, Davitt MF, Griffin M, Borrelli MR, Moore AL, et al. Preventing Engrailed-1 activation in fibroblasts yields wound regeneration without scarring. Science (80-) Internet. 2021 Apr 23;372(6540):eaba2374. Available from: http://science.sciencemag.org/content/372/6540/eaba2374.abstract
56. Liu F, Lagares D, Choi KM, Stopfer L, Marinković A, Vrbanac V, et al. Mechanosignaling through YAP and TAZ drives fibroblast activation and fibrosis. Am J Physiol Cell Mol Physiol Internet. 2014 Dec 12;308(4):L344–57. Available from: https://doi.org/10.1152/ajplung.00300.2014
57. Meng Z, Moroishi T, Guan K-L. Mechanisms of Hippo pathway regulation. Genes Dev Internet. 2016 Jan 1;30(1):1–17. Available from: https://pubmed.ncbi.nlm.nih.gov/26728553
58. Noguchi S, Saito A, Nagase T. YAP/TAZ Signaling as a Molecular Link between Fibrosis and Cancer. Int J Mol Sci Internet. 2018 Nov 20;19(11):3674. Available from: https://pubmed.ncbi.nlm.nih.gov/30463366
59. Piersma B, Bank RA, Boersema M. Signaling in Fibrosis: TGF-β, WNT, and YAP/TAZ Converge Internet. Vol. 2, Frontiers in Medicine . 2015. p. 59. Available from: https://www.frontiersin.org/article/10.3389/fmed.2015.00059
60. Shi-Wen X, Leask A, Abraham D. Regulation and function of connective tissue growth factor/CCN2 in tissue repair, scarring and fibrosis. Cytokine Growth Factor Rev Internet. 2008;19(2):133–44. Available from: http://www.sciencedirect.com/science/article/pii/S135961010800004X
61. Plikus M V, Guerrero-Juarez CF, Ito M, Li YR, Dedhia PH, Zheng Y, et al. Regeneration of fat cells from myofibroblasts during wound healing. Science. 2017 Feb;355(6326):748–52.
62. Festa E, Fretz J, Berry R, Schmidt B, Rodeheffer M, Horowitz M, et al. Adipocyte lineage cells contribute to the skin stem cell niche to drive hair cycling. Cell. 2011 Sep;146(5):761–71.
63. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998 Nov;282(5391):1145–7.
64. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007 Nov;131(5):861–72.
65. Grove JE, Bruscia E, Krause DS. Plasticity of bone marrow-derived stem cells. Stem Cells. 2004;22(4):487–500.
66. Ding D-C, Shyu W-C, Lin S-Z. Mesenchymal stem cells. Cell Transplant. 2011;20(1):5–14.
67. Lee DE, Ayoub N, Agrawal DK. Mesenchymal stem cells and cutaneous wound healing: novel methods to increase cell delivery and therapeutic efficacy. Stem Cell Res Ther. 2016 Mar;7:37.
68. Huang S-J, Fu R-H, Shyu W-C, Liu S-P, Jong G-P, Chiu Y-W, et al. Adipose-derived stem cells: isolation, characterization, and differentiation potential. Cell Transplant. 2013;22(4):701–9.
69. Duscher D, Atashroo D, Maan ZN, Luan A, Brett EA, Barrera J, et al. Ultrasound-Assisted Liposuction Does Not Compromise the Regenerative Potential of Adipose-Derived Stem Cells. Stem Cells Transl Med. 2016 Feb;5(2):248–57.
70. Hassan WU, Greiser U, Wang W. Role of adipose-derived stem cells in wound healing. Wound repair Regen Off Publ Wound Heal Soc and Eur Tissue Repair Soc. 2014;22(3):313–25.
71. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002 Dec;13(12):4279–95.

72. Dai R, Wang Z, Samanipour R, Koo K, Kim K. Adipose-Derived Stem Cells for Tissue Engineering and Regenerative Medicine Applications. Ferraro GA, editor. Stem Cells Int Internet. 2016;2016:6737345. Available from: https://doi.org/10.1155/2016/6737345

73. Desai VD, Hsia HC, Schwarzbauer JE. Reversible modulation of myofibroblast differentiation in adipose-derived mesenchymal stem cells. PLoS One. 2014;9(1):1–12.

74. Desmoulière A, Geinoz A, Gabbiani F GF. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol Internet. 1993 Jul 1;122(1):103–11. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2119614/

75. Tredget EE, Nedelec B, Scott PG, Ghahary A. Hypertrophic scars, Keloids and Contractures. Surg Clin North Am Internet. 1997;77(3):701–30. Available from: http://www.sciencedirect.com/science/article/pii/S0039610905705764

76. Gabrielli A, Avvedimento E V, Krieg T. Scleroderma. N Engl J Med Internet. 2009 May 7;360(19):1989–2003. Available from: https://doi.org/10.1056/NEJMra0806188

77. Franco OE, Shaw AK, Strand DW, Hayward SW. Cancer associated fibroblasts in cancer pathogenesis. Semin Cell Dev Biol Internet. 2010;21(1):33–9. Available from: https://www.sciencedirect.com/science/article/pii/S1084952109002134

78. El Ayadi A, Jay JW, Prasai A. Current Approaches Targeting the Wound Healing Phases to Attenuate Fibrosis and Scarring. Vol. 21, International Journal of Molecular Sciences . 2020.

79. Stein EA, Dufour R, Gagne C, Gaudet D, East C, Donovan JM, et al. Apolipoprotein B synthesis inhibition with mipomersen in heterozygous familial hypercholesterolemia: results of a randomized, double-blind, placebo-controlled trial to assess efficacy and safety as add-on therapy in patients with coronary artery disease. Circulation. 2012 Nov;126(19):2283–92.