CHAPTER 1

Introduction and outline
GENERAL INTRODUCTION

Lipofilling is the grafting of autologous fat, usually obtained by liposuction from subcutaneous adipose tissue in the abdominal region and legs. Grafted fat (often called lipoaspirate) has proven to be beneficial for restoring scarred skin with subcutaneous volume defects either via augmenting adipose tissue or via stimulation of tissue and wound healing processes. The use of lipofilling to correct soft tissue defects in damaged tissues, e.g. in breast reconstruction after mastectomy and irradiation, led to the observation that injection of lipoaspirate may have far greater influences than just volume restoration, since irradiated, fibrotic skin seemed to soften and scars seemed to disappear after lipofilling. The observations that lipofilling led to improved skin quality subsequently led to extensive use of lipofilling as a regenerative treatment, e.g. to treat wrinkled or scarred skin. Unfortunately, substantial scientific evidence elucidating the mechanisms of lipofilling-mediated repair is missing and the lack of controlled studies in this area needs to be addressed.

In the past decade lipofilling has emerged as a new treatment modality for dermal scarring. Dermal scarring is a fibrotic process of the skin which is the result of healing after injury. In physiological wound healing, the skin restores to a near normal shape, resulting in the formation of a physiological scar. If wound healing is dysregulated, the end result of this healing process will be a pathological scar that deviates significantly more from normal skin, since these scars can have a different texture, color and height than normal skin. Importantly, pathological scars cause clinical symptoms (e.g. pain, itching or movement restriction) more often than physiological scars. Both physiological and pathological scars may be accompanied by soft tissue defects, e.g. when the subcutis is lacking after deep burns or degloving injuries. The obvious mechanism of action of lipofilling is restoration of the soft tissue defects, i.e. restoring the volume of the subcutis with the grafted adipose tissue. However, based on observations in case series and case reports, lipofilling also seems to be able to induce the reversal fibrosis of the skin, to reduce scar-related pain and to improve existing movement restrictions.

Before it is possible to discuss the mechanisms underlying the potential anti-scarring properties of adipose tissue, it is important to understand the different types of scarring and the different ways in which adipose tissue and its components might be used as a therapy. In the course of this introduction, the reader will be introduced to the many entities of scarring and adipose tissue-based therapy, both from a biological as well as from a clinical point of view. We will discuss different scar types, including their clinical and histological presentation. Furthermore, we will discuss adipose tissue-based therapy: therapeutic use of adipose tissue as well as its separate components, such as the stromal vascular fraction (SVF) or adipose derived stromal cells (ASC) and adipose derived stromal cell conditioned medium (ASC CM).

With the use of adipose tissue-based therapy for dermal scars, the fields of regenerative medicine and reconstructive plastic surgery coalesce. On the one hand by definition of the Royal Netherlands Academy of Arts and Sciences ‘regenerative medicine focuses on functional regeneration of damaged tissues and organs by making use of the characteristics of natural tissues and cells’. On the other hand, renowned plastic surgeons Millard and Gillies described their central executional principles for reconstructive plastic surgery. These principles dictate among others that ‘tissue losses should be replaced in kind’, meaning soft tissue defects should ideally be replaced with soft tissue, as is the case when lipofilling is used to reconstruct soft tissue defects that accompany dermal scars. Furthermore, they describe that a surgeon should ‘invoke a Scot’s economy’, meaning that leftover tissue should never be discarded unless it is certain it could not be used. Also, they urge their colleagues to ‘Use a Robin Hood’s tissue appointment, signifying that graft tissue should be harvested there where it is most plentiful.

The use of lipoaspirate, or its components (such as SVF or ASC) to regenerate scarred skin aligns well with the philosophies of regenerative medicine as well as plastic surgery. However, it remains to be firmly elucidated if these adipose tissue-based therapies can truly prevent or reverse dermal fibrosis. In the past, the efficacy of lipofilling has been ‘affirmed’ by unsubstantiated clinical observations and the positive outcomes were attributed to the presence of ‘stem’ cells in the lipoaspirate. A better understanding from a basic scientific viewpoint would not only lead to a greater knowledge of the mechanisms underlying the regenerative capacity of adipose tissue, but is needed to improve clinical treatment and possibly to enable the development of more effective treatment strategies. Therefore the dialogue and collaboration between clinical and biological research fields is of vital and crucial importance to bridge the gap between these disciplines to improve adipose tissue-based therapy for dermal scarring.

Adipose tissue-based therapy

A brief history

In 1893, the first free fat transplantation for soft tissue reconstruction was reported by the German surgeon Gustav Neuber. He transplanted a small piece of adipose tissue to fill out a depressed scar in the face of a young man. In the following decades, while the technique of fat grafting quickly became infamous, harvesting and mincing adipose tissue to obtain small cubes was labor intensive and the resorption rates of transplanted adipose tissue were extremely high. Adipose tissue transplantation became obsolete in subsequent years, until the invention of liposuction in the 1970s revived interest in it. Liposuction allowed the harvesting and reinjection of large volumes of harvested fat and subsequently endless possibilities for application were opened up. A procedure ensuring optimal survival of the fat graft – consisting of manual adipose tissue aspiration under low pressure, brief centrifugation of the collected lipoaspirate and re-injection of the lipoaspirate in small tissue particles by means of 1cc syringes – was developed by the American dermatologist Sydney Coleman. Discovery of the presence of multipotent stem cells in adipose tissue reinforced the idea and belief that lipofilling was more than just the addition of volume to the regenerated tissue. In the following years, a plurality of stories emerged that adipose tissue and specifically ASC had regenerative and rejuvenating properties.
Adipose tissue biology
During embryonic development, adipose tissue is formed from the mesodermal germ layer. Embryonic mesenchymal stem cells (MSC) differentiate into adipocytes during embryonic development when given the appropriate stimuli for adipose tissue formation. However, some of the MSC remain in the form of pre-adipocytes or ASC and remain there during adulthood. Two different types of adipose tissue are found in humans: brown and white adipose tissue. The former is the ‘high energy’ fat that generates body heat and largely disappears after birth\(^\text{10}\). The latter remains throughout life: white adipose tissue is the energy storehouse of the body, which provides thermal insulation, protection and shock absorption and structural support. Adipose tissue also produces many different hormones, cytokines and growth factors that play an important role in endocrine and immune system regulation\(^\text{10}\). Fat is comprised of parenchyme (adipocytes) and stromal tissue, which consists of vessels, connective tissue or extracellular matrix (ECM), fibroblasts and ASC and cells of the immune system (mainly tissue resident macrophages)\(^\text{11}\) (Fig. 1). In terms of volume, the majority of adipose tissue is made up by mature adipocytes. The ASC reside mainly around the vasculature, functioning as vascular support cells\(^\text{12}\). As in all tissues, ECM holds adipose tissue together. In order to isolate SVF and ASC, these cell-ECM connections can be disrupted by enzymatic dissociation, which will be addressed in the paragraph ‘Stromal vascular fraction’.

Lipofilling
Harvested adipose tissue can thus be used as a therapy in the form of lipofilling: injection of harvested and processed adipose tissue into the site of soft tissue defects. How the different steps of harvesting, processing and injection of adipose tissue definitely influence the end results of lipofilling, as will be discussed in the following sections.

Liposuction
Liposuction is the method of choice for obtaining adipose tissue in a form that can be used as an injectable ‘filler’ as during lipofilling. Several liposuction techniques are available for this purpose: for example manual lipospiration (e.g. Coleman method), power-assisted liposuction (PAL), water jet assisted liposuction (WAL) or ultrasound-assisted liposuction (UAL). The main difference between these techniques is the way in which the adipose tissue is fragmented, i.e. mechanical/shear forces (manual aspiration, PAL and WAL) or ultrasound waves (UAL)\(^\text{13,14}\). In all these liposuction techniques, the adipose tissue is pre-treated with an infiltration solution to reduce bleeding by addition of vasoconstrictive agents (e.g. adrenalin), to reduce pain by addition of a local anesthetic (e.g. lidocaine) and to enhance removal of the adipose tissue fragments by aspiration\(^\text{13,17}\). After fragmentation, a mixture of adipose tissue and infiltration fluid is removed by negative pressure suction and the lipoaspirate is then collected in a bag or collector\(^\text{12}\).

Figure 1 | Adipose tissue. (A) Schematic overview of the components of adipose tissue. (B) Masson’s Trichrome staining (Cytoplasm = red; ECM = blue; nuclei = black) of human lipoaspirate, showing that human adipose tissue is mainly made up by adipocytes (honeycomb structures, stained red) with spare amounts of ECM (blue) in between (C) but also contains vessels (endothelial cells and smooth muscle cells stained red) and that there is more ECM present around these vascular structures. Scale bars represent 500µm.

Adipose tissue processing
Lipofilling is the injection of adipose tissue that has been harvested by liposuction into the dermis and/or subcutis of the desired body area. The percentage of the transplanted adipose tissue that survives after injection is dependent upon many factors. Harvesting, processing, injection and storage methods all influence fat graft viability and ASC number. There is no clear evidence which
liposuction harvesting technique is superior with regard to fat graft survival. Which harvesting technique is used, often depends on the personal preference of the surgeon. Controlled studies comparing different liposuction techniques side by side have not been conducted to date. However, use of large bore cannulas and moderation of the amount of negative pressure (above -700mmHg) increases cell viability of the lipoaspirate\(^1\). Fresh lipографs lack functional perfusion and therefore their ongoing viability depends on passive diffusion of nutrients and oxygen. The limit for passive diffusion has been estimated to be approximately 300mm\(^2\): Above that diameter, adipocytes in the center of large (>300mm) cell clusters will die due to hypoxia or necrosis. ASC are more resilient to hypoxia as compared to adipocytes and survive in deeper zones (>300mm from the edge of adipose tissue fragments)\(^1\). Thus, fat tissue particle size influences lipoaspirate survival and resorption after lipofilling\(^1\), since smaller fat tissue particles lead to higher fat graft take. Processing methods for the harvested lipoaspirate include washing, centrifugation, decantation, gauze or towel drying and sieving. No side-by-side comparison of these processing techniques has been performed in a clinical trial. In vitro data and animal studies suggest that, in terms of adipocyte and ASC viability and fat graft survival, the gauze/towel (a technique using the principle of gravity to pass the adipose tissue through a gauze, mesh gauze, towel or fabric) gives the best results compared to decantation, centrifugation, fat processing devices or metal sieves\(^1\). Yet, for clinical application the gauze/towel drying of adipose tissue would be an impractical and time-consuming method for preparing larger quantities of injectable lipoaspirate. Furthermore, systematic review of the literature reveals that the other processing techniques in clinical procedures such as facial, hand, breast and buttock augmentation, also lead to satisfactory results in terms of patient satisfaction\(^11\). After liposuction, the lipoaspirate can be stored at room temperature for several hours, or at 4°C for one day without major effects on adipocyte morphology and ASC vitality. In contrast, freezing adipose tissue without the addition of a cryoprotectant results in a non-viable lipoaspirate\(^13,18\). In addition, fat graft survival is improved when excess liquid volume is removed prior to administration.

**Injection of lipoaspirate into scars**

Lipofilling of scars is technically challenging. Scar tissue is largely acellular, stiff, tough and fibrotic with little space for the injection of lipoaspirate. Hallmark features of scarred skin are excessive ECM, in particular collagen fiber deposition with aberrant organization. In normal skin, the ECM is organized in a basket-weave pattern, whereas in scarred skin the ECM consists of thick, parallel aligned fibrils\(^1\). Therefore, when lipofilling is used to treat scars it is often combined with a percutaneous scar release where needles are used to break up the fibrous ECM to create space for the lipoaspirate\(^2\). The action of scar release and the needling procedure will provoke a significant effect on skin tissue histology and collagen quantity and organization of the scar\(^1\). Thus, this treatment might lead to histological normalization of scar tissue and thus might improve scar-related symptoms. In Chapter 2, we review what happens to the lipoaspirate after injection into scars and how this may alter the scar. The state of the art of current research regarding lipofilling as an anti-scarring agent is reviewed in this chapter.

**Stromal vascular fraction**

In Chapter 4, we discuss the composition of SVF and we systematically review different methods to isolate SVF. In summary, SVF containing ASC, endothelial cells, supra-adventitial cells, lymphocytes, monocytes/macrophages, fibroblasts and pericytes, can be isolated by enzymatic or mechanical dissociation of adipose tissue\(^2\). When SVF is isolated by means of enzymatic dissociation of adipose tissue, a single cell suspension, or cellular SVF (cSVF) is produced. When adipose tissue is mechanically disrupted, intact cell-cell interactions and adipose tissue ECM are retained, resulting in tissue SVF (tSVF). The Stromal Vascular Fraction (both tSVF and cSVF) can be isolated from a patient and then applied as a therapeutic tool within the same surgical procedure; as opposed to ASC, which have to be isolated from the patient and expanded in culture before they can then be therapeutically applied in a subsequent surgical procedure. For this reason, use of SVF may have a clinical advantage. Isolating SVF for direct clinical application is a ‘hot topic’ and since this field is rapidly advancing, no uniform procedure or device has been agreed upon thus far to produce SVF in a standardized fashion.

**Adipose derived stromal cells and their conditioned medium**

In Chapter 2 we characterize the phenotype of ASC and the composition of ASC CM, and we review the current status of literature with regard to the use of ASC as an anti-scarring therapy.

**CLINICAL ASPECTS OF SCARRING**

**Health care costs**

Severe scars which significantly impact quality of life often result from burn wounds, trauma or surgical interventions\(^1\). For the year 2004, it was estimated that approximately 11 million patients worldwide suffered burn injuries that required medical attention\(^1\). In the Netherlands, an average of 531 patients per year were admitted in specialized burn units in the period 1995-2011\(^1\). For these patients, health care costs of acute care within the first three months after burn injury was calculated to be approximately €26,000,- per patient\(^2\). Mean average costs for late reconstructive corrections up to 10 years after injury have been estimated to be €8,000,- per patient\(^2\). The number of patients with symptomatic scars resulting from trauma or surgical interventions is less well documented in the scientific literature. Therefore, the true prevalence and incidence of the costs of treatment of symptomatic scars are most likely underestimated in the numbers mentioned above.

**Scar types**

Scars are divided into different types: normotrophic, hypotrophic, hypertrophic and keloid scars\(^1\) (Fig. 2). The clinical differences between these scar types are listed in Table 1. The
differences in macroscopic appearance of the scar types is the consequence of microscopic differences in morphology, which will be discussed more extensively in the paragraph ‘wound healing and scar formation’. In general, all scar types differ from the surrounding normal skin in color (darker or lighter) and pliability (stiffer, but lower tensile strength). They may also differ in height: Hypertrophic and keloid scars are raised above the level of the surrounding skin, whereas hypotrophic scars accompanied by soft tissue defects present as a depression. Normotrophic scars are considered to be the best possible outcome after dermal wound healing in the adult, because pathological scar types such as hypertrophic and keloid scars, cause serious clinical symptoms more often than normotrophic scars.

Symptoms of scars

Patients with symptomatic scars can develop diverse problems. First of all, aesthetic problems results from the differences in appearance of a scar as compared to the normal skin, e.g. in color, relief or thickness. These differences can give rise to aesthetical dissatisfaction and abnormal tactile sensations of the scar. Furthermore, scars may be painful or itch, or when located in the proximity of a joint, may lead to movement restriction. Psychologically, the scar severity that is perceived by patients is closely linked to the amount psychosocial distress that experienced:

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**Figure 2 | Clinical presentation of the four different scar types: normotrophic, hypotrophic, hypertrophic and keloid scars. *Picture derived from publication by Ogawa.**

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Table 1 | Clinical differences between scar types

<table>
<thead>
<tr>
<th></th>
<th>Normotrophic scar</th>
<th>Hypotrophic scar</th>
<th>Hypertrophic scar</th>
<th>Keloid scar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consequence of trauma/injury?</td>
<td>Always</td>
<td>Always</td>
<td>Always</td>
<td>Not necessarily</td>
</tr>
<tr>
<td>Difference with normal skin level?</td>
<td>No</td>
<td>Below skin level</td>
<td>Above skin level</td>
<td>Above skin level</td>
</tr>
<tr>
<td>Scar can extend beyond borders of original wound?</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Accompanied by soft tissue defect?</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Can be accompanied by scar contractures?</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

* Table modified from publication by Verhaegen et al.

As a result of these scar-related symptoms, patients with scars may experience a significantly lower quality of life. The combination of physical and psychological factors related to the scars ultimately determines the burden of disease for the patient.

Conventional scar treatments

Treatment options for dermal scars either focus on prevention of scars during wound healing and early scar formation, or on reduction of existing scars in the remodeling phase or in mature scars. Accepted evidence based treatments to prevent scar formation are tension relief, silicone sheets or gels, pressure or compression garments and corticosteroid injections. For reduction of scar severity, both non-invasive and invasive treatments can be considered. Non-invasive approaches for managing existing scars include same therapies to those used for scar prevention, i.e. silicone and pressure therapy. Invasive treatments used for existing scars are injections with corticosteroids or other drugs, laser therapy, cryotherapy, or surgical scar revision. Operative removal of scars involves the direct excision of the scar tissue followed by primary closure. However, when primary closure is not possible, skin grafts or tissue flaps are additionally introduced. After surgical excision, optimal scar preventative measures should be taken to prevent recurrence of pathological scar formation. With the exception of surgical excision followed by a tissue flap, none of the mentioned treatments are able to restore a soft tissue defect that can accompany scars.

Scar evaluation tools

To evaluate the efficacy of novel and existing scar treatments, several measurement instruments have been developed and evaluated in clinical studies. Roughly, these instruments can be divided into two groups. The first option is the quantification of scar severity on validated scar scales or questionnaires in which patients and/or medical professionals score different parameters of scarring, e.g. color difference, height difference or pliability as compared to normal, non-scarred skin. The more severe the patient thinks his or her scar to be, the stronger the psychosocial distress. As a result of these scar-related symptoms, patients with scars may experience a significantly lower quality of life. The combination of physical and psychological factors related to the scars ultimately determines the burden of disease for the patient.
total score on these scales indicates scar severity. The second option is the use of tools or devices that measure defined parameters, e.g. color, stiffness or scar surface area or volume. Changes in these parameters can then be used to estimate changes in scars over time or after treatment.

**BILOGICAL ASPECTS OF SCARRING**

**The normal skin**

The skin is the largest organ of the human body. It functions as a barrier that protects the body from the environment. The skin helps to regulate body temperature and protects against dehydration. Histologically, the skin consists of two layers: the epidermis and the dermis (Fig. 3). The epidermis is separated from the dermal layer by a basal membrane. The epidermis is composed of layers of cells, mainly made up by keratinocytes. The keratinocytes closest to the basal membrane divide and renew the epidermis from below. Tight connections between the keratinocytes and their environment (e.g. desmosomes, hemi-desmosomes and integrins) help maintain the barrier function of the skin. The dermis consists mainly of extracellular matrix (ECM). Within this ECM, fibroblasts and blood vessels are the main cellular components of the dermis. ECM of the normal skin is mainly made up of collagens, elastins, laminins, fibronectins and proteoglycans. These components form a meshwork that supports adhesion and survival of the resident cells and allows binding of growth factors and water molecules. Together, these properties give the skin its strength and elasticity. Below the normal skin, there is the hypodermis, or the subcutaneous adipose tissue. Even though the hypodermis is not part of the skin, it plays an important role by providing padding for the underlying muscle and bone. When this layer is lacking – as is the case in hypotrophic scars – this causes an abnormal appearance and feeling of the overlying skin. Also, the skin appendages play an important role in the function of the skin. Hair follicles and sweat glands help regulate body temperature. Furthermore, nerves in the skin supply sensory information about the outside environment. In scars, skin appendages and innervation are often lacking, thus leading to abnormal function of the scarred skin.

**Wound healing and scar formation**

After damage to any organ in the human body, the wound healing cascade is initiated. Normal adult wound healing consists of three overlapping stages: inflammation, tissue generation and remodeling (Fig. 4).

**Inflammation**

During the inflammatory phase, blood loss is prevented by hemostasis, which is achieved by platelet aggregation and the formation of a fibrin clot. The provisional fibrin scaffold generates an environment, which supports immune cell invasion: in the early inflammatory stage, neutrophils and mast cells (within hours) followed by monocytes (within days) are recruited into the wound. Neutrophils are needed to neutralize invading microbes by means of phagocytosis or by a respiratory burst resulting in release of free radicals. Mast cells secrete cytokines that cause endothelial cell changes, leading increase vascular permeability to allow immune cell extravasation, help degrade the provisional fibrin scaffold to allow migration of fibroblasts into the wound environment, induce proliferation of and ECM production by these fibroblasts and stimulate angiogenesis. Monocytes differentiate into macrophages, acquiring the capability to phagocytose cell and tissue debris. Furthermore, the classically (M1) and alternatively (M2) activated macrophages secrete mediators that are important for immune activation and tissue regeneration.

**Tissue generation**

Growth factors secreted by the platelets, e.g. Transforming Growth Factor beta (TGF-β) during hemostasis activate resident tissue cells to begin proliferating and producing ECM proteins.
and immune cells, the regenerated tissue returns to a state of quiescence. Many activated resident tissue cells, e.g. myofibroblasts, macrophages and endothelial cells, migrate away from the former wound site or undergo apoptosis. Simultaneously, the composition of the ECM is normalized to reflect the usual tissue ECM composition at the site, among others by degradation of excessive collagens by matrix metalloproteinases (MMP), replacement of collagen type III by collagen type I and realignment and crosslinking of collagen fibers. The remodeling phase can take up to a year before the process is completed. In the ideal situation in dermal wound healing, the epidermal and dermal layers are restored to near normal, resulting in macroscopic and microscopic similarity to the normal skin. Yet, wound healing always leads to a certain degree of scar formation. Visually, as well as functionally, scarred skin will never be repaired to be identical to normal skin. For example, dermal scar tissue has lower tensile strength than normal skin, and also lacks nociception, sweat glands and hair follicles.

The myofibroblast and scar formation

Keratinocytes start to proliferate and migrate in order to restore the integrity of the epidermis. Meanwhile, fibroblasts migrate into the provisional fibrin matrix and proliferate. In the wound environment, these fibroblasts are activated by certain cytokines (mainly TGF-β1) and differentiate into myofibroblasts, producing even more ECM than a normal fibroblast. During wound healing, the myofibroblasts produce primarily collagen (most abundantly type III), elastin, proteoglycans and hyaluronic acid. Also, myofibroblasts acquire a contractile cytoskeleton, indicated by expression of alpha smooth muscle actin (αSMA), and thus a contractile phenotype. Hence, myofibroblasts are key players in the contraction that reduces the wound surface area and in the replacement of the provisional thrombin matrix by more permanent ECM components, e.g. collagens and elastins. Meanwhile, the newly deposited ECM is vascularized by endothelial cells driving the process of angiogenesis. This tissue generation phase usually lasts for about two to three weeks.

Remodeling

After completion of the tissue generation phase, the remodeling phase commences. In this phase, the regenerated tissue returns to a state of quiescence. Many activated resident tissue cells and immune cells, e.g. myofibroblasts, macrophages and endothelial cells, migrate away from the site and undergo apoptosis or are stimulated by mechanical tension, certain cell-matrix interactions and/or pro-fibrotic growth factors or cytokines, e.g. TGF-β. Myofibroblasts are essential during wound repair. Normally, this cell undergoes apoptosis after completion of regeneration and remodeling of the wound. However, when the myofibroblasts persist after wound healing is completed, their excessive proliferation, ECM production and contraction results in fibrosis. Importantly, myofibroblast differentiation and activation is dependent on pro-fibrotic signal transduction pathways. Thus, myofibroblast differentiation and activation are possible targets for therapeutic intervention as a means to modulate, inhibit or perhaps reverse scarring and fibrosis.

Regulatory mechanisms of scarring and fibrosis

In this section, we discuss regulatory mechanisms of scarring and fibrosis that are relevant to this thesis: TGF-β signaling and microRNAs.

TGF-β signaling

Without a doubt, TGF-β signaling is the most studied pro-fibrotic signal transduction pathway: TGF-β binds to the canonical TGF-β type 2 receptor (TGFBR2), after which the TGF-β type 1 receptor (TGFBR1, also known as Activin-like kinase 5 or ALK5) is recruited to form a complex on the cell surface. This ligand-activated complex phosphorylates (activates) Smad2/3, that is the transcription factor that, upon intranuclear localization, activates mesenchymal genes that relate to wound healing (Fig. 5A). In addition, chronic TGF-β stimulation of fibroblasts initiates a pro-fibrotic response via activation of canonical (Smad dependent) or non-canonical (e.g. extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase/p38 (JNK/p38), phosphoinositide 3-kinase/Akt (PI3K/Akt) or small GTPase dependent) TGF-β-induced signal transduction pathways.

Figure 4 | Schematic overview of the three stages of the wound healing process. Minutes after wounding, a fibrin clot is formed in which platelets become trapped. In the following minutes-hours, the inflammation phase commences as neutrophils and macrophages invade the wound and produce cytokines. These cytokines activate the resident tissue cells surrounding the wound: days-weeks after wounding, the tissue generation phase takes place when angiogenesis, keratinocyte proliferation and migration and fibroblast proliferation, activation and ECM production take place. When the wound area is closed, the remodeling process starts. The haphazardly deposited collagen produced by (myo)fibroblasts during tissue generation is remodeled, which – in the best case scenario – results in a near normal appearance of the skin, or a normotrophic scar. Remodeling can take up to a year to be completed.

Legend

- Fibrin clot
- Blood vessel
- Platelet
- Neutrophil
- Keratinocyte
- Myofibroblast
- Collagen

Visually, as well as functionally, scarred skin will never be repaired to be identical to normal skin. Yet, wound healing always leads to a certain degree of scar formation.
the context of this thesis, we will also investigate the role of non-canonical TGF-β signaling via small GTPases in fibroblast activation and fibrosis. When TGF-β binds to the TGFBR1 and 2, the GTPase intermediates Growth Factor Receptor-Bound 2 (Grb2) and son-of-sevenless homologues (SOS) 1 and 2 are recruited to the receptor tyrosine kinase and activate downstream GTPases by catalyzing the GDP for GTP exchange\textsuperscript{46}. Moreover, Grb2 can activate p38 MAPK via an unidentified mechanism. Ultimately, TGF-β induced activation of small GTPases results in fibroblast activation and ECM production\textsuperscript{46} (Fig. 5B). Previously, it has been shown that inhibition of signaling via the small GTPase Ras blocks fibrosis in vivo\textsuperscript{45,46}.

**microRNAs**

miRNAs (miR) are small, non-coding RNA that regulate gene expression at a post-transcriptional level, since they interact with complementary mRNA by base pairing. miR binding sites are usually located in the 3‘ untranslated region (3’UTR) of mRNA. When a miR-mRNA complex is formed, this leads to translational repression of the mRNA, e.g. by degradation of the mRNA or by blockage of initiation or elongation of protein translation (extensively reviewed by Filipowicz et al.\textsuperscript{20}). During scarring and fibrosis, miR expression patterns are altered and can induce or maintain the pathological process\textsuperscript{41}.

**Adipose tissue-based anti-scarring therapy: which, when and where?**

Scar formation is the result of the staged process of wound healing. Wound healing always results in a scar, but if the wound healing cascade progresses properly, this results in normotrophic scar formation, which is often non-symptomatic. But, if the wound healing process is dysregulated, pathological and symptomatic scars can be the end result. Thus, to limit pathological scar formation one could argue that optimization of repair in all stages of the wound healing process – i.e. inflammation, new tissue formation and remodeling – would help to limit scar formation. In the inflammatory phase, excessive inflammation resulting in tissue damage and excessive TGF-β availability should be prevented and the immune cell phenotype should be steered towards a pro-regenerative phenotype. In the tissue formation phase, fibroblasts and keratinocytes should be activated just enough to close the wound and replace the provisional fibrin matrix, but no more collagen than necessary should be deposited to prevent fibrosis at a later time point. During remodeling, the amount of ECM degradation should equal or exceed the amount of ECM that was deposited in the previous stages. When the process of wound healing has been completed and a scar has been formed and matured, this scar may give rise to symptoms and complaints. Every scar can cause symptoms, but this is more common in pathological scars than in physiological, normotrophic scars. These complaints can result from or be accompanied by a soft tissue defect and adherence of fibrotic scar tissue to the underlying structures, e.g. muscle fascia. An ideal therapy would be able to restore lost volume, to reduce scar related pain and to normalize the macroscopic scar appearance towards appearance.

**Figure 5. Schematic overview of (A) Canonical TGF-β signaling and (B) non-canonical TGF-β signaling via Ras**

**OUTLINE OF THIS THESIS**

Adipose tissue has been used since the nineteenth century to treat soft tissue defects that accompany scars\textsuperscript{6}. However, until now there has only been anecdotal evidence that adipose tissue indeed can decrease the visibility and symptoms of dermal scars. From a biological point of view, these observations were corroborated by the finding of Zuk and colleagues, who reported that adipose tissue is a source of pro-regenerative cells: adipose stromal cells\textsuperscript{51}. The objective of this thesis is to investigate the anti-scarring and possibly anti-fibrotic effects of adipose tissue and the role of the individual cellular fractions, i.e. SVF, ASC or ASC CM. We evaluate which adipose tissue component should be used during which stage of wound healing to achieve the optimal anti-scarring effects in vitro and in vivo. We investigate the molecular mechanisms underlying the anti-scarring effects of adipose tissue-based therapy. These studies aim to contribute to improving adipose tissue-based therapy, bridging the gap between the science and the clinic by dissecting the mechanisms driving the anti-scarring effects.

To evaluate the current level of evidence for lipofilling as a treatment for dermal scars, we review the literature relating to the use of lipofilling and ASC to treat existing dermal scars (Chapter 2). In our own therapeutic, non-randomized clinical study, we investigate the effect of two consecutive sessions of autologous lipografting in patients with symptomatic scars. Clinical efficacy of
lipofilling is evaluated using the patient and observer scar assessment scale (POSAS). Mechanical underpinning for the scar remodeling properties of lipofilling is investigated in scar tissue biopsies before and after lipofilling treatment (Chapter 3).

One of the adipose tissue components of interest in this thesis is the SVF. In a systematic review, we investigate all available literature about the procedures and devices suitable for intra-operative isolation of clinical grade SVF. Devices and procedures are evaluated for cell yield and viability, SVF composition, duration and costs (Chapter 4). To evaluate the use of SVF for scar prevention, we develop a design for and establish a randomized controlled clinical trial of breast reduction surgery. Patient inclusion for this study has commenced at five locations in the Netherlands (Chapter 5).

Another adipose tissue component of interest are ASC. To obtain a sufficient amount of these cells (in animal models, e.g. for wound healing and scar formation, often >1x10^6 ASC are used for cell therapy), in vitro culture expansion is often implemented. When ASC are cultured with xenogeneic sera and reintroduced into a patient, this carries the risk of contamination of the patient with xenogeneic infectious agents and of immune reactions against the sera. This risk would be avoided if human serum products, such as platelet-rich plasma (PRP) or platelet poor plasma (PPP), were used. Furthermore, in clinical applications, lipofilling or ASC skin rejuvenation therapies are sometimes combined with platelet-rich plasma (PRP), which is believed to boost the rejuvenating effects of these treatments. Therefore, we investigate if human ASC can be cultured with PPP and PRP. Furthermore, dose-dependent effects of PRP on ASC proliferation, phenotype, trophic factor production and pro-angiogenic capacity, are evaluated (Chapter 6).

Often, the anti-scarring effects of adipose tissue are attributed to ASC. In a series of in vitro experiments, we investigate if trophic factors from ASC, in the form of ASC CM, can inhibit TGF-β-mediated normal dermal and keloid fibroblast activation. Proliferation, cytoskeletal components and contractility and collagen I and III and MMP-1, -2 and -14 production of dermal fibroblasts treated with ASC CM are investigated (Chapter 7).

Part of the effects of ASC CM is mediated by microRNA in microvesicles (MV). MV are small cell membrane derived vesicles that play an important role in intercellular communication, since they contain proteins, mRNA, long noncoding RNA and miR. It has been shown that ASC produce MV containing miR that induce dermal fibroblast proliferation and speed up dermal wound healing. In the final experimental chapter, we examine an alternative approach for preventing organ fibrosis: In silico analysis identified miR-15b as a possible modifier of non-canonical TGF-β signaling via small GTPases, since miR-15b putatively targets its upstream intermediates Grb2, SOS 1 and SOS 2. Therefore, these data led us to investigate the role of microRNA-15b during cardiac fibrosis and the inhibitory effect of this microRNA on non-canonical TGF-β signaling in cardiac fibroblast activation (Chapter 8).

In conclusion, the results of the previous chapters are summarized and their future implications discussed (Chapter 9). This thesis concisely illustrates where we stand currently with regards to the application of adipose tissue-based therapy and where are we heading in the future: clearly addressing the question can we bridge the gap?
REFERENCES


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