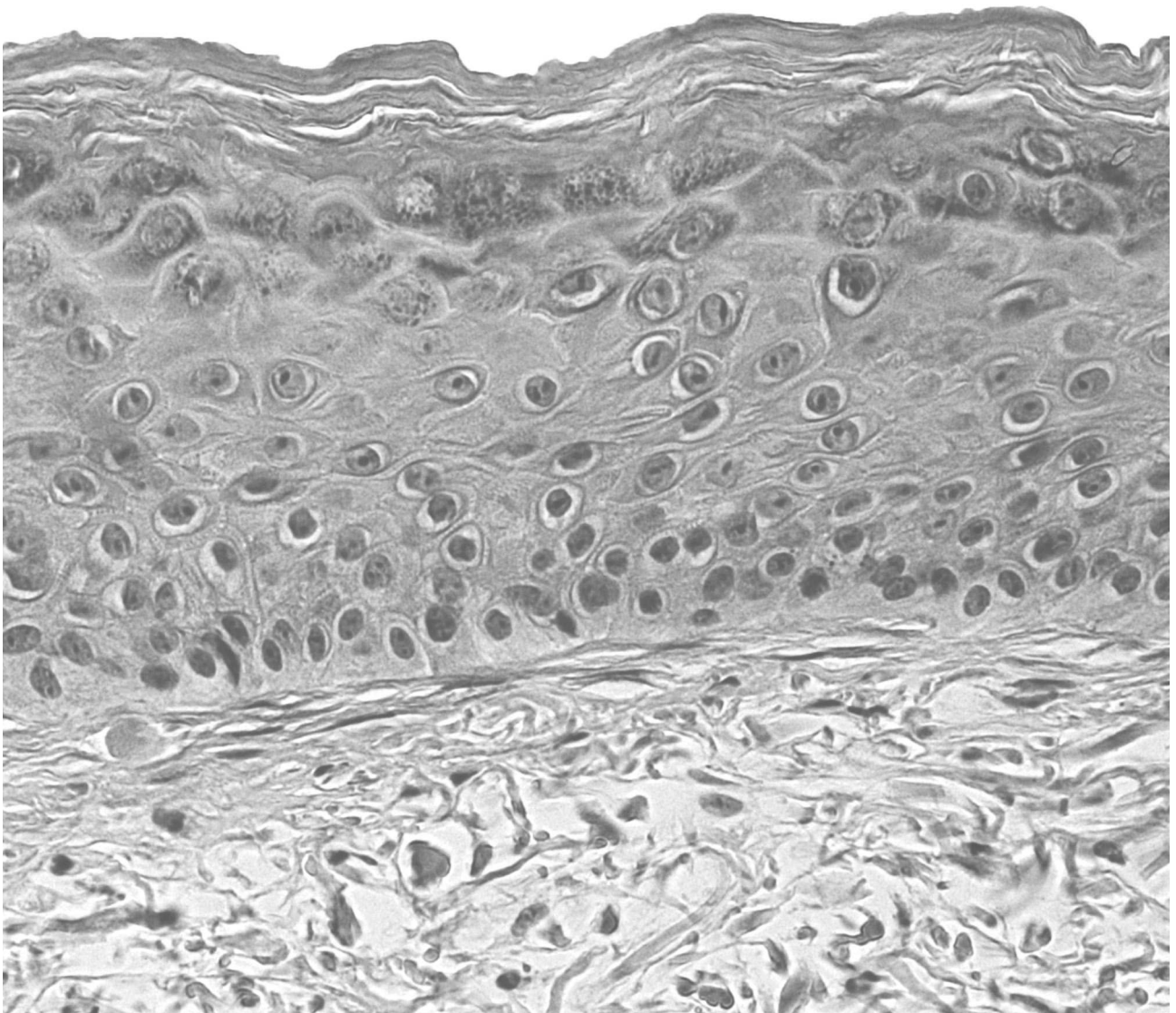


Chapter 1

General introduction



This thesis focuses on the development of an *in vivo* like hypertrophic scar model. In this first chapter human skin, scar formation and the existing scar models are introduced. Our own findings are described in the following chapters.

Tissue engineering

Tissue engineering is an emerging multi-disciplinary field that aims to regenerate, replace or restore diseased or damaged tissues with cellular and / or biological and synthetic materials¹. Over the last years there have been huge advancements in reconstructing tissues and organs *in vitro* (e.g. skin, heart valves, bladder, cartilage)²⁻⁸. The skin is the most advanced tissue-engineered construct, since organotypic reconstructed skin consisting of both epidermal and dermal layers already exists. Although achieved skin constructs may even contain immune cells e.g. Langerhans Cells in the epidermis and endothelial cells as well as fibroblasts in the dermis⁹⁻¹³, they do not yet contain more complex structures for example sweat glands and hair follicles^{1,4}. Tissue-engineered skin has *in vivo* and *in vitro* applications. In the clinic it is already used for many years as a replacement for lost skin (e.g. burns, ulcer)^{4,14}. As an *in vitro* model reconstructed skin is not only used to study fundamental skin biology processes such as mechanisms involved in wound closure, but it is now also widely implemented in Europe and America as an animal alternative *in vitro* model to identify irritative and corrosive chemicals that can come into contact with the skin^{4,15,16}. During the last 10 years, due to increasing pressure from the EU ("Cosmetic Directive") who demanded the replacement, reduction, and refinement of the use of animals models in the cosmetic industry, many new tissue-engineered skin based assays have been developed (e.g. models to distinguish sensitizers and to determine sensitizer potency)^{17,18}. *In vitro* skin models can also be modified to mimic skin diseases (e.g. psoriasis, melanoma)¹⁹⁻²¹. These can be used to study the pathogenesis, identify novel drug targets and test new therapeutic strategies. In this thesis we describe the development of a human tissue-engineered skin model to investigate hypertrophic scar formation, which may be used to study the pathogenesis, to test novel drugs and therapeutic strategies. Furthermore such a human physiologically relevant scar model will diminish the use of animals models.

Skin

The skin is the largest organ of the human body and it is constantly exposed to external influences. It is not only a barrier that protects the body against pathogens (e.g. microorganisms, viruses) and UV radiation, but also has an important function in temperature control, water loss and sensation. Depending on the location, the total skin thickness varies from 0.5 mm (eyelids) to 5 mm (the soles of your feet). The skin can be divided into three layers, the epidermis and two layers of connective tissue, the dermis and the

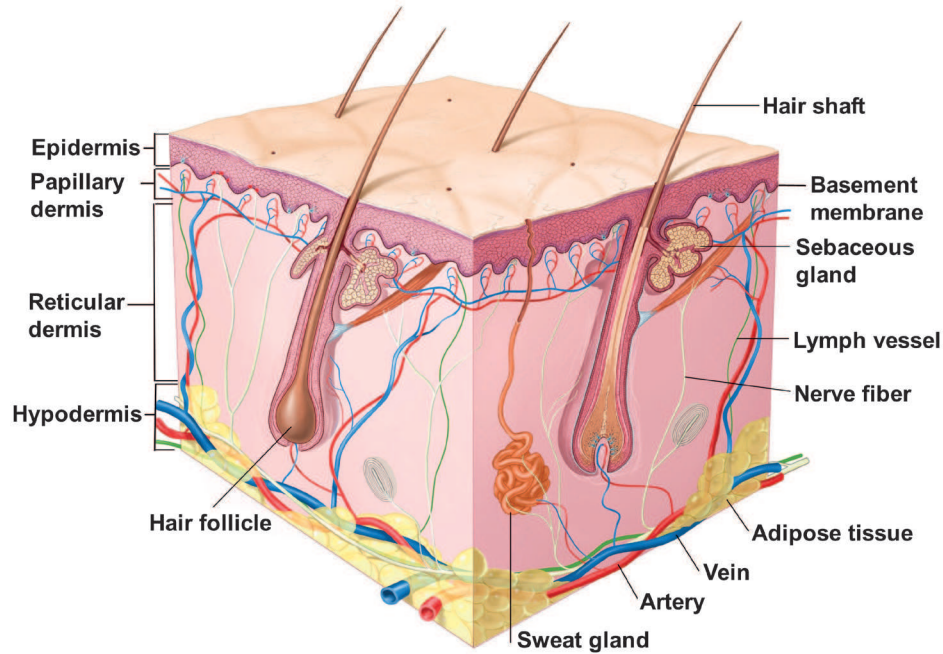


Figure 1. Schematic structure of human skin. (Adapted from <http://www.uchospitals.edu/online-library/content=CDR258035>)

hypodermis (or called adipose tissue). The epidermis is separated from the dermis by the basement membrane (Figure 1).

The epidermis is the outermost layer of the human skin and protects the body from the environment. It is a multi-layered cellular structure composed mainly of keratinocytes (up to 95%). Keratinocytes are keratin producing cells which are distributed in layers of increasing differentiation (stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum) (Figure 2). Dividing keratinocytes are found directly on top of the basement membrane in the stratum basale. Merkel cells (sensory cell) and melanocytes are located between the dividing keratinocytes. Melanocytes produce melanin, a pigment that is responsible for the skin colour and protects skin cells from UV radiation. Keratinocytes differentiate, move up to the more outer layers and lose their nucleus (stratum lucidum) during this process. Finally differentiated keratinocytes are hardened, flattened dead cells (stratum corneum) that overlap and create a tough, waterproof protective layer. These cornified keratinocytes are constantly shed from the outer surface of the skin. In the suprabasal layers of the epidermis (stratum spinosum and granulosum) the immune competent Langerhans cells are present.

The dermis lying beneath the epidermis consists mainly of extracellular matrix with cells and vessels distributed throughout it. The extracellular matrix consists predomi-

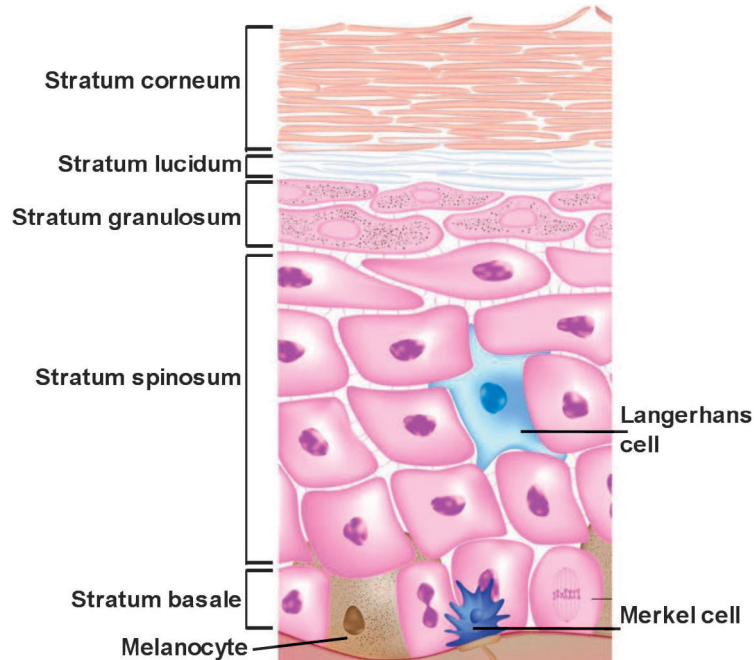


Figure 2. Schematic structure of epidermis. (Adapted from <http://silverbotanicals.com/assets/images/illustrations/structure-of-the-epidermis.jp>)

namely of collagen (70%), elastin (4%), glycosaminoglycans and proteoglycans, which give the dermis strength and elasticity. The dermis can be divided into the papillary dermis (or upper dermis) and the reticular dermis (deeper dermis). The papillary dermis (thickness 300–400 μm) consists of a network of relatively thin fibers of connective tissue, is more crowded with cells (e.g. endothelial cells, fibroblasts) than the reticular dermis and the main function is supporting the epidermis²². The reticular dermis consists of a more dense and thick connective tissue. The main cells that populate the dermis are the connective tissue producing mesenchymal cells, called fibroblasts. It also contains nerves, sweat glands, sebaceous glands, hair follicles, lymphatic vessels, microvascular vessels (composed of endothelial cells) and immune cells (e.g. macrophages, dermal dendritic cells, lymphocytes).

The hypodermis lies between the dermis and underlying tissues (e.g. bone, muscle) and organs. It serves to fasten the skin to the underlying tissues, provides thermal insulation, energy reserve and absorbs shocks from impacts to the skin. It consists of adipose tissue, hair follicles (roots), nerves, blood vessels and lymphatic vessels. Important cell types are the adipocytes (fat cells) and adipose tissue-derived mesenchymal cells.

Human cutaneous wound healing

Cutaneous wound healing is an interactive process characterized by a sequence of events that begin directly after injury. It can be divided into four phases; hemostasis, inflammation, proliferation and remodeling (respectively Figure 3a, b, c, d). It should

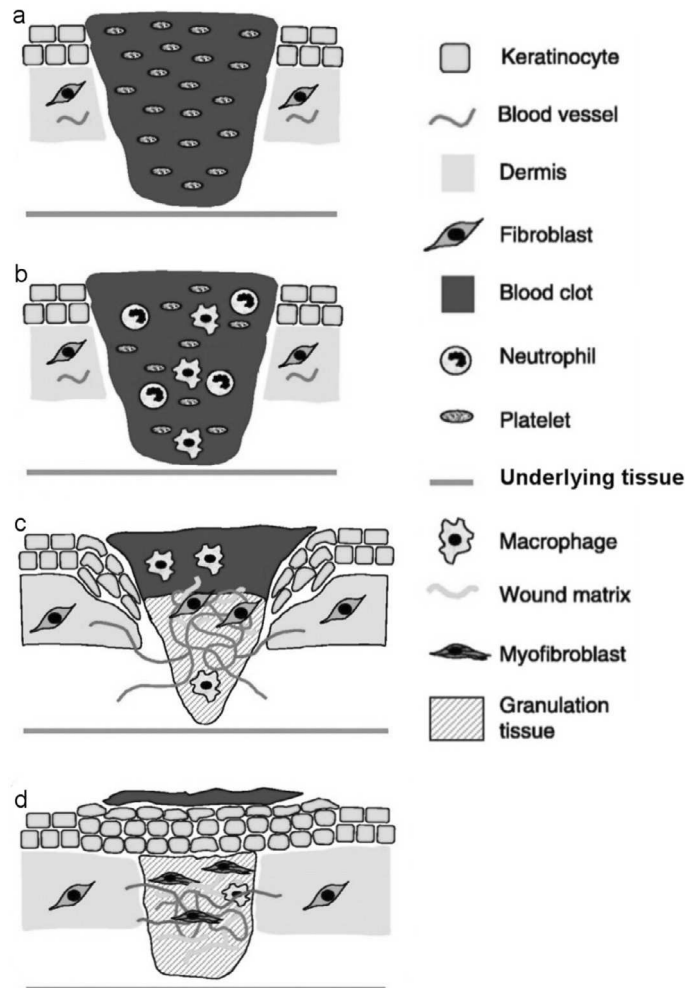


Figure 3. Schematic overview of human cutaneous wound healing. (a) A blood clot is formed. Platelets release inflammatory mediators which attract immune cells into the wound bed (Hemostasis phase). (b) Neutrophils and macrophages migrate into the wound bed to kill bacteria and to secrete more mediators (Inflammation phase). (c) Fibroblasts and endothelial cells are attracted to the wound site, proliferate and deposit extracellular matrix and form new blood vessels, respectively. Keratinocytes start to migrate and proliferate to re-epithelialize the wound (proliferation phase) (d) Fibroblasts differentiate into myofibroblasts, leading to wound contraction and increased extra cellular matrix deposition. The wound is completely closed by a new epidermis. In order to further repair and strengthen the dermis, the newly formed collagen is reorganized and cross-linked (proliferation and remodelling phase). (Adapted from Werner et al¹¹⁴)

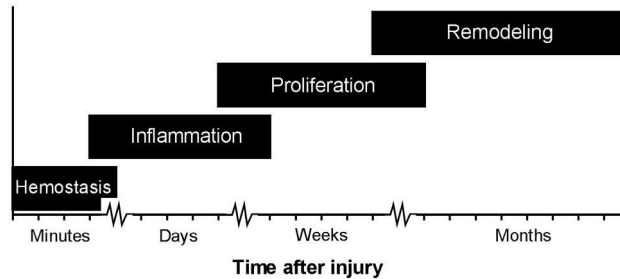


Figure 4. Overlapping phases of human cutaneous wound healing.

be noted that these phases overlap in time (Figure 4) and are characterized by multiple interactions.

The first phase hemostasis starts directly upon injury when bleeding occurs as a result of the disruption of blood vessels. To prevent blood loss the vessels constrict within seconds. Platelets are activated and undergo adhesion, aggregation and at the same time release soluble wound healing mediators. These mediators induce further platelet aggregation and activation of the coagulation pathway. Prothrombin is converted into thrombin and this in turn converts soluble fibrinogen to insoluble fibrin, which leads to a fibrin clot^{23,24}. The fibrin clot further contains fibrinectin, vitronectin and thrombospondin. Besides forming a temporary cover for the wound, the clot also serves as a network for cells migrating into the wound bed and as a reservoir of growth factors and cytokines which are required during the later stages of the wound healing process. Platelets influence wound repair by releasing chemotactic factors for infiltration of leukocytes and factors strongly implicated in wound repair (e.g. TGF- β 1, PDGF and VEGF)²³⁻²⁵.

Inflammation, the second phase, is crucial to neutralize infections. As mentioned earlier, cytokines and growth factors released by platelets and injured skin resident cells attract leukocytes into the wound bed. Neutrophils are the first immune cells that arrive at the wound bed in high numbers. Once in the wound bed, neutrophils produce a wide variety of proteinases and reactive oxygen species to kill bacteria and clear damaged matrix proteins^{23,26}. Neutrophil infiltration normally lasts for only a few days. At a later stage during inflammation (day 3-5), when the number of neutrophils declines, macrophages (blood-derived monocytes) are the predominate cell type. Monocytes infiltrate the wound bed in response to chemoattractants (e.g. CCL2 and IL-1) released by platelets and endothelial cells and differentiate into macrophages^{27,28}. Macrophages can become activated in response to signals present in the wound bed or to pathogens. Macrophages have been described to display two different functional phenotypes which can broadly be divided into M1 (classically activated) and M2 (alternatively activated) macrophages. However this is an oversimplification since *in vivo* macrophages have dynamic and plastic phenotypes (continuum between the M1 and M2 extremes)

that change with the local environment^{28,29}. During inflammation, macrophages are described to have a more M1 phenotype²⁸. They remove senescent cells and debris in the wound bed (innate immune system), present antigens of pathogens to T-lymphocytes (adapted immune system) and produce large amounts of cytokines and growth factors to further amplify the inflammatory response^{23,28,29}. M2 macrophages are more associated with tissue repair and fibrosis.

During the third phase (proliferation) damaged and/or lost tissue is regenerated. In contrast to the inflammation phase, where M1 macrophages predominate, during the proliferation phase M2 macrophages predominate. They suppress inflammatory responses by secreting factors like IL-10 and TGF- β 1 and promote angiogenesis, tissue remodeling and repair^{28,29}. Fibroblasts in surrounding intact dermis begin to proliferate, migrate into the fibrin clot (provisional matrix) and start to deposit their own extracellular matrix (called granulation tissue, which mainly consists of collagen III, collagen I, fibronectin, glycosaminoglycans and proteoglycans)³⁰. Under influence of TGF- β and mechanical stiffness in the wound bed a subgroup of the fibroblasts differentiate into myofibroblasts, which express α -smooth muscle actin in their stress fibres³¹. Myofibroblasts induces wound contraction (contributing to wound closure) and also deposit extracellular matrix^{23,32}. At the same time, wound microvasculature is reconstructed in order to re-establish the nutrient and oxygen supply to regenerated tissue. Endothelial cells sprout from pre-existing vessels into the wound matrix. This sprouting is stimulated by e.g. VEGF, FGF2 and TGF- β 1, which are mainly produced by keratinocytes, macrophages, platelets and fibroblasts, to form new vessels³⁰. The formation of granulation tissue into an open wound allows keratinocytes (coming from the basal layer of the wound edge and/or epidermal progenitors cells from hair follicles) to proliferate and migrate across the new tissue and form a new barrier between the wound and the environment resulting in re-epithelialization. This is mainly stimulated by factors (e.g. EGF, KGF TGF- α) secreted by macrophages, platelets and fibroblasts^{23,33}.

During remodeling, the last phase of wound healing, cell proliferation decreases and the levels of collagen production and degradation equalize. As a result the nutrient and oxygen demand decreases and unnecessary microvessels formed in granulation tissue regress³⁰. Cells that are no longer needed (e.g. myofibroblasts and macrophages) undergo apoptosis or leave the wound region^{34,35}. Collagen III, which is prevalent in granulation tissue, is replaced by the stronger collagen I, which results in increased strength of the extracellular matrix. The extracellular matrix is further strengthened by rearranging, cross-linking and aligning originally disorganized collagen fibers^{23,36}. Matrix metalloproteinases (MMPs) and their natural inhibitors (TIMPs) are described to be important regulators of proteolytic activity during this process and are mainly produced by macrophages, fibroblasts and endothelial cells³⁷. At the end the remodeling phase, restoration of skin integrity has been achieved. However, the tensile strength of the

healed wound is only approximately 80% that of normal unwounded tissue. This tissue is called a normotrophic scar.

Abnormal scar formation

Wound healing of full thickness wounds generally leads to the formation of a normotrophic scar (Figure 5a). The normotrophic scar is hardly visible since it is smooth, pale and flattened. This is considered to be the best clinical endpoint. However abnormal wound healing can lead to the development of an abnormal raised scar (hypertrophic scar or keloid scar (Figure 5b&c)). Both abnormal scars can cause significant physiological (limited joint mobility in particular with hypertrophic scars) and psychological (especially the face) problems leading to diminished quality of life^{38,39}. Unfortunately both scars are therapy-resistant with high recurrence rates after excision⁴⁰⁻⁴². Below, these two types of scar will be described followed by the pathogenesis of scar formation.

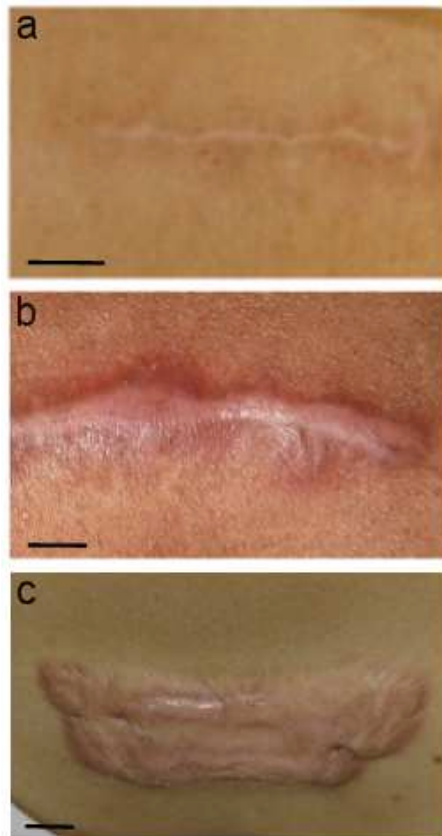


Figure 5. Macroscopic photographs of different scar tissues. (a) Normotrophic scar developed after incision wound (sternum) (b) Hypertrophic scar developed after incision wound (sternum) (c) Keloid scar formed from pustule (sternum). Bars = 1 cm

Keloid scar versus Hypertrophic scar

The differentiation between young hypertrophic and keloid scars remains clinically difficult. Both scars are red, firm and raised lesions, that arise from an overproduction of extracellular matrix⁴³. However there are clear clinical, morphological and epidemiological differences described between hypertrophic and keloid scars. Morphologically, collagen fibers are flatter and thinner and more microvessels are present in hypertrophic compared to keloid scars^{39,44,45}. A clinical difference between the two scars is that hypertrophic scars generally remain confined to the borders of the original lesion, whereas keloid scars show an invasive growth in the surrounding normal skin⁴⁶. Hypertrophic scars are more often formed on locations with high tension (e.g. shoulders, knees, presternum)⁴⁷⁻⁴⁹, but can form anywhere on the human body. Generally they start to develop within 4-8 weeks after injury, continue to grow for up to 6 to 12 months, mature and then may diminish in time (years). They are found in almost all patients when trauma is extensive and / or deep enough (34-64% undergoing standard surgical procedures^{50,51} and even up to 91% following large deep burn injury^{52,53}). In contrast, keloid scars can develop after minor injury (e.g. pustule or piercing), even years after the initial injury and almost never regress. Keloid scars have clear predilection sites (shoulders, chest, earlobes, cheeks and upper arms)^{44,46} and the recurrence rates of keloid scars after incision are higher than of hypertrophic scars⁵⁴. Keloid scars are less prevalent than hypertrophic scars, but are more common among the darker pigmented skin population (up to 6-10% in African populations) than north European Caucasian population (0.1%)^{39,55}. In addition to this, genetic predisposition of keloid scar formation is supported by reports that >50% of people developing keloid scars have a family history of keloids and that there is a prevalence of keloid scars in twins^{38,56}.

Pathogenesis

The differences between hypertrophic and keloid scars suggest a difference in the underlying pathology. Despite abundant literature the pathogenesis underlying hypertrophic and keloid scars remains largely unknown. In literature a clear distinction between the pathogenesis of the two adverse is rarely made. The pathogenesis described below focuses mainly on hypertrophic scar formation.

Normally during hemostasis, fibronectin (component fibrin clot) expression decreases within a few days after injury. However during hypertrophic scar formation fibronectin remains elevated for several weeks⁵⁷. In line with this, reports show that inadequate degradation of the fibrin clot might lead to fibrosis^{58,59}. An increased and prolonged inflammatory response has been linked to hypertrophic scar formation^{60,61}, but literature often shows contradictory results. For instance mast cells are described to be both increased⁶² or present in equal numbers⁶³ in hypertrophic scars compared to normotrophic scars. The development of a T-helper2 response is strongly linked to fibrosis^{52,60}. Furthermore

an increased number of Langerhans cells are found in epidermis of hypertrophic scars⁶³. Hypertrophic scars are more often seen after delayed wound closure⁴⁸ and the resulting hypertrophic scar shows increased numbers of microvessels⁴⁵, increased extracellular matrix (ECM) deposition and decreased apoptosis especially of fibroblasts and myofibroblasts compared to normotrophic scars^{61,64}. Abnormal expression of proteoglycans and MMPs/TIMPS (e.g. decreased decorin, MMP1/9 and increased TIMP-1, biglycan and versican) which may lead to increased proliferation and matrix production and reduced matrix breakdown also suggests an altered ECM remodeling in hypertrophic compared to normotrophic scar formation^{60,61}. Especially the increased levels of fibrogenic growth factors TGF β 1, and to lesser extent PDGF and IGF-1 have been linked to adverse scar formation^{46,52}. TGF β 1 is a chemoattractant for immune cells and fibroblasts, mitogenic for fibroblasts and facilitates differentiation of fibroblasts into myofibroblasts probably leading to increased matrix production^{52,60,65}. It has been found to be increased in keloid and hypertrophic scar fibroblasts^{66,67} and the transition from scar-less fetal wound healing to adult scarring is also thought to be TGF β 1 dependent (also TGF β 3)^{61,68}.

Hypertrophic and keloid scar models

Naturally, *in vivo* human patient studies most accurately reflect human wound healing. However exploring the pathogenesis of adverse scar formation in human is cumbersome. This is due to ethical issues and logistical problems with regards to a defined experimental set-up and obtaining samples for analysis and also due to patient variation with regards to extent and duration of trauma. Research into the pathogenesis of adverse scar formation has been further complicated by the lack of suitable adverse scar models. Adverse scar models are used with varying degrees of success to represent human scars and are discussed below.

Animal models

There are a large number of studies describing pigs, mice, rats, rabbits, and other animals as models to investigate hypertrophic scarring or keloid scar formation⁶⁸⁻⁷⁸. Some studies induce a scar by standardized trauma (e.g. excision wounds^{1,4,79}). Unfortunately, most often no clear distinction between normotrophic scar formation, hypertrophic scar formation and keloid scar formation is made⁵⁵. Also the skin physiology, immunology and therefore the wound healing process is markedly different in humans compared to animals⁸⁰⁻⁸³. In line with this, animals do not develop scars which are comparable to adverse scars in humans^{81,82}. Other studies describe animal models where human skin or scar tissue is grafted onto the animal. This led to the development of a hypertrophic scar model in which a healthy human split-thickness skin graft is grafted onto the back of a nude mouse where it developed into a red and thickened scar showing similarities with hypertrophic scars in humans^{69,84,85}. An animal model to study keloid pathogenesis

was constructed in a similar manner by transplanting human keloid scar onto nude mice^{72,74,86,87}. A limitation for the keloid scar models is the limited availability of human keloid scar. A limitation for both adverse scar models is that the immune component of wound healing and scar formation is severely compromised due to the immune deficiency of the nude mouse. This is supported by reports showing that mouse models in general poorly mimic human inflammatory events (e.g. burn wound trauma)⁸³. The only human immune cells present are derived from the transplanted skin itself as human immune cells from the blood are absent⁸⁸. Until now there is no optimal animal model to study adverse scar formation.

In vitro scar models

In vitro cell culture models have also been used to study scar pathogenesis. The most basic models consist of conventional monolayers of cells where normal and scar derived fibroblasts or keratinocytes are compared⁸⁹⁻⁹⁸. By introducing a scratch (wound) in the monolayer migration could be studied⁹⁹ next to proliferation and production of soluble wound healing mediators. These monolayer cultures are simple, fast, easy to perform and inexpensive but skin consists of more than one cell type. This led to (in)direct co-cultures of keratinocytes and fibroblasts which enabled the study of keratinocyte-fibroblast interactions on adverse scar formation^{93,94,100,101}. However, the cultures do not mimic the 3D *in vivo* like situation and therefore missed physiological relevance. Indeed, it was noticed that the introduction of a more physiologically relevant 3D environment (collagen or fibrin gel) and mechanical load positively influenced the behavior of fibroblasts towards the scar phenotype¹⁰²⁻¹⁰⁵. A more *in vivo*-like situation was created by enabling fibroblasts to produce their own matrix with on top a reconstructed fully differentiated epidermis of hypertrophic scar derived keratinocytes^{106,107}. This model showed a few characteristics of an adverse scar (e.g. dermal thickness, epidermal thickness, collagen I) and emphasize the role of keratinocytes in hypertrophic scar formation. Also a 3D skin equivalent model has been described using keloid scar fibroblasts in combination with normal skin derived keratinocytes showing increased collagen production and contraction compared to skin equivalent using normal skin derived fibroblast and keratinocytes^{108,109}. This is a limitation in the model since keloid keratinocytes have been described to be intrinsically different to normal skin derived keratinocytes^{90,93,100,110-113}. Major limitations of these models are their dependence on excised scar tissue, the lack of robust validated biomarkers for testing therapeutics and the lack of an immune component.

Outline and aim

Many attempts have been performed to develop an *in vivo* like hypertrophic scar model. Until now no robust and physiologically relevant *in vitro* hypertrophic scar model ex-

ists for *in vitro* testing of therapeutics with multiple defined scar forming parameters. With increasing pressure from the EU (Directive 86/609/EEC) who strongly stimulate the replacement, reduction, and refinement of the use of animals models, there is an urgent need to develop such a hypertrophic scar model. Once established the model could be used to study the pathogenesis of hypertrophic scar formation. This in turn should facilitate identifying and testing new therapeutics, and thus lead to novel treatment strategies. Therefore the aim of this study was to develop and validate an *in vitro* full-thickness human tissue-engineered hypertrophic scar model.

In chapter 2 we compared the transcription of genes and proteins involved in inflammation, angiogenesis and granulation tissue formation in patients forming hypertrophic scars rather than normotrophic scars to gain more knowledge of the pathogenesis of hypertrophic scar formation and to identify markers for the hypertrophic scar model. In chapter 3 we studied the influence of burn wound exudates (which mimic the burn wound bed) on adipose tissue-derived mesenchymal stem cells, dermal fibroblasts and keratinocytes which may be important for the use in skin tissue engineering constructs and wound healing. In particular CCL27, a skin specific chemokine highly present in burn wound exudates, was investigated (Chapter 3&4).

During wound healing a hypoxic environment (< 5% oxygen) is present until neo-vascularization occurs and this hypoxia may influence scar formation. In order to study this in chapter 5 adipose tissue-derived mesenchymal stem cells were chosen since they reside deep in the cutaneous wound bed which, when exposed, often heals with hypertrophic scar formation. Furthermore these stem cells maintain their capacity to differentiate into osteogenic, chondrogenic, myogenic and cardiomyocytic lineages in culture. Importantly, due to their availability and wide range of applications, adipose tissue-derived mesenchymal stem cells have great potential within the field of (skin) tissue engineering. Also, *in vivo*, fibrin forms a temporary extracellular matrix for neo-vascularization. Naturally occurring fibrinogen variants alter functional and molecular mechanisms of endothelial cells. High molecular weight fibrin increases neo-vascularization *in vitro* and *in vivo* compared to low molecular weight fibrin and unfractionated fibrin. The question arises whether the naturally occurring fibrinogen variants might alter mesenchymal stem cell expansion and differentiation and therefore scar forming properties. If this is the case then they need to be considered as a component of the dermal matrix when constructing the hypertrophic scar model. Therefore in chapter 5 the effect of oxygen tension (1% compared to 20%) and naturally occurring fibrinogen variants on adipose tissue-derived mesenchymal stem cell expansion and differentiation was studied.

Whereas chapters 2–5 present a detailed selection of potential biomarkers and cell types for incorporation into a hypertrophic scar model, chapters 6 and 7 describe two different tissue-engineered hypertrophic scar models. Chapter 6 describes a model of

primary cells isolated directly from excised scar tissue, which can be used to reconstruct 3D scar *in vitro*. Using this tissue-engineered scar model parameters were identified which distinguish normal skin and normotrophic scars from abnormal scars, and hypertrophic scars from keloid scars. These models are dependent on excised scar tissue, which is limited. Therefore the second hypertrophic scar model described in chapter 7 describes the use of keratinocytes and ASC obtained from healthy adult abdominal skin which is routinely removed in standard corrective surgical procedures and is a readily available source of cells for tissue engineering (Chapter 7).

In chapter 8 the progress, limitations and the future challenges in the field of human hypertrophic and keloid scar models are discussed. The major findings from this thesis are discussed in chapter 9.

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