CHAPTER 8

General Discussion
Wound healing is complex and accurate repair requires a well regulated system. Throughout the world many people suffer from inappropriate wound healing which results in either chronic wounds or fibrotic scars. In chronic wounds the healing process is impaired, while in fibrotic scars repair is exaggerated. Fibrotic or hypertrophic scars are often the result of burns and characterized by a rigid, raised, red, itchy, and contractile appearance. Consequently, patients experience functional and cosmetic problems, such as impaired mobility and pain. Scars often develop after deep dermal skin injury, but scar formation is uncommon in the oral mucosa. Furthermore, oral wounds heal faster compared to dermal wounds. Several differences between dermal and oral healing have been described, although the exact mechanisms responsible for scar forming or scarless healing are not yet defined. In this thesis I have tried to gain more insight in the differences between dermal and oral wound healing. The final goal is to reduce scar formation by modulation of dermal repair.

In chapter 2, factors already known from literature to differ between oral and dermal tissue and wounds have been reviewed. With respect to normal dermal and oral tissue, most of the differences were found on the level of the extracellular matrix (ECM). Some ECM proteins, but also matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases were higher or lower expressed in oral tissue compared to dermal tissue. Furthermore, dermal and oral fibroblasts differed in phenotype. For example, proliferation rate, production of hepatocyte growth factor and keratinocyte growth factor, and contraction ability were higher in oral than dermal fibroblasts. By contrast, in wounds, less ECM differences were found, but differences in immune mediators dominated in the comparison between dermal and oral wounds. Most of the immune cells, as well as cytokines, chemokines and growth factors were decreased in oral wounds. In subsequent chapters, relation between and expression of immune cells, fibroblasts, and ECM was further investigated.

Identification of differently activated macrophages in vitro
One of the immune cells thoroughly investigated during repair is the macrophage. These cells can adopt a variety of functional phenotypes in response to endogenous or exogenous factors. The best defined phenotypes are the classically (M1) and alternatively (M2) activated macrophages, representing the two extremes of activation. Macrophages were shown to be important during wound healing since
depletion resulted in delayed reepithelialization and vascularisation, decreased collagen deposition and inappropriate wound contraction.\textsuperscript{4, 5} M1 macrophages were reported to predominate in chronic wounds, while M2 macrophages were associated with fibrotic repair.\textsuperscript{6-8} When the function of M1 or M2 macrophages is studied, these specific phenotypes must be identified accurately. Regrettably, distinctive markers for M1 or M2 macrophages have not been established yet. Furthermore, various protocols are used by different research groups to generate these cells \textit{in vitro}. The effect of different maturation factors (normal human serum (NHS), macrophage colony-stimulating factor (M-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF) and activation methods (interferon-γ and lipopolysaccharide (IFN-γ/LPS), interleukin (IL)-4, dexamethason, and IL-10) on macrophage phenotype was determined in chapter 3. Regarding macrophage morphology, IFN-γ/LPS stimulated cell elongation, whereas IL-4, dexamethason, and IL-10 induced a circular appearance. Several markers that were described to be enhanced by either M1 or M2 macrophages were determined and we found significant upregulation of CD40, CD64 and pro-inflammatory cytokines by activation with IFN-γ/LPS. The anti-inflammatory activators IL-4 and dexamethason increased mannose receptor (MR) or CD163 expression respectively. Therefore, it is recommended to use CD40 and CD64 as markers for M1, and MR and CD163 expression for M2 identification.

\textbf{Macrophage-fibroblast interactions}

During wound healing fibroblasts are recruited to the site of injury. In normal conditions their main task is to maintain structural tissue integrity by regulating the turnover of ECM proteins. During wound healing, fibroblast proliferate and several cells differentiate towards α-smooth muscle actin (SMA) expressing myofibroblasts, induced by factors such as transforming growth factor (TGF)-β. Myofibroblasts produce collagen abundantly and are responsible for wound contraction. During the remodelling phase, these cells become apoptotic.\textsuperscript{9} In fibrotic tissues, however, these cells persist in large quantities.\textsuperscript{10} Since M2 macrophages have been associated with fibrosis,\textsuperscript{6, 8} we investigated their relation with myofibroblasts. This, in order to reveal whether M2 macrophages may play a role in scar formation observed in dermal but not in oral wounds. In chapter 4, we observed that α-SMA expression was upregulated by M2 but not by M1 macrophages. This effect, however, was found in both dermal and oral fibroblasts. TGF-β would be an obvious candidate for α-SMA induction in
fibroblast. However no difference in production was detected between M1 and M2 macrophages. M2 macrophages produced higher levels of platelet derived growth factor (PDGF)-CC which was responsible for the α-SMA induction in both dermal and oral fibroblasts (Fig. 1).

**Distribution of immune cells in skin and oral mucosal tissue and scars**

We established that M2 macrophages had similar effects on both dermal and oral fibroblasts, with respect to α-SMA expression. From this observation it may seem that M2 macrophages do not contribute to fibrosis directly, although the number of these cells may differ between dermal and oral wounds. A reduced distribution of M2 macrophages may result in a diminished effect of these cells. Biopsies derived from human wounds (oral and dermal) were difficult to obtain and therefore in chapter 5 we used scar tissue. In addition to M1 and M2 macrophages, other immune cells (mast cells, neutrophils, T-cells) and blood vessels were determined in dermal and oral tissue and scars. Remarkable, presence of immune cells did not differ between scars and healthy tissue. Healthy oral mucosa however, had significantly fewer neutrophils, macrophages, M2 macrophages, although more blood vessels than skin (Fig. 1). Reduced numbers of immune cells may induce a diminished immune reaction when wounding occurs, and could give rise to improved repair.

**Models to study human wound healing**

Most of the studies investigating wound healing are performed in animals. Because of the human-animal differences these results can not always be translated to humans directly. Anatomical conditions such as skin thickness, presence of rete ridges and sweat glands differ between humans and mice. In addition, mice heal by contraction while human repair is based on reepithelialization and formation of granulation tissue. In order to mimic human repair in vivo, a mouse model was developed in chapter 6A. Initially, the surgery procedure was optimized by transplantation of autografts on NOD-scid IL2Rgamma null (NSG) mice. Since these mice are severely immunocompromized, allografts or xenografts should not be rejected. Therefore, human skin was subsequently transplanted on these mice. After 90 days, human skin was successfully transplanted on the backs of NSG mice. However, most of the transplants still contained a crust, and the transplant that had shed the crust contracted more than 60% of the original size. Because of the crust and contraction, these transplants are not usable for subsequent wound
healing studies yet. In order to be useful, parameters such as skin thickness, or transplantation size could be adapted for improvement of this model.

Therefore, and because of ethical considerations it would be preferable to develop an alternative model in which the human repair process can be studied. In previous chapters we examined the effect of M1 and M2 macrophages on one specific cell type (fibroblast) or its presence in scars. In chapter 6B the effect of these cells was investigated during human ex vivo wound healing. An earlier study already showed that human skin can be maintained in culture for 21 days\textsuperscript{12}, and we applied these culture conditions in our experiments. Ex vivo human skin was wounded by means of a puncher. M1 and M2 macrophages or control (buffered saline solution) were applied to the wounds every 4 days. Interestingly, M2 macrophages elicited wound closure much faster than M1 or control (Fig. 1). Furthermore, increased numbers of fibroblasts migrated out of the tissue and/or proliferated when M2 macrophages were inserted. On mRNA level, PLOD2 was increased in wounds treated by M2 macrophages. This underlines a relation between M2 macrophages and fibrosis, since PLOD2 is related to fibrotic tissues.

Comparison of ECM components between skin and oral mucosa

Finally, we addressed possible differences on ECM level between oral and dermal tissue. Synthesis of ECM is a major feature of wound repair. The ECM provides a scaffold for cell recruitment, and stimulates cells to proliferate, differentiate and synthesize new ECM.\textsuperscript{13} Chronic wounds exhibit degraded ECM, while in fibrotic wounds ECM components, such as collagen and biglycan, are elevated.\textsuperscript{14, 15} In chapter 7 we compared the presence of several ECM components between oral and dermal tissue, since the fate of scarless or scar forming healing may already be defined by ECM components in unaffected tissue. Expression of fibronectin, its splice variant ED-A, and chondroitin sulphate was elevated in oral tissue, whereas elastin was higher expressed in the skin (Fig. 1). Remarkably, a study comparing fetal and adult tissue found identical expression regarding ECM components.\textsuperscript{16} Other tissue structures were also examined, revealing that oral mucosa contained more blood vessels than skin, as earlier observed in chapter 5. Oral keratinocytes proliferated more, while dermal keratinocytes demonstrated higher differentiation. Since ECM composition between oral and fetal tissue is comparable, expression may be involved in the mode of healing.
CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis I have shown that oral tissue differs from skin by means of immune cell numbers, ECM composition, and keratinocyte and fibroblast phenotype (Fig. 1). These architectural differences may give rise to different healing outcomes. Throughout the thesis I mainly focussed on macrophages, since these cells were shown to contribute to repair but could also have harmful effects on wound healing.\textsuperscript{5,17,18} We found similar effects of M2 macrophages on dermal and oral fibroblasts, regarding induction of α-SMA expression. In scars however, less M2 macrophages were found in oral compared to dermal scars. These results may indicate that M2 macrophages are associated with the burden of scar formation, although in our human \textit{in vitro} model, we found a beneficial effect of M2 macrophages on wound closure. Furthermore, non healing chronic wounds harbour mainly M1, while M2 macrophages were found in fibrotic tissues.\textsuperscript{6-8}

With respect to other inflammatory factors, inflammatory triggering molecules were very low expressed during scarless fetal repair.\textsuperscript{19} Moreover, in oral pig wounds inflammation occurred early and ceased rapidly,\textsuperscript{20} stressing the concept that extended inflammation may increase scar formation. Therefore, I conclude that M2 macrophages are necessary for wound healing, but prolonged presence may lead to fibrosis.

In addition, several ECM components were differently expressed between oral and dermal tissue. Expression of various ECM components may have an effect on the macrophage phenotype (Fig. 1). For instance, heavy-chain hyaluronic acid was shown to induce M2 polarization, and chondroitin-6-sulphate attenuated inflammatory responses by M1 macrophages.\textsuperscript{21,22} These results may explain the difference in numbers of immune cells found between skin and oral mucosa (chapter 5). In addition, the ECM can function as reservoir for growth factors that can be released during injury.\textsuperscript{23} Chondroitin sulphate and fibronectin likely bind other growth factors compared to elastin, which can have different effects during repair. ECM components were also shown to have effects on fibroblasts. Elastin increased fibroblast secretion of the pro-inflammatory mediators MMP-13, CXCL1, CXCL5, and CXCL8.\textsuperscript{24} Small fragments of hyaluronic acid induced fibroblast migration, but did not increase myofibroblast differentiation (Fig. 1).\textsuperscript{25}
Additionally, fibroblast and keratinocyte phenotypes differed between the two tissues. Oral keratinocytes showed higher proliferation, while dermal fibroblasts were more differentiated. Oral and dermal fibroblasts were previously shown to differ on many features such as proliferation rate, growth factor production, and ECM gene expression.\textsuperscript{26-30} Altogether, these results clearly point to a different tissue environment between skin and oral mucosa.

**Figure 1:** Overview of the main results obtained in the thesis.
In Chapter 4, it was shown that M2 macrophages induce α-SMA expression in both dermal and oral fibroblasts. This induction was mediated by PDGF-CC, secreted by these macrophages. Reduced numbers of M2 macrophages, together with total macrophage population and neutrophils, were observed in oral tissue, compared to skin (chapter 5). More blood vessels however, were found in oral mucosa. M2, but not M1 macrophages had the capacity to induce faster skin wound closure (chapter 6B). Moreover, wounds treated with M2 macrophages had increased PLOD2 mRNA expression. Dermal keratinocytes differentiated more, while proliferation was higher in oral keratinocytes (chapter 7). The ECM component elastin predominates in skin, while chondroitin sulphate (CS), fibronectin (FN) and its splice variant ED-A prevail in oral mucosa (chapter 7).
From the results obtained in this thesis, I conclude that faster resolution of immune responses is important for effective repair. M2 macrophages mediated faster wound closure, although persistence may lead to exaggerated scar formation. Therefore, a correct balance between M1 and M2 macrophages must be preserved. Environmental factors such as ECM components may be responsible for maintaining the accurate balance between M1 and M2 macrophages.

To further unravel the effect of M1 and M2 macrophages during dermal versus oral repair, oral tissue must be included in the human ex vivo wound healing model. In addition, prolonged administration of M2 macrophages may reveal whether M2 macrophages will indeed give visible features of fibrosis. Next to macrophages, other cells or factors can be studied in the model that we developed in chapter 6B.

Since we and others showed that environmental factors such as ECM components, fibroblasts, and keratinocytes differ between oral and dermal tissue, it is worthwhile to investigate their interaction with M1 and M2 macrophages in more detail.

For clinical applications it is recommended to skew macrophage activation towards an M2 phenotype in chronic wounds, and M1 phenotype in fibrotic tissue. This skewing can be induced by M1 or M2 polarizing factors (e.g. IFN-γ or IL-4 respectively) or by direct application of autologous M1 or M2 macrophages into the wounded area. Moreover, wounds that are highly predisposed to develop fibrotic scars (for example burns) may directly upon injury be treated with M2 macrophage inhibitory factors, in order to prevent exaggerated scar formation.

Consequently, this thesis contributed to possible new clinical approaches in order to improve scar formation. I propose that skewing of macrophage phenotypes will be prominent for this kind of treatment and additionally, fast resolution of these and other immune cells will be important for effective repair.
REFERENCE LIST


