MATRIX METALLOPROTEINASES
IN SCARLESS WOUND HEALING

by

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ABSTRACT

Objectives: Wound healing in skin often results in scar formation, whereas wound healing in oral palatal mucosa is fast and rarely results in scarring. Understanding the mechanisms that promote oral scarless wound healing may provide novel approaches to prevent scar formation in skin. The goal of the study was to compare the abundance of the major collagenases MMP-1 and MMP-13 and gelatinases MMP-2 and MMP-9 in normal unwounded oral mucosa and skin and in experimental excisional wounds in skin (healing results to scar formation) and oral mucosa (wounds heal with minimal scar formation) at various time points post-wounding at the protein level. We hypothesized that the abundance of MMPs will be higher in scarless oral mucosal wound healing, compared to skin wound healing.

Methods: Experimental wounds were created in oral palatal mucosa and dorsal skin of red Duroc pigs. Wound biopsies were collected before wounding and at various time points after wounding. The abundance of MMPs at the protein level was assessed by Western blotting and zymography.

Results: All studied MMPs showed a significantly increased accumulation in the wound tissue already at day three post-wounding. Their abundance remained high until day 28 when MMP-9 and MMP-13 returned to the level of unwounded tissue, while MMP-1 and MMP-2 remained significantly elevated. Oral mucosal wounds showed in general a robust early up-regulation of MMP-1, MMP-2 and MMP-9 as compared to skin wounds already at day 3 after wounding. In contrast, the peak abundance of these MMPs occurred at day 14 in skin wounds. Unwounded oral mucosa showed significantly higher abundance of total MMP-2 and active MMP-9 as compared to unwounded skin. Thus, MMPs needed for early wound
healing response are already present in higher abundance in oral mucosa as compared to skin before tissue injury possibly allowing a fast wound healing response.

**Conclusions:** Results suggest that oral mucosal wound healing is associated with fast and robust regulation of MMPs. Rapid controlled processing of wound extracellular matrix may play a key role in scarless palatal wound healing. In addition, MMPs may regulate inflammatory reaction that plays a central role in scar formation.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP</td>
<td>Activator Protein</td>
</tr>
<tr>
<td>APMA</td>
<td>Aminophenylmercuric Acetate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ETS</td>
<td>Erythroblastosis Twenty Six</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl-Phosphatidyl-Inositol</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic Acid</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>HPX</td>
<td>Hemopexin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte Growth Factor</td>
</tr>
<tr>
<td>MCP</td>
<td>Macrophage Chemoattractant Protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane Type Matrix Metalloproteinase</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T-cell Expressed, and Secreted</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphisms</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitors of Metalloproteinase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tissue Necrosis Factor</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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To My Parents Giota and Nikos:

I want to thank you for all your sacrifices to make me happy, believing in me, and for your unwavering love and support. You are the greatest people in the universe.
I hope one day to pay you back...

Στους γονείς μου Γιώτα και Νίκο:

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Ελπίζω μία μέρα να ανταποδώσω όλα αυτά που έχετε κάνει για μένα και ακόμη πολλά...

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I want to thank you for all your love, patience and encouragement, during the last three years that we have been apart. Without your support, I could not have succeeded. I promise to make it up to you in all the years to come. I know you are “the one”.
Σ’αγαπώ...
1. INTRODUCTION

Wound healing of damaged skin or mucosa is a critical process for the survival of the organisms. Wound healing involves four overlapping phases including inflammation, re-epithelialization, granulation tissue formation and tissue remodeling. The goal of wound healing is to completely restore the function and the structure of the damaged tissue (regeneration) (Toriseva and Kähäri, 2009). Only fetal skin wounds have the potential to heal completely with regeneration, whereas adult oral mucosa, liver and skeletal muscle have some limited ability to regenerate (Häkkinen et al., 2000). In most cases, higher vertebrates heal through repair. In the reparative type of wound healing, cutaneous wounds achieve dermal integrity at the expense of the unwanted outcome of scar formation (Takata, 1994). Several cellular and molecular differences between fetal and adult skin have been suggested as potential factors contributing to scarless wound healing. Briefly, fetal wound healing is characterised by higher collagen type III to type I ratio, expression predominantly of TGF-β3 isoform, rapid re-epithelialization, more hyaluronic acid, less inflammation, and myofibroblast absence compared to skin wound healing (Bullard et al., 2003; Namazi et al., 2011). Adult oral mucosal wounds heal more rapidly and with less or no scarring compared to cutaneous wounds. The presence of saliva and oral bacteria, the earlier resolution of the inflammatory reaction or a mild inflammatory reaction and the reduced wound contraction seem to be important parameters for scarless wound healing in oral mucosa (Häkkinen et al., 2000; Mak et al., 2009). A characteristic that fetal skin and adult oral mucosal wounds share in common is the higher matrix metalloproteinase (MMP) (MMP-1, -9 and -14) to tissue inhibitor of metalloproteinase (TIMP) (TIMP-1 and -3) ratio (Toriseva and Kähäri, 2009).
MMPs constitute a family of proteases that are capable of degrading most components of the extracellular matrix (ECM) (Hadler-Olsen et al., 2010). MMPs are key regulators of multiple aspects of tissue repair and they are involved in all the stages of wound healing. MMPs participate in the regulation of inflammation through the control of chemokine and cytokine activity, promote epithelial cell and fibroblast migration in the wound area, and are involved wound contraction and in tissue remodeling through ECM degradation (Gill and Parks, 2008). The present study compares the abundance of major collagenases MMP-1 and MMP-13 and gelatinases MMP-2 and MMP-9 in normal unwounded oral mucosa and skin and in experimental excisional wounds in skin (wound healing results to scar formation) and oral mucosa (wound healing results to minimal scarring). The results demonstrate that the abundance of MMPs is higher in scarless oral mucosal wound healing, compared to skin wound healing. Besides, MMPs accumulate earlier in scarless oral mucosal wounds as compared to skin wounds.
2. REVIEW OF THE LITERATURE

2.1 Overview of wound healing

Wound healing is a critical process for the organism and its major goal is to stop bleeding and to restore completely the function and the structure of the damaged tissue and to prevent microbial access into the tissues (Takata, 1994). Wound healing can be divided into four phases: hemostasis and inflammation, re-epithelialization, granulation tissue formation and tissue remodeling. Although these phases are histologically and functionally distinct, they show considerable overlap (Toriseva and Kähäri, 2009).

2.1.1 Hemostasis

Tissue injury results in blood vessel disruption and blood extravasation. The hemostasis mechanisms start with vasoconstriction and the exposure of the subendothelial type IV and V collagen promotes the adhesion and aggregation of the platelets (Legrand et al., 1986). Degranulation of the platelets follows and the release of serotonin, platelet-derived growth factor (PDGF), platelet-activating factor and thrombaxane A₂ results in further vasoconstriction, platelet aggregation and growth factors’ release (Clark, 1996). The cascade of coagulation factors is then activated, which results in increased thrombin production, leading to the cleavage of fibrinogen and formation of fibrin fibers. These fibers together with the platelets form a fibrin-rich clot, which acts as a physical barrier, preventing further bleeding and bacterial invasion. Besides, the clot can act as a reservoir of growth factors and as a chemotactic matrix for cell migration (Toriseva and Kähäri, 2009).
2.1.2 Inflammation

The first inflammatory cells arriving at the wound are the neutrophils. Neutrophils appear at the site a few hours after the injury attracted by various chemoattractants (fibrinopeptides cleaved from fibrinogen, fibrin degradation products, C5a and C3a complement components, bacterial products and others) (Smith et al., 1979; Tonnesen et al., 1984; Gimbrone et al., 1984; Egozi et al., 2003). Their main function is to cleanse the wound of foreign particles and infectious agents by phagocytosis and by enzymatic and oxygen radical mechanisms (Eming et al., 2007).

About one day after the injury, blood monocytes immigrate to the wound site and they become activated macrophages by contact with the wound extracellular matrix (Clark, 1996). Their main functions are wound area debridement by phagocytosis, antigen-presentation and growth factor secretion, including interleukin-1 (IL-1), PDFG, basic fibroblast growth factor (bFGF) and transforming growth factors (TGF-α and TGF-β) (Nathan, 1987; Eming et al., 2007). Mast cells and lymphocytes are also involved in the inflammatory stage of wound healing, but to a lesser extent (Gillitzer and Goebeler, 2001).

2.1.3 Re-epithelialization

Keratinocytes start migrating into the wound site about 24 hours after wounding (Woodley, 1996). In the mucosa, the major source for migrating epithelial cells is the basal layer of the wound periphery epithelium. On the other hand, cutaneous wounds have additional sources of epithelial cells, since they can arise also from hair follicles and sweat glands (Häkkinen et al., 2000).

As soon as the basal keratinocytes are exposed to the new fibrin matrix (“free edge effect”), their phenotype changes from stationary to migratory (Odland and Ross, 1968; Singer and Clark, 1999). The migratory epithelial cells dissolve their hemidesmosomal
complexes, get released from the basement membrane, withdraw their intercellular desmosomal attachments and have a more flattened and elongated morphology allowing cell movement (Gabbiani et al., 1978; Goliger and Paul, 1995; Larjava et al., 1996). Migrating keratinocytes are highly phagocytotic, allowing them to either penetrate through the clot or to migrate below the clot (Woodley, 1996; Häkkinen et al., 2000). This can be facilitated by the increased expression of MMP-1, MMP-9, MMP-10 and plasmin. All these enzymes can degrade most of the clot components (Saarialho-Kere et al., 1993; Salo et al., 1994; Bugge et al., 1996; Steffensen et al., 2001).

The epithelial cells stop migrating when they meet each other over the middle of the wound surface (“contact inhibition”) (Stenn, 1992). The deposition of basement membrane components (type IV and VII collagen, laminin-1 and heparan sulfate proteoglycan) occurs in a different pattern in epidermal and gingival wounds. In small gingival wounds, the nucleation of the basement membrane takes place in multiple sites at the same time (Larjava et al., 1993; Häkkinen et al., 2000). On the other hand, skin wounds show basement membrane deposition that starts from the wound margin, moving inwards in a “zipper-like” fashion (Clark et al., 1982). The re-establishment of the basement membrane is followed by re-formation of the cellular contacts, epithelial cell differentiation and multi-stratified pattern formation (Toriseva and Kähäri, 2009).

2.1.4 Granulation tissue formation

Approximately 4 days after tissue injury, wound granulation tissue begins to form. This granulation tissue contains macrophages, fibroblasts, new blood vessels and extracellular matrix (ECM) molecules, forming a loose connective tissue (Clark, 1996; Toriseva and Kähäri, 2009). Macrophages release copious amounts of cytokines and growth factors, which further stimulate fibroblastic proliferation and activation, as well as
angiogenesis (Singer and Clark, 1999). The movement of fibroblasts within the provisional matrix is facilitated by MMPs, which create a pathway for migration (Mignatti and Rifkin, 1996). Growth factors, like PDGF and TGF-β, promote ECM deposition and remodeling by fibroblasts. The ECM consists initially of fibronectin and hyaluronic acid, both of which stimulate further cell migration (Singer et al., 1988; Toriseva and Kähäri, 2009). The composition of the ECM changes at later stages, consisting mostly of type I and III collagen and proteoglycans (Clark, 1996).

2.1.5 Angiogenesis

Tissue destruction and hypoxia stimulate the secretion of vascular endothelial growth factor (VEGF), bFGF and TGF-β, by macrophages, fibroblasts, endothelial and epithelial cells. These cytokines induce further release of plasmin and MMPs. The last proteases are responsible for the digestion of the basement membrane of the surrounding vessels, the migration of the endothelial cells into the wound site and the formation of new blood vessels (also called angiogenesis). Angiogenesis starts on the 4th day after tissue injury (Singer and Clark, 1999).

2.1.6 Tissue remodeling

During the second week of healing, the fibroblasts obtain myofibroblast phenotype, which is characterized by increased expression of α-smooth muscle actin. The differentiation of myofibroblasts is induced mostly by TGF-β, in the presence of fibronectin. At the end of the second week, approximately 70% of the fibroblasts show a myofibroblast phenotype (Darby et al., 1990; Welch et al., 1990; Hinz et al., 2001; Toriseva and Kähäri, 2009). Myofibroblasts can attach to fibronectin and collagen and transmit contraction across the wound, pulling the wound edges closer to each other (Singer et al., 1984). When wound contraction is completed, myofibroblasts are eliminated by apoptosis. The tissue maturation
is characterized by decreased numbers of fibroblasts and disintegration of blood vessels. Collagen remodeling then follows for several months. Collagen will be accumulated and the tensile strength will be increased, however, it will never reach the original strength of intact tissue (Darby et al., 1990; Desmouliere et al., 1995; Singer and Clark, 1999).

2.2 Scar formation

The previous review of wound healing represents mostly the regenerative type of healing, which restores completely the damaged tissue both functionally and structurally. Only fetal skin wounds have the potential to heal completely with regeneration. Besides, adult oral mucosa, liver and skeletal muscle have some limited ability to regenerate (Michalopoulos and DeFrances, 1997; Häkkinen et al., 2000; Ferguson and O’Kane, 2004; Stoick-Cooper et al., 2007). In most cases, higher vertebrates heal through repair. In the reparative type of wound healing, cutaneous wounds achieve dermal integrity at the expense of the unwanted outcome of scar formation (Takata, 1994; Clark, 1996). Therefore, scar formation is defined as the macroscopic disturbance of normal skin structure and function as a consequence of wound repair, with such changes arising from alterations in epidermal, dermal and subcutaneous tissues (Ferguson et al., 1996). Microscopically, scar is a permanent excessive accumulation of abnormally organized collagen-rich ECM that lacks large blood vessels and elastin (Wijdeveld et al., 1991; Wong et al., 2009).

Different kinds of scars have been described. Small, shallow wounds can lead to flat scars that may have different color than surrounding skin. On the other hand, more extensive, deep wounds can lead to atrophic, hypertrophic, or keloid-like scars. Keloids are described as thick scar tissues, escaping the boundaries of the original wound and they seem to have a genetic predisposition. Hypertrophic scars remain within the original boundaries of the
wound, they are often erythematous and nodular and they form most commonly after burn injuries and occur most often in children (Rudolph, 1987; Datubo-Brown, 1990; Bayat et al., 2003; Slemp and Kirschner, 2006).

It seems that almost every dermal wound results in scar formation, although there are some exceptions, including tattoos, superficial scratches and venepunctures (Bayat et al., 2003). Regarding the extent and the type of scar, there are various contributing factors published. In general, it seems that there is considerable quantitative and qualitative variation in scar formation between individuals and even within the same individual (Sommerlad and Creasey, 1978). The age of the subject affects scar formation. Wounds in adolescents and young adults normally result in worse scarring compared to elderly people (Ashcroft et al., 1998). Individuals with pigmented skin seem also to be more prone to severe skin scarring than white people (Murray et al., 1981). The depth of the skin injury seems to be another factor contributing to scar formation. Deep and superficial skin wounds were produced on humans. It was shown that the deep wounds healed with visible scars, whereas the superficial skin wounds healed with no detectable scar. The threshold depth for scarring was calculated as 0.56 mm (Dunkin et al., 2007). Previous findings indicate that wound size affects scar formation in that larger wounds result in more frequent and severe scarring (Cass et al., 1997; Harunari et al., 2006; Zhu et al., 2007; Toriseva and Kähäri, 2009). The location on the body, tension attributable to motion, the gender, delayed closure and genetics seem to be important factors for scar formation as well (Bayat et al., 2003; Toriseva and Kähäri, 2009).

The scar formation has been partly attributed to impaired myofibroblast apoptosis and different cytoskeletal protein expression. In normal healing, the myofibroblasts undergo apoptosis after wound contraction, but in scarred healing, myofibroblast apoptosis is
diminished or aberrant (Desmouliere et al., 1995; Gabbiani, 2003). Regarding the cytoskeletal proteins, granulation tissue myofibroblasts lack in desmin and SM-myosin heavy chains, whereas hypertrophic scar myofibroblasts show increased expression of these proteins (Baur et al., 1975; Darby et al., 1990).

Scars are sometimes considered trivial, but they can be aesthetically unpleasant and cause tenderness, pain, sleep disturbance, anxiety, depression, and disruption of daily activities (Bell et al., 1988; Bayat et al., 2003). Scarring can impair normal function, as scars are less elastic and they are about 70% as strong as intact skin even after extended times. The growth in children might slow down (Levenson et al., 1965). Joint mobility (e.g., joint contractures) can be affected and that physical deformity as a result of skin scar contractures can sometimes be disabling (Wou and Seul, 2001). Development of post-traumatic stress reactions, loss of self esteem and stigmatisation are some of the psychosocial consequences of scar formation that lead to diminished quality of life (Taal and Faber, 1988).

It has been estimated that each year in the developed world 100 million patients acquire scars. Out of them, 11 million are keloid scars and four million burn scars, 70% of which occur in children. Global numbers are unknown but probably a lot higher (Bayat et al., 2003). It was reported that at least 45 million patients in the US alone undergo procedures each year that could benefit from therapies that reduce scar formation (Ocleston et al., 2008). Unfortunately, current standard of care is unable to prevent scar formation, and various treatment modalities (e.g., use of skin substitutes) may result in additional scarring (Durani et al., 2008; Ocleston et al., 2008; Tanaka and Galliot, 2009)
2.3 Scarless wound healing

2.3.1 Fetal wound healing

Fetal cutaneous wounds heal without or with minimal scar formation (Toriseva and Kähäri, 2009). This concept was first observed in 1979 on a 20-week human fetus (Rowlatt, 1979). That initial observation was subsequently confirmed by both human and animal experimental models, including rabbits (Somasundaram and Prathap, 1970), sheep (Longaker et al., 1990), mice (Whitby and Ferguson, 1991), rats (Ihara et al., 1990) and monkeys (Lorenz et al., 1993).

The fetal period in humans begins on the 8th week of gestation. From the 8th to the 24th week, the embryonic dermis changes from a highly cellular to a more fibrotic tissue. After the 24th week of the gestation, fetal skin matures fast and resembles adult skin both histologically and physiologically and heals by scarring (Lane, 1986; Bullard et al., 2003).

During those 24 weeks (first two trimesters), fetal skin wounds heal in a scarless pattern, with normal appendage (hair follicles, sweat and sebaceous glands) and normal dermal collagen architecture. A transition period follows, during which fetal skin wounds heal with a normal collagen pattern, similar to that of unwounded skin, but they cannot restore appendages. After this “transition wound period” (second half of third trimester), wounds heal with no appendages and disorganised collagen bundles, resembling typical adult scars (Lorenz et al., 1993; Bullard et al., 2003; Namazi et al., 2011).

Initially, the scarless wound healing characteristic of fetuses was attributed to their unique environment, since they bath in the amniotic fluid that is warm, sterile and rich in growth factors (Häkkinen et al., 2000; Bullard et al., 2003; Toriseva and Kähäri, 2009). A significant amount of studies, though, showed that the amniotic fluid is not essential or
sufficient for scarless healing. Fetal marsupials (Monodelphis domestica), developed outside
the uterus in the maternal pouch, demonstrated cutaneous wound healing without scar
formation (Armstrong and Ferguson, 1995). In another study by Longaker and coworkers in
1994, adult sheep skin was transplanted onto the backs of second trimester fetal sheeps,
bathed in amniotic fluid. It was shown that the wounds in the adult skin grafts healed with
scar formation (Longaker et al., 1994). Therefore, there is evidence that scarless fetal skin
healing properties are intrinsic to fetal skin (cell biology and gene expression) and are not
dependant on the fetal environment.

A vast amount of studies has focused on intrinsic differences between fetal and adult
tissues, attributing the scarless pattern to various components, including collagen, hyaluronic
acid, TGF-β and others.

2.3.1.i Collagen

In early-gestational fetal wounds, collagen deposition follows a fine, reticular pattern
that resembles unwounded skin. As gestational age increases, wounds heal with significant
collagen crosslinking, imitating adult skin scarring (Merkel et al., 1988; Burd et al., 1990;
Lin et al., 1994). Although type I collagen is the predominant collagen in both adult and fetal
extracellular matrix, fetal skin has a higher proportion of type III to type I collagen. In human
fetal skin, collagen type III represents 30% to 60% of the total collagen and in adult skin 10%
to 20%. With maturation, the amount of collagen type III relative to type I decreases and
reaches the adult ratio (Whitby and Ferguson, 1991; Hallock et al., 1993; Bullard et al., 2003;
Nazami et al., 2011).

Collagen type III is characterised by smaller and finer fibers compared to type I
fibers, which may predispose to a more reticular pattern of fiber deposition and prevent
increased collagen crosslinking, resulting in a more flexible matrix and scarless repair (Bullard et al., 2003).

2.3.1.ii Hyaluronic acid

Hyaluronic acid (HA) is considered a major component of the fetal extracellular matrix, resulting in increased fluidity, which enhances influx of fibroblasts. Besides, fetal fibroblasts tend to have more surface receptors for HA than do adult cells, which promotes fibroblast migration (Bullard et al., 2003; Nazami et al., 2011).

HA has the ability to regulate biomechanically the inflammatory process and to inhibit the chemotactic and phagocytic activity of the white blood cells. Besides, HA lowers fibrin formation and prevents excessive collagen deposition. Therefore, high levels of HA can potentially play a role in the decreased inflammation of fetal wounds (Balazs and Larsen, 2000; Bullard et al., 2003; Nazami et al., 2011).

2.3.1.iii TGF-β and other growth factors

Transforming growth factor-β (TGF-β) is the most widely studied growth factor in fetal wound healing. TGF-β is secreted by the majority of the cells involved in wound healing (i.e. neutrophils, macrophages, keratinocytes, fibroblasts and platelets) (Namazi et al., 2011). Three TGF-β isoforms have been identified, TGF-β1, -β2 and -β3. TGF-β1 promotes extracellular matrix synthesis and inhibits its degradation through increased expression of tissue inhibitors of metalloproteinases (TIMPs) and downregulation of the expression of MMPs (Bullard et al., 2003; Toriseva and Kähäri, 2009).

Wound healing studies on fetal wounds treated with TGF-β1 showed increased scar induction (Krummel et al., 1988). On the other hand, wounds treated with TGF-β3 showed significant down-regulation of TGF-β1 and TGF-β2 (Shah et al., 1995). Furthermore, fetal wound experiments showed that a high ratio of TGF-β3 to TGF-β1 and TGF-β2 is associated
with less scar formation, suggesting that TGF-β3 may have antifibrotic properties (Hsu et al., 2001; Eslami et al., 2009).

Except for the differences in TGF-β isoform expression, differences were noticed in the expression of TGF-β modulators between fetal and adult wounds. Fibromodulin seems to be the predominant TGF-β activity modulator in scarless period of fetal wounds. In later stages of gestation, the expression of fibromodulin decreases, suggesting a transition to adult-type scarring (Soo et al., 2000).

Other growth factors have different expression in fetal wounds compared to adult wounds. For instance, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) demonstrate higher expression in scarring wounds (Bullard et al., 2003; Namazi et al., 2011). Vascular endothelial growth factor (VEGF), on the other hand, is up-regulated in scarless wounds. The VEGF up-regulation in scarless wounding might be associated with an increase in angiogenesis and vascular permeability, resulting in faster healing (Bullard et al., 2003).

2.3.1.iv Fibroblasts

Fibroblasts might play a key role in scarless wound healing, since they are responsible for ECM remodeling through synthesis and degradation of the ECM macromolecules. Number of studies suggests differences between fetal and adult fibroblasts.

In general, fetal fibroblasts are capable of producing more total collagen than adult fibroblasts and especially collagen type III and V (Thomas et al., 1988). Fetal fibroblasts show faster migratory rate compared to adult fibroblasts. It has also been suggested that fetal fibroblasts can proliferate and produce collagen simultaneously. The fast migration and early collagen deposition of fetal fibroblasts might contribute to the scarless healing pattern (Thomas et al., 1988; Bullard et al., 2003).
Myofibroblasts and their role in scarless wound healing have also been investigated. Myofibroblasts are a subset of fibroblasts that contain smooth muscle a-actin and can cause wound contraction and indirectly, fibrosis and scar formation. In adults, myofibroblasts appear on the 5th to 7th post-wounding day. On the other hand, studies on fetal wound healing have shown that myofibroblasts were absent in the 75-day fetus and present after 100 days of gestation, when scar formation begins (Estes et al., 1994; Bullard et al., 2003).

2.3.1.v Inflammation

It has been shown that fetal wounds have less inflammatory infiltrate. This lower inflammatory response could be partly attributed to the poor fetal platelet aggregation and degranulation and to the lower levels of TGF-β and PDGF compared to adult platelets (Olutoye et al., 1995; Olutoye et al., 1996).

Low expression of early inflammatory cytokines may lead to decreased inflammatory response and subsequently regenerative repair. It has been shown that interleukin (IL)-6, IL-8, IL-1 and TNF-α are significantly lower in early fetal fibroblasts (Namazi et al., 2011). These cytokines are responsible for chemotaxis and activation of the inflammatory cells. Fetal PMNs seem to lack chemotactic activity of adult neutrophils (Kong et al., 2008). Later in the healing process, fetal macrophages are found in the wound, but they are present in lower numbers than in adults (Adzick et al., 1985).

2.3.1.vi Homeobox genes

More recent studies focus on the genetic level of the fetal tissue regulation and more specifically on the homeobox genes. Homeobox genes are transcription factors that seem to be responsible for pattern formation and cell type specification during development (Scott and Goldsmith, 1993).
During fetal skin development, eight homeobox genes are highly expressed. Fetal scarless repair is associated with two of them (HOX B13 and PRX-2) (Scott and Goldsmith, 1993; Stelnicki et al., 1997). Decreased expression of HOX B13 and increased expression of PRX-2 has been described in fetal skin (White et al., 2003; Mack et al., 2003; Namazi et al., 2011).

It is still unclear how these genes are associated with scarless wound healing, but their downstream targets include the promoter regions of genes of the TGF superfamily, cellular adhesion molecules, and cell surface proteins (Stelnicki et al., 1997).

2.3.2 Oral mucosal wound healing

Although healing in oral mucosal wounds hasn’t been studied as extensively as fetal wound healing, many observations have suggested that adult oral mucosal wounds heal more rapidly and with less or no scarring compared to cutaneous wounds. Recent studies have shown that oral mucosal wounds in pigs heal with significantly reduced clinical and histological scar formation when compared to similar wounds in skin (Mak et al., 2009; Wong et al., 2009). Differences in both extrinsic and intrinsic factors between adult oral mucosal and adult skin wounds have been documented (Toriseva and Kähäri, 2009).

2.3.2.1 Extrinsic factors

Saliva

Oral mucosal wounds are continuously bathed in saliva, which provides a unique environment in the mouth potentially promoting rapid tissue repair (Häkkinen et al., 2000). Saliva has features such as proper pH, ionic strength, and the presence of ions like calcium and magnesium that are beneficial for wound healing (Edgar, 1992). Saliva is a source of cytokines and growth factors, including epidermal growth factor and vascular endothelial
growth factor. The growth factor content of saliva together with its lubrication properties seem to prevent tissue dehydration and cell death, allow accelerated angiogenesis and increased breakdown of fibrin and tissue debris (Noguchi et al., 1991; Field and Kerstein, 1994; Zelles et al., 1995; Kilcullen et al., 1998). Results from studies on people with xerostomia or sialadenectomized animals show delayed healing of oral wounds, confirming the importance of saliva in the wound healing process (Epstein and Scully, 1992; Bodner and Dayan, 1995).

**Bacteria**

The oral cavity hosts more than 500 bacterial species. It seems that low bacterial concentrations can increase the rate of wound healing. Bacteria attract macrophages into the wound, promote cytokine secretion and accentuate blood supply and granulation tissue formation. This may lead to increased connective tissue synthesis and greater tensile strength of the contaminated wound (Laato et al., 1985; Kilcullen et al., 1998; Häkkinen et al., 2000).

**2.3.2.ii Intrinsic factors**

**Fibroblasts**

It seems that oral mucosa possesses a distinct fibroblast phenotype, which exhibits similar characteristics to fetal fibroblasts. Adult oral fibroblasts can remodel three-dimensional collagen matrices more efficiently than adult skin fibroblasts (Sloan, 1991). Besides, oral fibroblasts secrete more hepatocyte growth factor (HGF), more keratinocyte growth factor (KGF) and increased levels of MMPs (Stephens et al., 2001; Gron et al., 2002).

**Inflammation**

The inflammatory cell infiltrate shows lower levels in oral mucosal wounds compared to skin wounds, with a reduced recruitment of neutrophils, macrophages, T-cells and mast cells (Szpaderska et al., 2003; Mak et al., 2009; Wong et al., 2009). Wounds in the oral
mucosa showed reduced levels of IL-6, IL-8 and TGF-β1, compared to skin. At the same time, the level of TGF-β3 was elevated in oral wounds (Yang et al., 1996; Szpaderska et al., 2003; Schrementi et al., 2008). The reduced inflammation may be a result of faster resolution or a milder initial inflammatory response in oral mucosal compared to skin wounds (Mak et al., 2009).

Wound contraction

In a pig-model study, skin wounds showed severe contraction and distortion during the healing process, while oral mucosal wounds contracted only temporally and relaxed to their original size by day 46 after wounding (Mak et al., 2009).

Angiogenesis

Oral mucosal wounds showed less dramatic increases in blood vessel density than did skin wounds and their density appeared to be normalized earlier than in the skin wounds, both in pig and mouse models (Szpaderska et al., 2005; Mak et al., 2009).

The reason for this differential wound healing outcome in oral mucosa and skin is not completely clear. Taken together the previous mentioned factors, it seems that the earlier resolution of the inflammatory reaction or a mild inflammatory reaction and the reduced wound contraction are important parameters for scarless wound healing in oral mucosa.

2.4 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) constitute a large family of structurally and functionally related zinc- and calcium-dependent proteinases found in all species ranging from hydra to humans. Collectively, MMPs are capable of degrading most components of the extracellular matrix (ECM), including interstitial and basement membrane collagens, proteoglycans, fibronectin and laminin. Recently, it has been shown that MMPs can also
process a large number of non-ECM proteins, such as growth factors, cytokines, chemokines, cell receptors, serine proteinase inhibitors and other MMPs, and thereby regulate their activity (Maskos and Bode, 2003; Butler and Overall, 2009; Hadler-Olsen et al., 2010).

MMPs were first detected in 1962 as the enzymes responsible for the dissolution of tadpoles’ tails (Gross and Lapiere, 1962). Since then, it has been shown that MMPs participate in various biological processes associated with embryonic development, pregnancy, ovulation, growth, morphogenesis, regeneration and wound healing. MMP function has also been associated with prevention or provocation of diseases such as cardiovascular, autoimmune, neurodegenerative and various connective tissue diseases, periodontitis and cancer (Hulboy et al., 1997; Brinckerhoff and Matrisian, 2002; Sorsa et al., 2006; Gialeli et al., 2010; Hadler-Olsen et al., 2010).

Vertebrate MMPs have been categorized by their substrate specificity or classified by sequential numbers, which run from MMP-1 to MMP-28, omitting numbers 4–6. The MMP gene family in humans encodes 23 enzymes. MMPs can be divided into distinct groups, including collagenases (MMP-1, MMP-8 and MMP-13) that degrade structural collagens of types I-III; gelatinases (MMP-2 and MMP-9) that degrade mostly type IV collagen; stromelysins (MMP-3, MMP-10, MMP-11 and MMP-19) that are effective against non-collagen matrix molecules; matrilysins (MMP-7 and MMP-26), membrane-type MMPs (MMP-14, MMP-15, MMP-16 and MMP-17) and a group of other MMPs (Table 1) (Sternlicht and Werb, 2001; Overall, 2002).

Despite the diversity in structure and substrate specificity, MMPs have several key characteristics in common. First, they are all members of the “metzincin” zinc group of endopeptidases, named after the catalytic essential zinc ion and the conserved methionine
(Met) residue at the active site (Bode et al., 1993). Second, all MMPs are synthesized as latent pro-enzymes, that become activated after cleavage of the pro-domain. Lastly, they are all inhibited by tissue inhibitors of metalloproteinase (TIMPs) (Van Wart and Birkedal-Hansen, 1990).

2.4.1 Structure

The domain structure of MMPs is formed by various modules. Moving from the NH$_2$-terminal end to the C-terminal end, most MMPs contain a pre-domain, a pro-domain, a catalytic domain, a linker and a hemopexin (HPX)-like domain. These domains, motifs and modules of the MMPs are involved in interactions with other molecules and therefore, they determine substrate specificity, cell and tissue localization and activation of MMPs (Figure 1) (Hadler-Olsen et al., 2010).

2.4.1.i Pre-domain

MMPs are synthesized with a hydrophobic N-terminal signal peptide, also called pre-domain, which directs the enzymes to the secretory pathway. The pre-domain is cleaved upon insertion into the endoplasmic reticulum, leaving the latent pro-enzyme (Maskos and Bode, 2003).

2.4.1.ii Pro-domain

The MMP pro-domains have an egg-like shape, attached on its rounded side to the active site of the catalytic domain. The propeptide essentially consists of three mutually perpendicularly packed $\alpha$-helices and a segment connecting it with the catalytic domain. The pro-domain contains a “cysteine switch” PRCXXPD consensus sequence, whose cysteine blocks the catalytic zinc of the catalytic domain. The pro-domain is responsible for maintaining the latency of the enzymes (Van Wart and Birkedal-Hansen, 1990; Maskos and Bode, 2003).
2.4.1.iii Catalytic domain
Adjacent to the pro-domain, there is a characteristic catalytic domain, which is responsible for substrate binding. The catalytic domain consists of a five-stranded β-sheet and three α-helices that form an ellipsoid topology or a small active-site cleft. This cleft harbours the catalytic zinc. The active-site cleft is highly consistent among MMP family members, however, the substrate-binding groove narrows into a well-defined pocket, and variations in the residues that form this pocket can distinguish one MMP from another (Brinckerhoff and Matrisian, 2002; Maskos and Bode, 2003). Specifically, the catalytic domain of MMP-2 and MMP-9 has a large, cloverleaf-like fibronectin-like domain, made of three copies of fibronectin II (FnII)-like inserts. These fibronectin domains do not alter the overall structure of the catalytic domain, because they are inserted outwards. The fibronectin domain mediates the recognition of large ECM proteins such as type IV collagen, gelatin and elastin (Pickford et al., 1997; Morgunova et al., 1999; Brinckerhoff and Matrisian, 2002; Maskos and Bode, 2003).

2.4.1.iv Hemopexin (HPX)-like domain
The two matrilysins (MMP-7 and MMP-26) and MMP-23 consist only of the pro- and the catalytic domain. All the other human MMPs possess an additional C-terminal HPX-like domain that is involved in substrate and inhibitor recognition and binding and mediates molecular interactions. The HPX domain is linked to the catalytic domain via a hinge region. The hinge region is a sequence of 10-30 amino acids. Regarding MMP-9, this region contains 64 amino acids and is heavily O-glycosylated. MT-MMPs are linked to the plasma membrane via a transmembrane domain or by glycosyl-phosphatidyl-inositol (GPI) anchor, which are extensions of the HPX domain (Cao et al., 1995; Itoh et al., 1999; Maskos and Bode, 2003).
Table 1. Classification of the mammalian matrix metalloproteinases.

<table>
<thead>
<tr>
<th>MMP number</th>
<th>Common name</th>
<th>Major substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Collagenase-1</td>
<td>Type I and II fibrillar collagens</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Gelatinase A</td>
<td>Gelatin, collagen IV, V, VII, elastin, fibronectin, latent TGF-β1</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Stromelysin-1</td>
<td>Laminin, type IV collagen, elastin, E-cadherin</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin</td>
<td>Elastin, E-cadherin, syndecan-1, latent TNF, pro-α-defensins</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Collagenase-2</td>
<td>Type I and II fibrillar collagens</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatinase B</td>
<td>Gelatin, collagen IV, V, VII, elastin, fibronectin, latent TGF-β1, latent VEGF</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Stromelysin-2</td>
<td>Same as stromelysin-1</td>
</tr>
<tr>
<td>MMP-11</td>
<td>Stromelysin-3</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Metalloelastase</td>
<td>Latent TNF</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagenase-3</td>
<td>Type I and II fibrillar collagens</td>
</tr>
<tr>
<td>MMP-14</td>
<td>MT1-MMP</td>
<td>Fibrin, Syndecan-1, proMMP-2, γ2 subunit of laminin 5</td>
</tr>
<tr>
<td>MMP-15</td>
<td>MT2-MMP</td>
<td>Fibrin</td>
</tr>
<tr>
<td>MMP-16</td>
<td>MT3-MMP</td>
<td>Fibrin, Syndecan-1</td>
</tr>
<tr>
<td>MMP-17</td>
<td>MT4-MMP</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-19</td>
<td>Stromelysin-4</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-20</td>
<td>Enamelysin</td>
<td>Amelogenin</td>
</tr>
<tr>
<td>MMP-21</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-22</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-23</td>
<td>CA-MMP (Cysteine Array)</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-24</td>
<td>MT5-MMP</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-25</td>
<td>MT6-MMP</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-26</td>
<td>Endometase</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-27</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-28</td>
<td>Epilysin</td>
<td>ND</td>
</tr>
</tbody>
</table>

*MMP-4, -5 and -6 were not unique, since they were identical to MMP-2 and MMP-3; No mammalian homologue has been found for MMP-18. ND: not determined. (A modification of a table by Hadler-Olsen et al., 2010).*
Figure 1. Domain structure of MMP-1, -2, -9 and -13.
2.4.2 MMP regulation

As MMPs are extremely potent, their catalytic activity is regulated at three levels: 1) by controlling their expression at the levels of transcription, post-transcriptional modulation and protein secretion, 2) by extracellular proteolytic activation upon their release and 3) through the action of their natural tissue inhibitors. It is also considered that MMP activity is controlled by substrate availability and affinity (Manuel and Gawronska-Kozak, 2006).

2.4.2.1 Regulation of gene expression

The regulation of MMP gene expression is complicated and it is not completely clear yet. The two most important MMP gene promoters are the activator protein-1 (AP-1) enhancer element, which binds Fos–Jun heterodimers or Jun–Jun homodimers and the ETS (erythroblastosis twenty six) site, which is located close to the AP-1 enhancer. These two promoters cooperate to enhance transcription, but their activation is not enough to regulate MMP gene expression, as also other upstream elements seem to be required (Crawford and Matrisian, 1996; Benbow and Brinckerhoff, 1997). In addition, single nucleotide polymorphisms (SNPs) in the promoters might regulate MMP expression. For instance, the 5A/6A SNP in the MMP-3 gene influences MMP-3 expression, and the 5A allele is thought to contribute to atherosclerosis (Ye et al., 1996; Ye, 2000).

2.4.2.2 Activation

The unpaired cysteine (Cys) residue in the highly conserved ‘Pro–Arg–Cys–Gly–X–Pro–Asp’ sequence in the pro-domain of the latent MMPs forms a bridge with the catalytic zinc, that blocks the active site and prevents enzymatic activity. When Cys is “on” the zinc, the activity of the enzyme is “off”. The dissociation of the Cys from the zinc atom and the activation of the MMP is referred as “cysteine-switch” (Van Wart and Birkedal-Hansen, 1990; Brinckerhoff and Matrisian, 2002).
A proMMP is, therefore, activated when the pro-domain is physically delocalized from the catalytic site. Activation of MMPs can be achieved either by proteolytic cleavage and removal of the pro-domain or by dislocation of the pro-domain, without being cleaved resulting in disruption of the Cys-Zinc bridge.

**Allosteric activation**

The allosteric activation of MMPs can be achieved by a variety of methods, including S-reactive agents, organomercurials, reactive oxygen species, heavy metals, chymase, trypsin, peroxynitrine, glutahnione, neutrophil gelatinase associated lipocalin (HNL) and p-aminophenylmercuric acetate (APMA). Another mechanism is the induction of conformational changes through binding of chaotropic agents and detergents such as sodium dodecyl sulphate (SDS). The above agents interact with the cysteine residue in the pro-domain and promote structural changes, which are followed by autocatalytic degradation of the prodomain (Van Wart and Birkedal-Hansen, 1990; Nagase, 1997; Hadler-Olsen et al., 2010).

**Proteolytic activation**

Proteinases can cleave the prodomain in one or several steps, producing an active MMP with reduced molecular size. A large number of proteinases and other enzymes can cooperate and activate the MMPs (Nagase, 1997; Hadler-Olsen et al., 2010).

One example of this cascade process is the activation of pro-MMP-2 through the precise intermolecular interaction with TIMP-2 and MT1-MMP. It is a two-step process, where TIMP-2 is the linkage between the two MMPs. In brief, TIMP-2 forms a complex with MT1-MMP on the cell-surface that results in inactivation of MT1-MMP. That complex acts as a cell-surface-bound “receptor” for pro-MMP-2, since the C-terminal part of TIMP-2
binds to the HPX domain of the pro-enzyme. An adjacent, uninhibited MT1-MMP molecule cleaves the pro-domain of MMP-2 and a 64kDa inactive intermediate is released. This intermediate will be autocatalyzed into a fully active 62kDa MMP-2 molecule (Kinoshita et al., 1996; Zucker et al., 1998; Itoh and Seiki, 2006). In addition, MT2-MMP and MT3-MMP can activate pro-MMP-2 without the involvement of TIMP-2 (Morrison et al., 2001; Hadler-Olsen et al., 2010).

**Autoactivation**

Spontaneous autoactivation of MMPs has also been reported (Van Wart and Birkedal-Hansen, 1990). It has been shown that when pro-MMP-9 binds to a gelatin or to a type IV collagen-coated surface, it can cleave gelatin even with an intact pro-domain. However, the activity of the pro-enzyme was 10% of that of the active MMP-9 (Bannikov et al., 2002; Hadler-Olsen et al., 2010).

**Intracellular activation**

All the membrane-type MMPs and MMP-11, -21 and -28 contain a special Arg-Xaa-Arg/Lys-Arg sequence at the C-terminal end of the predomain. Furin, a serine proteinase belonging to the convertase family can cleave the pro-domain at this sequence. This intracellular cleavage of these MMPs in the trans-Golgi network leads to secretion of active enzymes at the cell surface (Maskos and Bode, 2003; Hadler-Olsen et al., 2010).

2.5 **Tissue inhibitors of matrix metalloproteinases (TIMPs)**

In mammals, there are four known endogenous tissue inhibitors of metalloproteinases (TIMPs) and they are designated as TIMP-1, -2, -3 and -4. TIMPs are natural inhibitors of the MMPs and they inhibit the MMP proteolytic activity with 1:1 stoichiometry (Toriseva and Kähäri, 2009). Apart from their inhibitory function, TIMPs form
complexes with the latent MMP forms (pro-MMPs) and they regulate their activation process. For instance, TIMP-2 and -4 are involved in the activation of pro-MMP-2, TIMP-1 in the activation of pro-MMP-9, and TIMP-3 in the activation of both above MMPs. Besides, TIMP-2 and -3 participate in the activation process of the membrane-type MMPs (MT-MMPs) (Wilhelm et al., 1989; Howard and Banda, 1991; Ward et al., 1991; Bigg et al., 1997; Buttler et al., 1999; Lambert et al., 2004).

Regarding their structure, TIMPs are 21 to 34 kDa proteins, consisting of two domains, the C-terminal and the N-terminal domain. The N-terminal domain is responsible for the inhibitory activity on the MMPs and the C-terminal domain takes part in the proenzyme activation process (Caterina et al., 1997; Maskos and Bode, 2003; Lambert et al., 2004).

TIMPs are secreted proteins, which are not bound to ECM. The only exception is TIMP-3, which is tightly bound to the ECM proteoglycans (Lambert et al., 2004; Toriseva and Kähäri, 2009). TIMPs are expressed by a variety of cell types and they are present in most tissues and body fluids. More specifically, TIMPs can be secreted by fibroblasts, epithelial and endothelial cells, osteoblasts, chondrocytes, smooth muscle cells and many tumor cells (Welgus et al., 1985; Cawston et al., 1986; Bord et al., 1999). Their secretion is stimulated by growth factors, including b-FGF, PDGF and EGF, serum, erythropoietin and cytokines, including IL-6 and IL-1 (Lambert et al., 2004).

The wedge-shaped TIMPs inhibit MMPs by inserting their conserved N-terminal segment into the entire length of the active-site cleft of catalytic domain of the MMP. The intermolecular contacts between the two proteins is restricted to the first five TIMP residues, Cys1 to Pro5, which bind to the MMP active-site cleft in a substrate/product-like manner,
rendering the MMP unable to bind to any other substrate (Gomis-Ruth et al., 1997; Fernandez-Catalan et al., 1998; Maskos and Bode, 2003).

TIMPs have been characterized as multifactorial proteins since they have a variety of other functions in addition to their interaction with MMPs. For instance, TIMPs show cell growth-promoting activity. TIMP-1 has been found to induce the growth of keratinocytes, chondrocytes, fibroblasts, epithelial, endothelial, lymphoid and myeloid cells (Bertaux et al., 1991; Hayakawa et al., 1992; Wang et al., 2002). Furthermore, TIMPs can modulate cell apoptosis, they have anti-angiogenic activity and they stimulate steroidogenesis (Takigawa et al., 1990; Boujrad et al., 1995; Lambert et al., 2004).

2.6 Matrix metalloproteinases and their inhibitors in wound healing

2.6.1 MMPs regulate the inflammatory process

MMPs are key regulators of multiple aspects of tissue repair and they are involved in all the stages of wound healing (Figure 2). MMPs participate in the regulation of inflammation through the control of chemokine activity, the establishment of chemotactic gradients and the extravasation of leukocytes into the wounded tissue.

2.6.1.i Direct chemokine activity regulation

Chemokines are chemotactic molecules that attract inflammatory cells, including neutrophils, monocytes and eosinophils, to the wound area (Borish and Steinke, 2003). There are two subfamilies of chemokines, the CC chemokines, which participate in monocyte chemotaxis and the CXC chemokines that are involved in neutrophil chemotaxis (Clark-Lewis et al., 1995; Zlotnik et al., 2006).
Figure 2. The MMPs that are involved in the different phases of wound healing. The most up-regulated MMPs in each phase are highlighted in bold.
Direct chemokine cleavage by MMPs can result in enhancement of their action, inactivation or antagonism of chemokine activities. MMP cleavage of CC chemokines most of the times leads to inactivation or reduction in chemokine activity. It has been shown that human recombinant MMP-2 can cleave chemically synthesized macrophage chemoattaractant protein-3 (MCP3) and alter its function by reducing its ability to promote chemotaxis (McQuibban et al., 2001; 2002). In a similar way, MMP-1, -3, -13 and -14 can cleave efficiently MCP1, MCP2, MCP3, MCP4 and RANTES and produce antagonist factors (Van den Steen et al., 2000; McQuibban et al., 2000; 2001; 2002).

On the other hand, CXC chemokines have more varied response to MMP cleavage. Several MMPs, including MMP-1, -2, -3, -9, -13 and -14 can cleave and process SDF1, resulting in a decrease in its chemotactic activity (Zhang et al., 2003). In contrast, when MMP-1, -2, -8 or -9 process and cleave IL-8 or LIX, it leads to potentiation of their chemotactic activity and increase in leukocyte influx (Van den Steen et al., 2000; 2003; Tester et al., 2007). It seems that of all MMPs examined, MMP-1, -3 and -9 can regulate most efficiently chemokine signalling and either augment or dampen inflammatory processes (Gill and Parks, 2008).

2.6.1.ii Establishment of chemokine gradients

MMPs can be involved in chemokine regulation indirectly by acting on other substrates that bind, retain or concentrate the chemotactic molecules (Li et al., 2002).

Chemotactic gradients are formed when chemokines bind to accessory macromolecules on the cell surface, i.e. the glycosaminoglycan side chains of proteoglycans. MMPs can act on these accessory molecules, release the chemokine-proteoglycan complex and provide directional cues to migrating leukocytes (Parks et al., 2004).
Tissue injury promotes chemokine secretion from epithelial cells. Epithelial cells, therefore, secrete CXCL8 in humans and CXCL1 in mice. These chemokines bind to the heparan sulphate chains of pre-existing syndecan-1 molecules. Epithelial cells also secrete MMP7, which can cleave the chemokine/syndecan-1 complex from the epithelial cell. The shed complex moves across the epithelium and creates a chemotactic gradient that guides neutrophils into the injured area (Dunsmore et al., 1998; Li et al., 2002).

2.6.1.iii Extravasation

MMPs can process components of the tight junctions of endothelial cells. Cleavage of occludin and VE-cadherin by MMP-2 and -9 can increase endothelial permeability and promote leukocyte extravasation (Alexander and Elrod, 2002).

2.6.1.iv Cytokine activation

MMPs can regulate the inflammatory process by activation of various cytokines, including VEGF, FGF, EGF and TGF-β1 (Parks et al., 2004). TGF-β is released in a latent form. Cleavage of the latent TGF-β binding protein (LTBP) results in releasing and activation of TGF-β. Extracellular matrix extracts from rat chondrocytes, containing TGF-β1, were treated with recombinant human active MMP-2 and -3. It was shown that both MMP-2 and -3 were able to activate TGF-β1 (Maeda et al., 2002). In similar studies on rat cells, it was shown that MMP-9 and MMP-14 are able to activate TGF-β1, as well (Yu and Stamenkovic, 2000; Karsdal et al., 2002). Studies have shown that MMP-2, -3 and -9 can activate IL-1β (Gearing et al., 1994). In contrast, other studies suggest that MMP-1, -2 and -9 can inactivate IL-1β, giving a dual role for MMPs in interleukin regulation (Ito et al., 1996).

2.6.2 MMPs in epithelial repair

Re-epithelialization requires the cells at the wound margin to disrupt their cell–cell and cell–ECM adhesions and to migrate across the wound (Singer & Clark, 1999). MMP-1, -
3, -7, -9, -10 and -14 have been associated with the re-epithelialization (Gill and Parks, 2008). It seems that MMP-1 is one of the most important MMP in re-epithelialization. The contact of the keratinocytes with type I collagen increases MMP-1 expression (Sudbeck et al., 1997). The expression of MMP-1 reaches its peak 24 hours after wounding and decreases by completion of re-epithelialization and establishment of new basement membrane (Saarialho-Kere et al., 1993; Inoue et al., 1995).

Studies with human keratinocytes have shown that MMP-1 facilitates keratinocyte migration over the dermal matrix by lowering the affinity of collagen–integrin contacts (Pilcher et al., 1997). The mechanism, briefly, involves integrin α2β1, which serves as a cell-surface receptor for extracellular matrix components, and it is highly adhesive for collagen type I, but less adhesive for gelatin. The binding of MMP-1 to cell surface integrin α2β1 in keratinocytes induces collagenolysis, leading to the cleavage of type I collagen and formation of protein fragments. These fragments at body temperature denature to gelatin, allowing for keratinocyte migration (Pilcher et al., 1997; Dumin et al., 2001).

Both stromelysins are highly expressed in epidermal cells during wound healing. More specifically, MMP-3 seems to be expressed mostly by the basal keratinocytes behind the tip of migrating cells, whereas MMP-10 is produced exclusively at the edge of the keratinocyte sheet, suggesting that these two MMPs serve distinct functions in re-epithelialization (Madlener et al., 1998; Rechardt et al., 2000; Gill and Parks, 2008).

MMPs can also expose cryptic functional sequences (matricryptins). For instance, laminin-5, collagen type IV or tenascin-C cleavage by MMP-14 can expose matricryptins, such as EGF-like sequences that can stimulate cell proliferation and migration in the wound area (Tran et al., 2005).
Studies on human lung tissue showed that MMP-7 is expressed by wound-edge epithelial cells and is involved in the cleavage of E-cadherin within the epithelial junctions, which facilitates the migration of epithelial cells (McGuire and Parks, 2003).

MMP-9 is produced mostly at the migrating epithelial front, promoting keratinocyte migration (Salo et al., 1994; Madlener et al., 1998; Mirastschijski et al., 2002).

2.6.3 MMPs in tissue remodeling

2.6.3.i Wound contraction/myofibroblasts
Defective wound contraction was observed in mice lacking MMP-3, together with abnormal organization of actin bundles in myofibroblasts. The decreased wound contraction led to increased wound size and slowed wound healing (Bullard et al., 1999).

2.6.3.ii ECM degradation
Originally, it was thought that the degradation of the ECM was the primary function of MMPs in wound healing, but recent studies on mice that express collagenase-resistant collagen have shown that wounds heal without differences in scar formation compared to similar wounds in control animals, suggesting that MMPs are not necessarily essential for remodeling during wound healing (Beare et al., 2003).

On the other hand, the ability of MMPs (mainly MMP-2, MMP-9 and MMP-14) to degrade ECM proteins has been demonstrated (Gill and Parks, 2008). MMPs are involved in remodeling the ECM either directly by proteolytic degradation of proteins, such as collagens, or indirectly via their ability to affect cell behaviour (Gill and Parks, 2008).

MMP-2 is expressed only in the connective tissue, by fibroblasts and endothelial cells and its expression seems to be long lasting and stable, indicating involvement in the prolonged ECM remodeling (Agren, 1994; Madlener et al., 1998). MMP-2 and -9 are localized to the epithelial-stromal interface behind the migrating epithelial cells, which
suggests that they may be involved in remodeling of the stroma and reformation of the basement membrane (Mulholland et al., 2005).

### 2.6.4 MMPs in scar tissue

The wound healing process in scars is abnormal, resulting in an imbalance between production and degradation of extracellular matrix (ECM) components (Tredget et al., 1997). However, the precise mechanisms by which scar tissue fibroblasts lead to an imbalance between production and degradation of ECM components through their own expression of MMPs and TIMPs have not been fully investigated (Imaizumi et al., 2009).

Human hypertrophic scar fibroblasts showed reduced levels of MMP-1 mRNA compared to normal tissue fibroblasts (Ghahary et al., 1996). Another study showed low expression of MMP-1 in human keloid tissues, while MMP-13 was up-regulated in the same samples. The expression of MMP-13 by keloid fibroblasts may reflect their attempt to remove the excess collagen in tissue and compensate for the low MMP-1 levels (Kuo et al., 2005; Toriseva and Kährä, 2009). In contrast, in another study it was shown that the production of type 1 collagen, MMP-1, MMP-2, and TIMP-1 by human keloid fibroblasts was 3-fold, 6-fold, 2.4-fold, and 2-fold greater than that of normal dermal fibroblasts, respectively (Fujiwara et al., 2005). In human tissue samples from hypertrophic scars, keloids and normal skin, it was shown that MMP-2 activity is significantly increased in hypertrophic scars and keloids, whereas very low MMP-9 activity is present in keloids and hypertrophic scars (Neely et al., 1999). A recent study showed no differences in MMP-9 expression between scarred and non-scarred human tissue, whereas at the same time MMP-2 and TIMP-1 and -2 were significantly increased in scar tissue compared to non-scarred tissue. The results indicated disturbed balance locally in scar tissue with a lower ratio of MMP to TIMP expression (Ulrich et al., 2010). A study on human scar tissue that took into consideration the
active to latent MMP form ratio showed that the highest active/latent ratio was found in keloids followed by hypertrophic scars and normal skin (Tanriverdi-Akhisaroglu et al., 2009).

The expression of MMPs and TIMPs in scar tissue seems to be affected by the time as well. In 2007, Xie et al. investigated the gene expression of MMP-2, MMP-9 and TIMP-1 in proliferative and mature hypertrophic scars in humans. Proliferative hypertrophic scars showed an increase in MMP-2, MMP-9 and TIMP-1 expression, whereas mature hypertrophic scars showed high expression of TIMP-1 and low expression of MMP-2 and -9. The high expression of TIMP-1 in scar tissue could be a key factor for pathological scar formation, while the lowering of MMPs could be associated with the maturation of hypertrophic scars. Similarly, another study on human keloid tissue samples demonstrated significantly increased expression of MMP-2, TIMP-2 and TIMP-3 in keloids compared with mature scars. Enhanced expression of MMP-2 specifically in collagen bundle regions suggests that the degradation of ECM is promoted in collagen bundle regions through increased MMP-2 activity in keloid fibroblasts. This could contribute to remodelling of collagen bundle regions and invasion of fibroblasts into peripheral normal regions through promoted degradation of ECM (Imaizumi et al., 2009).

2.6.5 MMPs in chronic wounds

Chronic wounds are characterized by delayed, insufficient or missing wound closure, pathologic inflammation and uncontrolled proteolysis. Factors that can increase the risk of chronic ulceration are increasing age, diabetic vasculopathy, venous insufficiency and arteriosclerosis (Menke et al., 2007). Studies on non-healing chronic ulcer have shown that the activity of MMPs is upregulated while the expression of TIMPs is decreased compared to acute wounds. MMP-1, -2, -8 and -9 levels are elevated in wound fluid of chronic ulcers.
(Wysocki et al., 1993; Yager et al., 1996; Rayment et al., 2008). Non-healing chronic ulcers also show lower TIMP-1 and TIMP-2 levels, as compared to normally healing wounds, resulting in defective inhibition of MMP activity and defective pro-MMP-2 activation (Bullen et al., 1995; Vaalamo et al., 1999). In contrast to fibroblasts in acute wounds, TIMP-4 is expressed in chronic wounds (Vaalamo et al., 1999).

2.6.6 TIMPs in wound healing
TIMPs and especially TIMP-3 seem to be involved in the regulation of the inflammatory response through control of cytokine signaling and inflammatory cell adhesion receptor processing. Briefly, TIMP-3 can inhibit ADAM17. In the absence of TIMP-3, ADAM17 activity is up-regulated leading to increased TNF-α release and an increase in the inflammatory cell infiltration (Amour et al., 1998; Mohammed et al., 2004). Absence of TIMP-3 also leads to an increase in vascular cell adhesion molecule-1 (VCAM-1) shedding from the endothelial cells and increased leukocyte recruitment (Singh et al., 2005). TIMPs can regulate aspects of cell migration, by restraining the activity of specific MMPs. Excess TIMP-1 levels can lead to impaired epithelial cell migration (Chen et al., 2006). Besides, TIMP-3 can inhibit angiogenesis (Qi et al., 2003). Altered collagen and fibronectin remodeling have also been reported in the absence of TIMP-3 (Gill and Parks, 2008).

2.7 MMPs in scarless wound healing

2.7.1 MMPs in fetal scarless wound healing
Regarding the contribution of MMPs to scarless wound healing, the results of the limited amount of existing studies are not conclusive and quite controversial. In unwounded fetal skin, it seems that an up-regulation of certain MMPs takes place during gestation, when
there is a transition from scarless healing to scar forming healing. For instance, Peled and coworkers (2002) compared MMP and TIMP expression on intact fetal rat skin during the scarless (until the 16th gestational day) and the scarring period (past the 18th gestational day). They found that during the transition to the scar forming period, MMP-1, MMP-3 and MMP-9 showed a 2- to 4-fold upregulation. At the same time, MMP-2, MT-MMPs and TIMPs remained relatively constant and did not change as a function of gestational age. A similar study was done by Chen and coworkers (2007) on humans. Human fetal upper back skins from 13 to 33 weeks of gestation were obtained from spontaneously aborted foetuses. The levels of gene expressions and protein content for MMP-2, -9 and -14 and TIMP-1 and -2 were significantly higher in late gestational and adult skins than that in early gestational skin (Chen et al., 2007).

On the other hand, in a study by Dang et al., (2003), excisional wounds were created on the dorsum of fetal rats both during the scarless and the scarring gestational period. An increase in MMP/TIMP ratio was associated with scarless wound healing. More specifically, the maximal increase in MMP-1 and MMP-9 mRNA expression occurred much more rapidly and was much greater in the scarless wound healing. Scarless wounds reached the maximum MMP-1 expression of 28-fold increase at 48h after wounding, whereas scarring wounds reached the maximum MMP-1 expression of 23-fold increase at 72h post-wounding. MMP-9 levels followed the same pattern with scarless wounds expressing a 18-fold increase at 48h when scarring wounds showed only a nine-fold increase at 72 post-wounding. MT-1 MMP presented a three-fold increase in its expression at 72h in scarless wounds, whereas it remained unchanged in scarring wounds. MMP-2 remained unchanged in scarless wounds, but a 4-fold decrease was noticed at 72h in scarring wounds. On the other hand, TIMP-1 and
TIMP-3 expression in scarring wounds increased six-fold and four-fold, respectively (Dang et al., 2003).

Other studies on rats confirmed the previous results. MMP gene expression in fetal scarless skin wounds showed greater increase compared to scarring wounds. Furthermore, the expression of TIMPs was higher in the scarred wounds, resulting in MMP/TIMP activity ratio that was higher in scarless wounds (Lorenz et al., 2001). A histological study, comparing human fetal skin (18th week of gestation) to adult skin, showed that MMP-1, -2 and -3 are expressed at similar locations both in fetal and adult skin, but fetal cells showed higher expression levels (Bullard et al., 1997).

To summarize, differences in MMP activity in fetal wounds can probably affect the MMP/TIMP ratio, allowing faster ECM turnover and promoting a scarless healing pattern (Bullard et al., 2003).

2.7.2 MMPs in oral mucosal scarless wound healing

Except for studies on oral mucosal wound healing alone or studies on the differential expression of MMPs between oral and skin fibroblasts (cell cultures), no studies comparing MMP expression in oral mucosal versus skin wounds have been published to our knowledge.

Salo and coworkers in 1994 created experimental wounds in human oral mucosa and biopsies were taken 1, 3, and 7 days after wounding. In situ hybridization demonstrated an exclusive expression of MMP-2 mRNA in the connective tissue fibroblasts and endothelial cells during all phases of wound healing. The epithelium gave no signal for MMP-2, while MMP-9 was expressed in mucosal epithelium during the entire experiment period. The basal cell layer of the migrating epithelial sheet was positive for MMP-9, whereas both basal and suprabasal cells were positive for MMP-9 in the nonwounded area. A strong MMP-9 signal was also detected within the granulation tissue seven days after wounding. The presence of
MMPs at protein level was tested with zymography. The relative amount of MMP-9 increased up to 2-fold from day 1 to 4. It was concluded that during oral mucosal wound healing, MMP-9 seems to promote keratinocyte migration and granulation tissue remodeling, whereas the expression of MMP-2 remains stable over time (Salo et al., 1994).

The expression of MMP-1 was also up-regulated during the entire healing period when experimental full thickness wounds were created in human oral mucosa. More specifically, MMP-1 was about 40 times up-regulated on the 1st post-wounding day, 22 times up-regulated on the 3rd post-wounding day and 17 times on the 7th day after wounding (Eslami et al., 2009).

In another study, the proteolytic activity of oral fibroblasts was compared to that of the adult skin fibroblasts. Oral fibroblasts exhibited markedly elevated activation of MMP-2 and lower expression of TIMP-1 and -2 compared to skin fibroblasts (Stephens et al., 2001).

Both gingival and fetal skin fibroblasts express MMP-13 when they are treated with TGF-β1, unlike adult skin fibroblasts, suggesting an important role for MMP-13 in rapid turnover of collagenous matrix during repair of gingival wounds which heal with minimal scarring (Ravanti et al., 1999).

2.7.3 MMPs in FOXN1 deficient nude mice scarless wound healing

FOXN1 is a transcription factor, regulating the development of hair and thymus. Mutations in its gene are characterized by lack of visible hair, athymia and immunodeficiency. Recently, it was shown that FOXN1 deficient (nude) mice exhibit a scarless skin wound healing process of skin that resembles regeneration. On a study conducted on FOXN1 deficient nude mice and control wild-type mice, full thickness dorsal skin wounds were created and followed for 36 days. Accelerated wound healing process, minimal granulation tissue formation and markedly diminished scarring was noticed in the
nude knock-out mice. MMP-9 and MMP-13 showed a distinctive, bimodal pattern of up-regulation during the early (day 1-5) and late (day 21-36) phases of wound healing in the nude mice, that was associated with scarless wound healing (Gawronska-Kozak, 2011).

### 2.7.4 MMPs in limb regeneration

Newts and other salamanders, hydra, and sea cucumbers show a remarkable ability to regenerate their limbs, tails, jaws, spinal cords, lenses, retinas, and heart ventricles (Butler and Ward, 1967; Brockes and Kumar, 2002). The regenerative ability has been attributed to an unusual degree of cellular plasticity near the site of injury. MMPs seem to be up-regulated and involved in the early phases of limb regeneration (Yang and Bryant, 1994; Park and Kim, 1999). Collagenolytic activity has been confirmed in the regenerating limbs of newts but absent in non-regenerating limbs (Grillo et al., 1968).

A recent study by Vinasrky and coworkers (2005) showed that all newts treated with the MMP inhibitor GM6001 either failed to regenerate their limbs or regenerated abnormal limbs, while newts treated with the vehicle alone exhibited no impairment in regeneration, suggesting that MMPs are required for normal newt limb regeneration. It was also shown that MMP-3 and -9 expression and activity were highly elevated within hours of limb amputation, suggesting that these proteases play an important role in the initial stages of limb regeneration.
3. AIM

The aim of the study was to compare the abundance of the major collagenases MMP-1 and MMP-13 and gelatinases MMP-2 and MMP-9 in normal unwounded oral mucosa and skin and in experimental excisional wounds in skin (wound healing results to scar formation) and oral mucosal (wounds heals with minimal scar formation) at day 3, 14 and 28 post wounding at the protein level. To this end, a well characterized pig wound healing model was used (Wang et al., 2000; Sullivan et al., 2001; Wong et al., 2009).

We hypothesized that:

- Unwounded oral mucosa and skin show distinct abundance of MMPs.
- The abundance of MMPs is higher in scarless oral mucosal wound healing, compared to skin wound healing.
- MMPs accumulate earlier in scarless oral mucosal wounds as compared to skin wounds.
4. MATERIAL AND METHODS

4.1 Experimental wounding

The study involved twelve juvenile, female red Duroc pigs that were obtained from the Neufeld Farm (Acme, AB, Canada). The pigs were group-housed at the University of Calgary Animal Care Facility with 12-hour light/dark cycles and were fed antibiotic-free food and water *ad libitum*. All the procedures were performed according to the guidelines of the Canadian Council of Animal Care and were approved by the Animal Care Committee of the Faculty of Medicine (University of Calgary). The experimental wounding was performed as previously described (Mak et al., 2009; Gallant-Behm et al., 2008; Wang et al., 2001). In brief, before wounding, the pigs were premedicated with ketamine (15 mg/kg, i.m.) and acepromazine (0.4 mg/kg, i.m.). General anesthesia was induced by administering 1-2% isofluorane by mask. Two identical full-thickness excisional wounds (15 mm x 27 mm) were prepared on the oral mucosal gingiva of the hard palate (one on the right and one on the left side) on each pig, by means of a scalpel. The incision was done near to the depth of the palatal bone and the tissue was removed leaving some soft tissue above the periosteum. The oral mucosal wounds were left to heal untreated without wound dressing. In addition, ten dorsal full-thickness skin wounds were prepared on each side of the spine of each pig, identical in size to those in the oral mucosa. The skin wounds were 20 mm apart from each other and included the epidermis, dermis and subcutaneous fat, leaving the underlying fascia exposed. The skin wounds were sprayed with gentamycin and were bandaged for 72 hours. The pigs were divided in two groups. Six pigs received the 3-day and 28-day wounds on the left and right side, respectively, whereas the other six pigs received the 14-day and 60-day wounds, respectively. That resulted in 6 parallel oral mucosal wounds and 60 skin wounds at
each time point in 6 different animals. The tissue sample were, therefore, collected on day 0 (unwounded tissue), day 3, day 14, day 28 and day 60 after wounding. The biopsies included 3mm of surrounding tissue and were embedded in Optimal Cutting Temperature Compound (Tissue-Tek®, Sakura Finetek Inc., Torrance, CA, USA) and were immediately frozen in liquid nitrogen. These samples were used for histological analysis. Another part of the biopsy sample was grinded into a powder form in liquid nitrogen and stored in cryotubes at -86°C until further use for MMP and protein analyses. Only the samples from days 0, 3, 14 and 28 were used for MMP and protein analyses, since previous gene-array experiments on these samples has shown that by day 60, the mRNA expression of MMPs is equal in skin and oral mucosal wounds samples and has returned to the levels of the unwounded tissue (unpublished data).

In order to assess wound healing and scar formation clinically, standardized digital images were taken of the unwounded tissue and from the wounds immediately after wounding and at days 3, 14 and 28 after wounding. The images were calibrated by using a standard ruler placed in the frame (Mak et al., 2009).

4.2 Sample preparation

For isolation of total proteins, three oral mucosa and three skin samples from parallel pigs were used for each time point, including day 0 (unwounded tissue), day 3, day 14 and day 28. Briefly, 10 mg (wet weight) of powdered frozen tissue sample was solubilized in 100μl of 1X SDS sample buffer (0.125 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 0.001% bromophenol blue), to the final concentration of 100 μg of wet weight tissue sample/μl of SDS buffer. The protein extraction was performed by sonication on ice for 2 minutes. The samples were then centrifuged at 5,000 rpm followed for 5 minutes to
sediment any residual undissolved tissue. The supernatant was collected, decanted to a fresh Eppendorf tube and stored at -86°C.

4.3 Fibroblast medium preparation
Red Duroc pig gingival fibroblasts were first cultured for 24 h in their regular growth medium (DMEM; Flow Laboratories, Irvine, CA, USA, containing 23 mM sodium bicarbonate, 20 mM HEPES (Gibco, Biocult, Paisley, UK), antibiotics (50 Pg/mL streptomycin sulphate, 100 U/mL penicillin), and 10% (v/v) heatinactivated fetal bovine serum (FBS) and then they were cultured for 48 h in DMEM without FBS, before the conditioned medium was collected. Five ml of conditioned medium was collected.

4.4 Zymography
Zymography was performed to evaluate the presence of gelatinolytic activity as previously described. To this end, 10% SDS-polyacrylamide gels impregnated with 1mg/ml gelatin were used. Equal amounts (5 μl) of the above described sample was loaded onto the gels and electrophoresed, without being heated, under non-reducing conditions. After electrophoresis, the gels were washed for 30 min in a 50 mM Tris buffer (pH 7.5) containing 2.5% Triton and 0.02% NaN₃. A second wash followed for 30 min in a similar solution further containing 5 mM CaCl₂ and 1mM ZnCl₂. The gels were then incubated in a 50mM Tris buffer (pH 7.5) containing 5 mM CaCl₂, 1 mM ZnCl₂ and 0.02% NaN₃ at 37°C overnight. The gelatin degradation was monitored and confirmed visually under ultraviolet light. The gels were fixed for 30 min with 7% acetic acid and 50% methanol in water and stained overnight with 0.2% Coomassie Blue. The zones of gelatinolytic activity appeared as negatively-stained bands in the dark background.
The different MMPs were identified based on their molecular weights as compared to a prestained protein molecular weight standard ladder (Precision plus protein prestained all-blue standard, Bio-Rad Laboratories, Hercules, CA, USA) that was run in each gel. Furthermore, 2.5 µl of red Duroc pig fibroblast conditioned medium, mixed with 2.5 µl of 1X SDS sample buffer, were run in each gel as a positive internal control for standardization of the results in the different zymograms.

The gelatin cleavage rate was quantified from the photographed gels with an image analysis software (Image J®, NIH) and it was expressed as relative pixel units that were normalized to the internal control (fibroblast conditioned medium).

4.5 Protein gels

In order to assess the total protein content, the samples were separated under non-reducing conditions on 4-15% Tris-HCl gradient gels (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts (5 µl) of the above described sample were loaded onto the gels and after electrophoresis, the gels were fixed for 30 min with 7% acetic acid and 50% methanol in water and stained overnight with 0.2% Coomassie Blue. An unstained protein molecular weight standard ladder (Precision protein unstained standard, Bio-Rad Laboratories, Hercules, CA, USA) was run in each gel.

The gels were photographed and protein amounts were quantified by densitometry using an image analysis software (Image J®, NIH). The total protein content of each sample was expressed as pixel units, relative to the 75 kDa and 50 kDa bands of the protein ladder in each gel.
4.6 Western-blot analysis

The samples were analyzed for the presence of MMP-1 and MMP-13 by Western blotting using specific antibodies. Equal amount (5 μl) of the above described sample was used. Before gel electrophoresis, 0.25 μl β-mercaptoethanol was added and then the samples were boiled for 5 min to denature the proteins. The samples were loaded on a 4-15% Tris-HCl gradient gels (Bio-Rad Laboratories) and after electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Hybond-ECL membrane, GE Healthcare Biosciences, Buckinghamshire, UK). The membrane was blocked with Odyssey blocking buffer (LI-COR Biosciences, Nebraska, USA) for 1 hour and then incubated overnight at +4°C with the relevant primary antibody. For detection of MMP-1, rabbit anti-human polyclonal antibody (1:500, in blocking buffer) was used ((N-17)-R: sc-8834-R, Santa Cruz Biotechnology, Santa Cruz, CA, USA). MMP-13 was detected by a sheep anti-human polyclonal antibody (1:1000, in blocking buffer) (L29/3, a generous gift by Dr. Murphy, Dept. of Oncology, Cambridge Research Institute, Cambridge, UK; Cowell et al., 1998). All the membranes were also probed with rabbit anti-human polyclonal antibody to beta-actin (ab8227, Abcam, Cambridge, USA) as an internal control. The membranes were then incubated for 1 hour at room temperature with the appropriate secondary antibody (1:20,000 in blocking buffer). The following secondary antibodies were used: IRDye® 680CW conjugated donkey anti-rabbit IgG (LI-COR Biosciences, Nebraska, USA), IRDye® 800CW conjugated donkey anti-sheep IgG (Rockland, Gilbertville, PA, USA) and IRDye® 800CW conjugated donkey anti-rabbit IgG (LI-COR Biosciences). The membranes were then washed with TBS-T (Tris-buffered saline with Tween-20) 4 times for 5 min each time at room temperature.
The fluorescence was detected with the Odyssey Infrared Imaging System (LI-COR Biosciences, Nebraska, USA) and the relative intensity of the protein signals was quantified with the Image J software (NIH). The different membranes were normalized to pixel density of the 75 kDa and 50 kDa bands of the protein ladder.

4.7 Statistical analysis
The results are presented as mean values ± SEM from the assessment of the three parallel wounds in each group. Comparisons between skin and oral mucosa at a given time point were performed using Student’s t-test, whereas multiple comparisons were performed by using one-way analysis of variance (ANOVA) (*p<0.05; **p<0.01).
5. RESULTS

5.1 Oral mucosal wounds show significantly reduced clinical scar formation as compared to skin wounds

Figure 3 represents standardized digital images of the unwounded tissue and from the wounds immediately after wounding and at days 3, 14 and 28 after wounding, that were taken for the assessment of the wound healing and the clinical scar formation. The healing of all wounds occurred without complications or wound infections. The third day after wounding coincides with the inflammatory phase of wound healing. At day 3, both oral mucosal and skin wounds were open and covered by a blood clot. The surrounding tissues were mildly erythematous. At day 14 (transition from the re-epithelialization/ granulation tissue phase to tissue remodeling phase), the oral mucosal showed no crust present and migrating epithelium had started to partially cover them. On the other hand, the skin wounds were still completely covered with a crust. Reduced erythema was noted compared to day 3. At day 28 (tissue remodeling phase of wound healing), the oral mucosal wounds were completely closed, and there were no clinical signs of inflammation. The skin wounds were still partially covered with a crust, whereas the uncovered wound area was covered with epithelium (Figure 3). The clinical and histological presentation of these wounds has been published previously (Mak et al., 2009).

5.2 Total protein content in oral mucosal and skin wounds

Equal wet-weight from pulverized frozen tissue samples solubilized in SDS-sample
Figure 3. Representative clinical images of wound healing over time in oral mucosa and skin. UW: unwounded tissue; TOW: images taken at the time of wounding. Numbers indicate time (days) post-wounding. The images were calibrated by using a standard ruler placed in the frame.
buffer were separated by electrophoresis using gradient SDS-Page gels (Figure 4). Densitometeric quantification of coomassie-stained gels showed that peak-abundance of proteins was reached at day three returning to the level of unwounded tissue at day 14 post-wounding in the oral mucosa. No significant difference in the total protein content was found between oral mucosa and skin at any time point (Figure 4 & 6).

Qualitative assessment of coomassie-stained protein bands in the gels (Figure 4) and densitometric scanning spectra (Figure 5) was then performed. Protein bands around 50 kDa and 15 kDa regions were consistently present in all samples at all time points (Figure 4 & 5). However, there was a notable reduction in the band intensities in all samples around 130 kDa region at day three compared to other time points. In addition, the intensity and number of protein bands at the 50 kDa region increased at day 14 and 28 samples compared to other time points (Figure 4 & 5). Comparisons between oral mucosa and skin samples showed a similar pattern of coomassie-stained protein bands, except for the presence of increased number of bands located at the 50 kDa region of oral mucosal day 14 and 28 wound samples (Figures 4 & 5).

5.3 The abundance of MMP-2 and MMP-9 in oral mucosal and skin wounds
The abundance of MMP-2 and MMP-9 was analyzed from the tissue extracts using gelatin zymography. In unwounded (day 0) oral mucosa and skin, the level of total MMP-9 was very low (Figure 7 & 8a). However, its abundance was significantly increased at day 3 and 14 after wounding, and returned to the level of unwounded tissue at day 28.
Figure 4. Coomassie-stained proteins from oral mucosa and skin samples obtained from unwounded tissue (day 0) and 3, 14 and 28 days after wounding. Equal amounts of wet weight from each sample were separated by gel electrophoresis on gradient SDS-Page gels followed by coomassie staining. The code of the animal and the wound are indicated at the bottom of each lane.
**Figure 5.** Densitometric scanning spectra of the total protein profiles from representative samples from unwounded oral mucosa and skin and from oral mucosal and skin wounds 3, 14 and 28 days post-wounding. Equal amounts of wet weight from each sample were separated by gel electrophoresis using gradient gels followed by coomassie staining and densitometry. The code of the animal and the wound are indicated at the bottom of each lane. OM: Oral mucosa; S: Skin.
Figure 6. Densitometric quantification of the total protein content in unwounded oral mucosa and skin (Day 0) and oral mucosal and skin samples collected 3, 14 and 28 days after wounding. Results show mean ± SEM of 3 parallel wounds at each time point. Statistical comparison between different samples was determined using ANOVA (**p<0.01).
Figure 7. Zymograms performed with samples from oral mucosa and skin, obtained from unwounded tissue (day 0) and 3, 14 and 28 days after wounding. Equal amounts of wet weight from each sample were separated by gel electrophoresis on 10% SDS-PAGE gels impregnated with 1 mg/ml gelatin, followed by coomassie staining. The latent and the active forms of MMPs are indicated, with three other areas of unknown gelatinolytic activity. The code of the animal and the wound are indicated at the bottom of each lane. FM: the fibroblast medium that was used as an internal control.
Figure 8. Densitometric quantification of the relative abundance of MMP-9 in oral mucosa and skin in the unwounded tissue and at 3, 14 and 28 days after wounding. (a) Total MMP-9; (b) Latent MMP-9; (c) Active MMP-9. Results show mean ± SEM of 3 parallel wounds at each time point. Statistical significance between oral mucosal and skin wounds at each time point was determined by ANOVA for multiple comparisons and student’s t-test for paired comparisons between oral mucosa and skin at each time point. (* p<0.05; ** p<0.01).
Figure 9. Densitometric quantification of the relative abundance of MMP-2 in oral mucosa and skin in the unwounded tissue and at days 3, 14 and 28 after wounding. (a) Total MMP-2; (b) Latent MMP-2; (c) Active MMP-2. Results show mean ± SEM of 3 parallel wounds at each time point. Statistical significance between oral mucosal and skin wounds at each time point was determined by ANOVA for multiple comparisons and student’s t-test for paired comparisons between oral mucosa and skin at each time point (* p<0.05; **p<0.01).
post-wounding. Oral mucosal wounds showed, however, the peak accumulation of total MMP-9 already at day 3, while in skin wounds it reached its highest level at day 14. The total abundance of MMP-9 was significantly elevated in oral mucosal compared to skin wounds at day three after wounding (Figure 7 and 8a). Similar pattern as for the total MMP-9 abundance was noted also for the latent and active forms of MMP-9 (Figure 7, 8b & 8c).

MMP-2 was present in unwounded tissue in both oral mucosal and skin wounds, but oral mucosa had a significantly higher content of total and latent MMP-2 compared with skin (Figure 7, 9a & 9b). For both oral mucosa and skin wounds, the level of total MMP-2 peaked on day 14. At day 28 post-wounding, the levels of MMP-2 remained significantly elevated for both skin and oral mucosal wounds compared to unwounded tissue (Figure 7 and 9). At day three, oral mucosal wounds showed significantly higher amount of total, latent and active MMP-2 as compared with corresponding skin wounds (Figures 7, 9a, 9b & 9c).

The total gelatinolytic activity, including total MMP-2 and -9 and the unknown gelatinolytic activity present at the 37, 150 and 250 kDa regions (Figure 7) was quantified by densitometry from the zymograms (Figure 10). Oral mucosal wounds exhibited maximum increase of gelatinolytic activity on day three post-wounding, and returned to the level of unwounded tissue at day 28 post-wounding. In contrast, gelatinolytic activity in skin samples reached its peak on day 14 post-wounding. The total gelatinolytic activity was significantly higher in the oral mucosal wounds compared to skin wounds at day three post-wounding (Figure 10).
Figure 10. Densitometric quantification of the relative total gelatinolytic activity in oral mucosa and skin in the unwounded tissue and at days 3, 14 and 28 days after wounding. Results show mean ± SEM of 3 parallel wounds at each time point. Statistical significance between oral mucosal and skin wounds at each time point was determined by ANOVA for multiple comparisons and student’s t-test for paired comparisons between oral mucosa and skin at each time point (* p<0.05; **p<0.01).
5.3 MMP-1 and MMP-13 abundance in oral mucosa and skin

5.3.1 MMP-1

Latent MMP-1 exists in 2 forms, the glycosylated 57 kDa pro-MMP-1 and the unglycosylated 52 kDa pro-MMP-1. Active MMP-1 exists in forms of 48 kDa and 42 kDa, with the 42 kDa MMP-1 representing the stable, active enzyme (Wilhelm et al., 1984; Wilhelm et al., 1986). In unwounded tissue (day 0), the latent forms of MMP-1 were mostly present in all samples. In 3-day and 14-day wounds, all the forms of MMP-1 were present and some of these bands were indistinguishable from each other. Unknown bands were also detected at 37kDa region, especially at day 14 and 28 after wounding (Figure 11).

The total MMP-1 abundance was quantified, including the latent forms and the active forms (Figure 11 & 12). Relatively low total MMP-1 levels were detected in the unwounded tissues. However, MMP-1 abundance was strongly increased during wound healing in both oral mucosa and skin. In the oral mucosal wounds, MMP-1 levels peaked already at day three while in skin wounds peak abundance was reached at day 14 post-wounding. Levels of MMP-1 remained significantly elevated still 28 days after wounding in both oral mucosa and skin wounds relative to unwounded tissue. At day three, oral mucosal wounds showed significantly higher levels of MMP-1 as compared to skin wounds (Figure 11 & 12).
Figure 11. Representative images of Western blots for MMP-1 in unwounded (day 0) oral mucosa and skin samples and at days 3, 14 and 28 postwounding. Equal amounts of wet weight from each sample were separated by gel electrophoresis on gradient SDS-PAGE gels, followed by probing with the appropriate primart and secondary antibodies. The 57 kDa latent form of MMP-1 is named as proMMP-1A, whereas the 52 kDa proMMP-1 is named as proMMP-1B. Similarly, the two active MMP-1 forms are named MMP-1A and MMP-1B. The unknown bands at 37 kDa are also indicated. The code of the animal and the wound are indicated at the bottom of each lane.
**Figure 12.** Densitometric quantification of the relative abundance of total MMP-1 in oral mucosa and skin in the unwounded tissue and at days 3, 14 and 28 after wounding. Results show mean ± SEM of 3 parallel wounds at each time point. Statistical significance between oral mucosal and skin wounds at the each time point was determined by ANOVA for multiple comparisons and student’s t-test for paired comparisons between oral mucosa and skin at each time point (**p<0.01).
5.3.2 MMP-13

The molecular weight of the proMMP-13 is 60–65 kDa. Active MMP-13 is 50–55 kDa, but is further cleaved into a final active form of 48 kDa (Freije et al., 1994; Knäuper et al., 1996). The latent forms of MMP-13 were mostly observed at day 0 and day 3. In contrast, multiple bands were detected at days 14 and 28 post-wounding, suggesting presence of all the forms of MMP-13. Unknown bands were detected by the specific antibody at 100kDa that were stronger on day 14. No significant differences were noticed in bands between oral mucosa and skin (Figure 13).

The total MMP-13 abundance was quantified, including the latent and the active MMP-13 forms. Both oral mucosal and skin wounds showed presence of MMP-13 in unwounded tissue (Figure 13 & 14). During wound healing, the abundance of total MMP-13 was significantly elevated at day three and 14 after wounding in both oral mucosa and skin, but returned to the level of unwounded tissue at day 28. No significant difference in the abundance of total MMP-13 between oral mucosa and skin wounds were noted at any time point (Figure 13 & 14).
Figure 13. Representative images of Western blots for MMP-13 in unwounded (day 0) oral mucosa and skin samples and at days 3, 14 and 28 postwounding. Equal amounts of wet weight from each sample were separated by gel electrophoresis on gradient SDS-PAGE gels, followed by probing with the appropriate primary and secondary antibodies. The MMP-13 bands were not easily distinguishable from each other and are indicated together as a group of total MMP-13. An unknown area is indicated as well. The code of the animal and the wound are indicated at the bottom of each lane.
**Figure 14.** Densitometric quantification of the relative abundance of total MMP-13 in oral mucosa and skin in the unwounded tissue and at days 3, 14 and 28 after wounding. Results show mean ± SEM of 3 parallel wounds at each time point. Statistical significance between oral mucosal and skin wounds at each time point was determined by ANOVA for multiple comparisons and student’s t-test for paired comparisons between oral mucosa and skin at each time point (* p<0.05; **p<0.01).
6. DISCUSSION

Proteases of the matrix metalloproteinase (MMP) family play an important role in regulation of several key cell functions in wound healing and scar formation, including inflammation, cell migration and growth, and extracellular matrix turnover (Bullard et al., 2003; Gill and Parks, 2008; Namazi et al., 2011). Importantly, altered inflammatory response and extracellular matrix turnover have been associated with scar formation following skin wound healing (Gill and Parks, 2008; Toriseva and Kähäri, 2009). In order to understand the role of MMPs in scar formation, we compared the abundance of key collagenases, MMP-1 and MMP-13, and gelatinases, MMP-2 and MMP-9, in scar-forming skin and scarless oral mucosal wounds in pigs. All studied MMPs showed a significantly increased accumulation in the wound tissue already at day three after wounding. Their abundance remained high until day 28 when they returned to the level of unwounded tissue. Interestingly, oral mucosal wounds showed in general a robust early up-regulation of MMP-1, MMP-2 and MMP-9 as compared to skin wounds already at day 3 after wounding. In contrast, the peak abundance of these MMPs occurred at day 14 in skin wounds. No differences were noted in the abundance of MMP-13 between these two tissues. In addition, unwounded oral mucosa showed significantly higher abundance of total MMP-2 and active MMP-9 as compared to unwounded skin. Thus, MMPs needed for early wound healing response are already present in higher abundance in oral mucosa as compared to skin before tissue injury possibly allowing a fast wound healing response.

MMPs are important in normal physiology and participate in various biological processes, including development, growth, regeneration and continuous normal modeling and
remodeling of connective tissues (Brinckerhoff and Matrisian, 2002; Hadler-Olsen et al., 2010). A consistent low level expression of MMPs has been detected in unwounded tissue (Toriseva and Kähäri, 2009). In our study, all studied MMPs were present in unwounded oral mucosa and skin. Additionally, the total MMP-2 and active MMP-9 amount was significantly higher in unwounded oral mucosa, compared to skin. This suggests that oral mucosa has higher baseline capacity for a faster wound healing response.

Originally, it was thought that the degradation of the ECM was the primary function of MMPs in wound healing. However, it has been shown that MMPs are involved in all the phases of wound healing, from the inflammatory phase and re-epithelialization to granulation tissue formation and matrix remodeling (Gill and Parks, 2008).

Inflammation is part of the first phase of wound healing, starting within hours from after wounding, reaches its peak around the third day after wounding and starts slowing down approximately after two weeks (Toriseva and Kähäri, 2009). Mostly MMP-1, and MMP-9 to a lesser extent, participate in the regulation of inflammation through the control of chemokine activity either by activating them and enhancing their action or by inactivating them (Van den Steen et al., 2000; McQuibban et al., 2000; 2001; 2002; Tester et al., 2007). MMP-2 and -9 are also involved in the extravasation of leukocytes into the wounded tissue (Alexander and Elrod, 2002). Various cytokines, including TGF-β, need the presence of MMP-2 for their activation, whereas IL-1β can be either activated or inactivated by MMP-1, -2 and -9 (Gearing et al., 1994; Ito et al., 1996; Karsdal et al., 2002; Maeda et al., 2002).

In the present study, MMP-1, -2 and -9 showed significantly increased accumulation in the wound tissue already at day three after wounding as compared to unwounded tissue. This early up-regulation of MMP-1, -2 and -9 at day three in oral mucosal wounds resulted in
MMP levels that were significantly higher compared to those of skin wounds as well. This increase coincides with the peak of the inflammatory process in wounds, suggesting that MMP-1, -2 and -9 may be involved with the regulation of the inflammation. MMPs have a dual function on chemokines (MCP1, MCP2, MCP3, MCP4, RANTES, IL-8) and cytokines (TGF-β1, IL-1β, VEGF, EGF) and they can either activate or inactivate them. Therefore, activation of specific chemokines might lead to earlier initiation of inflammation in the oral mucosal wounds as compared to skin, whereas inactivation of other chemokines could result in faster resolution and/or milder initial inflammatory response (Gearing et al., 1994; Ito et al., 1996; Van den Steen et al., 2000; Yu and Stamenkovic, 2000; McQuibban et al., 2000; 2001; 2002; Karsdal et al., 2002; Maeda et al., 2002; Parks et al., 2004; Tester et al., 2007).

These results are in accordance with the findings by Mak and coworkers (2009), who studied the wound healing process on the same animals that were used for the present study. It was shown that the macrophage numbers were significantly higher in the deep granulation tissue of the oral mucosal wounds, as compared to skin wounds at day 3. However, skin wounds reached the maximum number of macrophages at day 14 and their level remained unchanged till day 28 post-wounding, especially in the deep wound connective tissue. These findings on macrophages, together with the increased number mast cells in skin wounds as compared to oral mucosal wounds, suggest an earlier and milder inflammatory response in oral mucosa. This could be explained by early MMP-induced chemokine cleavage in the wound, causing their inactivation and impaired macrophage chemotaxis. This reduced inflammation could be a result of either faster resolution and/or milder initial inflammatory response (Mak et al., 2009). However, chemokine contents need to be verified.
Minimal and rapid inflammation, resulting in minimal scar formation in the oral mucosa has also been reported by Spaderska and coworkers (2003). They used a model of excisional wounds of equivalent size prepared on the tongue and skin of rats and it was found that significantly lower numbers of neutrophils, macrophages, and T cells, as well as reduced levels of the cytokines IL-6 and IL-8 were present in the rapidly healing oral wounds, suggesting a role for these mediators of inflammation in the scar production of wound healing (Spaderska et al., 2003).

There are three isoforms of TGF-β. TGF-β1 and β2 are the key factors to promote scar formation, whereas TGF-β3 appears to reduce scarring (Toriseva and Kähäri, 2009). Mak and coworkers (2009) showed that wound healing leads to a strong increase in TGF-β positive cells in both oral mucosal and skin wounds on days 14 and 28. No distinction was made regarding the relative abundance of the individual isoforms. On day 14 and 28, MMP-2 abundance was significantly high in both oral mucosal and skin wounds, suggesting possible contribution to TGF-β activation. It is unclear whether MMPs in oral mucosa favoured the activation of TGF-β3 or not.

Keratinocyte proliferation and migration starts one day after wounding and re-epithelialization is complete approximately in one to three weeks after wounding, depending on the size of the wound (Woodley, 1996; Toriseva and Kähäri, 2009). MMP-1 especially, but MMP-9 as well, have been associated with the re-epithelialization (Gill and Parks, 2008). The expression of MMP-1 by epithelial cells reaches its peak 24 hours after wounding and decreases by completion of re-epithelialization (Saarialho-Kere et al., 1993; Inoue et al., 1995). MMP-1 facilitates keratinocyte migration over the dermal matrix by lowering the affinity of collagen–integrin contacts (Pilcher et al., 1997). MMP-9 is produced mostly at the
migrating epithelial front, promoting keratinocyte migration (Salo et al., 1994; Madlener et al., 1998; Mirastschijski et al., 2002).

The robust early upregulation of both MMP-1 and MMP-9 in oral mucosal wounds compared to skin in the present study suggests that MMP-1 and -9 play an important role in the earlier re-epithelialization and cell migration. Mak and coworkers (2009) confirmed the earlier the re-epithelialization of the oral mucosal wounds compared to skin. More specifically, at day 14, no crust was present and the migrating epithelium had started to partially cover the oral mucosal wounds, whereas, skin wounds were completely covered with a crust. At day 28, the oral mucosal wounds were completely closed, in contrast to skin wounds that were still partially covered with a crust and migrating epithelium.

Tissue remodeling is the last phase of wound healing. It starts approximately on day 10 after wounding and follows for several months (Singer and Clark, 1999; Toriseva and Kähäri, 2009). Although it hasn’t been confirmed in vivo, MMPs seem to be involved in the remodeling phase of healing, since they are capable of degrading the existing collagen fibrils (Singer and Clark, 1999; Gill and Parks, 2008). MMP-2 is expressed only in the connective tissue, by fibroblasts and endothelial cells and its expression seems to be long lasting and stable, indicating involvement in the prolonged ECM remodeling (Agren, 1994; Madlener et al., 1998). MMP-9 may be involved in remodeling of the stroma and reformation of the basement membrane, but to a lesser extent compared to MMP-2 (Mulholland et al., 2005).

In the present study, MMP-2 reached its peak abundance on day 14 after wounding for both oral mucosal and skin wounds. Then, in both oral mucosal and skin wounds the amount of MMP-2 decreased on day 28 but to a level that was significantly higher compared to that of unwounded tissue. These findings suggest that MMP-2 may be involved in tissue
remodeling and collagen degradation. The fact that there was no significant difference at day 14 and 28 after wounding between oral mucosa and skin suggest that tissue remodeling is not the most important contribution of MMPs in scarless wound healing. Regulation of the inflammation and re-epithelialization may be the key factors in scarless wound healing.

MMP-1 abundance on day 28 is significantly decreased compared to day 14 for both skin and oral mucosa, but its level on day 28 remains significantly higher compared to that of the unwounded tissue. This suggests that MMP-1 may be involved in collagen degradation and tissue remodeling, besides its participation in the inflammation and re-epithelialization at the earlier stages of wound healing.

Another observation was that there was no quantitative difference in the peak abundances of MMP-1, -2 and -9 between oral mucosa and skin wounds. The only difference was that oral mucosal wounds reached the maximum MMP level on day three, whereas skin wounds reached it on day 14. This suggest that MMPs are equally important in the wound healing process in both oral mucosa and skin wounds, but it is the earlier up-regulation of MMPs in oral mucosa that may be associated with the scarless wound healing pattern.

Dang and coworkers (2003) used a different model to compare fetal skin wounds created in the scarless and in the scar forming period of the fetal development and measure the mRNA expression of various MMPs. MMP-1 and -9 expression level reached their maximum at 48 hours in the scarless group, whereas the scar forming group showed its maximal up-regulation in a later stage (72 hours). These findings agree with the results of the present study that showed, as well, that MMP-1 and MMP-9 reach their maximum levels first in oral mucosa scarless healing and skin scarred healing follows in later stages.
Proteins from frozen tissue samples need to be extracted efficiently and without degradation to ensure that an accurate representation of the proteins in the living tissue is obtained. Regarding the extraction technique, sonication and ball mill grinding seem to provide higher extraction yield than other means of tissue disintegration. The disadvantage of sonication is the aerosol that forms during sonication, resulting in a loss of about 7.5% of the material. Frozen tissue solubilization with SDS is the most common method to solubilise proteins for identity and quantity determination. A main disadvantage of the method is that it gives a rough measure of the amounts of different proteins in the mixture, without the ability to distinguish between proteins with similar apparent molecular weight. In addition, solubilization of tissue in SDS results in extraction of proteases as well. If the protein is sensitive to proteolysis, it is usually desirable to proceed quickly, and keep the extract cooled, to slow down proteolysis (Laemmli, 1970; Laemmli and Favre, 1973; Dignam, 1990; Harder et al., 1999).

Equal wet-weights from pulverized frozen tissue samples solubilized in SDS-sample buffer were separated by electrophoresis using gradient SDS-Page gels. The results from this protein analysis showed that fairly similar protein profiles were obtained from all parallel samples within given tissue. Some differences were detected between oral mucosa and skin, which could be expected based on our hypothesis. In addition, there were some differences that were consistent between parallel samples from the given tissues that occurred over time during wound healing. This was expected as well and it shows that the extraction of the proteins was appropriate.

Studies comparing oral mucosal and skin wound healing have been conducted on rodents or pigs. Rodents have loose skin and wound closure is achieved by contraction with
little scarring. On the other hand, pig’s skin structure is similar to human skin and the healing seems to follow the same mechanisms (Wang et al., 2000; Sullivan et al., 2001; Wong et al. 2009). In a recent publication, human palatal mucosal wounds were compared to pig palatal mucosa wounds. It was shown that human palatal mucosal wounds presented similar scarring both clinically and histologically with pig palatal mucosa wounds, suggesting that this animal model mimics human wound healing in the palatal mucosa. Red Duroc pigs could provide a useful model to study the regulation and the principles of scarless wound healing for finding novel ways for scar prevention in skin, with findings possibly applicable to humans (Wong et al., 2009).

One of the drawbacks of the present study could be considered the small number of samples per group. Another disadvantage is that the TIMP levels were not investigated. Since TIMP is the counterpart of MMP and responsible for both their activation and inhibition, studying TIMPs and their interaction with MMPs could result in better understanding of the MMP contribution in scarless wound healing. Furthermore, finding out the levels of the other MMPs could elucidate more in detail the participation of MMPs in scarless wound healing.

Regulation of inflammation by MMPs, together with faster re-epithelialization could be beneficial for the wound healing outcome and could be a key element of scarless wound healing. Differences in the local concentrations of chemokines or proteases, binding of MMPs to GAGs, environmental conditions e.g., pH, and the fact that other chemokines are activated by MMPs and others are inactivated are factors that need to be considered as well in the MMP induced chemokine cleavage process (Wolf et al., 2008).
7. CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

1. Unwounded oral mucosa showed significantly higher abundance of total MMP-2 and active MMP-9 as compared to unwounded skin. Thus, MMPs needed for early wound healing response are already present in higher abundance in oral mucosa as compared to skin before tissue injury, possibly allowing a fast wound healing response.

2. All studied MMPs showed a significantly increased accumulation in the wound tissue already at day three after wounding. Their abundance remained high until day 28 when MMP-9 and -13 returned to the level of unwounded tissue or close to that, while MMP-1 and -2 remained significantly elevated.

3. Oral mucosal wounds showed in general a robust early up-regulation of MMP-1, MMP-2 and MMP-9 as compared to skin wounds already at day 3 after wounding. In contrast, the peak abundance of these MMPs occurred at day 14 in skin wounds. No differences were noted in the abundance of MMP-13 between these two tissues.

4. MMP-1 and -9 may regulate the inflammatory reaction in the early stage of healing, resulting in faster resolution and/or milder initial inflammatory response to oral mucosal wounds. The regulation of the inflammation together with the faster MMP-1 and -9-induced re-epithelialization may be associated with the scarless healing pattern that was noticed in oral mucosal wounds.
7.2 Future directions

Comparing expression of MMPs in scar forming skin and scarless oral mucosal wound healing may provide novel therapeutic targets to prevent scar formation in skin. For a better understanding of the contribution of MMPs in wound healing, more MMPs need to be studied. MMP-3, -7 and -14 are involved in the regulation of the inflammatory process. MMP-3 is also involved in re-epithelialization, fibroblast differentiation and wound contraction. Therefore, future research on these MMPs and function in wound healing might benefit the understanding of scarless wound healing.

TIMP is the counterpart of MMP and responsible for both their activation and inhibition. Research towards this direction may benefit as well the field of scarless wound healing. Areas like MMP gene expression and their transcription factors are still unclear as well.

Furthermore, MMPs are involved in various physiological procedures and, therefore, their up- or down-regulation has been associated with various diseases. A better understanding of how MMPs influence complex biological processes will make it possible to use them as therapeutic targets for diseases like cancer, rheumatoid arthritis, periodontitis and others.
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