GENE EXPRESSION PROFILES DURING SCARLESS HEALING OF HUMAN PALATAL MUCOSA

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ABSTRACT

Scarless healing of adult wounds is not well understood. Unlike human skin, oral masticatory mucosa heals largely by regeneration rather than scar formation. For a better understanding of healing by regeneration, we compared gene expression profiles of human mucosal wounds 1-, 3-, and 7-day-postwounding to that of normal non-wounded mucosa in three healthy volunteers. Clinical and histological healing was followed in additional volunteers for up to 60 days. The majority of the mucosal wounds healed with regeneration as the wounds at 60 days or later were no longer clinically or histologically detectable. At day one, a large number of genes showed a change in expression (988 genes). Expression levels of extracellular matrix proteins were down-regulated while genes in several categories including cytokines, cell motility and immune response were up-regulated. Gene ontology analysis of 3-day-old wounds revealed up-regulation of many categories including proteinase inhibitors, calcium binding categories. In 7-day-old wounds, genes for the extracellular matrix proteins and cytoskeletal structural proteins were highly expressed. Interestingly, some genes with the largest increase in expression showed no major decline from day one to seven. These included matrix metalloproteinase-1, serine proteinase inhibitor B1 and a novel gene in the context of wound healing, carcinoembryonic antigen-related cell adhesion molecule-6 (Ceacam-6). In early wounds, Ceacam-6 protein was expressed by basal and suprabasal keratinocytes at the wound margin. After 7 days post-wounding, expression of Ceacam-6 in keratinocytes declined but was induced in granulation tissue fibroblasts that continued to express Ceacam-6 until day 28. The function of Ceacam-6 in oral cells during wounding remains unravelled but could involve regulation of self-defence against bacteria and

inhibition of anoikis. To conclude, the present study represents one of the first large-scale gene expression studies comparing different phases of regenerating human mucosal wounds. Future studies are needed to directly compare gene expression profiles of healing scar-forming skin wounds to regenerating oral wounds.

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ABBREVIATIONS

Ceacam-6	carcinoembryonic antigen-related cell adhesion molecule-6
TGF-α, -β	transforming growth factor -α, -β
SB	stratum basale
SS	stratum spinosum
SG	stratum granulosum
SC	stratum corneum
PDGF	platelet derived growth factor
FMLP	N-formyl-methionyl-leucyl-phenylalanine
TNF-α	tumor necrosis factor-α
IL-1, -4	interleukin -1, -4
FGF	fibroblast growth factor
MMP	matrix metalloproteinase
TIMP	tissue inhibitor of matrix metalloproteinase
EGF	epidermal growth factor
KGF	keratinocyte growth factor
CTGF	connective tissue growth factor
ECM	extracellular matrix
VEGF	vascular endothelial growth factor
GAG	glycosaminoglycan
IGF	insulin growth factor
HA	hyaluronic acid
HGF	hepatocyte growth factor

INTRODUCTION

Wound healing of damaged skin or mucosa is a complex multi-step process that is essential for the survival of all organisms. Human adult wound healing involves four major overlapping processes including inflammation, re-epithelialization, granulation tissue formation and angiogenesis, and tissue remodelling. The ultimate goal of wound healing is complete restoration of both the structure and function of the damaged tissues (regeneration). This type of healing can only be seen in certain adult vertebrates, such as newts, where complete regeneration of a missing appendage is indeed possible [1]. Interestingly, the early fetus of many mammals have the ability to heal skin wounds without scar formation [2]. In contrast, however, adult cutaneous wound healing results in scar-formation characterised by an abundance of collagen and disorganized fiber structure. Several cellular and molecular mechanisms have been proposed to explain why adult wounds form scars compared to scarless fetal wound healing [3]. These include increased levels of hyaluronan, less differentiated fibroblasts, lack of fibrin clot and a different inflammatory response between fetal and adult wounds. Fetal wounds demonstrate fewer inflammatory cells that are also cleared faster than in adult skin wounds [4]. As a consequence of this altered inflammatory response and the fetal tissue itself, fetal wounds contain less profibrotic cytokines such as TGFß-1 and -2 and more anti-fibrogenic cytokines such as TGFß-3, that could explain in part the scarless fetal wound healing [3]. Very little is known about cellular mechanisms and gene expression in adult tissues that do not scar. Gingival tissue and palatal masticatory mucosa have maintained their ability to regenerate and heal without forming a scar [5-7]. Some potential reasons for preferential oral wound healing may include a unique and moist

environment, the presence of oral bacteria, and fetal-like properties of oral cells [8, 9]. In the present study, we performed transcriptional profiling of human masticatory mucosal wounds at early time points (1-7 days) to screen thousands of genes without prior knowledge or bias for which genes may be involved in scarless healing. In addition, we followed healing for up to 60 days by histology, and clinically for up to 6 months. The results demonstrate that gingival wounds heal both histologically and clinically without scars. Hundreds of genes including well-known wound-associated genes and novel genes in this context showed significantly altered temporal expression during early healing.

CHAPTER I

Review of the Literature

1.1. Normal composition and function of oral mucosa

Oral mucosa can be divided into three basic categories: lining mucosa, which is found over mobile structures such as lips, cheeks, soft palate, alveolar mucosa, the vestibular fornix and floor of the mouth; masticatory mucosa, which covers the gingiva and the hard palate; and specialized mucosa, which is located on the dorsum of the tongue [10]. This review will focus specifically on masticatory mucosa.

Oral masticatory mucosa is comprised of a stratified squamous epithelium and underlying connective tissue, including a lamina propria and submucosa. A basal lamina is interposed between the epithelium and the lamina propria. The mucosal epithelium forms rete ridges alternating with projecting connective tissue papillae. Much of the research on stratified squamous epithelium and basal laminae are based on the cutaneous model, which hold many similarities to masticatory mucosa. In the cutaneous model, the epithelium, or epidermis can be divided into four distinct layers. These layers are: the stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG) and the stratum corneum (SC). The stratum basale is a single layer of columnar basal cells that attaches to a basement membrane by hemidesmosomes. These cells are primarily undifferentiated epidermal stem cells characterized by a high nuclear to cytoplasmic ratio, and cell organelles such as mitochondria and keratin filaments (primarily cytokeratin 5 and 14) [11]. The basal cells synthesize and secrete type IV collagen, type VII collagen, laminin, perlecan, and cytokines [11, 12].

The stratum spinosum has a spiny appearance due to an abundance of desmosomes[11]. In addition to the organelles found in the SB, the SS also contains lipid-rich lamellar bodies known as membrane-coating granules. There is an increase in cellular keratin filaments with cytokeratins 1 and 10 being the biological markers for this layer [11, 12]. In the more superficial layers of the SS, the cells begin to flatten and elongate.

The stratum granulosum is characterized by dark-staining keratohyalin granules. These granules consist of several proteins including filaggrin, loricrin, and involucrin (precursors of the cell envelope), while synthesis of cytokeratins 1 and 10 decline [12]. Keratohyalin granules become progressively larger in the upper granulocytes thus reflecting a quantitative increase in keratin synthesis[11]. The cells in this layer become more differentiated as indicated by an increase in protein synthesis and an increase in membrane-coating granules in the outer layers of the SG. These lamellar bodies play a role in the formation of the lipid permeability barrier of epidermis [13].

In vitro studies have shown that keratinocyte differentiation appears to be induced by an increase in cytosolic calcium [14]. It is thought that a rapid influx of calcium causes the cells of the stratum granulosum to transform into corneocytes (terminally differentiated keratinocytes), which are the cells that make up the last and most superficial layer of the epidermis, the stratum corneum [15]. Corneocytes are chemically and physically resistant cells that are flat, anucleated and packed with keratin filaments. They are surrounded by a cell envelope of cross-linked proteins (involucrin, loricrin, filaggrin, desmoplakin-like protein, and cysteine-rich protein) and covalently bound lipids [16]. The corneocytes are interconnected by desmomal attachments. The human

skin often has about 18-21 cell layers in the SC. Each cell may differ in their thickness, keratin packing, and number of desmosomes depending on the location in the body and the SC.

The structure and differentiation of mucosal epithelium may hold many similarities to skin; however, differences do exist. Most obviously, mucosa is devoid of hair follicles and sweat glands. In addition, there is considerable site variability of oral mucosal differentiation ranging from a fully keratinized epithelium on the hard palate and gingiva (epidermal-like), to a non-keratinized epithelium covering the buccal mucosa [17]. Variations in epithelial thickness, cell proximity and arrangement, as well as the depth and number of rete ridges and connective tissue papillae also exist [18]. Cell turnover is much faster in the oral cavity than in the epidermis, showing over four to five times the rate of epithelial turnover than in the skin [12].

Interposed between the epithelial layer and connective tissue is the basement membrane or basal lamina. This region is a highly specialized interface functioning as a barrier to cells and some large molecules and as mechanical support for the adhesion of the epithelium to the underlying dermis or lamina propria [19]. The basal lamina also has important roles for the migration of keratinocytes during wound healing (refer to section 1.2). Under electron microscopy, this zone can be divided into four layers from superficial to deep: the basal cell plasma membrane with hemidesmosomes, an electron lucent region known as the lamina lucida, an electron dense region referred to as the lamina densa, and a reticular layer consisting of various fibrillar and nonfibrillar proteins and anchoring fibrils. Currently, the basal lamina has been well defined with over twenty macromolecules characterized at the biological and genomic level. Some key molecules

in each layer include: bullous pemphigoid antigen of 230 kDa and 180kDa and the $\alpha 6\beta 4$ integrin in the hemidesmosome complex, laminin 5 in the lamina lucida, type IV collagen in the lamina densa, and type VII collagen in the anchoring fibrils [20].

The connective tissue layer, or lamina propria, of masticatory mucosa is mainly fibrous in nature. The primary cell type is the fibroblast, which plays a major role in the development, maintenance and repair of gingival connective tissue [9]. The primary function of the fibroblast is to synthesize the extracellular matrix components of the connective tissue. In addition, fibroblasts play a role in tissue homeostasis through collagen phagocytosis and production of collagenases, which allows tissue remodelling. Type I collagen is the primary collagen found in the connective tissue and is arranged in either large, dense bundles of thick fibers or loose, short, thin fibers arranged in a fine reticular network [21]. Type III collagen is also found in this fine reticular pattern and mostly adjacent to the basement membrane. Type V and VI collagen has also been localized in the gingival connective tissue [22]. Several proteoglycans have been identified in gingival connective tissue and include decorin, biglycan, and versican [23-25]. These proteoglycans function as organizers of extracellular matrix, space fillers, and cell surface components [26]. Other key components of the gingival connective tissue include glycoproteins such as fibronectin, osteonectin, vitronectin, elastin, and tenascins [27-30].

When describing specifically masticatory or palatal mucosa, it has been shown that palatal mucosa has approximately two times the number of desmosomes than lining mucosa [31]. This is likely a response to a greater need for resistance against forces of mastication. The epithelial-connective tissue interface in the hard palate is also

characterized by more lamina propria papillae [12]. Although the hard palate epithelium more closely resembles epidermis than non-keratinized oral epithelium, quantitative comparison of epidermal and palatal stratum corneum have shown that palatal epithelium shows fewer lipid lamellae than epidermis, which can account for the relatively greater permeability of the palatal mucosa compared to skin [32]. The basal cells of the hard palate were also found to contain significantly more ribosomes and mitochondria than epidermal basal cells [31]. The ultrastructural appearance of epidermal cornified squames revealed keratin filaments appearing less dense than the surrounding matrix, whereas the keratin filaments of the hard palate could not be distinguished from the surrounding matrix [31].

In addition to the hard palate, the masticatory mucosa also includes oral gingival epithelium. Macroscopically, gingiva can be anatomically divided into three distinct regions: the free marginal gingiva, the interdental gingiva and the attached gingiva (which is continuous with the mucosa of the hard palate). Histologically, the gingiva consists of three different types of epithelium based on location and phenotypic differences. The junctional epithelium is located from the base of the gingival sulcus to a point approximately 2 mm coronal to the alveolar bone. The sulcular epithelium lines the gingival sulcus and the oral epithelium is located from the tip of the gingival crest to the mucogingival junction, comprising part of the free marginal and attached gingival (for a review on gingiva see Bartold et al., 2000) [9].

Similar to skin, the function of masticatory mucosa is largely protective in nature. In particular, the unique junctional epithelium plays a role in host defence against

bacterial infection as well as allowing the movement of substances between the connective tissue and the oral cavity [33].

1.2 Overview of Wound Healing

Most tissues and organs of the body are capable of healing or repairing injuries. Healing can be divided into two different types: regeneration and repair. Regeneration, the most desirable healing outcome, is defined as the complete functional and structural restoration of the damaged tissue [34]. Repair is healing that occurs by replacement of the lost tissue by non-specialized connective tissue (granulation tissue) that can lead to scar formation [34]. Some animals, such as newts, are capable of epimorphic regeneration, or replacement of an amputated appendage [1]. Higher vertebrates, however, do not possess this ability for epimorphic regeneration and in most cases healing occurs through repair. Wound healing is a dynamic, interactive process that involves soluble mediators (growth factors and cytokines), blood elements, extracellular matrix molecules, and the participation of several cell types including inflammatory, epithelial, endothelial cells and fibroblasts [35]. Wound healing will occur as a response to tissue injury incurred by trauma, microbes or foreign materials, and can be described in four phases: hemostasis and inflammation, re-epithelialization, granulation tissue formation and angiogenesis, and tissue remodeling. These phases do not occur in sequential order, but instead show considerable overlap. In skin and mucosal wound healing, the goal is to restore the protective function of these tissues in an efficient manner in order to prevent microbial access into the tissues.

1.2.i Inflammation

The inflammatory phase can be divided into early and late inflammation. Early inflammation begins immediately upon tissue injury resulting in the damage of blood vessels and the extravasation of blood constituents. Blood clotting following extravasation will occur due to the surface activation of Hageman factor that can generate the fragments bradykinin and potent vasoactive agents as well as initiate the complement cascade [35]. These fragments will promote vasopermeability and vasodilation [36]. In addition, blood clotting will occur because of tissue pro-coagulant factors released from damaged cells, and surface membrane coagulation factors and phopholipids expressed on activated platelets and endothelial cells [37]. Following injury, the exposure of the subendothelial tissue and types IV and V collagen will promote the binding and aggregation of platelets [38]. Following activation, platelets will secrete adhesive proteins such as fibronectin, fibrinogen, thrombospondin and von Willebrand factor VIII [35]. The first three proteins act as ligands for platelet aggregation while von Willebrand factor functions as a mediator of adhesion between platelets and fibrillar collagen [39, 40]. In addition, platelets release other soluble mediators such as serotonin, platelet-derived growth factor (PDGF), transforming growth factor- α (TGF-α), adenosine phosphate, platelet-activating factor, factor V, 1,2-hydroxy eicosatetranoic acid, and thromboxane A2, which promote vasocontriction, additional platelet aggregation and further growth factor release [35, 41]. Platelets will undergo a series of functional and structural changes following activation, such as acquiring a spherical shape with spinous pseudopod projections that promotes increased aggregation with other platelets [41, 42]. The activated platelets undergo a rearrangement of their membrane phospholipids, which allow the interaction of clotting factors V and X [43]. This interaction will increase the

production of thrombin several fold, which catalyzes the formation of fibrin from fibrinogen and forms a meshwork between the aggregated platelets [43]. The aggregation of platelets and the formation of this fibrin-rich clot will fill in the discontinuity of the wounded tissue, preventing further bleeding and bacterial infection [35]. In addition, this clot will act as a provisional matrix for cell migration and a reservoir for growth factors, proteases, and protease inhibitors. Despite the important role that platelets play in the process of wound healing, a recent study comparing wound healing in thrombocytopenic to control mice revealed that despite a significant alteration in wound inflammation, the rate of the proliferative aspects of repair, including epithelialization, collagen synthesis and angiogenesis, were almost identical [44]. The level of key growth factors was also similar between the two groups of mice, which may suggest that other sources of cytokines and growth factors (such as the increased macrophage and T-cell population) compensated for the lack of platelets in this study.

It is important that clot formation stay limited to the area of injury, and this occurs via intrinsic blood vessel activities. These include the production of prostacylin that inhibits platelet aggregation [45]; the binding of antithrombin III to thrombin, which stops its activity [46]; the production of protein C, which is an enzyme that degrades clotting factors V and VIII [47]; and the release of plasminogen activator, which initiates clot lysis through the conversion of plasminogen to plasmin [48].

Neutrophils are the key cell type involved in the early inflammatory phase and arrive at the wound site following platelets. Neutrophils cleanse the wounded area of foreign particles and bacteria by phagocytosis, enzymatic and oxygen radical mechanisms [49]. Neutrophils arrive from the surrounding broken blood vessels and are

recruited to the site of injury by various chemoattractants. Chemoattractants for neutrophils and other leukocytes include: fibrinopeptides cleaved from fibrinogen, fibrin degradation products, complement components C5a and C3a, bacterial products characterized by the synthetic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP), leukotriene B4, platelet activating factor, PDGF and platelet factor 4 [50-52]. Many of these chemoattractants also function in the adherence of neutrophils to endothelial cells by increasing the expression of CD11/CD18 receptors on the neutrophil's surface. In conjunction with sialyl lewis X, the CD11/CD18 complex mediates the adherence of the neutrophils to the blood vessel endothelium [53]. By way of a concentration gradient of chemoattractants, the adherent neutrophils begin the process of diapedesis or migration through the endothelial cells and into the site of inflammation. Neutrophil activation by chemoattractants also stimulates the release of elastase and collagenase, which facilitate cell penetration through the blood vessel basement membranes [35]. This action of diapedesis is further enhanced by the increased capillary permeability caused by various vasodilating agents such as serotonin, bradykinin, histamine and arachidonic acid metabolites [43].

The early inflammatory phase usually lasts for about three days, after which time the neutrophils become trapped within the wound clot and dessicated tissue. This eschar sloughs during tissue repair. Any neutrophils remaining within viable tissue will be phagocytosed by macrophages during the late inflammatory phase [54]. In cases of excessive wound contamination with foreign particles or bacteria, a persistent neutrophil-inflammatory response may exist [35].

The late inflammatory phase overlaps with the early phase, beginning about one day after injury and persisting for about seven days. The primary cell type involved in this phase is the monocyte/macrophage. These cells may remain at the wound site for days to weeks [43]. Monocytes arrive after the neutrophils and are attracted to the site by chemoattractants. These chemoattractants include collagen fragments[55], elastin [56], fibronectin [57], enzymatically active thrombin [58] and TGF-β [59]. The method of recruitment of monocytes into the site of injury is similar to the recruitment of neutrophils (adhesion to endothelium and migration through the blood vessels). The metamorphosis of monocyte into macrophage occurs by contact with the wound extracellular matrix [35]. The function of the wound macrophage is to debride the tissue through phagocytosis and digestion of pathogenic microbes, tissue debris, and remaining neutrophils [54]. Phagocytosis is stimulated by the interaction of the macrophages with the extracellular matrix proteins through integrin receptors. Campbell found that cultured macrophages and presumably wound macrophages also release proteolytic enzymes such as collagenase, which can facilitate wound debridement [60]. In addition, macrophages can induce the membrane-associated enzyme phospholipase which releases arachidonic acid from the cell membrane. Arachidonic acid metabolites such as prostaglandins and leukotrienes play a role in modulating the inflammatory response.

Macrophages play an important role in growth factor and cytokine release. They are considered a major secretory cell with the ability to secrete more than 100 different molecules. Some of these include: colony stimulating factor-1 which is necessary for monocyte-macrophage survival, tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, PDGF, TGF-β, TGF-α, interleukin-1 (IL-1), and fibroblast growth factor

(FGF)[61]. Macrophage-derived growth factors are necessary for the initiation of new tissue formation in wounds [35]. Through the release of soluble mediators, macrophages act on other inflammatory cells and on mesenchymal cells in order to regulate their phenotype and function [43]. It has been shown previously that macrophage-depleted animals have defective wound repair [43, 62]. In a more recent study, however, it was shown that the PU.1 null mouse, which lacks functioning neutrophils and macrophages, was still capable of repairing skin wounds, with reduced scarring, yet in a similar time frame as the wild type mouse [63]. In this situation, other cells, such as fibroblasts, presumable took over some of the roles of the missing inflammatory cells, such as cytokine release and phagocytosis.

Other inflammatory cell types involved in the late inflammatory phase include mast cells and lymphocytes. Both of these cells are involved in the release of various mediators. Lymphocytes arrive at the wound site at the same time as the monocyte. They are of more importance in the wound healing process if injury was caused by a foreign antigen or subject to secondary infection [64].

1.2.ii Re-epithelialization

Re-epithelialization of a wound will begin within hours after injury [65]. In order to cover the denuded wound, the epithelial cells will begin to migrate over the site. Migrating epithelial cells will arise from the wound periphery, and in cutaneous wounds, from the residual hair or sweat gland structures at the wound base [66]. During early epithelial cell migration, cell proliferation does not occur. In the later stages of re-epithelialization (1-2 days after injury), proliferation of the epithelial cells will occur in order to provide an increased number of cells to cover the wound [66]. The basal cells of

the epithelium will respond to the wounding by dissolving their hemidesmosomal complexes and withdrawing their tonofilaments, which will release them from the basement membrane. The epithelial cells will also lose intercellular desmosomal attachments and form peripheral cytoplasmic actin filaments which will allow for cell movement [67, 68]. The migrating keratinocytes also have a more flattened and elongated morphology [69]. The signals for these actions will arise from various soluble mediators released from the damaged cells, blood clot, and inflammatory cells.

In order to migrate, keratinocytes will express matrixmetalloproteinases (MMP's), which are capable of degrading components of the extracellular matrix in order to facilitate migration below or through the clot. Epidermal cells, and other cell types such as fibroblasts and macrophages, can produce MMP's which function in the degradation of collagen, fibrin and fibronectin [70]. MMP activity is of great importance in re-epithelialization and studies in cell culture have shown that blocking MMP activity prevents keratinocyte migration [70, 71]. In addition, the epithelial cells produce plasmin, which is activated by plasminogen activator, and functions in the degradation of the fibrin-fibronectin clot [72]. The presence of the plasminogen gene is critical for wound healing, as animals that lack this gene do not re-epithelialize [73]. Plasminogen activator further activates collagenase and therefore further enhances the degradation of extracellular matrix proteins [74].

The migration of epithelial cells along a provisional wound matrix composed mainly of fibrin, fibronectin, and vitronectin is highly regulated by integrin cell-surface receptors [66, 75]. Integrins are cell-surface transmembrane $\alpha\beta$ heterodimers that are responsible for most cell-extracellular matrix interactions in keratinocytes [76]. Their

functions include regulation of cellular function during growth, development, differentiation, and the immune response [77, 78]. During wound healing, there is a change in the expression of existing integrins of epithelial cells and increased expression of other integrins that are able to adhere to and migrate along a provisional wound matrix [78]. The common components of the basement membrane, such as collagen type IV and VII, laminin-1 and heparan sulphate proteoglycan are missing from beneath the migrating keratinocytes [78]. In their place, the migrating keratinocytes appear to be depositing fibronectin, tenascin-C and laminin-5 into the provisional wound matrix [79-81]. Fibronectin is a key component in the formation of the initial clot and granulation tissue during wound healing. In wounds, fibronectin can exist in three alternatively spliced variants that have different roles. Fibronectin containing the EDA or EDB regions (which are omitted from plasma fibronectin), are found to be upregulated in embryonic development and wounding, with EDA fibronectin being expressed by migrating keratinocytes [76]. Laminin-5 is thought to function in keratinocyte migration [82]. Tenascin-C is found in the connective tissue underneath the migrating epithelial front which suggests a role in re-epithelialization during wound healing [79]. Vitronectin is also present in the provisional matrix and has roles in cell adhesion, cell migration and invasion [83].

Migrating keratinocytes have been found to express 3 new fibronectin receptors, namely $\alpha5\beta1$, $\alpha v\beta6$, and $\alpha v\beta1$ [78]. $\alpha5\beta1$ and $\alpha v\beta6$ are absent in normal healthy adult epithelium. The increased expression of fibronectin receptors is likely induced by cytokines such as TGF-$\beta1$ [84]. Keratinocytes can also utilize other extracellular matrix

molecules for migration including tenascin-C and laminin-5 [85]. The keratinocytes recognize these molecules by other specific integrin receptors.

There are two proposed models for epithelial cell migration. In the sliding model, the epithelial cells move as a sheet. In this sheet, the cells at the margin appear to be actively motile while the cells behind these appear to be dragged along [86]. If the attachment of the marginal cells to the wound matrix substrate is disturbed, the migrating sheet will withdraw. This model of epithelial cell migration has been demonstrated in tissue culture, for embryonic epithelial movement, amphibian wound closure and corneal wound closure [66]. It is currently accepted that most simple epithelium moves by the sliding model. For multilayered epithelium such as the skin and mucosa, a different model, known as the leapfrog model, has been proposed [87]. This model suggests that cells at the migrating front adhere to the substrate and the cells above and behind this front crawl over these newly adherent basal cells. The data for this model is mostly indirect, however there are many studies supporting this model [88, 89].

The specific stimulus for migration and proliferation during epithelialization has not yet been determined; however, it is known that migration is not dependent on cell proliferation [90]. It is thought that the absence of marginal neighbor cells or the "free edge" effect can signal both migration and proliferation of epithelial cells [65]. The local release of growth factors and cytokines by various cell types including epithelial cells, and the increased number of growth factor receptors may also stimulate these processes. Some of the key growth factors implicated in migration and proliferation of epithelium are: epidermal growth factor (EGF), TGF-α, keratinocyte growth factor (KGF), and TGF-β, which stimulates cell motility but inhibits epithelial cell proliferation [35]. TGF-

β plays a very important role in re-epithelialization as well as other important phases of wound healing. Three mammalian isotypes have been identified (TGF-β1, 2, 3) and all have been found in healing wounds of animals [91]. TGF-β1 is the predominant isoform in wound keratinocytes while both TGF-β1 and 2 have been found in wound fibroblasts and macrophages adjacent to the wound [92].

Once the migrating epithelial cells meet each other over the middle of the wound, the migration will stop according to the concept of "contact inhibition" [66]. This will occur around five to seven days following injury. Basement membrane proteins (type IV and VII collagen, laminin-1 and laminin-5 and heparan sulfate proteoglycans) will then begin to reappear in an ordered sequence from the margin of the wound inward in a "zipperlike" fashion in epidermal wounds [93]. In contrast, small gingival wounds show nucleation of the basement membrane in multiple sites at the same time [85]. Epithelial cells will revert to their normal stationary phenotype and firmly attach to the re-established basement membrane through hemidesmosomes and type VII collagen fibrils [94]. The epithelial cells will differentiate and then reform their stratified structure. The basement membrane is usually completely reorganized by four weeks following injury [76].

1.2.iii Granulation Tissue Formation and Angiogenesis

New stroma, which is also called granulation tissue, will begin to form beneath the epithelium approximately four days following injury [35]. Clinically this tissue has a somewhat granular appearance that bleeds easily [95]. This initial restructuring of the damaged tissue functions as a temporary barrier against the external environment. Granulation tissue is primarily composed of inflammatory cells (mainly macrophages),

fibroblasts, newly formed and forming blood vessels, and loose connective tissue. The provisional extracellular matrix promotes granulation tissue formation by providing a scaffold for contact guidance (fibronectin and collagen), low impedance for cell mobility (hyaluronic acid), a reservoir for cytokines, and direct signals to the cells through integrin receptors [96, 97]. Macrophages, blood vessels, and fibroblasts will occupy the wound space at the same time [98]. Macrophages function in providing a continuous source of cytokines, which are necessary to stimulate fibroplasia and angiogenesis [65]. Fibroblasts are the major cellular component that supply the new extracellular matrix necessary to support cellular ingrowth, while blood vessels will carry oxygen and nutrients to the cells to sustain their metabolism [35].

Fibroplasia is defined as the components of the granulation tissue that are derived from fibroblasts [95]. Fibroplasia is therefore a mixture of fibroblasts and extracellular matrix (ECM). The stimulus for fibroplasia arises from the chemotactic, mitogenic, and modulatory activities of cytokines; many of which are released from platelets and macrophages. Fibroblasts themselves are also capable of producing cytokines and these cells can respond in an autocrine manner [99]. As fibroblasts respond to cytokines, many biological responses occur, including the induction of additional cytokines [100], and the modulation of cytokine receptor number or affinity [101]. Some of the key cytokines involved in fibroplasia include: PDGF, basic fibroblast growth factor (b-FGF), TGF-α, TGF-β, and connective tissue growth factor (CTGF), which is induced by TGF-β and modulates fibroblast growth and extracellular matrix secretion [65, 102]. Fibroblasts synthesize, deposit, and remodel the ECM, while the ECM regulates this function of the fibroblasts. Fibroblasts can use fibronectin in the provisional matrix for movement

through the wound. In addition, fibroblasts also use fibrin and vitronectin as a substratum for movement [103]. The ability of fibroblasts to bind to fibronectin, fibrin and vitronectin are mediated by integrin receptors. In studies on fibroblast-like cells lines, it was found that the $\alpha 4\beta 1$ integrin receptor facilitates fibroblast movement while the fibronectin receptor $\alpha 5\beta 1$ retards movement, especially when overexpressed [104, 105]. It was also found that $\alpha 5\beta 1$ was maximally expressed in wound fibroblasts after they ceased migration and established links to the extracellular matrix [106].

Both PDGF and TGF-β can stimulate fibroblasts to migrate [107] and upregulate integrin receptors [108]. These growth factors are likely responsible for promoting a migratory fibroblast phenotype. In vitro fibroblast migration has also been observed in response to other chemoattractants including: fragments of the 5^{th} component of complement, types I, II, and III collagen-derived peptides, fibronectin fragments, elastin-derived peptides and interleukin 4 (IL-4) [55, 109-111]. Fibroblasts will move towards a chemotactic gradient by extending lamellipodia in the direction of the stimulus while their opposite pole will then be released. This process of movement is very similar to haptotaxis [112, 113]. In haptotaxis, the fibroblast will extend lamellipodia randomly and one lamellipodium will become dominant so that the cell will spread in its direction. The cell will ooze forward and break old adhesions and the excess membrane at the trailing edge will then become available to resume random protruding lamellipodia. Extracellular matrix fibrils strongly influence the direction of fibroblast migration as the fibroblasts tend to align and migrate along and not across the substrata fibrils [114]. This process is called contact guidance [115]. Fibroblast migration can therefore be influenced by chemotactic, haptotactic, and contact guidance signals.

The movement of fibroblasts into the provisional matrix also requires proteases to create a pathway for migration. These enzymes are derived from fibroblasts and serum and include plasminogen activator and MMP's including collagenases, gelatinase A, and stromelysin [74]. Once the fibroblasts have migrated into the wound, their major function changes from migration to protein synthesis [106]. The provisional matrix is gradually replaced with a collagenous matrix, which is likely the result of TGF-β1 [116]. The profibrotic phenotype of the fibroblast is characterized by an abundant rough endoplasmic reticulum and golgi apparatus [35]. Production of collagen appears maximal at seven days following injury [95].

The formation of new blood vessels, a process called angiogenesis, is necessary to sustain the formation of granulation tissue. This process relies upon the migration and mitogenesis of endothelial cells as well as the appropriate extracellular matrix in the wound bed. Initially, the acidic and basic forms of fibroblast growth factor were thought to initiate the induction of angiogenesis [65]. Many other molecules have also been found to have angiogenic properties including: vascular endothelial growth factor (VEGF), TGF-β1, angiogenin, angiotropin, angiopoietin 1, thrombospondin, interleukin-8, and lactic acid [35, 117, 118]. In addition, low oxygen tension may also stimulate angiogenesis [119]. Most of these molecules induce angiogenesis by stimulating the production of b-FGF or VEGF by macrophages or endothelial cells. The action of bFGF is critical during the first three days of wound repair whereas VEGF plays a more critical role in angiogenesis during the formation of granulation tissue [120]. bFGF is released by macrophages and injured tissue cells and stimulates endothelial cells to release

plasminogen activator and procollagenase [35]. VEGF can stimulate the proliferation of endothelial cells and cause increased vasopermeability [121].

In addition to the factors which induce angiogenesis, appropriate extracellular matrix and endothelial receptors for the provisional matrix are necessary. Proliferating endothelial cells adjacent to and within wounds deposit fibronectin within the vessel wall [122]. Angiogenesis also requires the expression of fibronectin receptors by endothelial cells [123] and therefore, a perivascular matrix abundant in fibronectin may act as a conduit for endothelial cell movement into the wound. The expression and activity of proteases are also necessary for angiogenesis [124].

A general hypothesis regarding the series of events leading to angiogenesis is as follows [65]: Injury causes tissue destruction and hypoxia and angiogenic factors, such as bFGF, are immediately released by macrophages (attracted to the wound site by fibronectin fragments and other degradation products). The production of VEGF from epidermal cells is stimulated by hypoxia. The production of plasminogen activator from endothelial cells will convert plasminogen to plasmin. Plasmin and activated collagenase will then digest the basement membrane. This fragmentation will allow endothelial cells to migrate into the wound site and form new blood vessels. Once the wound site is filled with new granulation tissue, angiogenesis will cease and many blood vessels will disintegrate by apoptosis.

1.2.iv Tissue Remodelling

The process of extracellular matrix remodeling, cell maturation, and cell apoptosis create the phase of wound healing known as tissue remodelling. This phase of wound repair overlaps granulation tissue formation and can be occurring at the margins of a

wound while granulation tissue is still invading the middle of the wound space [35]. By day seven following injury, actin fibrils begin to appear within the cytoplasm of the fibroblasts [65]. In addition, intercellular and cell-matrix connections begin to appear [106]. There is a change in integrin expression on the fibroblast surface from αV containing receptors to α5β1 fibronectin receptors [95]. After day nine, the fibroblasts begin to arrange themselves in a radial array across the wound and lose their collagen-synthesis phenotype to become the myofibroblast phenotype [95].The myofibroblast phenotype is characterized by abundant cytoplasmic microfilament bundles and α-smooth muscle actin [125]. The differentiation of myofibroblasts occurs between day six and fifteen after wounding and is induced by TGF-β [125, 126]. After day fifteen, approximately 70% of fibroblasts in mature granulation tissue express α-smooth muscle actin [125]. Myofibroblasts are often present in tissues under mechanical stress, but are only occasionally found in normal tissue [127]. The attachment of myofibroblasts to fibronectin and collagen by integrins, the intercellular adherens junctions between myofibroblasts, and the cross-links between collagen bundles allow contraction by the myofibroblasts to be transmitted across the wound [128]. Myofibroblasts are aligned along the lines of contraction in the wound. Wound contraction by myofibroblasts parallels myofibroblast differentiation as it occurs between days seven and fourteen following injury and is stimulated by TGF-β and PDGF [129, 130].

When wound contraction ceases, myofibroblasts will disappear by apoptosis and the scar tissue will become less cellular while new fibroblasts, typical of normal connective tissue, will emerge [125, 131]. It is still not entirely known whether these new fibroblasts

arise from connective tissue adjacent to the wound or from a primitive fibroblast population that originally populated the wound.

Apoptosis of myofibroblasts will begin at day twelve, peak at day twenty and resolve by day sixty following wounding [131]. Since myofibroblasts can persist in certain pathologic conditions (fibrotic lesions), it is important that they undergo apoptosis when wound contraction is completed.

Collagen remodelling occurs during the transition from granulation tissue to scar formation and is dependant on both a low rate of synthesis and degradation of collagen [65]. The deposition of new extracellular matrix proteins during granulation tissue formation is stimulated by TGF-β [76]. Another potent modulator of protein synthesis is mechanical tension. Signals initiated by tension in a three dimensional collagen matrix require integrin-collagen interactions. Tension mediated from the matrix seems to balance production versus degradation. For example, collagen gels under tension exhibit a relatively high protein synthesis rate [132] while stress relaxation down-regulates type I collagen expression and up-regulates MMP-1 and MMP-13 expression [133]. A healing wound will generally gain about 20% of its strength by the end of the third week of healing, at which time fibrillar collagen has accumulated rapidly [65]. After this time, wounds will accumulate collagen and increased tensile strength at a much slower rate. Further increase in tensile strength also has more to do with collagen remodelling and formation of larger collagen bundles and alteration of intermolecular cross-links rather than increased collagen deposition [134]. In skin, wounded tissue will fail to gain the same strength as uninjured tissue, with a scar being about 70% as strong as intact skin [135].

1.3 Fibrotic and Anti-fibrotic Mechanisms in Wound Healing

The healing of both cutaneous and oral mucosal wounds will proceed through the same basic phases of wound healing, including inflammation, epithelialization, granulation tissue formation and angiogenesis, and tissue remodelling. Adult cutaneous wound healing is considered reparative in nature as dermal integrity is re-established at the expense of scar formation. Scar formation can be 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 [136]. Cutaneous scarring is characterized by inflammation, a disorganized dermal collagen pattern, and failure of hair follicles and other dermal appendages to regenerate [3, 35]. In contrast to normal skin , where collagen bundles show a basket-weave like pattern with a random structure, scar tissue shows smaller bundles aligned in a parallel fashion to the epidermis [3]. Scar tissue following trauma and surgical procedures can be considered a significant clinical problem as it can impair normal growth and function and cause disfigurement. In contrast, fetal cutaneous wound healing is fundamentally different from the adult because early fetal wounds heal without scarring [137]. Oral mucosal wounds also differ from adult cutaneous wounds as healing is often more rapid and show reduced scar formation [138].

1.3.i Fibrogenic Mechanisms

Currently, there is no method to measure the exact amount of scarring either clinically or histologically. The difference between "normal" scarring and excessive scarring (such as keloids or hypertrophic scars) may only be the degree or extent of the

same basic process [139]. Scar formation can be essentially described as the permanent deposition of connective tissue made up of extracellular matrix components, with collagen being most abundant, and a lack of large blood vessels and elastin [140]. Initially the dermal wound is composed mainly of fibrin, fibronectin, and hyaluronan [141]. Following this, sulphated glycosaminoglycans (GAGs) and collagens are synthesized [35, 142]. The nature of the collagen in the later stages of the wound is predominantly type I [142].The final amounts of collagen and GAGs seem to contribute to the resilience and tensile strength of scar tissue [141]. Various fibrotic lesions and hypertrophic scars may accumulate an excess of extracellular matrix several years beyond initial injury [143].

Myofibroblasts, which arise from fibroblasts and are characterized by α-smooth muscle actin fibers, are believed to play a role in wound contraction and scarring [144]. The transformation of fibroblast to myofibroblast is influenced by the presence of mechanical stress, TGF-β1, and cellular fibronectin (ED-A splice variant) [145]. Myofibroblasts are abundant in the wound between six and fifteen days [125] and function in extracellular matrix production and wound contraction [145]. Following wound contraction, the granulation tissue myofibroblasts, along with vascular cells, undergo apoptosis following the completion of epithelialization [131]. During pathologic fibrotic phenomena, such as keloids and hypertrophic scars, apoptosis of myofibroblasts may be diminished or aberrant in comparison with normal healing wounds [145]. This aberrant response may be due to altered expression of p53 and Bcl family proteins [146, 147]. Keloids are described as thick scar tissues which have escaped the boundaries of the original wound [148]. They rarely exhibit erythema and are often asymptomatic.

Keloids can occur in all races, but a predilection for African Americans has been established [149]. Hypertrophic scars are different from keloids as they are often erythematous, nodular, and itchy. They also remain within the confines of the original wound [150]. Both of these conditions result in a prolonged inflammatory state, which therefore prolong the myofibroblast population. Eosinophils, in particular, will produce abundant inflammatory cytokines (most notably TGF-β), which will promote the differentiation of myofibroblasts and synthesis of extracellular matrix [151].

Different cytoskeletal protein expression has been found in myofibroblasts during wound healing and fibrocontractive disorders. For example, vimentin is present in early granulation tissue myofibroblasts whereas both vimentin and α-SM actin –type cells are found primarily during the retraction phase [125]. Granulation tissue myofibroblasts are devoid of desmin and SM-myosin heavy chains, whereas hypertrophic scar myofibroblasts express these proteins [125, 152]. In addition, keloids do not have α-SM actin –expressing myofibroblasts when compared to hypertrophic scars [153], which may mean that α-SM actin is a key element in the pathogenesis of contraction in hypertrophic scars. It has also been shown that α-SM actin-expressing myofibroblasts are the key cells involved in collagen synthesis during pulmonary fibrosis [154].

The turnover of extracellular matrix is regulated by matrix metalloproteinases, which are secreted by keratinocytes, macrophages, and other cells [155]. MMP's are secreted as inactive proenzymes and are zinc dependant proteinases capable of degrading a wide variety of extracellular matrix components [156]. The activity of MMP's is counteracted by tissue inhibitors of MMP's (TIMP's). The expression of MMP's and TIMP's must be carefully regulated during normal wound healing. In the case of keloids

and hypertrophic scars, excess collagen production has been attributed to a decrease in MMP-9 and no change in the TIMP expression [157]. An increase in TIMP expression and a decrease in MMP expression has also been implicated in excessive fibrosis such as in scleroderma [158]. In a recent study by Dang, it was shown that scars formed in fetal rats after nineteen days gestation whereas scarless wound healing was found at sixteen days gestation [155]. In this study, there was an increase in TIMP-1 and TIMP-3 in the scar-forming wounds where as the scarless wounds exhibited no change in the expression of these molecules.

It is well known that TGF-β plays a very important role in scarring. Reduced or more transient expression of TGF-βs and their receptors have been found in non-scarring fetal wounds as compared with adult wounds [159, 160]. A strong and persistent expression of TGF-βs and their receptors have been found in fibroblasts of postburn hypertrophic scars [161]. Both keloid tissues and keloid-derived fibroblasts have been associated with an overexpression of TGF-β1 and -β2 [162]. When fetal wounds were treated with TGF-β1, a large increase in scarring occurred in these cutaneous wounds [163]. Conversely, when incisional rat wounds were treated with neutralizing antibodies to TGF-β1 and TGF-β2, a marked reduction in extracellular matrix deposition and scarring was evident [138]. In this study, the neutralizing antibodies reduced the magnitude of monocyte and macrophage infiltration, but in doing so, healing progressed more slowly. The application of antibodies was most effective in the early stages of wound healing, which indicates that the fibroproliferative phenotype of the fibroblast may be established in early healing. Transgenic mice lacking intracellular Smad3, which is part of the signalling cascade after TGF-β binds to its receptor, have been found to

have reduced monocyte recruitment and a shorter inflammatory phase [164]. Mice lacking Smad3 have shown significantly decreased fibrosis in both cutaneous and pulmonary fibrosis [165, 166]. A study by Sumiyoshi et al. found that over-expression of Smad3 caused increased contraction of collagen gels by dermal fibroblasts, possibly by regulating TGB-β signalling [167].

There are currently five known isoforms of TGF-β, but only three of these, TGF-β1, TGF-β2, and TGF-β3, are expressed in mammalian tissues [168]. These three isoforms behave similarly in most assays; however, their expression during embryogenesis, fibrosis, and wound healing are different [138]. All three isoforms are expressed within wounds, with TGFβ1 making up 85% of this expression [151]. While TGF-β1 and TGF-β2 are most often associated with scarring, TGF-β3 is associated with fetal wound healing and antagonizing the scarring effects of the other TGF-β isoforms [138, 169].

There are other cytokines with fibrogenic properties that have been implicated in pathologic scarring. These included insulin-like growth factors (IGF), which act in synergy with TGF-β to increase fibroblast proliferation and matrix synthesis [170]. IGF has also been found in large quantities in fibrotic dermis [171]. Other cytokines that increase proliferation and differentiation of fibroblasts and synthesis of matrix include: fibroblast growth factor, epidermal growth factor, IL-1, IL-4 and CTGF [151]. CTGF is expressed in many different tissues and organs and can stimulate the proliferation and chemotaxis of fibroblasts directly [172]. CTGF is considered a downstream mediator of TGF-β1, and has been implicated in the pathogenesis of fibrotic

processes [173]. The degree of CTGF expression has been correlated with the degree of fibrosis [174].

Activins, which are members of the TGF-β superfamily, are involved in various aspects of cell growth and differentiation and have also been implicated in enhanced wound repair and scarring. Over-expression of activins in transgenic mice revealed epidermal thickening and dermal fibrosis, possibly by inducing expression of growth factors in dermal fibroblasts with stimulation of keratinocyte proliferation [175].

1.3.ii Antifibrotic Mechanisms: Fetal Wound Healing

Extensive research has shown that the embryo and early fetus respond to wounding in a fundamentally different mechanism than the adult. In general, the fetal wound will show rapid closure, lack of inflammation, a reduction in cytokine expression, and a change in the ratio of different cytokines [176]. The fetal wound environment is much different than in an adult, as the wound is bathed in fetal serum and amniotic fluid rich in growth factors, hyaluronic acid, and fibronectin. Hyaluronic acid (HA) has been found in high concentrations in regions of tissue repair, proliferation, and regeneration [176]. Although the factor that causes persistent elevation of HA in the fetus is unknown, the presence of HA cause fibroblasts to migrate more rapidly, thereby accelerating fetal wound healing [177]. Fetal wounds also heal without the formation of a fibrin clot, which is thought to be the result of healing in a fluid environment [178]. It is not entirely known whether the presence of an eschar in the adult plays a role in scarring, although it most likely serves as a reservoir for growth factors. Amniotic fluid has been found to regulate matrix degrading enzymes. It has been shown to enhance collagenase activity, and inhibit

hyaluronidase, elastase, and cathepsin-B, suggesting a role in scar-free healing [179]. Many other studies have shown, however, that scarless fetal healing can proceed in the absence of amniotic fluid, such as when human fetal skin was transplanted to a subcutaneous location on an adult athymic mouse [180]. In addition, when adult sheep skin was wounded in the intrauterine environment, the skin continued to heal with a scar [181]. Many studies now suggest that scarless repair is intrinsic to fetal tissue rather than being dependant on the environment.

Scarless healing in the fetus is dependant on both the gestational age and the size of the wound. In humans, an early fetal incisional wound (less than 24 weeks) will heal without a scar whereas a late fetal wound will heal with fibrosis [182]. At each gestational age, it has been found that increasing the wound size also increases the frequency of scarring [183]. Scarless healing has also been found to be dependant on the tissue type. Skin and bone heal without a scar during early gestational age; however, other regions such as nerve tissue and intestinal wounds do not [184, 185].

The fetal wound is exposed to a reduced early inflammatory signal when compared to adult wounds. It has been demonstrated that fetal platelets aggregrate poorly in response to collagen and contain less TGF-β and PDGF than adult platelets [186, 187]. Fetal wounds also lack the large neutrophil infiltrate that characterizes the early stage of adult wounds [188]. As healing progresses, fetal macrophages do enter the wound, but they are present in lower numbers [189].

The fetal fibroblast plays a crucial role in scarless healing and it exhibits many differences from its adult counterpart. Fetal fibroblasts synthesize more total collagen, exhibit greater prolyl hydroxylase activity (a rate limiting enzyme in collagen synthesis),

synthesize greater proportions of types III and V collagen, and migrate faster than adult fibroblasts [2, 190, 191]. The high amount of HA and the increased amount of HA receptors on fetal fibroblasts may be related to the increased migration of these fibroblasts [192]. The myofibroblast has been implicated in wound contraction and scarring. In fetal lambs, the myofibroblast was found to be absent in the 75-day fetus, and present after 100 days gestation, when scar formation begins [193]. This suggests that the absence of these cells may have a role in scarless fetal healing.

The fetal extracellular matrix also contributes to scarless wound healing. Fetal wounds heal with collagen in a fine reticular pattern which is indistinguishable from the surrounding uninjured dermis [194]. In addition, fetal wounds have a higher proportion of collagen type III in the early gestational age, which is smaller and finer than type I collagen. Cell adhesion molecules such as fibronectin and tenascin, and cell surface receptors (integrins) seem to appear earlier in fetal wounds than in adult wounds [195]. This may allow for a more rapid cellular infiltration and a faster rate of fetal fibroblast migration thus accelerating fetal wound healing. Higher proportions of MMP's to TIMP's have been found in early gestational wounds when compared to later gestational wounds [155]. This ratio of MMP's and their inhibitors in early fetal wounds favors extracellular matrix turnover and fetal cell migration, thus promoting scarless repair.

Growth factors have also been studied in the fetal environment. TGF-β, including all three isoforms, has been found in unwounded fetal skin. At early gestational ages, however, lower levels of TGF-β1 and high levels of TGF-β3 are expressed [169]. Fetal wound experiments have shown that a high TGF-β3 to TGF-β1 expression ratio is associated with scarless healing [196].

The unique properties of fetal tissue have also been studied at the genetic level. Some recent studies have demonstrated the activity of specific genes in human fetal skin known as homeobox genes. These genes are transcription factors that regulate pattern formation, such as the formation of limbs. After wounding, two of these homeobox genes, HOXB13 and PRX-2, show changes in expression that correlate with healing [197, 198]. In addition, these two genes are not found in adult skin. This may suggest a role for such genes in fetal skin regeneration.

1.3.iii Comparison of Oral and Cutaneous Wound Healing

Many observations have been made to suggest that oral wounds heal more rapidly and with less scarring than cutaneous wounds. The characteristics which lead to this type of healing in the oral cavity may include a unique environment and intrinsic differences in the cells involved.

Oral mucosal wounds are continuously bathed in saliva, which may be favorable for more rapid tissue repair. Studies have shown that desalivation of mice results in slowed healing of cutaneous wounds suggesting a favourable role of saliva in wound healing [199]. In addition, individuals with xerostomia have shown delayed oral wound healing [200]. Features of saliva such as proper pH, ionic strength, and the presence of ions seem to favour oral mucosal healing [201]. Saliva provides a moist environment which prevents tissue dessication, allows accelerated angiogenesis, and increased breakdown of fibrin and tissue debris [202]. In addition to a moist ionic environment, the majority of the healing potential of saliva is due to the presence of growth factors and bacteria [203]. Epidermal growth factor and vascular endothelial growth factor have both been found in saliva and play important roles in the repair of oral mucosa and

angiogenesis respectively [204, 205]. The presence of bacteria in the oral cavity may also increase the rate of wound healing by attracting macrophages into the wound site followed by the release of cytokines. The formation of granulation tissue and blood supply will therefore be accentuated in the wound, and a greater tensile strength of the contaminated wound will result due to increased connective tissue synthesis [206].

Studies have indicated that gingival fibroblasts, unlike adult skin fibroblasts, exhibit similar characteristics to fetal fibroblasts in three dimensional extracellular matrices [207, 208].Oral fibroblasts were able to contract a three dimensional collagen matrix faster than dermal fibroblasts [209]. In addition, gingival fibroblasts secrete different proteolytic enzymes in comparison to dermal fibroblasts, such as MMP -13 and increased levels of tissue plasminogen activator [210, 211]. Oral mucosal fibroblasts were also found to exhibit increased levels of active MMP-2 and lattice contraction when compared to skin fibroblasts [6]. These differences in the proteolytic enzyme profile of oral and skin fibroblasts likely contribute to the preferential remodelling of oral wounds. Oral fibroblasts have also been found to produce more hepatocyte growth factor and keratinocyte growth factor than skin fibroblasts [212]. The heterogeneity of HGF and KGF expression in oral and skin fibroblasts may play a role in the different rates of repair in the two tissues.

In a recent study by Szpaderska et al, it was found that oral wounds contained significantly less interleukin-6 and interleukin-8 (pro-inflammatory cytokines), and TGF-β1 in mucosa versus skin [7]. In addition the inflammatory cell infiltrate consisting of neutrophils, macrophages, and T-cell was reduced in oral versus dermal wounds. This

study suggests that a reduced inflammatory response leads to an improved healing response in oral versus dermal wounds.

1.4 Microarray Techniques and Wound Healing

Over the past decade, the use of large scale gene expression studies utilizing DNA microarrays has grown in popularity. These measurement techniques include the two most commonly used DNA microarrays: complementary DNA microarrays [213] and oligonucleotide microarrays [214]. All microarrays consist of distinct DNA sequences known as "probes" which are fixed to an immobile surface such as glass or nylon. This probe is then exposed to a labelled "target" of complementary RNA (cRNA) derived from mRNA of an experimental sample, which will then hybridize to the immobilized DNA. The amount of label associated with each DNA probe location will allow the inference of the abundance of each mRNA species from the experimental sample; therefore measuring gene expression. Microarray technology is useful because the expression of thousands of genes can be measured simultaneously in a single assay. This technique is in contrast to more traditional gene expression analyses, such as Northern blot, which can analyze up to 20 mRNAs at one time [215]. Microarray studies are essentially considered as "discovery research" and therefore are not considered as "hypothesis driven" research [216]. The information derived from microarrays, however, can help to narrow the search for genes that can later be used in hypothesis driven studies. To help better understand the function of genes, the analysis of the expression of mRNA under a certain set of conditions, known as functional genomics, has become important [215]. Using new large scale gene expression technologies, functional genomics will allow the comparison of different sets of genes used in different

conditions, such as in the comparison of health and disease, or healthy and wounded tissues.

In addition to the use of microarrays in functional genomics, this technology has also proven useful for: biomarker determination, finding genes that correlate with disease progression; pharmacological research, by exposing tissues to varying doses or types of compounds and determining differences in gene expression; toxicogenomics, by finding gene expression patterns in a model organism exposed to a compound and their use as a predictor for adverse effects; target selectivity, to define a compound by the gene expression profile that it evokes in a target tissue and then compare it to other compound gene expression profiles: prognostic tests, to determine sets of genes that can distinguish one disease from another: and disease subclass determination, to find multiple subcategories of tumours in a single clinical diagnosis [217].

The use of microarray techniques in the area of wound healing is growing, although it is still currently limited in the area of cutaneous and especially oral wound healing.

1.4.i Microarray Studies in the Area of Cutaneous Wound Healing

In 2000, Tsou et al compared gene expression of hypertrophic scar tissue to normal scar tissue and uninjured skin in humans [218]. It was found that 44 genes were over-expressed and 124 genes were under-expressed in hypertrophic scars compared with normal scars. Differential expression was also found between normal scars and uninjured skin and hypertrophic scars and uninjured skin. A more recent study compared cultured fibroblasts from hypertrophic scars and normal skin, and the response of these cells to interleukin-6 stimulation [219]. Comparison of the gene expression profiles, using

Affymetrix microarrays, revealed an increase in expression of 12 genes and a decrease in expression of 14 genes in the hypertrophic scar fibroblasts versus normal skin fibroblasts. It was also found that there was an absence of upregulation of matrix-metallo proteinases in hypertrophic scar fibroblasts in response to IL-6, which may play a role in excess collagen formation in these scars. This finding was in contrast to the effect of IL-6 on normal fibroblasts. Other recent studies have also found different gene expression patterns in hypertrophic scars and in keloids when compared to normal skin, including the upregulation of TGF-β1 in keloids [220, 221].

Studies in 2001 utilizing cDNA microarrays, were conducted to determine the baseline gene expression in uninjured skin, and to determine the gene expression at thirty minutes and one hour following initial injury [222, 223]. It was found that when comparing uninjured normal human skin in nine different individuals, seventy one genes (1.7%) were substantially variable in their expression [223]. Included in this list of genes was expression variability for many regulatory proteins such as DNA binding proteins. Mitochondrial genes were among the seventy-one variable genes, which may suggest differences in energy and oxidative metabolism. Although gene expression across the nine individuals was 98% similar, this study indicates that differential gene expression in normal skin exists between individuals, and such variation therefore must be considered when comparing normal skin to injured skin. The follow-up study comparing uninjured skin to early injury (at thirty minutes and 1 hour), showed a consistent increase in gene expression in 124 of 4000 genes (3%) at thirty minutes [222]. No significant down regulation was noted for any gene at thirty minutes. Most of the up-regulated genes were involved in transcription and signalling. By one hour, only 46 of 4000 genes (1.15%)

were significantly increased; however, 264 of 4000 (6.6%) of genes were significantly down-regulated, which included many structural genes and many other genes with unknown functions.

Affymetrix oligonucleotide microarrays have been used to analyze burn wounds in rats and mice. Spies utilized this microarray technique to determine gene expression at various time points following a burn wound (2, 6, 24, and 240 hours) as compared to uninjured skin in rats [224]. In this study, differential expression of genes were associated with events such as cell survival and death, cell growth regulation, cell metabolism, inflammation and immune response. The time of appearance of various genes matched the known time sequence of a healing burn wound. A similar study was conducted on mice with scald wounds, and revealed significant alterations in the expression of genes, with the greatest changes between 3 and 14 days after wounding [225]. Upregulation of gene expression in this study was both transient and time-dependant. Gene ontologies showed an integrated upregulation of inflammatory and protease genes at early time points in the scald wound.

Nowinski et al. utilized the affymetrix microarray technique to determine how keratinocytes may affect the activity of fibroblasts during the process of re-epithelialization [226]. Cocultures of these two cell types, physically separated by a membrane, were carried out for 48 hours after which fibroblasts were analyzed for gene expression. 243 genes were up-regulated two –fold or more, while 100 genes were down-regulated two-fold or more. This in-vitro model demonstrated that keratinocytes regulated the expression of a total of 343 fibroblasts genes, with the majority of these genes being stimulated.

Another recent study investigated the differences in gene expression between human neutrophils in peripheral blood, and those that have migrated to the site of a skin wound [227]. This study demonstrated that the migration of neutrophils into a skin lesion is associated with an extensive change in gene expression, including up-regulation of anti-apoptotic genes, down-regulation of pro-apoptotic genes, and up-regulated genes for cytokines and chemokines critical for chemotaxis. The cellular fate and function of neutrophils in skin wounds is therefore partially regulated at the transcriptional level.

Many of the cutaneous wound healing studies incorporating microarray techniques are still preliminary studies only. These studies have dealt with differences between baseline and injured tissue, injuries of various forms, and cell types involved in wound healing. Many of the studies utilize animal models, in vitro models, and have small sample sizes. In addition, not all of the studies use the same microarray technique. There are critical differences between oligonucleotide and cDNA microarrays. cDNA microarrays provide the amount of each transcript relative to another sample, while oligonucleotide arrays provide an absolute amount for each transcript [228]. This basic difference creates challenges in the ability to compare observations between the two techniques [229]. In the past, there have been few attempts to replicate and/or compare expression data between different microarray techniques, and there is a general lack of standardized controls for such experiments. Some studies that do compare the two techniques [230, 231] have found large discrepancies in the quantification of gene expression. Caution should therefore be exercised when attempting to compare results between different microarray platforms. Currently, recommendations do exist to increase the reliability of microarray data [216]. These include: adequate sample size, pooling

samples before hybridization, and using other statistical methods besides arbitrary fold increase to analyze the data. Confirmational studies are recommended (such as Northern blot, quantitative RT-PCR, in situ hybridization or immunohistochemistry) to verify expression data. It would not be feasible to verify all genes from a microarray data sheet; however, key genes of interest should be verified in some way.

While more microarray studies are slowly emerging in the area of wound healing, very limited data has been gathered specifically on oral wound healing. In addition, comparisons between oral and cutaneous tissues and wounds using microarray techniques have not been conducted.

CHAPTER II

Aim of the study

The aim of this study was to characterize the gene expression profiles of early human masticatory mucosal wounds after one, three, and seven days of healing. In addition, histologic and clinical analysis was carried out for these wounds for sixty days and six months, respectively. Based on the transcriptional profiling of the mucosal wounds, a novel gene product in the context of oral wound healing was further assessed by localization of the protein within the wounds.

CHAPTER III

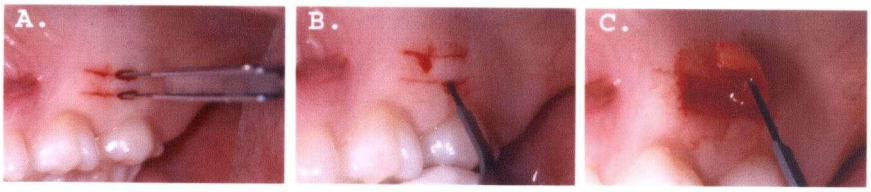
Materials and methods

Experimental wounding

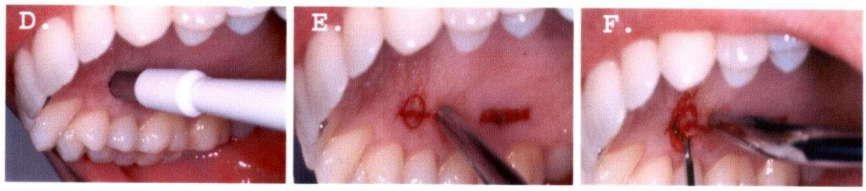
The experimental protocol for this study was approved by the Clinical Research Ethics Board of the University of British Columbia. The study involved twenty-three volunteer subjects. All subjects signed a written consent form to participate in the study. The subjects ranged between 24 and 66 years of age (mean 40.8 years) and were all healthy, non-smoking individuals. Full thickness wounds were made in the palatal masticatory mucosa at least 3 mm away from the gingival margins of the teeth and distal to the canine. The wounds were 10 mm long with the standard width of 2 mm, made with parallel blades. The tissue was dissected off at the depth of 2 mm using mini-blades (6900N Hartzell) (Fig. 1). The tissue harvested from the initial wound (control sample) was rinsed in physiologic saline and placed in either a cryotube (Nalge Nunc Int, Rochester, NY) or embedded in Tissue-Tek O.C.T. (Sakura Finetek Inc, Torrance, CA) and immediately frozen in liquid nitrogen (0 day or control sample). All control tissue specimens were stored at -86°C until further use. The wounds were then left uncovered to heal for a period of 1, 3, 7, 14, 28, or 60 days. Two punch biopsies (4 mm diameter) were taken from each wound and rinsed with physiologic saline. One biopsy was placed into a cryotube and immediately frozen in liquid nitrogen. The second biopsy was embedded in Tissue-Tek O.C.T. and immediately frozen in liquid nitrogen. All samples were then stored at -86°C until further use. Tissue samples (control, 1, 3, and 7 day wounds) that were stored in cryotubes were used for transcriptional profiling (see below).

Tissue samples (control and all wound time points) embedded in Tissue-Tek O.C.T. were prepared for routine histology and immunohistochemistry (see below).

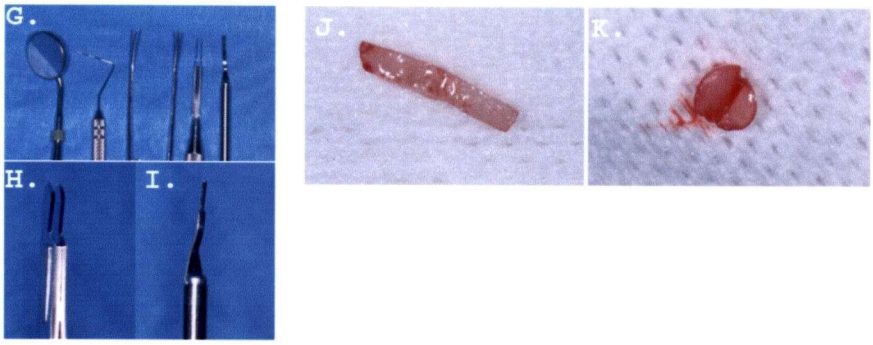
For evaluation of clinical scarring, a survey was conducted at the end of the experiment. Eight randomly organized palatal clinical photos (four pre-wounding samples and four wound sites after 6 months of healing) were shown to three dentists unaware of the purpose of this study. Each dentist was asked to describe whether palatal masticatory mucosa in the photographs was normal or abnormal in clinical appearance.



Initial wound made on healthy palatal gingiva away from the gingival margins of teeth with a standardized double blade holder. Initial tissue sample then removed (A,B,C).



After initial wound has healed for appropriate time, a 4 mm punch biopsy is placed over wound and the wound sample is removed carefully (D,E,F).



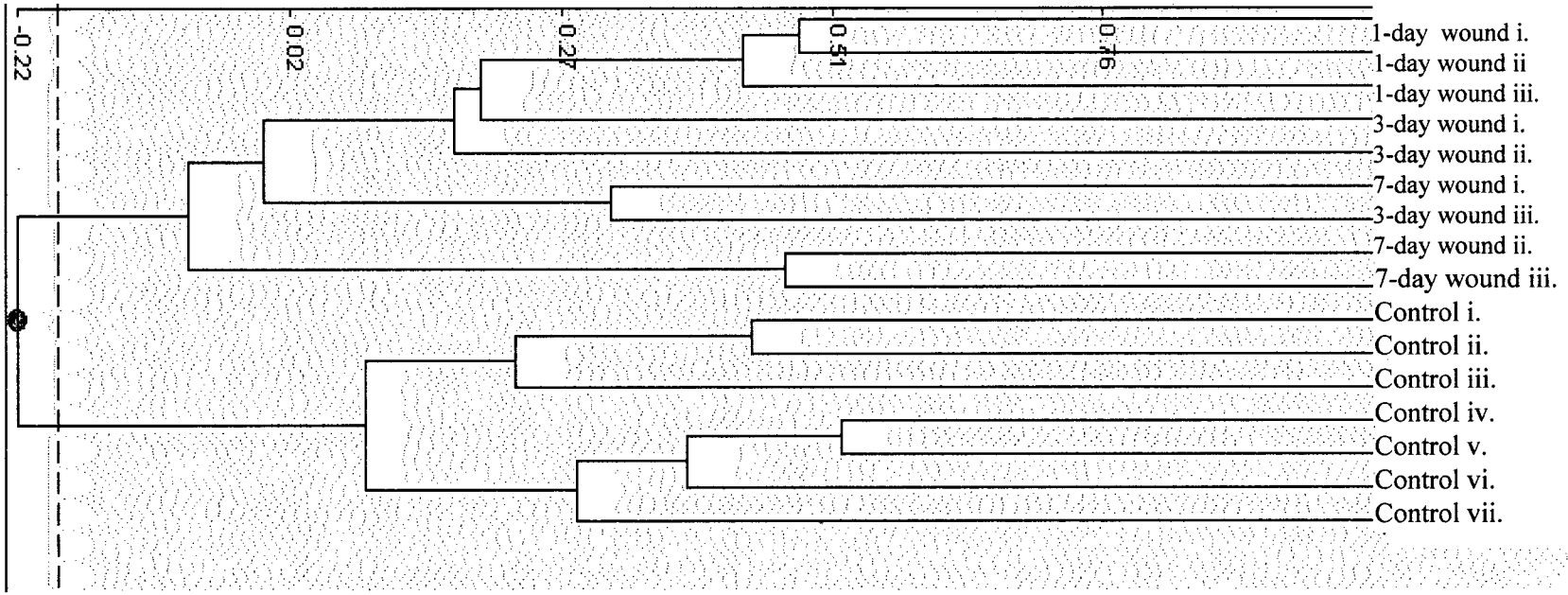
Armamentarium (G) includes mirror, periodontal probe, tissue forceps, double blade holder with # 15 blades, and 6900N mini-blade. Double blade Holder (H) and 6900N mini-blade (I). Initial tissue sample with 10mm X 2mm X 2mm dimensions (J) and biopsy of wound sample with 4 mm diameter and 2 mm depth (K).

Figure 1. Wound Preparation, Biopsy Technique and Armamentarium Used

Transcriptional profiling

Preparation of labelled cRNA was performed as recommended by the manufacturer of the microarrays (Affymetrix Inc). Total RNA was extracted from each gingival sample using TRIzol reagent. Poly(A) mRNA was isolated by reverse transcription with a T7-Oligo (dT) promoter primer to generate double stranded cDNA. The cDNA was then used as a template for in-vitro transcription (IVT) to yield biotin-labelled cRNA's. The biotinylyated cRNA's were then fragmented for hybridization to a gene chip HG-U95Av2 array for 16 hours at 45°C. After hybridization, the probe arrays were then washed in non-stringent buffer and stained with streptavidin phycoerythrin (SAPE) followed by an antibody amplification step. Arrays were then scanned for fluorescence intensities using Affymetrix protocols. Scanned output files were analyzed using Microarray Analysis Suite version 5.0. Agglomerative hierarchical clustering was performed to show correlation of gene expression profiles between individuals (Fig. 2). Results are presented as the fold changes between non-wounded control samples and test samples (for each 1-, 3-, and 7- day palatal wound). Analysis of variance was then conducted to determine the significantly up-regulated and down-regulated genes for all patients at each wounding time point (p-value$\leq$0.01). Genes whose expression did not differ from the background level were omitted. In addition, functional clustering of genes at each wound time-point was determined using the Gene Ontology (GO) Consortium. The "observed" gene ontology classes at each time point were compared to the "expected gene ontology classes that would occur by chance alone using bootstrapping statistical methods (p-value$\leq$0.01).

Figure 2 . Agglomerative Hierarchical Clustering of Gingival Wounds



Routine histology and immunohistochemistry

The control and wound specimens were sectioned (8 μm) in a cryostat and stored at -86°C until used. After fixing with -20°C acetone for 5 minutes, a set of the sections were stained by hematoxylin and eosin for morphological analysis of the wounds. These sections were then mounted with Krazy glue (Elmer's Products Can Inc, Brampton, ON). For immunolocalization studies, sections were fixed with -20°C acetone for five minutes, after which sections were rinsed in phosphate buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA) for five minutes. Sections were then incubated in normal blocking serum (Vectastain;Vector Laboratories Inc; Burlingame, CA) for 30 minutes at room temperature and then incubated with primary monoclonal antibody against carcino-embryonic antigen-related cell adhesion molecule 6 (Ceacam-6) (Genovac; Freiberg, Germany). After rinsing the sections with PBS/BSA (1mg/ml), sections were then incubated with biotinylated anti-mouse secondary antibody for 1 hour at room temperature and then reacted with ABC avidin/peroxidase reagent (Vectastain Elite kit; Vector Laboratories Inc; Burlingame, CA). Sections were rinsed again and reacted with Vector VIP substrate. The reaction was stopped when the desired colour was detected in the cells under a light microscope by immersing the sections in distilled water for five minutes. All sections were then dried and mounted with Vectamount (Vector Laboratories, Burlingame, CA). Control staining was performed without the primary antibodies and no specific reaction was obtained (data not shown).

CHAPTER IV

Results

Clinical healing

All palatal mucosal wounds healed uneventfully and with no complications. Clinical observations of 1-day to 28-day wounds show a continuous progression of healing (changes in the color, outline and size of the wound) (Fig. 3 and 4). The outline of the wound margin could still be delineated for certain individuals after 28 days of healing (Fig. 4). Clinical comparison of same-day wounds between different individuals revealed some variation in healing speed (data not shown). Observations of the palatal mucosa were made following at least 6 months after the biopsy procedure (second wounding) and compared to the baseline palatal mucosa. The majority of the wounds (about 75%, see below) healed by regeneration and wound sites were indistinguishable from the surrounding tissue (Fig. 5). In some cases, however, the wound site demonstrated an outline consistent with a biopsy wound. The clinical survey comparing pre- and post-wounded palates revealed that clinical changes associated with the experimental wounds were not obvious to dental-trained individuals. The only observation regarding four pre-wound and post-wound palates was a possible fibrosis or scar tissue noted in one palate (Fig. 5) by two out of the three examiners.

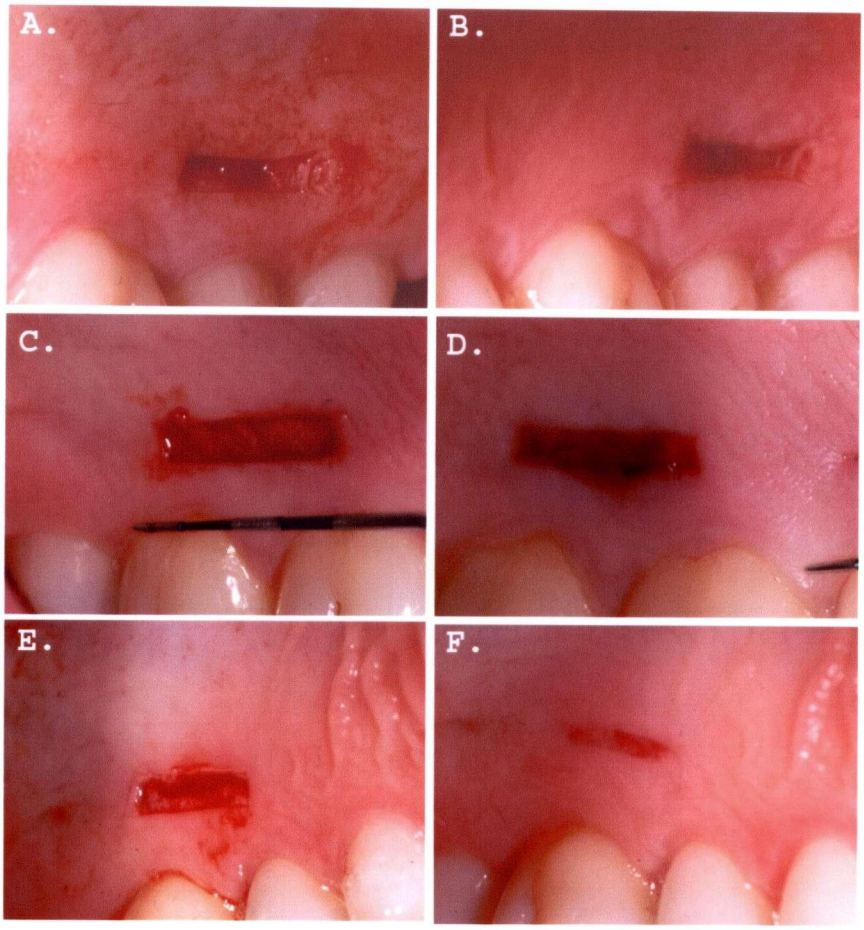


Figure 3. Initial palatal wound (A,C,E) and corresponding 1-day-old wound (B), 3-day-old wound (D), and 5-day-old wound (F).

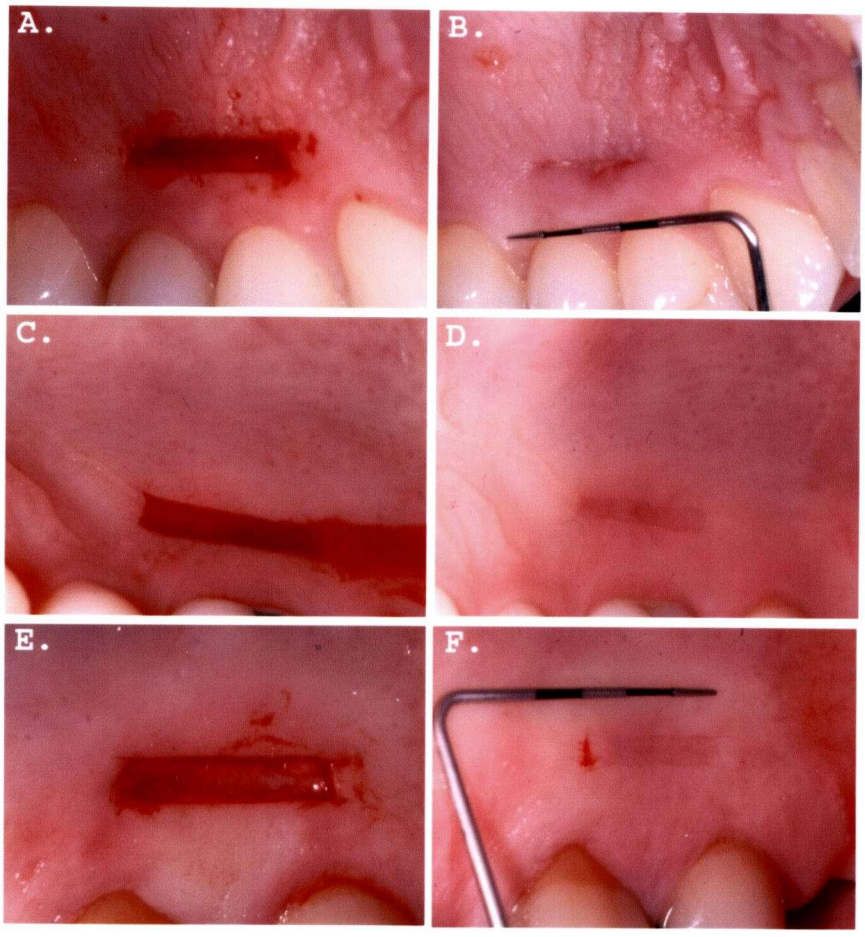


Figure 4. Initial palatal wound (A,C,E), and corresponding 7-day-old wound (B), 14-day-old wound (D), and 28-day-old wound (F).

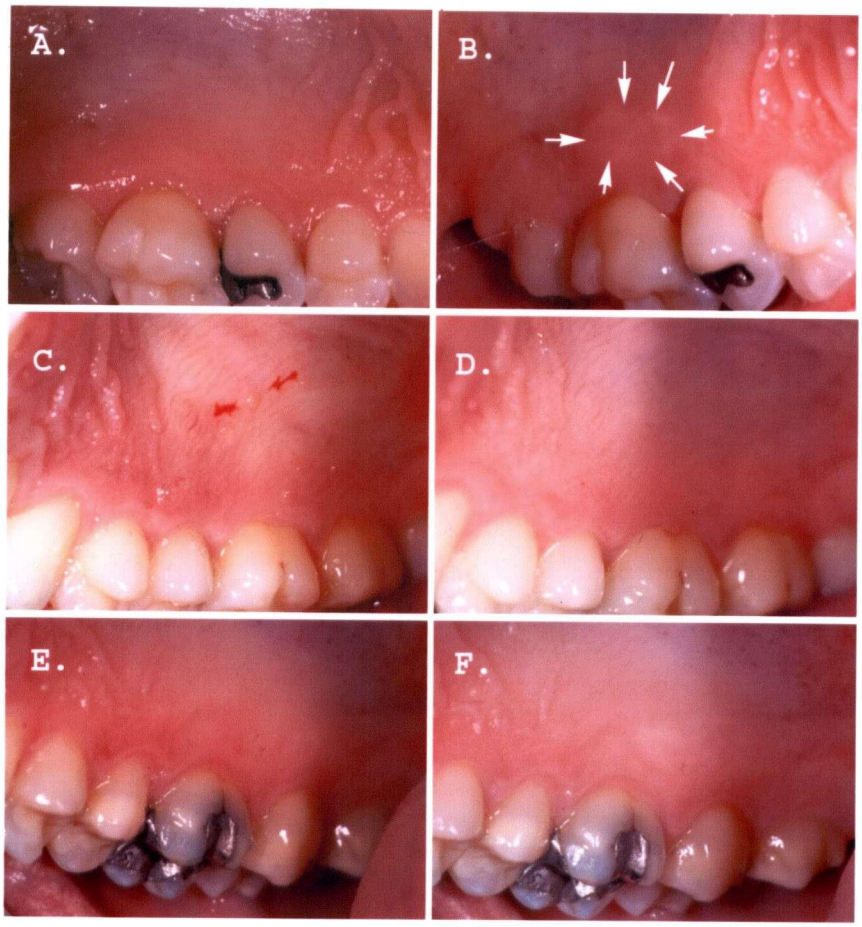


Figure 5. Baseline palatal gingiva prior to initial wound preparation (A,C,E), and corresponding area following more than 6 months healing after wound biopsy (B,D,F). Clinical differences are evident between A and B (area surrounded by arrows), but not evident between C and D, or E and F.

Transcriptional profiling

Seven control tissue samples (0 day) and three samples for each wound time point (1-, 3-, and 7-day) were analyzed by Affymetrix protocols as described above. A total of 988 genes showed a significant fold change in expression in the one-day wound samples compared to baseline non-wounded gingiva. Of this total, 471 (47.7%) were up-regulated and 517 (52.3%) were down-regulated (Fig. 6). At day three, 421 genes showed a change in expression with 270 of these (64.1%) showing up-regulation and 151 (35.9%) showing down-regulation (Fig. 6). By day seven, 543 genes showed a change in expression with 298 (54.9%) being up-regulated and 245 (45.1%) being down-regulated (Fig. 6). No wound time points beyond seven days were analyzed.

Table 1 and 2 list the twenty most up-regulated and down-regulated genes at the 1-day wound. The largest increase in expression was seen for serine proteinase inhibitor B1, matrix metalloproteinase-1 (MMP-1) and carcinoembryonic antigen-related cell adhesion molecule-6 (Ceacam-6) at the 1-day wound. In addition, an increase in expression of certain growth factors and cytokines was seen (IL-6, IL-8, endothelial cell growth factor-1). In contrast, a down-regulation in the expression of collagen type I, III, VI and cytokeratin 2 and 15 were seen at the 1-day wound. Trends in ontology analysis revealed an up-regulation of genes in 24 categories in the 1-day wound including cytokines, negative control of cell proliferation, cell motility, inflammatory and immune response categories, as well as many others (Fig. 7). A general downward trend in 13 categories was seen in the 1-day wound including such categories as extracellular matrix, epidermal differentiation, and collagen categories (Fig. 8).

The 3-day wounds showed some similar changes in gene expression to the 1-day wound in that MMP-1, Ceacam-6, and serine proteinase inhibitor B1 showed the greatest increase in expression (Table 3). Gene ontology analysis showed an upward trend of 10 categories in the 3-day wound including proteinase inhibitors, calcium binding and cytoplasm categories (Fig. 9). Table 4 reveals the most significantly down-regulated genes at the 3-day wound. Ontology analysis revealed a downward trend in six groups in the 3-day-old wounds including epidermal differentiation (as in the day-1 wound), and embryogenesis and morphogenesis categories (Fig. 10).

Tables 5 and 6 reveal the most significantly up-regulated and down-regulated genes expressed following 1 week of healing. Some notable changes in the gene ontology trends were seen at the 7-day wound including both a downward and upward trend in epidermal differentiation, an upward trend in extracellular matrix genes, collagen and collagen binding genes and cytoskeletal structural proteins (Fig. 11 and 12). An upward trend for proteinase inhibitors (with serine proteinase inhibitor B1 still being highly expressed) persisted in the 7-day wound. In total, ontology analysis revealed an upward trend in gene expression for 23 categories and a downward trend in 5 categories.

Figure 6. Significant Alterations in Gene Expression During Wound Healing

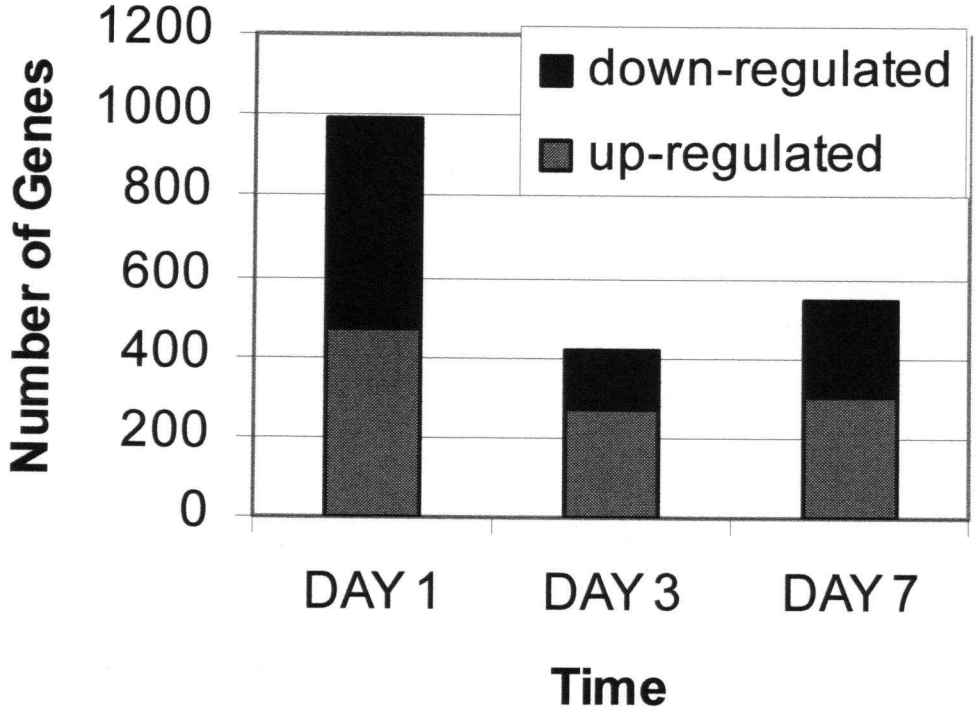


Figure 6. The total number of genes showing a significant change in expression is denoted by the entire bar for each wound time point. Each bar is then divided into up-regulated or down regulated genes.

Figure 7. Over-represented Ontologies (p≤0.01)
Day 1 – Up-regulated genes

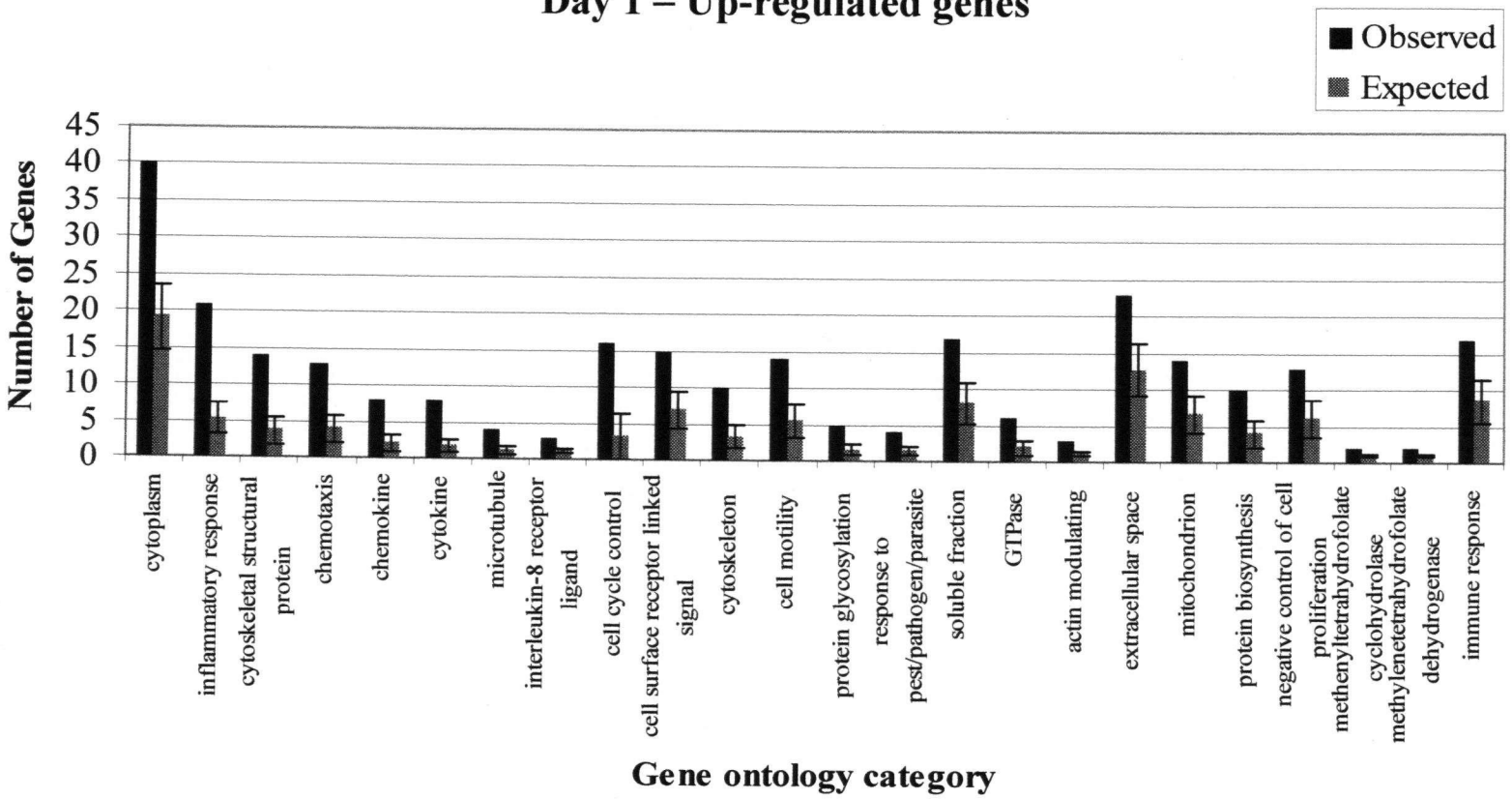


Figure 7. Each gene ontology category consists of a certain number of up-regulated genes. The expected results are the number of genes expressed in each GO category that would occur by chance alone.

Figure 8. Over-represented Ontologies (p≤0.01)
Day 1 – Down-regulated genes

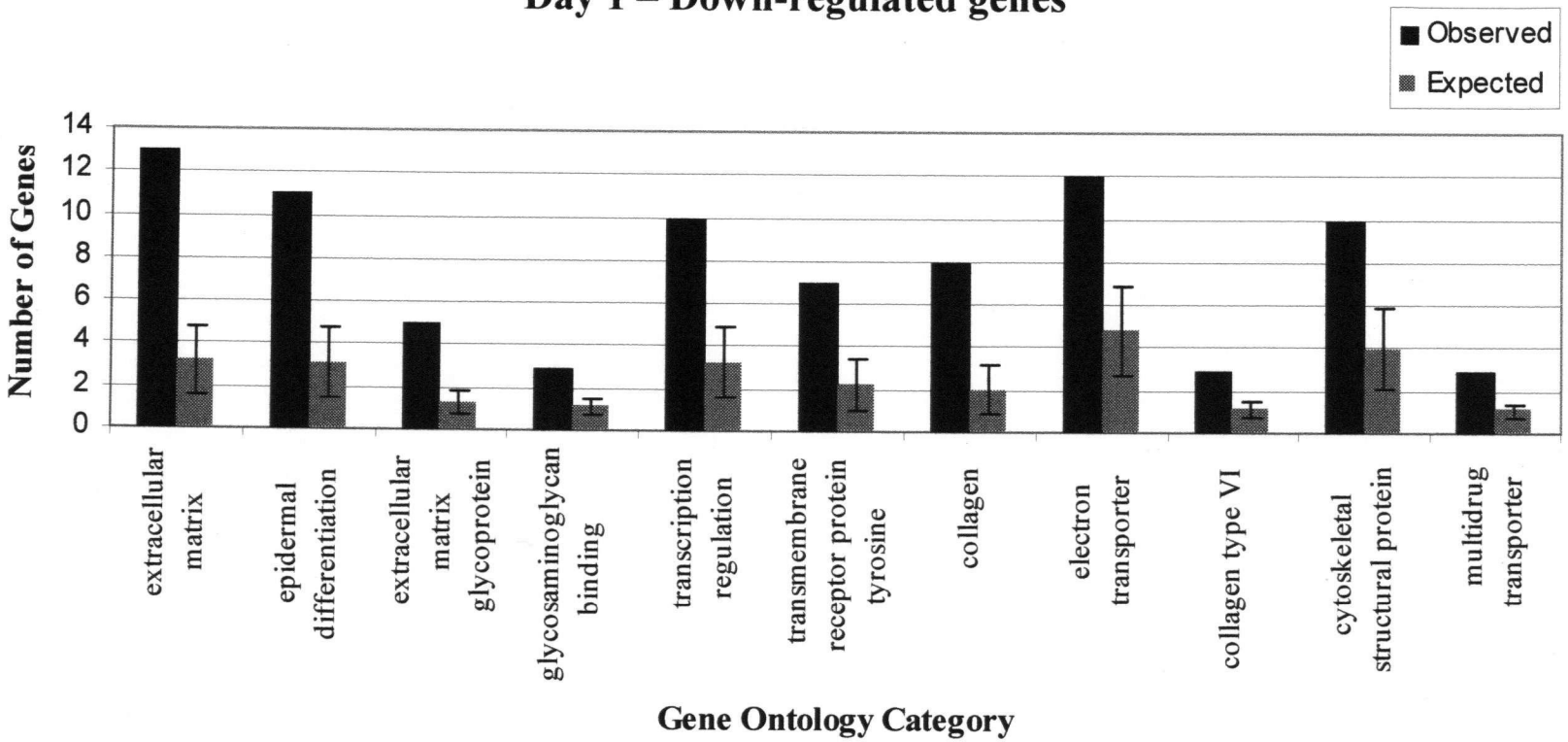


Figure 8. Each gene ontology category consists of a certain number of down-regulated genes. The expected results are the number of genes expressed in each GO category that would occur by chance alone.

Figure 9. Over-represented Ontologies (p≤0.01)
Day 3 – Up-regulated genes

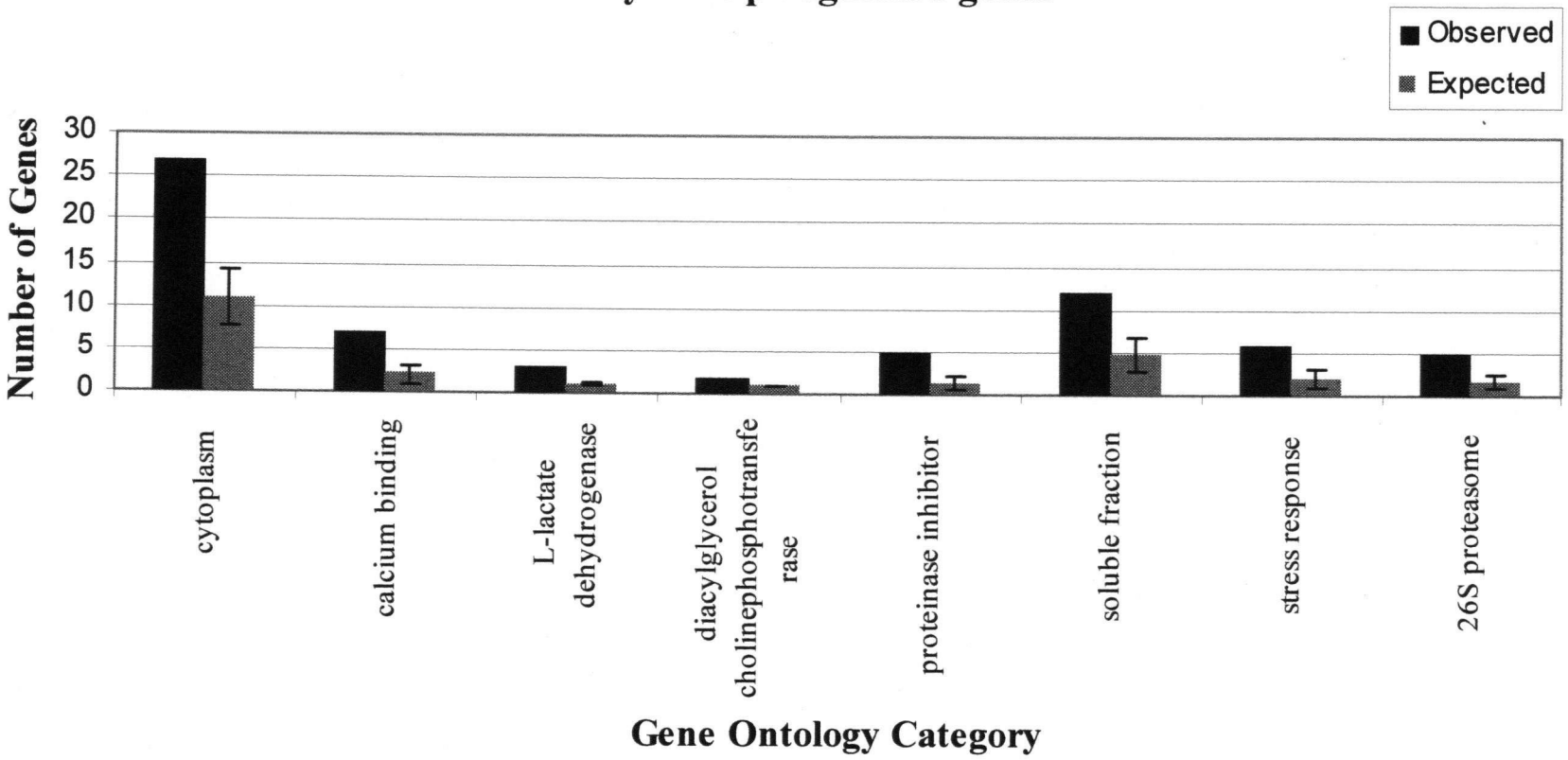


Figure 9. Each gene ontology category consists of a certain number of up-regulated genes. The expected results are the number of genes expressed in each GO category that would occur by chance alone.

Figure 10. Over-represented Ontologies (p≤0.01)
Day 3 – Down-regulated genes

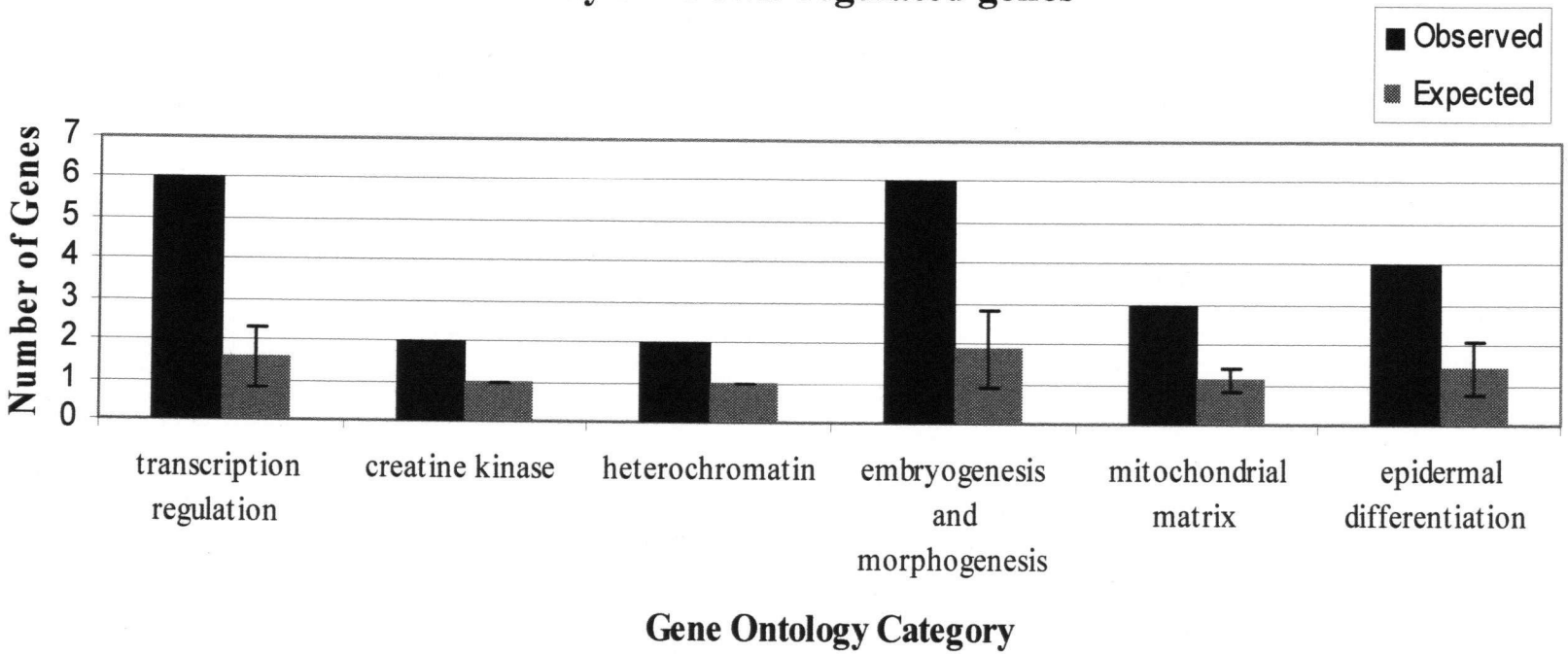


Figure 10. Each gene ontology category consists of a certain number of down-regulated genes. The expected results are the number of genes expressed in each GO category that would occur by chance alone.

Figure 11. Over-represented Ontologies (p≤0.01)
Day 7 – Up-regulated genes

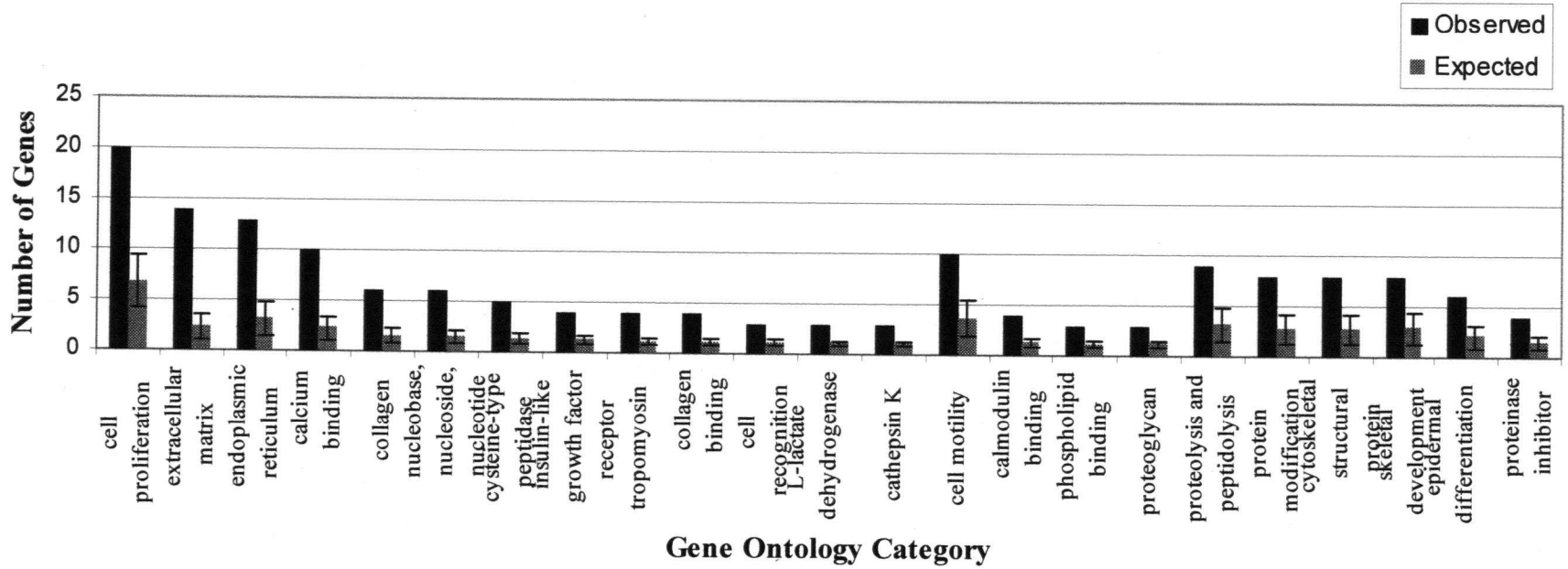


Figure 11. Each gene ontology category consists of a certain number of up-regulated genes. The expected results are the number of genes expressed in each GO category that would occur by chance alone.

Figure 12. Over-represented Ontologies (p≤0.01)
Day 7 – Down-regulated genes

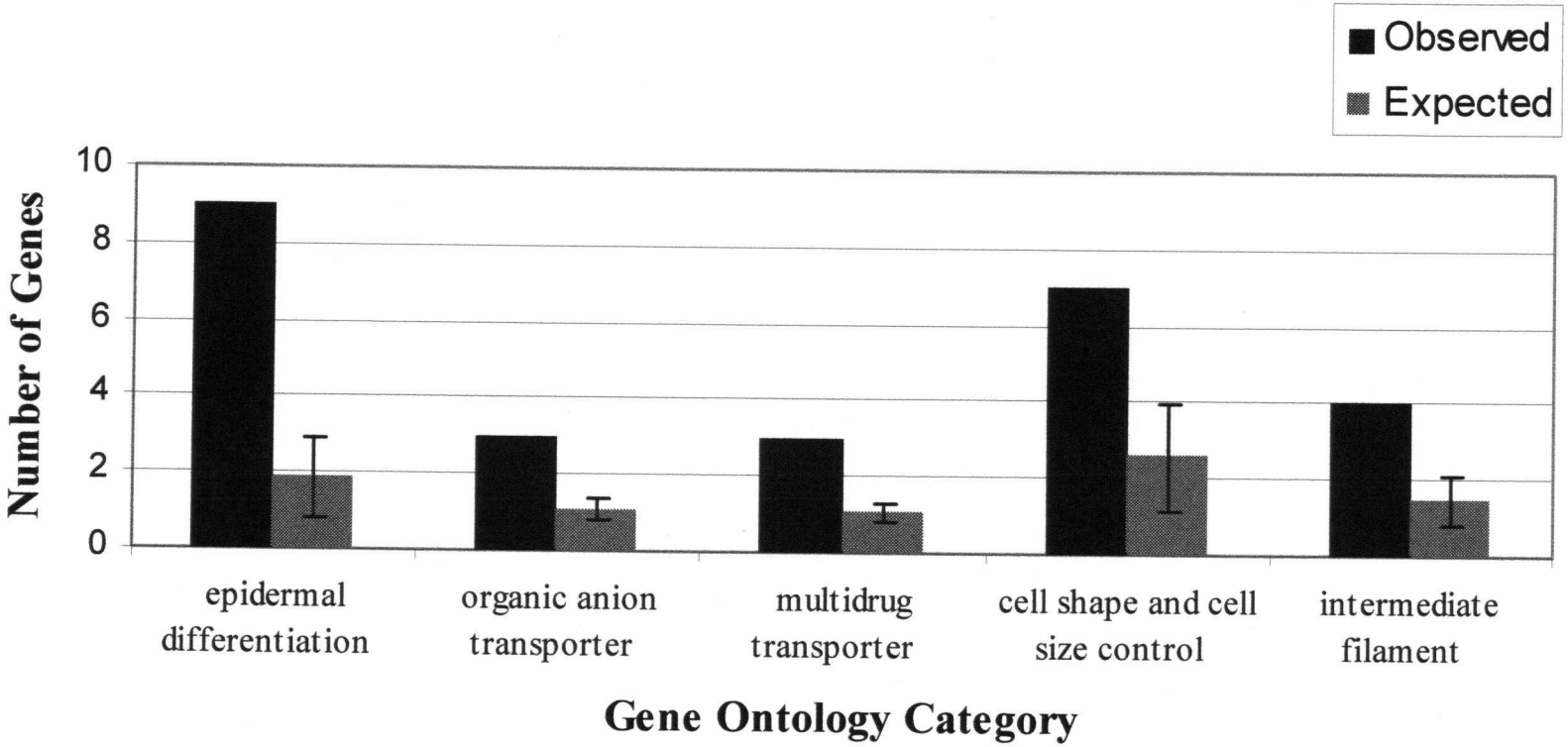


Figure 12. Each gene ontology category consists of a certain number of down-regulated genes. The expected results are the number of genes expressed in each GO category that would occur by chance alone.

Table 1. The Twenty Most Upregulated Genes at Day 1 Wound Compared to Baseline

Gene	Fold Change	Gene Description
SERPINB1	100	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1
MMP1	40.11	matrix metalloproteinase 1 (interstitial collagenase)
NCA	**33.67**	**carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)**
IL6	21.66	interleukin 6 (interferon, beta 2)
DEFB2	18.43	EMBL: Homo sapiens beta defensin 2 (HBD2) gene, complete cds.
MMP9	16.71	matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
GRO1	15.4	EMBL: Human gene for melanoma growth stimulatory activity (MGSA)
GPR37	13.1	Human putative endothelin receptor type B-like protein mRNA, complete cds
G0S2	12.98	EMBL: Human G0S2 protein gene, complete cds.
UP	12.74	uridine phosphorylase
IL8	12.34	interleukin 8
GRO2	11.18	chemokine (C-X-C motif) ligand 2
ANXA3	9.61	annexin A3
SERPINB4	9.39	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 4
ECGF1	9.24	endothelial cell growth factor 1 (platelet-derived)
SLC16A1	8.31	solute carrier family 16 (monocarboxylic acid transporters), member 1
T1A-2	7.93	lung type-I cell membrane-associated glycoprotein
LTBR	7.78	lymphotoxin beta receptor (TNFR superfamily, member 3).
NNMT	7.35	nicotinamide N-methyltransferase
ACTN1	7.32	actinin, alpha 1

Table 2. The Twenty Most Downregulated Genes at Day 1 Wound compared to Baseline

Gene	Fold Change	Gene Description
COL1A2	-21.13	collagen, type I, alpha 2
HSA8SEQ	-13.88	EMBL: Human 3' nucleotide sequence mRNA.
AZGP1	-12.47	alpha-2-glycoprotein 1, zinc
MT4	-9.83	EMBL: Human metallothionein IV (MTIV) gene, complete cds.
FHL1	-9.26	four and a half LIM domains 1
RNASE4	-6.82	ribonuclease, RNase A family, 4
HPGD	-6.81	hydroxyprostaglandin dehydrogenase 15-(NAD)
MYH11	-6.72	myosin, heavy polypeptide 11, smooth muscle
AQP1	-6.61	aquaporin 1 (channel-forming integral protein, 28kDa)
DSIPI	-6.43	delta sleep inducing peptide, immunoreactor
COL3A1	-6.01	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
HUMCYT2	-5.81	cytokeratin 2
IGHG3	-5.59	immunoglobulin heavy constant gamma 3 (G3m marker)
MN1	-5.51	meningioma (disrupted in balanced translocation) 1
FBLN2	-5.5	fibulin 2
AADAC	-5.37	arylacetamide deacetylase (esterase)
KRT15	-5.06	keratin 15
THBS2	-5.05	thrombospondin 2
PPAP2B	-4.94	phosphatidic acid phosphatase type 2B
COL6A1	-4.84	collagen, type VI, alpha 1

Table 3.The Twenty Most Upregulated Genes at Day 3 Wound Compared to Baseline

Gene	Fold Change	Gene Description
SERPINB1	55.89	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1
NCA	**48.12**	**carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)**
MMP1	21.72	matrix metalloproteinase 1 (interstitial collagenase)
UP	18.21	uridine phosphorylase
KLK6	17.2	kallikrein 6 (neurosin, zyme)
SERPINB4	16.17	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 4
S100A7	15.14	S100 calcium binding protein A7 (psoriasin 1)
SLC16A1	11.56	solute carrier family 16 (monocarboxylic acid transporters), member 1
HBB	9.32	hemoglobin, beta
PI3	7.75	EMBL: Homo sapiens elafin precursor, gene, complete cds.
ODC1	7.62	EMBL: Human gene for ornithine decarboxylase ODC (EC 4.1.1.17)
DEFB4	7.13	EMBL: Homo sapiens beta defensin 2 (HBD2) gene, complete cds.
TIMP1	6.76	EMBL: Homo sapiens TIMP gene for tissue inhibitor of metalloproteinases, partial cds.
PRSS3	6.55	protease, serine, 3 (mesotrypsin)
PDXK	6.28	pyridoxal (pyridoxine, vitamin B6) kinase
F2RL1	5.6	coagulation factor II (thrombin) receptor-like 1
S100A13	5.52	S100 calcium binding protein A13
HBAP1	5.03	EMBL: Human alpha globin psi-alpha-1, alpha-2 and alpha-1 genes, complete cds.
MYH11	4.98	myosin, heavy polypeptide 11, smooth muscle
TCRBC1	4.9	human germline T-cell receptor beta chain

Table 4. The Twenty Most Downregulated Genes at Day 3 Wound Compared to Baseline

Gene	Fold Change	Gene Description
APOD	-8.4	apolipoprotein D
MT4	-6.15	EMBL: Human metallothionein IV (MTIV) gene, complete cds.
DSIPI	-3.72	delta sleep inducing peptide, immunoreactor
NR1D2	-3.52	nuclear receptor subfamily 1, group D, member 2
CYP3A5	-3.49	cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 5
MAPT	-3.47	microtubule-associated protein tau
CLU	-3.24	clusterin
MFAP4	-3.22	microfibrillar-associated protein 4
KIAA0802	-3.2	KIAA0802 protein
WEE1	-3.19	WEE1+ homolog (S. pombe)
DBP	-3.11	EMBL: Human D-site binding protein gene, exon 4 and complete cds.
ID2	-2.91	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
RPL23AP1	-2.89	EMBL: Human DNA sequence from clone RP3-377H14 on chromosome 6p21.32-22.1.
PTN	-2.65	pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)
FAT2	-2.59	FAT tumor suppressor homolog 2 (Drosophila)
KIAA0318	-2.54	KIAA0318 protein
D4S234E	-2.49	EMBL: Homo sapiens 21 kDa protein gene, complete cds, clone D4S234.
CKB	-2.43	EMBL: Human gene for creatine kinase B (EC 2.7.3.2)
ABCC5	-2.39	ATP-binding cassette, sub-family C (CFTR/MRP), member 5
IGFBP5	-2.38	insulin-like growth factor binding protein 5

Table 5. The Twenty Most Upregulated Genes at Day 7 Wound Compared to Baseline

Gene	Fold Change	Gene Description
SERPINB1	22.54	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1
MMP1	17.07	matrix metalloproteinase 1 (interstitial collagenase)
KLK6	17.03	kallikrein 6 (neurosin, zyme)
NNMT	15.62	nicotinamide N-methyltransferase
SPP1	14.97	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)
MMP9	14.48	matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
DKFZP586l	13.77	DKFZP586L151 protein
FN1	12.67	fibronectin 1
NCA	**12.46**	**carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)**
PTHLH	12.17	parathyroid hormone-like hormone
T1A-2	11.71	lung type-I cell membrane-associated glycoprotein
FAP	11.57	fibroblast activation protein, alpha
SLC16A1	10.9	solute carrier family 16 (monocarboxylic acid transporters), member 1
UP	8.87	uridine phosphorylase
FCGR3B	8.7	Fc fragment of IgG, low affinity IIIa, receptor for (CD16)
IL6	8.65	interleukin 6 (interferon, beta 2)
SLC16A3	7.65	solute carrier family 16 (monocarboxylic acid transporters), member 3
COL4A1	7.33	EMBL: Human alpha-1 collagen type IV gene, exon 52.
LILRB1	7.12	Homo sapiens monocyte/macrophage Ig-related receptor MIR-7 (MIR cl-7) mRNA, complete cds
NT5	6.29	5'-nucleotidase, ecto (CD73)

Table 6. The Twenty Most Downregulated Genes at Day 7 Wound Compared to Baseline

Gene	Fold Change	Gene Description
MT4	-22.99	EMBL: Human metallothionein IV (MTIV) gene, complete cds.
HSA8SEQ	-11.01	EMBL: Human 3' nucleotide sequence mRNA.
ARG1	-10.58	arginase, liver
DSC1	-8.36	desmocollin 1
KRT2A	-7.98	EMBL: Homo sapiens keratin 2e (KRT2E) gene, complete cds.
CYP3A5	-5.66	cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 5
HTR3A	-5.27	5-hydroxytryptamine (serotonin) receptor 3A
COCH	-5.1	coagulation factor C homolog, cochlin (Limulus polyphemus)
FLG	-4.84	filaggrin
SLAC2-B	-3.91	SLAC2-B
ABCC5	-3.8	ATP-binding cassette, sub-family C (CFTR/MRP), member 5
EPHB6	-3.73	EphB6
EPHA4	-3.69	EphA4
PNUTL1	-3.68	peanut-like 1 (Drosophila)
COMP	-3.54	cartilage oligomeric matrix protein (pseudoachondroplasia, epiphyseal dysplasia 1, multiple)
BMP7	-3.17	bone morphogenetic protein 7 (osteogenic protein 1)
LY6G6C	-3.01	EMBL: Homo sapiens genes encoding RNCC protein, DDAH protein, Ly6-C protein and immunoglobulin receptor
DSIPI	-2.97	delta sleep inducing peptide, immunoreactor
RNASE4	-2.89	ribonuclease, RNase A family, 4
KRT10	-2.83	EMBL: Human gene for acidic (type I) cytokeratin 10

Histology and immunohistochemistry

All wounds were processed for histological analysis with hematoxylin and eosin. The 1-day wound margins were clearly defined with a fibrin clot and a multitude of inflammatory cells established within the wound (Fig. 13). The 3-day-old wound still showed evidence of a fibrin clot with some apparent migration of epithelium along the wound margins (Fig. 13). In some of the 5-day wounds, fibrin clot was still apparent at the top of the wound. Migration of epithelium was clearly evident at the wound edge and epithelium showed increased thickness at the wound margin consistent with strong proliferation. In some, but not in all 5-day-old wounds epithelial sheets were fused in the middle of the wound. Granulation tissue was present in the centre of the wound below the fibrin clot and migrating epithelium (Fig. 13). By day 7, the epithelium had covered all the wounds. Granulation tissue filled the wound below the newly formed epithelium. The wound outline could still be demarcated (Fig. 14). The 14-day-old wounds showed still an increase in the layers of epithelium and rete ridges were being restored. The granulation tissue is still apparent beneath the new epithelium, although the wound margins are not well defined (Fig. 14). By 28 days, the outline of the wound was not readily apparent and the cellularity of the new connective tissue was reduced (Fig. 14). By day 60, the connective tissue is totally regenerated and the wound site cannot be distinguished from the surrounding tissue (Fig. 14). Epithelial rete ridges have been re-established in these wounds (Fig. 14).

To verify the transcriptional profiling at the protein level, we conducted immunolocalization of Ceacam-6 (one of the mostly up-regulated novel genes in all time

points) in healthy gingiva and all gingival wounds. No Ceacam-6 was present in either epithelium or connective tissue in the non-wounded healthy gingiva (Fig. 15). Ceacam-6 was localized in keratinocyte cell membranes of the basal and suprabasal layers of epithelium especially at the wound margin but also at a distance from the margin in the 1-day wound (Fig. 15). Ceacam-6 expression was reduced in the basal layer of epithelium in the 3-day and 5-day wounds with more notable localization of this protein in the suprabasal epithelial layers and in the proliferating and migrating epithelium (Fig. 15). In addition, the staining is seen in closer proximity to the wound margin as compared to the 1-day wound (Fig. 15). No Ceacam-6 is evident in the connective tissue of any of the 1, 3, and 5- day wounds (Fig. 15). By day 7, Ceacam-6 is still noted in the suprabasal layers of the newly formed epithelium (Fig. 16). Positive staining for Ceacam-6 is now evident in the fibroblasts of the newly forming connective tissue in the 7-day wound (Fig. 16). Ceacam-6 staining expression persists in the fibroblasts in the 14-day wound but it is no longer expressed in the keratinocytes (Fig. 16). By day 28, Ceacam-6 expression was no longer seen in either the epithelium or connective tissue (Fig. 16).

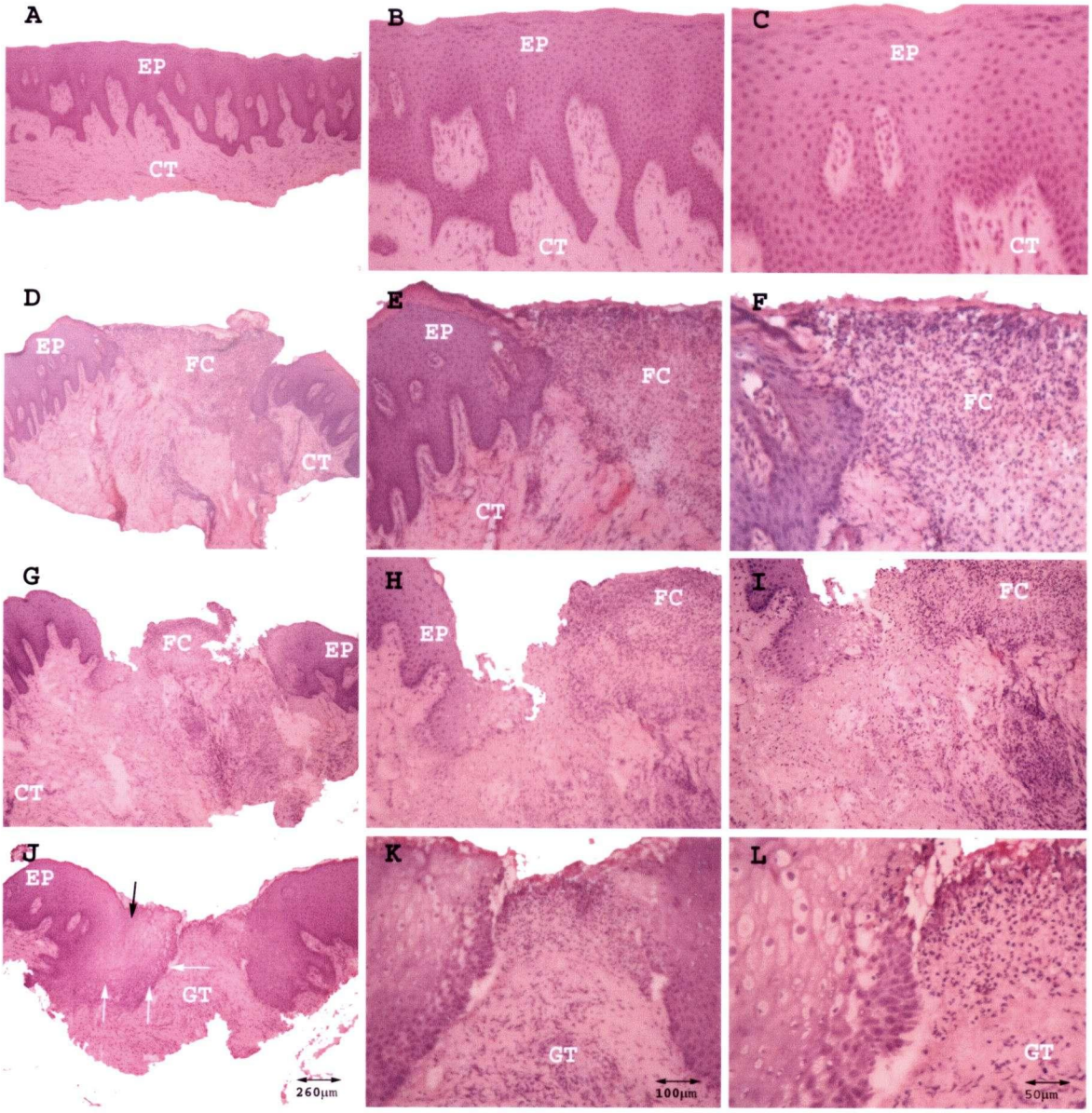


Figure 13. Hematoxylin & Eosin staining of healthy gingiva (A,B,C), 1-day-old wound (D,E,F), 3-day-old wound (G,H,I), and 5-day-old wound (J,K,L). Arrows in panel J mark epithelium that has started to proliferate prior to migration over the wound bed. EP, epithelium; CT, connective tissue; FC, fibrin clot; GT, granulation tissue. Bar 260 µm: A, D, G, J; Bar 100 µm: B, E, H, K; Bar 50 µm:C, F, I, L.

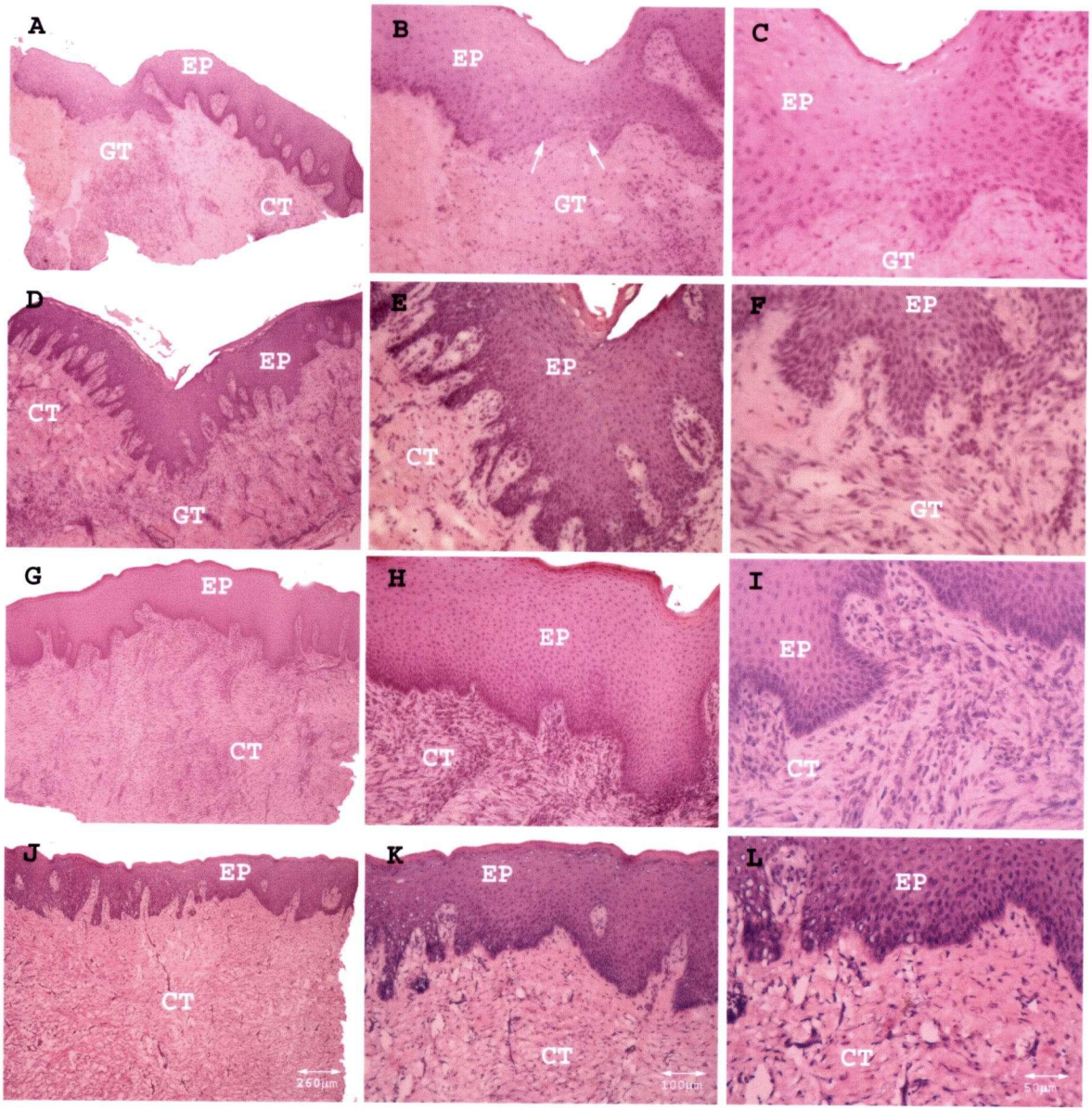


Figure 14. Hematoxylin and Eosin Staining of 7-day-old wound (A,B,C), 14-day-old wound (D,E,F), 28-day-old wound (G,H,I), and 60-day-old wound (J,K,L). Arrows in panel B show that the epithelium has completely covered the underlying granulation tissue at 7 days. The wound is no longer evident by 60 days. EP, epithelium; CT, connective tissue; GT, granulation tissue. Bar 260 mm: A, D, G, J; Bar 100 mm: B, E, H, K; Bar 50 mm: C, F, I, L.

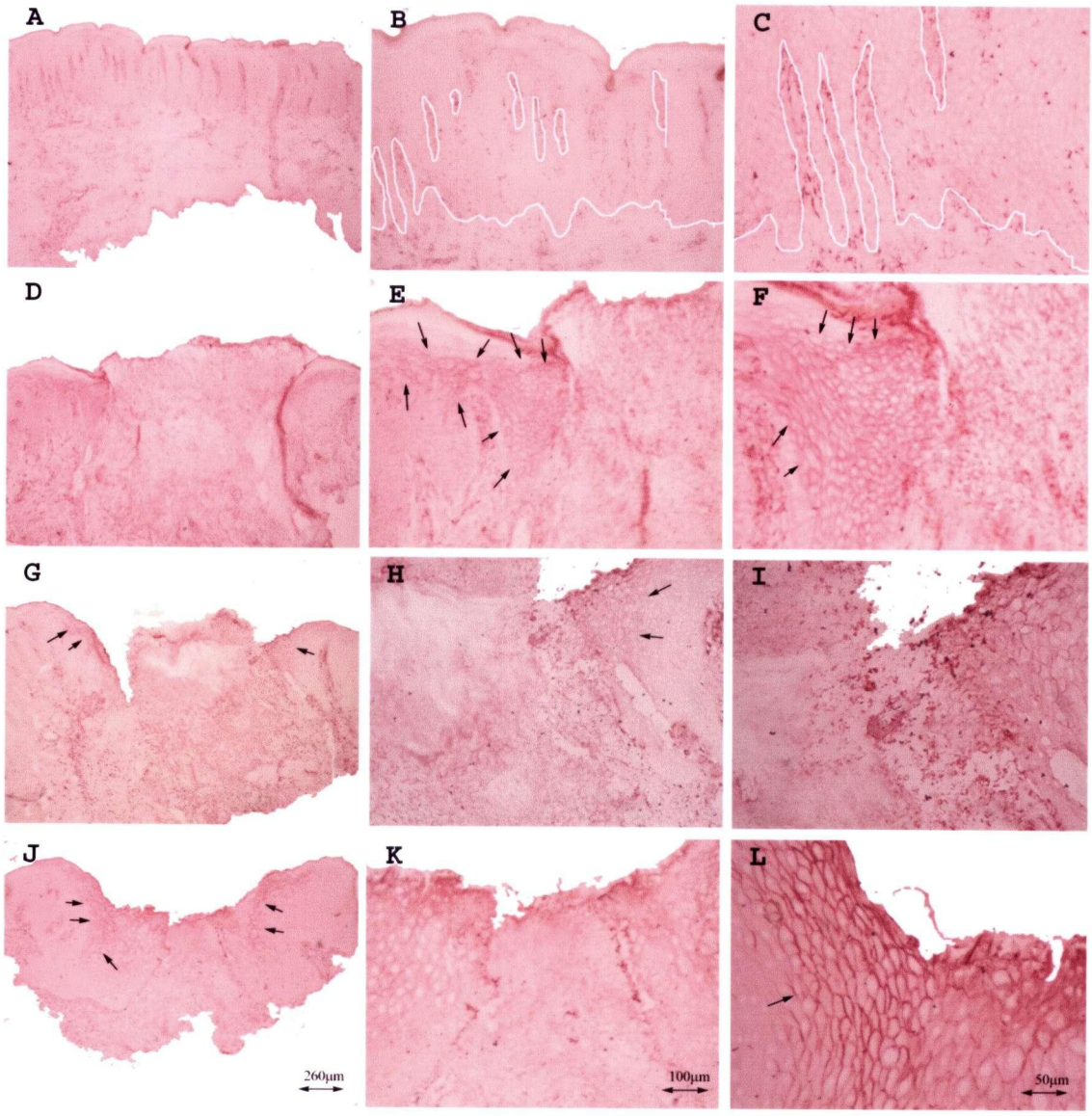


Figure 15. ***Immunostaining of Ceacam-6 in oral gingival wounds.*** Healthy gingiva (A,B,C) does not express Ceacam-6 in either epithelium or connective tissue. In panel B and C, the white line indicates the separation of epithelium and connective tissue. The 1-day-old wound (D,E,F) shows positive staining for Ceacam-6 in the basal and spinous layers of the epithelium (denoted by arrows in E and F). This staining begins distant from the wound margin and continues to the epithelium adjacent to the wound margin where epithelial cells are beginning to proliferate. The 3-day -old wound (G,H,I) and the 5-day-old wound (J,K,L) reveal less staining in the basal layer of epithelium, but persistent staining in the suprabasal layers and the proliferating epithelium adjacent to the wound margin (denoted by arrows in G, H, J, and L). Bar 260 μm: A, D, G, J; bar 100 μm: B, E, H, K; bar 50 μm: C, F, I, L.

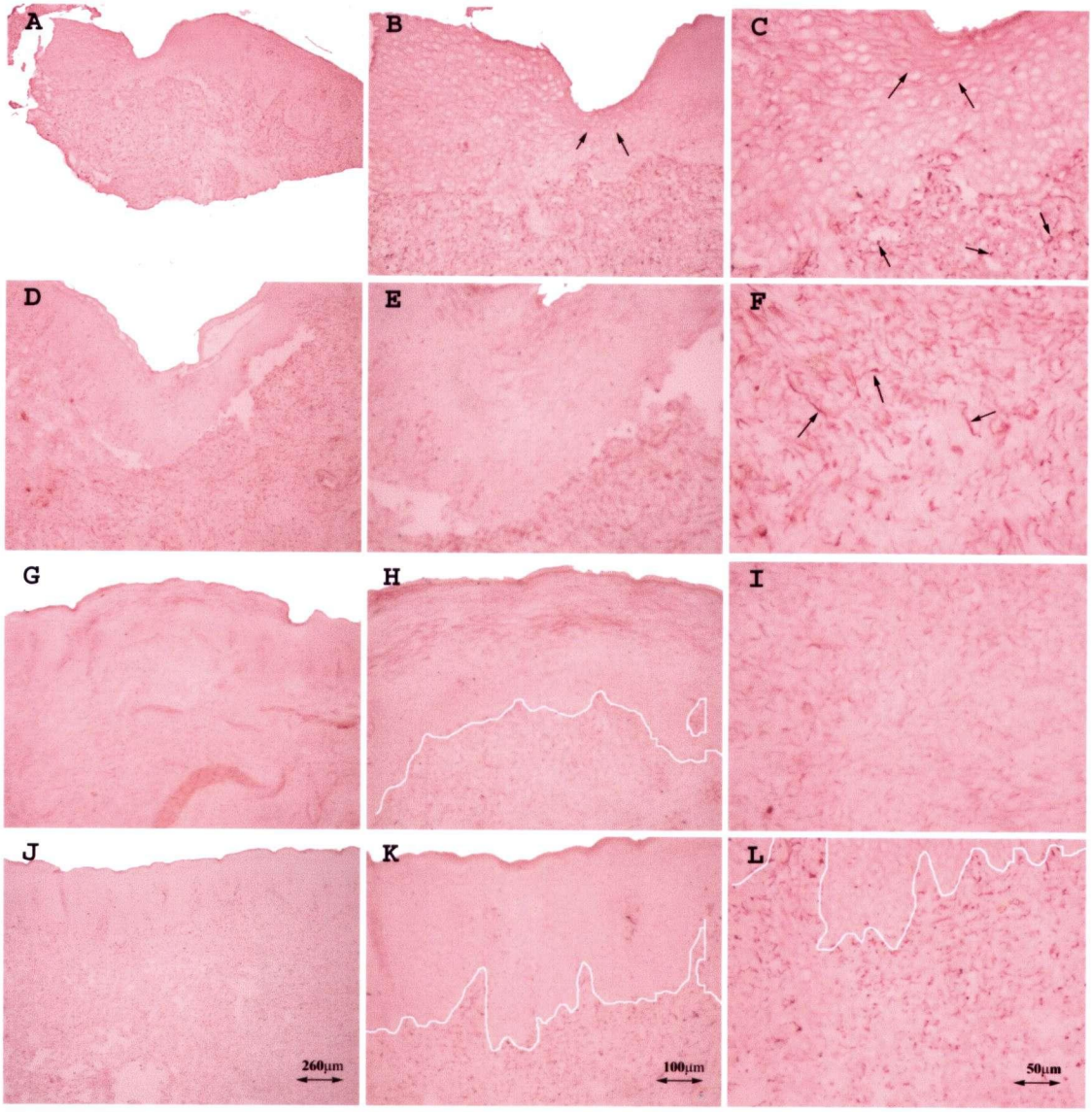


Figure 16. ***Immunostaining of Ceacam-6 in oral gingival wounds.*** The 7-day-old wound (A,B,C) shows some positive staining for Ceacam-6 in the new suprabasal layers of epithelium and an increase in staining of cells in the connective tissue (denoted by arrows in B and C). The 14-day-old wound continues to show expression of Ceacam-6 in cells of the connective tissue (denoted by arrows in F), but staining is no longer positive in the epithelium. The 28-day-old wound (G,H,I) and the 60-day-old wound (J,K,L) no longer exhibit positive staining for Ceacam-6 in either the epithelium or the connective tissue. The outline in panels H, K, and L show the separation between epithelium and connective tissue. Bar 260 μm: A, D, G, J; bar 100 μm: B, E, H, K; bar 50 μm: C, F, I, L.

CHAPTER V

Discussion

The mechanism of adult scarless wound healing is not well understood. As demonstrated in this study, healing of palatal masticatory mucosa in the early stages (1-7 days) involved up-regulation and down-regulation of several genes in a highly coordinated fashion. This study revealed that the gene expression profiles between individuals showed good correlation even though some expected clinical variation in the healing speed was noted between individuals. Wound healing rate and histological healing of this study was comparable to those we have documented previously using the same type of experimental setting [79, 85]. Following more than six months of healing, most wounds healed by regeneration i.e. did not show clinical evidence of scarring. This agrees with the clinicians' observations and some data that even relatively large wound sites in masticatory mucosa heal rapidly and with limited or lack of scar formation [7, 76].

Gene-ontology analysis in this study is consistent with general knowledge of the phases of wound healing. Expected early upregulation of genes involved in processes such as inflammation, cell motility and chemotaxis, and a downregulation in extracellular matrix and collagen formation and epidermal differentiation was observed. Genes involved in cell proliferation, extracellular matrix formation, and epidermal differentiation were up-regulated at a later stage which is consistent with a lower abundance of epithelial cells and fibroblasts at early stages of healing, an increase in layers of epithelium seen after one week and the early stages of fibroplasia. Some of these changes in gene expression found during the first week of wound healing have been documented previously in gene

and protein expression studies over several decades. Therefore, it was not surprising to observe the up-regulation of many extracellular matrix genes, such as collagen type I, III, VI and several proteoglycans that associate with granulation tissue formation in 7-day-old wounds. Equally expected was the relative down-regulation of these genes in earlier wounds that can be explained by the low abundance of mRNA derived from fewer fibroblasts present in the wound biopsy relative to that from inflammatory cells. Indeed, inflammatory cells are likely to contribute to up-regulation of various cytokines and growth factors during early wound healing. For example, the present study revealed IL-8 co-expression with GRO-α/CXC ligand 1 one day after injury in the superficial wound bed, which has been previously determined in cutaneous wound healing [232]. GRO1 and IL-8 are both potent regulators/activators of neutrophil chemotaxis and therefore play a critical role in the early inflammation phase of wound healing [233, 234].

Expression of matrix metalloproteinases (MMP-1, MMP-3, MMP-9, and MMP-10) was highly up-regulated in 1-day-old wounds suggesting the need for active matrix degradation to initiate cell migration, clot degradation and clearance of wound debris. This data agrees with previous reports demonstrating MMP expression profiles in both connective tissue and epithelium during 1, 3, and 7 days post- wounding [235] . Interestingly, the expression of MMP-1 was one of the highest of all genes in all time points studied. The simplest explanation is that the levels in non-wounded tissue are negligible but this should be true for many genes in non-wounded tissue. One would have also expected the expression of MMP-1 to show decline in the 7-day-old wounds in which high synthesis of extracellular matrix is a characteristic feature. Several previous reports have indicated that MMP-1 expression is strongly induced by migrating

keratinocytes in human skin wounds and gradually declines when re-epithelialization is complete [236, 237]. Many different cell types are likely to contribute to the total MMP-1 mRNA up-regulation and further in situ hybridization studies are necessary to pinpoint the cellular origin of MMP-1 in regenerating mucosal wounds. For normal wound healing, it is important that MMP activity stays well controlled as chronic non-healing wounds are characterized by excessive MMP activity [238]. In palatal wounds, an up-regulation of TIMP-1 was seen only in 3-day-old wounds. MMP-1 activity is regulated at various levels and, therefore, no conclusions can be drawn from the relative expression imbalance of MMP-1 and TIMP-1. General proteolytic control in these wounds was sustained as evidenced by a large increase in serine protease inhibitors and proteinase inhibitors in all wounds.

High expression of TGFß3 has been linked to scarless wound healing, and TGFß1 for scar formation, respectively [3]. TGFß1 and TGFß3 were both up-regulated at the same time in 7-day-old wounds (4.7 and 2.6 fold increase respectively). Interestingly, CTGF was up-regulated at all time points (2.7, 4.2, 4.7 fold increase for 1, 3, 7 day wounds, respectively). CTGF has been considered as a downstream mediator of TGFß1 [173] but certainly their gene expression profiles are not linked in oral wounds. More studies are needed to compare gene expression levels in non-wounded and wounded tissue that heal with scars or with regeneration, to unravel whether these processes are inbuilt to the tissue itself.

The gene expression findings of the present study are largely confirmatory which is of great importance in order to lend validity to the use of microarray technologies. The major goal of this gene profiling study was to uncover novel genes that may play key

roles in scarless oral wound healing. To explore whether the alterations in mRNA levels seen in the array would reflect an induction of respective protein levels and to find out which cells are involved, we investigated the tissue distribution of Ceacam-6 protein that is a novel gene in the wound healing context. This gene was chosen for further study based on such a significant increase in expression at the early time points in the wound. Ceacam-6 is a member of the carcinoembryonic antigen family together with Ceacam-1 and Ceacam-7. Ceacam-6 is expressed in normal tissues including the epithelium lining the gastrointestinal tract, eccrine sweat glands, lung epithelia, testes, granulocytes and monocytes but its expression in wounds has not been investigated [239]. Ceacam-6 is up-regulated, however, in colorectal tumors [240]. Biological functions of Ceacam-6 include homotypic or heterotypic cell adhesion [241], inhibition of anoikis [242] and binding to a variety of bacteria [243-245]. Ceacam-6 may therefore play a role in the innate immune response by binding and trapping microorganisms and preventing bacterial invasion [246]. It is possible that induction of Ceacam-6 expression in epithelial cells at the wound margin could play a role in self-defence against bacterial challenge of the oral cavity. Interestingly, Ceacam-6 was also detected in connective tissue cells (fibroblasts) in the later phases of wounding (1-2 weeks post-injury), where it may also play a role in inhibiting anoikis when the cells need to detach from the matrix for migration. Further studies of Ceacam-6 in oral wound healing will be necessary to clarify the function of this protein.

Previous gene profiling studies in human wounds have been limited. The baseline gene expression profiles in uninjured skin, and the gene expression changes at thirty minutes and one hour following initial injury have been determined [222, 223]. It was

found that gene expression between different individuals was largely identical except for variable expression of 71 genes (1.7%), including many regulatory proteins such as DNA binding proteins and mitochondrial genes[223]. The follow-up study comparing uninjured skin to early injury (at thirty minutes and 1 hour), showed a consistent increase in gene expression in 124 of 4000 genes (3%) at thirty minutes [222]. No significant down regulation was noted for any gene at thirty minutes. Most of the up-regulated genes were involved in transcription and signalling. By one hour, only 46 of 4000 genes (1.15%) were significantly increased; however, 264 of 4000 (6.6%) of genes were significantly down-regulated, which included many structural genes and many other genes with unknown functions. Tsou et al. compared gene expression of hypertrophic scar tissue to normal scar tissue and uninjured skin and found that 44 genes were over-expressed and 124 genes were under-expressed in hypertrophic scars compared with normal scars [218]. Other recent studies have also found different gene expression patterns in hypertrophic scars and in keloids when compared to normal skin, including upregulation of TGF-β1 in keloids [220, 221]. In addition to humans, Affymetrix oligonucleotide microarrays have been used to analyze burn wounds in rats [224, 225]. Differential expression of genes was associated with events such as cell survival and death, cell growth regulation, cell metabolism, inflammation and immune response. The time of appearance of various genes matched the known time sequence of a healing burn wound. A study conducted on mice with scald wounds, revealed significant alterations in the expression of genes, with the greatest changes between 3 and 14 days after wounding [225]. It was also found that gene expression was a transient and time-dependant upregulation. Gene ontologies in this study showed an integrated upregulation of

inflammatory and protease genes at early time points in the scald wound. The present study is currently the only human large-scale gene expression study comparing the different phases of oral wound healing for greater than 48 hours. A recent study by Warburton et al. utilized cDNA microarrays to characterize early human mucosal wound healing at 2-4 hours and 48 hours post-wounding [247]. Constitutive gene expression was also determined for the normal, pre-wounded mucosa. After 2 to 4 hours of healing, an upregulation of genes involved in transcription, such as c-fos and jun-D, was noted. After 48 hours, it was found that genes involved in tissue remodelling, such as MMP's, were significantly upregulated, while genes involved in cell adhesion were downregulated. This latter finding is consistent with the one and three day wounds of the present study.

Some of the limitations of the present study include the small sample size analyzed for each gingival wound and the use of microarrays for the early phases of wound healing only. Further studies are needed to corroborate the findings of this study and should incorporate gene expression profiles of later wounds. In addition, it is imperative to compare gene expression profiles of regenerating mucosal wounds to skin wounds that heal by scar formation in an animal model that closely reflects human skin and oral mucosa.

CHAPTER VI

Conclusions and Future Directions

6.1 Conclusions

1. Clinical healing occurred with regeneration or lack of scarring in the masticatory mucosa of the majority of the individuals in the study.
2. Morphologic analysis of all wounds revealed consistent progression in healing from 1 to 60 days, with the final wound (60 days of healing) showing complete regeneration (restored epithelium, rete ridges, and connective tissue).
3. Microarray data revealed large changes in gene expression during the early phases of wound healing, many of which were confirmatory of previous wound healing studies, and others which were novel in the context of wound healing.
4. The expression of one such novel gene, Ceacam-6, was confirmed by the localization of the protein in 1-day to 14-day wounds and may play important roles in the oral wound healing process.

6.2 Future Directions

Future studies will be necessary to corroborate the findings of the present study and should include microarray analysis of later wound time points. It will be important to compare gene expression profiles in regenerating oral mucosal wounds to scar forming skin wounds in both an animal model and in humans. Further studies clarifying the role of Ceacam-6 in both oral and cutaneous wounds should also be conducted. Finally, it may be of interest to compare wound healing of regenerating masticatory mucosa to other oral tissues that have been shown to scar (such as alveolar mucosa).

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