

# **Differences Between Oral and Skin Keratinocytes During Wound Healing**

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THESIS

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## **LIST OF ABBREVIATIONS**

DETCs	Gamma Delta T Cells or Dendritic Epidermal T cells
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FGF-2	Fibroblast Growth Factor-2
FGF-7	Fibroblast Growth Factor-7
FGF-10	Fibroblast Growth Factor-10
FGFR2-IIIb	Fibroblast Growth Factor Receptor-IIIb
FRS-2	Fibroblast Growth Factor Receptor substrate-2
IL-1 $\alpha$	Interleukin-1 $\alpha$
IL-1 $\beta$	Interleukin-1 $\beta$
IL-6	Interleukin-6
IL-8	Interleukin-8
IP-10	Interferon Inducible Protein-10
IRB	Institutional Review Board
KGF-1	Keratinocyte Growth Factor-1
KGF-2	Keratinocyte Growth Factor-2
MCP-1	Macrophage Chemoattractant Protein
MDCK	Madin-Darby Canine Kidney
MMPs	Metalloproteinases
MMP-1	Metalloproteinase-1
MMP-2	Metalloproteinase-2

## **LIST OF ABBREVIATIONS (continued)**

MMP-3	Metalloproteinase-3
MMP-9	Metalloproteinase-9
MMP-10	Metalloproteinase-10
MMP-14	Metalloproteinase-14
MMP-28	Metalloproteinase-2
PCR	Polymerase Chain Reaction
PLC $\gamma$	Phospholipase C- $\gamma$
ROS	Re-active oxygen species
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SD	Standard Deviation
SDF-1	Stromal cell-Derived Factor-1
TGF $\alpha$	Transforming Growth Factor-alpha
TGF $\beta$ 1	Transforming Growth Factor-beta1
TGF $\beta$ 2	Transforming Growth Factor-beta2
TGF $\beta$ 3	Transforming Growth Factor-beta3
TIMPs	Tissue Inhibitors of Metalloproteinases
TNF- $\alpha$	Tumor Necrosis Factor- $\alpha$
VEGF	Vascular Endothelial Growth Factor
WRR	Wound Repair and Regeneration

## Summary

Epithelial cells provide barrier protection throughout the body. Although oral mucosal and skin epithelium share many morphological similarities they also exhibit distinct capacities.

One significant functional difference is the wound healing response, as mucosal wounds heal more rapidly than skin (Szpaderska 2005). The structure of the oral mucosa resembles the skin in many ways. It is composed of two layers, the epithelium and connective tissue. The epithelium of the oral mucosal membrane may be keratinized or nonkeratinized depending on location. In humans and mice, the dorsum of the tongue, gingiva and hard palate are keratinized tissues. Keratinized tissue in the oral mucosa is very similar to skin, making it a good model to study healing.

One important aspect of wound healing is re-epithelialization, which is restoration of the epidermis by keratinocytes. Upon injury, epithelial cells from wound edges dissolve their hemidesmosomal connections, detach from the basement membrane and move quickly across the exposed connective tissue. In the normal situation, cells just behind the wound edge undergo a proliferative and migratory burst and effectively replace keratinocytes lost as a result of injury. This tremendous proliferative and migratory capacity of keratinocytes is critical for effective wound re-epithelialization.

In a study where equivalent full thickness 1mm diameter wounds are created on both the dorsal skin and tongue of the mouse, oral mucosa wounds



exhibit re-epithelialization very rapidly, with 100% completion at 24 hours post-injury. In contrast, cutaneous wounds are less than 25% re-epithelialized at the 24 hour time point (Schrementi 2008). These data strongly suggest that the proliferative capacity of oral keratinocytes is greater than that of dermal keratinocytes. As migration is another critical aspect of re-epithelialization, in this study we investigate whether the migratory capacity of oral keratinocytes is different in comparison to skin.

Evidence to support the hypothesis that differences responsible for rapid repair in oral mucosa could be intrinsic keratinocyte characteristics is available. Since keratinocytes are the major source of VEGF in the wound and produce pro-angiogenic factors in response to hypoxia, investigation of the VEGF mRNA and protein levels in isolated oral and skin keratinocytes was conducted. When subjected to hypoxia, epidermal keratinocytes produced higher levels of both VEGF protein and mRNA than did oral keratinocytes (Szpadarska 2005). These findings imply that the superior repair in oral mucosa is likely to be related to intrinsic characteristics of mucosal tissue and not to environmental factors such as temperature, salivary flow or microflora.

The goal of this study was to identify innate positional differences in epithelial cells that might contribute to the site-specific response to injury.

## **CHAPTER 1 INTRODUCTION**

### **1.1 Overview**

Tissue injury is an occurrence that happens to almost everyone during his or her lifetime. It can be as light as getting a burn while eating a hot pizza or it can involve severe laceration of the skin and deeper tissues. In all cases, after an injury occurs, tissue integrity is re-established through a wound healing process. Healing consists of four overlapping stages: hemostasis, inflammation, proliferation, and remodeling. Without the proper sequence, timing, duration and extent of each stage, the overall and final healing outcome may be adversely affected. The healing process is similar for diverse array of wounds but the rate of healing depends on location, size, bacterial infection, age and overall health of the host (Ashcroft 2002).

Skin wound healing nearly always ends with scar formation. These scars range from having little or no impact on physiologic function to hypertrophic scarring that interferes with the function of soft tissue. Impaired wound healing contributes to physical and psychosocial complications for patients (Occleston 2008). There are an estimated 3 to 6 million people in the United States suffering from non-healing wounds (Mathieu 2006).

Oral mucosa undergoes a similar wound healing process as skin but results in minimal scarring to scarless repair. Studies have shown that skin transposed into the oral cavity maintains its morphologic characteristics (Bussi 1995), and Reilly and co-workers observed an intraoral keloid in transposed skin (Reilly 1980). These findings imply that the repair in oral mucosa is likely to be related to intrinsic characteristics of mucosal tissue and not to environmental factors such

as temperature, salivary flow, or microflora. Because mucosal healing is efficient, it may provide clues as to the critical elements that make skin wounds heal more slowly.

After an injury occurs, epithelial (skin) cells, keratinocytes, need to restore their function as the protective barrier against the outside world, and restoration of lost tissue must occur. This stage of wound repair occurs 2–10 days after injury and is characterized by cellular proliferation and migration of different cell types. The first event is the migration of keratinocytes over the injured dermis. New blood vessels then form, and the sprouts of capillaries, along with fibroblasts and macrophages, replace the fibrin matrix with granulation tissue. Granulation tissue then serves as a new substrate for keratinocyte migration at later stages of the repair process. Keratinocytes that are behind the leading edge proliferate and mature and, finally, restore the full barrier function of the epithelium.

To explore the mechanisms of swift oral wound healing, our laboratory established a model to study wound healing in equivalently sized excisional skin and oral wounds (Szpadarska 2003). Oral wounds exhibited accelerated re-epithelialization as compared to skin wounds. Since keratinocytes are the cell type responsible for re-epithelialization, the data from these studies suggest that there are intrinsic differences in oral versus epidermal keratinocytes. Global gene expression in baseline keratinocyte oral and skin tissues, unwounded tissues, has not yet been studied to determine if intrinsic differences are present even at that level, predisposing these tissues to different wound healing outcomes. Oral

keratinocytes could also mediate faster closure than skin keratinocytes via a higher turnover and a higher proliferative rate than skin. Another potential mechanism of superior oral repair might be that oral keratinocytes produce higher levels of mitogenic and motogenic growth factors in comparison to skin keratinocytes.

We propose to examine regional differences in keratinocyte cell function by using *in vivo* mouse models that allow concurrent analysis of both oral and skin wounds as well as *in vitro* primary cultures to observe keratinocytes independently. The hypothesis of this study is that oral keratinocytes proliferate and migrate at a faster rate than skin keratinocytes, thus wound closure occurs faster in oral mucosa. The current goal of this research is to compare oral and skin wound repair in order to determine which features of oral keratinocytes support rapid wound healing. We also explore whether there are differentially expressed genes in unwounded/normal skin and oral epithelial tissues. The object of the study is to identify innate positional differences in epithelial cells contributing to the site-specific response to injury. Our long-term goal is to discover molecular mechanisms that lead to more rapid oral mucosal healing compared to the skin.

### **1.2 Cutaneous wound healing**

After an injury occurs, wound healing is a physiological process that is essential for tissue homeostasis. However, this process is not perfect, and most cutaneous wounds end with scar formation. Skin wound healing has been studied as far back as 100 years. Skin is a complex tissue that consists of many

structures, cell layers and lineages (Kanitakis 2002). When a “full-thickness” wound occurs on the skin, the damage affects the structures from the outside in. First, the epidermal keratinocyte layer (the body’s barrier to the outside world), together with associated epidermal appendages, such as hair follicles and sweat glands, are disturbed; followed by the basement membrane that underlies the epidermis; and last the dermis, which is an intricate structure that consists of fibroblasts, extracellular matrix (ECM), nerves, blood and lymphatic vessels (Shaw and Martin 2009).

Wound healing is the outcome of many events, including coagulation, inflammation, angiogenesis, collagen synthesis, epithelialization, wound contraction, and remodeling (Diegelmann and Evans 2004). The process is divided into four overlapping phases that coincide with the temporal sequence: hemostasis, inflammation, proliferation and remodeling (Gosain and DiPietro 2004).

Hemostasis is the immediate process after injury characterized by vascular constriction and fibrin clot formation. Following vascular injury, platelets aggregate and degranulate, initiating the clotting cascade. Infiltration of neutrophils, monocytes, and lymphocytes takes place during inflammatory phase. Bacteria and debris are phagocytized and factors are released, causing the migration and division of cells in the proliferative phase. The proliferative phase includes re-epithelialization, angiogenesis, synthesis of collagen, and the formation of extracellular matrix (Mathieu 2006). The remodeling phase involves

maturation and remodeling of cells and tissue, and continues from months to years.

### **A). Hemostasis**

When an injury occurs, cells and blood vessels at the wound margins and its vicinity are ruptured. To alert other body systems to the creation of a wound, mechanical and chemical damage signals are sent off. First, the broken blood vessels need to stop local hemorrhage immediately. This is achieved by platelet activation and aggregation, which results in formation of a fibrin clot consisting of a group of insoluble fibrin fibers (Shaw and Martin 2009).

There are two separate pathways that activate clotting cascade during wound healing. There are called: intrinsic and extrinsic pathways. They do not act independently but rather take place at the same time. Both pathways eventually combine into the common pathway resulting in the formation of fibrin clot (Larjava 2012)

The extrinsic pathway is defined by the involvement of tissue (TF). In damaged blood vessels, circulating factor VII complexes with tissue factor and forms an active protease (VIIa) that eventually results in prothrombin being converted into thrombin (Larjava 2012).

The intrinsic pathway is activated when Factor XII (Hageman Factor) interacts with collagen on the subendothelium of damaged blood vessels and is activated to Factor XIIa. This also eventually results in prothrombin being converted into thrombin (Larjava 2012).

The common pathway initiates when Factor X is changed Factor Xa from either or both extrinsic and intrinsic pathway (Larjava 2012). Once thrombin is formed, it manipulated the formation of a fibrin gel. After an injury has occurred, the blood that has been lost in the extravascular space has undergone the clotting cascade through the extrinsic and common pathways, Blood vessels within the injury undergo the clotting cascade through the intrinsic and common pathways (Larjava 2012).

Besides blocking the vessels, the clot also provides the means of a provisional matrix to which growth factors bind and through which cells can crawl (Nurden 2008). Platelets also release many growth factors and cytokines such as transforming growth factor  $\beta$  (TGF- $\beta$ ), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and endothelial growth factor (EGF) (Le 2005). Platelets press various aspects of the repair process forward through this growth factor release, including angiogenesis, inflammation, and migration of both keratinocytes and fibroblasts. In addition to the platelet-derived growth factors, cells are bathed in other serum factors that leak out of the injured capillaries. Serum, the fluid component of clotted blood, contains many interleukins, colony-stimulating factors, tumor necrosis factor- $\alpha$  (TNF-  $\alpha$ ), interferon- $\gamma$  (INF-  $\gamma$ ) and other components that together lead to induction of serum response factor (SRF). SRF binds to and induces transcription of immediate early genes and early growth response genes (Chaind and Tarnawski 2002; Grose 2002). Other factors that might also contribute to the behavior of wound cells *in vivo* include mechanical signals such as “stretch”, which occurs in response to changing

tissue tensions (Kippenberger 2000); electrical currents that result from membrane damage and breaks in the epithelial barrier (Nuccitelli 2008); and exposure to various microorganisms whose epitopes are largely recognized by Toll-like receptors (TLRs) (Shaykhiev 2008). Activation of epithelial cell TLRs triggers the expression and release of pro-inflammatory mediators and antimicrobial peptides (Shaw 2009).

After bleeding is controlled through vascular constriction and clotting, vascular permeability increases, a circumstance that facilitates an increase in tissue levels of serum proteins and the migration of inflammatory cells into the wound site.

## **B). Inflammation**

### **The innate immune response in wounds**

Immediately following injury, innate immune cells at the site of injury initiate an inflammatory response (Shaw 2009). Cells of the innate immune system stand at the ready, acting as sentinels to respond quickly to tissue damage or microbes. The innate immune cells within the skin are of several types, and include mast cells (Noli 2001), dendritic cells, fixed macrophages (Cumberbatch 2000), and some specialized gamma-delta T cell populations (Jameson 2004). In the skin, keratinocytes are often considered a part of the immune sentinel system as well, as this cell type can quickly respond to stimuli and produces several pro-inflammatory mediators (Kupper, 2004). The response of the innate immune cells to injury is rapid, with cells such as keratinocytes and macrophages able to produce inflammatory mediators within the first hour following insult. Resident



mast cells respond even more rapidly. Mast cells can be activated by numerous stimuli, including trauma, antigen, superoxides, complement, neuropeptides and lipoproteins (Marshall 2004). Once activated, mast cells immediately degranulate, releasing preformed proinflammatory mediators, including histamine, leukotrienes, prostanoids, and cytokines. The mediators produced by resident innate immune cells in response to injury or insult trigger vascular responses, including vasodilation, endothelial cell activation, and increased vascular permeability. Further, early mediators from resident innate immune cells assist in recruiting the first wave of circulating leukocytes from the blood stream into the injured tissue. Thus the resident innate immune cells trip the inflammatory switch, initiating the robust cellular inflammatory response that is observed in wounds.

### **Inflammatory cell infiltration into wounds**

Following tissue injury and innate immune cell activation, the early inflammatory phase is marked by neutrophil infiltration. Neutrophils, a cell type that is rarely found in normal tissue, begin to accumulate in the wound within minutes and continue to increase in number for several days after injury. Similar to all immune cells, the recruitment of neutrophils into wounds is mediated by chemotactic factors generated at the site. As mentioned above, there are many sources of chemotactic factors in the wound, including innate immune cells. Additional chemoattractants can be released by platelets, are produced during complement activation, and may also derive from microorganisms (Robson 2001, Szpadarska 2005).

Sometime after neutrophil infiltration is well underway, the macrophage population within the wound begins to increase. Resident macrophages become activated, and their numbers increase as circulating monocytes move into the extravascular space and differentiate into mature tissue macrophages (Martinez 2006). Although it has been commonly thought that activated macrophages behave uniformly, studies that are more recent show that macrophages are capable of developing diverse phenotypes. In vitro studies suggest that macrophages readily acquire at two different phenotypes that are dependent upon the method of activation. Classically activated macrophages, or the M1 phenotype, are pro-inflammatory cells that produce an array of proinflammatory cytokines. Alternatively activated macrophages, or the M2 phenotype, are anti-inflammatory and support proliferation by producing growth factors (Martin 2009). While these dichotomous phenotypes are well described in some systems, the situation with wound macrophages themselves seems much more complex. Within acute skin wounds, multiple and perhaps intermediate phenotypes are found within the macrophage population. Some studies suggest that macrophage activity during wound inflammation is excessive and promotes fibrosis during healing (Daly 2010, Koh 2011, Koh 2009).

Another cell type that also increases in density in the wound bed is mast cells, with most of the infiltrating mast cells originating in the adjacent tissue (Artuc 1999). As the leukocyte density within the wound increases, the leukocytes that have been recruited into the wound produce large amounts of cytokines and chemoattractants, amplifying the inflammatory response.

In the late inflammatory phase of wound repair, T lymphocytes appear in the wound bed, and may support the resolution and remodeling of the wound (Barbul 1989a; Barbul, 1989b). T-cells may be attracted by interleukin-1 (IL-1), complement components and immunoglobulin G (IgG) breakdown products (Hart 2002; Broughton 2006; Hunt 2000).

### **Inflammatory cell function in wounds**

#### **Neutrophils**

Inflammatory cells have multiple functions in both early and late phases of inflammation (Velnar 2009). The first inflammatory cells to arrive to the site of injury are neutrophils. In the early response, the main function of neutrophils is considered to be prevention of infection by killing of microbes (Broughton 2006). Neutrophils are highly phagocytic, and are important to decontamination of the wound. However, in the absence of microbial insult, neutrophils are not essential for proper wound healing in the skin (Simpson 1972). In a classic study by Simpson and Ross, no discernable differences in fibrin content or distribution were observed in wounds of neutropenic and control guinea pigs. More contemporary studies suggest that in the absence of microbial contamination, neutrophils may be detrimental to repair. The depletion of neutrophils has been shown to accelerate wound closure in both normal and diabetic mice (Dovi 2004). This depletion had no significant effect on collagen deposition, wound disruption strength, or macrophage infiltration in wounds of neutropenic mice in comparison to control.

The negative role for neutrophils in wound healing is thought to derive from the bystander damage caused by these cells as they attempt to clear microbes and debris. Neutrophils achieve the clearance of bacteria, foreign materials and damaged cells in the wound by phagocytosis (Robson 2001; Hart 2002). Neutrophils may also attack microbes by trapping them in extruded nets of histones and DNA (Brinkmann 2004). To perform this function, neutrophils move through intact capillary walls into surrounding body tissue and to the bacteria. However, this fight against infectious organisms does not come without casualties. Activated neutrophils release a set of microbicidal substances that are essential in fighting of invading pathogens. Many of these factors, such as reactive oxygen species, cationic peptides, eicosanoids, and proteases not only kill microbes but also affect healthy host cells (Martin 2005; Dovi 2004; Richardson 2004; Broughton 2006). Neutrophil elastase, for instance, can degrade virtually every component of the extracellular matrix, as well as proteins as diverse as clotting factors, complement, immunoglobulins, and cytokines (Weiss 1989).

Once the bacteria and foreign material have been cleared from the wound site, neutrophils are no longer needed and the task of the host becomes to eliminate them from the wound site. However, clearing of neutrophils from the wound must neither initiate an inflammatory response nor damage the tissue. This is achieved by their extrusion to the wound surface as slough and by apoptosis (Velnar 2009; Hart 2002; Hunt 2000). Apoptotic neutrophils are

recognized by wound macrophages, and are thought to stimulate the macrophages to adopt the M2, or alternative phenotype.

### **Mast cells**

Mast cells are part of the resident innate immune population. They are thought to be critical for the maintenance of tissue integrity and function (Maurer 2003). Once an injury occurs, mast cells degranulate and release a variety of pre-stored mediators from their granules (Puxeddu 2003). Mast cell deficient mice have been described to exhibit impaired wound healing of large sized wounds, although smaller wounds heal equally well in these mice (Egozi 2003; Weller 2006). The depletion of mast cells also leads to a decrease in neutrophils in wounds (Egozi 2003; Weller 2006; Wilgus 2008). However, the number of macrophages and CD-3-positive T cells in wounds are similar to those in wild-type wounds (Egozi 2003; Wilgus 2008). In addition to the resident population, mast cells are recruited to wounds, albeit in a slower time-course than neutrophils. Martin (2005) has suggested that the small numbers of resident mast cells in tissues of injury play a surprisingly important role in the immediate-early activation events, especially to draw in neutrophils. What happens to mast cells in wounds after their job is complete is not well understood. Some studies suggest that they might disperse into the surrounding tissues by the lymphatic drainage (Burman 2005).

Mast cells produce several factors that affect: fibroblast proliferation (Skold 2001), contractility and collagen production (Garbuzenko 2002), and collagen fiber maturation (Kischer 1972). However, an excessive amount of mast cells has

been linked to fibrosis and scar formation. In a pig model of hypertrophic scar formation, the inhibition of mast cell function results in a decrease in wound contraction to normal levels as well as a reduction of wound fibrosis, but without an effect on ECM deposition or remodeling (Corrie Gallant-Behm 2008).

### **Macrophages**

Macrophages have been quoted as “essential”, “indispensable”, and “most important to a successful outcome of wound healing”, as these cells have been shown to produce growth factors that stimulate angiogenesis and fibrogenesis (Leibovich 1975b; Danon 1989; Hunt 1984). Meszaros et al. (2000b) have demonstrated that macrophages, which arrive in the wound bed just after the neutrophils and just prior to neutrophil disappearance, are capable of inducing neutrophil apoptosis *in vitro*. Further, neutrophil-derived fragments have been found in phagosomes inside of macrophages (Meszaros 1999a). It seems that one of the most important function of macrophages to wound healing is to accelerate the regression of the inflammatory response via the elimination of neutrophils.

Macrophages also appear to be an important source of proliferative factors in wounds. Macrophage depletion was first shown to lead to delayed wound healing by Leibovich and Ross, who performed such a depletion study in guinea pigs in 1975 (Leibovich 1975b). More recent studies have used sophisticated genetic manipulations to very specifically deplete macrophage populations in wounds. These studies reinforce the concept that macrophages play an integral

role for a successful outcome of wound healing through the generation of growth factors that promote not only cell proliferation and protein synthesis (Mirza 2009; Goren 2009). Macrophages have different functions during various stages of wound healing. Repair mechanisms controlled by macrophages during the early stages include granulation tissue formation and myofibroblast differentiation (Lucas 2010). During the mid-stage of the repair response, macrophages may stabilize vascular structures and play a role in scar tissue formation (Lucas 2010).

Macrophages release many growth factors within wounds (Rappolee 1988). Growth factors that are important to wound healing, including (transforming growth factor-alpha [TGF $\alpha$ ], transforming growth factor-beta [TGF $\beta$ ] and platelet-derived growth factor are expressed by wound macrophages. Wound macrophages can also stimulate re-epithelialization and angiogenesis by secreting such growth factors as fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) (Kovacs and DiPietro 1994; Bingle 2002). The fate of macrophages in the wound is not completely known. A study by Bellingan *et al* (1996) suggests that the inflammatory macrophages do not die in the wounds but emigrate to the draining lymph node.

The balance of neutrophils and macrophages is also critical to wound healing outcomes. If the inflammatory process is altered such that the recruitment of neutrophils is increased, this may stimulate increased recruitment of macrophages to remove them. The end result of excessive inflammation is local tissue damage and hypoxia, and ultimately, delayed healing.

### **Natural Killer T cells**

Natural Killer T cells are innate lymphocytes. Their role in cutaneous wound healing is adverse, in the sense that they slow down the rate of healing (Schneider 2007). They do not express clonally derived antigen receptors such as those found on B and T cells and migrate to sites of injury during early phases of inflammation (Schneider 2007, Moretta 2002).

In excisional punch wound model, early wound closure is accelerated in the absence of Natural Killer T cells (NKT cells) (Schneider 2011). NKT cells are part of the early wound inflammatory infiltrate with their kinetics of accumulation occurring similar to neutrophil influx (Schneider 2011). NKT cells also influence local TGF- $\beta$ 1 production and in their absence, early wound collagen deposition is increased (Schneider 2011). Due to this finding, it is believed that NKT cells negatively regulate both inflammatory and fibroproliferative signals in the early wound healing (Brubaker 2011).

### **T lymphocytes**

T lymphocytes arrive in the wound after neutrophils and macrophages at about day 7 after injury. Their presence is at its peak during the late proliferative/early remodeling phase.

While the function of T lymphocytes in wounds is not well understood, a reduced level of T cells has been shown to impair healing outcomes. The specific type of T cell appears to be critical to function, as CD 4+ cells (T-helper cells)



have been suggested to accelerate wound healing while CD8<sup>+</sup> cells (T-suppressor-cytotoxic cells) inhibit wound healing (Park 2004; Barbul 1989a; Barbul 1989b). Studies in mice deficient in both T and B cells have shown that scar formation is diminished in the absence of lymphocytes (Gawronska-Kozak 2006).

### **Gamma delta T Cells or Dendritic Epidermal T cells (DETCs)**

The resident gamma delta T-cell populations found in epithelial tissues are different from anywhere else in the body (Allison 1991) due to their differences in expression of tissue-restricted T cell Receptors, TCRs (Jameson 2007). These cells are also called dendritic epidermal T cells (DETC) due to their unique dendritic morphology. DETCs are activated by stressed, damaged, or transformed keratinocytes.

The role of DETCs in wound healing was shown by Jameson *et al* (2002). Full-thickness wounds of TCR delta <sup>-/-</sup> knockout mice, exhibited delayed wound closure that could be rescued by the addition of either activated DETCs or recombinant KGF-1 (Jameson 2007; Jameson 2002).

A role for DETCs in wound inflammation has also been demonstrated. The inflammatory cell content of full-thickness wounds in TCR delta <sup>-/-</sup> knockout mice is normal as far as neutrophils are concerned, yet exhibits delayed infiltration of macrophages in comparison to wild type mice (Jameson 2005; Jameson 2007). The difference in the level of macrophage infiltration was seen in the upper dermal compartment, where wild type mice exhibit large numbers of macrophages by day 2 after injury (Jameson 2005). This absence of

macrophages was not believed to be from a defect in extravasation of macrophages from the blood stream but from a defect in macrophage migration within the damaged dermis (Jameson 2005).

In mice there are different subsets of gamma/delta T cells, depending on such factors as time of appearance in ontogeny, anatomical location, TCR repertoires, and thymus dependence (Haas 1993). Each subset expresses unique adhesion proteins that are responsible for the differences in their migration capacity (Haas 1993). Two different subsets are concentrated in skin epithelia and in the mucosal surfaces of the tongue (Itohara 1990), however they are both derived from the gamma delta T cells which appear first in the fetal thymus (Haas 1993).

### **Cytokines and chemokines in wounds**

Pro-inflammatory cytokines play a critical role in wound healing. Neutrophils and macrophages, along with activated keratinocytes, are major producers of pro-inflammatory cytokines in wounds. These include: Interleukin-1 $\alpha$  (IL-1 $\alpha$ ), IL-1 $\beta$ , IL-6, IL-8 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). IL-1, IL-6, and TNF- $\alpha$  are up-regulated during the inflammatory phase of wound healing (Singer 1999). Together, cytokines prevent infection, stimulate cellular recruitment, and activate immune cells. In addition, cytokines regulate the ability of fibroblasts and epithelial cells to remodel damaged tissue.

Chemokines (chemotactic cytokines) are small heparin-binding proteins that direct the movement of circulating leukocytes to sites of inflammation or injury. They are active participants in the wound healing process because they

stimulate the migration of multiple cell types in the wound site especially inflammatory cells (Barrientos 2008). The CXC, CC, and C families of ligands act by binding to G protein-coupled surface receptors, CXC-receptors and the CC-receptor. Discussed below are select group of mediators chosen due to their direct impact on keratinocytes.

### **Interleukin-1 (IL-1)**

IL-1 is highly up-regulated during the inflammatory phase of wound healing (Singer 1999). This cytokine is produced by diverse groups of cells: keratinocytes, neutrophils, monocytes, and macrophages. IL-1 plays a role in the proliferation of keratinocytes and endothelial cells, activation of neutrophils, natural killer cells, T-cells, B-cells, and macrophages at the wound site (Barrientos 2008).

Upon injury, pre-stored IL-1 is immediately released by keratinocytes, acting as the initial signaling for barrier damage and calling for neutrophils to the damaged site (Barrientos 2008). In addition, neutrophils further stimulate the inflammatory response by secreting IL-1 $\alpha$  and IL-1 $\beta$  (Werner 2003). This is achieved by recruiting monocytes, inducing adhesion molecule expression in blood vessels to promote diapedesis, supporting macrophage survival and retention at the wound site.

IL-1 has both paracrine and autocrine effect on migration and proliferation of keratinocytes. It induces the expression of K6 and K16 keratins in migrating keratinocytes (Komine 2000), activates fibroblasts, and increases secretion of fibroblast growth factor 7(FGF-7)(Tang 1996).

Interleukin-1 signaling plays major contribution to fibrotic healing in multiple systems such as liver, kidney, lung and heart. IL-1 $\alpha$  expression is significantly upregulated in both skin and vaginal mucosa following injury. However, IL-1 $\alpha$  expression is much greater in skin than in mucosa, 15-fold and 3-fold respectively at 12 hours (Gallant-Behm 2011). IL-1 $\alpha$  upregulation is then followed by a significant increase in skin IL-1 $\beta$  expression following re-epithelialization (Gallant-Behm 2011). When wounds in IL-1 receptor knockout mice were compared wounds in wild type mice, the cutaneous wounds in knock out mice exhibited better restoration of normal skin architecture and a marked reduction in fibrosis without compromise in tensile strength. Deep tissue wounds in IL-1 receptor knockout mice also showed reduced collagen levels (Thomay 2009). In a study by Gallant-Behm *et al*, an IL-1 receptor antagonist that inhibits both IL-1 $\alpha$  and IL-1 $\beta$  signaling was administered locally to a subset of wounds following complete reepithelialization. The results showed that IL-1 receptor antagonist treatment reduced skin wound fibrosis (Gallant-Behm 2011).

### **Interleukin-6 (IL-6)**

IL-6 is another pro-inflammatory cytokine and has been shown to be crucial in initiating the healing response. Studies have shown that IL-6 expression increases and remains high after wounding (Grellner 2000; Di Vita 2006). Sources of IL-6 in the wound include neutrophils, monocytes, fibroblasts, and activated mature macrophages.

IL-6 has proliferative effect on keratinocytes and is chemo-attractive to neutrophils (Gallucci 2004). IL-6 can also act as an endogenous pyrogen and

augments immunoglobulin production by B-cells and enhances B-cell growth and differentiation.

The importance of IL-6 in healing has been shown by studies in mouse model systems. Mice genetically deficient for IL-6 exhibit a healing time that is three times of that of wild-type controls. In these knockouts, re-epithelialization was delayed and the granulation tissue formation was also impaired (Gallucci 2000). However, this impaired healing would be rescued through administering recombinant murine IL-6 protein 1 h prior to wounding. Therefore, IL-6 may be more crucial at the beginning of the healing process, which is in accordance with its chemo-attractive effect on neutrophils and mitogenic effects upon wound edge keratinocytes.

Interestingly, IL-6 is also associated with severe scar formation, such as that seen in keloid formation. A study by Xue *et al* 2000 showed that there is increased IL-6 expression at both gene and protein levels in keloids. This is suggested to be through interferon-gamma (IFN-gamma) regulation of IL-6 secretion from fibroblasts (Yellin 1995; Maruyama 1995).

### **Interleukin-8 (IL-8 or CXCL8)**

IL-8 is a member of the CXC family (Raja 2007) and its expression is increased in acute wounds (Engelhardt 1998). IL-8 is a strong chemoattractant for neutrophils, thus participating in the inflammatory response (Rennekampff 2000). A direct homologue of IL-8 is not present in mice, creating some confusion as to its function in rodent systems.

### **Macrophage Chemoattractant Protein (MCP-1 or CCL2)**

MCP-1 is a CC family chemokine. It is induced in keratinocytes upon wounding. It is a chemoattractant for monocytes, macrophages, T-cells, and mast cells (DiPietro 1995).

In MCP-1 knockout mice, wound healing is delayed, especially with the emphasis on re-epithelialization, angiogenesis, and collagen synthesis (Low 2001), however this phenotype is not seen in humans (Engelhardt 1998). When exogenous MCP-1 is applied to wounds in animals, there is only moderate improvement in wound healing (DiPietro 2001).

### **Interferon Inducible Protein 10 (IP-10 or CXCL10)**

IP-10 is a member of the alpha or cysteine-X amino acid-cysteine (CXC) chemokine family of chemotactic cytokines. It is produced late in wound healing, becoming evident 4 d after wounding (Engelhardt 1998). IP-10 is a chemoattractant for monocytes and T lymphocytes. IP-10 can inhibit the proliferation of endothelial cells, and inhibit the growth of tumors *in vivo* (Luster 1998). An IP-10 transgenic mouse, which constitutively expresses IP-10, demonstrates an abnormal wound healing response. It displays an intense inflammatory phase and a prolonged and disorganized granulation phase with impaired blood vessel formation (Luster 1998). The  $\alpha$ -helix domain of IP-10 is able to inhibit endothelial cell motility, vessel formation and induce vessel dissociation via direct binding and activation of the CXCR3B receptor (Yates-Binder 2012).

*In vitro* studies show that IP-10 delays re-epithelialization and prolongs the granulation phase (Shiraha 1999). It inhibits the migration of dermal fibroblasts by blocking their release from the substratum regulated by IP-10 inhibition of EGF and heparin-binding EGF-like growth factor receptor-mediated calpain activity (Shiraha 1999). Also, studies have shown that IP-10 inhibits angiogenesis (Sgadari 1996).

### **Stromal cell-Derived Factor SDF-1 (CXCL12)**

The chemokine SDF-1 (CXCL12), stromal cell-derived factor-1, is a member of the CXC family and is angiogenic (Salcedo 1999). It binds to CXCR4 receptors on endothelial cells, induces endothelial cell chemotaxis and formation of blood vessels (Salcedo 1999). Also, SDF-1 is constitutively abundant in many tissues (Shirozu 1995). Mice lacking SDF-1 or its receptor CXCR4 present with severe developmental defects in the immune, circulatory and central nervous system (Tachibana 1998; Ma 1998; Zou 1998).

SDF-1 plays a role in the inflammatory response by recruiting lymphocytes to the wound and promoting angiogenesis. Endothelial cells, myofibroblasts, and keratinocytes express SDF-1. When homeostasis is disturbed in an acute wound, SDF-1 expression increases at the wound margin in humans (Toksoy 2007). However, starting from day 10 after wounding, SDF-1 is almost completely suppressed in the fibrous neostroma that replaces the provisional matrix covering the initial wound defect whereas endothelial cells, major producers of SDF-1, are abundant at this site (Toksoy 2007). However, in a mouse wound healing model, there is downregulation of SDF-1 (Fedyk 2001). Further, in incisional wounding

there is no expression of SDF-1 mRNA expression during the first 5 days (Florin 2005). However, there is an increase in the expression of SDF-1 in diabetic *db/db* at the wound margin (Asai 2006). In addition, during the initial phase of skin recovery following burns an increase of SDF-1 protein is observed in rats (Avniel 2006).

### **C. Proliferation – Epithelial Reconstitution**

Re-epithelialization is a crucial initial stage of the proliferative phase. It is the re-formation of a barrier as keratinocytes migrate and proliferate from the wound margins. The first response to injury is the coverage of the denuded wound surface by a monolayer of keratinocytes. Once that is complete, epidermal migration ceases and a new stratified epidermis with underlying basal lamina is re-established from the margins of the wound inward. Suprabasal cells cease to express integrins and basal keratins and instead undergo the standard differentiation program of cells in the outer layer of unwounded epidermis. Little is known about keratinocyte “stop” signals except that they probably include contact inhibition arising from mechanical signals (Streker-McGraw 2007).

Keratinocytes migrate in concerted steps: 1) protrusions develop at the leading edge driven by Rac- and Cdc42, 2) integrins come into play and stabilize these protrusions, 3) movement of the cells occurs, and 4) adhesion and cytoskeleton disassembly such as actomyosin mediated retraction takes place (Ridley 2003). Epidermal tissue consists of epithelium and underlying connective tissue. The major cell components of epithelium are keratinocytes while fibroblasts are the primary cell type within the connective tissue. These two types



of cells are constantly influencing each other by secreting autocrine and paracrine growth factors. Other cellular components within epithelium are melanocytes, Langerhans cells, Merkel cells and dendritic epidermal T-cells. Of these, keratinocytes represent the largest population (Feliciani 1996).

The epithelium is separated from connective tissue by a basement membrane. Basal layer keratinocytes are connected to the basement membrane by hemidesmosomes. Adjacent keratinocytes form tight connections between each other by desmosomes, adherens junctions and gap junctions. During epithelium renewal, basal keratinocytes detach from the basement membrane and move toward the outer layers of epithelium while undergoing terminal differentiation (Blanpain and Fuchs 2009 from the book). The neoepithelium differentiates into a stratified epidermis and restoration of an intact basement membrane occurs (Li 2007).

Keratinocytes start to move from edges of the wounds within 24 hours. The flattening and elongation of keratinocytes, development of pseudopod-like projections of lamellipodia and formation of actin filaments are the initial changes in keratinocytes in preparation for migration. Other elements implicated in keratinocyte migration include the extracellular matrix, integrin receptors, matrix metalloproteinase (MMPs) and growth factors. Fibrin, fibronectin and type V collagen provide an initial matrix to allow keratinocyte migration. Surface integrins allow keratinocytes to interact with fibronectin within the matrix, providing the path and direction for migration. Migrating keratinocytes produce MMPs, which degrade type IV collagen and laminins in the basement membrane.

The degradation allows cells to leave basement membrane and migrate into the wound. MMP1 also disrupts attachment to fibrillar collagen and allows continual keratinocytes migration into the wound. Keratinocyte proliferation is mediated by locally released factors such as epidermal growth factor, keratinocyte growth factor, and FGF-7 (auf DemKeller 2004).

Different models have been proposed of the mechanism by which keratinocytes migrate onto the wound. Stenn and Malhotra (1992) have described their model as basal keratinocytes migrating on the wound matrix while pulling along the suprabasal cell layers. In studies of Madin-Darby canine kidney (MDCK) epithelial cells, Farooqui and Fenteany (2004) have shown that submarginal cells of the wounded MDCK cell sheet extend 'cryptic' lamellipodia basally under cells in front of them and crawl actively against the substratum while maintaining cell-cell contacts. Their study showed a delayed transient increase in cell proliferation after wounding, although a complete pharmacological blockage of proliferation did not affect the rate of wound closure. Another model that is often discussed is called "purse-string". Studies both *in vivo* and *in vitro* have shown that epithelial cells entering the wound site in a process that resembles locomotion or invasion (Kiehart 1999). These cells can assemble a supracellular contractile ring or purse string composed of actin and myosin-II that contracts to draw the wound margins inward (Kiehart 1999). Another model, demonstrated by Laplante *et al* (2001) in organotypic model of wound healing, is that of a "leap-frog" mechanism of movement where keratinocytes migrate individually over each other. Suprabasal keratinocytes

crawl over the basal cells and attach to the wound bed forming a new leading front. The organotypic model used by Laplante is in concert with the original studies by Garlick and Taichman (1994); these studies also suggested the “leap-frog” model. In multicellular organisms, the combination of multiple different mechanisms is most likely responsible for effective keratinocyte migration and re-epithelialization in wounds.

#### **D. Proliferation - Angiogenesis**

Angiogenesis is the formation of new blood vessels. Angiogenesis is necessary in the healing wound, because when an injury occurs, blood vessels are severed and hypoxia results. A continuous supply of nutrients and oxygen is needed by rapidly proliferating reparative cells. During the angiogenic process, uninjured endothelial cells proliferate in the extracellular matrix to form new capillaries. This process is mediated by cytokines, growth factors, and inflammatory cells that are present within the hypoxic wound (Bauer 2005). Most importantly, endothelial cells are recruited by a hypoxia-induced secretion of angiogenic factors from macrophages and the presence of fibronectin in the clot (Hunt 1984). The sprouting, migration and formation of blood vessels is regulated by integrin interaction with collagen (Rupp 2001). As tissue heals, these newly formed capillaries eventually regress, resulting in a vascular density similar to normal tissue (Wietecha 2012). This vascular pruning is part of the wound remodeling phase, described in detail below.

One of the most important pro-angiogenic mediators in the healing wound is VEGF (Nissen et al, 1998). Both macrophages and keratinocytes release

Vascular Endothelial Growth Factor (VEGF) (Brown 1992) which aids in the breakdown of the basement membrane and cross-linked fibrins by inducing the production of plasmin and matrix metalloproteinase (Prager 2004). VEGF stimulates endothelial cell migration and capillary formation, a process that ultimately supplies wound bed with oxygen and nutrients (Wilgus 2005). Deodato (2002) showed that human VEGF-encoded recombinant AAV vectors enhanced wound healing, while Roman et al (2002) showed that studies with several anti-angiogenic agents results in decreased vascularity in skin wound. In pig wound models, the application of neutralizing VEGF antibodies has displayed significant reduction in wound angiogenesis, formation of granulation tissue and fluid accumulation (Howdieshell 2001).

VEGF has two high-affinity tyrosine kinase receptors, VEGFR-1 (flt-1) and VEGFR-2 (Ferrera 2003). Even though these receptors are found on endothelial cells, it has also been shown that other cell types, including keratinocytes, express functional VEGFR-1. This finding suggests that keratinocyte-derived VEGF may promote wound repair through actions other than purely angiogenic (Wilgus 2005).

VEGF and the angiogenic process have also been suggested to play a role in the outcome of wound healing, the amount of scar produced. Fetal wounds heal without scar formation. However, when total VEGF levels are increased in fetal skin wounds, a transition from scarless to fibrotic healing is observed (Wilgus 2008). Conversely, in adult incisional wounds, antibody

neutralization of VEGF led to reduced scar size and improvement in the quality of the deposited collagen (Wilgus 2008).

### **E. Remodeling**

The remodeling phase of wound healing takes from months to years to complete. In this stage of wound healing, tissue is restored to optimal strength, function and minimal scarring. At the beginning of the remodeling phase, capillary regression occurs, restoring the vasculature to baseline levels. Capillary regression appears to result from both a decrease in pro-angiogenic stimuli as well as an increase in anti-angiogenic factors in the wound (Wietecha, 2011, 2012).

Changes in the content and architecture of the extracellular matrix are another important element of wound remodeling. This process begins as soon as temporary extracellular matrix is produced in the wound bed. During wound remodeling,, immature extracellular matrix is broken down by metalloproteinases, elastase, and collagenases. During wound healing, collagen type III is produced in the dermal tissue. This collagen has poorly organized structure and is not optimal for final skin architecture. As wound healing proceeds, the poorly organized type III collagen fibers are replaced with well-organized type I collagen fibers, resulting in improved tensile strength (Ballas and Davidson 2001, Gosain and DiPietro 2004). The final result of the remodeling phase depends on the length of the inflammatory response, the development of infection, and the quality of the granulation tissue that is formed (Baum and Arpey 2005).

### **1.3 Keratinocyte–Fibroblast Interactions in Wound Healing**

The epidermis consists of two layers: epithelium and dermis. Cross talk between the major cell constituents of these two layers has been a source of much investigation and must be considered an important element of skin wound healing. Keratinocytes influence fibroblasts by inducing expression of growth factors that are needed for its proliferation and migration during wound healing. This cross talk has been explored in reconstructed *in vitro* epidermis by el-Ghalbzouri *et al* (2002). In cultures where fibroblasts were absent, a complete cessation of keratinocyte proliferation was observed after 2 weeks of culture. The epidermis formed contained only three or four viable cell layers. In contrast, in the presence of fibroblasts, the reconstructed epidermis showed a histologically high similarity to native tissue, indicating the stimulatory role of fibroblasts on keratinocyte proliferation.

Epithelial cells also influence fibroblasts, a process that has also been examined *in vitro* by Nowinski *et al* (2004). Subconfluent keratinocytes were co-cultured *in vitro* with fibroblasts for 48 hours, and large-scale gene expression in fibroblasts was analyzed by a microarray. The results demonstrated that keratinocytes regulate the expression of 343 fibroblast genes in an *in vitro* co-culture model. A majority of these genes were stimulated, supporting the view that keratinocytes act to stimulate fibroblast activity during the re-epithelialization process.

One factor that has been shown to be important to keratinocyte-fibroblast cross talk is IL-1. IL-1, expressed by keratinocytes, enhances expression of Keratinocyte

Growth Factor (KGF-1) in fibroblasts and in this way feeds back to direct keratinocyte proliferation (Maas-Szabowski 1999). Although IL-1 $\alpha$  and -1 $\beta$  themselves had no immediate effect on keratinocyte growth in monocultures, inhibition of their function in co-cultures diminished keratinocyte proliferation to an extent comparable to that obtained by neutralization of KGF-1 (Maas-Szabowski 1999).

One level of control of epithelial-fibroblast interactions is the AP-1 transcription factor subunit JUN, a factor shown to be crucial in the trans-regulatory control of keratinocyte proliferation (Szabowski et al, 2000). In an *in vitro* heterologous organotypic skin equivalent composed of murine fibroblasts and primary human keratinocytes, wild-type fibroblasts allowed keratinocytes to proliferate and to develop into a well stratified epithelium resembling normal human skin. AP-1-dependent KGF-1, GM-CSF, pleiotrophin, and stromal cell-derived factor 1 expression were important regulators of keratinocyte proliferation and differentiation in keratinocyte–fibroblast cocultures (Florin 2005).

During remodeling, keratinocytes balance and modulate the content of extracellular matrix (ECM) in the dermis via the release of Matrix Metalloproteinases (MMP)-2 and -9 and their inhibitors, a process described in detail in the following section. MMP production by keratinocytes seems likely to involve cellular cross talk, as the level of MMP-9 activity has been shown to be higher in the keratinocyte and fibroblasts co-cultures when compared to separate cultures. Thus the keratinocytes and fibroblasts autocrine/paracrine

control mechanism seems involved in ECM modulation in wounds (Sawicki 2005).

#### **1.4 Matrix Metalloproteinases (MMPs) and Keratinocytes**

As mentioned above, epidermal cells in healing wounds secrete matrix metalloproteinases (MMPs), a class of extracellular matrix (ECM)-degrading proteinases, which play a critical role in keratinocyte migration (Martins 2012), and may also influence the dermal ECM. During re-epithelialization, basal keratinocytes dissolve their hemidesmosomes, disrupting their contact with the basement membrane and allowing migration through the wound matrix. MMPs play a crucial role in this process by changing the wound matrix during keratinocyte migration. Keratinocytes can migrate through the provisional wound matrix made of fibronectin and fibrin or “under” this matrix coming into contact with the underlying dermis. Depending on the interaction with different types of ECM, migrating keratinocytes can activate several different integrins:  $\alpha 5\beta 1$  and  $\alpha V\beta 1$  integrins, both fibronectin receptors;  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  integrins that bind to laminin-332;  $\alpha 2\beta 1$  integrin, a collagen receptor; and  $\alpha 9\beta 1$ , a tenascin receptor and integrin  $\alpha V\beta 5$ , a vitronectin receptor (Martins 2012). In this section we will focus on those MMPs that are known to take part in keratinocyte migration and proliferation during re-epithelialization.

##### **Matrix Metalloproteinase-1 (MMP-1)**

Metalloproteinase-1 (MMP-1) is only expressed in basal keratinocytes at the migrating epithelial front in wounds, where there is no basement membrane (Saarialho-Kere 1993). MMP-1 expression is induced only if cells are in contact



with native type I collagen (Martins 2012). Keratinocyte migration on type I collagen is dependent on the interaction between MMP-1,  $\alpha 2\beta 1$  integrin and type I collagen. Acute wounds in human skin, followed for 14 days, demonstrate that MMP-1 expression peaks in migrating basal keratinocytes at the wound edge at day 1 followed by a gradual decrease, being undetectable at the time of complete re-epithelialization (Sudbeck 1997).

### **Matrix Metalloproteinase-3 and -10 (MMP-3 and -10)**

Metalloproteinase-3 and -10 degrade several collagens as well as non-collagenous connective tissue molecules such as proteoglycans, gelatin, laminin and fibronectin (Murphy 1991). They are expressed by epidermal cells during wound repair in both human and mouse wounds. MMP-3 is expressed by keratinocytes proliferating adjacent to wound edge. However, MMP-10 is expressed at the leading edge of the wound (Martins 2012). Keratinocytes expressing MMP-3 are in contact with an intact basement membrane, while MMP-10 expression is induced in keratinocytes migrating on type I collagen (Saarialho-Kere 1994). MMP-10 expression is mostly likely regulated by cytokines such as EGF, TGF- $\beta 1$  and TNF- $\alpha$  (Rechardt 2000). When MMP-10 was overexpressed in the epidermis of wounded skin, the result was a disorganised migrating epithelium with degradation of a newly formed matrix (including laminin-332), aberrant cell–cell contacts of the migrating keratinocytes, and an increased rate of apoptosis of wound edge keratinocytes (Krampert 2004). MMP-3 also regulates wound contraction by influencing the formation of organized actin bundles within dermal fibroblasts (Bullard 1999).

### **Matrix Metalloproteinase-14 (MMP-14)**

MMP-14 is expressed at the migrating front in migrating keratinocytes (Seiki 2002). Disruption of this gene in mice causes defects in type I collagen turnover as well as MMP-2 activation (Holmbeck 1999, Zhou 2000). It has been suggested that MMP-14 may be involved in the regulation of epithelial cell proliferation after tissue injury through a mechanism involving KGF receptor expression (Atkinson 2007). *In vitro* studies have shown that MMP-14 can cleave syndecan-1, CD44 and laminin-332, accelerating migration of different epithelial cells (Martins 2012).

### **Matrix Metalloproteinase-28 (MMP-28)**

Metalloproteinase-28 is the most recently described member of the MMP enzyme family. MMP-28 expression is tightly regulated during wound repair, and is produced *in vivo* by non-migrating, proliferating keratinocytes in response to skin injury (Saarialho-Kere *et al* (2002). Unlike most other MMPs, MMP-28 does not seem to be induced in aggressive subtypes of basal or squamous cell carcinoma of the skin (Saarialho-Kere 2002). MMP-28 is not generally expressed by the same keratinocyte population that synthesizes MMP-1, MMP-9, and MMP-10 (Saarialho-Kere 2002).

### **1.5 Integrins and keratinocytes**

Integrins are molecules that act as glue, binding cells to the extracellular matrix. The basic integrin structure consists of two subunits:  $\alpha$  and  $\beta$ . Their role in wound healing is multi-faceted and includes a role in keratinocyte migration during wound healing. Keratinocytes normally express integrins  $\alpha3\beta1$ ,  $\alpha2\beta1$ ,

$\alpha 9\beta 1$  and  $\alpha 6\beta 4v$ ; during injury these integrins are upregulated. Also during injury, keratinocytes interact with fibronectin filled environment and a new profile of integrins are expressed:  $\alpha 5\beta 1$ ,  $\alpha v\beta 1$  and  $\alpha v\beta 6$  (Larjava 2012, Cavani 1993). In particular,  $\beta 1$  integrins play an important role in keratinocyte migration and wound healing. In  $\beta 1$ -null keratinocyte mice, re-epithelialization is delayed yet proliferation is not affected, (Grose 2002). The role of each integrin in wound re-epithelialization is still incompletely understood, although it seems that redundancy exists. In addition, discrepancies in *in vitro* and *in vivo* studies add confusion to the picture. For example, when  $\beta 5$  and  $\beta 6$  knockout mice were studied, it was observed re-epithelialization was not affected *in vivo*, even though *in vitro* studies suggested otherwise (Huang 1996, Huang 2000).

### **1.6 Growth Factors Essential for Re-epithelialization**

Wound healing and re-epithelialization are known to involve a large number of growth factors that may signal through autocrine or paracrine mechanisms. Growth factors impact cellular function by binding to specific cell surface receptors or ECM proteins (Barrientos 2008). Once a growth factor binds to a receptor on the cell surface, a sequence of intracellular actions occurs. The end result involves the activation of factors which regulate the transcription of proteins controlling such functions as cell cycle, motility and differentiation (Raja 2007). Growth factors are key players in epithelial migration and proliferation. Several essential growth factors that regulate keratinocytes during the proliferative phase of wound healing, as well as growth factors that are produced by keratinocytes during this phase, are discussed below.

### **The Epidermal Growth Factor Family: EGF and TGF-alpha**

The EGF family consists of members that are key players in wound healing and especially in re-epithelialization. Two members of this family are discussed here: Epidermal Growth Factor (EGF) and Transforming Growth Factor  $\text{TGF}\alpha$  ( $\text{TGF}\alpha$ ). EGF is secreted by platelets, macrophages, and fibroblasts. Its effect on keratinocytes is mediated through paracrine and autocrine signaling (Schultz 1991).  $\text{TGF}\alpha$  is secreted by platelets, keratinocytes, macrophages, fibroblasts, and lymphocytes and affects keratinocytes in autocrine fashion (Coffey 1987).

The receptor for both of these growth factors is EGF receptor (EGFR), a receptor that is mainly expressed in the basal layer of epidermis. EGFR is a tyrosine kinase transmembrane protein which upon ligand binding results in dimerization of the receptor, autophosphorylation, and tyrosine phosphorylation of downstream proteins (Oda 2005). EGF and  $\text{TGF}\alpha$  binding to EGFR promotes cell motility in *in vitro* re-epithelialization studies (McCawley 1998). In wounded skin, the EGF receptor is transiently up-regulated and is necessary to the proliferative and migratory aspects of re-epithelialization (Hudson 1998). *In vitro* studies show that shedding of EGFR ligands is a critical event in keratinocyte migration (Tokumaru 2000).

Both EGF and TGF-alpha have been shown to stimulate epithelial cell movement both *in vitro* and *in vivo*. Ando and Jensen used phagokinetic assay, which shows cell migration path as a gold-particle-free area. They observed that the addition of EGF to defined medium in the absence of any other growth factor

induced an increase in migration of 2.5-4.5 fold after overnight incubation (Ando and Jensen 1993). Similarly, a single application of TGF $\alpha$  and EGF exhibited mitogenic effect *in vitro* keratinocyte studies (Li 2006).

*In vivo* studies of the topical application of EGF and/or TGF- $\alpha$  show the effect of these factors on wound healing. The topical application of EGF stimulates epithelialization of partial-thickness wounds and produces a positive impact on the underlying dermis during the early phases of wound repair (Nanney 1990). Exogenous application of EGF or TGF- $\alpha$  to burn wounds on the backs of pigs enhances re-epithelialization (Martin 1997). TGF $\alpha$  knockout mice had significantly delayed epithelialization of partial-thickness ear wounds. However, no difference was observed between TGF $\alpha$  null and control mice in epithelialization of full-thickness wounds (Kim 2001). Feedback loops also exist, as the exogenous application of TGF $\alpha$  or EGF further enhances the expression of TGF $\alpha$  in these cells (Coffey 1987). Together, these studies suggest an important role for members of the EGF family in wound re-epithelialization.

### **Keratinocyte Growth Factor 1 and 2/Fibroblast Growth Factor 7 and 10 (KGF-1, KGF-2/ FGF-7, FGF-10)**

Keratinocyte Growth Factor 1 (KGF-1), a member of the Fibroblast Growth Factor Family, is mitogenic specifically for epithelial cells (Finch 1989). It is expressed by fibroblasts and acts on keratinocytes in a paracrine manner (Smola 1993). In addition, dendritic epidermal gamma delta T cells (DETCs), a cell that

is in (Bergstresser 1983, Tschachler 1983) contact with epithelial cells, express KGF-1 (Boismenu 1994). The receptor for KGF-1, FGFR2-IIIb, is expressed by epithelial cells. Thus, KGF-1 expressed by mesenchymal cells and gamma delta T cells can bind to a receptor that is located on epithelial cells, acting on keratinocytes in a paracrine manner (Rubin 1995, Rubin 1989).

From previous studies, it seems that inflammation has an effect on KGF-1. KGF-1 expression in wounds is greatly stimulated by IL-1 (Chedid 1994) and anti-inflammatory steroids have an inhibitory effect on KGF-1 expression during wound repair (Brauchle 1995). Surprisingly, non steroidal anti-inflammatory molecules have no effect on KGF expression (Chedid 1996).

The role of KGF-1 in wound healing was emphasized with the finding of its upregulation more than 100-fold within 24 hours by dermal fibroblasts at the wound margin (Werner 1992). The role of KGF and its receptor was studied in transgenic mice where a dominant-negative receptor was expressed (Werner 1994). The response to injury in these mice was reduced proliferation rate of epidermal keratinocytes at the wound edge (Werner 1994).

In an experiment where full thickness incisional back wounds were made on KGF-1 null and control mice, no significant differences in healing were observed between the two types of mice (Guo 1996). This result suggested KGF-1 is replaceable during the wound-healing process, possibly by KGF-2 or other growth factors.

Several studies suggest that (Sato 1995, Tsuboi 1993) KGF-1 mediates its effect on healing via the promotion of keratinocyte migration and proliferation.

Beyond the interaction with FGFR2-IIIb, KGF1 is believed to interact with EGFR signaling pathways to induce its mitogenic role (Dlugosz 1994). Moreover, KGF-1 influences keratinocyte function in wounds through multiple other mechanisms, including indirect ones. For example, the expression of Stromelysin-2 (MMP-10), a member of the metalloproteinase gene family, (Windsor 1993) is upregulated by KGF-1 in cultured keratinocytes and is expressed in migrating keratinocytes at the wound edge (Madlener 1996). KGF-1 induces the expression of TGF- $\alpha$  in keratinocytes, a factor that in turn increases keratinocyte proliferation (Dlugosz 1994).

The many effects of KGF-1 suggest that the addition of KGF-1 to wounds might enhance repair. In fact, the topical application of KGF-1 and KGF-2 improves wound healing by accelerating re-epithelialization (Abraham 1996, Jimenez 1999). Staiano-Coico *et al.* showed increased re-epithelialization of split-thickness porcine donor sites treated with KGF-1 (Staiano-Coico 1993). In this study, increased re-epithelialization was believed to occur only through increased keratinocyte proliferation. However, in another injury model, the denuded ear cartilage, a model in which keratinocyte migration is necessary for closure, increased re-epithelialization was not observed with treatment of KGF-1 (Pierce 1994).

Keratinocyte Growth Factor 2 is highly homologous to KGF-1 (57% analogous at the amino acid level) and is also expressed in the dermis (Yamasaki 1996). Expression of KGF-2 is not upregulated in wounds but it's

been suggested that bioactive levels of it might be increased in wounding (Beer 2000). KGF-2 acts by binding to epithelial cell receptor FGFR2-IIIb, the same receptor that recognizes KGF-1, and acts on keratinocytes in a paracrine manner (Ornitz 1996, Igarashi 1998 ). This has led many to believe that KGF-2 serves to compensate the role of KGF-1 in its absence.

The direct role of KGF-2 cannot be assessed in knockout mice, as KGF-2 deficient mice have significant organ development defects leading to death (Ohuchi 2000). Similar to KGF-1, KGF-2 is also expressed by dendritic epidermal gamma delta T cells (DETCs) (Jameson 2002, Boismenu 1994). Mice lacking DETCs show altered re-epithelialization phenotype which is believed to be due to lack of KGF-1 and KGF-2 (Jameson 2002, Boismenu 1994).

In therapeutic uses of KGF-2, the topical application of KGF-2 improves the healing of venous ulcers with success (Robson 2001). Further, its use accelerated the rate of epithelialization in the meshed skin graft model and improved the breaking strength of incisional wounds (Soler 1999).

Both, KGF-1 and KGF-2, increase the production of transcription factors contributing to detoxification of re-active oxygen species (ROS). This reduces apoptosis of keratinocytes so they can be more available for re-epithelialization (Raja 2007).

### **Fibroblast Growth Factor Receptor2-IIIb (FGFR2-IIIb)**

Fibroblast Growth Factor Receptor2-IIIb (FGFR2-IIIb), the receptor for KGF-1 and KGF-2 described above, is a tyrosine kinase receptor and a splicing transcript variant of Fibroblast Growth Factor Receptor 2 (FGFR2) (Capone



2000, Nagy2006). The IIIb splice variant of Fibroblast Growth Factor receptor 2 (FGFR2) is specifically expressed in epithelial cells (Ornitz 1996). It binds Fibroblast Growth Factor-1, Fibroblast Growth Factor-3, Fibroblast Growth Factor-7 (Keratinocyte Growth Factor; KGF-1), and Fibroblast Growth Factor-10 (Keratinocyte Growth Factor -2) (Igarashi 1998; Steiling 2003).

Ligand binding to FGFR2-IIIb results in dimerization, followed by autophosphorylation on tyrosine residues within the intracellular domain and recruitment and phosphorylation of substrate proteins, such as phospholipase C- $\alpha$  (PLC $\alpha$ ) and FGFR substrate 2 (FRS2). FRS2 is a lipid-anchored docking protein that becomes tyrosine phosphorylated and binds to Grb/Sos in response to KGF stimulation (Capone 2000, (Ostrovsky 2002, Marchese 2003). FGFR2-IIIb regulates keratinocyte proliferation and differentiation, and is found mostly within the suprabasal layers (Capone 2000). When FGFRIIIb is stimulated, production of the differentiation-specific keratins, such as K1 and K10 occurs in suprabasalar layers (Dlugosz 1994, Marchese 2001) When FGFR2-IIIb is ablated, keratinocyte proliferation is reduced (Petiot 2003).

### **Transforming Growth Factor- $\beta$ (TGF- $\beta$ )**

The Transforming Growth Factor-  $\beta$  family includes TGF-  $\beta$  1-3, bone morphogenic proteins (BMP), and activins. From this family of growth factors TGF-  $\beta$ 1 has received the most experimental attention in adult cutaneous wound healing. TGF-beta 1 can be produced by macrophages, fibroblasts, keratinocytes, and platelets (Lee 1997, Eppley 2004, Wu 1997, Mani 2002, Rolfe 2007). TGF-  $\beta$ 1 plays role in inflammation, angiogenesis, re-epithelialization, and

connective tissue regeneration in wound healing. Its expression is increased upon injury (Kopecki 2007, Kane 1991).

TGF- $\beta$  is chemotactic for fibroblasts, keratinocytes, and inflammatory cells. The TGF- $\beta$  family of proteins consists of several structurally related but functionally distinct isoforms (Schrementi 2008). In mammals, three isoforms, TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3, are identified (Massague 1990). Studies have suggested specific roles for each of the TGF- $\beta$  isoforms in inflammation, proliferation, and the remodeling stages of wound healing (Roberts 1988, Wahl 1987). TGF- $\beta$ 1 promotes inflammatory cell recruitment. Inflammatory cells recruited to the wound produce additional TGF-  $\beta$ 1, increasing the inflammatory cell response (Moses 1990). TGF-  $\beta$ 1 is also believed to be a regulator in the development of hypertrophic scarring and keloid formation (Russell 1988; Ghahary 1993), while increased levels of TGF- $\beta$ 3 are associated with improved healing and reduced scar formation.

In the process of re-epithelialization, TGF-  $\beta$ 1 has been shown to induce keratinocyte integrin expression from stationary to a more migratory phenotype (Li 2006). Transforming Growth Factor-  $\beta$  (TGF- $\beta$ ) has an interesting effect on wound re-epithelialization. Studies have shown that re-epithelialization is accelerated in mice expressing a gene encoding a dominant- negative TGF- $\beta$  receptor in the epidermis (Mazzieri 2005, Sellheyer 1993, Amendt 1998) *In vivo* studies have shown that mice deficient in Smad3, a protein which transduces signals from TGF- $\beta$  (Smad3<sup>ex8/ex8</sup>) have enhanced cutaneous wound healing when compared to wild-type. Wound healing in Smad-3 null mice is

characterized by an increased rate of re-epithelialization and significantly reduced local infiltration of monocytes (Ashcroft 1999). Interestingly, the overexpression of TGF-  $\beta$ 1 increases the proliferative phenotype of keratinocytes during the late stages of wound healing but not re-epithelialization rates (Zamburno 1995, Bottinger 1997).

In addition to a role in epithelial repair, TGF-  $\beta$ 1 influences collagen type I and type III production, inhibits metalloproteinase MMP-1, MMP-3, and MMP-9 production, and promotes metalloproteinase TIMP-1 synthesis, inhibiting collagen breakdown (White 2000, Mauviel 1996, Papakonstantinou 2003, Zeng 1996). In fetal wounds, TGF-  $\beta$ 1 transcription is decreased, a factor which is believed to be important to scarless fetal healing (Lin 1995, Whitby 1991, Adzick 1994). The exogenous administration of TGF-  $\beta$ 3 has been shown to inhibit scarring and promote better collagen organization *in vivo* (Shah 1995). Together, these findings suggest that the TGF $\beta$ 1- $\beta$ 3 ratio may be an important factor in the regulation of dermal scar formation in wounds.

### **1.7 Oral Mucosal Healing**

The oral surface is a vulnerable location and subjected to constant mechanical injury. The structure of the oral mucosa resembles the skin in many ways. It is composed of two layers, the epithelium and connective tissue. The epithelium of the oral mucosal membrane may be keratinized or nonkeratinized depending on location. In humans and mice, the dorsum of the tongue, gingiva

and hard palate are keratinized tissues. Keratinized tissue in the oral mucosa is very similar to skin making it a good model to study healing.

Oral mucosal wounds undergo the same healing process as dermal wounds. However, wound healing in the oral mucosa is clinically distinguished from skin healing in terms of both its rapidity and relatively minimal to no scar formation. Studies in at least three different models of oral mucosal wound healing demonstrate that reduced inflammation and reduced scar formation are universal features of the superior healing phenotype that is observed in the oral cavity (Mak 2009; Wong 2009, Szpaderska 2003). The one exception that has been seen are excisional wounds placed on the hard palate of the mouse. In this model, the underlying connective tissue is extremely thin, so the wound depth reaches the periosteal bony surface and healing slows (Graves 2003). Nearly all other oral mucosal wounds, including palatal wounds in humans and pigs, heal more quickly than skin (Mak 2009; Wong 2009, Szpaderska 2003).

Very little is known regarding the cellular and molecular mechanisms that are responsible for superior wound repair in oral tissues. Previously, it was believed that saliva played a major role in superior mucosal repair, but further studies have generated conflicting results. Several investigators have proposed that saliva, containing abundant amounts of cytokines, growth factors, and protease inhibitors, is the key factor that accounts for rapid oral wound healing (Zelles 1995, Sporn 1983, Yamano 1999, Ashcroft 2000). Yang *et al* (1996) has shown specific components of saliva, such as TGF- $\alpha$ , in oral wounds accelerate the healing process. This growth factor supply may contribute to the quicker healing

as numerous studies have shown growth factors can accelerate normal healing process (Sonis 1994; Girdler 1995; Jahovic 2004). Other studies also provide evidence that the presence of saliva cannot alter the capability of adult skin to heal. Bussi et al. have reported that skin transposed into the oral cavity maintained its morphologic characteristics such as keratinization, hair follicles, and sweat glands, and showed an intense inflammatory reaction in the dermis (Bussi 1995), while Reilly and co-workers observed an intraoral keloid in transposed skin (Reilly 1980), suggesting intrinsic differences in the healing of oral mucosa and skin exist that are independent of environment.

Inflammation is an integral part to successful wound healing. A delicate balance exists between the necessary amount of inflammatory cells and an excessive amount which leads to delayed wound healing. It is an improvement in this balance that may be critical to oral mucosal healing, as oral wounds exhibit seem reduced inflammatory response in comparison to skin (Wetzler 2000; Szpaderska 2003). In comparison to skin wounds, there is a lower inflammatory response in oral sites with lower neutrophil, macrophage, and T-cell infiltration (Szpaderska 2003). Further, cytokine production is decreased in oral wounds, including diminished production of IL-6 and KC (Szpaderska 2003). Similar to changes in inflammatory cytokines, oral and skin wounds have differences in the expression of TGF- $\beta$ 1, a pro-inflammatory, pro-fibrotic cytokine that has been implicated in hypertrophic scars (Wang 2000, Schrementi 2008).

Other groups have also shown that the inflammatory process in oral mucosal wounds is different than skin. In a study performed by Mak *et al* (2009) oral mucosal wounds showed significantly fewer mast cells than did the skin wounds at 60-day post-wounding in the red Duroc pig model. T-lymphocyte cell content in wounds is also significantly decreased in oral mucosal wounds on day 7 post-injury in comparison to skin wounds in mice (Szpaderska 2003).

The reduced inflammation seen at sites of oral mucosal injury may derive partially from faster repair processes, such as accelerated cellular proliferation and faster migration. Yet, an innate decrease in the inflammatory response in the oral mucosal wound seems to be involved. The low inflammatory response in mucosal wounds is similar to the findings in fetal wound healing models. Wounds produced in the early to mid-gestation fetus display very little, if any, inflammatory response while healing in a scarless fashion (Adzick 1985, Cowin 1998, Liechty 1998).

All inflammation is not harmful in the process of wound healing. For instance, macrophages seem to support healing through the generation of growth factors that promote cell proliferation and protein synthesis during late inflammatory phase (Leibovich 1975, Hunt 1984, Rappolee 1988, Mirza 2009, Goren 2009). Inflammation process in the mucosal environment seems to be at a balance where it leads to fast and minimal scarring wound healing outcome.

One of the differences responsible for rapid repair in oral mucosa could be intrinsic keratinocyte characteristics. In a study where equivalent full thickness 1mm diameter wounds are created on both the dorsal skin and tongue

of the mouse, oral mucosa wounds exhibit rapid re-epithelialization, with 100% completion at 24 hours post-injury. In contrast, cutaneous wounds are less than 25% re-epithelialized at the 24 hour time point (Schrementi 2008). These findings led to the hypothesis of the current study: oral keratinocytes proliferate and migrate at a faster rate than skin keratinocytes, thus wound closure occurs faster in oral mucosa. This suggests that keratinocytes have differential responses to wounding depending on their location in the body. Some previous studies support this concept. For example, when individual primary cultures of human epithelial cells from skin and oral mucosa were stimulated with IL-1 $\beta$ , a major inflammatory cytokine that is increased in the wounded tissues, the amount of IL-6 and TNF- $\alpha$  mRNA that was produced by cells from oral mucosa was much lower than the amount produced by cells from skin (Chen 2010). This data also strongly points to the conclusion that keratinocytes from skin and mucosa have different regulatory pathways in response to injury.

Previous work from our laboratory has provided a comprehensive and dynamic study of gene expression profiles in skin and mucosal wounds over all stages of wound healing. Using microarray technology we have identified similarly expressed, as well as significantly differentially expressed, genes in cutaneous and mucosal wounds (Chen 2010). We have shown even though the expression patterns are similar in both types during healing, they are not identical. Specifically, the results demonstrate that in oral mucosa the response to injury is more rapid, shorter in duration, and of lesser intensity than the

response of skin. Not surprisingly, the level of hypoxia has been shown to be significantly lower in oral mucosal versus skin wounds (Chen 2012).

The current knowledge about oral mucosal wound healing suggests several important concepts. Oral mucosal wounds heal by kinetics that are quite different from skin. The superior healing ability of the oral mucosa most likely derives from both intrinsic and extrinsic factors. The study of oral mucosal healing seems likely to provide strategies to improve the healing of skin. Ultimately, such knowledge may assist in the design of therapeutic strategies to enhance tissue repair. While most people exhibit adequate and appropriate wound healing, an estimated one million persons in the U.S. suffer from wound healing problems each year. The medical benefits from improved healing may include shorter hospitalization time, reduced requirements for split-thickness autografts, and reduced long-term morbidity after recovery.

### **1.8 Purpose of the Present Study**

The purpose of the current study is to examine regional differences in keratinocyte cell function by using *in vivo* mouse models that allow concurrent analysis of both oral and skin wounds and *in vitro* primary cultures to observe keratinocytes independently. **The hypothesis of this study is oral keratinocytes proliferate and migrate at a faster rate than skin keratinocytes, thus wound closure occurs faster in oral mucosa.** The current goal of this research is to compare oral and skin wound repair mechanisms in order to determine which features of oral keratinocytes support rapid wound healing. Our long-term goal is to discover molecular mechanisms



that lead to more rapid oral mucosal healing compared to the skin. The following specific aims will elucidate how oral keratinocytes mediate the swift re-epithelialization of oral wounds:

**I. Identify characteristics that distinguish the swift re-epithelialization of oral mucosa wounds from more slowly healing skin wounds.**

- A. Compare global gene expression profiles in normal/unwounded skin and mucosal epithelium.
- B. Compare growth factor and growth factor receptor expression in excisional mouse wounds at both oral mucosal and cutaneous sites.

**II. Isolate key intrinsic differences between human oral and skin keratinocytes that might mediate differential wound closure.**

- A. Compare the intrinsic proliferative capability of isolated oral versus skin keratinocytes in an *in vitro* wound model.
- B. Determine the intrinsic migratory ability of isolated oral and skin keratinocytes using an *in vitro* wound model.

## **CHAPTER 2**

### **Intrinsic Differences between oral and skin keratinocytes**

#### **2.1 INTRODUCTION**

Cutaneous wound healing is a multi-step process that nearly always ends with scar formation in adults. These scars range from having little or no impact on physiologic function to hypertrophic scarring that interferes with the function of soft tissue. The healing process entails overlapping but distinct processes that can be categorized into four major groups: hemostasis, inflammation, proliferation and remodeling (Guo and DiPietro 2010). One important aspect of wound healing is re-epithelialization, restoration of epithelium by keratinocytes during the proliferative phase. Upon injury cells in the immediate vicinity of the wound edges undergo proliferative and migratory burst and effectively replace keratinocytes lost as a result of the injury (Martin 1997). This tremendous proliferative and migratory capacity of keratinocytes is critical for effective wound re-epithelialization.

The healing of oral mucosal wounds proceeds through similar stages as that of skin wounds (Sciubba 1978, Walsh 1996). However, wound healing in the oral mucosa is clinically distinguished from skin healing in terms of both its rapidity and relatively minimal to no scar formation (Whitby and Ferguson 1991). Studies in our laboratory have shown that in comparison to skin wounds there is a lower inflammatory response in oral sites with lower neutrophil, macrophage, and T-cell infiltration (Szpaderska 2003, Chen 2010). Further, cytokine expression is decreased in oral wounds including diminished production of IL-6 and KC, a murine homolog of human interleukin-8 (Szpaderska 2003, Chen 2010).

Similar to changes in inflammatory cytokines, oral and skin wounds also have differences in the expression of TGF- $\beta$ 1, a pro-inflammatory, pro-fibrotic cytokine known to be implicated in hypertrophic scars (Wang *et al.*, 2000, Schrementi 2008).

The production of Vascular Endothelial Growth Factor (VEGF), a dominant mediator of wound angiogenesis, is significantly less

in oral vs. skin wounds and wound angiogenesis in oral wounds was much less than in skin (Szpaderska 2005).

Previous work from our laboratory has shown systemic, comprehensive and dynamic study of gene expression profiles in skin and mucosal wounds over all stages of wound healing. Using microarray technology we have identified similarly expressed, as well as significantly differentially expressed, genes in cutaneous and mucosal wounds (Chen 2010). We have shown even though the expression patterns are similar in both types during healing, they are not identical. Specifically, their results demonstrate that in oral mucosa the response to injury is more rapid, shorter in duration, and of lesser intensity than the response of skin. These observations suggest that in adults oral wounds heal by kinetics different from skin. One obvious explanation suggested for this difference is the environmental differences of two sites, such as temperature, salivary flow, or microflora. However studies have shown that skin transposed into oral cavity maintains its morphologic characteristics (Bussi 1995), and transposed skin results in an intraoral keloid (Reilly 1980). These findings imply the repair in oral mucosa is likely to be related to intrinsic characteristics of mucosal tissue and not to environmental factors. Anatomic variation between oral mucosa and skin epithelium can also be considered in explaining the healing differences noted between these two sites. Even though both oral

mucosa and skin are stratified epithelium, structural differences between these two sites do exist. For example, the presence of hair follicles and sweat glands in skin but not in mucosa and presence of taste buds in mucosa but not in skin.

One of the differentiating factors responsible for rapid repair in oral mucosa could be intrinsic keratinocyte characteristics. Notably in excisional skin and oral mucosal wounds of equivalent size (1mm), oral mucosa wounds, as quantified by histomorphometric analysis, exhibited re-epithelialization very rapidly, with 100% closure at 24 hours post-injury. In contrast, cutaneous wounds were less than 25% re-epithelialized at a 24 hour time point (Schrementi 2008). This data strongly suggests the proliferative capacity of oral keratinocytes is greater than that of skin keratinocytes. Since keratinocytes are the major source of VEGF in the wound and produce pro-angiogenic factors in response to hypoxia, investigation of the VEGF mRNA and protein levels in isolated oral and skin keratinocytes was conducted. When subjected to hypoxia, epidermal keratinocytes produced higher levels of both VEGF protein and mRNA than did oral keratinocytes (Szpaderska 2005). Comparable wounds on murine dorsal skin and tongue (5mm) were made to determine levels of hypoxia and HIF-1 $\alpha$ . Skin wounds were found to be significantly more hypoxic and had higher levels of HIF-1 $\alpha$  than mucosal wounds (Chen 2012).

In addition, epithelial cells are a major source of pro-inflammatory cytokines during wound healing. Chen *et al* (2010) have shown that when individual primary cultures of human epithelial cells from skin and oral mucosa were stimulated with IL-1 $\beta$ , a major inflammatory cytokine that is increased in the wounded tissues, the amount of IL-6 and TNF- $\alpha$  mRNA that was produced by cells from oral mucosa was much lower than in skin.

This data also strong points to the conclusion that keratinocytes from skin and mucosa have different regulatory pathways in response to injury.

These findings further suggest that the superior repair in oral mucosa is likely related to mucosal intrinsic characteristics. Because mucosal healing is more efficient than skin, it may provide clues to the critical elements that make adult skin wounds heal more slowly.

In the current study we focus on identifying key differences inherent between oral and skin keratinocytes which mediate differential wound closure. Chang *et al* (2002) have shown human fibroblasts from different anatomic sites exhibit systematic differences. However global gene expression in isolated keratinocytes of normal/unwounded tissue has not been studied. Our microarray analysis demonstrated gene expression between oral and skin tissues is drastically different, however global gene expression in isolated keratinocytes of normal/unwounded tissue has not been studied. Migration and proliferation are primary keratinocyte functions and our point of interest in relation to wound healing and previous microarray analysis informed us of inherent different proliferative and motogenic capacity present between oral and skin keratinocyte tissues. Proliferation and migration functional assays with primary keratinocyte cell cultures demonstrated that oral keratinocytes have different proliferative and migrative functional expression patterns than their skin counterparts. Further, oral keratinocytes proliferate and migrate at a significantly faster rate than skin.

## **2.2 Materials and Methods**

### **Isolation of human primary skin and oral mucosal keratinocytes**

Paired human skin (arm) and oral mucosal (palate) tissues (2mmX10mm) were obtained from healthy adult donors after consent under a protocol approved by Institutional Review Board at the University of Illinois at Chicago. The tissues were rinsed in 70% alcohol, washed with PBS containing 50 µg/mL gentamycin, and 0.5 µg/mL amphotericin B, and incubated for 2 hours at room temperature with 0.2% dispase solution. Separated epithelium was incubated for 10 min at 37°C in 0.05% trypsin and 0.53 mM EDTA (Invitrogen, Carlsbad, CA, USA) so that a single cell suspension would be prepared. Following incubation, trypsin was neutralized with PBS containing 10 mg/mL Soybean Trypsin Inhibitor (Invitrogen, Carlsbad, CA, USA). The cells were suspended in KBM-2 and transferred into a 60-mm Petri dish at a density of  $1 \times 10^5$  cells *per* dish. Briefly skin and oral mucosal keratinocytes were cultured at 37°C and 5% CO<sub>2</sub> in a humid atmosphere and grown in keratinocyte basal medium-2, KBM-2 (Cambrex, Walkersville, MD, USA).

### ***In Vitro* keratinocyte scratch and proliferation assay**

Keratinocytes were seeded in 12-well tissue culture plates. *In vitro* wounds were created by a scratch assay which involved the scraping of a 75-80% confluent keratinocyte monolayer by a 200 µl (yellow) pipette tip both horizontally and vertically across the plate, creating a grid form. 4x4 scratches were made. Six independent human keratinocyte cultures of both skin and oral derived cells at time points of 0, 24, and 48 hours after scratch were examined with CellTiter96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin), which utilizes a colorimetric

indicator to measure cell proliferation. Proliferation ratio was determined by the formula:

$OD_{X \text{ hrs post-scratch}} / OD_{0 \text{ hrs post-scratch}}$ . OD was read at an absorbance of 490nm.

### ***In Vitro* keratinocyte migration assay**

The gold salt phagokinetic migration assay, which was first described by Albrecht-Buehler in 1977, has been routinely used to directly evaluate keratinocyte motility without the confounding the possibility of cell proliferation (Albrecht-Buehler 1977). Tissue culture slides were dipped in 1% solution of bovine serum albumin and drained, then dipped in 100% ethanol, and rapidly dried. 1.8 ml  $\text{HAuCl}_4$  (14.5 mM) (Fisher Scientific, Pittsburg, PA) was added to 6 ml of 36.5 mM  $\text{Na}_2\text{CO}_3$  (Sigma, St. Louis, MO) and diluted with 11 ml  $\text{H}_2\text{O}$ . The solution was heated just to boiling and 1.8 ml of 0.1% formaldehyde was added. Two ml of the hot (80-90°C) gold particle suspension was added to culture slides. After 45 min the gold solution was aspirated, and slides re-coated for 1 hour at room temperature with fibronectin in HBSS (50 ug/ml). Freshly trypsinized subconfluent keratinocytes were seeded at a density of  $2.5 \times 10^3$  per slide. The cells were incubated for 18 hours at 37°C to allow adherence and migration. Afterwards, slides were washed gently to remove nonadherent cells and fixed in 0.1% formaldehyde in PBS. The phagocytic tracks were observed and photographed under dark field illumination. Under these conditions, the gold coat appeared as a finely-granulated light-gray background, and the particle-free areas appeared black. To assess cell migration, we used computer analysis to monitor microscopic images of the tracks made by cells and measured the area of tracks. Twenty non-overlapping fields from each slide were digitally imaged using AmScope acquisition software (American Scope) and analyzed using ScionImage software (Maryland). Since the migration tracks of the

cells were visible as black empty spaces against the background of bright gold-salt particles, the colorization tool in the software was used to identify and measure the area of each track in each field. The sum of the track areas in the field was divided by the total area of the field and multiplied by 100 to yield the percentage of each field taken up by tracks. This percentage was called the migration index (MI).

### **Animals for microarray analysis**

Four (6- 8 week old) female Balb/c mice (Harlan Inc., Indianapolis, IN) were sacrificed and skin epidermal tissues were obtained from the tail of mice, and oral epidermal tissues were obtained from the hard palate. Epithelium was separated from the dermis by 0.2% dispase treatment for 2 hrs at room temperature. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

### **Total RNA preparation**

Total RNA was extracted from the whole epithelial sheets using TriZol (Invitrogen, Carlsbad, CA) and purified by RNeasy kit (QIAGEN, Valencia, CA). The integrity (18S/28S) and concentrations of RNA was determined using an Experion (Bio-Rad, Hercules, CA) per the manufacturer's instruction.

### **Microarray analysis**

For genomic analyses, paired mRNA from mouse skin and palate (n=4) epithelium was subjected to Affymetrix GeneChip Mouse Genome 430 v 2.0 Hybridizations x 8. The data was analyzed to identify genes whose expression differed between the two tissues. It was analyzed in Partek Genomics Suite statistical package. Hybridization signal intensities were normalized by



quantiles and summarized using the Robust Multi-array Average. A False discovery rate (FDR) of  $<0.05$  was used to identify significant positional differences in gene expression. Ingenuity Pathways Analysis computes a score for each network according to the fit of the user's set of significant genes. The score is derived from a p-value and indicates the likelihood of the Focus Genes in a network being found together due to random chance. A score of 2 indicates that there is a 1 in 100 chance that the Focus Genes are together in a network due to random chance. Therefore, scores of 2 or higher have at least a 99% confidence of not being generated by random chance alone. Biological functions are then calculated and assigned to each network.

Functional analysis of the data was done through the use of Ingenuity Pathways Analysis. The Functional Analysis identified the biological functions that were most significant to the data set. Molecules from the dataset that met the  $> 10$  fold cutoff of  $\text{FDR} < 0.01$  and were associated with biological functions in Ingenuity's Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function assigned to that data set is due to chance alone. A heat map was generated by hierarchical clustering with squared Euclidean distance between the samples and complete linkage. The packages "hclust" and "heatmap" in R (<http://www.r-project.org>) were used.

### **Total RNA preparation**

Total RNA was extracted using TriZol (Invitrogen, Carlsbad, CA) and purified using by RNeasy kit (QIAGEN, Valencia, CA). The integrity (18S/28S) and concentrations of RNA was determined using an Experion (Bio-Rad, Hercules, CA) per the manufacturer's instruction.

## Real time PCR

1 µg of total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA); reverse transcription was performed with Omniscript Reverse Transcriptase (Qiagen, Valencia, CA, USA). cDNA concentration was assessed using a NanoDrop ND-1000 Spectrophotometer (Nanodrop), 25ng of cDNA was used for each sample. cDNA samples, primers, and [AmpliTaq Gold® Fast PCR Master Mix](#) (Applied Biosystems, Foster City, CA, USA) were loaded onto MicroAmp 96-well PCR reaction plates (Applied Biosystems), and the amplification protocol was run using the StepOnePlus Real Time PCR system (Applied Biosystems). Internal control genes were selected after performing geNorm analysis program version 3.4 developed by Vandesompele *et al.* in 2002. We identified TBP, TATA-box binding protein, as the internal control for skin wounds.  $\beta$ 2m,  $\beta$ -2-microglobulin, was selected as the internal control for mucosal wounds. The following primers were purchased from Applied Biosystems:  $\beta$ -2-microglobulin-Mm00437762\_m1, TATA box binding protein-Mm01277043\_m1, KGF-1-Mm00433291\_m1, KGF-2-Mm01297079\_m1, EGF- Mm01316968\_m1. The results were quantified using the comparative  $2^{-\Delta\Delta Ct}$  method. Normal skin and tongue tissues were normalized to 1.

## Statistical analysis

GraphPad software (GraphPad Software, San Diego, CA) was used to analyze quantitative data. ANOVA was used to evaluate grouped data, and a Bonferroni's post-test was used to determine differences between groups. Comparisons were considered statistically significant when  $p < 0.05$ .

## **2.3 RESULTS**

### **Significant differences in gene expression exist between normal (unwounded) oral and skin epithelium tissues**

We first investigated if there were baseline differences in global gene expression between oral and skin keratinocyte tissues. Mouse epithelial tissues were chosen for microarray analysis due to their availability and low variance between samples as opposed to the difficulty of obtaining these samples from humans. These cultures were primary, isolated and independent of their underlying fibroblast layer (see Material and Methods). 13,710 genes were differentially expressed between oral and skin epithelium out of the differentially expressed genes, 107 were up-regulated more than 10-fold in oral epithelium at a threshold of  $FDR < 0.01$ . In contrast, 216 genes were up-regulated more than 10-fold in skin epithelium at the same threshold. A heatmap was generated to cluster genes up-regulated more than 10-fold (Fig. 1), which demonstrates that epithelial gene expression is dramatically different between mucosa and skin with well defined clustering of the genes by tissue type.

To understand biological processes and molecular functions of the differentially expressed genes between oral and skin epithelium, we analyzed the dataset through the use of Ingenuity Pathway Analysis (Ingenuity® Systems). Genes that are used as starting point for generating biological networks are referred to as focus genes in Ingenuity Pathways Analysis. Focus genes, are then used as the starting point for generating biological networks. The application is optimized to generate highly connected networks based on physical and functional interactions between focus genes and all other genes (and gene products) stored in the Ingenuity Pathways Knowledge

Base (Supplemental data: Table II-VII). Out of the 107 genes upregulated in oral epithelium 42 focus genes were identified as the starting point for generating biological networks. Of the 216 genes upregulated in skin epithelium, 70 focus genes were used as the starting point for generating top biological networks (Supplemental data: Table II-VII).

We generated a diagram to demonstrate some biological functions they were shared, by both oral and skin epithelium, and others they were distinct to each location (Fig. 2). Although functions such as cellular development, cellular assembly and organization, cell-to-cell signaling and interaction, organ development, and cellular movement were biological functions that were shared by both oral and skin epithelium, hair and skin development was a biological function of the skin epithelium, whereas cellular growth and proliferation was a biological function for oral epithelium. We focused our analysis on genes related to keratinocyte proliferation and migration due to their critical role in wound closure. At significant threshold of  $FDR < 0.01$ , in baseline unwounded/normal epithelial tissues, different proliferative and migrative genes were upregulated in oral and skin epithelium (Table I). This suggested to us that these tissues have different inherent capacity in proliferation and migration, which contributes to their differential response to wound healing. To investigate this, we performed proliferation and migration functionality assays described below.

### **Intrinsic proliferative capability of oral keratinocytes is higher than that of skin keratinocytes**

We have shown oral mucosal wounds re-epithelialized at a much faster rate than skin wounds (Szpaderska 2003). Our current goal was to understand the proliferative character of human oral keratinocytes independent of their environment and interactions

with other cell types. To most faithfully reproduce the *in vivo* response, we studied primary keratinocytes from healthy, adult skin and palate tissues rather than established cell lines, since cell lines are often characterized by significant changes in growth regulation that occur during immortalization. Cells were isolated and plated as described in Materials and Methods. To study the proliferative response, we utilized an *in vitro* wounding model in which a confluent keratinocyte monolayer is scratched with a pipette tip in a grid form. Six independent keratinocyte cultures of both skin and oral derived cells at time points of 0, 24, and 48 hours after scratch were examined. Keratinocyte proliferation was compared in oral and skin *in vitro* scratch wounds. We observed that oral keratinocyte proliferation ratio was significantly faster at 24hr wounds in comparison to skin (Fig. 3).

#### **Intrinsic migratory ability of isolated oral keratinocytes is higher than that of skin keratinocytes**

Since re-epithelialization includes both keratinocyte proliferation and migration, we next compared migration between oral and skin tissues. The migration of keratinocytes across the wound bed is an early and critical event in re-epithelialization of a wound. To identify tissue specific differences, we studied the migration capacity of human paired oral and skin keratinocytes. We performed a gold salt phagokinetic migration assay which has been routinely used to directly evaluate keratinocyte mobility without confounding the possibility of cell proliferation. A representative paired human oral and skin keratinocyte migration assay is shown in Fig. 4A. We quantified that oral keratinocytes migrate at an average rate of 2.6 fold faster than skin keratinocytes (Fig. 4B).

### **Critical growth factors for re-epithelialization are differentially expressed in oral and skin wounds**

We have shown that oral keratinocytes proliferate and migrate at a much faster rate than skin wounds. Consequently, we compare the expression patterns of mitogenic, responsible for proliferation, and motogenic, involved in migration, growth factors in oral and skin *in vivo* wounds.

Equivalent sized circular excisional wounds (1mm diameter) were produced in the oral mucosa and dorsal skin of mice. To assess mRNA expression levels, RT-PCR analysis was carried out. Wounds were harvested after 24 hours, 48 hours, 72 hours, and 5 days after injury. Normal/uninjured skin and mucosal tissues were used for baseline normalization of the wounds. KGF-1, KGF-2 and EGF mRNA expression levels were examined by RT-PCR to test the hypothesis whether or not critical growth factors of re-epithelialization are differentially expressed in oral and skin wounds (Fig. 5). We observed that KGF-1 and 2 expressions increased significantly in 24 and 48 hours wounds in comparison to normal/unwounded skin tissue. No significant increase in KGF-1 and 2 expressions were observed in oral wounds in comparison to normal/unwounded mucosal tissue. EGF expression was decreased in skin wounds in comparison to normal skin tissues, whereas its expression increased slightly in oral wounds over time but not enough to show significance.

This data indicates that KGF-1 and 2, but not EGF are important in skin re-epithelialization. Our observations also suggest that either KGF-1, 2 and EGF are not the critical growth factors for mucosal re-epithelialization or that it is not growth factor dependent.

## **2.4 DISCUSSION**

The healing of oral mucosal and skin wounds proceeds through similar stages (Sciubba 1978, Walsh 1996). However, studies in at least three different models (human, pig, and mouse) demonstrate that oral wound healing occurs faster and with less scarring than skin wound healing (Mak 2009, Wong 2009, Szpaderska 2003, Chen 2010).

Intrinsic differences in growth factor production, stem cell levels, and cellular proliferation capacity have all been suggested to support oral mucosal wound repair (Sciubba 1978, Hill 1981, Angelov 2004, Yang 1996). Studies in our laboratory have shown that in comparison to skin wounds, there is a lower inflammatory response and reduced angiogenesis in oral sites (Szpaderska 2003, Szpaderska 2005, Chen 2010).

Previously, we have shown that oral mucosal wounds re-epithelialized at a much faster rate than skin wounds in a murine model (Szpaderska 2003). Re-epithelialization consists of keratinocyte migration and proliferation (Woodley 1985) and their mechanisms are independent of each other (Woodley 1993).

We focus on determining whether or not intrinsic differences in keratinocyte function between oral and skin wounds lead to the a faster oral re-epithelialization phenotype. We examined the baseline gene expression profiles of these two tissues in comparison to each other. The result of 13, 710 significant ( $FDR < 0.05$ ) differentially expressed genes between the two tissues led us to conclude that gene expression profiles of oral and skin epithelium are different. From our analysis, we observed different proliferative and migrative genes were upregulated in oral and skin epithelium (summarized in Table I). Previous findings about these molecules indicate their role in proliferation and migration

of keratinocytes. As shown in Table I, Keratin 13 gene was upregulated in oral epithelium and it has been shown previously that this protein is expressed in mouse suprabasal tongue and oral mucosa (Caulin 2004). Keratin 13 mRNA is also associated with the development of malignant squamous-cell carcinoma in TPA-treated mice (Hummerich 2006). Mouse Keratin 4 is involved in the differentiation of epithelial cells, maintenance of their internal integrity and plays a role in their proliferation (Ness 1998). Mouse Pitx2 is involved in migration and proliferation of cells (Liu 2002, Kioussi 2002). In mice, mouse pro-epithelin protein increases healing of wound that is decreased by mutant mouse Slp1 gene (homozygous knockout) (Zhu 2002). In mice, a mutant mouse Slp1 gene (homozygous knockout) increases inflammation of wounded mouse skin involving biologically active form of mouse Tgfb (Ashcroft 2000) and upregulation of Slp1 is associated with the development of malignant squamous-cell carcinoma in TPA-treated mice (Hummerich 2006).

As shown in Table I, other genes have been identified as playing a role in cell migration. AHSG has been reported to increase the migration of keratinocytes (Wang 2010), while human CD36 protein increases migration of melanoma cells treated with laminin and fibronectin (Thorne 2000). Human Salcam protein increases the migration of yolk sac cells that is decreased by human Alcam protein. In wound, interference of human MMP 3 protein by neutralizing antibody decreases migration of HaCaT cells that is increased by human MEKK1. Transgenic mouse KLK6 protein in mouse increases migration of epidermal keratinocytes in mouse skin.

Identifying a subject of genes that are critical to proliferation and migration of oral mucosal and skin keratinocytes will allow us to focus on identifying molecules that



contribute to the superior oral mucosal wound healing. To identify site specific molecules that will contribute to improving wound healing is especially significant in such clinical cases as diabetes, where there is delayed re-epithelialization (Goodson and Hunt 1979, Fahey et al 1991).

An additional consideration in the comparison of oral mucosal and skin wound healing is the interaction of fibroblasts and epithelial cells. Several studies suggest that fibroblasts might influence epithelial cells, and vice versa, and that such epithelial-connective tissue interactions might be important to wound healing outcomes (O'Shaughnessy 2009). For example, in *in vitro* co-culture experiments, signals from overlying keratinocytes influence collagen synthesis and the activity of underlying fibroblasts (Colwell 2007, Chang 1995). The reverse effect, dermal influence on epithelial phenotype, has also been demonstrated (Rinn 2008, Schweizer 1984, Mackenzie and Hill 1981, Mackenzie and Hill 1984, El Ghalbzouri 2004).

Evidence that fibroblasts from different anatomical sites maintain intrinsic differences is also strong (Sandulache 2005, Stephens 1996, Chang 2002). As compared to skin, oral mucosal fibroblasts exhibit enhanced proliferative capacity and an altered contractility profile (al-Khateeb 1996, al-Khateeb 1997, Lee 1995, Stephens 2001). Thus, both fibroblasts and epithelial cells appear to maintain positional identities that contribute to the superior repair of oral mucosa. The data in this report suggest that differential epithelial responses seem highly likely to be important to the site-specific differences in repair of the mouth and skin wound. Overall, the divergent healing responses of oral mucosa and skin probably reflect intrinsic differences in epithelial cell and fibroblast function as well as interactions between these cells.

Studies have reported that oral mucosa has a higher proliferative rate than skin (Hill et al 1981, Hata 1995). The current study is the first to compare human oral and skin keratinocyte proliferation independent of their underlying connective tissue and environment. In Figure 2, we show oral keratinocytes proliferate at a faster rate than skin keratinocytes after *in vitro* wounding. This data suggests that epithelial cells have an intrinsic proliferative capacity that is independent of modulation by the underlying connective tissue.

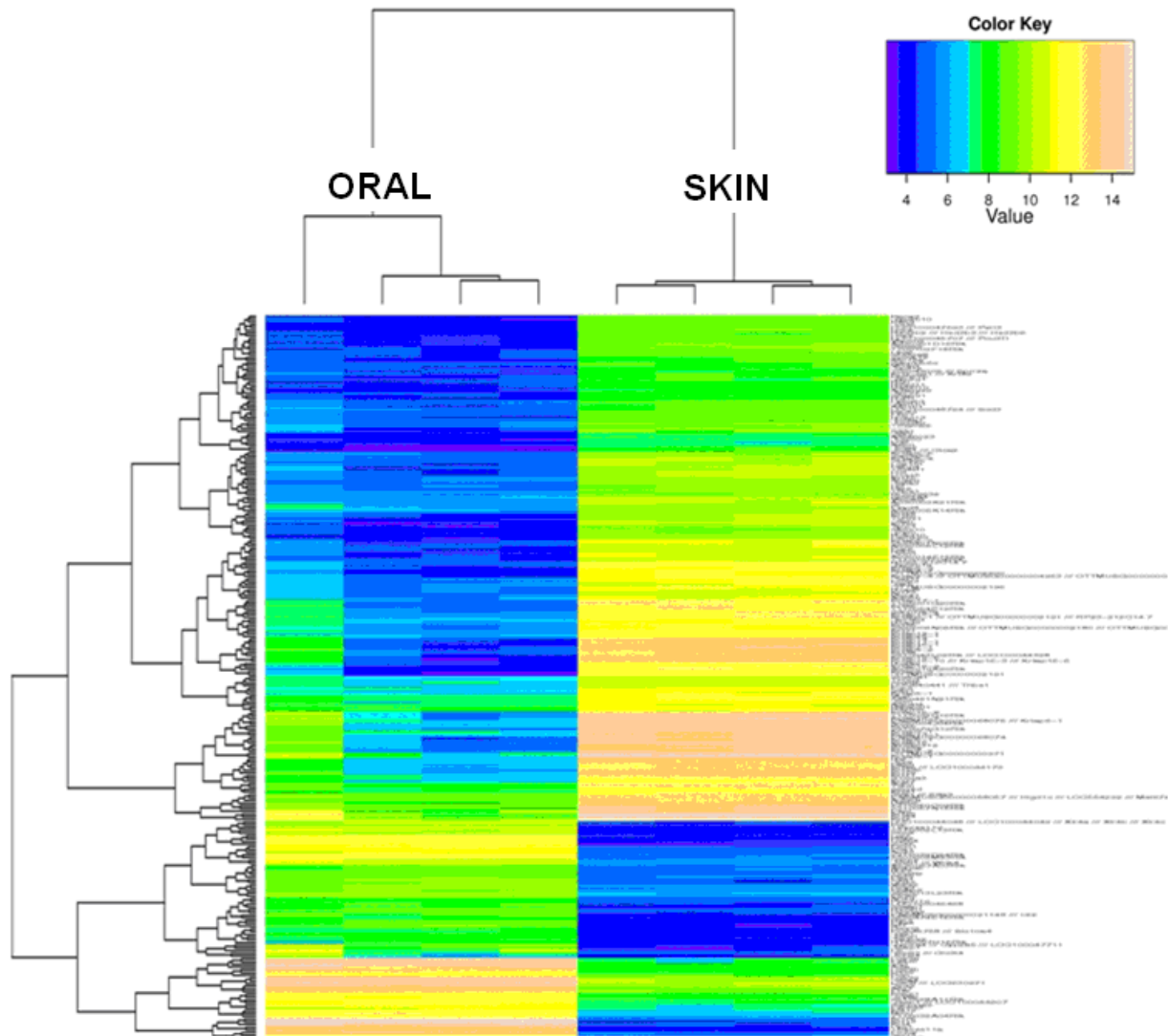
Further, we demonstrate that oral keratinocytes migrate 2.6 times faster than skin keratinocytes in paired human primary cultures. It must be noted again that this is independent of underlying fibroblasts, which are the source of critical paracrine motogenic factors, such as KGF-1 and 2, and dermal matrix metalloproteases (MMPs). Since we have eliminated sources from the connective tissue, faster oral keratinocyte migration seems to be intrinsic. This suggests that between oral and skin keratinocytes, there could be differential expression of motogenic autocrine growth factors, matrix metalloproteases and integrins. For example, MMP 9 is a type IV collagenase that is expressed by migrating keratinocytes. It is not expressed in quiescent human skin keratinocytes, but transient upregulation is observed in the migrating keratinocytes of acute wounds, decreasing as re-epithelialization is complete (Madlener 1998). In contrast to the epidermis, MMP-9 is expressed in the quiescent keratinocytes of oral mucosa (Salo 1994). MMP-9 expression is also detected in the migrating epithelial outgrowth of mucosal wounds (Salo 1994).

Oral and skin keratinocytes could also have differential patterns of interaction with extracellular matrix proteins. In our experiments (Figure 3) tissue culture slides were

coated with fibronectin, which is one of the most critical extracellular matrix proteins on which keratinocytes migrate *in vivo*. Different oral and skin keratinocytes integrins could mediate cell migration on fibronectin. Migrating keratinocytes are also believed to produce their own extracellular matrix molecules to support their migration, which could also be different in oral and skin keratinocytes (Häkkinen 2000).

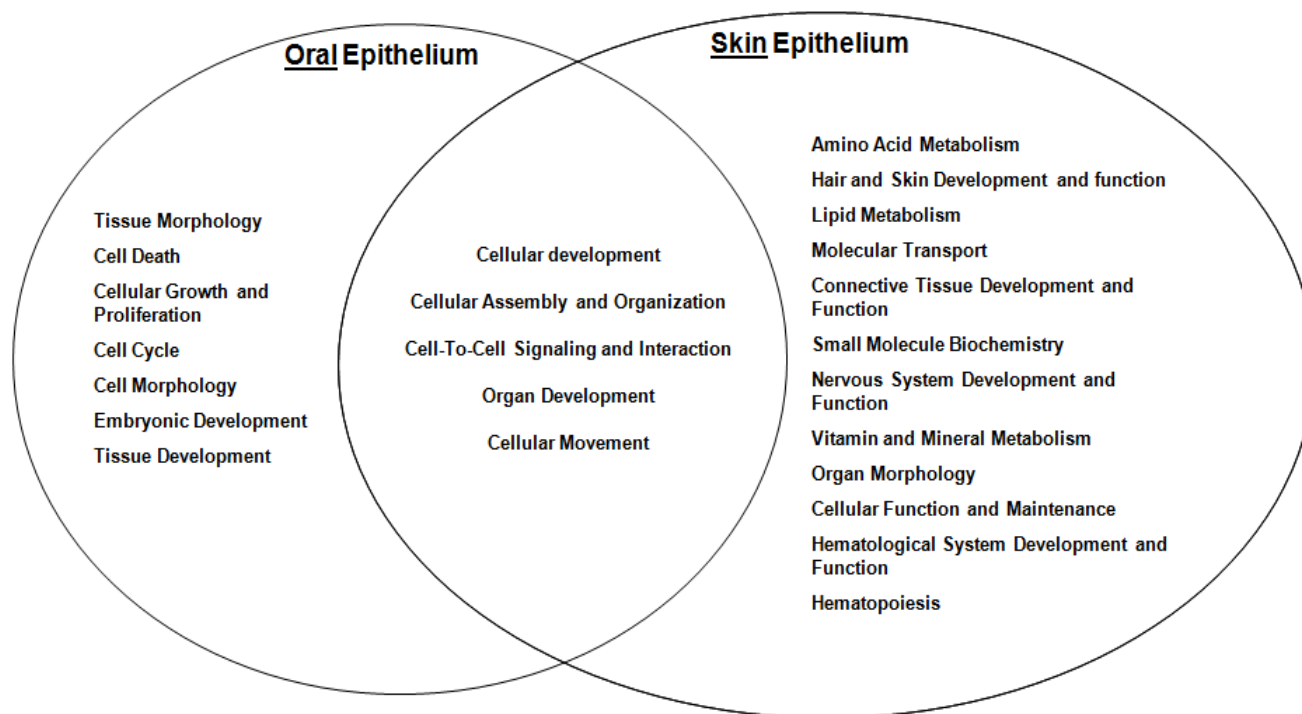
Ultimately, such knowledge may assist in the design of therapeutic strategies to enhance skin tissue repair, as well as allowing for the development of tissue specific treatment strategies. While most people exhibit adequate and appropriate wound healing, an estimated one million patients in the U.S. suffer from wound healing related problems annually. The medical benefits from improved healing may include shorter hospitalization time, reduced requirements for split-thickness autografts, and reduced long-term morbidity after recovery.

## 2.5 Figures and Table



**Figure 2.1. Hierarchical clustering of genes up-regulated more than 10-fold in oral and skin epithelium.** The heatmap indicates that gene expression in the skin and oral epithelium is significantly different. The color key shows the assignment of color to the expression intensity value. FDR< 0.01, N=4.

### Top Biological Functions for Genes Up-regulated > 10-fold



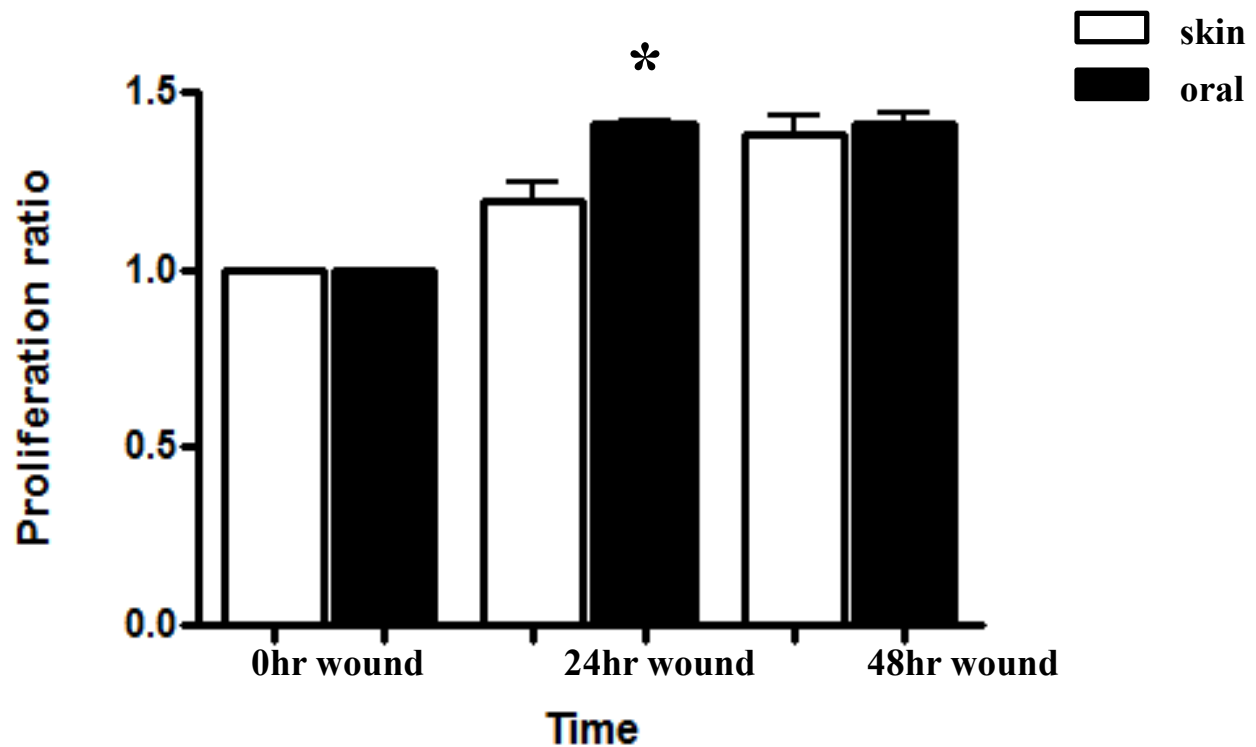
**Figure 2.2. Top Biological Functions at each location.** Fischer's exact test was used to calculate a p-value ( $< 0.01$ ) determining the probability that each biological function assigned to that data set is due to chance alone. Ingenuity Pathway Analysis software.

TABLE I

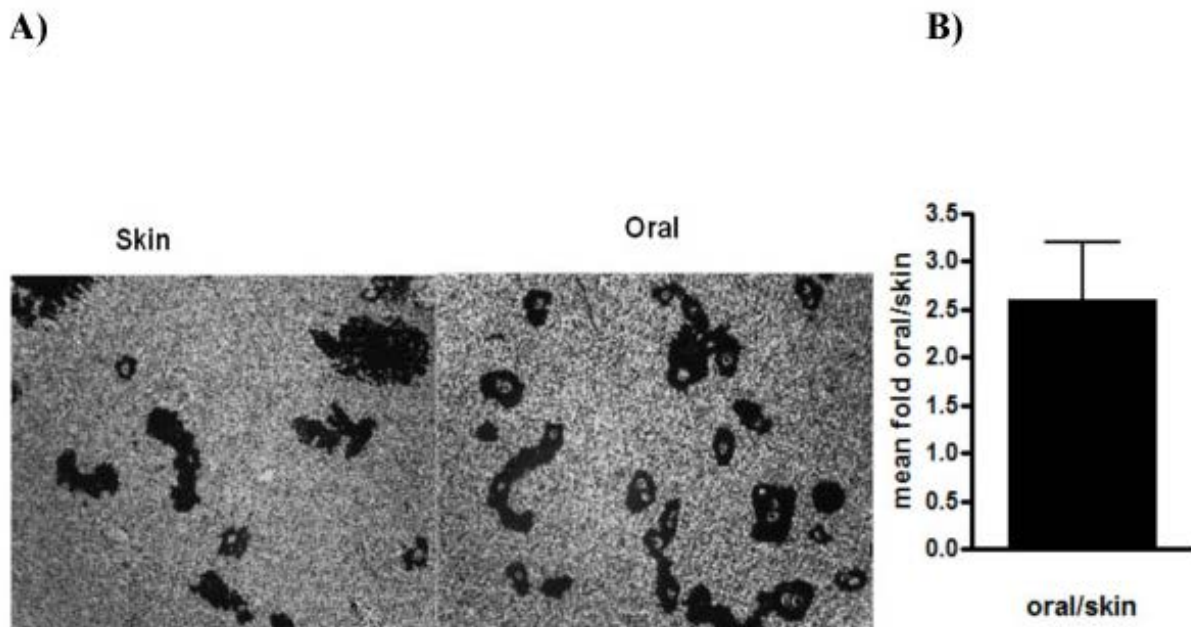
**UP-REGULATED MOLECULES RELATED TO CELLULAR MOVEMENT AND  
PROLIFERATION**

Oral epithelium		Skin epithelium	
<u>Molecules*</u>	<u>Fold Change</u>	<u>Molecules*</u>	<u>Fold Change</u>
<b>KRT13</b>	<b>1211</b>	<b>CD36</b>	<b>37</b>
<b>KRT4</b>	<b>637</b>	<b>ALCAM</b>	<b>20</b>
<b>PITX2</b>	<b>243</b>	<b>MMP3</b>	<b>14</b>
<b>SLPI</b>	<b>80</b>	<b>KLK6</b>	<b>8</b>
<b>AHSG</b>	<b>11</b>		

\*Keratin 13 (KRT13), Keratin 4 (KRT4), Paired-like homeodomain 2 (PITX2), Secretory leukocyte peptidase inhibitor (SLPI), Alpha-2- HS-glycoprotein (AHSG) , CD36 molecule (thrombospondin receptor) (CD36), Activated leukocyte cell adhesion molecule (ALCAM), Matrix Metalloproteinase 3 (MMP3), Kallikrein-related peptidase 6 (KLK6).



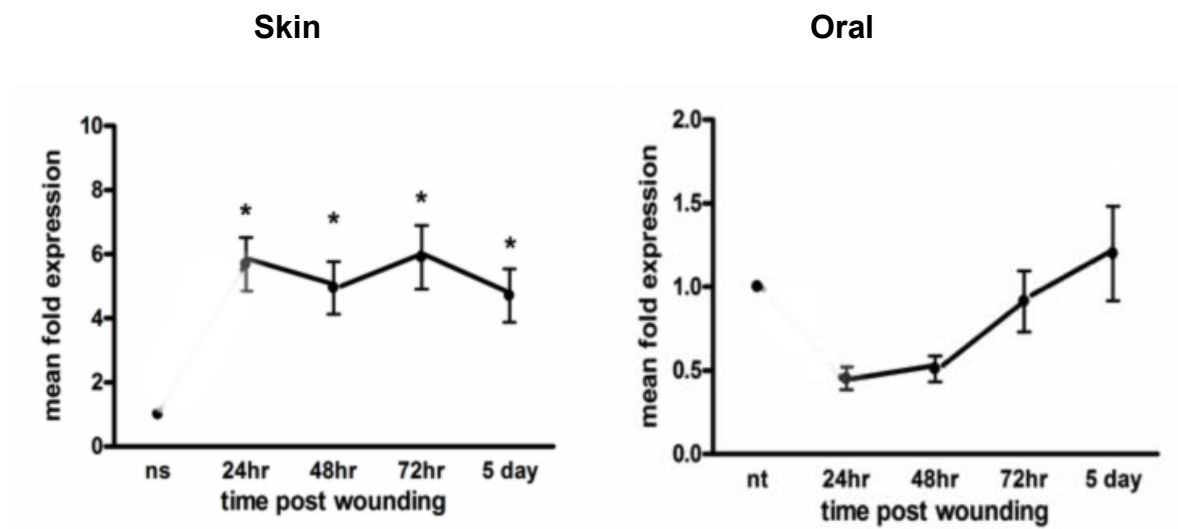
**Figure 2.3. Comparison of skin and oral keratinocyte proliferation after *in vitro* wounding.** Proliferation of keratinocytes derived from skin and palate at time points of 0, 24, and 48 hours after scratch were examined with CellTiter96 Aqueous One Solution Cell Proliferation Assay. \*  $p < 0.05$  by one-way ANOVA and Bonferroni post-test in skin vs oral wounds. N=3. \* Compared to 24hr skin.



**Figure 2.4. Comparison of skin and oral keratinocyte migration.** Oral keratinocytes migrate faster than skin keratinocytes as shown by Gold-Colloidal Migration Assay.

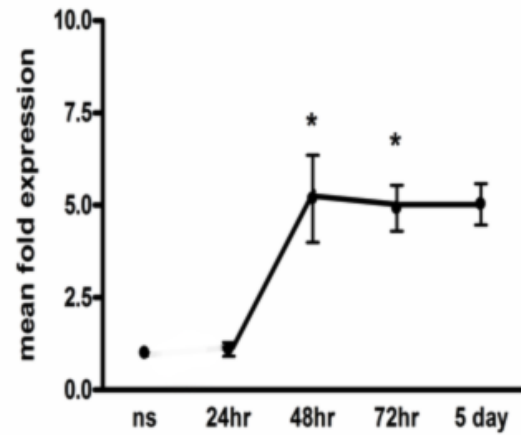
Photographs are representative examples of the tracks made by single cells. The sum of the track areas in the field was divided by the total area of the field and multiplied by 100 to yield the percentage of each field taken up by tracks. This percentage was called the migration index (MI). A) Representative migration assay slides of paired human skin and oral keratinocytes, MI  $5.1 \pm 1.5$  and  $13.0 \pm 2.7$  respectively, B) Average migration fold ratio of (2.6 fold) oral/skin of 3 independent paired oral and skin keratinocyte migration assays.



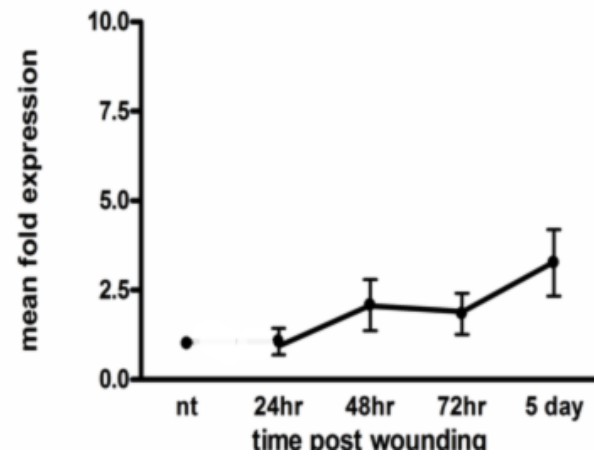


**A) Keratinocyte Growth Factor-1 Expression over time in *In Vivo* Wounds**

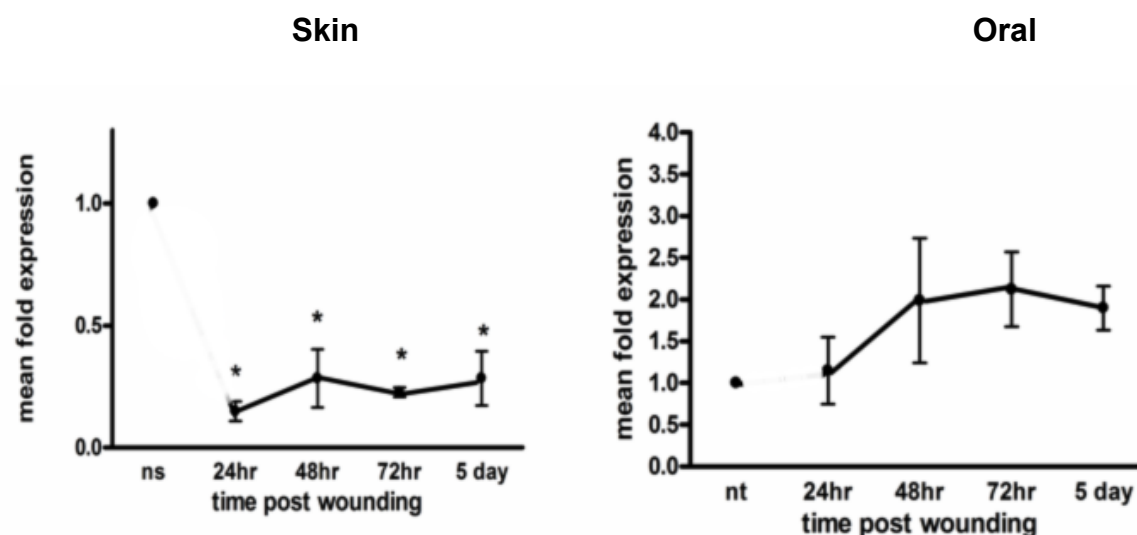
## Skin



## Oral



B) Keratinocyte Growth Factor-2 Expression over time in *In Vivo* Wounds



### C) Epidermal Growth Factor Expression over time in *In Vivo* Wounds

**Figure 2.5. Comparison of KGF-1, KGF-2 and EGF expression in skin and oral mucosa wounds.** RT-PCR was performed on RNA isolated from normal tissues or wound samples at indicated times post-injury. n=5. A) KGF-1, B) KGF-2, C) EGF. To determine relative changes in mRNA levels during development, we normalized to either TBP for skin samples and  $\beta$ 2M for oral mucosa samples. Normal skin (ns) and normal tongue (nt) is normalized to 1.  $p < 0.05$  by one-way ANOVA and Bonferroni post-test in wounds vs normal tissues.

## 2. 6 Supplemental Data

**TABLE II**

INGENUITY PATHWAYS ANALYSIS: TOP NETWORKS—ASSOCIATED NETWORK FUNCTIONS FOR GENES UPREGULATED MORE THAN 10-FOLD IN ORAL EPITHELIUM

Top Functions	Score*	Focus	Molecules**	Molecules
Cellular Development, Organ Development, Tissue Development	26	13		<b>ADA, AHSG, ALOX15, ARG1, CALCRL</b> , cyclic AMP, DLX2, EGR1, FGF10, <b>FMO1</b> , Focal adhesion kinase, GCM1, GH1, <b>GPX2, GSTA4</b> , IL6, <b>ISL1</b> , NANOG, NEUROD1, NFE2L2, <b>NMU</b> , NR5A1, <b>PALLD, PITX1, PITX2</b> , POMC, POU1F1, PRL, <b>SOX2</b> , STAT3, TCF3, TNF, TWIST1, VEGFC
Gene Expression, Cellular Growth and Proliferation, Nervous System Development and Function	25	13		<b>BDNF</b> , beta-estradiol, <b>CADPS2, CASP1</b> , Ck2, COL1A1, <b>CRISP3 (includes EG:10321)</b> , <b>CRYAB</b> , CSF1R, <b>DCN, DEFB4 (includes EG:56519)</b> , dopamine, EGR1, ERBB4, <b>FMO2</b> , GNRH1, HMGB1 (includes EG:3146), Hsp90, IGF1, IL1, <b>IL1F6</b> , IRS1, L-glutamic acid, NCOA1, NFkB (complex), NRG1, <b>ODZ3, PLAGL1</b> , PML, POMC, SIN3A, <b>SLPI</b> , SUMO1, <b>TMEFF2</b> , TP53
Gene Expression, Organ Development, Cellular Function and Maintenance	25	13		AHR, amino acids, APP, CDX1, CREB1, <b>CSRP2</b> , CTNNB1, CYP19A1, <b>CYP2A13, DSC2</b> , EGR1, <b>EYA1, EYA4, FETUB</b> , FGF3, HNF4A, <b>HS3ST1</b> , ID2, <b>IQGAP2, MEIS2</b> , NR5A1, <b>PITX2</b> , PML, POMC, retinoic acid, <b>RYR3, SIM2</b> , SMAD2, SMAD3, SMAD4, SUMO1, TCF3, TRIM24, <b>WNK4</b>
Reproductive System Development and Function, Hair and Skin Development and Function, Organ Development	4	3		beta-estradiol, CYP19A1, Cytokeratin, dopamine, EGF, EGFR, ESR1, GNRH1, KRT1, KRT2, <b>KRT4</b> , KRT5, KRT7, KRT9, KRT10, KRT12, <b>KRT13</b> , KRT14, KRT15, KRT16, KRT17, KRT19, KRT20, KRT23, KRT6A, Mapk, MAPK1, NCOA1, NCOA2, NCOA3, <b>PGR</b> , progesterone, SRC, TRIM24

\* The network Score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's Exact Test. The score is the  $-\log$  (Fisher's Exact test result). Networks are scored for the likelihood of finding the focus molecule(s) in that given network. The higher the score, the lower the probability that one would find the focus molecules(s) in a given network by random chance.

\*\* Networks are preferentially enriched for focus molecules (shown in bold) with the most extensive interactions, and for which interactions are specific with the other molecules in the network (rather than molecules that are promiscuous--those that interact with a broad selection of molecules throughout Ingenuity's knowledge base). Additional non-focus molecules from the dataset and from Ingenuity's knowledge base are then recruited and added to the growing networks.

TABLE III

INGENUITY PATHWAYS ANALYSIS: TOP NETWORKS—ASSOCIATED NETWORK FUNCTIONS FOR GENES UPREGULATED MORE THAN 10-FOLD IN SKIN EPITHELIUM

Top Functions	Score*	Focus Molecules**	Molecules
Hematological System Development and Function, Cellular Development, Cellular Growth and Proliferation	27	15	<b>ADH1C (includes EG:126), ALCAM</b> , Calmodulin, CYTH3, Cytokeratin, dihydrotestosterone, EGF, EPO, GATA1, GATA2, <b>HOXA9, HOXC13, HOXD8, HOXD9, HOXD10, HSD3B1</b> , JUN, <b>KRT2, KRT5, KRT7, KRT33A, KRTAP6-3 (includes EG:337968)</b> , MEIS1, NFATC2, NFE2, <b>PADI3</b> , PBX1, PHF1, PKNOX1, <b>RGN, S100A3</b> , SIM1, <b>THBS1</b> , VAV1, VIP
Cellular Development, Developmental Disorder, Cardiovascular System Development and Function	22	13	<b>ASPN</b> , CDH1, COL2A1, Collagen(s), CSF1, CSF1R, <b>DCT, DDR2, FBP1</b> , IL1B, KIT, KLF10, LIF, MITF, MITF-p300/CBP, <b>MMP3</b> , MMP13, MMP14, <b>ORM2</b> , PVR, PVRL1, <b>PVRL3</b> , REN, RUNX1, <b>SAA2, SCD3, SILV, SLC39A8, STFA3</b> , testosterone, TFE3, TGFB1, TGFB2, TGFB3, <b>THBS1</b>
Lipid Metabolism, Small Molecule Biochemistry, Molecular Transport	20	12	ACLY, Ap1, <b>CD36, CD200, CXCL12</b> , DDX20, <b>EGR2, EGR3, FABP4</b> , Gata, <b>GATA3</b> , HDL, IL1RL1, Jnk, linolenic acid, LIPE, <b>LPL</b> , LTBP1, Mek, <b>MMP3</b> , MMP17, NCOR-LXR-Oxysterol-RXR-9 cis RA, NFkB (complex), octanoic acid, P38 MAPK, PVR, Rxr, <b>SCD</b> , STAT1/3/5 dimer, Tgf beta, TH2 CYTOKINE, <b>THBS1</b> , Timp, <b>TIMP3</b> , TLR6
Cellular Assembly and Organization, Organismal Development, Lipid Metabolism	17	11	ADIPOQ, AGT, AHR, aldosterone, beta-estradiol, CDH1, CLDN6, EPO, ESR2, FASN, <b>FXYD4, GJA3, GPR37</b> , HGF, HSPA5, IGF1R, <b>KRT35</b> , LPIN1, MED1, <b>MGLL</b> , nitric oxide, NR1H3, PLCG1, PPARG, <b>RDH16, SNAP91, SPRR2D, SPRR2G (includes EG:6706)</b> , <b>SUSD2</b> , TJP1, TLR2, TYR, <b>TYRP1</b> , Vegf, VIP

TABLE III cont.

Top Functions	Score*	Focus Molecules**	Molecules
Gene Expression, Lipid Metabolism, Molecular Transport	15	10	ADIPOQ, BMP2, BMPR2, <b>CA6</b> , CAR ligand-CAR-Retinoic acid-RXR $\alpha$ , <b>COL4A1</b> , CSF1R, <b>CYP1B1</b> , Cyp2b, <b>CYP2B6</b> , <b>CYP2B19</b> , <b>CYP2G1P</b> , DCN, DDIT3, EPO, ERBB4, ethanol, FASN, fluoride, HAMP, IL10, LDL, <b>LRAT</b> , Ncoa-Nr1i2-Rxra, Ncoa-Nr1i3-Rxra, NOS2, NR1I2, <b>PDZRN3</b> , PXR ligand-PXR-Retinoic acid-RXR $\alpha$ , retinoic acid, Rxr, RXRA, <b>SLC40A1</b> , <b>TCHH</b> , Unspecific monooxygenase
Nervous System Development and Function, Tissue Morphology, Cellular Movement	14	9	ADCYAP1, ATN1, <b>ATP1B1</b> , BCL6, <b>CCDC80</b> , CNP, <b>CRYM</b> , DLG4, EBF1, <b>ELOVL3</b> , ERBB2, ERBB4, FYN, <b>GGCT</b> , HBEGF, HTT, IGFBP5, KRT7, <b>KRT27</b> , MPZ, NAB2, norepinephrine, NR1H3, NRP1, PMP22, <b>POU3F1</b> , progesterone, PTK2B, RGS16, SEMA3A, <b>SEMA3D</b> , SEMA3E, SMAD7, <b>TM4SF1</b> , Vegf

\* The network Score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's Exact Test. The score is the  $-\log$  (Fisher's Exact test result). Networks are scored for the likelihood of finding the focus molecule(s) in that given network. The higher the score, the lower the probability that one would find the focus molecules(s) in a given network by random chance.

\*\* Networks are preferentially enriched for focus molecules (shown in bold) with the most extensive interactions, and for which interactions are specific with the other molecules in the network (rather than molecules that are promiscuous--those that interact with a broad selection of molecules throughout Ingenuity's knowledge base). Additional non-focus molecules from the dataset and from Ingenuity's knowledge base are then recruited and added to the growing networks.

TABLE IV

**INGENUITY PATHWAYS ANALYSIS: TOP BIO FUNCTIONS - MOLECULAR AND CELLULAR FUNCTIONS FOR GENES UPREGULATED MORE THAN 10-FOLD IN ORAL EPITHELIUM**

<b>Category</b>	<b>P-value*</b>	<b># Molecules</b>	<b>Molecules</b>
Gene Expression	7.49E-05 - 3.75E-02	13	<u>ADA</u> , <u>BDNF</u> , <u>CYP2A13</u> , <u>DCN</u> , <u>EYA1</u> , <u>ISL1</u> , <u>MEIS2</u> , <u>PGR</u> , <u>PITX1</u> , <u>PITX2</u> , <u>PLAGL1</u> , <u>SIM2</u> , <u>SOX2</u>
Cell Morphology	1.25E-04 - 3.16E-02	6	<u>ADA</u> , <u>ARG1</u> , <u>BDNF</u> , <u>ISL1</u> , <u>PALLD</u> , <u>PITX2</u>
Cellular Development	2.60E-04 - 3.85E-02	16	<u>ADA</u> , <u>AHSG</u> , <u>ARG1</u> , <u>BDNF</u> , <u>CADPS2</u> , <u>CASP1</u> , <u>CRYAB</u> , <u>DCN</u> , <u>EYA1</u> , <u>ISL1</u> , <u>KRT4</u> , <u>PALLD</u> , <u>PGR</u> , <u>PITX1</u> , <u>PITX2</u> , <u>SOX2</u>
Cell Death	8.41E-04 - 3.85E-02	9	<u>ADA</u> , <u>BDNF</u> , <u>CADPS2</u> , <u>CASP1</u> , <u>CRYAB</u> , <u>DCN</u> , <u>EYA1</u> , <u>ISL1</u> , <u>PALLD</u>
Cellular Growth and Proliferation	9.10E-04 - 3.18E-02	15	<u>ADA</u> , <u>AHSG</u> , <u>BDNF</u> , <u>CALCRL</u> , <u>CASP1</u> , <u>CRYAB</u> , <u>DCN</u> , <u>ISL1</u> , <u>KRT4</u> , <u>PGR</u> , <u>PITX2</u> , <u>PLAGL1</u> , <u>SLPI</u> , <u>SOX2</u> , <u>TMEFF2</u>

\* Fischer's exact test was used to calculate a p-value determining the probability that each biological function assigned to that data set is due to chance alone.

TABLE V

**INGENUITY PATHWAYS ANALYSIS: TOP BIO FUNCTIONS - MOLECULAR AND CELLULAR FUNCTIONS FOR GENES UPREGULATED MORE THAN 10-FOLD IN SKIN EPITHELIUM**

<b>Category</b>	<b>P-value*</b>	<b># Molecules</b>	<b>Molecules</b>
Cellular Development	1.14E-05 - 4.93E-02	15	Wdnm1-like, ATP1B1, CD200, CXCL12, EGR2, EGR3, FABP4, GATA3, HOXA9, HOXD11, MMP3, PU3F1, SCD, THBS1, TIMP3
Amino Acid Metabolism	3.14E-05 - 3.31E-02	4	DCT, HAL, SILV, TYRP1
Lipid Metabolism	3.14E-05 - 4.93E-02	18	ADH1C, CD36, CXCL12, CYP1B1, CYP2B19, CYP2G1P, ELOVL3, FABP4, FAR2, LPL, LRAT, MGLL, MMP3, RDH16, SAA2, SCD, SLC22A4, THBS1
Molecular Transport	3.14E-05 - 4.93E-02	13	ADH1C, CD36, CXCL12, ELOVL3, FABP4, LPL, LRAT, MMP3, RDH16, SAA2, SCD, SLC22A4, THBS1
Small Molecule Biochemistry	3.14E-05 - 4.93E-02	23	ADH1C, CD36, CXCL12, CYP1B1, CYP2B19, CYP2G1P, DCT, ELOVL3, FABP4, FAR2, GATA3, HAL, LPL, LRAT, MGLL, MMP3, RDH16, SAA2, SCD, SILV, THBS1, TYRP1, SLC22A4

\* Fischer's exact test was used to calculate a p-value determining the probability that each biological function assigned to that data set is due to chance alone.



TABLE VI

**INGENUITY PATHWAYS ANALYSIS: TOP BIO FUNCTIONS - PHYSIOLOGICAL  
SYSTEM DEVELOPMENT AND FUNCTION FOR GENES UPREGULATED MORE  
THAN 10-FOLD IN ORAL EPITHELIUM**

Category	P-value*	# Molecules	Molecules
Tissue Morphology	5.13E-06 - 3.16E-02	11	ADA, ARG1, BDNF, CALCRL, DCN, IL1F6, PGR, PITX1, PITX2, RYR3, SIM2,
Embryonic Development	1.25E-05 - 3.47E-02	11	ADA, BDNF, ETNK2, EYA1, ISL1, KRT4, PALLD, PITX1, PITX2, SIM2, SOX2
Tissue Development	1.25E-05 - 3.45E-02	9	BDNF, CRYAB, EYA1, PALLD, PGR, PITX1, PITX2, SIM2, SOX2
Organ Development	2.03E-05 - 3.85E-02	14	ADA, BDNF, CALCRL, CRYAB, DSC2, ETNK2, EYA1, ISL1, MEIS2, PGR, PITX1, PITX2, SIM2, SOX2
Organismal Development	2.36E-05 - 2.12E-02	12	ADA, ARG1, BDNF, ETNK2, EYA1, ISL1, KRT4, PGR, PITX1, PITX2, SIM2, SLPI

\*Fischer's exact test was used to calculate a p-value determining the probability that each biological function assigned to that data set is due to chance alone.

TABLE VII

**INGENUITY PATHWAYS ANALYSIS: TOP BIO FUNCTIONS - PHYSIOLOGICAL  
SYSTEM DEVELOPMENT AND FUNCTION FOR GENES UPREGULATED MORE  
THAN 10-FOLD IN SKIN EPITHELIUM**

Category	P-value*	# Molecules	Molecules
Connective Tissue Development and Function	1.14E-05 - 4.93E-02	13	Wdnm1-like, CXCL12, EGR2, FABP4, HOXD8, HOXD10, HOXD11, LHX2, LPL, MMP3, RDH16, SCD, THBS1
Hair and Skin Development and Function	3.14E-05 - 4.12E-02	12	ADAMTS9, DCT, ELOVL3, HOXC13, KRT25, KRT27, KRT71, KRTAP16-7, SILV, THBS1, TIMP3, TYRP1
Organ Development	6.10E-05 - 4.12E-02	11	CYP1B1, GJA3, HOXC13, HOXD11, KRT25, KRT27, KRT71, KRTAP16-7, MMP3, THBS1, TYRP1
Organismal Development	1.41E-04 - 3.31E-02	11	COL4A1, CXCL12, HOXA9, HOXC13, HOXD8, HOXD9, HOXD10, HOXD11, LHX2, MMP3, THBS1
Nervous System Development and Function	1.97E-04 - 4.93E-02	11	ALCAM, ATP1B1, CD36, CXCL12, EGR2, GATA3, HOXD10, LHX2, POU3F1, SEMA3D, THBS1

\*Fischer's exact test was used to calculate a p-value determining the probability that each biological function assigned to that data set is due to chance alone.

## **CHAPTER 3**

### **Importance of Housekeeping Gene Selection for Accurate RT-qPCR in a Wound Healing Model**

#### **3.1 Introduction**

To investigate the molecular mechanisms of wound healing, quantitative PCR (RT-qPCR) has been widely used to analyze the expression of candidate genes. In recent years the utilization of the technique has grown, with an increasing number of published papers citing its use for the study of gene expression (VanGuilder 2008). RT-qPCR is popular mainly due to its ability to efficiently amplify small quantities of RNA in a relatively short period of time. For example, gene expression results can be obtained in under 40 minutes using reagents such as Taqman Fast Universal PCR Master Mix (Applied Biosystems).

Despite its advantages, RT-qPCR has certain drawbacks which affect the interpretation of its results: 1) reliable extraction of equal amounts of non-degraded RNA from each sample; 2) consistent reverse transcriptase efficiency resulting in equal amounts of cDNA in all samples; 3) adequate primer specificity; 4) presence of inhibitors in samples. In order to overcome these drawbacks, results are most often normalized to the expression of “housekeeping genes” that do not change their expression levels across sample or treatment groups (Nygard 2007). Housekeeping genes are recognized as cellular maintenance genes which regulate basic and ubiquitous cellular functions, and therefore are valid candidates to act as internal controls for gene expression. The stable expression

of a housekeeping gene in any RT-qPCR reaction is mandatory since it plays a critical role in the interpretation of the final result (Suzuki 2000).

Studies in the field of wound healing have utilized a variety of different housekeeping genes for RT-qPCR analysis. However, nearly all of these studies assume that the selected normalization gene is stably expressed throughout the course of the repair process. Generally the stability of the housekeeping gene is not assessed, leaving questions about whether normalization is accurate. The purpose of our current investigation is to identify the most stable housekeeping genes for studying gene expression in mouse wound healing using RT-qPCR. To identify which housekeeping genes are optimal for studying gene expression in wound healing, we examined all articles published in Wound Repair and Regeneration (WRR) that cited RT-qPCR during the period of Jan/Feb 2008 until July/August 2009. Twenty-six such articles were identified. From the methods, we determined that *ACTIN*, *GAPDH*, *18S* and  $\beta 2M$  were the most frequently used housekeeping genes in human, mouse, and pig tissues in these studies. These genes are typically used because they are believed to be stable; however the discussion of why specific housekeeping genes were chosen for the RT-qPCR normalization was generally absent.

Several Microsoft Excel based applications are available to assess the degree of variation in the candidate housekeeping genes. In the current study, we analyzed the stability of expression of 13 different housekeeping genes using geNorm analysis program version 3.4 developed by Vandesompele *et al.* in 2002. We selected this specific application due to its general acceptance in the

literature. The program uses an algorithm to determine the stability of the candidate housekeeping gene across samples. This measurement is denoted  $M$ , which is the average paired variation of a particular gene against all other candidate genes. Genes with the lowest  $M$ -values are considered to have the most stable expression ( Vandesompele 2002, Minner 2009).

We investigated nine commonly used housekeeping genes that are not generally used in wound healing models: *GUS*, *TBP*, *RPLP2*, *ATP5B*, *SDHA*, *UBC*, *CANX*, *CYC1*, and *YWHAZ*. Validation was carried out by normalizing the target gene, *keratinocyte growth factor-2 (kgf-2)*, to the most stable housekeeping gene, which we determined to be TATA-box binding protein, *TBP*.

### **3.2 Materials & Methods**

#### **Animals and Wound Models**

All animal procedures were approved by University of Illinois Institutional Animal Care and Use Committee. BALB/c-strain female mice, 2-3 months of age were used for the described experiments. This strain has been well-characterized in wound healing studies. Mice were anesthetized using a 100 mg/kg ketamine and 5mg/kg xylazine solution. The dorsal skin was shaved and cleansed with 70% isopropyl alcohol. Six excisional full-thickness dermal wounds were made on the dorsal surface of each mouse using a sterile 1-mm punch-biopsy instrument (Acu-Punch,Acuderm Inc., Ft. Lauderdale, FL), with three oneach side of the midline. The excised skin derived at the time of wound placement was used as normal, uninjured skin control for subsequent experiments. At 24hr, 48hr, 72hr, and 5 days after injury, five mice per time point were sacrificed and the wounds

harvested; samples were placed in RNAlater (Ambion) solution for real timeRT-PCR analysis.

### **Real time PCR**

Normal skin and wound samples stored in RNAlater solution (Ambion, USA) were homogenized in TRIzol reagent (Invitrogen, USA). Total RNA was isolated according to protocol, checked for purity and the concentration quantified spectrophotometrically. 1 µg of total RNA was isolated and treated with DNase I (Invitrogen, Carlsbad, CA, USA); and reverse transcription was performed with Omniscript Reverse Transcriptase (check on this source) (Qiagen, Valencia, CA, USA). cDNA concentration was assessed using a NanoDrop ND-1000 Spectrophotometer (Nanodrop), to determine 25ng of cDNA for each sample. List of housekeeping genes used and their source catalog number are shown in Table 2A, B. cDNA samples, primers, and AmpliTaq Gold® Fast PCR Master Mix (Applied Biosystems) were loaded onto MicroAmp 96-well PCR reaction plates (Applied Biosystems), and the amplification protocol was run using the StepOnePlus Real Time PCR system (Applied Biosystems). Genorm analysis was performed to determine housekeeping gene stability across samples and tissues. However, before Ct values from the quantitative real-time PCR were input into geNorm, all CT values were transformed into relative quantification data. Highest Ct values were subtracted from all other Ct values for each gene measured. This is called the delta Ct value with the highest delta Ct value as 0. All other values are less than 0. For each data point,  $2^{(-\text{delta Ct})}$ , equation was applied. All data was expressed relative to the expression of the least expressed

gene. Keratinocyte Growth factor 2 primers were purchased from Applied Biosystems: KGF-2- Mm01297079\_m1. The results were quantified using the comparative  $2^{-\Delta\Delta Ct}$  method. Normal/unwounded skin and tongue tissues were normalized to 1.

### **Data analysis**

For analysis of gene stability we used the geNorm application, a Microsoft Excel program available at <http://medgen.ugent.be/~jvdesomp/genorm/>. The premise of this program is that ratios between constantly expressed, non-normalized housekeeping genes should remain regular. M, the average pair-wise variation of a single housekeeping control gene was calculated from the raw expression data. The lowest M value is the most stable, so the gene with the highest instability (highest M value) is removed at each step. A new M value for each remaining gene is calculated until only two genes remain. Since these calculations are based on ratios, the final two genes cannot be resolved from each other. Normalization factors were calculated using the geometric mean to control for changes in relative gene expression and outlying values. Pair-wise variation ( $V_n/nC1$ ) was calculated by geNorm between two sequential normalization factors for all the samples (Vandesompele 2002).

### **Statistical analysis**

GraphPad software (GraphPad Software, San Diego, CA) was used to analyze quantitative data. Comparisons were considered statistically significant when  $p < 0.05$  by one-way ANOVA and Bonferroni post-test in wounds vs normal tissues.

### **3.3 Results**

#### **Selection of candidate housekeeping genes**

Housekeeping genes are cellular maintenance genes which regulate basic and ubiquitous cellular functions. In many RT-qPCR reactions, these genes are used as internal control genes without proper validation. We researched articles, from 2008 to 2009: Volume 17, Issue 4, in Wound Repair and Regeneration to identify which housekeeping genes are commonly used (Table VIII) in wound healing models. We observed that *GAPDH*, *ACTIN*, *18S*, and  $\beta 2M$  are four most commonly used housekeeping genes in wound healing experiments. However, validation of these genes was generally not shown. Nine housekeeping genes which were not typically used in wound healing related experiments were selected for our studies: *GUS*, *TBP*, *RPLP2*, *ATP5B*, *SDHA*, *UBC*, *CANX*, *CYC1* and *YWHAZ*. Pre-designed primer/probe sets for  $\beta 2M$ , *GUS*, *TBP*, *GAPDH*, *RPLP2*, *18S*, and *ACTIN* were purchased from Applied Biosystems (Table IXA). Primer/probe sets for *ATP5B*, *SDHA*, *UBC*, *CANX*, *CYC1*, and *YWHAZ* were obtained from geNorm™ Housekeeping Gene Selection Kit with Perfect Probe (Table IXB). Special attention was paid to selecting genes that belong to different functional classes, which significantly reduces the chance that genes might be co-regulated.

#### **Identification of the most stable housekeeping genes in normal skin**

Normal skin (unwounded tissue) was harvested from the dorsum of mice and RNA was extracted. Single strand cDNA was synthesized simultaneously from each extract in order to minimize any variation during this step of the

process. The expression of the transcripts of 13 potential housekeeping genes was then assayed using this cDNA.

Transformed expression data were analyzed with the geNorm pairwise analysis (Vandesompele 2002) which determines a gene expression stability measure, *M*, for each candidate housekeeping gene based on the average pairwise variation between a particular gene and all other control genes. The expression stability was as follows from the most stable to the least stable: *UBC*, *SDHA*, *CYC1*, *CANX*, *ATP5B*, *TBP*, *YWHAZ*,  $\beta 2M$ , *RPLP2*, *GAPDH*, *GUS*, *18S*, and *ACTIN* (Fig. 1).

#### **Identification of the most stable housekeeping genes in wounded skin**

Six 1mm wounds were placed on the dorsum of each mouse using a biopsy punch. Wounds were harvested after 24h, 48h, 72h, and 5 days. To identify the most stable housekeeping genes for each wounded time point, RNA was extracted from samples. Single strand cDNA was synthesized simultaneously from each extract in order to minimize any variation during this step of the process. The expression of the transcripts of 13 potential housekeeping genes was then assayed using this cDNA. As described above, transformed expression data were analyzed with the geNorm software (Vandesompele 2002).

In 24h wounds, the order of expression stability from the most stable (lowest *M* values) to the least stable (highest *M* values) was: *RPLP2*, *TBP*,  $\beta 2M$ , *GAPDH*, *YWHAZ*, *ACTIN*, *GUS*, *18S*, *CYC1*, *CANX*, *SDHA*, *ATP5B*, *UBC* (Fig.



2A). As noted, at this time point *UBC* is the least stable gene which is in contrast to its superior stability in normal, uninjured skin tissue.

In 48h wounds, the expression stability from the most stable to the least stable was: *ACTIN*, *TBP*, *CANX*,  $\beta$ 2M, *YWHAZ*, *GUS*, *GAPDH*, *CYC1*, *RPLP2*, *18S*, *UBC*, *SDHA*, *ATP5B* (Fig. 2B). As opposed to normal skin in which *ACTIN* was the least stable gene, in 48h wounds it was one of the most stable genes.

In 72h wounds, the expression stability calculated in the genes analyzed was from the most stable to the least stable:  $\beta$ 2M, *GUS*, *TBP*, *GAPDH*, *RPLP2*, *18S*, *UBC*, *YWHAZ*, *CYC1*, *CANX*, *ACTIN*, *SDHA*, *ATP5B* (Fig. 2C). Once again, wounded tissues exhibited contrasting gene stability data to normal skin. Gene *SDHA* was one of the least stable genes in 72h wounds, as opposed to being one of the most stable in normal skin.

In 5 day wounds, the expression stability from the most stable to the least stable was:  $\beta$ 2M, *TBP*, *GAPDH*, *ACTIN*, *RPLP2*, *18S*, *YWHAZ*, *CANX*, *CYC1*, *UBC*, *GUS*, *SDHA*, *ATP5B* (Fig. 2D). 5 day wounds also had contrasting gene stability data to normal skin.

In all wound tissues, the gene with the most stable expression was *TBP* with its order changing slightly in each group. We observed that wounded and unwounded tissues have contrasting housekeeping gene expression stability (Table X). We provide a summary of the 3 most stable housekeeping genes for each tissue in Table X. According to Vandesompele *et al* (2002) using the 3 best housekeeping genes is in most cases a valid normalization strategy, and results in much more accurate and reliable normalization compared to the use of only

one single housekeeping gene. A normalization factor based on the expression levels of the 3 most stable housekeeping genes is calculated.

### **Normalization of Keratinocyte Growth Factor-2 (KGF-2) in skin wound samples**

Keratinocyte Growth Factor-2 (KGF-2) is a potent mitogenic factor for keratinocytes (Finch 1989) and its level of mRNA has been reported to increase by as much as 160 times upon skin injury (Werner 1992). We analyzed its expression in wounds harvested from 24h, 48h, 72h and 5 days. We normalized *KGF-2* to *TBP* (Fig. 3A), which we determined to be the most stable housekeeping gene across the spectrum of the healing stages (Table X).  $\beta 2M$  is the next “most suitable” candidate housekeeping gene for skin wound samples (Table X). In Figure 3B, we show its expression profile is similar to KGF-2 normalized to *TBP*. In contrast, we normalized *KGF-2* to *GAPDH*, which is generally used in wounding related RT-PCR reactions (Fig. 3C ).

*GAPDH* “M” value traced closely behind *TBP* in most cases (Figure 2A thru 2D). However, there is a drastic difference between the expression profiles of KGF-2 normalized to *GAPDH* versus to *TBP*. The reason for this difference lies in the calculation of relative quantitation, meaning that it is best to select for a housekeeping gene that has a comparable number of copies per cell as the target gene, allowing for measurement of the genes to be performed on the same linear scale. KGF-2 is low-to mid-range expressing gene in normal skin, thus it is best to normalize it to a housekeeping gene, such as *TBP*, which also is a low-to mid-range expressing gene. Alternative housekeeping genes, such as *GAPDH*

which is high-range expressing in normal skin tissue and in skin wounds, is best to be used as a normalizing gene for comparable target gene expression levels.

In a multiple housekeeping gene approach, three most stable housekeeping genes are chosen according to their “M” value. Normalization factor is determined for these 3 genes by using the geometric mean of housekeeping gene expression as a normalization factor instead of the arithmetic mean, because it helps to control for possible outlying values and abundance differences among the different genes. In Figure 3D, we demonstrate how single vs multiple housekeeping gene approach affects KGF-2 expression at each wound healing time point. For each time point, its 3 most stable housekeeping genes were chosen according to their “M” values shown in Table 3 and a normalization factor was determined for each time point.

This data demonstrates importance of selecting appropriate housekeeping gene, and the affect of inappropriate selection on the data output.

### **3.4 Discussion**

In this study, we investigated for the first time the variability in the expression of a panel of housekeeping genes used for the normalization of real-time quantitative RT-qPCR in a murine wound healing model. When the results from the normal skin were compared to those of wounded tissue, we identified unique housekeeping genes that were most stable in each tissue and therefore could be validated as the most suitable internal controls for these RT-qPCR reactions. Our results indicate that there is a difference in housekeeping gene expression stability between wounded and unwounded tissues.

The candidate housekeeping genes that we have analyzed are generally used as internal controls, because they are believed to have relatively stable expression. We paid special attention to select genes from different functional families. This is required for geNorm analysis, the currently preferred software program for selection of the best housekeeping gene (Vandesompele 2002). The software calculates the internal control gene-stability measure “M”, which is defined as the average pairwise variation of a particular gene with all other control genes (Vandesompele 2002). This measure relies on the principle that the expression ratio of two ideal internal control genes is identical in all samples, regardless of the experimental condition or cell type. Variation of the expression ratios of two housekeeping genes reflects the fact that one of them is not constantly expressed. An increasing variation in ratios corresponds to decreasing expression stability. The 1.5 cut-off for “M” is optimally determined by the geNorm program. It measures deviation from standard, so 1.5 means deviation of 50%. If the deviation is higher than 50%, we consider them statistically insignificant for a scientifically valuable correlation to emerge. In essence, it is a way to quickly sort out those genes which deviate too far for further analysis. The remainder ( $< 1.5$ ) are not automatically considered to exhibit statistically important correlation, and they are further analyzed using stepwise exclusion method. Stepwise exclusion of the gene with the highest M value results in a combination of two constitutively expressed housekeeping genes that have the most stable expression in the tested samples.

We must also mention the significance of testing the quality of RNA must not be overlooked. From previous experiments in our laboratory, we have realized that RNA quality has a profound impact on the results, in terms of the significance of differential expression and variability of reference genes. Thus, we test the integrity (18S/28S) and concentrations of RNA by using an Experion per the manufacturer's instruction. Besides testing the purity and integrity of RNA, we also consider the requirement related to the input RNA material; it should be free of contaminating DNA. We performed a proper DNase treatment on all of our RNA samples to eliminate the presence of DNA.

Our results indicated that the *TBP* can be used as a reference gene for relative gene quantification and normalization in 1mm 24h, 48h, 72h and 5 day wounds as determined by geNorm program. However, in normal skin *UBC*, *SDHA*, and *CYC1* were found to be the most stable housekeeping genes. Although ubiquitin is not commonly used as a normalization gene, in our normal skin studies it was found to be quite suitable. Previously, Czechowski et al. (2005) pointed out that genes with fairly low levels of expression, such as genes of the ubiquitin complex, may be of particular interest for normalizing expression levels of genes which have moderate to low levels of expression. Previous studies have also mentioned paying special attention to housekeeping genes in diseased skin, such as an increase in expression of *GAPDH* in all areas of epidermis of psoriatic plaque compared to normal skin (Wu 2000). Interestingly, *GAPDH* and *ACTIN* genes are the most widely used housekeeping genes. *ACTIN* has been described as a high expression housekeeping gene in

skin tissues (Bar 2009). However, expression stability of *ACTIN* and *GAPDH* has been shown to vary significantly across tissues, cell types, and during cell proliferation and development (Radonic 2004, Selvey 2001, Glare 2002, Deindl 2002). De Jonge (2007) et al. identified candidate housekeeping genes in human gene array samples, which had enhanced stability among different cells types and experimental conditions, with none of the commonly used housekeeping genes found in the top 50 stable expressed genes. The current study supports the idea that *GAPDH* and *ACTIN* may not be highly suitable as internal controls for wound healing studies that utilize RT-qPCR. Thus, caution should be applied in the selection of an appropriate housekeeping gene for such investigations. As we observed in our study, the selection of an appropriate internal control is essential for obtaining meaningful results in RT-qPCR studies. We tested the need for careful housekeeping gene selection for normalization in two additional wound healing models including a 3mm skin wound and a 1mm tongue wound (unpublished data). These studies have confirmed that best housekeeping gene for normalization must be independently selected for each model. For example, in 3mm skin wounds, we performed analysis of several target genes that are significant in wound healing. These genes are: Epidermal Growth Factor (EGF), Transforming Growth Factor beta 1 and 3 (TGF $\beta$ 1 and 3), Vascular Endothelial Growth Factor (VEGF), and Keratinocyte Growth Factor 1 (KGF 1). We normalized these target genes to 3 different housekeeping genes, such as *GAPDH*, *TBP*, and  $\beta$ 2M. This unpublished data also verified our conclusion of the

importance of selecting appropriate housekeeping gene, and the affect of inappropriate selection on the data output.

Furthermore, although our results have identified suitable control genes for wound healing, this analysis is likely specific to the particular model that we utilized. Therefore, control gene selection must be validated for each experimental model, cell and tissue type or in healing vs non-healing diabetic wounds. In the latter case, the above discussed normalization analysis might not be sufficient due to the absence of set time course and might require additional/alternative methods.

### **3.5 Figures**

**TABLE VIII**  
**TYPICAL HOUSEKEEPING GENES USED IN HUMAN, MOUSE, AND PIG WOUND MODELS**

<b>Gene</b>	<b>Number of articles referencing each gene*</b>
<i>ACTIN</i>	8
<i>GAPDH</i>	8
<i>18S</i>	6
<i>β2M</i>	1
<i>HPRT</i> **	1
<i>RPS9</i> **	1
<i>CYCLOPHILIN A</i>	2
<i>YWHAZ</i>	1

\* 26 articles in the issues of 2008 and upto July/August issue of Wound Repair and Regeneration were analyzed to determine the most commonly used housekeeping genes.

\*\**HPRT*- hypoxanthine ribosyltransferase; *RPS9*- ribosomal protein S9.

TABLE IXA

## CANDIDATE REFERENCE GENES (SOURCE: APPLIED BIOSYSTEMS)

Gene Symbol	Gene Name	Cellular Function
<b>B2M</b>	$\beta$ -2-microglobulin	histocompatibility complex
<b>TBP</b>	TATA box binding protein	transcription factor
<b>GAPDH</b>	Glyceraldehyde-3-phosphate dehydrogenase	
<b>GUSb</b>	Glucurodinase,beta	glycosidase
<b>RPLP2</b>	Ribosomal protein, large p2	translation, ribosomal protein
<b>ACTB</b>	Actin,,beta	cytoskeleton
<b>18S</b>	Ribosomal RNA 18S	member of ribosome RNA

TABLE IXB

## CANDIDATE REFERENCE GENES (SOURCE: PRIMERDESIGN LTD\*)

Gene Symbol Cellular Function	Gene Name
<b>UBC</b> ubiquitination	Ubiquitin C
<b>YWHAZ</b> signal transduction	Phospholipase A2
<b>ATP5B</b> ATP production	ATP synthase subunit
<b>CANX</b> retain protein in ER	Calnexin
<b>CYC1</b> electron transport chain	Cytochrome c-1
<b>SDHA</b> dehydrogenase	Succinate dehydrogenase complex, subunit A

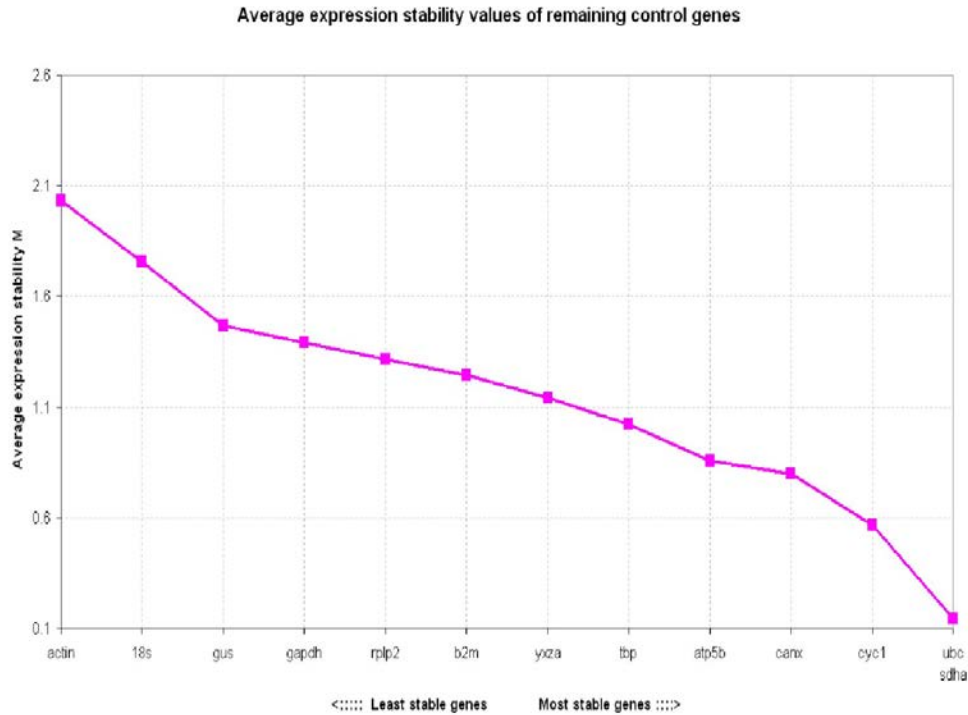
- The above listed genes are provided in geNorm™ Housekeeping Gene Selection Kit with PerfectProbe.

TABLE X



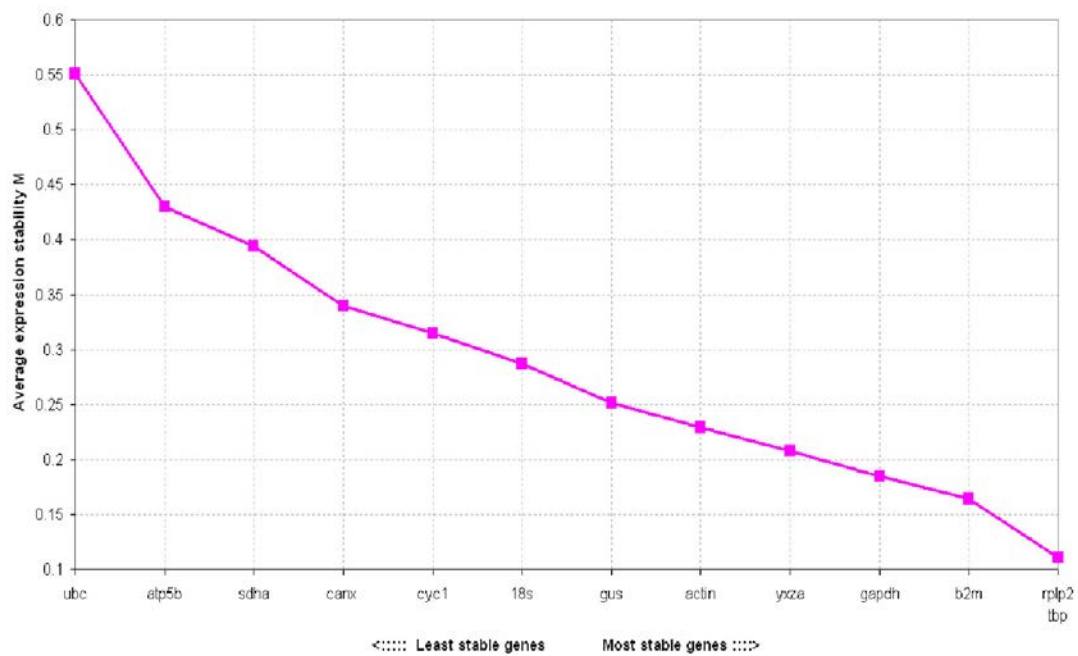
## SUMMARY OF 3 MOST STABLE HOUSEKEEPING GENES FOR EACH TISSUE

[illegible]



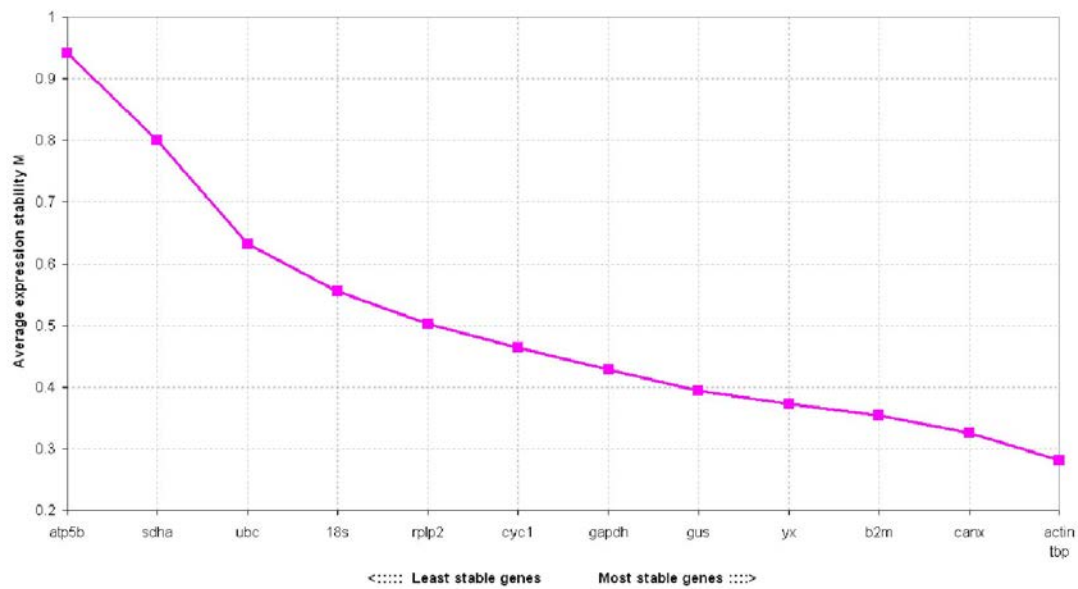
**Figure 3.1. Gene expression stability of candidate housekeeping genes in normal skin.** Values of M with geNorm software that compares gene expression without accounting for experimental groups and proceeds to the stepwise exclusion of the genes whose relative expression levels are more variable among tissues samples. Threshold for eliminating a gene as unstable was  $M \geq 1.5$ . Lower values of M correspond to the most stable genes, thus the most suitable for normalization.

Average expression stability values of remaining control genes

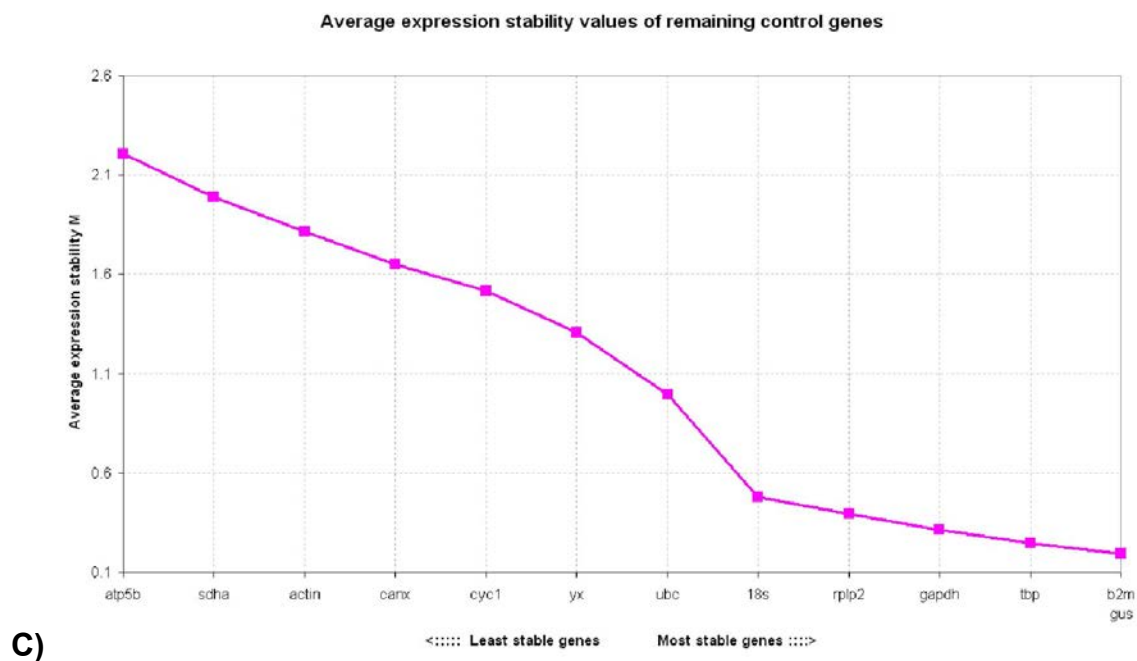


A)

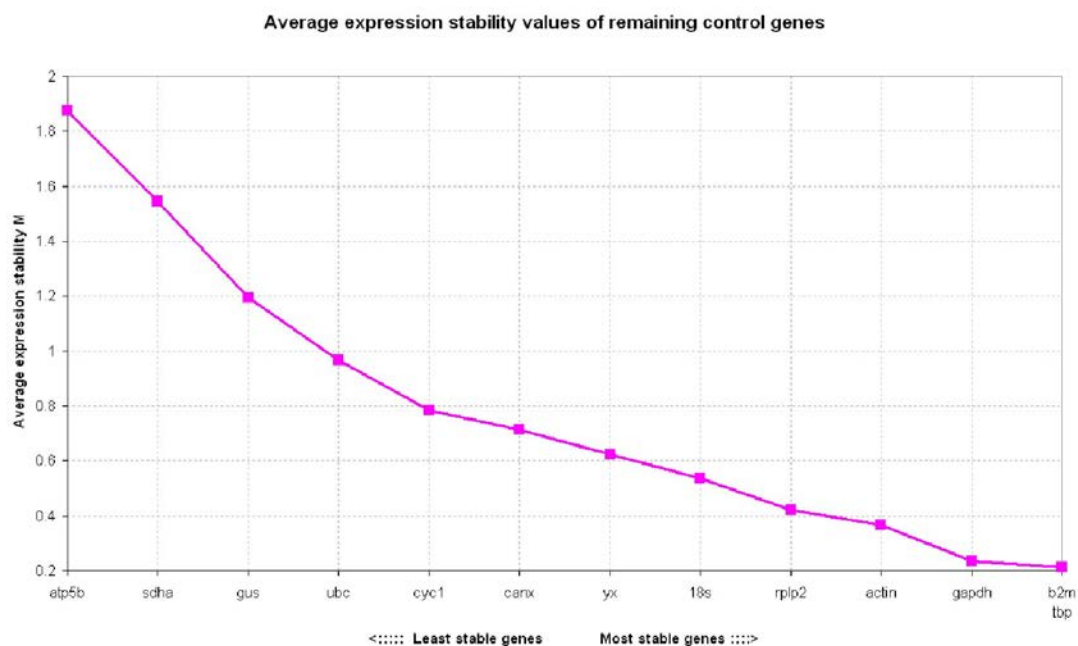
Average expression stability values of remaining control genes



B)



C)

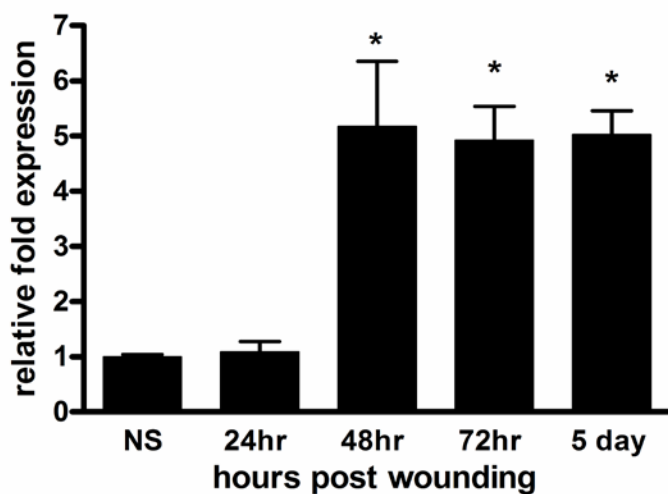


D)

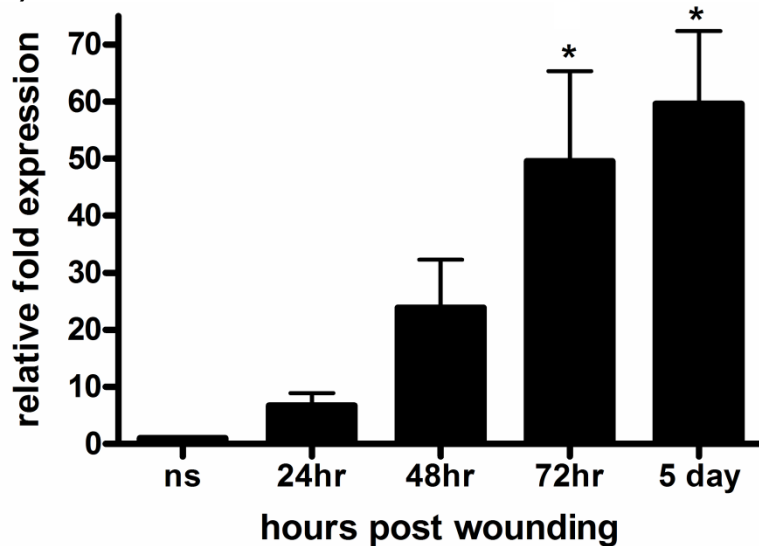
**Figure 3.2. Gene expression stability of candidate housekeeping genes in skin wounds.** 2A) Gene expression stability of candidate housekeeping genes

in 1mm 24h skin wounds; 2B) Gene expression stability of candidate housekeeping genes in 1mm 48h skin wounds; 2C) Gene expression stability of candidate housekeeping genes in 1mm 72h skin wounds; 2D) Gene expression stability of candidate housekeeping genes in 1mm 5 day skin wounds. Threshold for eliminating a gene as unstable was  $M \geq 1.5$  Lower values of M correspond to the most stable genes, thus the most suitable for normalization.

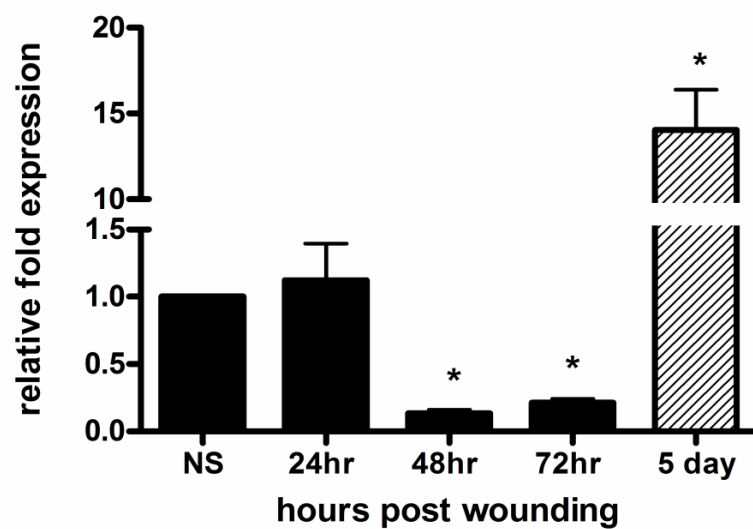
A)



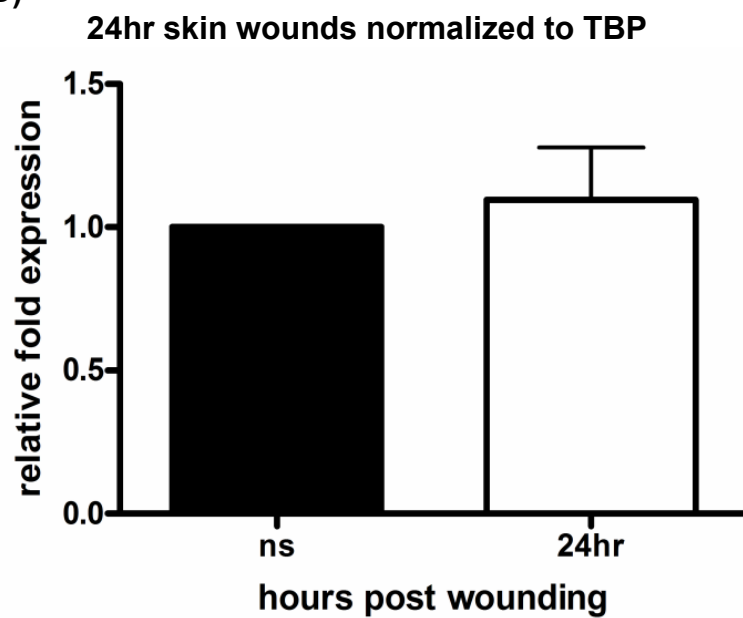
B)

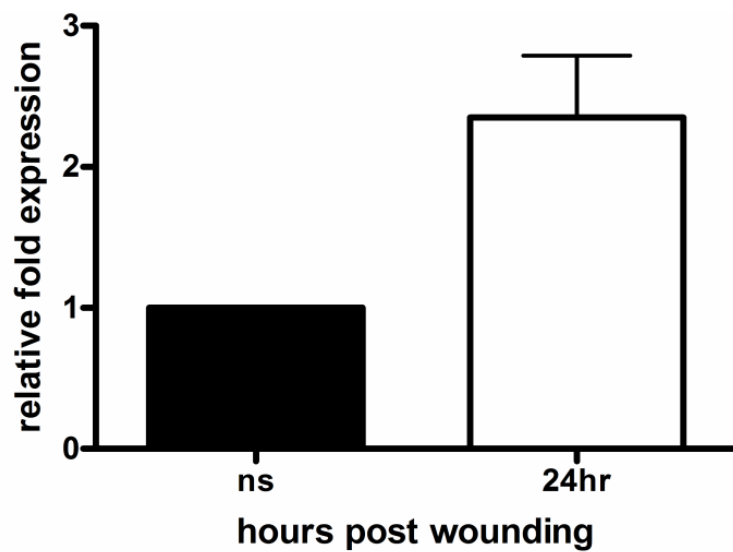


C)

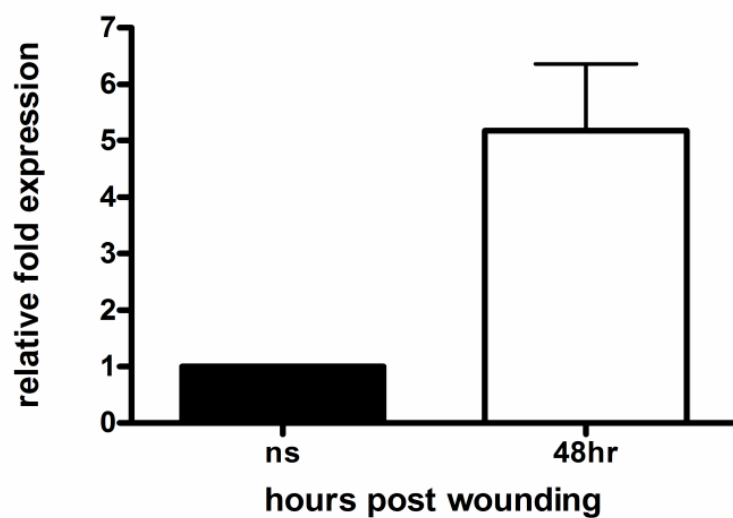


D)

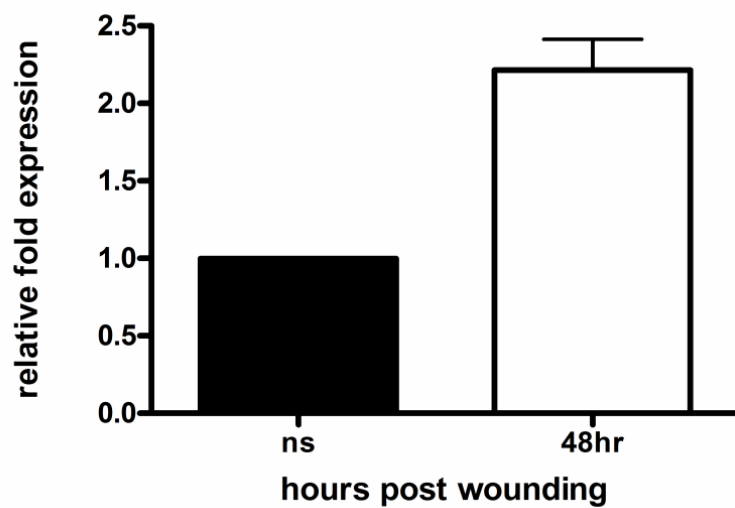




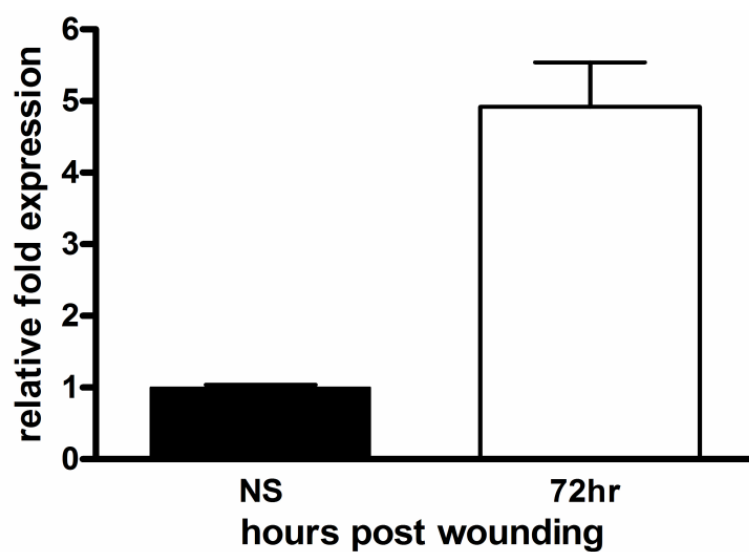
48hr skin wounds normalized to TBP



48hr skin wounds normalized to 3 most stable housekeeping genes

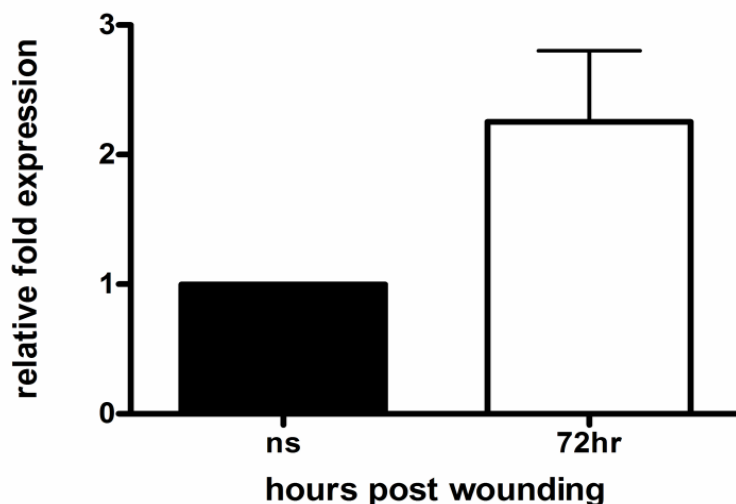


72hr skin wounds normalized to TBP



72hr skin wounds normalized to 3 most stable housekeeping genes





**Figure 3.3. KGF-2 expression normalized in wounds.** RT-PCR was performed on RNA isolated from normal tissues or wound samples at indicated times post-injury. To determine relative changes in mRNA levels during development, we normalized **A)** KGF-2 normalized to *TBP*, \* $P < 0.0003$  by One-way analysis of variance; **B)** KGF-2 normalized to  $\beta 2M$ , \* $P < 0.0004$  by One-way analysis of variance **C)** KGF-2 normalized to *GAPDH*, \* $P < 0.0001$  by One-way analysis of variance; **D)** KGF-2 normalized to single housekeeping gene, *TBP*, vs to 3 most stable housekeeping genes for each time point, chosen according to their M values from Table 3. A normalization factor was determined for each time point. Normal skin was normalized to 1 (A-D).

## DISCUSSION

### **4.1 Discussion**

The healing of oral mucosal and skin wounds proceeds through similar stages (Sciubba 1978, Walsh 1996). However studies in at least three different models (human, pig, and mouse) demonstrate that oral wound healing occurs faster and with less scarring than skin wounds (Mak 2009, Wong 2009, Szpaderska 2003).. Intrinsic differences in growth factor production, stem cell levels and cellular proliferation capacity have all been suggested to support oral mucosal wound repair (Sciubba 1978, Hill 1981, Angelov 2004, Yang 1996). Studies in our laboratory have shown that in comparison to skin wounds, there is a lower inflammatory response and angiogenesis in oral sites (Szpaderska 2003, Szpaderska 2005).

Previously, we have shown that oral mucosal wounds re-epithelialized at a much faster rate than skin wounds (Szpaderska 2003). Re-epithelialization consists of keratinocyte migration and proliferation (Woodley 1985) with their mechanisms independent of each other (Woodley 1993). In this study we focus on determining whether or not the differences in keratinocyte function between oral and skin wounds lead to faster oral re-epithelialization phenotype. Studies have reported that oral mucosa has a higher proliferative rate than skin (Hill et al 1981, Hata 1995) *in situ*. The current study is the first to compare human oral and skin keratinocyte proliferation independent of their underlying connective tissue and environment. We show that oral keratinocytes proliferate at a faster rate than skin keratinocytes after *in vitro* wounding. This data suggests

that epithelial cells have an intrinsic proliferative property that is independent of modulation by the underlying connective tissue.

Further we demonstrate that oral keratinocytes migrate 2.6 fold faster than skin keratinocytes in paired primary cultures. It must be noted again that this is independent of underlying fibroblasts, which are the source of critical paracrine motogenic factors, such as KGF-1 and 2, and dermal matrix metalloproteases (MMPs). Since we have eliminated sources from the connective tissue, faster oral keratinocyte migration seems to be intrinsic.

The intrinsic differences in proliferation and migration that were observed in skin and oral keratinocytes might be derived from several underlying mechanisms. First, oral and skin keratinocytes may exhibit differential expression of motogenic autocrine growth factors or matrix metalloproteases. For example, MMP-9 is a type IV collagenase that is expressed by migrating keratinocytes. It is not expressed in quiescent human skin keratinocytes, but transient upregulation is observed in the migrating keratinocytes of acute wounds, decreasing as re-epithelialization is complete (Madlener 1998). In contrast to epidermis, MMP-9 is expressed in the quiescent keratinocytes of oral mucosa (Salo 1994). MMP-9 expression is also detected in the migrating epithelial outgrowth of mucosal wounds (Salo 1994).

A second explanation for the observed difference in migration may derive from differential integrin expression or differential patterns of interaction with extracellular matrix proteins by oral and skin keratinocytes. In our migration experiments, keratinocyte migration was tested on slides that were coated with

fibronectin, which is one of the most critical extracellular matrix proteins on which keratinocytes migrate *in vivo*. Different oral and skin keratinocytes integrin expression might influence cell migration on fibronectin. While integrins are known to be critical to cellular migration, little is known about how integrin expression varies between skin and oral epithelial cells. Migrating keratinocytes are also believed to produce extracellular matrix molecules themselves. This ECM, which is thought to support their own migration, could also be different in oral and skin keratinocytes (Häkkinen 2000).

Our studies show that oral and skin keratinocytes are different, and some possible mechanisms are suggested above. If differential patterns of expression of growth factors, proteases, ECM and/or adhesion molecules do exist, the question remains as to how these differences are maintained. One obvious example is epigenetic regulation. Epigenetic regulation has now been demonstrated to dictate site-specific differences in skin fibroblasts, suggesting that similar mechanisms might also occur in epithelium (Chang 2002 ). In this context, the influence of connective tissue on epithelial function, discussed more extensively below, should be considered. Since fibroblasts from mucosa and skin are known to be epigenetically distinct, connective tissue cells may imprint location specific patterns of behavior on epithelial cells.

In addition to studying isolated keratinocytes, we also compared the the expression patterns of mitogenic and motogenic growth factors in oral and skin *in vivo* wounds. These studies provide new information about site specific roles for KGF-1 and 2, factors that are ligands for the IIIb variant of FGF receptor 2

(FGFR2-IIIb), in wound healing. Prior studies have suggested that KGF-1 and 2 are critical growth factors for re-epithelialization of skin wounds (Braun 2004, Werner 1994). Our results support this idea, and suggest that KGF-1 and 2, but not EGF, are important in skin re-epithelialization. Our observations also suggest that KGF-1, 2 and EGF are not the critical growth factors for mucosal re-epithelialization. Skin wounds demonstrate higher proliferative growth factor expression levels than oral wounds. This high level of proliferative growth factor expression could be a possible explanation of why skin wounds often heal with hypertrophic epidermis and thickened stratum corneum. One explanation for higher levels of growth factors in skin versus mucosa derives from the relative populations of dendritic epidermal T-cells, a cell type that is a potent source of KGF-1 and KGF-2 in skin (Jameson 2002) but absent in mucosa. Mice lacking dendritic epidermal T-cells show decreased keratinocyte proliferation and wound closure after skin injury. The contribution of KGF-1 and 2 from these cells could also explain their higher expression in skin wounds.

Our results also suggest the possibility that, due to intrinsically higher proliferation rates, epithelial proliferation in oral wounds is growth factor independent. Additional studies are needed to examine how growth factors influence oral mucosal healing. Future studies might investigate if there is differential expression of FGFR2-IIIb in oral and skin keratinocytes. Future studies to learn the specific cellular mechanisms that lead to enhance proliferative rate of oral epithelial cells are also needed.

As mentioned briefly above, one important additional consideration in the comparison of oral mucosal and skin wound healing is the interaction of fibroblasts and epithelial cells. Several studies suggest that fibroblasts might influence epithelial cells, and vice versa, and that such epithelial-connective tissue interactions might be important to wound healing outcomes (O'Shaughnessy 2009). For example, in *in vitro* co-culture experiments, signals from overlying keratinocytes influence collagen synthesis and the activity of underlying fibroblasts (Colwell 2007, Chang 1995). The reverse effect, dermal influence on epithelial phenotype, has also been demonstrated (Rinn 2008, Schweizer 1984, Mackenzie and Hill 1981, Mackenzie and Hill 1984, El Ghalbzouri 2004).

Evidence that fibroblasts from different anatomical sites maintain intrinsic differences is also strong (Sandulache 2005, Stephens 1996, Chang 2002). As compared to skin, oral mucosal fibroblasts exhibit enhanced proliferative capacity and an altered contractility profile (al-Khateeb 1996, al-Khateeb 1997, Lee 1995, Stephens 2001). Thus both fibroblasts and epithelial cells appear to maintain identities that contribute to the superior repair of oral mucosa. The data in this report suggest that differential epithelial responses seem highly likely to be important to the site-specific differences in repair of the mouth and skin. Overall, the divergent healing responses of oral mucosa and skin probably reflect intrinsic differences in epithelial cell and fibroblast function as well as interactions between these cells.

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## APPENDIX A

### UNIVERSITY OF ILLINOIS AT CHICAGO

Office for the Protection of Research Subjects (OPRS)  
Office of the Vice Chancellor for Research (MC 672)  
203 Administrative Office Building  
1737 West Polk Street  
Chicago, Illinois 60612-7227

#### Approval Notice Continuing Review

January 9, 2013

Anna Turabelidze, BS  
Periodontics  
801 South Paulina  
M/C 859  
Chicago, IL 60612  
Phone: (312) 996-1407

**RE: Protocol # 2008-0988**  
**“Oral Mucosa and Skin Characteristics in Wound Healing”**

Dear Ms. Turabelidze:

Your Continuing Review was reviewed and approved by the Convened review process on January 9, 2013. You may now continue your research.

Please note the following information about your approved research protocol:

**Protocol Approval Period:** January 30, 2013 - January 30, 2014

**Approved Subject Enrollment #:** 30 (20 subjects enrolled to date)

**Additional Determinations for Research Involving Minors:** These determinations have not been made for this study since it has not been approved for enrollment of minors.

**Performance Sites:** UIC

**Sponsor:** NIH-National Institutes of Health

**PAF#:** 2008-00171

**Grant/Contract No:** F30 DE019054

**Grant/Contract Title:** Individual Predoctoral Dental  
Scientist Training Fellowship

**Research Protocol(s):**

- a. Comparison of oral and skin keratinocytes during re-epithelialization, Version #2, 01/07/2009

**Recruitment Material(s):**

- a. Research Participant Recruitment Guide; Version 1; 01/07/2009
- b. Research Participants Needed; Version 4; 10/01/2012

**Informed Consent(s):**

- a. Mucosa/Skin Healing, Version #3, 02/03/2009

**HIPAA Authorization(s):**

- a. Mucosa/Skin Healing, Version #2, 01/07/2009 (Please continue to use the Authorization form, which was stamped and approved on February 6, 2009).

**Please note the Review History of this submission:**

Receipt Date	Submission Type	Review Process	Review Date
12/14/2012	Continuing Review	Convened	01/09/2013

Please remember to:

à Use your **research protocol number** (2008-0988) on any documents or correspondence with the IRB concerning your research protocol.

à Review and comply with all requirements on the enclosure, **"UIC Investigator Responsibilities, Protection of Human Research Subjects"**

**Please note that the UIC IRB has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.**

**Please be aware that if the scope of work in the grant/project changes, the protocol must be amended and approved by the UIC IRB before the initiation of the change.**

We wish you the best as you conduct your research. If you have any questions or need further help, please contact OPRS at (312) 996-1711 or me at (312) 996-9299. Please send any correspondence about this protocol to OPRS at 203 AOB, M/C 672.

Sincerely,

Marissa Benni, M.S.  
IRB Coordinator, IRB # 1

Office for the Protection of Research Subjects

Enclosure(s):

1. **UIC Investigator Responsibilities, Protection of Human Research Subjects**
2. **Informed Consent Document(s):**
  - a. Mucosa/Skin Healing, Version #3, 02/03/2009
3. **Recruiting Material(s):**
  - a. Research Participant Recruitment Guide; Version 1; 01/07/2009
  - b. Research Participants Needed; Version 4; 10/01/2012

cc: Phillip T. Marucha DDM, Ph.D, Periodontics, M/C 859  
OVCR Administration, M/C 672  
IDS, Pharmacy Practice, M/C 883

Phone: 312-996-1711 <http://www.uic.edu/depts/ovcr/oprsl> FAX: 312-413-2929

## VITA

### EDUCATION

Ph.D., Oral Sciences. Expected May 2013. Department of Periodontics, University of Illinois, Chicago.

DDS., Expected May 2013. College of Dentistry, University of Illinois, Chicago.

B.S., Bachelor of Sciences, May 2003. University of Missouri, Columbia.

### RESEARCH EXPERIENCE

#### Doctoral Research:

Advisor: Dr. Luisa DiPietro.

Thesis project: Differences between oral and skin wound healing.

Key thesis objectives:

Identify characteristics and key intrinsic differences that distinguish swift re-epithelialization of oral mucosa wounds from more slowly healing skin wounds.

Methods used: obtained human oral and skin epithelial tissues from young healthy donors at UIC College of Dentistry.

### Publications

#### Articles:

**Turabelidze A**, DiPietro LA. "Inflammation and wound healing". Endodontic Topics. 2012. Accepted 15 October 2012. Pages: 1601-1538.

**Turabelidze A**, Guo S, DiPietro LA. "Importance of housekeeping gene selection for accurate reverse transcription-quantitative polymerase chain reaction in a wound healing model". Wound Repair Regen. 2010 Sep-Oct;18(5):460-6. doi: 10.1111/j.1524-475X.2010.00611.x. Epub 2010 Aug 19.

#### Book Chapter:

DiPietro LA, **Turabelidze A**. Oral Wound Healing: Cell Biology and Clinical Management by Hannu Larjava. (Editor). Inflammation and Immunity chapter.

### Presentations:

Guo S, Yen AS, A. **Turabelidze A** and L.A. DiPietro. Differential gene expression in human oral mucosal and skin keratinocytes. Presented at IADR/AADR General Session and Exhibition, San Diego Convention Center, San Diego, California. March 18, 2011. Poster presentation.

**Turabelidze A**, Guo S, Marucha PT and Luisa A. DiPietro. Differences in epithelial cells of oral and skin tissues. Presented at the AADR Annual Meeting, Washington, DC. March 3, 2010. Oral presentation; Hatton Finalist. Gordon Conference 2009: poster presentation.

**Turabelidze A**, Guo S, Marucha PT and Luisa A. DiPietro. Behavior of epidermal versus oral keratinocytes in *in vitro* wounds. Presented at the Gordon Research

Conference on Tissue Repair & Regeneration, Colby-Sawyer College in New London, New Hampshire. June 14-19, 2009; poster.

**Turabelidze A.** Differences in oral and skin keratinocytes. Session Title: Epithelial Cell Biology. Presented at IADR/AADR General Session and Exhibition Miami, Miami Beach Convention Center, Miami, Florida. April 4, 2009. Oral presentation.

**Turabelidze A.** Oral Session Chair for Epithelial Cell Biology Session during the IADR/AADR General Session and Exhibition Miami, Miami Beach Convention Center, Miami, Florida April 4, 2009

#### **Awards:**

F30 –DE019054-01A1 (**Turabelidze, Anna**, Trainee) 08/01/08 – 07/31/13.  
NIH/NIDCR. Individual Predoctoral Dental Scientist Training Fellowship.  
2009 AADR Block Travel grant  
2010 Hatton Award finalist

#### **Teaching Experience:**

Teaching Assistant, University of Missouri, Columbia. Microbiology, Fall Semester 2002  
Assisted in instruction of lab sections and lead a small group discussion.

#### **Professional Affiliations:**

American Dental Association (ADA)  
American Association for Dental Research (AADR)

#### **Miscellaneous**

Fluent in: French, Russian, Georgian