Summer 7-29-2016

FOXO1 Differentially Regulates Both Normal and Diabetic Gingival Wound Healing

Hyeran H. Jeon
University of Pennsylvania School of Dental Medicine, hjeon@upenn.edu

FOXO1 Differentially Regulates Both Normal and Diabetic Gingival Wound Healing

Abstract
We have previously demonstrated that keratinocyte-specific forkhead box O1 (FOXO1) deletion interferes with keratinocyte migration in normal skin wounds. However it has an opposite effect in diabetic skin wounds, significantly improving the healing response. In addition we found that skin epithelium regulates connective tissue healing mediated by FOXO1, which is strongly associated with wound angiogenesis in our microarray results. However, a role for keratinocytes in this complex process has yet to be investigated. To this end, we investigated possible involvement of gingival keratinocytes in connective tissue healing under both normal and diabetic conditions. We found that keratinocyte-specific FOXO1 deletion interfered with normal gingival connective tissue healing by decreasing granulation tissue formation and angiogenesis, which were mediated by vascular endothelial growth factor A (VEGF-A). In particular this is the first evidence that avascular epithelium regulates angiogenesis involving the VEGF-A secretion mediated by FOXO1. Furthermore, we investigated the possible role of epithelial to mesenchymal transition (EMT) during wound healing using the lineage tracing in transgenic mice. But we did not find any keratinocyte-specific reporter activity in the connective tissue indicating that there was no apparent trans-differentiation of keratinocytes into typical fibroblasts or myofibroblasts during wound healing. These results establish an important role of epithelial cells in accelerating wound angiogenesis and connective tissue healing through a FOXO1-dependent mechanism.

Degree Type
Thesis

Degree Name
DScD (Doctor of Science in Dentistry)

Primary Advisor
Dana T. Graves, DDS, DMSc

Keywords
Forkhead box protein O1 (FOXO1), wound healing, diabetes, angiogenesis, Vascular Endothelial Growth Factor A (VEGF-A), Epithelial to mesenchymal transition (EMT)

Subject Categories
Dentistry

FOXO1 DIFFERENTIALLY REGULATES BOTH NORMAL AND DIABETIC GINGIVAL WOUND HEALING

Hyeran Helen Jeon, DDS, MSD

A DISSERTATION

Presented to the Faculties of the University of Pennsylvania in Partial Fulfillment of the Requirements for the Degree of Doctor of Science in Dentistry

2016

Supervisor of Dissertation

Dana T. Graves, DDS, DMSc

Interim Chair and Professor, Department of Periodontics
Vice Dean for Scholarship and Research
Director, Doctor of Science in Dentistry Program

Chairperson of Dissertation Committee

Faizan Alawi, DDS

Associate Professor of Pathology
Assistant Dean for Curriculum
Director, Penn Oral Pathology Services

Dissertation Committee

Hyun-Duck Nah-Cederquist, DMD, MSD, PhD
Research Associate Professor, Department of Plastic and Reconstructive Surgery University of Pennsylvania School of Medicine; Clinical Associate Professor, Department of Orthodontics University of Pennsylvania School of Dental Medicine

Sarah E. Millar, PhD
Albert M. Kligman Professor, Department of Dermatology University of Pennsylvania School of Medicine

Sunday O. Akintoye, BDS, DDS, MS
Associate Professor, Department of Oral Medicine
Director, Oral Medicine Research Program University of Pennsylvania School of Dental Medicine

FOXO1 DIFFERENTIALLY REGULATES
BOTH NORMAL AND DIABETIC GINGIVAL WOUND HEALING

ACKNOWLEDGMENT

I would like to express my sincere appreciation to my advisor, Dr. Dana T. Graves. You have been the best mentor, PI and boss ever for the last three years. I would like to thank you for providing several critical opportunities, challenging myself all the time and finally enabling myself to extend my capacity and become a better person. Your guidance and advice on my research as well as on my career have been invaluable.

I would also like to thank my committee members, Dr. Faizan Alawi, Dr. Hyun-Duck Nah-Cederquist, Dr. Sarah E. Millar and Dr. Sunday O. Akintoye for serving as my committee members. Especially I would like to thank Dr. Faizan Alawi for taking the position of my Committee Chair even though it was the sudden and last-minute request. I thank Dr. Hyun-Duck Nah-Cederquist for her invaluable guidance not only for my thesis project but also for my future career as a clinician-scientist in Orthodontic field with several nice dinners. I thank Dr. Sarah E. Millar for her keen comments and the fastest response always. I thank Dr. Sunday O. Akintoye for his support and endless encouragement.

I would especially like to thank our wound healing project members, Evelyn Spencer, Quan Yu, Olga Stepanchenko, Satoko Yamaba and Rameen Vafa. I was so lucky and feel blessed to work with you. Thank you very much for your perfect works, time, efforts, encouragement and your friendship. Without your help I cannot imagine how I could have done this project. I thank Eunice Han, Matthew Gavin and Gabrielle James for doing the genotyping and taking care of my precious K14.Cre FOXO1 mice. Also I thank all our lab members for your support and happy hours together.

My deepest appreciation is extended to Dr. Chung and Dr. Coby for their invaluable support and advice on my career. I would like to thank my co-residents, faculty and staffs at the Department of Orthodontics. They always encourage myself and help me to be a good orthodontist.

My last but not least, my special thanks go to my family and friends. You are the sources of my energy. My mom and dad, you make me the positive and happy person. My two sisters, my brother-in-law and my cutest niece—Thank you very much for your endless love and prayer. Especially I would like to thank Sumin Lee and Jieun Shin for sharing your experience and providing tremendous advices with endless encouragement.

Finally I thank my God for everything. I have experienced your guidance every moment. You are the ONE who guides my path. Thank you, Lord.

This work was supported by a grant from the National Institute of Dental and Craniofacial Research (DE019108).

ABSTRACT

FOXO1 DIFFERENTIALLY REGULATES
BOTH NORMAL AND DIABETIC GINGIVAL WOUND HEALING

Hyeran Helen Jeon

Dana T. Graves

We have previously demonstrated that keratinocyte-specific forkhead box O1 (FOXO1) deletion interferes with keratinocyte migration in normal skin wounds. However it has an opposite effect in diabetic skin wounds, significantly improving the healing response. In addition we found that skin epithelium regulates connective tissue healing mediated by FOXO1, which is strongly associated with wound angiogenesis in our microarray results. However, a role for keratinocytes in this complex process has yet to be investigated. To this end, we investigated possible involvement of gingival keratinocytes in connective tissue healing under both normal and diabetic conditions. We found that keratinocyte-specific FOXO1 deletion interfered with normal gingival connective tissue healing by decreasing granulation tissue formation and angiogenesis, which were mediated by vascular endothelial growth factor A (VEGF-A). In particular this is the first evidence that avascular epithelium regulates angiogenesis involving the VEGF-A secretion mediated by FOXO1. Furthermore, we investigated the possible role of epithelial to mesenchymal transition (EMT) during wound healing using the lineage tracing in transgenic mice. But we did not find any keratinocyte-specific reporter activity in the connective tissue indicating that there was no apparent trans-differentiation of keratinocytes into typical fibroblasts or myofibroblasts during wound healing. These results establish an important role of epithelial cells in accelerating wound angiogenesis and connective tissue healing through a FOXO1-dependent mechanism.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AGEs: Advanced glycation end products

α-SMA: α-smooth muscle actin

bFGF: Basic fibroblast growth factor

CreER: Fusion protein between Cre recombinase and the tamoxifen responsive hormone-binding domain of the estrogen receptor

CTGF: Connective tissue growth factor

EC: Endothelial cell

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EMT: Epithelial to mesenchymal transition

FOXO1: Forkhead box protein O1

FSP1: Fibroblast-specific protein 1

H & E: Hematoxylin and eosin

HIGK: Human immortalized gingival keratinocyte

IHC: Immunohistochemistry

K14.Cre: Transgene containing the Cre coding sequence 3' from the human K14 promoter/enhancer

MFI: Mean fluorescence intensity

MMPs: Matrix metalloproteinases

NHEK: Normal human epidermal keratinocytes

PBS: Phosphate-buffered saline

SEM: Standard error of the mean

TGF-β: Transforming growth factor-β

TNF-α: Tumor necrosis factor-α

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

VPF: Vascular permeability factor

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER 1

INTRODUCTION

1.1 Wound Healing, Diabetes and Angiogenesis

Wound healing is a dynamic interactive process that begins at the moment of wounding and involves soluble mediators, many cell types, and extracellular matrices (5-7). The healing of oral wounds proceeds through similar stages as that of skin wounds including three phases: 1) inflammatory phase, 2) proliferative phase, and 3) maturation phase (8). A number of cell subsets contribute to healing, including keratinocytes, fibroblasts, endothelial cells and inflammatory cells. The activity of these cells is regulated by cytokines acting in both autocrine and paracrine fashion to bring about efficient healing (9). The goal of the inflammatory phase is to obtain hemostasis and to provide an influx of neutrophils and macrophages into the wound bed in a milieu of cytokines and growth factors. At the end of the inflammatory phase apoptosis of inflammatory cells appears concurrently with re-epithelialization of the wound and may be a signal of healing at that site in the wound (10). The second phase, the proliferative phase, is characterized by the granulation tissue formation, angiogenesis, deposition of new extracellular matrix (ECM), and re-epithelialization. Although granulation is classically assigned to the proliferative stage, angiogenesis is initiated immediately upon wounding and is mediated throughout the entire wound healing process. In order to manufacture the extracellular matrix fibroblasts require oxygen and nutrients so there is a requirement for angiogenesis (11). The third phase, the maturation phase, involves wound contraction and remodeling. Collagen is realigned along tension lines and cells that are no longer needed are removed by programmed cell death or apoptosis.

Diabetes has a major effect on wound healing. It is a chronic disease characterized by elevated blood sugar levels resulting from either lack of insulin production or resistance to insulin (12). It is one of the most challenging health problems of the 21st century. Some 382 million people worldwide (8.3% of adults) are estimated to have diabetes (13). If these trends continue, by 2035, some 592 million people, or one adult in 10, will have diabetes. The largest increases will take place in the regions where developing economies are predominant. In addition, diabetes imposes a large economic burden on individuals and families, national health systems, and countries. Health spending on diabetes accounted for 10.8% of total health expenditure worldwide in 2013. There are numbers of complications caused by diabetes, including increased heart disease, stroke, blindness, kidney failure, and delayed wound healing (14). Diabetes affects the oral cavity by increasing the prevalence of caries, gingivitis, periodontal disease, implantitis, fibromas, and traumatic ulcers (15). A number of factors may contribute to impaired healing in diabetics, such as decreased or impaired growth factor production (16-18), angiogenic response (18, 19), macrophage function (20), collagen accumulation, epidermal barrier function, quantity of granulation tissue (18), keratinocyte and fibroblast migration and proliferation, number of epidermal nerves (21), bone healing, and balance between the accumulation of ECM components and their remodeling by matrix metalloproteinases (MMPs) (22) (Fig. 1). One of the major cellular components of wound granulation tissue relevant to healing and angiogenesis is macrophages (23, 24). Wound macrophages have been established as key players in the maintenance of tissue homeostasis through tissue remodeling and repair via the release of mediators at the wound site (25). In support of this view, suppressed recruitment of macrophages into granulation tissue impairs wound healing (26, 27). Decreased numbers of macrophages and insufficient macrophage activation contribute to the impaired healing of diabetic

wounds (20). Increased fibroblast apoptosis has been proposed to be a mechanism for diabetes-impaired dermal wound healing (28-30). Patients with diabetes are characterized by a reduction in angiogenesis, which is an important part of the proliferative phase of healing. Limited penetration of new blood vessels into the wound would restrict entry of inflammatory cells, and high amounts of distinct angiogenic factors

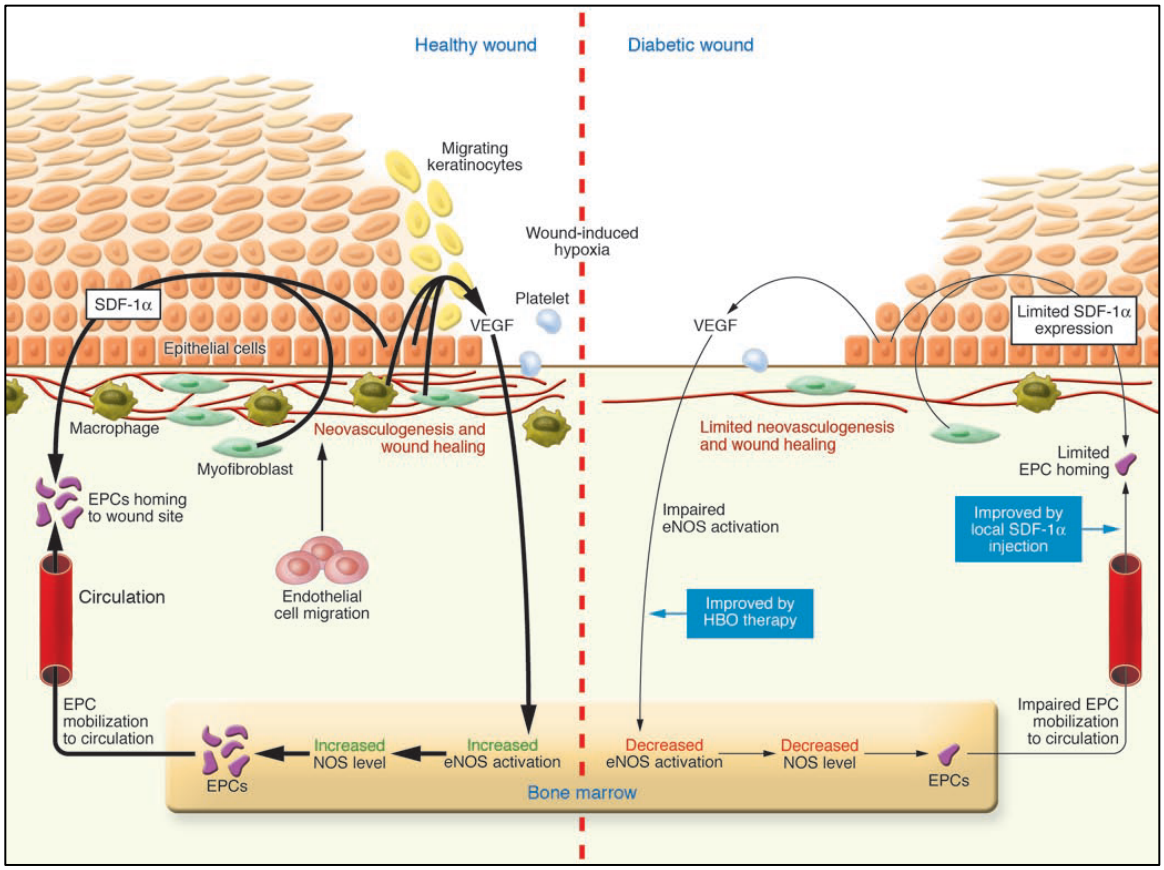


Figure 1. Mechanisms of wound healing in healthy people versus people with diabetes (31) In healthy individuals (left), the acute wound healing process is guided through multiple signals released by keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. During wound-induced hypoxia, VEGF released by keratinocytes, macrophages and fibroblasts induces the phosphorylation and activation of Endothelial Nitric Oxide Synthase (eNOS) in the bone marrow, resulting in an increase in NO levels, which triggers the mobilization of bone marrow EPCs to the circulation. On the contrary, in people with diabetes (right) keratinocytes show less migration and incomplete differentiation. Fibroblasts demonstrate the decreased migration and proliferation. Also, the amount of VEGF released by several different cell types is reduced, resulting in impaired eNOS activation in bone marrow, which directly limits endothelial progenitor cells (EPC) homing. Therefore it impairs wound healing.

produced by immune cells are limited (32). Factors that play a decisive role in diabetes-associated wound healing disorders comprise augmented inflammatory responses, impaired granulation tissue formation, reduced growth factors and a disturbed angiogenesis (13). In particular, hyperglycemia-related microvascular pathology is thought to be a critical contributor to impaired diabetic healing (33). Hyperglycemia can affect endothelial function via several major mechanisms, including toxic effects on endothelial cells via nitric oxide, oxidative stress and inflammation (34, 35), and accumulation of advanced glycation end products (AGEs) that results in altered extracellular matrix and disrupted angiogenic growth factor signaling (36, 37). In vitro studies in high glucose culture conditions demonstrated increased endothelial apoptosis (38, 39); impaired proliferation, adhesion, and tube formation (40, 41); and changes in cytokine production (16).

Streptozotocin (STZ) is most widely used to induce experimental diabetes in rodents because of its simplicity and reproducibility (42). STZ (2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose) is a broad-spectrum antibiotic which is produced by *Streptomyces achromogenes*. The diabetogenic effect of STZ was first reported in 1963 by Rakieten et al. after injection of a single intravenous dose in rats and dogs (43). STZ is similar enough to glucose (Glu) and N-acetyl glucosamine (GlcNAc) (Fig. 2) to be transported into the pancreatic β cells by the glucose transport protein GLUT2, but is not recognized by the other glucose transporters. This explains its relative toxicity to β cells, since these cells have relatively high levels of GLUT2 (44, 45). Once it is taken it causes β-cell death by DNA fragmentation due to the nitrosourea moiety. Three major pathways associated with cell death are: (i) methylation of DNA by the formation of carbonium ion (CH3+) resulting in the activation of the nuclear enzyme poly ADP-ribose synthetase as part of the cell repair mechanism and therefore NAD+ depletion; (ii) free radical

generation as hydrogen peroxide and (iii) nitric oxide production (46, 47). The toxic effects of STZ are not restricted to pancreatic β-cells. STZ also causes renal, cardiac and adipose tissue damage and increases oxidative stress, inflammation and endothelial dysfunction (48). Moreover, several studies have described immune-modifying effects of STZ both in vitro and in vivo (49-51).

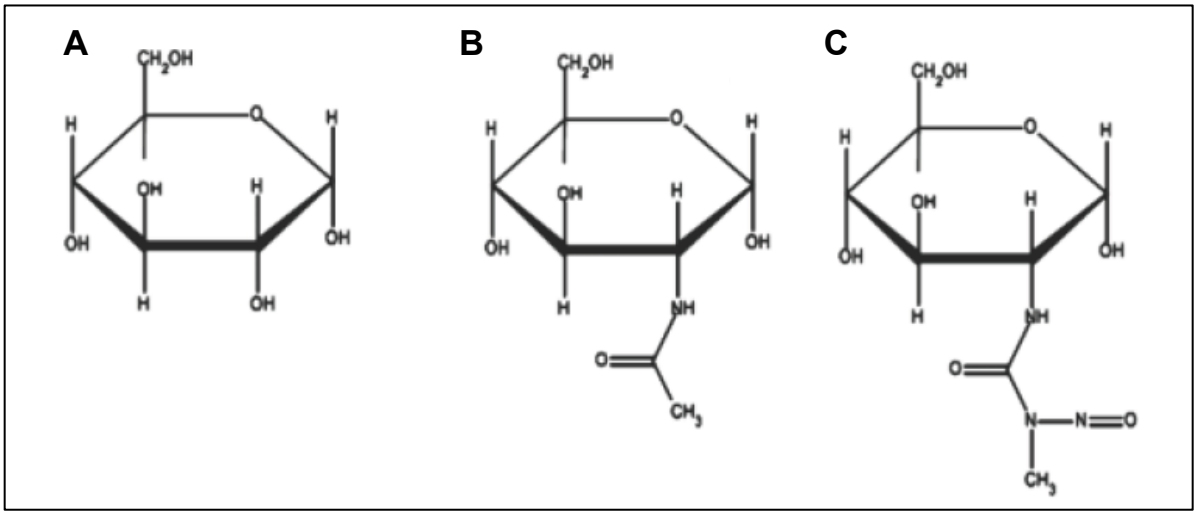


Figure 2. Chemical structures of (A) glucose, (B) N-acetyl glucosamine and (C) streptozotocin

Wound angiogenesis is critical to wound healing. Angiogenesis is initiated upon wounding by multiple molecular signals, including hemostatic factors, inflammation, cytokine growth factors, and cell-matrix interactions. Initially, there is no vascular supply to the wound center and the appearance of new viable tissue is limited to wound margins that are in contact with uninjured tissue through direct contact with uninjured blood vessels and through the process of short-distance diffusion through the uninjured interstitium. Under normal conditions, a tissue or tumor cannot grow beyond 1 to 2 mm in diameter without neovascularization. This distance is defined by limits in the diffusion of oxygen and metabolites, such as glucose and amino acids (52). The vascular

component depends upon angiogenesis, in which new vessels appear as early as d 3 after wounding (53). The formation of new blood vessels in the developing acute granulation tissue (angiogenesis) occurs by a budding or sprouting mechanism from intact vessels at the wound borders. The development of vascular outgrowths requires endothelial cell proliferation. Numerous tissue growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) play central regulatory roles in neovascularization and subsequent tissue repair (54). Macrophages also appear to be necessary for wound neovascularization to occur, especially along wounded tissue oxygen gradients (55). As collagen accumulates in the granulation tissue to produce scar, the density of blood vessels diminishes (56). New capillaries proliferate via a cascade of biological events to form granulation tissue in the wound bed. Injured tissue releases growth factors that bind to their receptors on endothelial cells, activating signal transduction pathways and stimulating endothelial proliferation, migration, and vascular tube formation. Bone marrow derived endothelial progenitor cells are mobilized and become incorporated into new blood vessels. Stabilization of the vasculature occurs through the recruitment of smooth muscle cells and pericytes (Fig. 3). Several evidences implicate VEGF as a significant factor in wound healing immediately after injury (53, 57). Maximal activity occurs during a period approximately 3 to 7 d after injury. Once the wound is granulated, angiogenesis ceases and blood vessels decline as endothelial cells undergo apoptosis.

Defects in the angiogenesis delay healing and these are evident in chronic wounds. In general, the process of angiogenesis is believed to be controlled by changes in the levels of proangiogenic and antiangiogenic molecules present within the microenvironment surrounding the vasculature (1). Many proangiogenic and antiangiogenic mediators have been identified (Table 1). Vascular endothelial growth

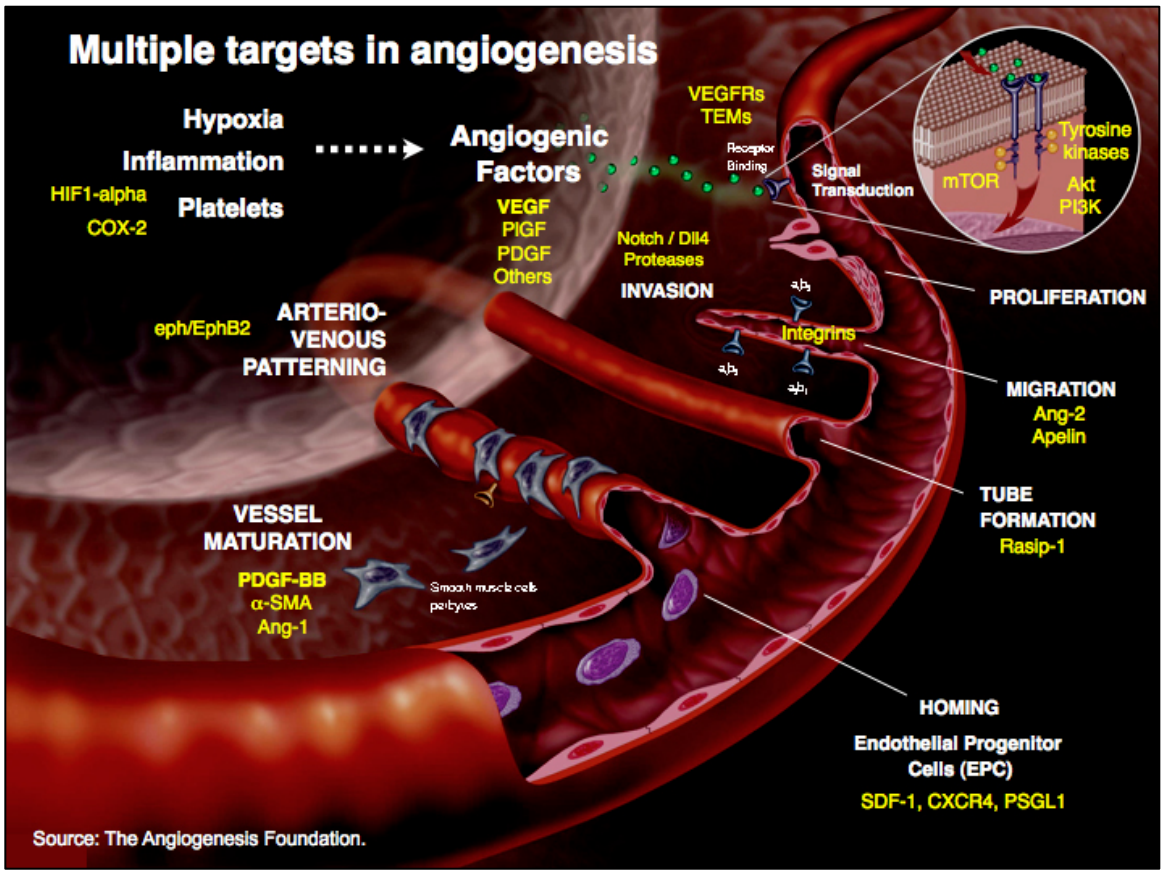


Figure 3. The Angiogenesis Cascade of Events and Multiple targets Injured tissues release growth factors that bind to their receptors on endothelial cells, activating signal transduction pathways and stimulating endothelial proliferation, migration, and vascular tube formation. Bone marrow derived endothelial stem cells are mobilized and become incorporated into new blood vessels. Stabilization of the vasculature occurs through the recruitment of smooth muscle cells and pericytes.

Table 1. Molecular Regulators of Angiogenesis (Source: The Angiogenesis Foundation)

Angiogenic Stimulators

Angiogenin
Angiopoietin-1
Del-1
Fibroblast growth factors: acidic (aFGF) and basic (bFGF)
Follistatin
Granulocyte colony-stimulating factor (G-CSF)
Hepatocyte growth factor (HGF) /scatter factor (SF)
Interleukin-8 (IL-8)
Leptin
Midkine
Placental growth factor
Platelet-derived endothelial cell growth factor (PD-ECGF)
Platelet-derived growth factor-BB (PDGF-BB)
Pleiotrophin (PTN)
Progranulin
Proliferin
Transforming growth factor-alpha (TGF-alpha)
Transforming growth factor-beta (TGF-beta)
Tumor necrosis factor-alpha (TNF-alpha)
Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF)

Angiogenesis Inhibitors

Angioarrestin
Angiostatin (plasminogen fragment)
Antiangiogenic antithrombin III
Arrestin
Chondromodulin
Canstatin
Cartilage-derived inhibitor (CDI)
CD59 complement fragment
Endostatin (collagen XVIII fragment)
Endorepellin
Fibronectin fragment
Fibronectin fragment (Anastellin)
Gro-beta
Heparinases
Heparin hexasaccharide fragment
Human chorionic gonadotropin (hCG)
Interferon alphagamma
Interferon inducible protein (IP-10)
Interleukin-12
Kringle 5 (plasminogen fragment)
Metalloproteinase inhibitors (TIMPs)
2-Methoxyestradiol
PEX
Pigment epithelium derived factor (PEDF)
Placental ribonuclease inhibitor
Plasminogen activator inhibitor
Platelet factor-4 (PF4)
Prolactin 16kD fragment
Proliferin-related protein (PRP)
Prothrombin kringle 2
Retinoids
Soluble Fms-like tyrosine kinase-1 (S-Flt-1)
Targeting fibronectin-binding integrins
Tetrahydrocortisol-S
Thrombospondin-1 (TSP-1) and -2
Transforming growth factor-beta (TGF-b)
Troponin I
Tumstatin
Vasculostatin
Vasostatin (calreticulin fragment)

factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor and placental growth factor (PlGF) are proteins that stimulate angiogenesis whereas angiostatin (a fragment of plasminogen), endostatin (a fragment of collagen XVIII) and thrombospondin-1 are examples of angiogenesis inhibitors. The maintenance of vessels in a quiescent state is thought to occur when the level of antiangiogenic signals outweigh proangiogenic signals; however, periods of active angiogenesis occur when endothelial cells sense a shift in the balance of these mediators, with proangiogenic signals predominating over antiangiogenic signals. The angiogenic switch concept was proposed by Folkman and Hanahan, who first used it to describe the regulation of tumor angiogenesis (58). These events are stimulated in part by a number of mitogens and chemotactic factors. The soluble factors shown to be mitogenic for endothelial cells in vitro and angiogenic in vivo include different members of the fibroblast growth factor family, transforming growth factor-α (TGF-α), epidermal growth factor (EGF) (5) and platelet-derived growth factor BB (6). Furthermore, transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) have been shown to be angiogenic in vivo, although they inhibit endothelial cell growth in vitro (7-11). Thus, they may be indirectly angiogenic under certain conditions. The vascular endothelial growth factor (VEGF) is the most potent and specific vascular growth factor. VEGF family comprises in mammals five members: VEGF-A, placental growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D. The latter ones were discovered later than VEGF-A and, before their discovery, VEGF-A was termed VEGF. It is a soluble protein secreted by a wide variety of cell types. The inhibition of VEGF by antibody has been shown to inhibit angiogenesis (59-61). VEGF-A levels are regulated through transcriptional control and mRNA stability (62). Four different human isoforms of VEGF-A including VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206}, have been isolated from various sources and arise by alternative splicing of

mRNA (12). The most abundant isoform found in human tissue is VEGF-A_{165}. Whereas all VEGF-A isoforms seem to have similar biological activities, only VEGF-A_{121} and VEGF-A_{165} are secreted in soluble form, whereas the higher-molecular-weight forms apparently remain cell associated (63). VEGF-A plays a pivotal role during the angiogenic response in tissue repair by regulating vascular permeability, the influx of inflammatory cells into the site of injury, migration and proliferation of pre-existing endothelial cells and the recruitment of marrow-derived endothelial progenitor cells to the local wound site (4, 19, 57, 64, 65). In skin major sources of VEGF-A are epidermal keratinocytes, macrophages, endothelial cells, fibroblasts and mast cells (1, 66-68). VEGF-A is up-regulated during the early days of healing, when capillary growth is maximal. Delayed wound closure and reduced vessel density have been reported in mice lacking VEGF-A in keratinocytes (65). Brown et al. demonstrated expression of VEGF mRNA in proliferating keratinocytes of the newly formed epithelium during wound healing (4) (Fig. 4). In contrast, expression of one of the receptors for VEGF-A (flt-1) was found to be up-regulated in the sprouting blood vessels at the wound edge (19) and in endothelial cells of the granulation tissue (20). These findings suggest that keratinocyte-derived VEGF-A stimulates angiogenesis during wound healing in a paracrine manner. Detmar et al. characterized the effects of several cytokines and growth factors on the expression and secretion of vascular permeability factor (VPF)/VEGF mRNA and protein by cultured human epidermal keratinocytes, as well as the effect of VPF/VEGF on the growth of cultured human dermal microvascular endothelial cells (69). These results suggest that the

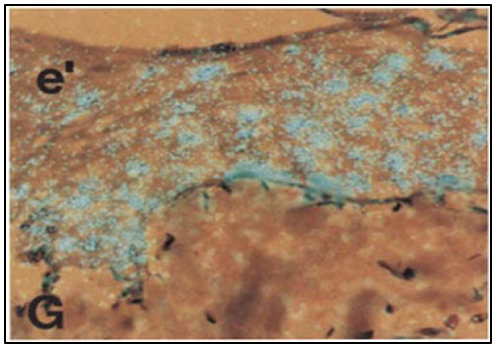


Figure 4. In situ expression of VEGF mRNA in healing rat. Epidermal labeling was maximal on days 2-3 (4).

avascular epidermis has the capacity to regulate dermal angiogenesis and microvascular permeability by a paracrine mechanism involving the secretion of VPF/VEGF.

VEGF-A transcription and secretion are elevated in partial (4) and full-thickness skin wounds (70, 71). In partial thickness wounds, keratinocytes at the wound edge express elevated VEGF-A as early as 1 d after injury and eventually migrate to cover the defect. Epidermal labeling for VEGF-A mRNA reaches a peak after 2–3 d, coincident with a peak in vascular permeability, and levels remain elevated until epidermal coverage is complete. Similarly maximal VEGF-A mRNA is found between 3 and 7 d after full-thickness wounding during the period of granulation tissue formation (70). The time course of VEGF-A expression provides insight into the progression of wound healing. During the proliferative phase of repair occurring approximately 3 to 7 d post-wounding capillary growth and differentiation are at a maximum. During this period, VEGF-A is up-regulated to promote the early stages of angiogenesis (i.e., vascular dilation, permeability, migration, and proliferation) (57). Antibody neutralization of VEGF-A diminishes the chemotactic and angiogenic properties of wound fluid, thus revealing further evidence for the importance of VEGF-A in wound repair (57). Additionally, wounds of streptozotocin-induced diabetic mice demonstrate diminished synthesis of several growth factors including VEGF-A (71).

To date, the majority of the effects of VEGF-A during wound repair have been attributed to its proangiogenic activity (1) (Fig. 5); however, recent studies have indicated that in addition to vascular endothelial cells, other cells such as keratinocytes and macrophages express VEGFRs and can respond directly to VEGF-A. VEGF-A likely promotes collagen deposition and epithelialization as well (53). Traditionally, VEGF-A produced by epidermal keratinocytes was thought to act in a paracrine manner,

stimulating endothelial cells within the granulation tissue. However, functional VEGFRs have been recently identified on keratinocytes (72-75), which suggests the possibility of autocrine VEGF-A signaling in keratinocytes as well as direct effects of VEGF-A derived from other cellular sources on keratinocytes. Direct effects of VEGF-A on keratinocytes have been described in vitro using cultured primary human keratinocytes (72) and primary mouse keratinocytes (73). Those studies suggested possible roles for VEGF-A in the regulation of keratinocyte proliferation, migration and survival (72, 73, 75-77). In addition to keratinocytes, myeloid cells (monocytes and macrophages) are another important cell type known to respond to VEGF-A during wound healing (1). Monocytes and macrophages express VEGFR-1 and VEGF-A has been shown to increase the migration of these cells in vitro through VEGFR-1 (78-80). A higher density of macrophages has been reported in wounds from VEGF-A transgenic mice (81).

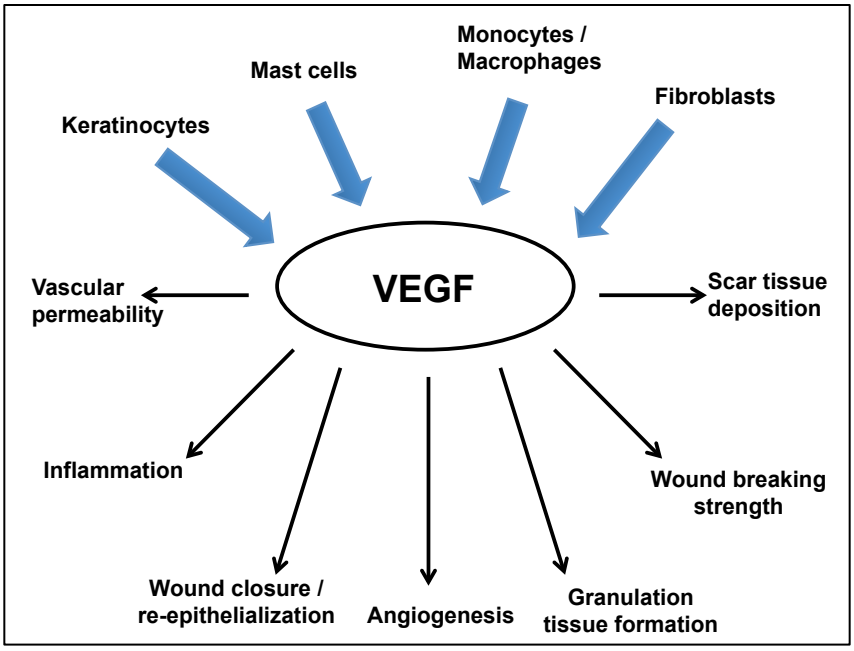


Figure 5. Cellular sources and actions of VEGF during wound healing (1)

1.2 Epithelial-Mesenchymal Transition (EMT) in Wound Healing

Epithelial to mesenchymal transition (EMT), originally identified as an essential differentiation and/or morphogenetic process during embryogenesis, may contribute to wound healing, tissue remodeling, fibrosis, and metastatic malignancies (82). Elizabeth Hay first described an “epithelial-mesenchymal transformation” using a model of chick primitive streak formation (83). In the intervening time, the term “transformation” has been replaced with “transition,” reflecting in part the reversibility of the process and the fact that it is distinct from neoplastic transformation (84). Three distinct manifestations of EMT have been proposed based on stages of development and associated biomarkers; 1) embryogenesis and organ development (Type I), 2) wound healing and organ fibrosis in mature tissue (Type II), and 3) tumorigenesis and cancer metastasis (Type III) (Fig. 6). EMT is a trans-differentiation process by which epithelial cells lose their epithelial characteristics and acquire a mesenchymal phenotype and is characterized by changes in cell morphology, and disruption of tight junctions and adherent junctions (85) (Fig. 7). Down-regulation of E-cadherin is considered a key step in EMT (Fig. 8). A growing number of transcriptional molecules have been found to be involved in the EMT process (Table 2) (86). Among them, ectopic expression of Snail has been reported to suppress E-cadherin expression, leading to a full EMT phenotype, whereas silencing of Snail expression reverses this process (87). The acquired mesenchymal phenotype is characterized by expression of cytoskeletal proteins such as α-smooth muscle actin (α-SMA) and vimentin, as well as matrix metalloproteinases (MMPs). As a result of these phenotypic changes, epithelial cells are released from the surrounding tissue and become migratory and invasive.

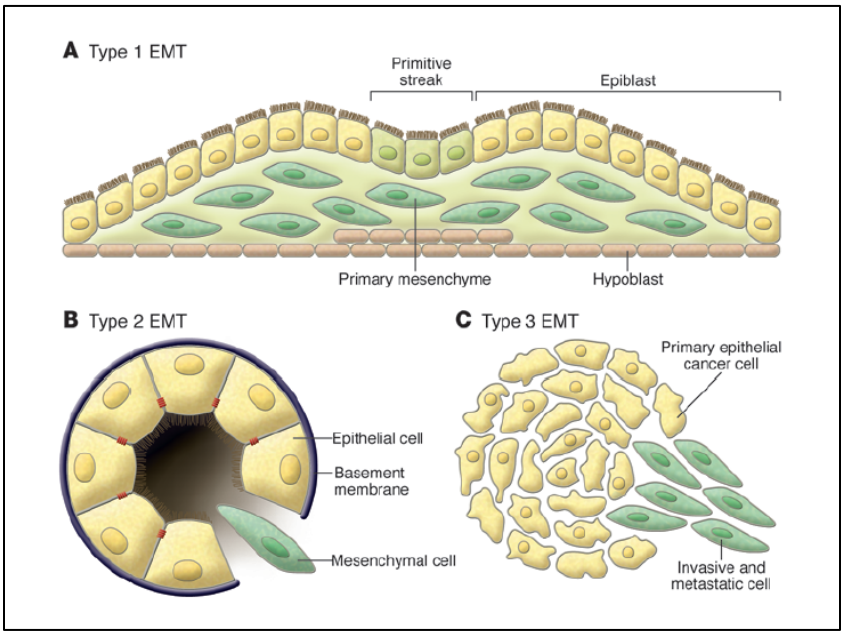


Figure 6. Different types of Epithelial to mesenchymal transition (88); (A) embryogenesis and organ development (Type I), (B) wound healing and organ fibrosis in mature tissue (Type II), and (C) tumorigenesis and cancer metastasis (Type III)

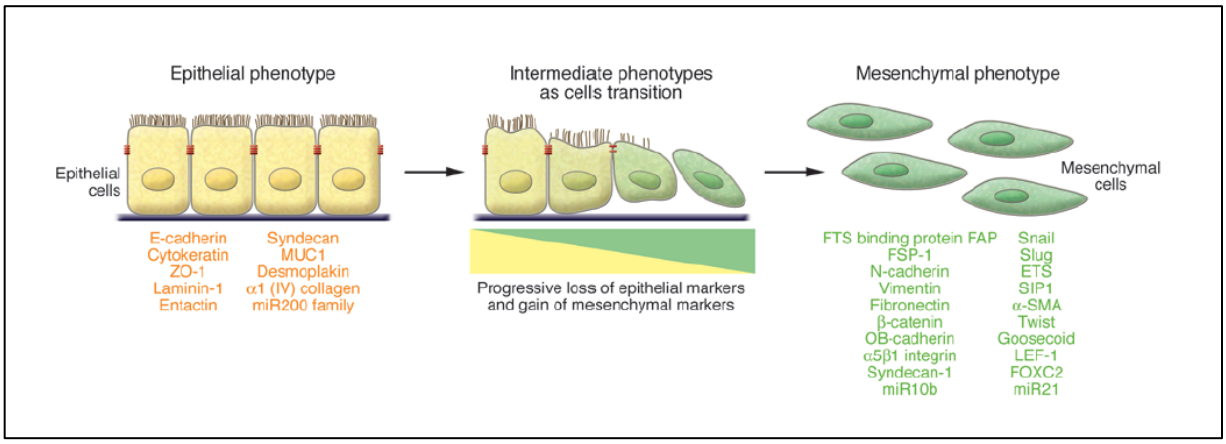


Figure 7. Epithelial to mesenchymal transition (EMT) (88). An EMT involves a functional transition of polarized epithelial cells into mobile and ECM component-secreting mesenchymal cells.

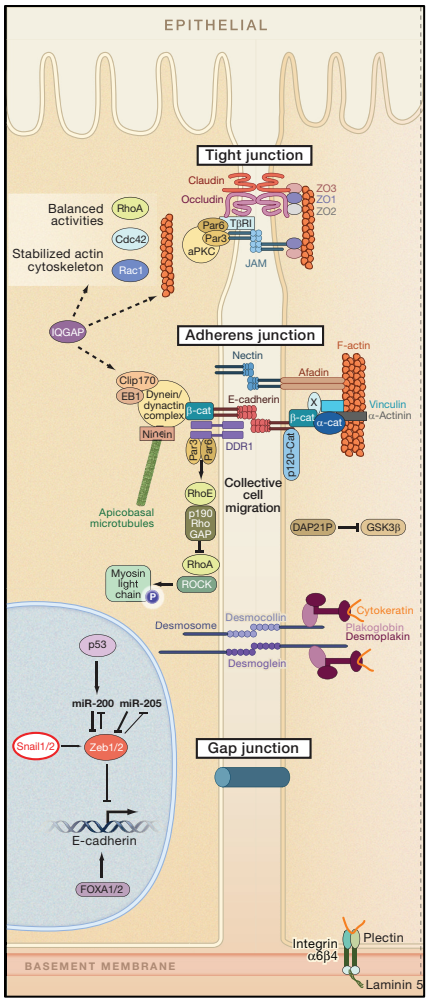


Figure 8. The Epithelial Phenotype. Polarized epithelial cells are typified by tight junctions, adherens junctions, desmosomes, and gap junctions. (3)

More recently, EMT-like processes have been described during tissue repair (89). Transforming growth factor β (TGF-β) is the most potent inducer of EMT (90) as well as an essential factor during normal wound healing. Transforming growth factor β (TGF-β) signaling at the wound site has been implicated in re-epithelialization, inflammatory cells infiltration, wound contraction, extracellular matrix deposition and remodeling. Successful wound healing is a complex process involving cells of the epidermis, dermis, vasculature, and the immune system (91). Two distinct cellular mechanisms directly contribute to the closure of skin wounds: keratinocyte-driven re-epithelialization and fibroblast-mediated contraction of the newly formed connective tissue bed beneath the wound site, pulling the edges of the wound closer together (92). During re-epithelialization, migrating keratinocytes undergo numerous phenotypic and functional alterations reminiscent of epithelial–mesenchymal transformation, including disruption of desmosomes and hemidesmosomes, alterations in the actin-based cytoskeleton, retraction of intermediate filaments and loss of cell polarity. Epithelial cells migrate from the edges of the wound very soon after the initial insult until a complete sheet of cells covers the wound and attaches to the matrix below. In wounds that are primarily closed, this phase can be completed within 24 hours. Changes in cytokine concentration result in epithelial cells switching from a motile phenotype to a proliferative one in order to

Table 2. Epithelial to Mesenchymal Transition Markers (86)

Proteins that increase in abundance	
N-cadherin	FOXC2
Vimentin	Sox10
Fibronectin	MMP-2
Snail1 (Snail)	MMP-3
Snail2 (Slug)	MMP-9
Twist	Integrin αvβ6
Goosecoid	
Proteins that decrease in abundance	
E-cadherin	Cytokeratin
Desmoplakin	Occludin
Proteins whose activity increases	
ILK	Rho
GSK-3β	
Proteins that accumulate in the nucleus	
β-catenin	Snail1 (Snail)
Smad-2/3	Snail2 (Slug)
NF-κβ	Twist
In vitro functional markers	
Increased migration	Elongation of cell shape
Increased invasion	Resistance to anoikis
Increased scattering	

repopulate epithelial cell levels and complete wound repair (93). In wounds that heal by secondary intention, the area lacking epithelial cells can be large and the wound must contract significantly before epithelialization can be completed. Wound contraction process is performed by myofibroblasts activated by TGF-β (70). The sources of myofibroblasts in a healing wound are numerous. Emerging evidence suggests that epithelial cells are also an important source of myofibroblasts in fibrosis and cancer (94). But, its role during wound healing is ambiguous. Several studies have examined Slug expression in skin injury models to provide evidence for an EMT-like process in cutaneous wound healing (95, 96). Savagner et al. analyzed the expression of Snai2 during the wound healing by in situ hybridization. Snai2 expression was elevated in keratinocytes bordering cutaneous wounds in mice in vivo, in keratinocytes migrating from mouse skin explants ex vivo, and in human keratinocytes at wound margins in vitro. Overexpression of Snai2 in keratinocytes caused increased cell spreading and desmosomal disruption in vivo and accelerated in vitro wound healing, suggesting a critical role for Snai2 in epithelial keratinocyte migration and wound healing. Furthermore, in Slug-null mice, a 1.7-fold decrease in re-epithelialization was seen in an excisional wound model. These studies indicate that an EMT-like process might occur in the re-epithelialization process of wound healing.

To date, several studies supporting EMT during wound healing have focused on re-epithelialization through keratinocyte migration. On the other hand, studies demonstrating EMT trans-differentiation from keratinocytes into myofibroblasts during wound healing are rare. Yan et al. characterized the EMT-like features in acute and fibrotic wounds in human skin (89). In tissue staining, they rarely noticed the co-localization of both E-cadherin and vimentin/fibroblast-specific protein 1 (FSP-1) cells in epithelial layers. This is understandable because gain of mesenchymal and loss of

epithelial markers occurs simultaneously, leaving very little window to trap the transition process. However, such dynamic transition has been recapitulated in vitro. With co-localization stain method they did not observe trans-differentiation of human keratinocytes into myofibroblasts. Trans-differentiation is ideally demonstrated by lineage tracing using transgenic mice in which epithelial cells have been “permanently” marked.

Among the major proteins exclusive to epithelial tissues are keratins, a family of >20 proteins whose members are differentially expressed in a tissue-, differentiation-, and developmental- specific fashion (97). Based on sequence homologies, these proteins can be subdivided into two distinct groups: type I keratins are smaller (40-56.5 kDa) and acidic (pKi = 4.5-5.5), and type II keratins are larger (53-67 kDa) and more basic (pKi = 5.5-7.5) (98). Type I and type II keratins are expressed as specific pairs. Keratin 14 (K14) and its partner K5 are the major proteins expressed by the mitotically active cells of the epidermis and its appendages (99, 100) and the genes encoding these keratins are abundantly transcribed in cultured human keratinocytes (101). For these reasons, the K14 and K5 promoters are especially attractive candidates for use in studies of keratinocytes. For our study we utilized the Cre-loxP recombination system for the introduction of a spatiotemporal gene ablation with human keratin 14 (K14) promoter. The system generally requires cross mating of two lines of genetically manipulated mice (Fig. 9). One line of mice carries alleles with a gene of interest flanked by two identically orientated loxP sites. The other line contains a Cre transgene in which the expression of Cre is controlled by a defined promoter. Recombination between the two loxP sites in the mated mice results in the catalysis of a deletion of the region flanked by the loxP sites; this is dependent on Cre transgene expression.

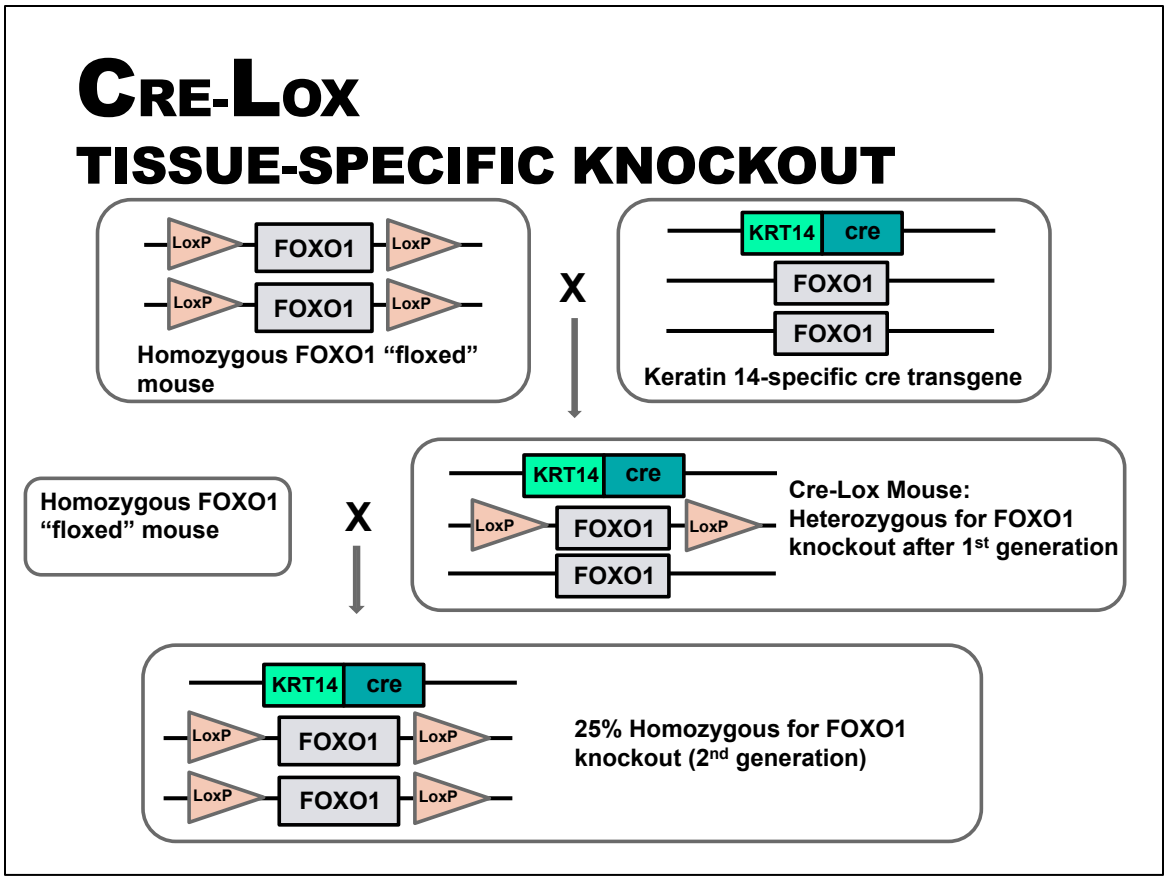


Figure 9. Cre-loxP recombination system

To study EMT we examined transgenic mice expressing Cre and a fusion protein between Cre recombinase and the tamoxifen responsive hormone-binding domain of the estrogen receptor (CreER). Expression of the fusion protein was under the control of the human keratin 14 (K14) promoter. The CreER recombinases are inactive, but can be activated by the synthetic estrogen receptor ligand 4-hydroxytamoxifen (OHT), therefore allowing for external temporal control of Cre activity. By combining tissue-specific expression of a CreER recombinase with its tamoxifen-dependent activity, the excision of floxed chromosomal DNA can be controlled in a time- and tissue-specific manner. In

order to label the fluorescence to keratinocyte, we crossed K14CreER transgenic mice with ROSA26 Cre reporter mouse (B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J). TdTomato is expressed following Cre-mediated recombination with tamoxifen administration. To our knowledge, this model is the first to assess the role of EMT as the mechanism of trans-differentiation of keratinocytes into typical myofibroblasts during wound healing.

1.3 FOXO1, Wound healing and Angiogenesis

The forkhead boxO (FOXO) transcription factors represent a subclass of a large family of forkhead proteins characterized by the presence of a winged-helix DNA binding domain called Forkhead box (102). In mammals this subclass comprises four members: FOXO1 (or FKHR), FOXO3 (or FKHRL1), FOXO4 (also called AFX) and FOXO6 (103). The four mammalian isoforms may have both distinct and overlapping functions and compensation of one member by another may mask the function of individual FOXOs. FOXO1, 3 and 4 are ubiquitously expressed and relatively abundant in bone and bone cells. FOXO6 expression is confined to the brain (104). FOXOs are master signaling integrators that translate environmental stimuli, like hormonal changes, inflammation, and oxidative stress, into dynamic gene expression programs involved in many physiological and pathological processes (Fig. 10). Among the many biological functions they serve, FOXO proteins are known best for three defining properties: survival, by means of resistance to oxidative stress; glucose metabolism, a property specific to FOXO1; and suppression of tumorigenesis, a property also mainly regulated by FOXO1 (105). Balancing the multiple avenues of FOXO activity are several opposing signaling pathways that regulate their localization to the nucleus, where they are active (106-108). Among them FOXO1 is particularly noteworthy in that it regulates the most diverse array of the FOXO's known biological activities, including organ growth, insulin action, tumorigenesis, and angiogenesis. It is a main target of insulin signaling and regulates metabolic homeostasis and organismal survival at many different levels.

FOXO proteins are deactivated by the insulin/PI3K/Akt signaling pathway (2). A major form of regulation is Akt-mediated phosphorylation of FOXO in response to insulin or growth factors. Phosphorylation at three conserved residues results in the export of

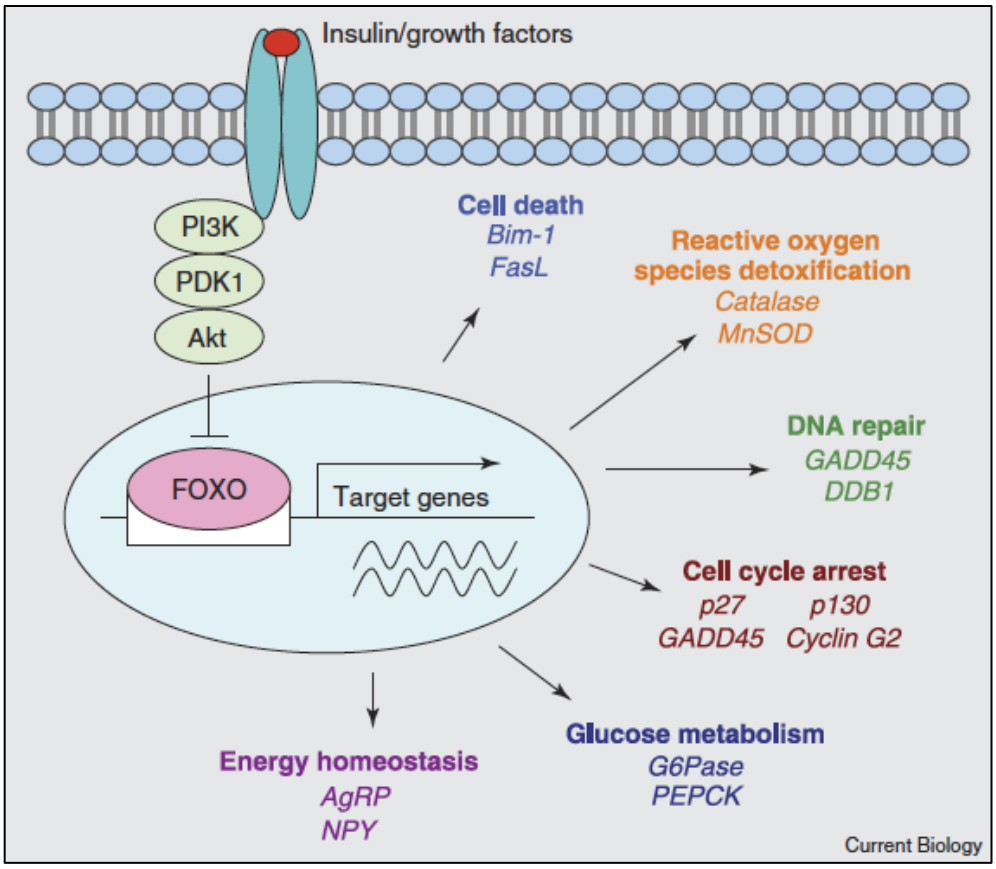


Figure 10. In the absence of insulin or growth factors, FOXO transcription factors are located in the nucleus, where they specify target gene expression (2).

FOXO factors from the nucleus to the cytoplasm, thereby inhibiting FOXO-dependent transcription. FOXO proteins are also phosphorylated by other protein kinases, including JNK or the mammalian ortholog of the St20-like protein kinase (Mst1), which phosphorylate FOXO under conditions of oxidative stress. This phosphorylation leads to translocation of FOXO into the nucleus, thus opposing Akt's action. In addition to being post-translationally modified by phosphorylation, FOXO proteins also bind to co-activator or co-repressor complexes and become acetylated or deacetylated.

Recent evidences indicate that FOXO1 plays a critical role in wound healing. Mori et al. performed immunohistochemistry (IHC) analysis to determine which cells express

FOXO1 protein during skin wound healing (109). By only 1 day after injury, FOXO1 was markedly present in the leading edge and basal layer of keratinocytes and hair follicles and in recruited neutrophils. Seven days after injury FOXO1 was present in macrophages, fibroblasts and endothelial cells at the wound site. Ponugoti et al. demonstrated that FOXO1 promotes wound healing through the up-regulation of TGF-β1 and prevention of oxidative stress in normal wounds (110). Lineage-specific FOXO1 deletion in keratinocytes interfered with wound healing and keratinocyte migration in normal skin and mucosal wounds (110-113). Surprisingly, the same deletion of FOXO1 in diabetic wounds had the opposite effect, significantly improving the healing response (111-113). In high glucose, FOXO1 enhanced expression of serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2), and chemokine (C-C motif) ligand 20 (CCL20). The impact of high glucose on keratinocyte migration was rescued by silencing FOXO1, by reducing SERPINB2 or CCL20, or by insulin treatment (111). Thus, FOXO1 expression can positively or negatively modulate keratinocyte migration and wound healing by its differential effect on downstream targets modulated by factors present in diabetic but not normoglycemic healing. In addition FOXO1 expression in vascular cells is necessary for vascular cell development as well as for the biological response to cellular mediators. Mice homozygous for a $Foxo1^{-/-}$ allele, but not $Foxo3a^{-/-}$ or $Foxo4^{-/-}$ mice, die during embryogenesis from defects in vascular development (16, 17). Endothelial cell colonies in Foxo1-deficient mice fail to respond to vascular endothelial growth factor in a manner similar to wild-type endothelial cells (114). Although these studies suggest an essential role of FOXO1 in the formation and maturation of the nascent vasculature, relatively little is known about the function and significance of the distinct FOXO family members for the angiogenic activity of endothelial cells and postnatal vessel formation (115). In addition our microarray results show that those

genes selectively decreased by FOXO1 knockdown in normal human epidermal keratinocytes (NHEK) are most strongly associated with angiogenesis, suggesting that FOXO1 activation in keratinocytes may have a strong relationship with wound angiogenesis (Table 3).

Table 3. Genes down-regulated by FOXO1 knockdown and related to angiogenesis

Gene name	Fold change by FOXO1 knockdown
Interleukin 18	-1.91197
Vascular endothelial growth factor C	-1.82767
Angiopoietin-like 4	-1.63326
Vascular endothelial growth factor A	-1.61825
Cysteine-rich, angiogenic inducer, 61	-1.53097
Connective tissue growth factor	-1.49486
Hypoxia inducible factor 1, alpha subunit	-1.38889
Fibroblast growth factor 2 (basic)	-1.35659

1.4 Hypothesis

Diabetes is a significant health concern in the United States, where 25.8 million people (8.3% of the population) have diabetes. Impaired wound healing is a major complication of diabetes. Factors that play a decisive role in diabetes-associated wound healing disorders comprise augmented inflammatory responses, impaired granulation tissue formation, reduced growth factors and a disturbed angiogenesis.

Wound angiogenesis is pivotal to wound healing and results from multiple signals acting on endothelial cells (ECs). VEGF is one of the most potent angiogenic mediators, with keratinocytes and macrophages being the major producers. As another possible factor that may affect connective tissue healing, epithelial-mesenchymal transition (EMT) is recognized as a process that contributes to embryogenesis, tissue remodeling, fibrosis, and metastatic malignancies. It is an orchestrated series of events during which epithelial cells lose many of their epithelial specific characteristics and acquire features typical of mesenchymal cells. Although this concept has been mentioned sporadically in the literature as an important aspect of wound healing, there is no in vivo evidence that EMT plays a direct role in oral repair processes.

Forkhead box O1 (FOXO1), which belongs to a large family of forkhead transcription factors, participates in a wide range of cellular processes, including cell cycle arrest, DNA repair, apoptosis, oxidative stress resistance, angiogenesis and glucose metabolism. Recent studies from our lab showed that lineage-specific FOXO1 deletion in keratinocytes interfered with re-epithelialization in normal skin and mucosal wounds. Surprisingly, the same deletion of FOXO1 in diabetic wounds had the opposite effect, significantly improving re-epithelialization. As keratinocytes are a major source of growth

factors such as VEGF, FOXO1 activity in keratinocytes may affect connective tissue healing, but this important question has not been previously investigated in vivo.

Based on these findings, my central hypothesis is that FOXO1 deletion in keratinocytes significantly affects both normal and diabetic gingival connective tissue healing and is associated with regulation of mediators that stimulate angiogenesis and EMT. The goals of this study are: 1) to evaluate the effect of lineage specific FOXO1 deletion in keratinocytes during both normal and diabetic gingival connective tissue wound healing; 2) to determine whether FOXO1 organizes keratinocyte activity to regulate angiogenesis during both normal and diabetic gingival connective tissue wound healing; 3) to evaluate the effect of lineage specific FOXO1 deletion in keratinocytes on epithelial-mesenchymal transition (EMT) during both normal and diabetic gingival connective tissue wound healing.

CHAPTER 2

MATERIALS AND METHODS

Animals and induction of diabetes

Animal experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Mice with floxed Foxo1 were provided by R.A. DePinho (MD Anderson Cancer Center, Houston, TX) as previously described (116). Mice expressing Cre recombinase under the control of keratin 14 promoter (K14-Cre; strain Tg(KRT14-cre)1Amc/J) were obtained from the Jackson Laboratory. Lineage-specific Foxo1 deletion was obtained by crossing these mice to generate experimental (K14.Cre^{+}.Foxo1$^{L/L}$) and control (K14.Cre^{-}.Foxo1$^{L/L}$) mice (Fig. 11). Two to five mice were housed per cage under standard conditions with a 14-h light/10-h dark cycle. All the experiments were performed with adult mice 10–20 weeks old. Type 1 diabetes was induced by multiple low dose i.p. injections of streptozotocin (50 mg/kg; Sigma-Aldrich) in 10 mM citrate buffer daily for 5 d. Control mice were treated identically with vehicle alone. The blood glucose levels were monitored after completion of multiple low dose streptozotocin or citrate buffer injections. Mice were considered to be hyperglycemic when serum glucose levels were >220 mg/dl. Experiments were performed when mice had been hyperglycemic for at least 10 days.

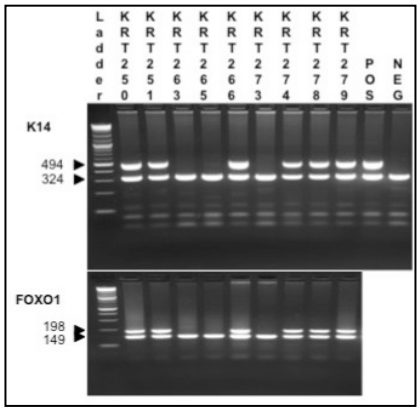


Figure 11. Genotype results We tested with K14 specific primer instead of generic Cre primer.

For the EMT study we purchased transgenic mice having a tamoxifen inducible Cre-mediated recombination system driven by the human keratin 14 (KRT14) promoter (K14CreER; strain Tg(KRT14cre/ER)20Efu/J) from the Jackson Laboratory. This promoter is strongly active in dividing cells of epidermis and other stratified squamous epithelia. In order to characterize the recombination properties of a Cre transgenic mouse line, we crossed K14CreER transgenic mice with ROSA26 Cre reporter mouse (B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J). To induce Cre activity, we administered tamoxifen (5 mg per mouse per day) orally to the K14CreER-ROSA26 mice for 5 consecutive days as described (117). Oral wound surgery was performed 1 week after the last administration (118).

Gingival wounding experiment

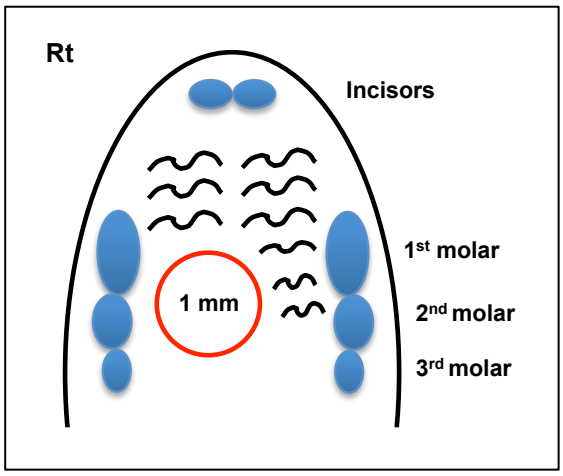


Figure 12. Gingival wound making 1 mm full-thickness wound was made in the palatal gingiva near the right first molar.

Mice were anaesthetized by i.p. administration of ketamine (80 mg/kg), xylazine (5 mg/kg) and acepromazine (1 mg/kg). Full-thickness wounds were made in the palatal gingiva near the right first molar by means of a 1-mm biopsy punch (Accu-Punch; Electron Microscopy Sciences, USA) as described previously (119) (Fig. 12). A 1 mm wound size is commonly used in oral wound studies with mice. Larger oral wounds may jeopardize the animal's wellbeing because of difficulty of eating. Wounds were assessed for both 4 and

7 days post-wounding (n = 7-9 for each group). For The EMT study we examined wounds after 4, 7 and 14 days (n = 2-4 for each group).

Histology

Specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h and decalcified in 10% EDTA solution. Wounds were bisected in caudocranial direction and embedded in paraffin. 4-μm paraffin sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome and histomorphometric analysis was performed with NIS-elements D image analysis software at the center of each lesion. Masson's trichrome stain produces red keratin and muscle fibers, blue or green collagen and bone, light red or pink cytoplasm, and dark brown to black cell nuclei. Mature collagen fibers take deep blue color and the new collagen fibers stain light blue. For quantification of newly formed collagen in Masson's trichrome-stained slides images were taken with a 20x objective from the middle and/or the two edges of the wound. Using NIS-elements software (Nikon), the intensity RGB threshold was set for the blue–green positive color of collagen and the area of positive pixels was counted. For the EMT study palatal gingival tissues were harvested and embedded in OCT medium (Sakura Finetek USA, Inc., Torrance, CA, USA), immediately frozen on dry ice, and sectioned at 10-μm thickness. Samples were stained with DAPI and assessed by fluorescence microscopy.

Immunohistochemistry in histological sections

Paraffin-embedded, formalin-fixed sample sections were processed for immunofluorescence analyses. Antigen retrieval was performed in 10 mM of citric acid, pH 6.0, at 120°C except for VEGFa, for which Proteinase K was used at room temperature for 30 minutes. Sections were incubated with primary antibody to FOXO1 (rabbit; Santa Cruz Biotechnology, Inc.), CD31 (rabbit; Abcam), α-SMA (rabbit; Abcam), VEGFa (rabbit; Abcam) and Ki67 (rat; eBioscience) overnight at 4°C as well as the appropriate isotype-matched negative control IgG. Biotinylated secondary antibody (Vector Laboratories) and ABC reagent (Vector Laboratories) were then used. Tyramide signal amplification (Adipogen) was also used to enhance the chromogenic signal. Finally, Alexa Fluor 546–conjugated streptavidin (Invitrogen) and/or fluorescein avidin (Vector Laboratories) and DAPI-containing mounting media were used to visualize the staining (Sigma-Aldrich). In order to rule out the pericytes, we excluded α-SMA positive cells if they are morphologically associated with blood vessels. This was assisted by use of immunofluorescence with antibody to CD31 in adjacent slides to detect blood vessels.

In order to further assess how keratinocytes through FOXO1 regulate connective tissue healing we analyzed fibroblasts/endothelial cell proliferation with an antibody to Ki67 and apoptosis with TUNEL assay with/without an antibody to CD31. Fibroblasts were identified by their typical spindle-shaped appearance. Apoptosis was quantified by TUNEL assay and samples were incubated at 37°C for an hour with fluorescein-12-dUTP (Thermo Fisher Scientific) catalytically incorporated by recombinant terminal deoxynucleotidyl transferase (Promega). Negative controls were similarly treated with the omission of rTDT.

Images were taken at 4x, 20x, and 40x magnification with a fluorescence microscope (ECLIPSE 90i; Nikon) with the same exposure time for experimental and negative control groups. Image analysis was performed using NIS Elements AR image analysis

software. The number of immunopositive cells divided by the area or number of DAPI-positive cells was used to measure the percentage of positive cells for each measured antibody. Mean fluorescence intensity was measured by NIS Elements AR image analysis software, with a maximum fluorescence intensity set at 3,000 arbitrary units to obtain results in the linear response range. For the EMT study we washed the samples with PBS and mounted with DAPI. Images were taken as previously described.

Cell culture and transfection

Human immortalized gingival keratinocytes (HIGK) cells were provided by Jeffrey J. Mans (University of Florida, Gainesville, FL), immortalized with human papilloma virus type 16, the E6/E7 gene and maintained in KGM-2 growth medium supplemented with human keratinocyte growth supplements (Lonza). Primary mouse epidermal keratinocytes were isolated from the neonates (0–2d) of experimental ($K14.Cre^{+}.Foxo1^{L/L}$) and control ($K14.Cre^{-}.Foxo1^{L/L}$) mice. In brief, mouse skin was collected and digested with 2.5 U/ml Dispase II (Roche) overnight at 4°C. The dermis was then separated from the epidermis by digesting with 0.1% trypsin and 0.02% EDTA in PBS for 15 min at 37°C. Keratinocytes from the epidermis were cultured in KGM-2 growth medium containing antibiotics. All cell cultures were maintained in a 5% CO_2 humidified incubator at 37°C. Keratinocytes were passaged in KGM-2 growth media with supplements including standard insulin (8.6×10^{-7} M). For assays, cells were transferred to KGM-2 media with supplements except the insulin. ON-TARGETplus SMARTpool siRNAs against human FOXO1 and control siRNA (ON-TARGETplus Non-targeting Control Pool) were obtained from GE Healthcare and transfection was performed using GenMute siRNA Transfection Reagent (SignaGen Laboratories). In most transfection

experiments, cells were incubated for 6 h with siRNA and transfection reagent 2 d before assay. Cells were then rinsed and transferred back to the indicated culture media for the remaining incubation period.

Immunofluorescence analysis in vitro

Primary keratinocytes isolated from K14.Cre^{+}.Foxo1$^{L/L}$ and K14.Cre^{-}.Foxo1$^{L/L}$ mice and HIGK cells were grown on 96-well plates. HIGK cells were transfected with FOXO1 or scrambled siRNA. After 24 hour incubation in KBM-2 media with/without supplements except insulin, both primary keratinocytes and HIGK cells were fixed with 10% formalin, permeabilized with 0.5% Triton X-100 and incubated overnight at 4^{o}C with primary antibodies to FOXO1 (rabbit; Santa Cruz Biotechnology, Inc.), VEGFa (rabbit; Santa Cruz Biotechnology Inc.) or negative control IgG. Primary antibody was localized with biotinylated secondary antibody and visualized with streptavidin-conjugated Alexa Fluor 546. Avidin-biotin peroxidase enzyme complex (Vector Laboratories) and tyramide signal amplification (Adipogen, San Diego, CA, USA) were used to enhance the fluorescent signal. Nuclei were stained with DAPI. Images were captured with a fluorescence microscope (Nikon) and the mean fluorescence intensity (MFI) was determined using NIS Elements AR software (Nikon).

Luciferase reporter assay

Transient transfection with luciferase reporter constructs was performed using Lipofectamine3000 (Invitrogen) in 48-well plates. In brief, HIGK cells were incubated in culture media with/without insulin for 5 d with 5 mM d-glucose. Cells were cotransfected

with VEGFa luciferase reporter (provided by Dr. Keping Xie, University of Texas M. D. Anderson Cancer Center, Houston, TX) (120) together with pRL-TK luciferase control vector, FOXO1-AAA plasmid that is constitutively transported to the nucleus, pcDNA3.1 control plasmid, scrambled or FOXO1 siRNA. 2 d after transfection, cells were lysed, and Firefly and Renilla luciferase activities were measured using Dual Luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions. Firefly luciferase activities were divided by Renilla activities to normalize for transfection efficiency. Experiments were performed three times with similar results. In some experiments, primary murine keratinocytes from K14.Cre^{-}.FOXO1$^{L/L}$ mice in low (5 mM d-glucose) glucose medium were cotransfected with VEGFa luciferase reporter together with pRL-TK luciferase control vector, FOXO1, FOXO1-AAA plasmid and pcDNA3.1 control plasmid. 2 days after transfection, cells were lysed and tested with the Dual Luciferase Reporter Assay (Promega).

Microarray analysis

Normal human epidermal keratinocytes (NHEK) cells were incubated in low (5 mM d-glucose) or high (25 mM d-glucose) glucose medium for 5 days and transfected with FOXO1 or scrambled siRNA. Total RNA was then isolated using an RNeasy kit (QIAGEN). RNA profiling was performed using a GeneChIP Human Gene 1.0 ST array (Affymetrix). The identification of genes that were up- or down-regulated by high glucose and FOXO1 knockdown was defined as those that were both up-regulated (>1.3-fold) by high glucose and down-regulated (<0.7-fold) by FOXO1 siRNA with $P < 0.05$, both when compared with the matched control group.

Statistics

Statistical analysis between Cre^+ and Cre^- groups was performed using 2-tailed Student's t test. In experiments with multiple time points or treatments, differences between the wild type and experimental groups were determined by ANOVA with Scheffe's post-hoc test. Results were expressed as the mean ± SEM. $P < 0.05$ was considered statistically significant.

CHAPTER 3

RESULTS

3.1 To evaluate the effect of lineage specific FOXO1 deletion in keratinocytes during both normal and diabetic gingival connective tissue wound healing

3.1.1 Keratinocyte-specific FOXO1 deletion impairs connective tissue healing in normoglycemic mice while showing no significant difference in diabetic mice

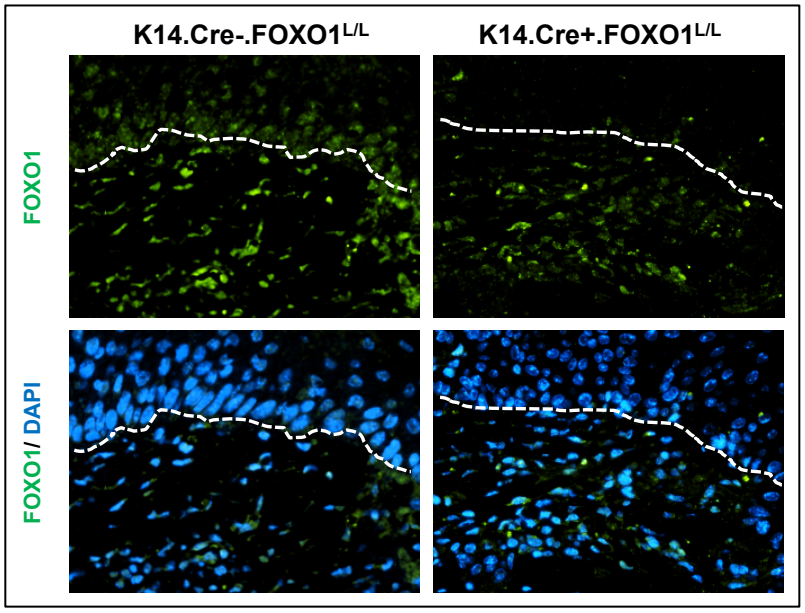


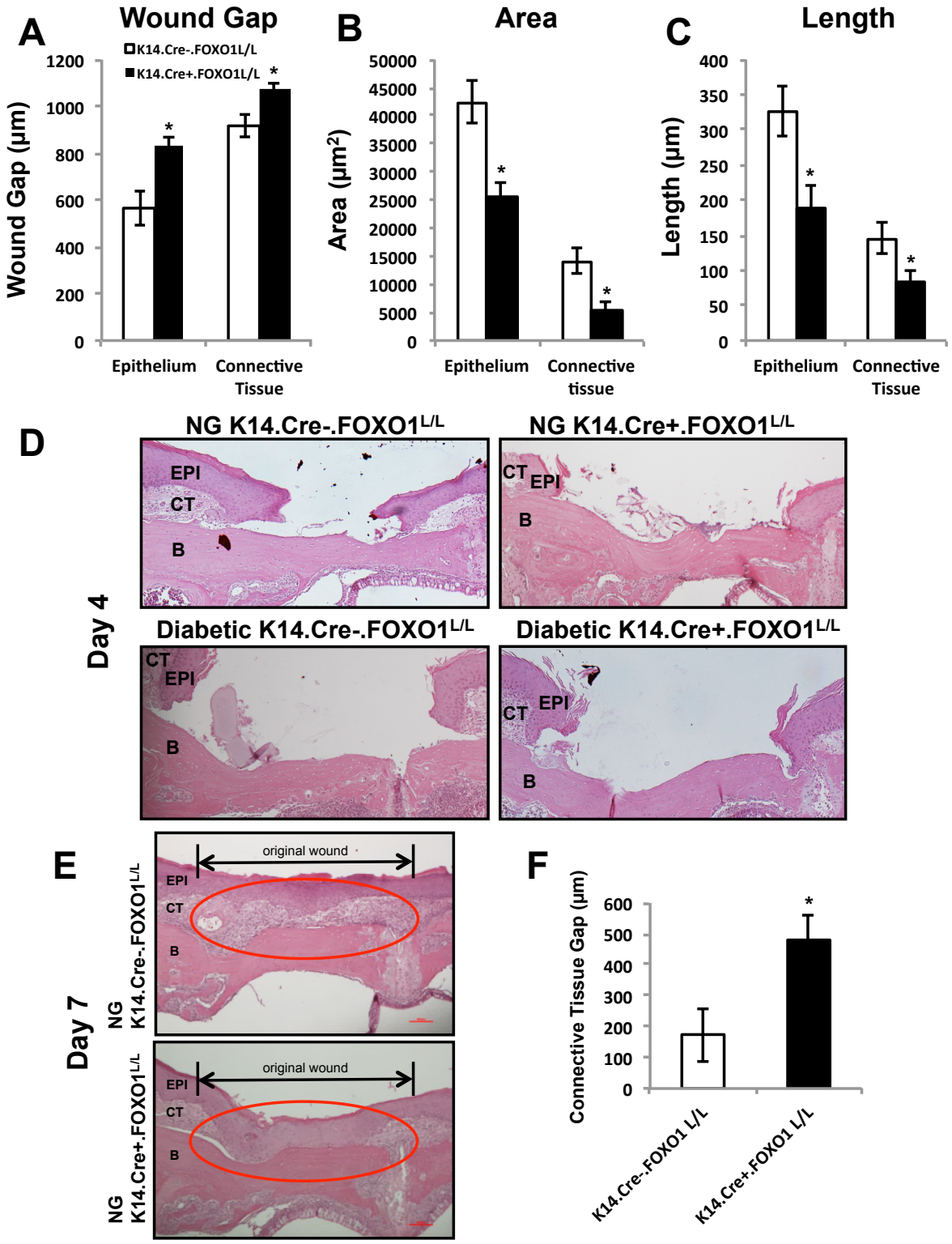
Figure 13. FOXO1 Immunofluorescence
demonstrating lineage-specific Foxo1 deletion in keratinocytes

1-mm full-thickness wounds were created in the palatal gingiva near the right first molar in experimental transgenic mice (K14.Cre^{+}.Foxo1$^{L/L}$) with keratinocyte-specific deletion of Foxo1 driven by keratin-14 Cre recombinase and littermate control (K14.Cre^{-}.Foxo1$^{L/L}$) mice without Foxo1 deletion. We confirmed that Foxo1 expression was decreased by Cre recombinase in the epithelium compared with matched control mice, consistent with our previous results (113). In contrast, there was little difference

observed in the connective tissue, demonstrating lineage-specific deletion in keratinocytes (Fig. 13).

Deletion of Foxo1 in keratinocytes of nondiabetic mice delayed connective tissue healing (day 4, Fig. 14 A-D). The gap between the edges of connective tissue was increased by keratinocyte-specific FOXO1 deletion in both day 4 and 7 nondiabetic wounds ($P<0.05$). Even though both normoglycemic experimental and control mice showed the complete re-epithelialization on day 7, there was the incomplete bridging of the wound by connective tissue in experimental K14.Cre^{+}.Foxo1$^{L/L}$ mice (Fig. 14 E and F, H & E). Both granulation tissue formation and collagen production in the normoglycemic wounds of experimental K14.Cre^{+}.Foxo1$^{L/L}$ mice was reduced, but diabetic mice showed the similar results between experimental and control mice on both day 4 and day 7 wounds (Fig. 15 A-D, Masson's trichrome stain). Thus, keratinocyte-specific Foxo1 deletion in normoglycemic mice has a pronounced effect on healing of connective tissue.

Figure 14. Keratinocyte-specific deletion of FOXO1 impairs gingival connective tissue healing responses in normoglycemic wounds. (A-C) Quantification of normoglycemic wound healing in H&E stained sections (day 4) including (A) wound gap, (B) area and (C) length. (D) Hematoxylin and eosin (H & E) staining of both normal and diabetic K14.Cre^{-}.Foxo1$^{L/L}$ and K14.Cre^{+}.Foxo1$^{L/L}$ mice wound biopsies on day 4 after wounding (10x). (E) Normoglycemic K14.Cre^{-}.Foxo1$^{L/L}$ and K14.Cre^{+}.Foxo1$^{L/L}$ mice wound biopsies on day 7. Red circles demonstrate the difference of connective tissue healing between the experimental and control mice. There was the incomplete bridging of the wound by connective tissue in experimental K14.Cre^{+}.Foxo1$^{L/L}$ mice (10x). (F) Connective tissue gap on day 7. EPI, epithelium; CT, connective tissue; B, bone. Each *in vivo* value is the mean ± SEM for n=7-9 mice per group. *, $P<0.05$ versus Cre^{-} group; #, $P<0.05$ versus matched normoglycemic control group.


A
Wound Gap
K14.Cre-.FOXO1L/L
K14.Cre+.FOXO1L/L
Wound Gap (µm)
1200
1000
800
600
400
200
0
Epithelium
Connective Tissue
B
Area
Area (µm²)
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Epithelium
Connective tissue
C
Length
Length (µm)
400
350
300
250
200
150
100
50
0
Epithelium
Connective Tissue
D
NG K14.Cre-.FOXO1L/L
NG K14.Cre+.FOXO1L/L
Diabetic K14.Cre-.FOXO1L/L
Diabetic K14.Cre+.FOXO1L/L
Day 4
EPI
CT
B
E
Day 7
NG K14.Cre-.FOXO1L/L
NG K14.Cre+.FOXO1L/L
original wound
F
Connective Tissue Gap (µm)
600
500
400
300
200
100
0
K14.Cre-.FOXO1 L/L
K14.Cre+.FOXO1 L/L

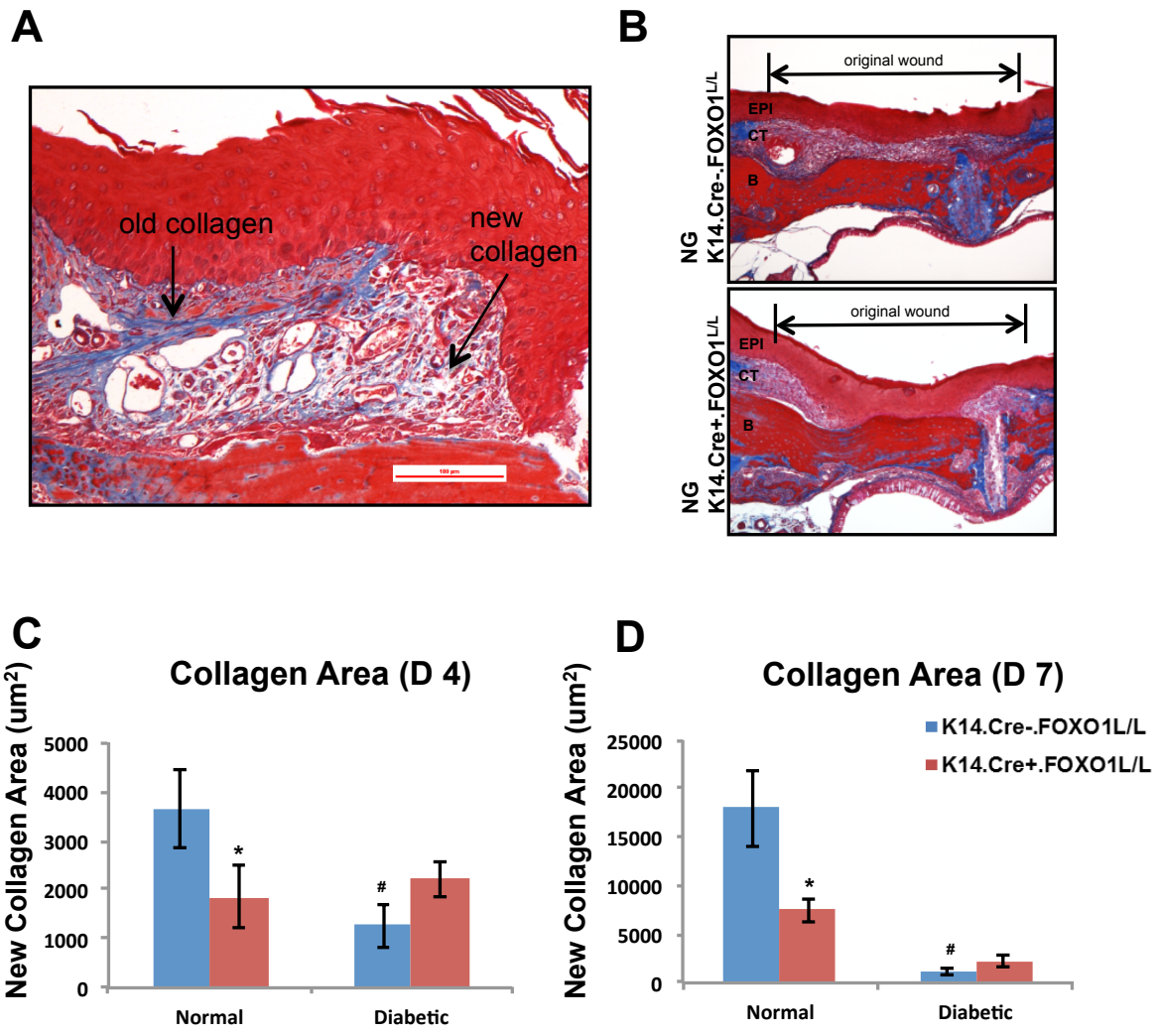


Figure 15. Keratinocyte-specific deletion of FOXO1 decreases collagen production in normoglycemic wounds. (A) Old collagen fibers take deep blue color and the new collagen fibers stain light blue, representing mature or early collagen (20x). (B) Representative images of Masson's trichrome staining (10x). (C and D) Newly formed collagen on both day 4 and 7 was measured in Masson's trichrome stained sections. Each *in vivo* value is the mean ± SEM for n=7-9 mice per group. *, P<0.05 versus Cre$^-$ group; #, P<0.05 versus matched normoglycemic control group.

3.1.2 Keratinocyte-specific FOXO1 deletion in normal wounds results in decreased fibroblast proliferation and increased apoptosis in vivo

The effect of keratinocyte-specific Foxo1 deletion on fibroblast numbers was assessed *in vivo*. Fibroblast density in connective tissue was reduced by 56% in normoglycemic wounds (day 4, $P<0.05$), but was similar with or without keratinocyte-specific FOXO1 deletion in diabetic wounds (Fig. 16 A-C). Myofibroblasts are crucial for the wound contraction and maturation. The number of myofibroblasts was reduced 57% by FOXO1 deletion in epithelium in normal wounds ($P<0.05$) but increased by 82% (Day 4) and 28% (Day 7) in diabetic wounds ($P<0.05$) (Fig. 17 A-D). The differentiation of fibroblasts to myofibroblasts is a key event in connective tissue wound healing (121). When tissues are injured, fibroblasts are activated and differentiate into myofibroblasts, which contract and participate in healing by reducing the size of wound and secreting extracellular matrix (ECM) proteins. We next investigated the effect of keratinocyte-specific Foxo1 deletion on fibroblast proliferation and apoptosis in vivo. The percentage of proliferating fibroblasts was measured by the number of Ki67–positive fibroblasts divided by total fibroblast number. Ki-67 is used as a marker for determining the growth fraction of a given population of cells. Ki-67 is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). During interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The percentage of proliferating fibroblasts was reduced by 68% (day 4 normal) in K14.Cre^{+}.Foxo1$^{L/L}$ mice compared to littermate control mice ($P<0.05$), indicating impaired fibroblast proliferation with epithelial-specific FOXO1 deletion (Fig. 18 A and B). In diabetic wounds the percentage of proliferating fibroblasts was similar on day 4, but showed a 2.7 fold

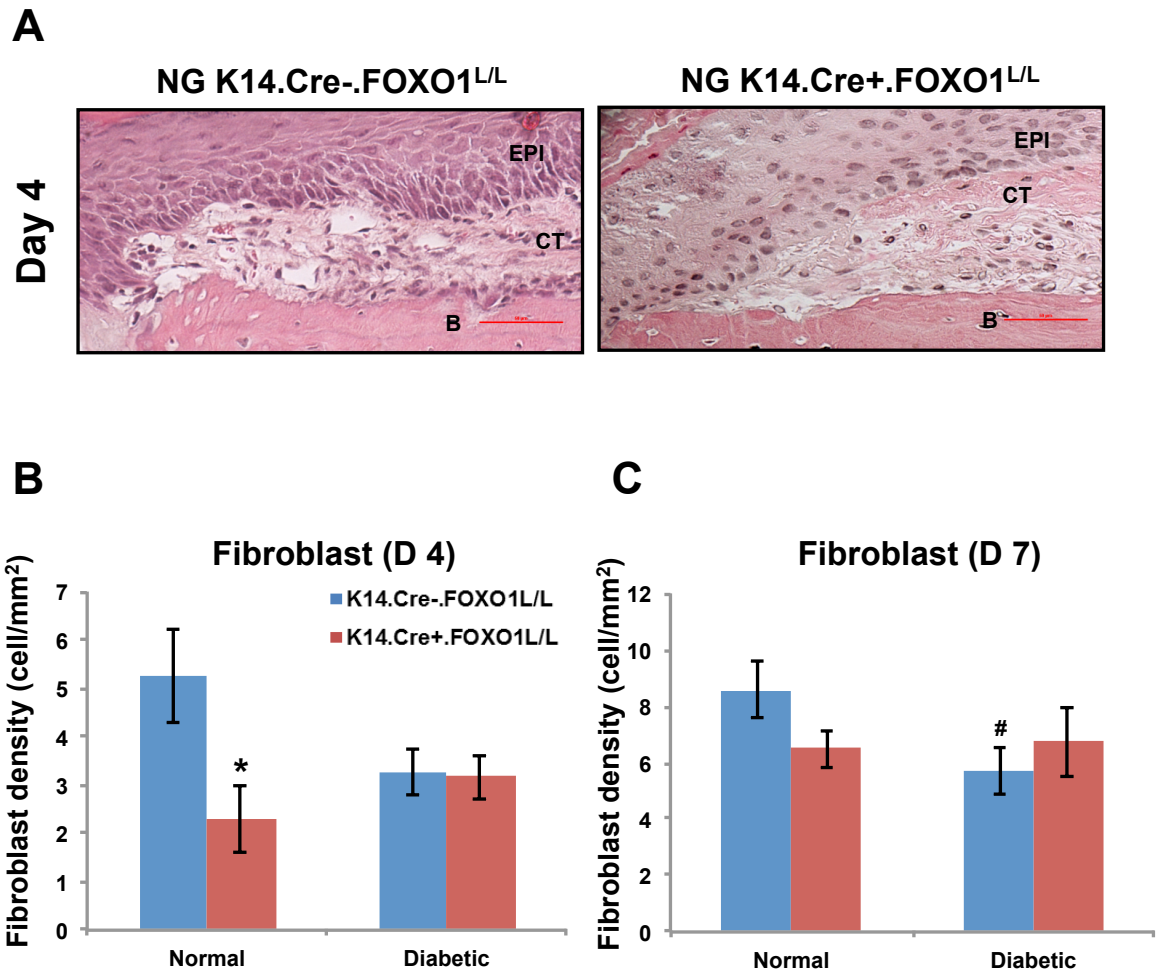


Figure 16. Keratinocyte-specific deletion of FOXO1 decreases fibroblast density in normoglycemic wounds. (A) Representative images of Hematoxylin and eosin (H & E) staining of K14.Cre^{-}.Foxo1$^{L/L}$ and K14.Cre^{+}.Foxo1$^{L/L}$ mice wound biopsies on day 4 after wounding (40x). Fibroblasts were identified by their typical spindle-shaped appearance. (B and C) Fibroblast density in newly formed connective tissue was measured in H & E stained histologic sections. Each *in vivo* value is the mean ± SEM for n=7-9 mice per group. *, P<0.05 versus Cre^{-} group; #, P<0.05 versus matched normoglycemic control group.

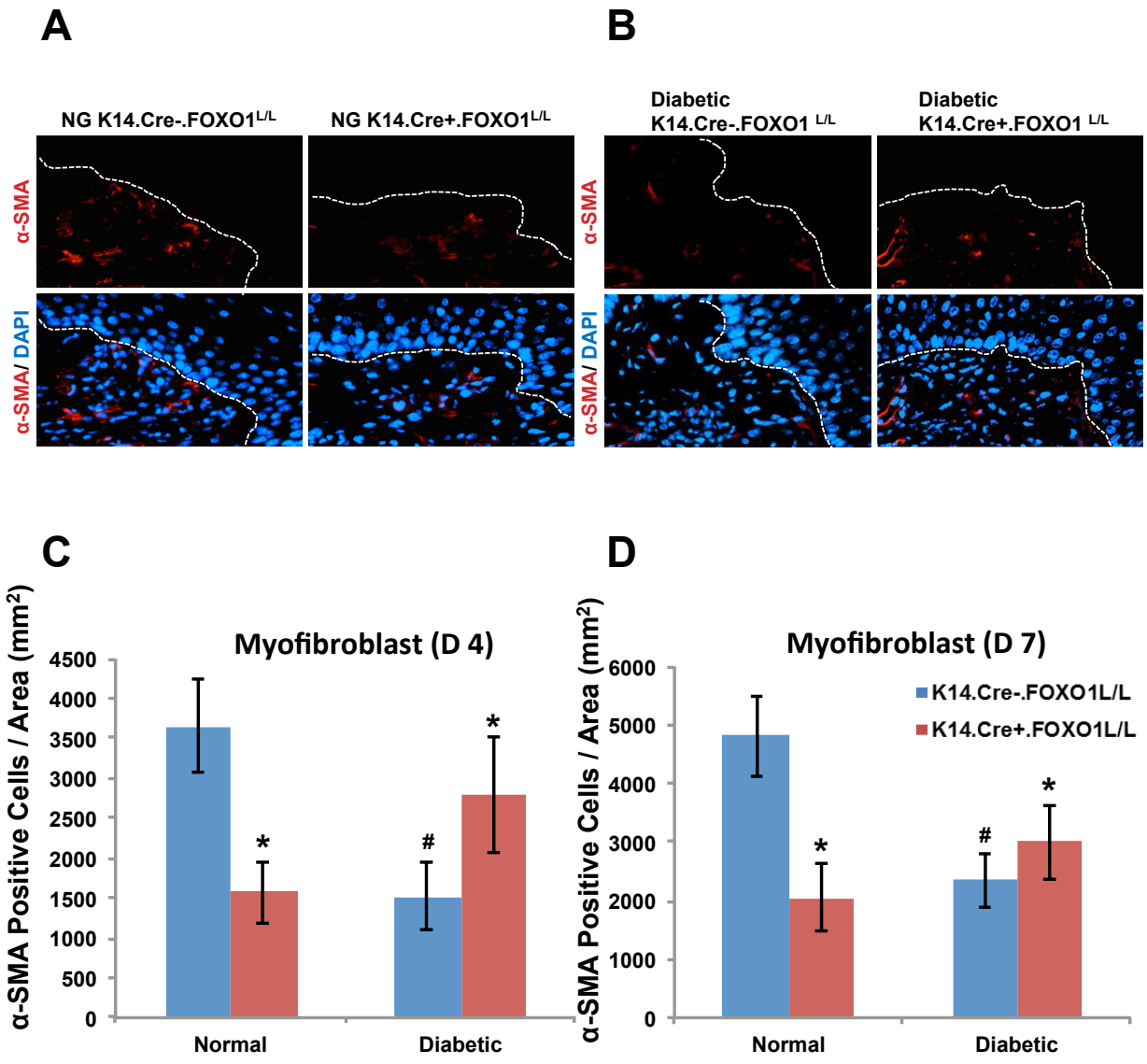
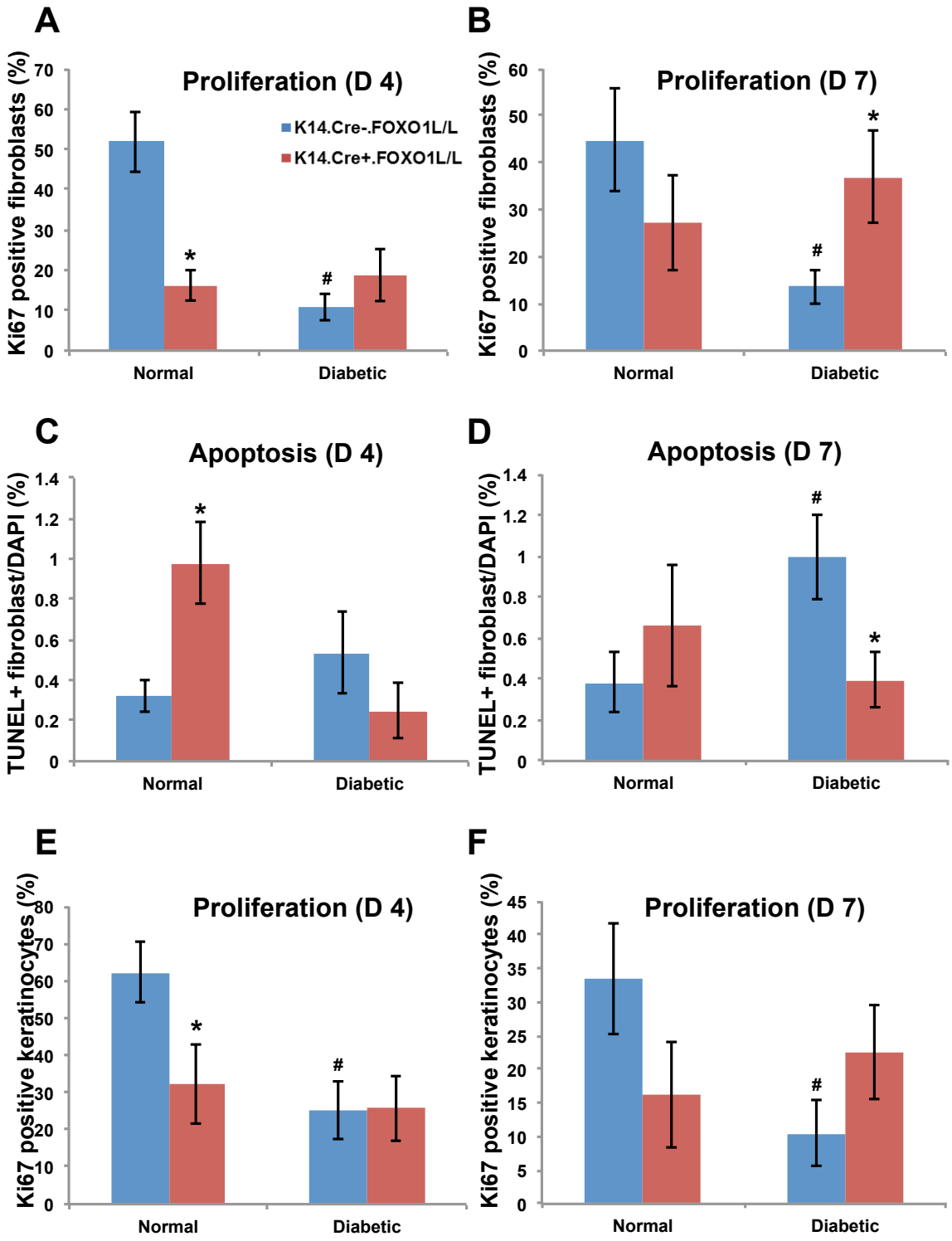


Figure 17. Keratinocyte-specific deletion of FOXO1 decreases the number of myofibroblasts in normoglycemic wounds but increases in diabetic wounds. (A and B) Representative images of α-SMA immunofluorescence (40x, day 4, normoglycemic and diabetic mice) (C and D) α-SMA immunofluorescence analyses of myofibroblasts in newly formed connective tissue. Each *in vivo* value is the mean ± SEM for n=7-9 mice per group. *, P<0.05 versus Cre^{-} group; #, P<0.05 versus matched normoglycemic control group.

increase in K14.Cre^{+}.Foxo1$^{L/L}$ mice on day 7 ($P<0.05$). The percentage of apoptotic fibroblasts increased by 3-fold in Foxo1-deficient normoglycemic wounds compared with control mice ($P<0.05$) on day 4 but decreased by 60% in FOXO1-deficient diabetic wounds on day 7 ($P<0.05$) (Fig. 18 C and D). In addition, we observed a 53% decrease in keratinocyte proliferation in the normoglycemic wound epithelium of Foxo1-deleted mice (day 4, $P<0.05$) (Fig. 18 E and F).

Figure 18. Keratinocyte-specific FOXO1 deletion in normal wounds reduces fibroblast proliferation but enhances apoptosis. (A and B) Ki67 immunofluorescence analyses of proliferating fibroblasts in connective tissue. Fibroblasts were identified by their typical spindle-shaped appearance. (C and D) Apoptotic fibroblasts were measured by TUNEL staining. (E and F) Quantification of Ki67 immunopositive keratinocytes. Each *in vivo* value is the mean ± SEM for n=7-9 mice per group. *, $P<0.05$ versus Cre^{-} group; #, $P<0.05$ versus matched normoglycemic control group.


A
Proliferation (D 4)
K14.Cre-.FOXO1L/L
K14.Cre+.FOXO1L/L
Ki67 positive fibroblasts (%)
Normal
Diabetic
B
Proliferation (D 7)
Ki67 positive fibroblasts (%)
Normal
Diabetic
C
Apoptosis (D 4)
TUNEL+ fibroblast/DAPI (%)
Normal
Diabetic
D
Apoptosis (D 7)
TUNEL+ fibroblast/DAPI (%)
Normal
Diabetic
E
Proliferation (D 4)
Ki67 positive keratinocytes (%)
Normal
Diabetic
F
Proliferation (D 7)
Ki67 positive keratinocytes (%)
Normal
Diabetic

3.2 To determine whether FOXO1 organizes keratinocyte activity to regulate angiogenesis during both normal and diabetic gingival wound healing.

3.2.1 Keratinocyte-specific FOXO1 deletion impairs neovascularization in normoglycemic mice while showing no significant difference in diabetic mice

Angiogenesis in the wound granulation tissue was compared between experimental ($K14.Cre^{+}.Foxo1^{L/L}$) and control ($K14.Cre^{-}.Foxo1^{L/L}$) mice based on the expression of the endothelial cell marker CD31 (Fig. 19 A and B). Vascular density was calculated as the number of CD31 immunopositive blood vessels/mm^2. Foxo1 ablation in keratinocytes of normoglycemic mice decreased vascular density in connective tissue by 52% (day 4) and 44% (day 7) compared with control littermates ($P < 0.05$) (Fig.19 C and D). In contrast, keratinocyte-specific Foxo1 ablation in diabetic mice did not affect vascular density in experimental compared to matched control mice ($P > 0.05$).

To investigate mechanisms through which FOXO1 deletion in epithelium affected angiogenesis during wound healing, endothelial cell proliferation and apotosis were assessed in vivo. Foxo1 deletion in normoglycemic mice caused a significant 40% (day 4) and 49% (day 7) reduction in the percentage of both Ki67 and CD31–positive proliferating EC in wounds ($P < 0.05$; Fig. 19 E and F). In contrast, Foxo1 deletion in keratinocytes of diabetic mice did not show the significantly different results. The percentage of apoptotic EC is very low in our study.

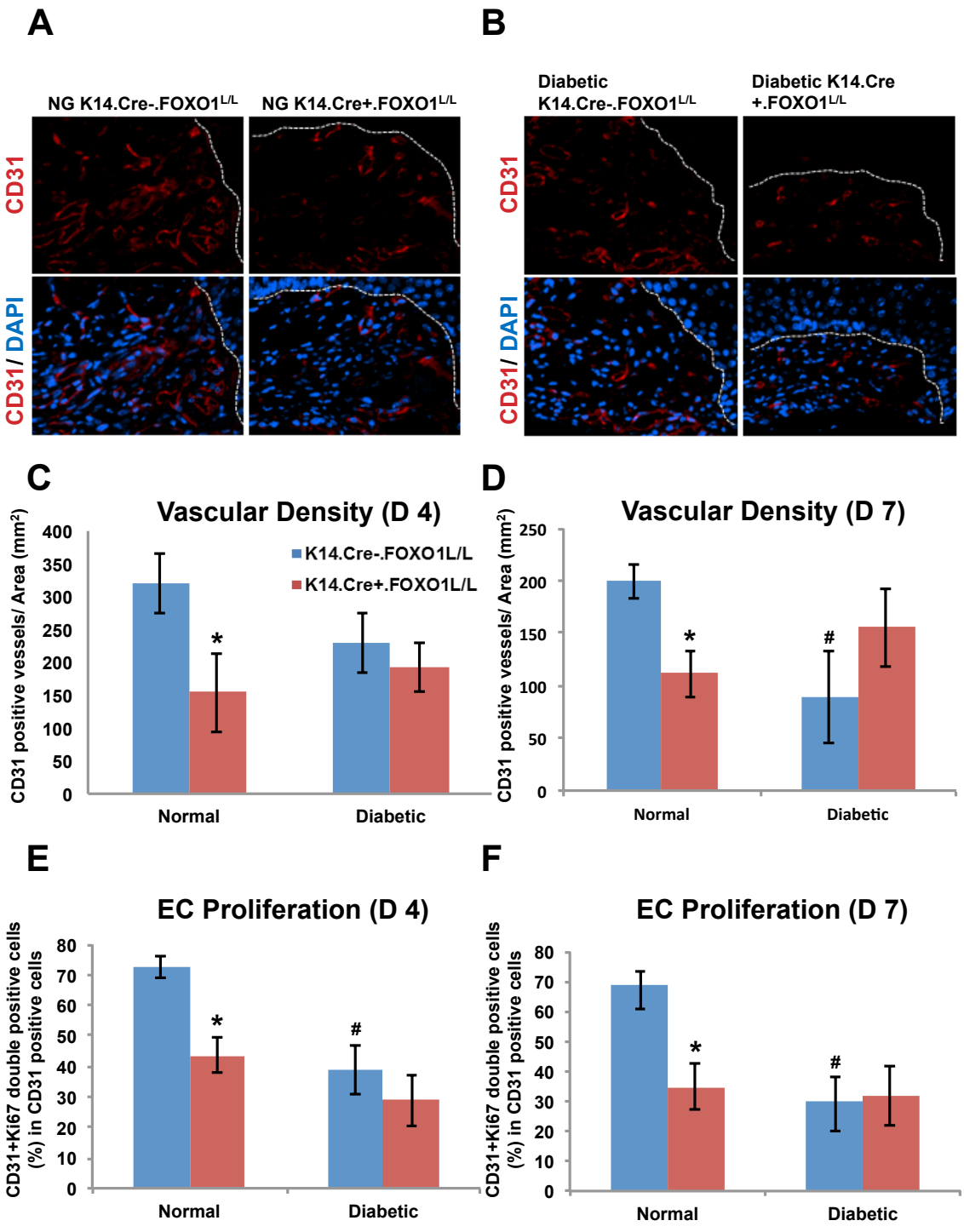

A
B
NG K14.Cre-.FOXO1L/L
NG K14.Cre+.FOXO1L/L
Diabetic K14.Cre-.FOXO1L/L
Diabetic K14.Cre +.FOXO1L/L
CD31
CD31/ DAPI
C
Vascular Density (D 4)
CD31 positive vessels/ Area (mm2)
K14.Cre-.FOXO1L/L
K14.Cre+.FOXO1L/L
Normal
Diabetic
D
Vascular Density (D 7)
E
EC Proliferation (D 4)
CD31+Ki67 double positive cells (%) in CD31 positive cells
F
EC Proliferation (D 7)

Figure 19. Keratinocyte-specific FOXO1 deletion in normal wounds reduces vascular density and endothelial cell proliferation. (A and B) CD31 immunofluorescence analyses in newly formed connective tissue (40x, day 4, normoglycemic and diabetic mice). (C and D) Vascualr density in newly formed connective tissue. (E and F) Quantification of CD31 and Ki67 double immunopositive cells for analyses of proliferating endothelial cells. Each *in vivo* value is the mean ± SEM for n=7-9 mice per group. *, P<0.05 versus Cre^- group; #, P<0.05 versus matched normoglycemic control group.

3.2.2 The effect of keratinocyte-specific FOXO1 deletion on angiogenesis is mediated by VEGF-A

Transfection of HIGK cells with FOXO1 siRNA significantly reduced VEGFa protein levels by 57% in standard glucose media (P<0.05; Fig. 20 A-C). Keratinocyte-specific FOXO1 deletion in vivo also significantly reduced VEGF-A protein levels by 53% (day 4, p<0.05) and 42% (day 7, p<0.1) in wounded epithelium from normoglycemic mice but had little effect on VEGF-A levels in diabetic mice (P>0.05) (Fig. 21 A-D). Surprisingly in normal wounds VEGF-A expression in the connective tissue was reduced by 58% (day 4, p<0.05) and 52% (day 7, p<0.05) when FOXO1 was deleted in epithelium, indicating that keratinocytes play an important role in VEGF-A expression in connective tissue. In diabetic wounds VEGF-A expression in both epithelium and connective tissue were similar between experimental ($K14.Cre^+.Foxo1^{L/L}$) and control ($K14.Cre^-.Foxo1^{L/L}$) mice on day 4 but there was a small but significant 1.7 fold increase of VEGF-A expression in connective tissue on day 7 (p<0.05). This is consistent with our vascular density results.

To determine whether FOXO1 can regulate VEGF-A transcriptional activity, we examined the luciferase activity of the VEGF-A reporter in HIGK and primary mouse

epidermal keratinocytes. The luciferase reporter analysis revealed that overexpression of constitutively active FOXO1 (FOXO1AAA) results in a 2.1-fold increase in VEGF-A transcriptional activity in HIGK cells and a 3.3-fold increase in primary mouse epidermal keratinocytes, whereas FOXO1 silencing produced a 20-43% decrease in VEGF-A promoter activity in HIGK cells ($P < 0.05$; Fig. 22 A-C). These results indicate that FOXO1 transactivates VEGF-A promoter activity and suggests that VEGF-A may be a downstream target of FOXO1.

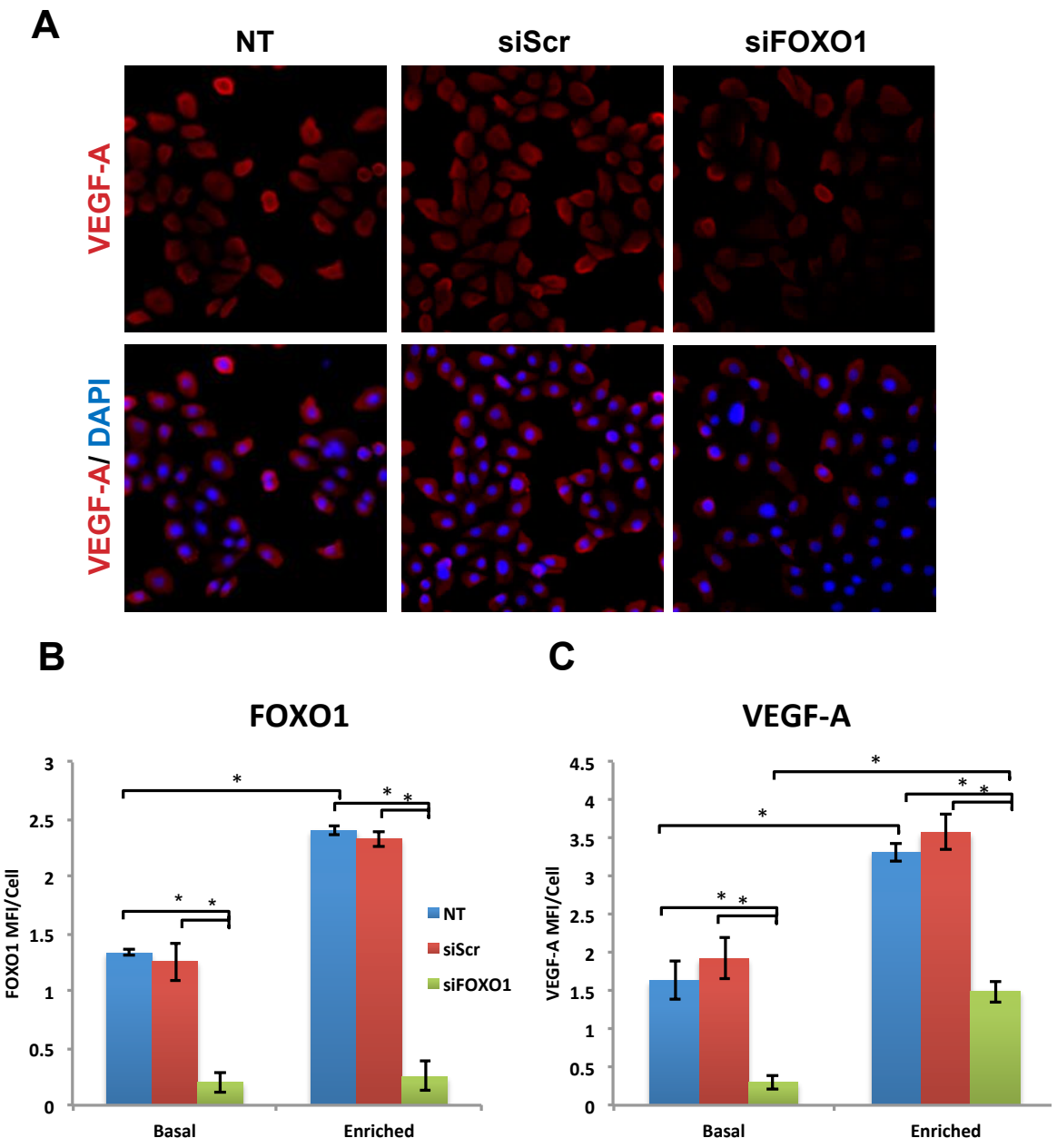


Figure 20. FOXO1 deletion in keratinocytes reduces VEGF expression in normal glucose media. (A) Representative images of VEGF-A immunofluorescence in human immortalized gingival keratinocytes (HIGK). (B) FOXO1 mean fluorescence intensity analysis. (C) VEGF-A mean fluorescence intensity analysis. In vitro values represent the mean ± SEM of three independent experiments. *P<0.05

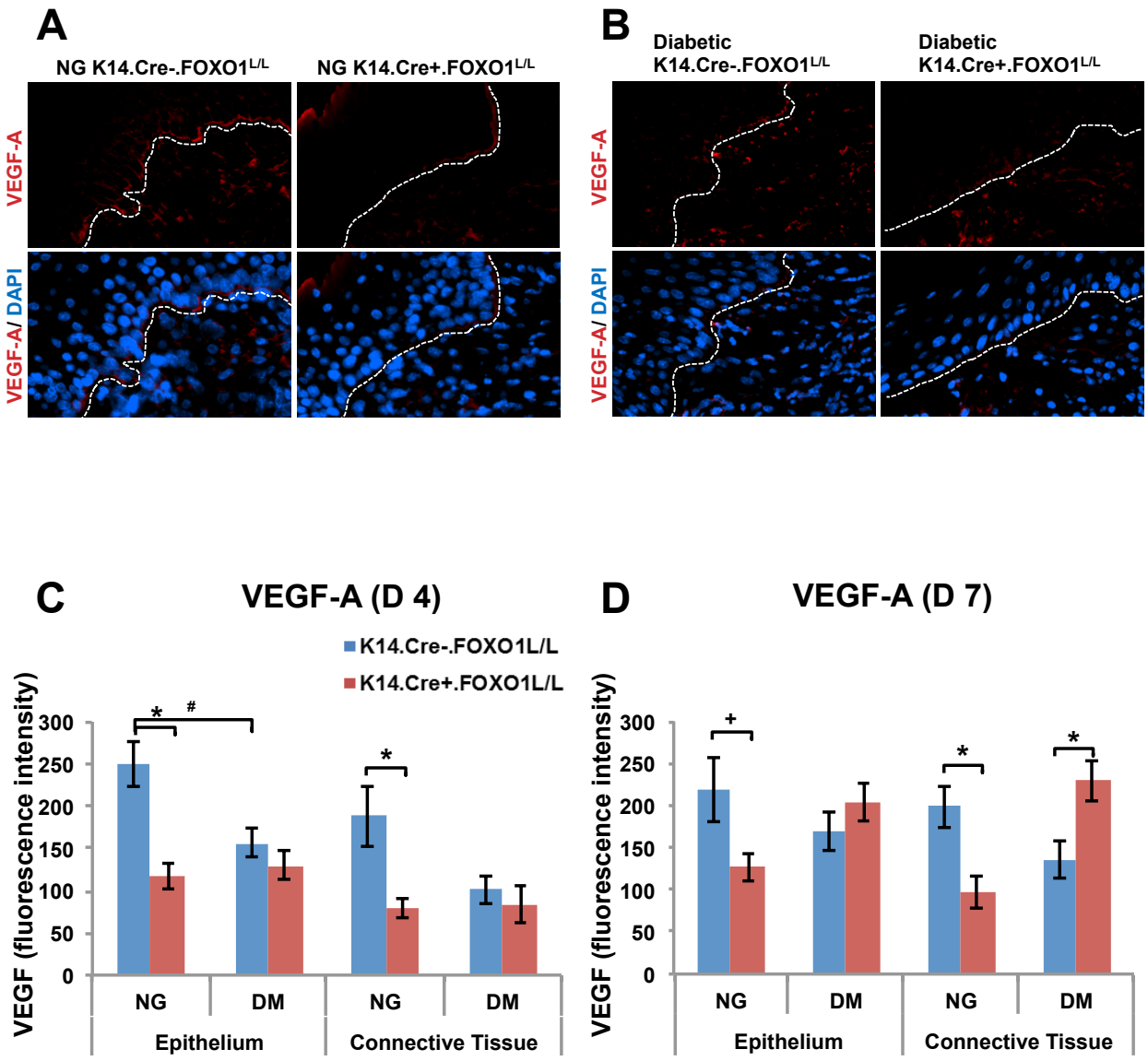


Figure 21. FOXO1 deletion in keratinocytes in normoglycemic wounds reduces VEGF expression in vivo. VEGF-A immunofluorescence on normoglycemic (A) and diabetic wounds (B) (day 4). VEGF-A mean fluorescence intensity analysis on day 4 (C) and day 7 (D). Each *in vivo* value is the mean ± SEM for n=7-9 mice per group. *, P<0.05 versus Cre$^-$ group; +, P<0.1 versus Cre$^-$ group; #, P<0.05 versus matched normoglycemic control group.

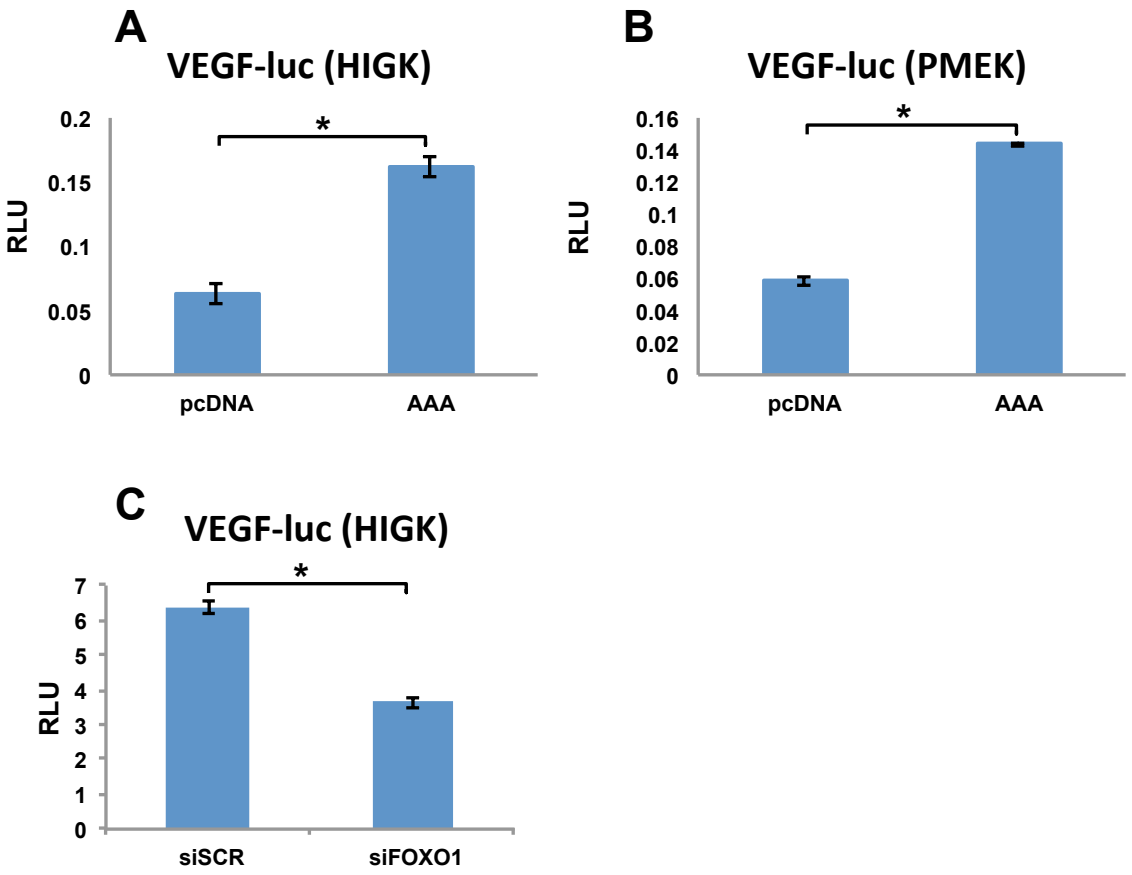


Figure 22. FOXO1 transactivates VEGF expression in normal glucose media. (A-C) VEGF-A luciferase reporter gene analyses. FOXO1 overexpression in HIGK cells (A) and primary mouse epidermal keratinocytes (PMEK) (B). FOXO1 silencing in HIGK cells (C). Data show mean ± SEM of at least three independent experiments. *P < 0.05

3.3 To evaluate the effect of lineage specific FOXO1 deletion in keratinocytes on epithelial-mesenchymal transition (EMT) during both normal and diabetic gingival connective tissue wound healing.

We examined K14CreER-ROSA26 mice in which keratinocytes were genetically altered to permanently express TdTomato with tamoxifen administration, allowing us to map the fate of these cells and definitively test in vivo whether EMT occurs during wound healing. TdTomato was strongly expressed following Cre-mediated recombination with tamoxifen administration. But, we did not find the reporter activity in the connective tissue indicating that there was no transition to typical fibroblasts or myofibroblasts with initial samples (n=2-4 per each group) (Fig. 23 A-E).

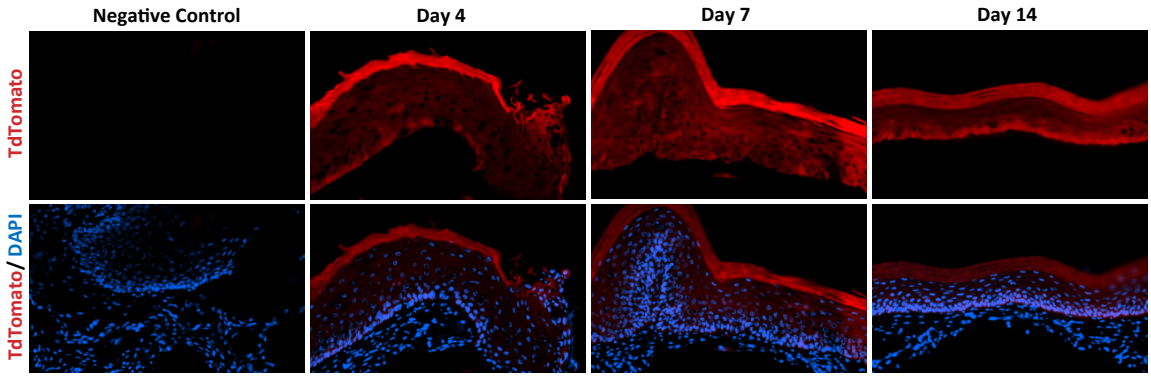


Figure 23 DAPI stain of oral wounds of K14CreER-ROSA26 mice. (left to right) Negative control, day 4 experimental, day 7 experimental and day 14 experimental wounds. (20x)

CHAPTER 4

DISCUSSION

VEGF-A is a key molecule that orchestrates the formation and function of vascular networks (122). It functions as an endothelial cell mitogen (123, 124), chemotactic agent (125), and inducer of vascular permeability (126). Unlike other angiogenic growth factors such as basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β), VEGF-A affects multiple components of the wound-healing cascade including angiogenesis, re-epithelialization and collagen deposition. The endothelium of the microvasculature exhibits several important responses to extracellular VEGF-A including increased endothelial proliferation and increased permeability, exerted through a change in endothelial cell structure and function (127, 128). Several lines of evidence indicate that FOXO1 deletion in keratinocytes in vivo led to deficient angiogenesis and substantially reduced the expression of VEGF-A during normal wound healing. In vitro HIGK cells with FOXO1 knockdown by siRNA substantially reduced VEGF-A expression and decreased VEGF-A promoter activity, and Cre recombinase deletion of Foxo1 in primary mouse keratinocytes reduced VEGF-A protein levels. Conversely, overexpression of constitutively active FOXO1 in both HIGK and primary murine keratinocytes significantly increased VEGF-A promoter activity in luciferase reporter assays. These results suggest that the avascular epidermis has the capacity to regulate angiogenesis by a paracrine mechanism involving the secretion of VEGF-A and FOXO1 is needed for adequate VEGF-A expression.

Interestingly we found that FOXO1 deletion in keratinocytes affected the VEGF-A expression in both epithelium and connective tissue. As a possible explanation one of

the major cellular components of wound granulation tissue relevant to healing and angiogenesis is macrophages (23, 24, 129). Macrophages in the wound tissue supply pro-inflammatory chemoattractants to recruit and activate additional macrophages, which participate in wound repair by secreting cytokines that induce angiogenesis, such as VEGF and TGF-β. Macrophages can also increase vascular permeability by releasing vasoactive substances such as vascular permeability factor (130), substance P (131), platelet activating factor and prostaglandins (132). Macrophage depletion during wound healing in mice results in decreased wound levels of TGF-β1 (27), suggesting that macrophages represent a major cellular source of TGF-β1 during normal wound healing (133). Evidence obtained in macrophage-deficient mice confirmed the essentiality of macrophages to the neovascularization of wounds (25, 27, 134, 135). Goren et al. showed that the altered expression patterns of vascular endothelial growth factor on macrophage reduction were associated with a disturbed neovascularization at the wound site (26). Leibovich et al. demonstrated that mice with non-functioning macrophages display retarded wound repair (134). This retarded wound repair includes delayed re-epithelialization, delayed neovascularization and aberrant granulation tissue formation (27, 135). Mirza et al. showed that macrophage depletion resulted in delayed re-epithelialization, decreased collagen deposition, impaired angiogenesis, and reduced cell proliferation associated with increased production of tumor necrosis factor (TNF)-α and decreased production of TGF-β1 and vascular endothelial growth factor (VEGF) (27). Also TGF-β1 is known for a chemoattractant for macrophages (136) and our previous study proved that FOXO1 regulates TGF-β1 in keratinocytes (110). However, most of studies still demonstrate that keratinocytes are the main producer of VEGF-A during wound healing (4, 9, 65). Brown et al. revealed that greatly increased amounts of vascular permeability factor (VPF) mRNA were expressed by keratinocytes, initially

those at the wound edge and at later intervals, keratinocytes that migrated to cover the wound surfaces; occasional mononuclear cells also expressed VPF mRNA (4). Rossiter et al. performed wound-healing studies in mice with VEGF-A-deficient keratinocytes and showed that the wounds contained fewer blood vessels beneath the epidermis and healed more slowly (65).

The contribution of VEGF-A to overall wound closure and epidermal repair has been examined extensively in animal studies (1). VEGF-A, which is normally expressed at low levels by epidermal keratinocytes, is up-regulated in these cells in injured skin (4). Studies in human wounds and animal models have indicated that VEGF-A is produced by keratinocytes early and later stages in the wound-healing process (64, 137). Activated fibroblasts, mast cells, and macrophages also express VEGF-A in injured skin (4, 138, 139). Traditionally, VEGF-A produced by epidermal keratinocytes was thought to act in a paracrine manner, stimulating endothelial cells in blood vessels within the underlying dermis (1). The expression of the flt-1 receptor for VEGF-A is up-regulated in the sprouting blood vessels at the wound edge and in endothelial cells of the granulation tissue (54). However VEGF-A has been shown to stimulate keratinocyte migration and collagen production via fibroblasts (53). Functional VEGFRs have been recently identified on keratinocytes (72-75), which suggests the possibility of autocrine VEGF-A signaling in keratinocytes as well as direct effects of VEGF-A derived from other cellular sources on keratinocytes. Several studies have also shown that VEGF-A enhances macrophage recruitment in vivo (140-143). Hong et al. demonstrated an increase in the density of macrophages has been observed in wounds created in transgenic mice that overexpress VEGF-A in the epidermis, suggesting that VEGF-A plays a role in recruiting macrophages to damaged skin (81). Reducing VEGF-A activity by treating with neutralizing antibodies or small molecule inhibitors of VEGF-A signaling or conditional

genetic deletion of VEGF-A lead to delayed healing (65, 72, 144, 145). Additionally, topical treatment with neutralizing antibodies to VEGFR-1 results in reduced re-epithelialization rates (72). Both keratinocyte-derived and myeloid cell–derived VEGF-A have been shown to affect some components of the repair process (65, 137, 144). For example, delayed wound closure, reduced vessel density, and decreased granulation tissue formation have been reported in mice lacking VEGF-A in myeloid cells (137, 144), and delayed wound closure and reduced vessel density have been reported in mice lacking VEGF-A in keratinocytes (65). Direct and indirect evidences implicate VEGF-A as a significant factor in wound healing immediately after injury.

Angiogenesis, the sprouting of new capillaries from the preexistent blood vessels, is of central importance in many biological processes, including embryonic vascular development and differentiation, wound healing, and organ regeneration (115, 146, 147). In addition, angiogenesis plays a major role in pathological conditions such as diabetic retinopathy, rheumatoid arthritis, psoriasis, cardiovascular diseases, tumor growth, and metastasis (148, 149). During angiogenesis, endothelial cells migrate, proliferate, organize into tube-like structures, and play an active role in tissue remodeling. In our study FOXO1 deletion in keratinocytes reduced endothelial cells proliferation in vivo. Endothelial cell apoptosis in vivo can be evaluated by one or more of several techniques, including immunofluorescence staining for endothelial specific markers, detection of DNA strand breaks by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), assay for caspase activation and measurement of phosphatidyl serine exposure by annexin V binding (150-152). Morphologic changes characteristic of apoptosis were also identified: cell shrinkage, nuclear and cytoplasmic condensation, and cellular budding into "apoptotic bodies”. In our study we could find TUNEL positive

cells but observe very little endothelial cell apoptosis. Generally apoptotic cell death and in particular endothelial cell apoptosis may be difficult to observe in vivo as apoptotic cells are rapidly phagocytosed and their digestion can be completed within 60 min (153, 154). Notably, endothelial cells can engulf apoptotic cells or apoptotic bodies (155, 156). Moreover, apoptotic endothelial cells may detach from the vessel wall into the circulation. Another possible reason is the time points for endothelial cell apoptosis. At the end of the proliferative phase fibroblasts start to undergo apoptosis. Endothelial cells and remaining fibroblasts disappear upon the completion of wound maturation (11). Desmouliere et al. examined apoptotic patterns in cells in open wounds created in rats (157). They found that apoptosis in myofibroblasts began on day 12, peaked at 20 d and resolved by 60 d suggesting that myofibroblast apoptosis begins about the same time as wound closure. Apoptosis patterns in the cells of the neovasculature similarly started at around 12 d and had maximal expression of apoptosis between 16 and 25 d. The parallel apoptosis patterns of fibroblasts and endothelial cells suggest that there is a coordinated decrease in cellularity with wound maturation that may involve cell to cell communication. In our study we used both day 4 and 7, when wound closure starts but it may be early for the endothelial cell apoptosis.

In our study we found that FOXO1 deletion in epithelium led to impaired connective tissue healing that included decreased formation of new connective tissue in normal mice. Fibroblast numbers and proliferation were both reduced when FOXO1 was deleted in keratinocytes consistent with impaired production of extracellular matrix. In addition keratinocyte-specific FOXO1 deletion led to reduced myofibroblast numbers. Our results agree with the previous papers (110). As a possible mechanism TGF-β1 and connective tissue growth factor (CTGF) are the key regulators of connective tissue healing

prompting the expression of ECM proteins such as collagen and initiating granulation tissue formation (158). TGF-β stimulates the expression of CTGF, which is thought to be responsible for many of the pro-fibrotic properties of TGF-β, particularly in the promotion of fibroblast proliferation and ECM production (159, 160). CTGF plays a physiological role in early wound healing (161). Previous studies in our lab found that TGF-β1 and CTGF expression in both epithelium and connective tissue were decreased by keratinocyte-specific deletion of FOXO1 during wound repair (submitted). Interestingly, they found that the same deletion of FOXO1 in diabetic wounds had the opposite effect, significantly improving the healing response (111, 113). However in our study we found that diabetic wound healing was impaired, compared with normal mice group but there was no significant difference between diabetic experimental and control groups. A possible explanation for those similar results is a relatively significant commensal flora in oral cavity that might mask the effect of FOXO1 deletion in keratinocytes. The bacteria of the oral cavity have the potential to alter the wound healing process by interacting with keratinocytes (162). Treatment with antibiotics may significantly diminish the persistent inflammatory infiltrate and improve healing in the diabetic mice (163).

Although EMT has been mentioned sporadically in the literature as an important aspect of wound healing, it is still unknown whether EMT occurs during cutaneous wound healing (164, 165). EMT was originally described as an important cellular programming that orchestrates formation of mesoderm during gastrulation (83, 166). In last decade accumulated evidence has uncovered EMT in many chronic diseases such as cancer progression and fibrotic diseases. Generally the purpose of EMT is believed to mobilize epithelial cells into motility or invasion through loss of cell-adhesion and polarity and simultaneous gain of mesenchymal phenotype. TGF-β is the key cytokine involved in

many elements of wound healing, including the induction of EMT via many pathways. Previous studies in our lab found that FOXO1 regulates TGF-β, which is one of the predominant factors that stimulate EMT, in keratinocytes during wound healing. Therefore we assumed that EMT occurred and it could be regulated by FOXO1. However, we did not observe trans-differentiation of keratinocytes into typical fibroblasts or myofibroblasts with lineage tracing in transgenic mice.

In summary, in this study we found that keratinocyte-specific deletion of FOXO1 interfered with normal gingival connective tissue healing by decreasing granulation tissue formation and angiogenesis, which were mediated by VEGF-A. In particular this is the first evidence that avascular epithelium regulates angiogenesis involving the VEGF-A secretion mediated by FOXO1.

BIBLIOGRAPHY

1. Johnson KE, Wilgus TA. Vascular Endothelial Growth Factor and Angiogenesis in the Regulation of Cutaneous Wound Repair. Advances in wound care. 2014;3(10):647-61. doi: 10.1089/wound.2013.0517. PubMed PMID: 25302139; PMCID: PMC4183920.
2. Carter ME, Brunet A. FOXO transcription factors. Curr Biol. 2007;17(4):R113-4. doi: 10.1016/j.cub.2007.01.008. PubMed PMID: 17307039.
3. Sleeman JP, Thiery JP. SnapShot: The epithelial-mesenchymal transition. Cell. 2011;145(1):162 e1. doi: 10.1016/j.cell.2011.03.029. PubMed PMID: 21458675.
4. Brown LF, Yeo KT, Berse B, Yeo TK, Senger DR, Dvorak HF, van de Water L. Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. J Exp Med. 1992;176(5):1375-9. PubMed PMID: 1402682; PMCID: PMC2119412.
5. Robson MC, Stenberg BD, Heggers JP. Wound healing alterations caused by infection. Clin Plast Surg. 1990;17(3):485-92. PubMed PMID: 2199139.
6. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med. 1999;341(10):738-46. doi: 10.1056/NEJM199909023411006. PubMed PMID: 10471461.
7. Nwomeh BC, Yager DR, Cohen IK. Physiology of the chronic wound. Clin Plast Surg. 1998;25(3):341-56. PubMed PMID: 9696897.
8. Walsh LJ, L'Estrange PR, Seymour GJ. High magnification in situ viewing of wound healing in oral mucosa. Aust Dent J. 1996;41(2):75-9. PubMed PMID: 8670037.
9. Mirza RE, Koh TJ. Contributions of cell subsets to cytokine production during normal and impaired wound healing. Cytokine. 2015;71(2):409-12. doi: 10.1016/j.cyto.2014.09.005. PubMed PMID: 25281359; PMCID: 4297569.
10. Brown DL, Kao WW, Greenhalgh DG. Apoptosis down-regulates inflammation under the advancing epithelial wound edge: delayed patterns in diabetes and improvement with topical growth factors. Surgery. 1997;121(4):372-80. PubMed PMID: 9122866.
11. Greenhalgh DG. The role of apoptosis in wound healing. The international journal of biochemistry & cell biology. 1998;30(9):1019-30. PubMed PMID: 9785465.
12. Ponugoti B, Dong G, Graves DT. Role of forkhead transcription factors in diabetes-induced oxidative stress. Exp Diabetes Res. 2012;2012:939751. doi: 10.1155/2012/939751. PubMed PMID: 22454632; PMCID: PMC3290826.
13. IDF. IDF DIABETES ATLAS Six edition. 2014.
14. Xiao E, Graves DT. Impact of Diabetes on the Protective Role of FOXO1 in Wound Healing. Journal of dental research. 2015;94(8):1025-6. doi: 10.1177/0022034515586353. PubMed PMID: 25978971; PMCID: 4530387.
15. Guggenheimer J, Moore PA, Rossie K, Myers D, Mongelluzzo MB, Block HM, Weyant R, Orchard T. Insulin-dependent diabetes mellitus and oral soft tissue pathologies: II. Prevalence and characteristics of Candida and Candidal lesions. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2000;89(5):570-6. PubMed PMID: 10807713.
16. Galkowska H, Wojewodzka U, Olszewski WL. Chemokines, cytokines, and growth factors in keratinocytes and dermal endothelial cells in the margin of chronic diabetic foot ulcers. Wound repair and regeneration : official publication of the Wound

Healing Society [and] the European Tissue Repair Society. 2006;14(5):558-65. doi: 10.1111/j.1743-6109.2006.00155.x. PubMed PMID: 17014667.
17. Goren I, Muller E, Pfeilschifter J, Frank S. Severely impaired insulin signaling in chronic wounds of diabetic ob/ob mice: a potential role of tumor necrosis factor-alpha. Am J Pathol. 2006;168(3):765-77. PubMed PMID: 16507892; PMCID: PMC1606528.
18. Falanga V. Wound healing and its impairment in the diabetic foot. Lancet. 2005;366(9498):1736-43. doi: 10.1016/S0140-6736(05)67700-8. PubMed PMID: 16291068.
19. Galiano RD, Tepper OM, Pelo CR, Bhatt KA, Callaghan M, Bastidas N, Bunting S, Steinmetz HG, Gurtner GC. Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. Am J Pathol. 2004;164(6):1935-47. doi: 10.1016/S0002-9440(10)63754-6. PubMed PMID: 15161630; PMCID: PMC1615774.
20. Maruyama K, Asai J, Ii M, Thorne T, Losordo DW, D'Amore PA. Decreased macrophage number and activation lead to reduced lymphatic vessel formation and contribute to impaired diabetic wound healing. Am J Pathol. 2007;170(4):1178-91. doi: 10.2353/ajpath.2007.060018. PubMed PMID: 17392158; PMCID: PMC1829452.
21. Gibran NS, Jang YC, Isik FF, Greenhalgh DG, Muffley LA, Underwood RA, Usui ML, Larsen J, Smith DG, Bunnett N, Ansel JC, Olerud JE. Diminished neuropeptide levels contribute to the impaired cutaneous healing response associated with diabetes mellitus. J Surg Res. 2002;108(1):122-8. PubMed PMID: 12443724.
22. Lobmann R, Ambrosch A, Schultz G, Waldmann K, Schiweck S, Lehnert H. Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. Diabetologia. 2002;45(7):1011-6. doi: 10.1007/s00125-002-0868-8. PubMed PMID: 12136400.
23. Kamoshita E, Ikeda Y, Fujita M, Amano H, Oikawa A, Suzuki T, Ogawa Y, Yamashina S, Azuma S, Narumiya S, Unno N, Majima M. Recruitment of a prostaglandin E receptor subtype, EP3-expressing bone marrow cells is crucial in wound-induced angiogenesis. Am J Pathol. 2006;169(4):1458-72. doi: 10.2353/ajpath.2006.051358. PubMed PMID: 17003499; PMCID: PMC1780188.
24. Kurashige C, Hosono K, Matsuda H, Tsujikawa K, Okamoto H, Majima M. Roles of receptor activity-modifying protein 1 in angiogenesis and lymphangiogenesis during skin wound healing in mice. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2014;28(3):1237-47. doi: 10.1096/fj.13-238998. PubMed PMID: 24308973.
25. Brancato SK, Albina JE. Wound macrophages as key regulators of repair: origin, phenotype, and function. Am J Pathol. 2011;178(1):19-25. doi: 10.1016/j.ajpath.2010.08.003. PubMed PMID: 21224038; PMCID: PMC3069845.
26. Goren I, Allmann N, Yogev N, Schurmann C, Linke A, Holdener M, Waisman A, Pfeilschifter J, Frank S. A transgenic mouse model of inducible macrophage depletion: effects of diphtheria toxin-driven lysozyme M-specific cell lineage ablation on wound inflammatory, angiogenic, and contractive processes. Am J Pathol. 2009;175(1):132-47. doi: 10.2353/ajpath.2009.081002. PubMed PMID: 19528348; PMCID: PMC2708801.
27. Mirza R, DiPietro LA, Koh TJ. Selective and specific macrophage ablation is detrimental to wound healing in mice. Am J Pathol. 2009;175(6):2454-62. doi: 10.2353/ajpath.2009.090248. PubMed PMID: 19850888; PMCID: PMC2789630.
28. Darby IA, Bisucci T, Hewitson TD, MacLellan DG. Apoptosis is increased in a model of diabetes-impaired wound healing in genetically diabetic mice. Int J Biochem Cell Biol. 1997;29(1):191-200. PubMed PMID: 9076954.

29. Liu R, Desta T, He H, Graves DT. Diabetes alters the response to bacteria by enhancing fibroblast apoptosis. Endocrinology. 2004;145(6):2997-3003. doi: 10.1210/en.2003-1601. PubMed PMID: 15033911.
30. Desta T, Li J, Chino T, Graves DT. Altered fibroblast proliferation and apoptosis in diabetic gingival wounds. Journal of dental research. 2010;89(6):609-14. doi: 10.1177/0022034510362960. PubMed PMID: 20354230; PMCID: 3318033.
31. Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. J Clin Invest. 2007;117(5):1219-22. doi: 10.1172/JCI32169. PubMed PMID: 17476353; PMCID: PMC1857239.
32. Tie L, An Y, Han J, Xiao Y, Xiaokaiti Y, Fan S, Liu S, Chen AF, Li X. Genistein accelerates refractory wound healing by suppressing superoxide and FoxO1/iNOS pathway in type 1 diabetes. J Nutr Biochem. 2013;24(1):88-96. doi: 10.1016/j.jnutbio.2012.02.011. PubMed PMID: 22819564.
33. Saito H, Yamamoto Y, Yamamoto H. Diabetes alters subsets of endothelial progenitor cells that reside in blood, bone marrow, and spleen. Am J Physiol Cell Physiol. 2012;302(6):C892-901. doi: 10.1152/ajpcell.00380.2011. PubMed PMID: 22159079.
34. Cooke JP, Losordo DW. Nitric oxide and angiogenesis. Circulation. 2002;105(18):2133-5. PubMed PMID: 11994243.
35. Efron DT, Most D, Barbul A. Role of nitric oxide in wound healing. Curr Opin Clin Nutr Metab Care. 2000;3(3):197-204. PubMed PMID: 10871235.
36. Pradhan L, Nabzdyk C, Andersen ND, LoGerfo FW, Veves A. Inflammation and neuropeptides: the connection in diabetic wound healing. Expert Rev Mol Med. 2009;11:e2. doi: 10.1017/S1462399409000945. PubMed PMID: 19138453; PMCID: PMC3708299.
37. Tamarat R, Silvestre JS, Huijberts M, Benessiano J, Ebrahimian TG, Duriez M, Wautier MP, Wautier JL, Levy BI. Blockade of advanced glycation end-product formation restores ischemia-induced angiogenesis in diabetic mice. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(14):8555-60. doi: 10.1073/pnas.1236929100. PubMed PMID: 12805564; PMCID: PMC166267.
38. Busik JV, Mohr S, Grant MB. Hyperglycemia-induced reactive oxygen species toxicity to endothelial cells is dependent on paracrine mediators. Diabetes. 2008;57(7):1952-65. doi: 10.2337/db07-1520. PubMed PMID: 18420487; PMCID: PMC2453610.
39. Kageyama S, Yokoo H, Tomita K, Kageyama-Yahara N, Uchimido R, Matsuda N, Yamamoto S, Hattori Y. High glucose-induced apoptosis in human coronary artery endothelial cells involves up-regulation of death receptors. Cardiovasc Diabetol. 2011;10:73. doi: 10.1186/1475-2840-10-73. PubMed PMID: 21816064; PMCID: PMC3161855.
40. El-Ftesi S, Chang EI, Longaker MT, Gurtner GC. Aging and diabetes impair the neovascular potential of adipose-derived stromal cells. Plast Reconstr Surg. 2009;123(2):475-85. doi: 10.1097/PRS.0b013e3181954d08. PubMed PMID: 19182604; PMCID: PMC2878769.
41. Jain M, LoGerfo FW, Guthrie P, Pradhan L. Effect of hyperglycemia and neuropeptides on interleukin-8 expression and angiogenesis in dermal microvascular endothelial cells. J Vasc Surg. 2011;53(6):1654-60 e2. doi: 10.1016/j.jvs.2011.02.019. PubMed PMID: 21609799.

42. Lenzen S. The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia. 2008;51(2):216-26. doi: 10.1007/s00125-007-0886-7. PubMed PMID: 18087688.
43. Rakieten N, Rakieten ML, Nadkarni MV. Studies on the diabetogenic action of streptozotocin (NSC-37917). Cancer Chemother Rep. 1963;29:91-8. PubMed PMID: 13990586.
44. Wang Z, Gleichmann H. GLUT2 in pancreatic islets: crucial target molecule in diabetes induced with multiple low doses of streptozotocin in mice. Diabetes. 1998;47(1):50-6. PubMed PMID: 9421374.
45. Schnedl WJ, Ferber S, Johnson JH, Newgard CB. STZ transport and cytotoxicity. Specific enhancement in GLUT2-expressing cells. Diabetes. 1994;43(11):1326-33. PubMed PMID: 7926307.
46. Tesch GH, Allen TJ. Rodent models of streptozotocin-induced diabetic nephropathy. Nephrology (Carlton). 2007;12(3):261-6. doi: 10.1111/j.1440-1797.2007.00796.x. PubMed PMID: 17498121.
47. Szkudelski T. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res. 2001;50(6):537-46. PubMed PMID: 11829314.
48. Valentovic MA, Alejandro N, Betts Carpenter A, Brown PI, Ramos K. Streptozotocin (STZ) diabetes enhances benzo(alpha)pyrene induced renal injury in Sprague Dawley rats. Toxicol Lett. 2006;164(3):214-20. doi: 10.1016/j.toxlet.2005.12.009. PubMed PMID: 16460892.
49. Gaulton GN, Schwartz JL, Eardley DD. Assessment of the diabetogenic drugs alloxan and streptozotocin as models for the study of immune defects in diabetic mice. Diabetologia. 1985;28(10):769-75. PubMed PMID: 2933286.
50. Nichols WK, Spellman JB, Vann LL, Daynes RA. Immune responses of diabetic animals. Direct immunosuppressant effects of streptozotocin in mice. Diabetologia. 1979;16(1):51-7. PubMed PMID: 153868.
51. Koulmanda M, Qipo A, Auchincloss H, Jr., Smith RN. Effects of streptozotocin on autoimmune diabetes in NOD mice. Clin Exp Immunol. 2003;134(2):210-6. PubMed PMID: 14616779; PMCID: PMC1808849.
52. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971;285(21):1182-6. doi: 10.1056/NEJM197111182852108. PubMed PMID: 4938153.
53. Bao P, Kodra A, Tomic-Canic M, Golinko MS, Ehrlich HP, Brem H. The role of vascular endothelial growth factor in wound healing. The Journal of surgical research. 2009;153(2):347-58. doi: 10.1016/j.jss.2008.04.023. PubMed PMID: 19027922; PMCID: 2728016.
54. Folkman J, Klagsbrun M. Angiogenic factors. Science. 1987;235(4787):442-7. PubMed PMID: 2432664.
55. Knighton DR, Silver IA, Hunt TK. Regulation of wound-healing angiogenesis-effect of oxygen gradients and inspired oxygen concentration. Surgery. 1981;90(2):262-70. PubMed PMID: 6166996.
56. Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. J Investig Dermatol Symp Proc. 2000;5(1):40-6. doi: 10.1046/j.1087-0024.2000.00014.x. PubMed PMID: 11147674.
57. Nissen NN, Polverini PJ, Koch AE, Volin MV, Gamelli RL, DiPietro LA. Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. Am J Pathol. 1998;152(6):1445-52. PubMed PMID: 9626049; PMCID: PMC1858442.

58. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell. 1996;86(3):353-64. PubMed PMID: 8756718.
59. Sirohi B, Smith K. Bevacizumab in the treatment of breast cancer. Expert Rev Anticancer Ther. 2008;8(10):1559-68. doi: 10.1586/14737140.8.10.1559. PubMed PMID: 18925848.
60. Socinski MA. Bevacizumab as first-line treatment for advanced non-small cell lung cancer. Drugs Today (Barc). 2008;44(4):293-301. PubMed PMID: 18536787.
61. Lien S, Lowman HB. Therapeutic anti-VEGF antibodies. Handb Exp Pharmacol. 2008(181):131-50. doi: 10.1007/978-3-540-73259-4_6. PubMed PMID: 18071944.
62. Roth D, Piekarek M, Paulsson M, Christ H, Bloch W, Krieg T, Davidson JM, Eming SA. Plasmin modulates vascular endothelial growth factor-A-mediated angiogenesis during wound repair. Am J Pathol. 2006;168(2):670-84. doi: 10.2353/ajpath.2006.050372. PubMed PMID: 16436680; PMCID: PMC1606492.
63. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. The Journal of biological chemistry. 1992;267(36):26031-7. PubMed PMID: 1464614.
64. Kishimoto J, Ehama R, Ge Y, Kobayashi T, Nishiyama T, Detmar M, Burgeson RE. In vivo detection of human vascular endothelial growth factor promoter activity in transgenic mouse skin. Am J Pathol. 2000;157(1):103-10. doi: 10.1016/S0002-9440(10)64522-1. PubMed PMID: 10880381; PMCID: PMC1850203.
65. Rossiter H, Barresi C, Pammer J, Rendl M, Haigh J, Wagner EF, Tschachler E. Loss of vascular endothelial growth factor a activity in murine epidermal keratinocytes delays wound healing and inhibits tumor formation. Cancer research. 2004;64(10):3508-16. doi: 10.1158/0008-5472.CAN-03-2581. PubMed PMID: 15150105.
66. Ballaun C, Weninger W, Uthman A, Weich H, Tschachler E. Human keratinocytes express the three major splice forms of vascular endothelial growth factor. The Journal of investigative dermatology. 1995;104(1):7-10. PubMed PMID: 7798644.
67. Weninger W, Uthman A, Pammer J, Pichler A, Ballaun C, Lang IM, Plettenberg A, Bankl HC, Sturzl M, Tschachler E. Vascular endothelial growth factor production in normal epidermis and in benign and malignant epithelial skin tumors. Lab Invest. 1996;75(5):647-57. PubMed PMID: 8941211.
68. Namiki A, Brogi E, Kearney M, Kim EA, Wu T, Couffinhal T, Varticovski L, Isner JM. Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. The Journal of biological chemistry. 1995;270(52):31189-95. PubMed PMID: 8537383.
69. Detmar M, Yeo KT, Nagy JA, Van de Water L, Brown LF, Berse B, Elicker BM, Ledbetter S, Dvorak HF. Keratinocyte-derived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. The Journal of investigative dermatology. 1995;105(1):44-50. PubMed PMID: 7615975.
70. Frank S, Hubner G, Breier G, Longaker MT, Greenhalgh DG, Werner S. Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. The Journal of biological chemistry. 1995;270(21):12607-13. PubMed PMID: 7759509.
71. Shukla A, Dubey MP, Srivastava R, Srivastava BS. Differential expression of proteins during healing of cutaneous wounds in experimental normal and chronic models. Biochemical and biophysical research communications. 1998;244(2):434-9. doi: 10.1006/bbrc.1998.8286. PubMed PMID: 9514941.
72. Wilgus TA, Matthies AM, Radek KA, Dovi JV, Burns AL, Shankar R, DiPietro LA. Novel function for vascular endothelial growth factor receptor-1 on epidermal

keratinocytes. Am J Pathol. 2005;167(5):1257-66. doi: 10.1016/S0002-9440(10)61213-8. PubMed PMID: 16251410; PMCID: PMC1603795.
73. Lichtenberger BM, Tan PK, Niederleithner H, Ferrara N, Petzelbauer P, Sibilia M. Autocrine VEGF signaling synergizes with EGFR in tumor cells to promote epithelial cancer development. Cell. 2010;140(2):268-79. doi: 10.1016/j.cell.2009.12.046. PubMed PMID: 20141840.
74. Beck B, Driessens G, Goossens S, Youssef KK, Kuchnio A, Caauwe A, Sotiropoulou PA, Loges S, Lapouge G, Candi A, Mascre G, Drogat B, Dekoninck S, Haigh JJ, Carmeliet P, Blanpain C. A vascular niche and a VEGF-Nrp1 loop regulate the initiation and stemness of skin tumours. Nature. 2011;478(7369):399-403. doi: 10.1038/nature10525. PubMed PMID: 22012397.
75. Riese A, Eilert Y, Meyer Y, Arin M, Baron JM, Eming S, Krieg T, Kurschat P. Epidermal expression of neuropilin 1 protects murine keratinocytes from UVB-induced apoptosis. PloS one. 2012;7(12):e50944. doi: 10.1371/journal.pone.0050944. PubMed PMID: 23251405; PMCID: PMC3518474.
76. Brem H, Kodra A, Golinko MS, Entero H, Stojadinovic O, Wang VM, Sheahan CM, Weinberg AD, Woo SL, Ehrlich HP, Tomic-Canic M. Mechanism of sustained release of vascular endothelial growth factor in accelerating experimental diabetic healing. The Journal of investigative dermatology. 2009;129(9):2275-87. doi: 10.1038/jid.2009.26. PubMed PMID: 19282838.
77. Zhu JW, Wu XJ, Luo D, Lu ZF, Cai SQ, Zheng M. Activation of VEGFR-2 signaling in response to moderate dose of ultraviolet B promotes survival of normal human keratinocytes. The international journal of biochemistry & cell biology. 2012;44(1):246-56. doi: 10.1016/j.biocel.2011.10.022. PubMed PMID: 22062947.
78. Shen H, Clauss M, Ryan J, Schmidt AM, Tijburg P, Borden L, Connolly D, Stern D, Kao J. Characterization of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes. Blood. 1993;81(10):2767-73. PubMed PMID: 8490183.
79. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. Blood. 1996;87(8):3336-43. PubMed PMID: 8605350.
80. Sawano A, Iwai S, Sakurai Y, Ito M, Shitara K, Nakahata T, Shibuya M. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. Blood. 2001;97(3):785-91. PubMed PMID: 11157498.
81. Hong YK, Lange-Asschenfeldt B, Velasco P, Hirakawa S, Kunstfeld R, Brown LF, Bohlen P, Senger DR, Detmar M. VEGF-A promotes tissue repair-associated lymphatic vessel formation via VEGFR-2 and the alpha1beta1 and alpha2beta1 integrins. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2004;18(10):1111-3. doi: 10.1096/fj.03-1179fje. PubMed PMID: 15132990.
82. Fukawa T, Kajiya H, Ozeki S, Ikebe T, Okabe K. Reactive oxygen species stimulates epithelial mesenchymal transition in normal human epidermal keratinocytes via TGF-beta secretion. Experimental cell research. 2012;318(15):1926-32. doi: 10.1016/j.yexcr.2012.05.023. PubMed PMID: 22664326.
83. Hay ED. An overview of epithelio-mesenchymal transformation. Acta Anat (Basel). 1995;154(1):8-20. PubMed PMID: 8714286.
84. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. The Journal of clinical investigation. 2003;112(12):1776-84. doi: 10.1172/JCI20530. PubMed PMID: 14679171; PMCID: 297008.

85. McCormack N, O'Dea S. Regulation of epithelial to mesenchymal transition by bone morphogenetic proteins. Cellular signalling. 2013;25(12):2856-62. doi: 10.1016/j.cellsig.2013.09.012. PubMed PMID: 24044921.
86. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. The Journal of cell biology. 2006;172(7):973-81. doi: 10.1083/jcb.200601018. PubMed PMID: 16567498; PMCID: 2063755.
87. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol. 2000;2(2):76-83. doi: 10.1038/35000025. PubMed PMID: 10655586.
88. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. The Journal of clinical investigation. 2009;119(6):1420-8. doi: 10.1172/JCI39104. PubMed PMID: 19487818; PMCID: 2689101.
89. Yan C, Grimm WA, Garner WL, Qin L, Travis T, Tan N, Han YP. Epithelial to mesenchymal transition in human skin wound healing is induced by tumor necrosis factor-alpha through bone morphogenic protein-2. Am J Pathol. 2010;176(5):2247-58. doi: 10.2353/ajpath.2010.090048. PubMed PMID: 20304956; PMCID: 2861090.
90. Zhang J, Tian XJ, Zhang H, Teng Y, Li R, Bai F, Elankumaran S, Xing J. TGF-beta-induced epithelial-to-mesenchymal transition proceeds through stepwise activation of multiple feedback loops. Science signaling. 2014;7(345):ra91. doi: 10.1126/scisignal.2005304. PubMed PMID: 25270257.
91. Nakamura M, Tokura Y. Epithelial-mesenchymal transition in the skin. Journal of dermatological science. 2011;61(1):7-13. doi: 10.1016/j.jdermsci.2010.11.015. PubMed PMID: 21167690.
92. Coulombe PA. Wound epithelialization: accelerating the pace of discovery. The Journal of investigative dermatology. 2003;121(2):219-30. doi: 10.1046/j.1523-1747.2003.12387.x. PubMed PMID: 12880412.
93. De Donatis A, Ranaldi F, Cirri P. Reciprocal control of cell proliferation and migration. Cell Commun Signal. 2010;8:20. doi: 10.1186/1478-811X-8-20. PubMed PMID: 20822514; PMCID: PMC2942889.
94. Selman M, Pardo A. Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers. Proceedings of the American Thoracic Society. 2006;3(4):364-72. doi: 10.1513/pats.200601-003TK. PubMed PMID: 16738202.
95. Hudson LG, Newkirk KM, Chandler HL, Choi C, Fossey SL, Parent AE, Kusewitt DF. Cutaneous wound reepithelialization is compromised in mice lacking functional Slug (Snai2). Journal of dermatological science. 2009;56(1):19-26. doi: 10.1016/j.jdermsci.2009.06.009. PubMed PMID: 19643582; PMCID: 3612935.
96. Savagner P, Kusewitt DF, Carver EA, Magnino F, Choi C, Gridley T, Hudson LG. Developmental transcription factor slug is required for effective re-epithelialization by adult keratinocytes. Journal of cellular physiology. 2005;202(3):858-66. doi: 10.1002/jcp.20188. PubMed PMID: 15389643.
97. Vassar R, Rosenberg M, Ross S, Tyner A, Fuchs E. Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. Proceedings of the National Academy of Sciences of the United States of America. 1989;86(5):1563-7. PubMed PMID: 2466292; PMCID: PMC286738.
98. Fuchs E, Tyner AL, Giudice GJ, Marchuk D, RayChaudhury A, Rosenberg M. The human keratin genes and their differential expression. Curr Top Dev Biol. 1987;22:5-34. PubMed PMID: 2443316.

99. Wang X, Zinkel S, Polonsky K, Fuchs E. Transgenic studies with a keratin promoter-driven growth hormone transgene: prospects for gene therapy. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(1):219-26. PubMed PMID: 8990189; PMCID: PMC19291.
100. Fuchs E, Green H. Changes in keratin gene expression during terminal differentiation of the keratinocyte. Cell. 1980;19(4):1033-42. PubMed PMID: 6155214.
101. Stellmach V, Leask A, Fuchs E. Retinoid-mediated transcriptional regulation of keratin genes in human epidermal and squamous cell carcinoma cells. Proceedings of the National Academy of Sciences of the United States of America. 1991;88(11):4582-6. PubMed PMID: 1711202; PMCID: PMC51709.
102. Almeida M. Unraveling the role of FoxOs in bone--insights from mouse models. Bone. 2011;49(3):319-27. doi: 10.1016/j.bone.2011.05.023. PubMed PMID: 21664311; PMCID: PMC3143252.
103. Greer EL, Brunet A. FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene. 2005;24(50):7410-25. doi: 10.1038/sj.onc.1209086. PubMed PMID: 16288288.
104. Jacobs FM, van der Heide LP, Wijchers PJ, Burbach JP, Hoekman MF, Smidt MP. FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. J Biol Chem. 2003;278(38):35959-67. doi: 10.1074/jbc.M302804200. PubMed PMID: 12857750.
105. Kousteni S. FoxO1: a molecule for all seasons. J Bone Miner Res. 2011;26(5):912-7. doi: 10.1002/jbmr.306. PubMed PMID: 21541992.
106. Nakae J, Park BC, Accili D. Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. The Journal of biological chemistry. 1999;274(23):15982-5. PubMed PMID: 10347145.
107. Essers MA, Weijzen S, de Vries-Smits AM, Saarloos I, de Ruiter ND, Bos JL, Burgering BM. FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. The EMBO journal. 2004;23(24):4802-12. doi: 10.1038/sj.emboj.7600476. PubMed PMID: 15538382; PMCID: PMC535088.
108. Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science. 2004;303(5666):2011-5. doi: 10.1126/science.1094637. PubMed PMID: 14976264.
109. Mori R, Tanaka K, de Kerckhove M, Okamoto M, Kashiyama K, Tanaka K, Kim S, Kawata T, Komatsu T, Park S, Ikematsu K, Hirano A, Martin P, Shimokawa I. Reduced FOXO1 expression accelerates skin wound healing and attenuates scarring. Am J Pathol. 2014;184(9):2465-79. doi: 10.1016/j.ajpath.2014.05.012. PubMed PMID: 25010393; PMCID: 4188279.
110. Ponugoti B, Xu F, Zhang C, Tian C, Pacios S, Graves DT. FOXO1 promotes wound healing through the up-regulation of TGF-beta1 and prevention of oxidative stress. The Journal of cell biology. 2013;203(2):327-43. doi: 10.1083/jcb.201305074. PubMed PMID: 24145170; PMCID: 3812981.
111. Zhang C, Ponugoti B, Tian C, Xu F, Tarapore R, Batres A, Alsadun S, Lim J, Dong G, Graves DT. FOXO1 differentially regulates both normal and diabetic wound healing. The Journal of cell biology. 2015;209(2):289-303. doi: 10.1083/jcb.201409032. PubMed PMID: 25918228; PMCID: PMC4411275.
112. <Foxo1 Inhibits Diabetic Mucosal Wound Healing but Enhances Healing of Normoglycemic Wounds.pdf>. doi: 10.2337/db14-0589/-/DC1.

113. Xu F, Othman B, Lim J, Batres A, Ponugoti B, Zhang C, Yi L, Liu J, Tian C, Hameedaldeen A, Alsadun S, Tarapore R, Graves DT. Foxo1 inhibits diabetic mucosal wound healing but enhances healing of normoglycemic wounds. Diabetes. 2015;64(1):243-56. doi: 10.2337/db14-0589. PubMed PMID: 25187373; PMCID: PMC4274809.
114. Furuyama T, Kitayama K, Shimoda Y, Ogawa M, Sone K, Yoshida-Araki K, Hisatsune H, Nishikawa S, Nakayama K, Nakayama K, Ikeda K, Motoyama N, Mori N. Abnormal angiogenesis in Foxo1 (Fkhr)-deficient mice. The Journal of biological chemistry. 2004;279(33):34741-9. doi: 10.1074/jbc.M314214200. PubMed PMID: 15184386.
115. Srivastava RK, Unterman TG, Shankar S. FOXO transcription factors and VEGF neutralizing antibody enhance antiangiogenic effects of resveratrol. Mol Cell Biochem. 2010;337(1-2):201-12. doi: 10.1007/s11010-009-0300-5. PubMed PMID: 20012470; PMCID: PMC4153854.
116. Paik JH, Kollipara R, Chu G, Ji H, Xiao Y, Ding Z, Miao L, Tothova Z, Horner JW, Carrasco DR, Jiang S, Gilliland DG, Chin L, Wong WH, Castrillon DH, DePinho RA. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell. 2007;128(2):309-23. doi: 10.1016/j.cell.2006.12.029. PubMed PMID: 17254969; PMCID: PMC1855089.
117. Anastassiadis K, Glaser S, Kranz A, Berhardt K, Stewart AF. A practical summary of site-specific recombination, conditional mutagenesis, and tamoxifen induction of CreERT2. Methods Enzymol. 2010;477:109-23. doi: 10.1016/S0076-6879(10)77007-5. PubMed PMID: 20699139.
118. Vasioukhin V, Degenstein L, Wise B, Fuchs E. The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(15):8551-6. PubMed PMID: 10411913; PMCID: PMC17554.
119. Tomikawa K, Yamamoto T, Shiomi N, Shimoe M, Hongo S, Yamashiro K, Yamaguchi T, Maeda H, Takashiba S. Smad2 decelerates re-epithelialization during gingival wound healing. Journal of dental research. 2012;91(8):764-70. doi: 10.1177/0022034512451449. PubMed PMID: 22699208.
120. Shi Q, Le X, Abbruzzese JL, Peng Z, Qian CN, Tang H, Xiong Q, Wang B, Li XC, Xie K. Constitutive Sp1 activity is essential for differential constitutive expression of vascular endothelial growth factor in human pancreatic adenocarcinoma. Cancer research. 2001;61(10):4143-54. PubMed PMID: 11358838.
121. Li B, Wang JH. Fibroblasts and myofibroblasts in wound healing: force generation and measurement. J Tissue Viability. 2011;20(4):108-20. doi: 10.1016/j.jtv.2009.11.004. PubMed PMID: 19995679; PMCID: PMC2891362.
122. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer. 2008;8(8):579-91. doi: 10.1038/nrc2403. PubMed PMID: 18596824.
123. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 1989;246(4935):1306-9. PubMed PMID: 2479986.
124. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science. 1989;246(4935):1309-12. PubMed PMID: 2479987.
125. Yoshida A, Anand-Apte B, Zetter BR. Differential endothelial migration and proliferation to basic fibroblast growth factor and vascular endothelial growth factor. Growth Factors. 1996;13(1-2):57-64. PubMed PMID: 8962720.

126. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science. 1983;219(4587):983-5. PubMed PMID: 6823562.
127. Mahdavian Delavary B, van der Veer WM, van Egmond M, Niessen FB, Beelen RH. Macrophages in skin injury and repair. Immunobiology. 2011;216(7):753-62. doi: 10.1016/j.imbio.2011.01.001. PubMed PMID: 21281986.
128. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol. 1995;146(5):1029-39. PubMed PMID: 7538264; PMCID: PMC1869291.
129. Okizaki S, Ito Y, Hosono K, Oba K, Ohkubo H, Amano H, Shichiri M, Majima M. Suppressed recruitment of alternatively activated macrophages reduces TGF-beta1 and impairs wound healing in streptozotocin-induced diabetic mice. Biomed Pharmacother. 2015;70:317-25. doi: 10.1016/j.biopha.2014.10.020. PubMed PMID: 25677561.
130. Berse B, Brown LF, Van de Water L, Dvorak HF, Senger DR. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. Mol Biol Cell. 1992;3(2):211-20. PubMed PMID: 1550962; PMCID: PMC275520.
131. Pascual DW, Bost KL. Substance P production by P388D1 macrophages: a possible autocrine function for this neuropeptide. Immunology. 1990;71(1):52-6. PubMed PMID: 1698717; PMCID: PMC1384220.
132. Middleton M, Thatcher N. G- and GM-CSF. Int J Antimicrob Agents. 1998;10(2):91-3. PubMed PMID: 9716285.
133. Eming SA, Hammerschmidt M, Krieg T, Roers A. Interrelation of immunity and tissue repair or regeneration. Seminars in cell & developmental biology. 2009;20(5):517-27. doi: 10.1016/j.semcdb.2009.04.009. PubMed PMID: 19393325.
134. Leibovich SJ, Ross R. The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. Am J Pathol. 1975;78(1):71-100. PubMed PMID: 1109560; PMCID: PMC1915032.
135. Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Muller W, Roers A, Eming SA. Differential roles of macrophages in diverse phases of skin repair. J Immunol. 2010;184(7):3964-77. doi: 10.4049/jimmunol.0903356. PubMed PMID: 20176743.
136. Kim JS, Kim JG, Moon MY, Jeon CY, Won HY, Kim HJ, Jeon YJ, Seo JY, Kim JI, Kim J, Lee JY, Kim PH, Park JB. Transforming growth factor-beta1 regulates macrophage migration via RhoA. Blood. 2006;108(6):1821-9. doi: 10.1182/blood-2005-10-009191. PubMed PMID: 16705092.
137. Willenborg S, Lucas T, van Loo G, Knipper JA, Krieg T, Haase I, Brachvogel B, Hammerschmidt M, Nagy A, Ferrara N, Pasparakis M, Eming SA. CCR2 recruits an inflammatory macrophage subpopulation critical for angiogenesis in tissue repair. Blood. 2012;120(3):613-25. doi: 10.1182/blood-2012-01-403386. PubMed PMID: 22577176.
138. Nissen NN, Polverini PJ, Gamelli RL, DiPietro LA. Basic fibroblast growth factor mediates angiogenic activity in early surgical wounds. Surgery. 1996;119(4):457-65. PubMed PMID: 8644013.
139. Shiota N, Nishikori Y, Kakizoe E, Shimoura K, Niibayashi T, Shimbori C, Tanaka T, Okunishi H. Pathophysiological role of skin mast cells in wound healing after scald injury: study with mast cell-deficient W/W(V) mice. Int Arch Allergy Immunol. 2010;151(1):80-8. doi: 10.1159/000232573. PubMed PMID: 19672099.
140. Murakami M, Iwai S, Hiratsuka S, Yamauchi M, Nakamura K, Iwakura Y, Shibuya M. Signaling of vascular endothelial growth factor receptor-1 tyrosine kinase promotes

rheumatoid arthritis through activation of monocytes/macrophages. Blood. 2006;108(6):1849-56. doi: 10.1182/blood-2006-04-016030. PubMed PMID: 16709927.
141. Murakami M, Zheng Y, Hirashima M, Suda T, Morita Y, Ooehara J, Ema H, Fong GH, Shibuya M. VEGFR1 tyrosine kinase signaling promotes lymphangiogenesis as well as angiogenesis indirectly via macrophage recruitment. Arterioscler Thromb Vasc Biol. 2008;28(4):658-64. doi: 10.1161/ATVBAHA.107.150433. PubMed PMID: 18174461.
142. Muramatsu M, Yamamoto S, Osawa T, Shibuya M. Vascular endothelial growth factor receptor-1 signaling promotes mobilization of macrophage lineage cells from bone marrow and stimulates solid tumor growth. Cancer research. 2010;70(20):8211-21. doi: 10.1158/0008-5472.CAN-10-0202. PubMed PMID: 20924106.
143. Kerber M, Reiss Y, Wickersheim A, Jugold M, Kiessling F, Heil M, Tchaikovski V, Waltenberger J, Shibuya M, Plate KH, Machein MR. Flt-1 signaling in macrophages promotes glioma growth in vivo. Cancer research. 2008;68(18):7342-51. doi: 10.1158/0008-5472.CAN-07-6241. PubMed PMID: 18794121.
144. Stockmann C, Kirmse S, Helfrich I, Weidemann A, Takeda N, Doedens A, Johnson RS. A wound size-dependent effect of myeloid cell-derived vascular endothelial growth factor on wound healing. The Journal of investigative dermatology. 2011;131(3):797-801. doi: 10.1038/jid.2010.345. PubMed PMID: 21107350.
145. Jacobi J, Tam BY, Sundram U, von Degenfeld G, Blau HM, Kuo CJ, Cooke JP. Discordant effects of a soluble VEGF receptor on wound healing and angiogenesis. Gene Ther. 2004;11(3):302-9. doi: 10.1038/sj.gt.3302162. PubMed PMID: 14737090.
146. Folkman J. Fundamental concepts of the angiogenic process. Curr Mol Med. 2003;3(7):643-51. PubMed PMID: 14601638.
147. Folkman J. Angiogenesis and proteins of the hemostatic system. J Thromb Haemost. 2003;1(8):1681-2. PubMed PMID: 12911577.
148. Folkman J. Angiogenesis inhibitors: a new class of drugs. Cancer Biol Ther. 2003;2(4 Suppl 1):S127-33. PubMed PMID: 14508090.
149. Folkman J. Angiogenesis and apoptosis. Semin Cancer Biol. 2003;13(2):159-67. PubMed PMID: 12654259.
150. Winn RK, Harlan JM. The role of endothelial cell apoptosis in inflammatory and immune diseases. J Thromb Haemost. 2005;3(8):1815-24. doi: 10.1111/j.1538-7836.2005.01378.x. PubMed PMID: 16102048.
151. Iwata A, Harlan JM, Vedder NB, Winn RK. The caspase inhibitor z-VAD is more effective than CD18 adhesion blockade in reducing muscle ischemia-reperfusion injury: implication for clinical trials. Blood. 2002;100(6):2077-80. doi: 10.1182/blood-2002-03-0752. PubMed PMID: 12200369.
152. Garcia-Barros M, Paris F, Cordon-Cardo C, Lyden D, Rafii S, Haimovitz-Friedman A, Fuks Z, Kolesnick R. Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science. 2003;300(5622):1155-9. doi: 10.1126/science.1082504. PubMed PMID: 12750523.
153. Savill J, Fadok V. Corpse clearance defines the meaning of cell death. Nature. 2000;407(6805):784-8. doi: 10.1038/35037722. PubMed PMID: 11048729.
154. Fadok VA, Bratton DL, Henson PM. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. The Journal of clinical investigation. 2001;108(7):957-62. doi: 10.1172/JCI14122. PubMed PMID: 11581295; PMCID: PMC200959.
155. Hess KL, Tudor KS, Johnson JD, Osati-Ashtiani F, Askew DS, Cook-Mills JM. Human and murine high endothelial venule cells phagocytose apoptotic leukocytes.

Experimental cell research. 1997;236(2):404-11. doi: 10.1006/excr.1997.3745. PubMed PMID: 9367624.
156. Dini L, Lentini A, Diez GD, Rocha M, Falasca L, Serafino L, Vidal-Vanaclocha F. Phagocytosis of apoptotic bodies by liver endothelial cells. Journal of cell science. 1995;108 (Pt 3):967-73. PubMed PMID: 7622623.
157. Desmouliere A, Redard M, Darby I, Gabbiani G. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. Am J Pathol. 1995;146(1):56-66. PubMed PMID: 7856739; PMCID: PMC1870783.
158. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society. 2008;16(5):585-601. doi: 10.1111/j.1524-475X.2008.00410.x. PubMed PMID: 19128254.
159. Leask A, Holmes A, Black CM, Abraham DJ. Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. The Journal of biological chemistry. 2003;278(15):13008-15. doi: 10.1074/jbc.M210366200. PubMed PMID: 12571253.
160. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature. 2003;425(6958):577-84. doi: 10.1038/nature02006. PubMed PMID: 14534577.
161. Alfaro MP, Deskins DL, Wallus M, DasGupta J, Davidson JM, Nanney LB, M AG, Gannon M, Young PP. A physiological role for connective tissue growth factor in early wound healing. Lab Invest. 2013;93(1):81-95. doi: 10.1038/labinvest.2012.162. PubMed PMID: 23212098; PMCID: PMC3720136.
162. Bhattacharya R, Xu F, Dong G, Li S, Tian C, Ponugoti B, Graves DT. Effect of bacteria on the wound healing behavior of oral epithelial cells. PloS one. 2014;9(2):e89475. doi: 10.1371/journal.pone.0089475. PubMed PMID: 24586806; PMCID: PMC3931835.
163. Graves DT, Nooh N, Gillen T, Davey M, Patel S, Cottrell D, Amar S. IL-1 Plays a Critical Role in Oral, But Not Dermal, Wound Healing. The Journal of Immunology. 2001;167(9):5316-20. doi: 10.4049/jimmunol.167.9.5316.
164. Willis BC, Borok Z. TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. Am J Physiol Lung Cell Mol Physiol. 2007;293(3):L525-34. doi: 10.1152/ajplung.00163.2007. PubMed PMID: 17631612.
165. Yang J, Liu Y. Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis. Am J Pathol. 2001;159(4):1465-75. doi: 10.1016/S0002-9440(10)62533-3. PubMed PMID: 11583974; PMCID: PMC1850509.
166. Shook D, Keller R. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. Mech Dev. 2003;120(11):1351-83. PubMed PMID: 14623443.