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TITOLO TESI

The role of p66Shc knockout in a murine model of diabetic ulcers and peripheral ischemia

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[…
A l'alta fantasia qui mancò possa;
ma già volgeva il mio disio e 'l velle,
sì come rota ch'igualmente è mossa,
l'amor che move il sole e l'altre stelle.]

Dante Alighieri, Commedia, Paradiso XXXIII, 143-146

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Most used abbreviations

ROS: reactive oxygen species

NO: nitric oxide

wt: wild-type

p66Shc KO: p66Shc knockout

PTP: permeability transition pore

ser36 = serine 36

AGE: advanced glycation end products

STZ: streptozoticin

EPCs: Endothelial Progenitor Cells.

NADPH: nicotinamide adenine dinucleotide phosphate

PKC: protein kinase C

GSH: glutathione

PARP: poly(ADP-ribose) polymerase

GAPDH: glyceraldehyde-3 phosphate dehydrogenase

MAP kinases: mitogen-activated protein kinases

CVDs: cardiovascular diseases

hpf: high-power fields

I. Riassunto

Presupposti: Nel diabete mellito l'arteropatia periferica e la ritardata guarigione delle ferite rappresentano una emergenza sanitaria ed un notevole costo sociale. I meccanismi molecolari alla base di queste complicanze sono complessi ma possono essere ascritti alle *pathway* che interessano tutte le complicanze che si verificano nel diabete: aumentata via dei polioli, attivazione della protein chinasi C (PKC), aumentata formazione di prodotti di glicazione avanzata (AGE) e aumentata via delle esosamine. Tutti questi meccanismi, comunque, sembrano essere attivati da un'eccessiva produzione di radicali liberi e specie ossigeno reattive (ROS).

p66Shc, proteina codificata dal gene ShcA, è un fine modulatore dello stato *redox* intracellulare ed un regolatore la lunghezza della vita: topi *knockout* per p66Shc hanno un'estensione di circa il 30% della lunghezza della vita e sono resistenti al danno indotto da stress ossidativo. La scoperta dell'attività *redox* di p66Shc ha, inoltre, confermato che i ROS rappresentano una importante via di segnale intracellulare, con effetti anche a livello sistemico nel regolare il metabolismo energetico. Diversi lavori hanno dimostrato che p66Shc, contribuendo alla produzione ROS, è coinvolta nella patogenesi di diverse complicanze diabetiche come disfunzione endoteliale, glomerulopatia indotta da AGE, cardiomiopatia diabetica ma anche nel danno da ischemia/riperfusione.

Materiali e metodi: Il diabete è stato indotto mediante una singola iniezione intraperitoneale di streptozotocina in topi Sv129; gli animali sono considerati diabetici dopo 4 settimane con glicemia $\geq$ 300 mg/dl. L'ischemia periferica è stata ottenuta mediante rimozione chirurgica del fascio vascolare femorale. I topi *wild type* (wt) e p66Shc *knockout* (KO) sono stati suddivisi nei seguenti gruppi sperimentali: non diabetici non ischemici, non diabetici ischemici, diabetici non ischemici e diabetici ischemici. In ciascun animale è stata praticata un'ulcera di 4mm sull'arto posteriore, monitorandone il tempo di guarigione. Campioni di ulcere a metà tempo di chiusura sono stati prelevati per analizzare la maturazione del tessuto di granulazione, l'apoptosi, la risposta vascolare e l'espressione di β-catenina e c-*myc*. Fibroblasti isolati da espianti cutanei sono stati utillizati in un modello di migrazione in vitro in condizione di iperglicemia e ipossia. I muscoli ischemici sono stati analizzati per apoptosi e risposta angiogenica, mentre la perfusione degli arti inferiori è stata monitorata con *laser-doppler*. Campioni di cute sono stati prelevati nei vari gruppi di animali per quantificare pentosidine nitrotirosine. In lisati di cute e di fibroblasti in vitro è stata valutata l'espressione di p66Shc. La quantifi-

cazione dei progenitori endoteliali (EPCs), caratterizzati come cellule $CD34^+/Flk\text{-}1^+$, è stata effettuata su campioni di sangue periferico prelevati 4 giorni dopo l'ischemia.

Scopo della tesi: lo scopo della tesi è studiare il contributo del *knockout* di p66Shc nel processo di guarigione di ulcere cutanee in un modello murino di diabete ed ischemia periferica.

Risultati e discussione: diabete ed ischemia periferica aumentano significativamente il tempo di guarigione delle ulcere in topi *wt*, ma il *knockout* di p66Shc ha un effetto protettivo: topi p66Shc KO mostrano una guarigione più veloce rispetto topi *wt* soprattutto in condizione di diabete associato ischemia. In topi *wt* con diabete ed ischemia il tessuto di granulazione è più sottile, riepitelizzazione e densità capillare sono ridotte, aumenta l'apoptosi e aumentano l'espressione di β-catenina e c-*myc* nella cute, mentre il *knockout* di p66Shc migliora favorevolmente questi parametri. Iperglicemia ed ipossia riducono le proprietà migratorie di fibroblasti cutanei *wt in vitro*, mentre fibroblasti p66Shc KO hanno risposte migratorie più efficaci. In accordo con questi dati, il *knockout* di p66Shc riduce in topi diabetici la formazione di pentosidine (*marker* di glicazione avanzata) e nitrotirosine (*marker* di stress ossidativo) nella cute.

La risposta all'ischemia, analizzata nei muscoli è migliore in topi p66Shc KO rispetto a *wt*: il *knockout* di p66Shc riduce apoptosi, infiammazione e aumenta la risposta angiogenica post-ischemica, stranamente di più in animali diabetici. La mobilizzazione di progenitori endoteliali (EPCs) dal midollo, stimolata dall'ischemia in topi *wt*, è annullata in topi *wt* diabetici. Nei topi p66Shc KO non si ha mobilizzazione di EPCs in risposta all'ischemia, nè in presenza nè in assenza di diabete. Questo dato potrebbe suggerire che la risposta compensatoria endogena dei muscoli p66Shc è sufficiente e non rischiede l'apporto di sorgenti cellulari "esterne" come le EPCs. In alternativa, i ROS che si producono in seguito all'ischemia potrebbero essere ridotti dal *knockout* di p66Shc, riducendo di conseguenza il segnale di mobilizzazione. Il *signaling* angiogenico legato a p66Shc richiede sicuramente indagini ulteriori.

Nel complesso, la delezione di p66Shc riduce lo stress ossidativo e la formazione di AGE nella cute diabetica, migliorando il tempo di guarigione delle ulcere. Questo è associato a ridotta apoptosi, una miglior maturazione del tessuto di granulazione e migliori proprietà migratorie dei fibroblasti. Concordemente, l'espressione di p66Shc è aumentata nella cute di topi *wt* diabetici ed in fibroblasti esposti ad ipossia ed iperglicemia.

II. Abstract

Background: in the settings of diabetes mellitus, peripheral vascular disease and delayed wound healing represent a real health threat and a social cost. The underlying molecular mechanisms are complex but they mirror the pathway of other diabetic complications: augmented polyol pathway, protein kinase C (PKC) activation, advanced glycation end (AGE) products formation and increases flux in the hexoxamine pathway. The common precursor of these pathways seems to be an increased production of free radicals and reactive oxygen species (ROS).

p66Shc, the product of gene ShcA, finely tunes intracellular redox status and regulates lifespan: p66Shc knockout mice showed 30% increase in lifespan and had increased resistance to oxidative stress. The discovery of p66Shc substantiated the role of ROS as pivotal intracellular signaling molecules with effects spanning over systemic metabolism. Several works demonstrated that p66Shc is linked to the development of diabetic complications such as AGE-induced glomerulopathy, endothelial dysfunction, diabetic-heart failure and ischemic-injury.

Aim of the thesis: This thesis aims to study the role of p66Shc in a murine model of diabetic-ischemic ulcers.

Material and methods: diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) in Sv129 mice. Animals were considered diabetic after 4 weeks of glycemia ≥ 300 mg/dl. Peripheral ischemia was achieved by surgical dissection of femoral artery and vein. We created four groups of animals both for wild-type (wt) and p66Shc knockout (KO) mice: non-diabetic non-ischemic, non-diabetic ischemic, diabetic non-ischemic and diabetic ischemic. Animals underwent hindlimb ulceration with a 4 mm-diameter biopsy punch and we quantified wound healing time. Wound at mid-closure time were harvested to analyze granulation tissue maturation, apoptosis, vascular development and c-*myc* and β-catenin expression. Skin fibroblasts were used to assess migration features in vitro under hypoxia and hyperglycemia. Ischemic muscles were analyzed for apoptosis, vascular responses and, by means of laser-doppler, for perfusion. Nitrotyrosine and pentosidine were quantified in skin samples, while in skin fibroblasts lysates we quantified p66Shc protein expression. Endothe-

lial progenitor cells (EPCs), defined as $CD34^{+}/Flk\text{-}1^{+}$ cells, were counted in peripheral blood 4 days after ischemia.

Results and discussion:diabetes and ischemia increased wound healing time in wt mice; p66Shc KO mice are, instead, protected from the detrimental effects of diabetes and ischemia on wound healing. Wt mice showed impaired granulation tissue thickness, reduced reepithelialization, poor vascular development and augmented apoptosis. Moreover, diabetic-ischemic ulcers had increased expression of β-catenin e c-myc, which is compatible with impaired healing. The knockout of p66Shc was associated with strong improvement in all these parameters. Wt fibroblasts exposed in vitro to high glucose and hypoxia had dampened migration features while p66Shc KO fibroblasts had improved migration. Accordingly, the knockout of p66Shc reduced nitrosityrosine (a marker of oxidative stress) and pentosidine (a marked of advanced glycation) concentration in the skin of diabetic mice.

Ischemia had a favorable outcome in skeletal muscle of p66Shc KO mice, especially in the settings of diabetes, with reduced apoptosis, inflammation and increased post-ischemic angiogenesis compared to wt. Ischemia triggered EPCs mobilization in wt mice but not in wt-diabetic mice. On the other hand, p66Shc KO mice mobilized EPCs neither without diabetes nor with diabetes. These data suggest that improved recovery of p66Shc KO muscles from ischemia reduced the stimulus for mobilization; moreover, reduced ROS production due to knockout of p66Shc could result in reduced bone marrow mobilization. p66Shc angiogenic signaling surely requires further investigation.

Collectively, the knockout of p66Shc reduced oxidative stress and AGE formation in the skin of diabetic mice, which also showed improved wound healing. These data are associated with reduced apoptosis, better granulation tissue maturation and improved migration features of fibroblasts. By consent, p66Shc expression is increased in the skin of diabetic wild-type mice and in fibroblasts exposed to hyperglycemia and hypoxia.

III. Introduction

1. The Skin

The skin, the largest organ in the body, is an indispensable barrier to protect the organism from several threats such as infections, temperature changes, mechanical injuries and dehydration. Besides the protective functions, the skin is a complex organ in which cellular and molecular events finely regulate responses to our environment. The skin is divided into epidermis, the outmost layer and dermis, the lowermost. (Fig. 1).

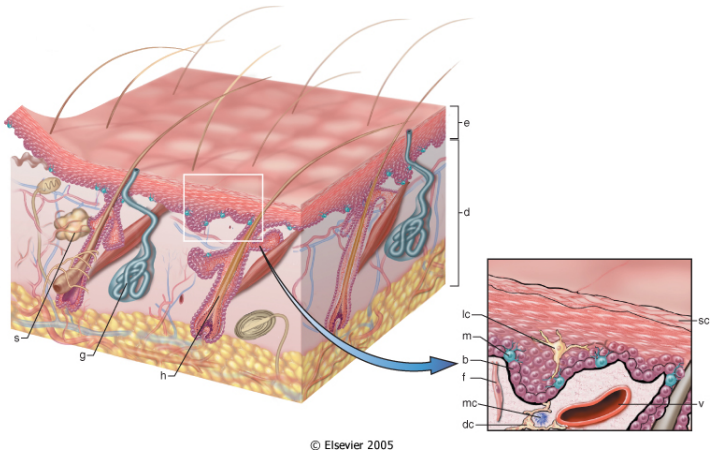


Figure 1: Schematic representation of human skin: The skin is composed of an epidermal layer (e) from which specialized adnexa (hair follicles, h; sweat glands, g; and sebaceous glands, s) descend into the underlying dermis and hypodermis (d). *The* projection of the epidermal layer (e) and underlying superficial dermis demonstrates the progressive upward maturation of basal cells (b) into cornified squamous epithelial cells of the stratum corneum (sc). Melanin-containing dendritic melanocytes (m) (and rare Merkel cells containing neurosecretory granules) and midepidermal dendritic Langerhans cells (lc) are also present. The underlying dermis contains small vessels (v), fibroblasts (f), perivascular mast cells (mc), and dendrocytes (dc), potentially important in dermal immunity and repair (from Robbins & Cotran, Pathologic Basis of Disease).

1.1. The Epidermis

The epidermis comprises a physical, a chemical/biochemical (antimicrobial, innate immunity) and an adaptive immunological barrier [1]. The physical barrier consists of the *stratum corneum*, which is a continuous sheet of protein-enriched cells called corneocytes. Corneocytes represent the final step in kearatinocytes differentiation (keratinization), where keratin accumulation leads to flat, anucleated cells. Keratin, in corneocytes, is organized into tight bundles because of the aggregation with filaggrin, another matrix protein. Corneocytes are connected by desmosomes (called corneodesmosomes) and embedded in a matrix enriched in

non-polar lipids organized in lamellar layers, which are produced by keratinocytes and released into intercellualr space by lamellar bodies [1]. Furthermore, the exterior of these cells is surrounded with a cornified cell envelope, that is a tough protein/lipid polymer structure. The corneocytes confer mechanical and chemical protection and, along with the lipid surroundings, water impermeability [1]. The nucleated cells of the epidermis are also crucial in barrier function, because they prevent both excessive water loss and the entry of harmful substances into the skin [2].

It is widely accepted that epidermis can act as an immunological barrier [3]. The so-called "epidermal immune unit", is made up of i) Langherans cells, ii) keratinocytes and iii) epidermotropic T-cells. Langheran cells derive from the monocytic lineage of the bone marrow and are the major antigen-presenting cells of the skin [3]; they present antigens in the context of class II major histocompatibility complex (MHC) molecules to T-cells. Keratinocytes have a role in prompting cell-mediated immune responses releasing cytokines, mostly interleukin-1α (IL-1α), IL-1β and tumor necrosis factor-α (TNF-α) [3, 4]. Moreover, after the stimulation with interferon γ (IFN-γ), they transiently express MHC class II molecules, even if the exact role of this phenomenon is unknown [3]. The concept that T lymphocytes home to the skin is supported by the observation that lymphocytic infiltrates within the skin differ from their concentration in the peripheral blood [5]. Finally, also melanocytes seem to have an immunological role, possessing dendritic features and secreting cytokines [3].

1.2. The Dermis

The dermis is a dense but irregular connective tissue with fibroblasts, adipose cells and macrophages. It is responsible for most of the structural strength of the skin and with its extended vascular network, it provides for the metabolic needs of the up-lying epidermis. The extracellular matrix is mainly composed of type I collagen, but elastin and reticular fibers are also present. The dermis is divided into two layers: the deeper is called reticular layer while the superficial one is called papillary layer. The reticular layer, the main fibrous layer of the dermis, is made of irregularly arranged fibers of elastin and collagen, which give to the skin its stretch-resistance feature. The papillary layer derives its name from projections called *papillae* that extend toward the epidermis. This layer is more cellular than the reticular layer, but contains fewer fibers, which are loosely arranged. Papillae features lymphatic and blood vessels, which supply the avascular epidermis with nutrients, remove waste products and con-

tribute to regulate body temperature [6]. The dermis includes several specialized *adnexa* such as hair follicles, sweat glands, sebaceous glands and nerve endings.

1.3. The Hypodermis

The hypordemis is not part of the skin but has the important function of arranging the dermis over the underlying structures. Moreover, the reticular layer of the dermis interdigitates with the hypodermis, making a clear-cut of the two layers difficult. It features lax connective tissue with abundant adipose cells, which confers insulation, padding and energy storage. The nerves and the vascular network of the hypodermis supply also the dermis [6].

2. Wound healing

Given the critical role of the skin as a protective barrier against outside hazards, any break in must be rapidly and efficiently repaired. Wound healing is a sequence of overlapping but tightly regulated steps: i) coagulation & inflammation, ii) new tissue formation and iii) remodeling. It is surprising that, despite the extreme complexity of the wound repair process, it rarely becomes uncontrolled, and malignant transformation is an infrequent event in the wound [7, 8]. Remarkably, in some eukaryotes the injury triggers a still poorly understood process known as regeneration, which could recapitulate the original tissue architecture. How it occurs and why humans lose this ability is unknown [9]. Studying wound healing in those eukaryotes, but also in embryos, where the repair is fast and efficient, close to a perfect regeneration of the tissue, could help to figure out how damaged skin can be reconstructed more precisely and improve our knowledge about pathological alteration of wound healing [8, 10].

2.1. Coagulation & Inflammation

This stage occurs immediately after tissue damage and it activates the components of the coagulation cascade, inflammatory pathways and immune system in order to reduce blood and fluid loss, remove dead and devitalized tissues and prevent infections (fig. 2). During the early inflammatory phase, blood vessel disruption triggers platelets aggregation and blood coagulation. The formation of the platelet plug, followed by a fibrin matrix composed of fibronectin, vitronectin and thrombospondin, serves as temporary shield for the denuded tissues; moreover it provides a scaffold for the infiltrating cells and a reservoir of cytokines and growth factors, released from the degranulating platelets, that prompt the following phas-

es of the wound closure process [8, 10, 11]. Within a few minutes after the injury, neutrophils gather in at the site of the wound [12], attracted by activation of the complement, the degranulation of platelets and the products of bacterial degradation [13]. They are the first line of defense against infection, phagocyting and killing microorganisms by means of oxygen radicals [14]. After 2-3 days, monocytes reach the wound and differentiate into macrophages. They act as strong phagocytes, clearing the wound from matrix and cell debris, but also from exhausted neutrophils [15]. Platelets, neutrophils and macrophages but also damaged blood vessels, secrete a plethora of mediators such as cytokines, growth factors and small molecules [11, 16]. They include, but are not limited to, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), IL-10, IL-6, ATP, adenosine [11, 17]. These factors sustain and propagate the inflammatory response but are also crucial to the other phases of the healing process such as angiogenesis, matrix deposition, scarring and reepithelialization [8, 11, 16-18]. After injury, the wound is a hypoxic environment, however, hypoxia increases keratinocyte migration, early angiogenesis, the recruitment of endothelial progenitors, the proliferation of fibroblasts and the synthesis of growth factors and cytokines [19].

A review from Martin and Leibovich analyzed the role of each immune cell that is recruited at the wound site, starting from pioneering experiments from the 70's, where depletion of macrophages resulted in a retarded wound healing [15, 20]. However, recent experiments with transgenic models questioned the fear that interfering with inflammatory responses at the wound site could stop the repair process: a selective depletion of each actor of the inflammatory response and a complete deletion of all the inflammatory cells during wound healing, did not dampen the repair process [16]. However, the inflammatory response is very complex and these findings urge for deeper investigations.

Within hours after the injury, reepithelialization begins with epidermal cells, which undergo marked phenotypic alteration: the dissolution of hemidesmosomes and the formation of peripheral actin filaments unchain cells from basament membrane, allowing migration [21]. Integrin receptors are very important for cell migration in this phase because, driving the movement of epidermal cells, they allow to dissect the wound, separating the viable tissue from the dessiccated eschar [22].

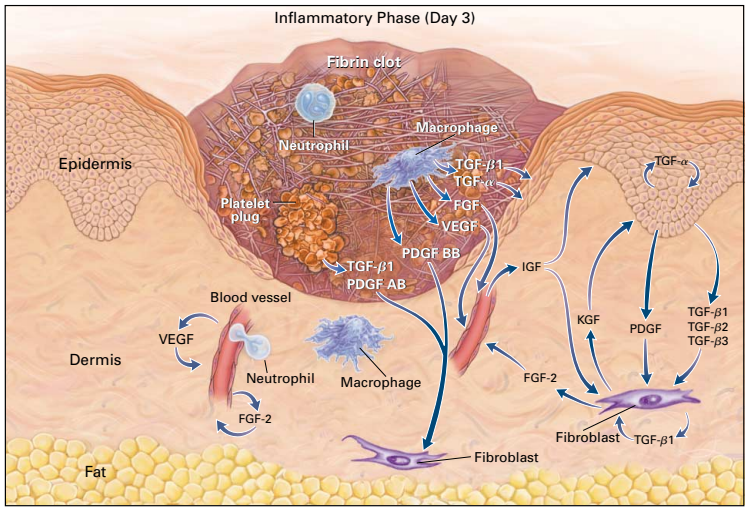


Figure 2: Wound healing: inflammatory phase. During the inflammatory phase macrophages and neutrophils are recruited to the wound site. A fibrin clot entraps the platelet plug and serves as a reservoir of growth factors. At the same time, keratinocyte migration begins. TGF-β1, TGF-β2, and TGF-β3 denote transforming growth factor β1, β2, and β3, respectively; TGF-α transforming growth factor α; FGF fibroblast growth factor; VEGF vascular endothelial growth factor; PDGF, PDGF AB, and PDGF BB platelet-derived growth factor, platelet-derived growth factor AB, and platelet-derived growth factor BB, respectively; IGF insulin-like growth factor; and KGF keratinocyte growth factor (from Singer and Clark, 1999).

2.2. New Tissue Formation

Approximately four days after wounding, new stroma begins to invade the wound area. Due to its granular appearance, is called "granulation tissue", which is composed of matrix-synthesizing fibroblasts and capillaries. Macrophages, at this stage, represent a continuos source of growth factors, which support fibroplasia and angiogenesis (fig. 3).

PDGF and TGF-β1 stimulate fibroblast proliferation, the expression of appropriate integrin receptors to interact with extracellular matrix molecules and migration into the wound space [23]. The provisional matrix is composed of fibrin, fibronectin and hyaluronic acid and provides the scaffold for cell migration [21]. Fibroblasts clear a path into the matrix secreting proteoltic enzymes including plasminogen activator, collagenases, gelatinase A and stromelysin (all belonging to the matrix metalloproteinases family) [21]. The products of matrix digestion contributes to the recruitment of peripheral blood monocytes, followed by their activation to macrophages. Once they have migrated into the wound, fibroblasts begin to synthesize a collagenous extracellular matrix which replace the provisional one [24]. The primary collagen type deposited in the wound bed are type I and type III, with initial increased amounts of type III during the first 3-4 days, after which there is a rapid increase of type I collagen [22]. TGF-β1 and PDGF are key molecules that regulate collagen deposition and there-

fore may be potential targets for therapeutical interventions. At the same time, new capillaries endow the granulation tissue. Acidic and basic FGFs produced by macrophages and endothelial cells seem to induce the angiogenic process during the first three days after wounding, subsequently the stage is taken by VEGF, produced by activated epidermal cells, which drive angiogenesis during the formation of granulation tissue [25]. The interaction between endothelial cells and extracellular matrix (via integrins) finely tunes the migration and proliferation of endothelial cells during angiogenesis; microvascular endothelial cells adjacent to and within the wounds transiently deposit increased amount of fibronectin within the vessel wall to prompt the migration of endothelial cells into the wound [26].

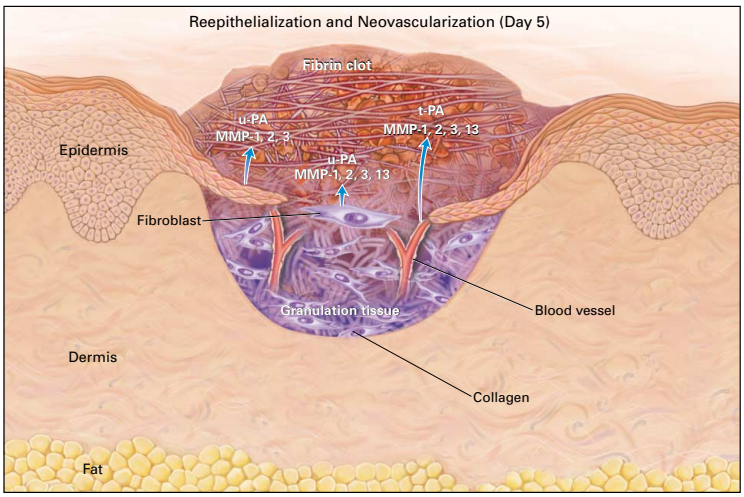


Figure 3: Wound healing: proliferation and new tissue formation. While the inflammatory response begins to fade, fibroblasts migrate into the wound synthesizing collagen and new vessels sprouts into the wound. These events lead to the formation of granulation tissue. Parallelly, epidermal cells cover the wound. u-PA denotes urokinase-type plasminogen activator; MMP-1, 2, 3, and 13 matrix metalloproteinases 1, 2, 3, and 13 (collagenase 1, gelatinase A, stromelysin 1, and collagenase 3, respectively); and t-PA tissue plasminogen activator (from Singer and Clark, 1999).

Moreover, the endothelial cells themselves secrete plasminogen activator and procollagenese, which activate plasminogen and collagenase respectively. These proteases digest the basement membrane, releasing endothelial cells from structural constrains, allowing migration and new vessel formation [21]. Recently, the discovery of endothelial progenitor cells (EPCs) boosted the investigation on wound healing because these cells seem to be recruited to the wound site, where they contribute to new vessels formation [27, 28], even if the contribute seems to be small, in non pathological conditions [29].

Once the new collagen-rich matrix has completely filled the wound, the angiogenic response begins to fade, and a lot of the new blood vessels disintegrate as a result of apoptosis [30]. In

the later part of this phase fibroblasts differentiate into myofibroblasts [31, 32], accumulating large bundles of actin-containing microfilaments and interacting with surrounding cells and matrix [21, 32]. This process is driven, at least in part, by PDGF and TGF-β1 [21, 32]. With the appearance of myofibroblasts, wound edges start contracting and the connective tissue begins to compact.

2.3. Tissue Remodeling

Tissue remodeling starts as collagen deposition ends; meanwhile, reepithelialization ensues and basement membrane proteins reappear in a very ordered sequence, from the margin to the center of the wound, in a zipper-like fashion [33]. During the transition from granulation tissue to scar, collagen remodeling relies upon continued synthesis and the catabolism of collagen at low rate. This stage begins 2-3 weeks after injury and may last for a year or more [8]. During this process the matrix composition shifts from a mostly type III collagen backbone to one predominately composed of type I collagen [8]. This process is superbly orchestrated by growth factors such as PDGF, TGF-β, EGF and FGF [22]. The degradation of collagen is controlled by matrix metalloproteinases, which are secreted by macrophages, epidermal cells, endothelial cells and fibroblasts [21]. In the first three weeks, wounds gain only 20 percent of their final strength. Thereafter, wounds slowly gain tensile strength, even though they never attain the same breaking strength as uninjured skin, reaching about 60-70 percent of the strength of normal skin [19, 34].

3. Wound Healing in Diabetes: The Role of Oxidative Stress

Hindlimb ulceration and impaired wound healing represent a serious threat to diabetic patients' life and a striking clinical and social problem [35, 36]. Foot ulcers affect about 15% of diabetic patients and with an annual amputation rate of about 1%, they are the leading cause of non-traumatic lower extremity amputation [37]. Chronic ulcers are associated with profound disabilities and strongly reduce patients' quality of life [38]. Moreover, after major amputation mortality rate is very high [39, 40].

The molecular and biochemical events that lead to diabetic chronic ulcers mirror the mechanisms of other chronic complications and include the polyol and hexosamine pathway, activation of protein kinase C (PKC) and mitogen-activated protein (MAP) kinases and formation of advanced glycation end products (AGE) [41]. One of the major hypotheses proposed to ex-

plain the onset of these complications is increased oxidative stress, which is now widely recognized as the common precursor of all these alterations [42-45].

3.1. Oxidative Stress

Free radicals are chemical species that have a single unpaired electron in their outer orbit, which makes them highly reactive. Oxidative stress is defined as the imbalance between the production of free radicals and the system's ability to scavenge these reactive products or to readily repair tissue damage. There are two major types of free radicals: reactive oxygen species (ROS) and reactive nitrogen species [46]. The nitric oxide radical (NO•) is produced by the oxidation of L-arginine by the enzyme nitric oxide synthase (NOS).

ROS are produced by enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, cyclooxigenases and xantine oxidases, but the vast majority of cellular ROS (about 90%) can be traced back to the mitochondria [46, 47]. In cells lacking mitochondria and NADPH oxidase (i.e. erythrocytes), a metal-catalyzed glucose autoxidation mechanism in the production of ROS has been proposed, but all data comes only from in vitro models [48]. The product of these reactions is the superoxide anion ($\bullet O_2$-), which could be non-enzymatically converted into hydrogen peroxide (H_2O_2). In the presence of reduced transition metals (e.g., ferrous or cuprous ions) hydrogen peroxide can be converted into the highly reactive hydroxyl radical (•OH). Alternatively, a plethora of antioxidant compounds are available to detoxify these reactive molecules: superoxide dismutase, catalase, glutathione peroxidase as well as nonenzymic compounds such as α-tocopherol, β-carotene, ascorbate and glutathione [46]. The effects of ROS are wide-ranging due to their high reactivity, but three reactions are particularly relevant for cell injury: i) lipids peroxydation, ii) oxidative modification of proteins, and iii) lesions in DNA. Free radicals, ROS in particular, have always been seen as by-products of metabolic pathways or toxic compounds, of which it was necessary to get rid before cell injury occurs; recent evidence, however, suggests that ROS, and especially H_2O_2 with its ability to induce fully reversible protein modifications, are actually important second messengers, regulating intracellular signaling pathways and gene expression [46, 49, 50].

3.2. ROS in diabetes

Oxidative stress propagation in diabetes is due to two distinct but overlapping mechanisms: increased ROS production and depletion of antioxidants.

Diabetes potently increases ROS production in mitochondria, changing the site where ROS are generated: electron transport chain perturbation shifts the site of production of ROS from complex I and the ubiquinone-complex III interface, to the complex II. [48]. Moreover, mitochondrial ROS seem to increase PKC activity, AGE formation and sorbitol accumulation [51-53]. Indeed, the overexpression of uncoupling protein 1 (UCP1), which facilitates proton leakage across the mitochondrial membrane, dissipating high membrane potential and thus limiting respiration rate, is sufficient to reduce PKC and nuclear factor-kappa B (NF-kB) activation, hexoxamine pathway activation, AGE formation, sorbitol accumulation and glyceraldehyde-3-phosphate dehydrogenase inhibition [51, 52].

Since the discovery of nonphagocytic isoforms of NADPH oxidase, several reports demonstrated that this enzyme is an alternative major source of ROS in diabetes, especially in vasculature and kidney [48, 54, 55]. NADPH oxidase is activated by PKC, which in turn is stimulated by ROS, creating a vicious circle that propagate oxidative stress [48].

Xanthine oxidase has a an undetectable activity in normal cells, but it has been proposed to be a major source of ROS in chronic disease such as atherosclerosis and diabetes [56], although conflicting data also exits [57].

Several antioxidants are reduced in the settings of diabetes (also by the means of ROS themselves), impairing cells' ability to cope with increased oxidative damage. Glutathione (GSH) is the primary intracellular free radical scavenger, but it is also an essential cofactor for several enzymes. There are clinical and experimental data that show a reduction of GSH levels in association with diabetes [48], but direct evidence in experimental models is still lacking. GSH is the cofactor for two antioxidant enzymes namely glutathione reductase and glutathione peroxidase. The first uses NADPH to reduce oxidized GSH replenishing the antioxidant reservoir of the cell; however, there is no clear evidence that diabetes affects the activity of this enzyme [48]. Glutathione peroxidase uses two molecules of GSH to reduce oxidants and, due to the prominent role as ROS scavenger this class of enzymes has been extensively studied. It is generally agreed that glutathione peroxidase activity is reduced in diabetes, but the extent to which this inhibition affects cell health is still uncertain [48].

Aldose reductase, despite not being an antioxidant enzyme, could indirectly increase the burden of oxidative stress. It is the first enzyme in the polyol pathway, converting aldose sugar to their respective alcohols at the expenses of NAPDH. Aldose reductase activation, which is reported in diabetes, causes NADPH depletion reducing GSH reservoir and, therefore, impairing cell's ability to face oxidative stress. Moreover, aldose reductase activity leads to the accumulation of potentially harmful by-products, such as alcohols [48].

There is supporting evidence that in the setting of diabetes superoxide dismutase and catalase are inhibited and vitamin C and E display reduced bioavailability [48]. Mitochondrial ROS production, however, seems to be generally the primary site of ROS production, and, according to Brownlee, the *primum movens* of diabetic complications [42]. ROS production primary targets DNA causing strand breaks. This activates poly(ADP-ribose) polymerase (PARP), which splits nicotinamide adenine dinucleotide (NAD^+) into its two components parts: nicotinic acids and ADP-ribose. The latter is used to make polymers of ADP-ribose to repair DNA breaks. PARP activity could affect also proteins, which could be modified by the accumulation of polymers of ADP-ribose. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH), a key enzyme for glycolysis, shuttles between nucleus and cytoplasm. PARP could modify also GAPDH, thus reducing its activity. This step is crucial because the reduction of GAPDH activity interrupts glycolysis, leading to intermediate accumulation and activating four major pathways involved in diabetic complications: glyceraldehyde-3 phosphate induces PKC activation and predisposes AGE accumulation; fructose-6 phosphate activates the hexoxamine pathway while glucose enters the polyol pathway [42] (fig. 4).

These pathways are overlapping and interconnected, and this creates vicious circles that propagate stress responses.

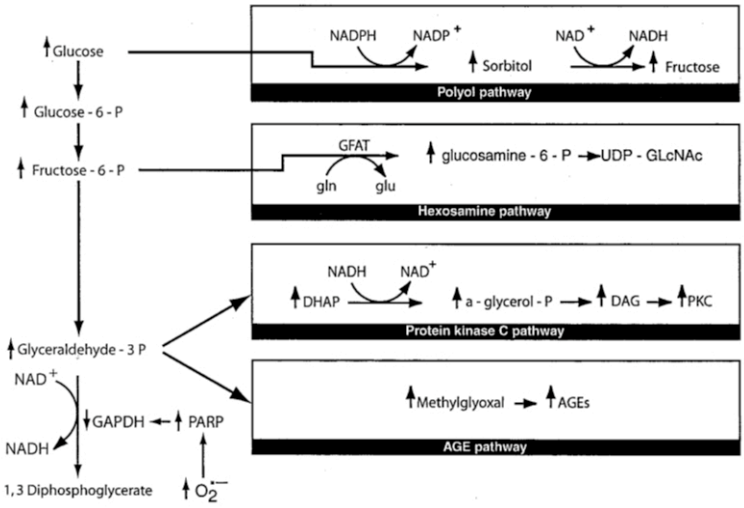


Figure 4: Unifying hypothesis of ROS-induced mechanism of cellular damage in diabetes. ROS

cause DNA strand breaks. PARP activation repairs DNA but could also modify protein such as GAPDH. This modification reduces GAPDH activity, which causes accumulation of intermediates and activation of the major pathway involved in diabetic complications (from Brownlee, 2005)

3.3. Wound Healing, ROS & Diabetes

Canonically, a set of predisposing abnormalities has been referred to the "pathogenic triad" of neuropathy, ischemia and trauma, with the urge to consider also infections, which play a major role in healing impairment, hospitalization and limb loss in diabetes [19, 40, 58]. According to clinical evidences, neuroischemic ulcers are common, if not the rule [59]. Micro- and macroangiopathy, hallmark symptoms in diabetic patients, occur early in diabetes but the molecular mechanisms are very complex. Endothelial dysfunction, which may precede the development of diabetes, is surely one of the leading causes. When endothelium loses its physiological properties, i. e. the tendency to promote vasodilation, fibrinolysis and antiaggregation, it becomes dysfunctional. Reduced nitric oxide (NO) bioavailability is one of the main causes of endothelial dysfunction in diabetes [60, 61]. Endothelial NOS (eNOS) when deprived of L-arginine or tetrahydrobiopterin (BH4) become uncoupled, diverging its activity from production of NO to the generation of $\bullet O_2^-$ and H_2O_2. Increased $\bullet O_2^-$ production further reduce NO bioavailability with the formation of peroxynitrite ($ONOO^-$), a highly reactive radical correlated with inflammation and atherosclerosis, which further worsen eNOS uncoupling [48]. Moreover, NADPH oxidase is a major source of ROS in endothelial cells [62], which contribute to NO scavenging and eNOS uncoupling in diabetes [61, 62]. Again, hyperglycemia, ROS themselves and proinflammatory cytokines secreted by adipose tissue activate PKC, which increases NADPH oxidase activity [63]. Hyperglycemia also triggers the formation of AGE, which accumulate in the vessel wall, altering the structural integrity of the basement membrane and quench NO activity [60]. Moreover, AGE are signal transduction ligands for their receptor (RAGE). RAGE is a member of the immunoglobulin superfamily of cell-surface molecules, which could activate an inflammatory-proliferative process that activates PKC, NF-kB, and NADPH oxidase [48, 60]. Recently, it has been proposed that endothelial progenitor cells (EPCs), a subset of bone marrow-derived progenitor cells, could contribute to vascular homeostasis maintaining endothelium integrity and supporting compensatory angiogenesis [64]; a dysfunction of these cells has been reported in subjects with diabetes mellitus and cardiovascular diseases [65-67]. These data have prompted to new pathophysiological hypothesis on endothelial dysfunction in diabetes [65, 68].

Neuropathy is linked to vasculopathy in diabetes, because it is tied mostly to microvascular defects [19]. Moreover, autonomic neuropathy leads to aberrant blood flow. Among the classic pathways involved in diabetic complications, oxidative stress, AGE formation are two key mechanisms which lead to nerve dysfunction [69, 70]. Additional mechanisms include a role of MAP kinases, activation of cyclooxygenase-2 and lipoxygenase, abnormal Ca^{2+} homeostasis and Na^{+}/H^{+} exchanger-1 activation [70]. With the onset of diabetic neuropathy, trauma can initiate the development of an ulcer; thereafter, reduced pain perception contributes to the pathogenesis of Charcot foot. Additional motor fiber abnormalities lead to the development of anatomical deformities and might increase the risk of infection [19, 70].

Diabetes dampens wound closure also impairing the healing process itself. The pathogenesis of chronic diabetic ulcers is complex and still unclear. The ordered and superbly orchestrated events that lead to wound healing are impaired in diabetic or chronic ulcers, because parts of the wound may have lost the synchrony that leads to healing, being stuck in different phases of the process [19]. Elevated AGE deposition in wound of diabetic mice is associated with a delayed inflammatory response [69]. AGE accumulation in the granulation tissue could severely impair migration, adhesiveness phenotype and interaction with extracellular matrix of keratinocytes and dermal fibroblasts [69]. Moreover, once again, AGE/RAGE interaction increases oxidative stress which further promotes AGE accumulation via a vicious circle. Oxidative stress is physiological at low levels in wounds, and it is useful to stimulate angiogenesis [71]. At higher concentrations, ROS induce hypoxia-inducible factor (HIF)-1α proteosomal degradation [46]; HIF-1α represents an essential factor for wound healing [72], but it is also of pivotal importance to recruit EPCs to the ischemic foci and improve angiogenesis [73-75]. Moreover, AGE and ROS affect the amount of matrix components secreted by fibroblasts, resulting in a poorly structured granulation tissue [69]. In addition, AGE interfere with the signaling network of growth factors and cytokine, such as bFGF and TGFβ, by disrupting the structure of either growth factor or receptors [69].

Collectively, clinical and experimental evidence strongly supports the pivotal role of oxidative stress in the pathogenesis of wound healing defects in diabetes.

4. p66Shc

Three ShcA genes have been found in the mammalian genome, which have been classified as ShcA, ShcB and ShcC [76]. ShcA proteins are expressed widely throughout tissues in humans

and mice but are replaced by ShcC in mature neurons [77]. ShcB gene is also expressed primarily in the brain, but its function is not characterized [76].

The ShcA gene encodes two mRNA species and three proteins: p46Shc, p52Shc, p66Shc. The p66Shc mRNA has an alternative transcription initiation from that of p46Shc and p52Shc isoforms. On the other hand, p46Shc and p52Shc arise from alternative translation site on the same mRNA [78]. These isoforms share three identical functional domains: an amino-terminal phosphotyrosine-binding domain (PTB), truncated in the p46 isoform, a central proline-rich domain (CH1) and a carboxy terminal Src homology 2 (SH2) domain. p66Shc harbors an additional N-terminal proline-rich domain (CH2) (Figure 5).

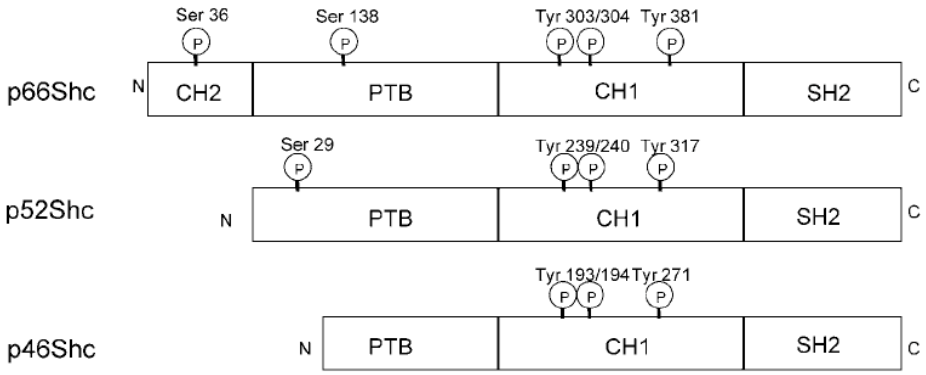


Figure 5: ShcA proteins domain scheme (from Purdom & Chen, 2003)

p46Shc and p52Shc are adaptors proteins involved in the receptor-activated MAP kinase signaling. The PTB domain of ShcA proteins could bind the phosphorylated intracellular domain of receptors such as epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR) and ErbB-2 [79]. Upon binding, CH1 domain becomes phosphorylate itself, allowing the binding of growth factor receptor-bound protein 2 (Grb2) adapter molecule, which then activates the MAP kinase pathway [77, 79]. On the other hand, p66Shc does not activate MAP kinase pathway, even though it binds phosphorylated receptors and forms stable complex with Grb2 upon tyrosine phosphorylation [80] (fig. 6). Therefore p66Shc could act as a decoy to regulate the activation of MAP kinases, as seen by Natalicchio *et al*, which showed that p66Shc through MAP kinases modulation regulated glucose transport in skeletal muscle [81].

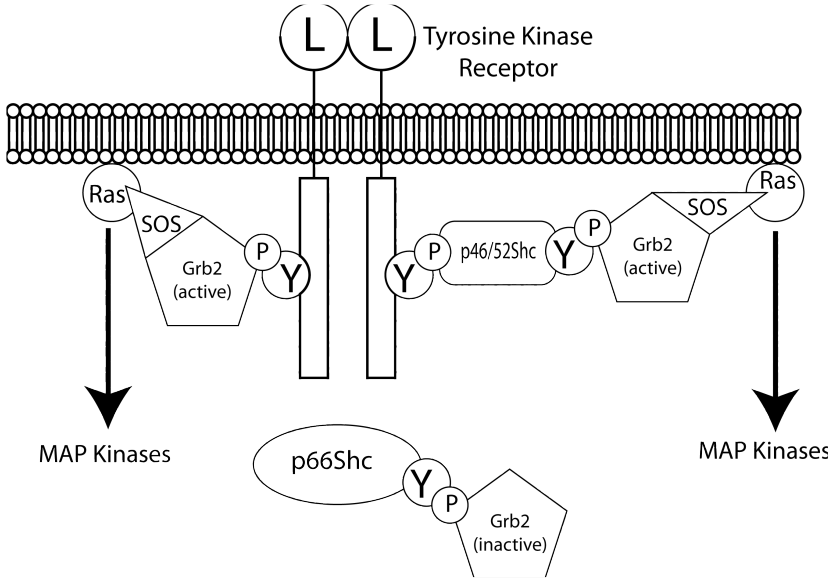


Figure 6: Differential role of ShcA proteins in tyrosine kinase receptor-mediated signaling. p46Shc and p52Shc participate in MAP kinase pathway as adaptors proteins. They bind tyrosine kinase receptors and bridge with Grb2 to transduce the signal. p66Shc binds Grb2 too, without activating the MAP kinase pathway. (adapted from Purdom & Chen, 2003).

Surprisingly, p66Shc plays a crucial role in the regulation of oxidative stress responses, apoptosis and lifespan [82]. Pelicci's group first reported that ablation of p66Shc conferred resistance to apoptosis induced by H_2O_2 or UV light and that p66Shc knockout mice had increased resistance to paraquat and 30% increase in life span [82]. This study was the first to link a deletion in single mammalian gene to prolonged longevity. Additional works from the same group and from Pinton *et al*., clarified the mechanism of action of p66Shc. p66Shc is actually a redox enzyme, which exists both in a cytosolic fraction and in a mitochondrial pool. Upon stress stimuli like oxidative stress, hyperglycemia or UV light cytosolic p66Shc becomes phosporylated on serine 36 (ser36) by activated PKC-β; thereafter, prolyl isomerase Pin1 recognizes phosphorylated p66Shc and triggers its mitochondrial accumulation [83]. Serine phosphorylation appears to be critical for cytosolic p66Shc activation [82]. Mitochondrial p66Shc localizes within the intermembrane space where it binds to a complex which includes the TIM-TOM import system and mitochondrial heat shock protein 70 (mtHSP70) [84, 85]. Both the cytosolic-activated p66Shc and oxidative stress destabilize this complex, releasing p66Shc in its activated form. Activated p66Shc oxidizes cytochrome c to generate H_2O_2 (p66Shc accounts for about one third of the total intracellular H_2O_2 pool), which triggers permeability transition pore (PTP) opening [84]. This event unleashes mitochondrial swelling and disruption with the release of pro-apoptotic factors such as cytochrome c, which finally lead to apoptosis [84, 86] (fig. 7). Gene delivery of Tim44, a component of the TIM-TOM

complex, was able to reduce increased ROS generation in vascular smooth muscle cells exposed to high glucose [87]. Moreover, p66Shc along with p53 constitutes a sensor of the levels of intracellular oxidative signals and, upon high intensity oxidative signals, it also mediates p53-induced apoptosis [88].

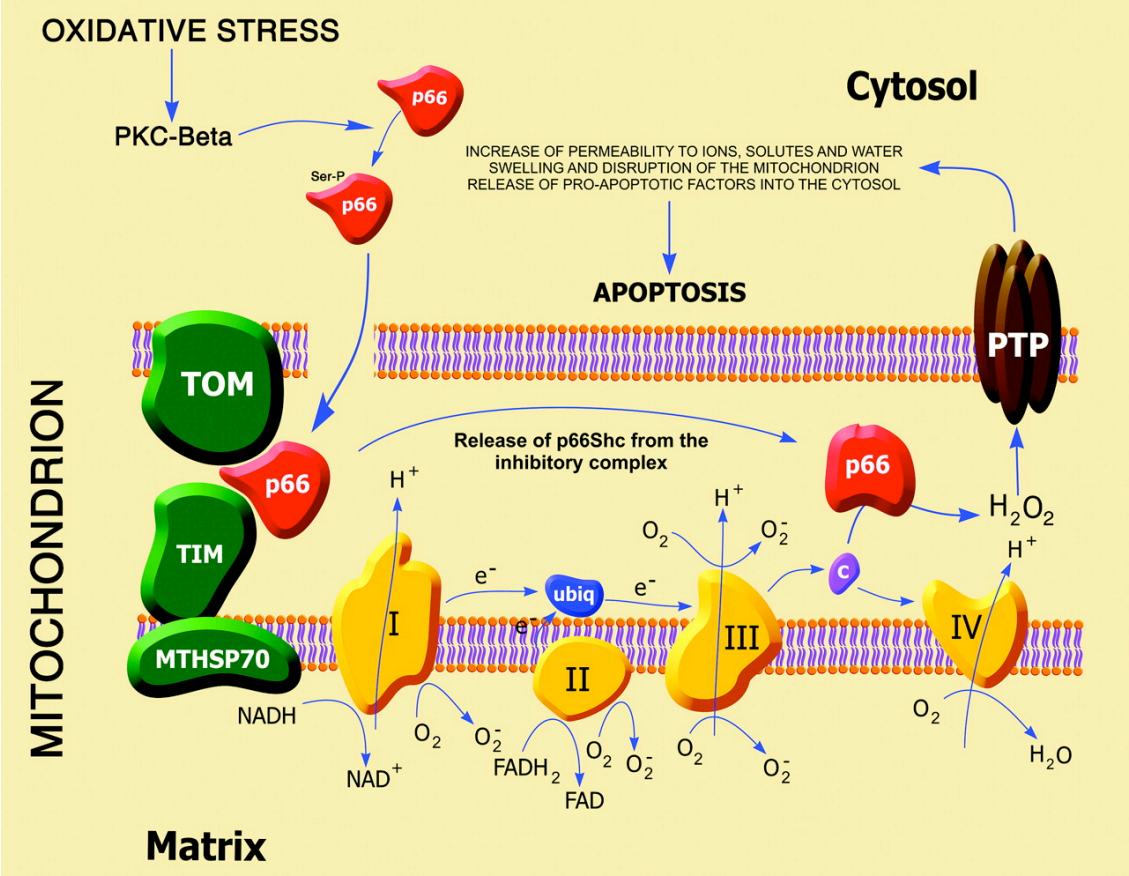


Figure 7: Proposed mechanism of action of p66Shc. p66shc in response to oxidative stress or hyperglycemia is phosphorilated by PKC at serine 36. Thereafter p66Shc translocates into the mitochondria within the intermembrane space. p66Shc could also be found anchored to the TIM/ TOM import complex. p66Shc oxidizes cytocrome c to generate H2O2 that triggers PTP opening thus inducing apoptosis (from Cosentino *et al*., 2008).

Parallelly, Nemoto and Finkel, showed that p66Shc regulate forkhead protein activity upon oxidative stress stimulation, demonstrating an important functional link between forkhead proteins that regulate longevity in *C. elegans* and p66Shc that is implicated in mammalian lifespan and intracellular oxidants, which are thought to play a role in aging across all species [89]. This concept was further remarked by a very recent paper from Berniakovich *et al*., which reported that p66Shc is part of an adipocyte-specific signal trasduction pathway; insulin activates p66Shc via ser36 phosphorylation and p66Shc-generated ROS tightly regulates

insulin signaling via several mechanisms, including Akt phosphorylation, Foxo localization and the regulation of specific genes [90]. p66Shc deletion caused profound metabolic changes including increased mitochondrial uncoupling and trygliceride accumulation in fat tissue, and, *in vivo*, increased life span and metabolic rate, reduced fat mass and resistance to diet-induced obesity [90]. Therefore, p66Shc regulates the effects of insulin in adipose tissue with consequences spanning over the energetic metabolism of the whole organism.

In the 50s Denham Harman proposed a "free-radical theory" of aging, theorizing that ROS generated and accumulating in cells could result in a pattern of cumulative damage, which could contribute to the process of aging. This theory originally implied that ROS, spreading in cells and tissues, target molecules in a random, indiscriminate and cumulative way [91]. The evidence reported here, along with researches in *Drosophila* and *C. Elegans*, significantly strengthens the mechanistic connection between oxidants, stress and aging, supporting the original theory of Harman [92]; however, it appears that although oxidants may act stochastically, ROS are actually specific signaling molecules both in physiological and pathophysiological processes [46, 47, 50, 93]. A logical consequence of the free-radical theory of aging, is that if we are able to reduce the amount of ROS production, we could theoretically reduce tissue damage and slow down the aging process. Importantly ROS are generated mainly in the mitochondria and therefore ROS production could mirror, at least in part, the metabolic rate of an organism. Indeed, the most outstanding relationship between metabolic rate and aging is the observation that manipulations that effect metabolism (i. e. caloric restriction, or IGF-1 pathway manipulation) can also lead to alterations in the life span [47, 94]. Notably, p66Shc KO mouse embryonic fibroblasts showed a reduction in oxygen consumption by about 30% to 50% [95]; moreover, p66Shc regulates mitochondrial metabolism diverging energy conversion from oxidative towards glycolytic pathways [95].

Collectively, there is cumulative evidence that strongly supports the idea that ROS play a primary role in the aging process; therefore, the discovery that a deletion in a single gene leads to increased lifespan in mammals has given new tools to understand why and how we age, but more importantly, could result in a new therapeutical target.

5. p66Shc in diabetes and cardiovascular diseases

The leading role of oxidative stress in the developing of cardiovascular diseases (CVDs) has prompted several groups to study the putative role of p66Shc in the pathogenesis of diabetic complications and ischemic injury.

One of the first reports showed that deletion of p66Shc protected high-fat fed mice from oxidative stress, vascular cell apoptosis and early atherogenesis [96]. By consent, p66Shc knockout exerts a protective function also in hypercholesterolemic ApoE knockout mice, reducing formation of advanced atherosclerotic lesions [97]. Francia *et al*. showed that age-related endothelial dysfunction was prevented in p66Shc KO animals, due to reduced accumulation of ROS [98]; moreover the same effect was reported in response to hyperglycemia-induced endothelial dysfunction, where p66Shc knockout diabetic mice showed reduced peroxynitrite generation, nitrotyrosine expression and lipid peroxidation in the aortic tissue [99]. Interestingly, p66Shc regulates endothelial NO production and endothelium-dependent vasorelaxation [100, 101].

p66Shc also contributes to the pathogenesis of diabetic glomerulopathy, a cause of chronic renal failure, mediating AGE formation, increasing oxidative stress and modulating NADPH oxidase [102, 103].

During ischemia and ischemia/reperfusion ROS formation is a leading cause of tissue injury, therefore it was not surprising that p66Shc played a role in tissue responses to acute ischemia: Zaccagnini and colleagues showed that ischemia increased p66Shc expression, while genetic ablation of p66Shc reduced fiber necrosis and apoptosis after ischemia in skeletal muscle [104]. These data were confirmed recently also in cardiac muscle, where deletion of p66Shc protected the heart from ischemia/reperfusion injury, confirming the striking role of uncontrolled mitochondrial ROS generation in tissue injury [105]. Moreover, p66Shc deletion protected the heart from diabetes-induce heart failure and cardiac stem cells aging [106]. Heart senescence is induced also by over activation of the renin-angiotensin system which also increases oxidative stress [107]; angiotensin II infusion in mice, indeed, resulted in left ventricular hypertrophy and myocyte apoptosis but p66Shc knockout protected animals from hypetrophic and proapoptotic effects of angiotensin [108].

It has been demonstrated recently that p66Shc knockout rescued mouse EPCs disfunction induced by hyperglicemia [109]. In 2005 our group reported that p66Shc is upregulated in peripheral blood mononuclear cells (PBMC), which are believed to harbor a subset of EPCs,

from diabetic patients and that this is positively correlated with total plasma 8-isoprostanes, a marker of oxidative stress [110]. This was the first study which demonstrates a potential association of p66Shc and diabetes also in human subjects. Notably, Pandolfi *et al.* showed that p66Shc is actually upregulated in fibroblasts from human centenaries, apparently contradicting common thought that p66Shc knockout improved lifespan [111]. Furthermore, p66Shc in aged humans did not appear to be regulated epigenetically as previously described [78] and it was downregulated in response to ischemia [111]. These data may underline that human p66Shc regulation could be more complex than previously described.

Interestingly, p66Shc stimulates T-cells to undergo apoptosis [112, 113] and p66Shc KO mice developed an age-related lupus-like autoimmune disease, with spontaneous activation and proliferation of T- and B-cells [114]. Consequently, these mice showed autoantibody production and immune complex deposition in kidney and skin, which lead to glomerulonephritis and alopecia, respectively. Recently, p66Shc emerged to be a critical regulator of B-cells proliferation because patients with chronic lymphocytic leukemia had profoundly impaired expression of p66Shc that could be associated with differential prognosis; in addition p66Shc might modulate Bcl-2 family expression towards anti-apoptotic stimuli [115]. Moreover, p66Shc knockout mice showed decreased superoxide production in macrophages, which if it reduced oxidative injury, on the other side it could predispose to infection due to reduced respiratory burst [116].

These data demonstrate that with a reduction of oxidative stress, p66Shc knockout animals showed remarkably improvement in several experimental models of CVDs. However, most of these studies only showed the effects of the knockout of p66Shc; very few, indeed, demonstrated that p66Shc is actually upregulated in such diseases. This additional information could provide further insight into p66Shc signaling, establishing a clear causative role. Moreover, all the studies were conducted in a model of genetic ablation of p66Shc, where animals lacked p66Shc in all tissue since birth, but we do not know whether a conditional knockout, a tissue specific deletion of p66Shc could have the same effects. A putative therapeutic strategy must be developed in a model where p66Shc could be inhibited or downregulated, even at adulthood stages, to distinguish global effects from tissue specific effects. Furthermore, studies on B and T cells showed an unexpected role of p66Shc in the regulation of autoimmune burden and in the control of B-cell proliferation in leukemia.

Finally, expanding our knowledge of p66Shc in humans may introduce novel pathophysiological mechanisms that could be exploited to develop new drugs.

IV. Aims of the thesis

This thesis aims to i) to investigate the effects of p66Shc knockout on wound healing in a model of diabetes and hind limb ischemia ii) to characterize which mechanisms of the wound healing process are affected by p66Shc iii) to study if diabetes modifies p66Shc expression.

V. Material And Methods

1. Animals

All the procedures involving animals and their care were conducted in accordance with international guidelines, laws and policies and with the National Institutes of Health Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985). The protocol was authorized by our local institutions. Wild-type and p66Shc KO mice were on a Sv129 genetic background. Generation of p66Shc KO mice was described previously [82].

Animals were divided into 4 groups: wild-type non-diabetic, wild-type diabetic, p66Shc KO non-diabetic and p66ShcKO diabetic. Each animal underwent ischemia on the right hind limb while controlateral hind limb was used as non-ischemic control (Table 1). For every group N≥3.

Wild-type	Non-Diabetic	Non-Ischemic
		Ischemic
	Diabetic	Non-Ischemic
		Ischemic
p66Shc KO	Non-Diabetic	Non-Ischemic
		Ischemic
	Diabetic	Non-Ischemic
		Ischemic

Table 1: Animals subdivision into experimental groups

Diabetes was induced with a single intraperitoneal injection of Streptozotcin (STZ, Sigma Aldrich, St. Louis, MO, USA), dosage 150 mg/kg, in citrate buffer 50 mM, ph 4.5, according to *Animal Models of Diabetic Complications Consortium* guidelines (www.admcc.org) . Glycemia was measured with a commercially available kit (Glucocard Gmeter, Menarini, Florence, Italy) on days 2, 7 and 14; animals with glycemia above 300 mg/dl in at least two measurements within the first week were classified as diabetic. Diabetic mice were allowed feeding and drinking *ad libitum* and caged in controlled conditions. Diabetic animals were

stabulated for 4 weeks before performing experiments in order to stabilize hyperglycaemia and diabetes.

2. Hindlimb ischemia

Hind limb ischemia was performed by surgical resection of femoral artery and vein. Animals were sedated with zolepam/tilamine (dosage 10 mg/kg, Zoletil®, Laboratories Virbac, Nice, France) and xylazine (dosage 7 mg/kg, Xilor®, Laboratories Carlier, Spain). The artery and the vein were surgically dissected from femoral nerve, then cauterized with low temperature cautery (FIAB, Florence, Italy) and excised between inguinal ligament and hackle. Tissue debries were removed, and the operating field was washed with sterile physiological solution. Skin was sutured. Fifteen days after surgery we performed Laser Doppler imaging on ischemic animals to measure hind limb microvascular perfusion; animals were sedated as previously described and hind limbs were scanned with Perimed Periscan-Pim II Laser Doppler System equipped with LPDIWin 2.5 software (both Perimed AB, Sweden). Each measure was repeated at least 5 times. Animals were killed by euthanasia 15 days after surgery, muscles (adductor and semimembranous) and skin samples were harvested and frozen in liquid nitrogen-cooled isopentane.

3. Nitrotyrosine and pentosidine quantifications

Skin was shaved and cleaned from fat and cellular debris and then processed according to Fallon *et al*. [117]. Tissue was homogenized and delipidated in 2:1 chloroform/methanol for 18 hours. After centrifugation, the pellet was resuspended in 4 ml 1:1 water/methanol solution for two hours at room temperature. After centrifugation the pellet was hydrolyzed in 4 ml HCl 6N for 18 hours. Once HCl was completely evaporated, the residual was resuspended in 500 µl of distilled water.

Nitrotyrosine concentration in mouse skin was measured with an ELISA assay. 65 µl of samples and standards were incubated overnight at dark with 135 µl of carbonate buffer (pH=9.6) in each well of the plate. After 2 washes with PBS-T (PBS + 0.05% Tween 20) and saturation with 200 µl PBS-T/BSA 0.1%, we added 100 µl of mouse α-nitrotyrosine monoclonal Ab (Millipore, Tecumela, CA, USA) dilution 1:250 in PBS-T/BSA 0.1%, at 37°C for 1 hour. After two washes, we added 100 µl of secondary antibody, rabbit α-mouse HRP conjugated Ab (Sigma-Aldrich), dilution 1:1000 in PBS-T/BSA 0.1% at 37°C for 1 hour. After two washes

150 µl of TMB substrate (Sigma-Aldrich) was added for 15 minutes. Colorimetric reaction was blocked with 50 µl of H_2SO_4 0.5 M and adsorbance was read at 450 nm with background at 620 nm.

Pentosidine was quantified using HPLC (High Performance Liquid Cromatography). Skin was prepared as above. We used Prostar 363 HPLC detector (Varian, Palo Alto, CA, USA) equipped with C-18 columns. Mobil phase (column eluition), a 5-28% gradient of acetonitrile in water with 0.01 M heptafluorobutyric acid, was applied for 35 minutes. Pentosidine peak was eluted after 20 minutes. Red cell haemoglobin A1c (HbA1c) was measured with an automated DCA2000$^+$ analyzer (Bayer, Milan, Italy).

We quantified at least 3 samples per group of animals.

4. Skin wounds

Animal was sedated with inhaled sodium isofluorane, then the skin of dorsal surface of hind limb was shaved with Veet cream and disinfected with alcohol/ether; a 4mm diameter wound was performed with a biopsy punch (H-S Medical Inc, Colton CA, USA). Daily photographs were taken to evaluate wounds closure time and wounds area was quantified through a commercially available image processing software ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA). Wounds on ischemic animals were performed 2 days after surgery. A patch of skin surrounding the wound with the underlying muscle was excised and frozen in liquid nitrogen cooled isopentane.

5. Progenitor cell counts

Blood samples were collected from the orbital sinus at basal condition and 4 days after ischemia for progenitor cells count. There is no consensus as to the phenotype required to identify EPCs both in humans and experimental models. We chose CD34 and Flk-1 to identify endothelial progenitor cells, as in other studies [118]. This phenotype resemble the $CD34^+KDR^+$ cell population, which is considered the best human EPC phenotype at flow cytometry [119].

All the samples were processed within 2 hours from the drawing. Briefly, 150 µl of blood were incubated with 10 µl of Alexa Fluor 647 rat α-mouse CD34 Ab (Beckton Dickinson, Franklin Lakes, NJ USA) and 10 µl of Alexa Fluor 488 rat α-mouse Flk-1 Ab (BioLegend, San Diego, CA, USA). The frequency of peripheral blood cells positive for the above reagents

was determined by a two-dimensional side scatter-fluorescence dot plot analysis of the samples stained with the different reagents, after appropriate gating to exclude granulocytes. Initially we gated $CD34^+$ peripheral blood cells and then examined the resulting population for dual expression of Flk-1. Data were processed using Macintosh CELLQuest software (Beckton Dickinson).

6. Cell culture

Fibroblasts were isolated from skin explants of wild-type and p66Shc KO non-diabetic mice. Briefly, mice were killed by euthanasia and a patch of skin was shaved, cleaned with alcohol and ether and excised. The patch was transferred into a sterile tissue culture-treated petri dish (Beckton Dickinson) and cut in about 1 mm^2 pieces that were placed with dermis adherent to bottom of the dish and covered with DMEM 5 mM glucose medium (Sigma Aldrich) + 10% FBS (Invitrogen, Carlsbad, CA, USA) and 1% L-Glutamine/Penicillin-Streptomycin (Sigma Aldrich). Fibroblasts sprouted from explants in about 5 days and when they reached confluence we passed them. We used cells from passage 3 to 7 and every experiment was repeated at least in triplicate.

In migration assay we used a commercially available device (Ibidi, Munich, Germany) following manufacture's instructions.

For high glucose experiments we used DMEM 25 mM glucose (Sigma Aldrich) + 10% FBS (Invitrogen, Carlsbad, CA, USA) and 1% L-Glutamine/Penicillin-Streptomycin (Sigma Aldrich). Cells were cultivated in high glucose for 4 days prior to perform the experiments and were, then, maintained in high glucose for the length of the whole experiment.

Hypoxia was achieved with a commercial available device (GasPack EZ, Beckton Dickinson) according to the manufacture's instructions; cells were cultivated in normoxia prior to place them in hypoxic condition during the whole migration assay.

H_2O_2 viability test was performed according to Migliaccio *et al*., [82]. Wt and p66Shc KO fibroblasts were treated with 400 μM H_2O_2 for 24 hours. Viability was assessed by 0.1% Tripan Blue exclusion. Counts were performed in ten random high-power field (hpf). Experiments were repeated in triplicate.

7. Histology and immunohistology

All the stainings were performed on 7 μm-thick cryosections.

Hematoxilyn/Eosin and Masson Trichrome stainings were performed using commercially available kits (Bio-Optica, Milan, Italy) according to manufacture's protocols. Counts and quantifications were performed on whole sections reconstrucion in at least 6 sections per sample, repeated for at least 3 animals per group.

Muscle sections were stained with rabbit α-laminin polyclonal Ab (dilution 1:25, Sigma Aldrich) and fluorescein labeled GSL-I-isolectin B4 (dilution 1:50, Vector Labs, Burlingame, CA, USA). Secondary antibody was swine α-rabbit TRITC-conjugated Ab (1:150 Dako-Cytomation, Denmark). Counts were performed in 10 random hpf repeated for at least 3 animals per group.

Skin sections were stained with fluorescein labeled GSL I – isolectin B4 (dilution 1:50, Vector Labs), mouse anti-c-*myc* monoclonal Ab (1:100, Roche Diagnostic, Basel, Switzerland) and mouse anti-β catenin monoclonal Ab (1:50, Beckton Dickinson). Secondary antibody was goat anti-rabbit TRITC-conjugated (1:150, Jackson Labs). Quantifications were performed in 5 random hpf and repeated for at least 3 animals per group. Images were acquired with a Leica TCS SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Nuclei were counterstained with Hoechst 33258 (Sigma Aldrich). Apoptotic cells were detected with Apoptag® Plus *In Situ* Apoptosis Detection Kit. (Millipore) and counted in 10 random hpf for at least 3 animals per group. Histological quantifications were performed using a commercially available image processing software ImageJ (NIH, Bethesda, MD, USA).

8. Western Blot

Tissue extracts (skin or muscle) were prepared by liquid-nitrogen tissue homogenization followed by lysis with extraction buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 10% glicine, 1% Triton, 10 mM NaP_2O_7) with Complete™ Protease Inhibitor Cocktail (Roche Diagnostic) and phosphatase inhibitor (Sigma Aldrich) for 15 minutes in ice. After sonication at 2 watts for 10 seconds with Vibra-Cell™ ultrasonic processor (Sonics Newtown, CT, USA), lysates were placed in ice for 10 minutes. After centrifugation we discarded the pellets and working aliquots of surnatants were stored at -80°C.

For cells extracts, 10^6cells were washed twice with PBS, detached with disposable scrapers (Sarstedt, Germany) and then lysated as described.

Muscle or cells protein concentration was quantified with Bradford reagent (Sigma Aldrich). 30 μg of proteins were heated in SDS sample buffer and separated by 10% SDS-PAGE. Protein were transfered on nitrocellulose membrane (Whatman, Kent, UK) for 1 hour at 4°C with fixed voltage at 80V. Membranes were saturated for 1 hour at room temperature in 50 mM Tris-HCl, pH 7.5/0.15 M NaCl/0.05% Tween (TBST) with 5% skimmed milk. Membranes were washed 3 times for 10 minutes in TBST and then incubated with rabbit polyclonal antibodies α-Shc (Upstate, Lake Placid, NY, USA), rabbit α-Shc/p66, phospho-specific (Ser36) (Calbiochem, Gibbstown, NJ, USA), rabbit α-Actin (Oncogene, San Diego, CA, USA) overnight at 4°C in TBST/5% milk. Then, the membranes were washed 3 times for 10 minutes in TBST and incubated for 1 hour at room temperature with goat anti-rabbit antibody conjugated with peroxidase (Santa Cruz Biotechnology) diluted 1:5000 with TPBS. After being washed again in TBST, the membranes were treated with a chemioluminescent reagent (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford USA) and immunoreactive bands were revealed by autoradiography film (GE- Healthcare, Uppsala, Svezia). Relative expression of p66Shc was normalized by detection of actin as housekeeping protein.

9. Statistical analyses

All data are expressed as mean±SEM. p values ≤ 0.05 were considered to represent statistical significance. Statistical analyses were performed by unpaired Student's t tests.

VI. Results

1. p66Shc deletion improves wound healing in diabetes and ischemia

We created a four-mm diameter wound on dorsal skin of the right hind limb in wt and p66Shc KO mice, in the settings of 4weeks STZ-induced diabetes or 2-weeks hind limb ischemia, or both. As show in figure 1A, in non-diabetic non-ischemic animals wound healing was not different in wt and p66Shc KO. Diabetes delayed wound healing by about 70% in wt animals and 30% in p66Shc KO (fig.1B), with a significant faster healing in p66Shc KO compared to wt (wound area at day 4: 23.6±2.2 vs 35.9±5.0%; p=0.028). Hind-limb ischemia further delayed wound healing by about 185% in wt and 70% in p66Shc KO mice (fig. 1C; $p<0.05$ from day 1 to 11). When diabetes is associated with ischemia the differences between wt and p66Shc KO are further exacerbated (fig. 1D, $p<0.05$ from day 1 to 15). Collectively, the knockout of p66Shc significantly improved wound healing in diabetes and ischemia but the effects were particularly evident when diabetes coexisted with hind-limb ischemia.

2. p66Shc deletion recovers defective granulation tissue in diabetic-ischemic wounds

Wound healing requires a well-development and vascularized granulation tissue. Here, we report that diabetes, especially when associated with ischemia, severely impairs granulation tissue maturation in wt mice. Collagen-stained area, which is expression of matrix deposition by fibroblast, was significantly reduced in wounds from diabetic ischemic mice as compared to control mice (18.7±4.0 vs 36.0±2.0 %; p=0.002, fig. 9A). At the same time, the thickness of granulation tissue (105.5±18.4 vs 383.5±35.0 μm; $p<0.0001$, fig. 9B) and the closure of epithelial gap was blunted in diabetic wounds (non-ischemic 11.3±3.6 vs 26.6±4.6 %; p=0.015; ischemic: 20.6±5.9 vs 29.4±6.8 %; p=0.33, fig. 9C). Within the granulation tissue of diabetic wounds, capillary network showed flawed development (non-ischemic: 91.2±12.7 vs 159.6±11.2 arbitrary units; p=0.002; ischemic: 42.8±6.6 vs 135.7±18.9 arbitrary units; p=0.002, fig. 10A), while apoptosis was greatly increased (non-ischemic 89.9±22.9 vs 19.2±6.0 cells/hpf; p=0.009 fig. 10B) when compared to non-diabetic controls. The knockout of p66Shc was able to revert these alterations: collagen-stained area (non-ischemic: 53.5±5.7 vs 32.6±3.6 %; p=0.01; ischemic: 68.6±2.4 % vs 18.6±3.9 % $p<0.001$, fig. 9A), granulation tissue thickness (non-ischemic: 413.1±64.9 vs 177.05±26.5 μm; p=0.003; ischemic:

435.5±33.7 vs 105.5±18.4 µm; p<0.0001, fig. 9B) and reepithelialization (non-ischemic 45.5±9.7 vs 11.3±3.4 %; p<0.005; ischemic: 49.7±8.8 vs 20.6±5.9%; p=0.01, fig. 9C) were increased in wounds from diabetic p66Shc KO mice compared to the corresponding wt.

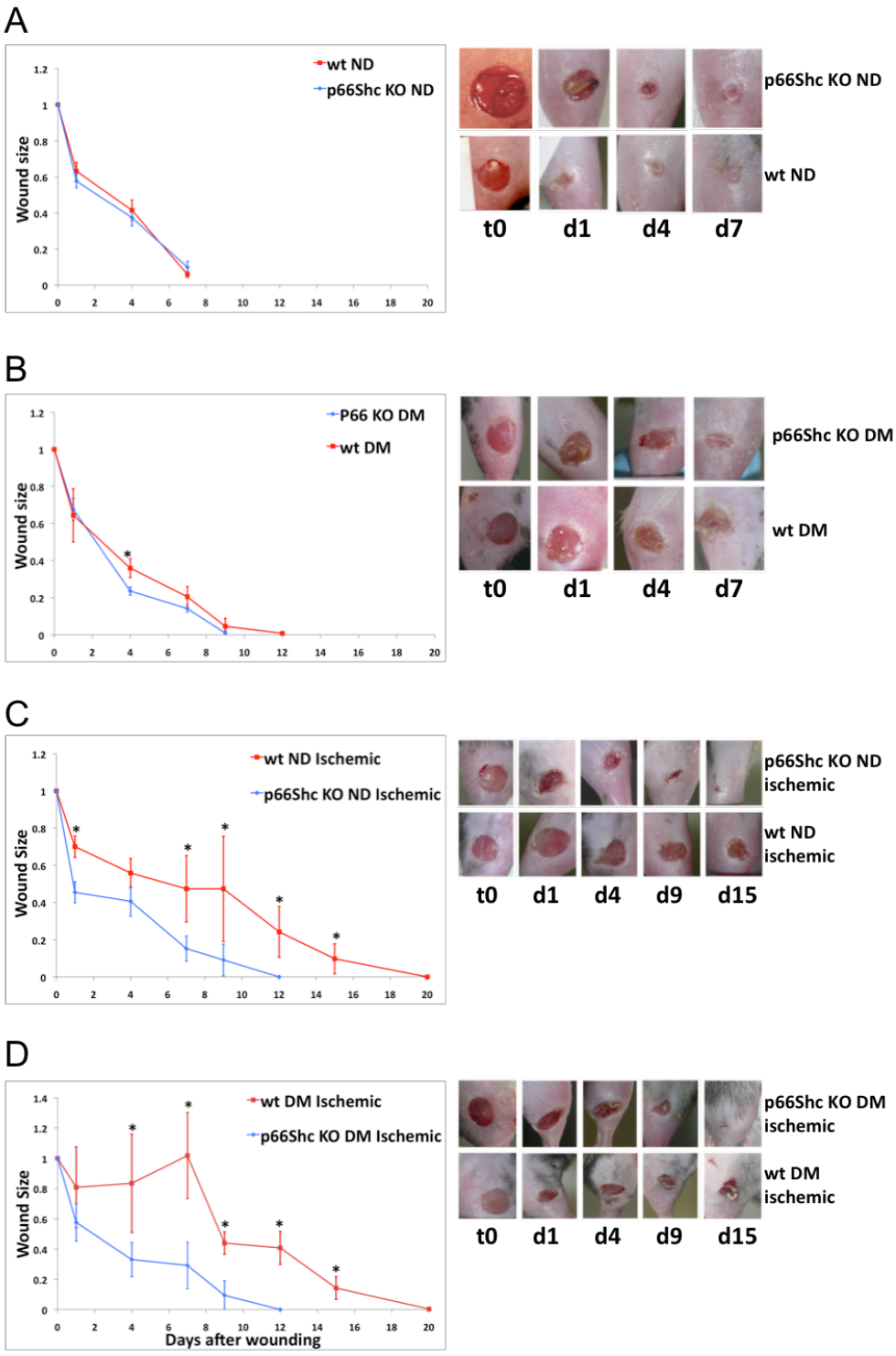


Figure 8: Wound Healing. Healing time-course of a 4 mm-diameter wound in wild-type (wt) and p66Shc knockout (KO) diabetic (DM) and non-diabetic (ND) mice, with or without ischemia. Fig. 1A non-diabetic non-ischemic; fig. 1B diabetic non-ischemic; fig. 1C non-diabetic ischemic; fig. 1D diabetic ischemic. Wound size is expressed as percentage of initial area. N≥3 mice for each group. * p<0.05 in KO vs wt.

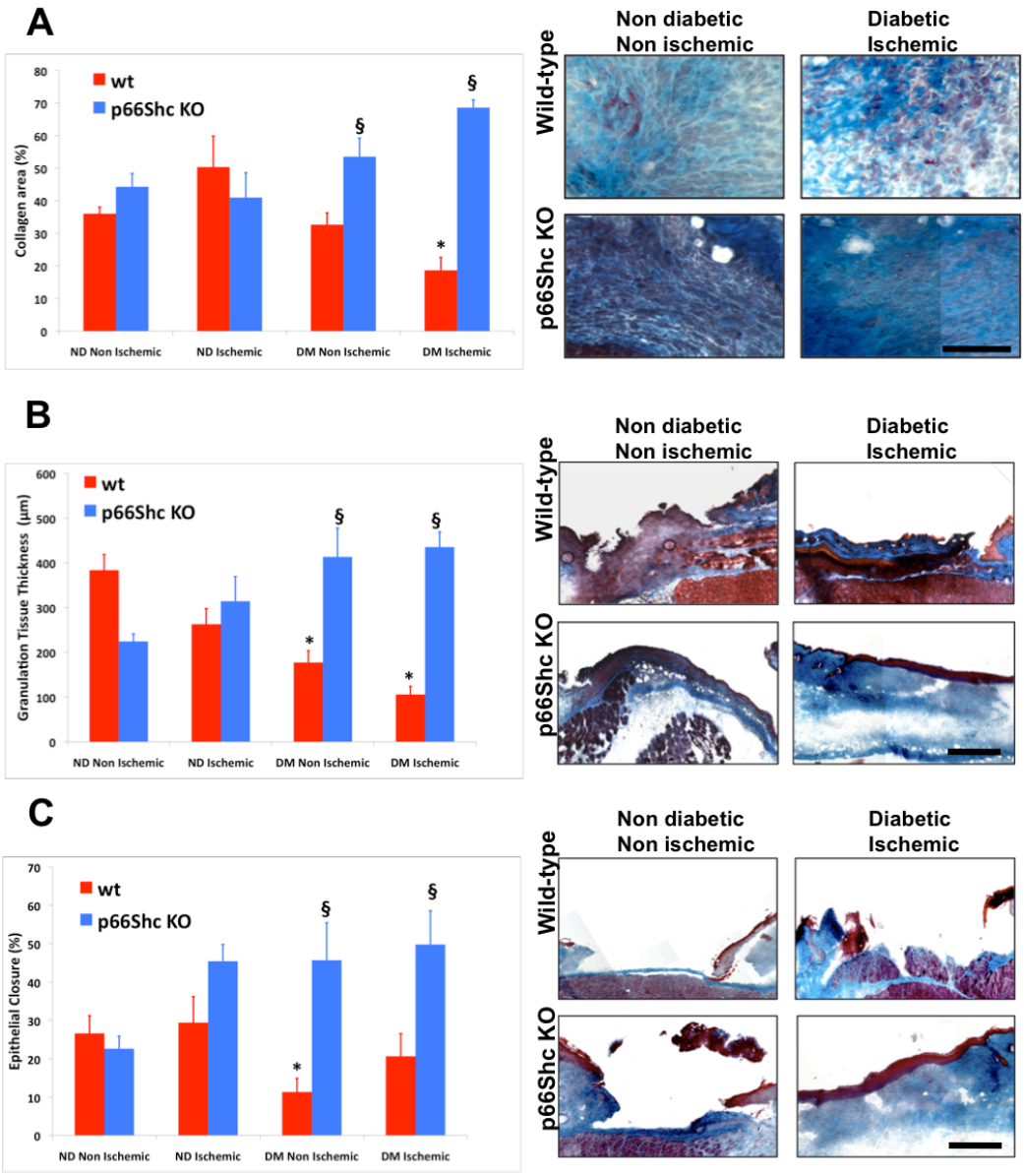


Figure 9: Characteristics of granulation tissue. Morphological features of the granulation tissue of wound from wild-type (wt) and p66Shc knockout (KO) mice, diabetic (DM) and non-diabetic (ND), with or without hind-limb ischemia. Masson's trichrome staining was performed to quantify collagen deposition, granulation tissue thickness and epithelial closure (fig. 2A, fig. 2B and fig. 2C, respectively). Collagen area is expressed as percentage of the examined area. Epithelial closure is expressed as fraction of the wound size. Representative images are shown for control (non-diabetic and non-ischemic) and diabetic ischemic wt and p66Shc KO mice. N≥3 mice for each group; N≥5 sections for each sample. * $p<0.05$ in diabetic vs the corresponding group non-diabetic . § $p<0.05$ in p66Shc KO vs the corresponding wt. Scale bar: fig 2A 100 µm, fig. 2B and 2C 400 µm.

Moreover apoptosis was greatly reduced both in wound edges ($p\leq0.05$ for every group, fig. 10C) and within the granulation tissue (fig. 10B). Interestingly, the capillary density of non-diabetic-non-ischemic p66Shc KO mice was significantly lower compared to wt corresponding mice (50.8±3.1 vs 159.6±11.2 arbitrary units; $p=0.0001$), but this difference was overcome in the presence of either diabetes or ischemia and was inverted in the presence of both

diabetes and ischemia (92.8±18.7 vs 42.8±6.6 arbitrary units; p=0.002). These data suggest that the deletion of p66Shc improves vascularization of granulation tissue, dampening the negative effects of diabetes and ischemia (fig. 10A).

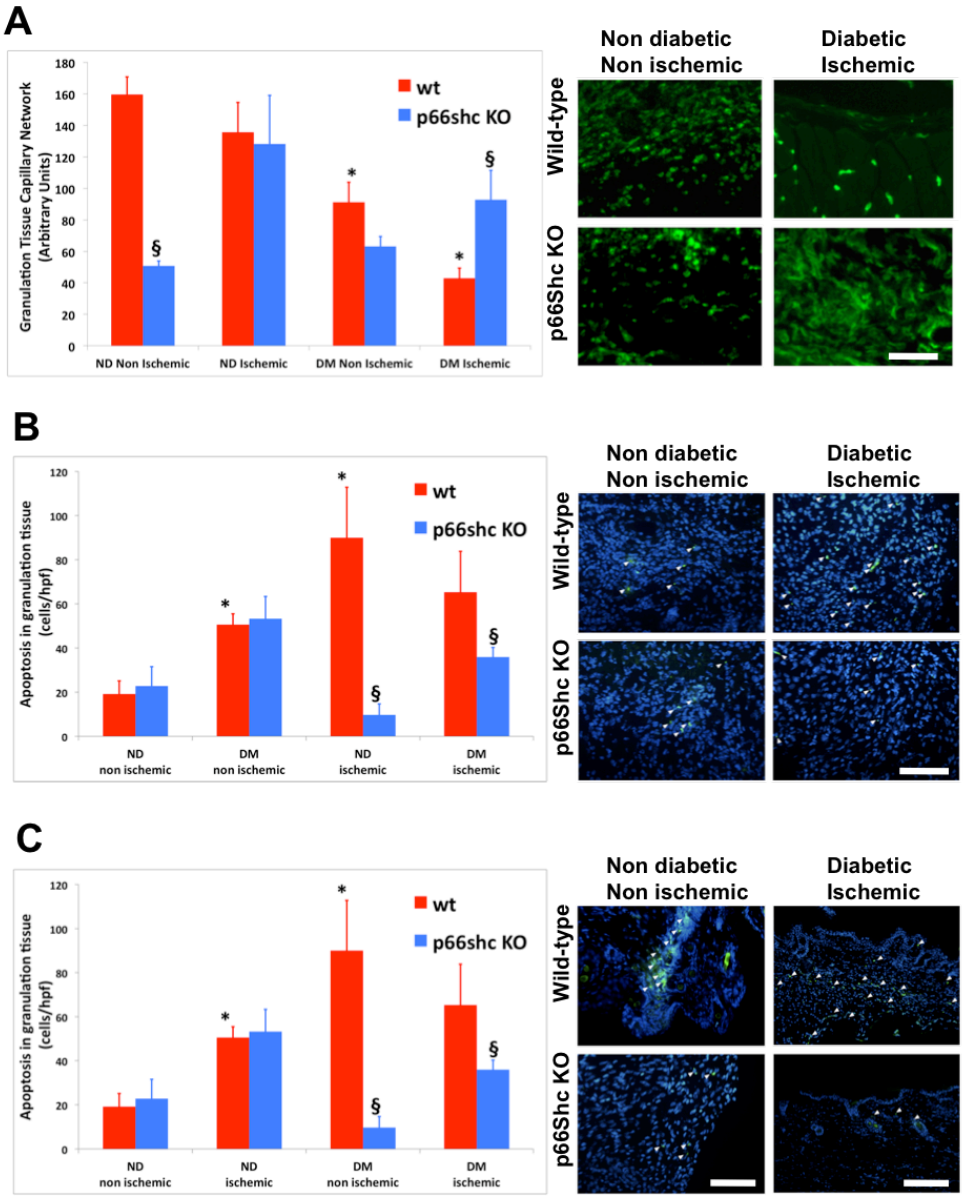


Figure 10: Characteristics of granulation tissue. Morphological features of the granulation tissue of wound from wild-type (wt) and p66Shc knockout (KO) mice, diabetic (DM) and non-diabetic (ND), with or without hind-limb ischemia. Lectin staining was performed to quantify capillary network development (Arbitrary Units, , fig. 10A). Apoptosis was quantified in wound edges and within the granulation tissue (cells/hpf, fig. 10B and 10C). Representative images are shown for control (non-diabetic and non-ischemic) and diabetic ischemic wt and p66Shc KO mice. N≥3 mice for each group; N≥5 sections for each sample. * p<0.05 in diabetic vs the corresponding group non-diabetic . § p<0.05 in p66Shc KO vs the corresponding wt. Scale bar: fig 3A 100 µm, fig. 3B and 3C 200 µm, except in the p66Shc KO diabetic ischemic image where scale bar is 100 µm.

3. p66Shc deletion reduces β catenin and c-*myc* activation in diabetic wounds

Higher expression of oncogene c-*myc* and nuclear localization of β catenin in epidermal cells at wound edges has been described as a molecular hallmark of chronic-non healing ulcers [120]. By consent, we show that ulcers from diabetic ischemic wt mice had increased expression of c-*myc* (27.3±7.3 vs 7.6±3.8 % of positive cells; p=0.03) and nuclear localization of β catenin (24.1±1.2 vs 9.4±3.5 % of positive nuclei; p=0.02) in epidermal cells at their edges compared to controls, where c-*myc* was virtually absent and β catenin was recognizable only in adherens junctions between cells (fig. 11 and 12). The deletion of p66Shc abrogated this non-healing footprint as diabetic ischemic ulcers showed both reduced activation of c-*myc* (4.93±4.29 vs 27.23±7.43 % of positive cells, p=0.047, fig. 11) and lower nuclear β catenin localization compared to wt (1.99±0.52 vs 24.09±1.22 % of positive nuclei, p=0.0007, fig. 12).

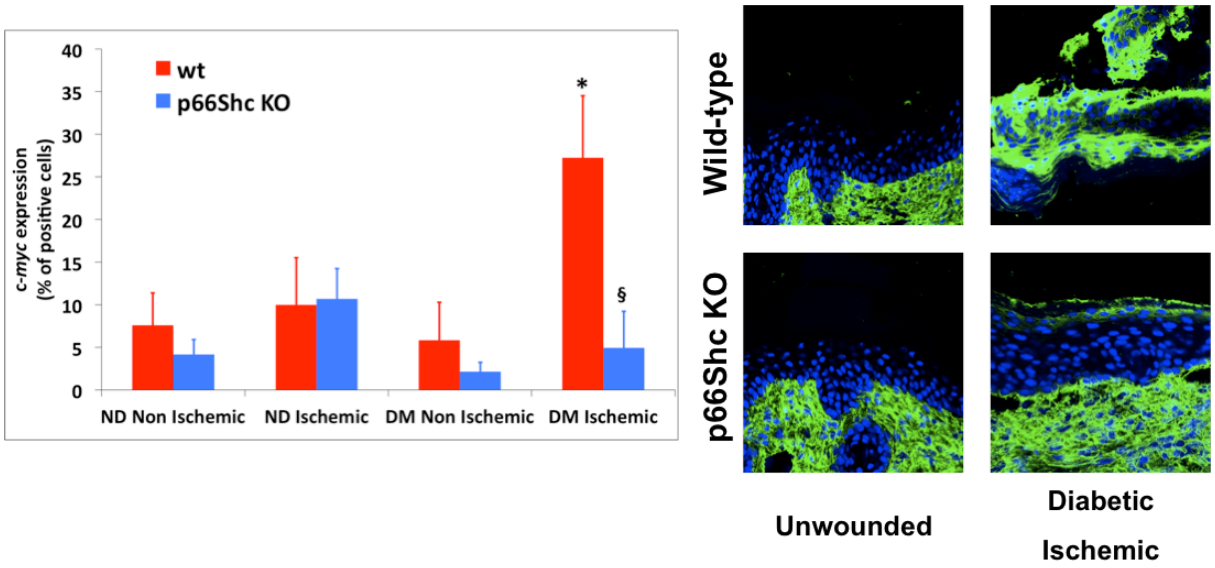


Figure 11: c-*myc* expression in the epidermid. expression of c-*myc* (% of positive cells) quantified in the edges of wounds of wild-type (wt) and p66Shc knockout (KO) mice, diabetic (DM) and non-diabetic (ND), with or without hind-limb ischemic. Herein are shown representative confocal images of immunofluorescence for c-*myc* in unwounded animals (non-diabetic and non-ischemic) and diabetic ischemic wt and p66Shc KO mice. N≥3 mice for each group; N≥5 sections for each sample. * p<0.05 in diabetic vs the corresponding group non-diabetic. § p<0.05 in p66Shc KO vs the corresponding wt. c-*myc* is showed in green, nuclei in blue.

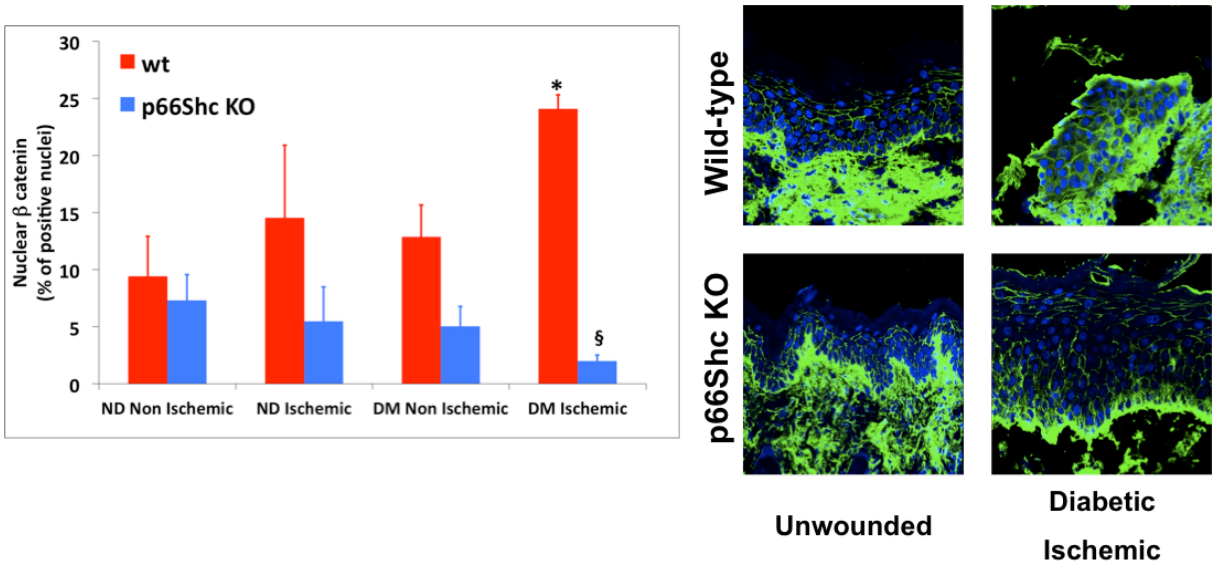


Figure 12: β catenin expression in the epidermid. Expression of β catenin (% of positive nuclei) quantified in the edges of wounds of wild-type (wt) and p66Shc knockout (KO) mice, diabetic (DM) and non-diabetic (ND), with or without hind-limb ischemic. Herein are shown representative confocal images of immunofluorescence for β-catenin in unwounded animals (non-diabetic and non-ischemic) and diabetic ischemic wt and p66Shc KO mice. In unwounded animals β-catenin is localized in adherens junctions, while wounded skin shows nuclear β-catenin localization. N≥3 mice for each group; N≥5 sections for each sample. * $p<0.05$ in diabetic vs the corresponding group non-diabetic . § $p<0.05$ in p66Shc KO vs the corresponding wt. β catenin is showed in green, nuclei in blue.

4. p66Shc deletion reduces nitrotyrosines and pentosidine accumulation in diabetic skin

Oxidative stress and AGE deposition are broadly recognized as hallmarks of diabetic complications and, in particular, could delay wound healing process [69, 121]. Herein, we show that the skin concentration of skin nitrotyrosines, a marker of oxidative stress, was increased by 2.5-fold in wt diabetic versus non-diabetic mice (0.74±0.05 vs 3.47±0.87 nM, p=0.03), while there were not significant differences between diabetic versus non-diabetic p66Shc KO mice (1.43±0.14 vs 1.88±0.24 nM, p=0.18); notably p66Shc KO diabetic mice showed reduced nitrotyrosine compared to wt diabetic mice (3.47±0.87 vs 1.88±0.24 nM, p=0.049) (fig. 13).

Similarly, skin pentosidine concentration, an index of advanced glycation, was increased by about 75% in wt diabetic mice compared to non-diabetic (6.70±0.60 vs 13.56±0.13 pg/ml, p=0.006), while, in p66Shc KO mice, it was not significantly different (6.46±1.85 vs 7.51±1.41 pg/ml, p=0.62); moreover, p66Shc KO diabetic mice showed reduced pentosidine accumulation compared to wt mice (13.56±0.13 vs 7.5±1.41 pg/ml, p=0.049, fig. 14). On the contrary, we reported no difference in fasting glucose levels between wt and p66Shc KO mice

(388±28 vs 401±34 mg/dL; p=0.77); consistently, glycated hemoglobin was equally increased in wt and p66Shc KO diabetic animals as compared to non-diabetic. (13.8±1.4 vs 6.6±0.7 % and 13.5±1.2 vs 6.5±0.6 %, p=0.87) (fig. 15)

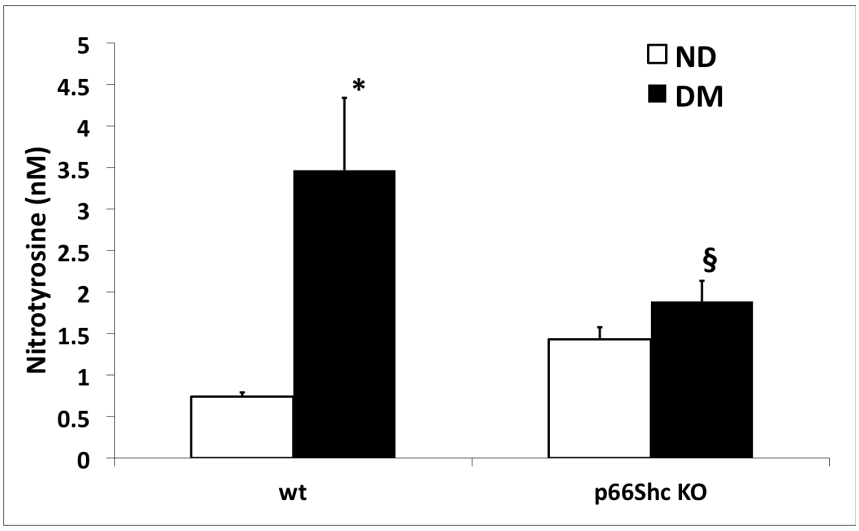


Figure 13: Nitrotyrosine in mice skin. Nitrotyrosines, a marker of oxidative stress, were quantified with an ELISA assay in skin from wild-type (wt) and p66Shc knockout (KO) mice, diabetic (DM) and non-diabetic (ND). Diabetes greatly increases nitrotyrosines formation in wt mice, while in p66Shc KO mice there is no difference between DM and ND. Moreover, DM p66Shc KO mice showed lower nitrotirosines compared to DM wt. N≥3 mice for each group. p<0.05 in diabetic vs the corresponding group non-diabetic . § p<0.05 in p66Shc KO vs the corresponding wt.

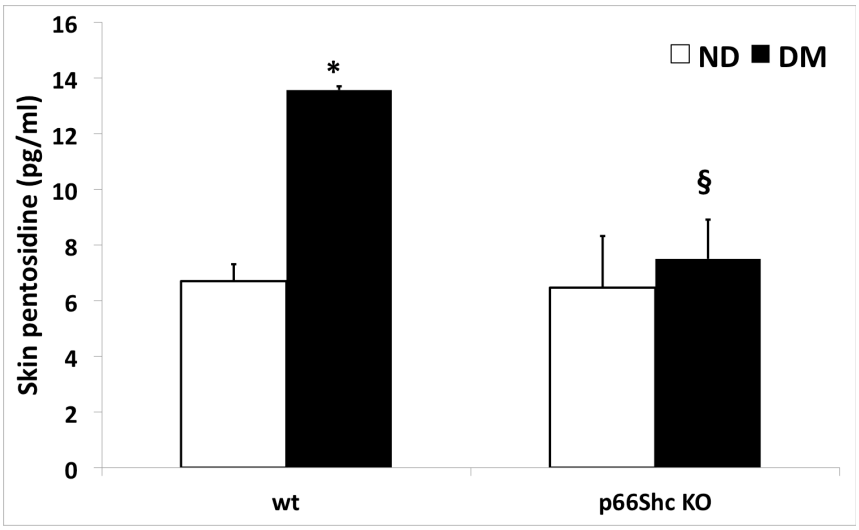


Figure 14: Pentosidine in mice skin. Pentosidine, a marker of advanced glycation, was quantified with an in skin from wild-type (wt) and p66Shc knockout (KO) mice, diabetic (DM) and non-diabetic (ND). In wt mice, diabetes increases pentosidine accumulation, whereas in p66Shc KO mice there is no difference between DM and ND. Moreover, DM p66Shc KO mice showed lower pentosidine compared to DM wt. N≥3 mice for each group.* p<0.05 in diabetic vs the corresponding group non-diabetic . § p<0.05 in p66Shc KO vs the corresponding wt.

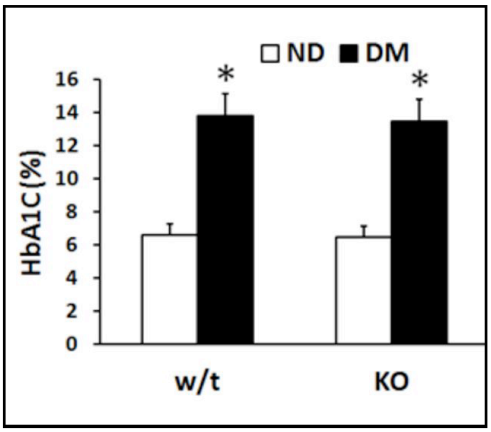


Figure 15: Glycated hemoglobin. Glycated hemoglobin (HbA1C) was equally increased in wt and p66Shc KO diabetic mice. N≥3 mice for each group.* p<0.05 in diabetic vs the corresponding group non-diabetic.

5. Improved migration of p66Shc KO dermal fibroblasts

Deletion of p66Shc protect from oxidative stress [82]; indeed we confirmed that p66Shc KO dermal fibroblasts were resistant to hydrogen peroxide exposure *in vitro*, while about 90% of wt fibroblasts died after H_2O_2 treatment (fig. 16).

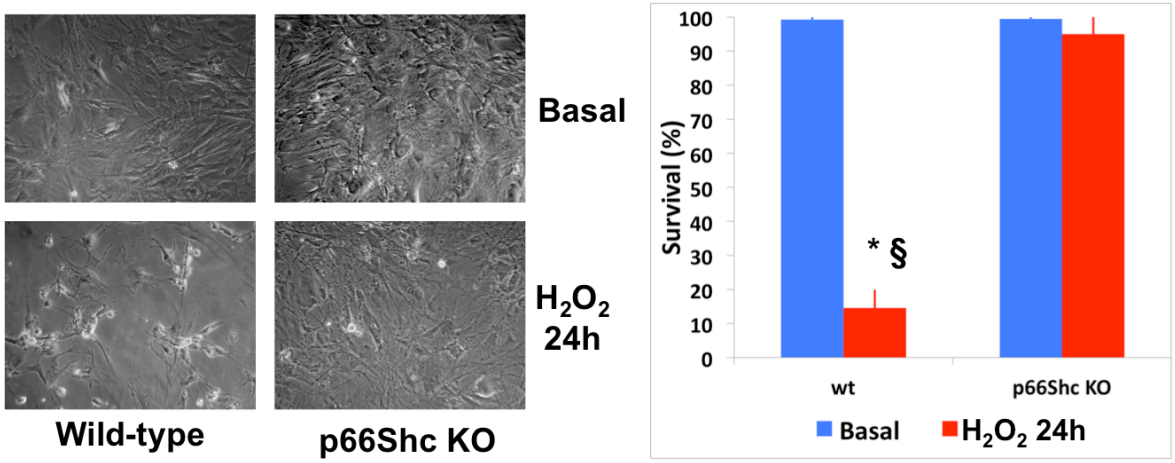


Figure 16: Viability of fibroblast after H_20_2 treatment in vitro. Dermal fibroblasts were exposed to H_20_2 400 µM for 24 hours. wt fibroblasts undergo massive death while p66Shc showed resistance to oxidative damage. Viability was assessed by tripan blue exclusion. Results are expressed as percentage of untreated culture. Experiments was repeated in triplicate. * p<0.05 in basal vs H_20_2 treatment . § p<0.05 in p66Shc KO vs wt.

Thereafter we set up an *in vitro* model to assess migration features. We isolated fibroblasts from dermal explants from wt and p66Shc KO non-diabetic non-ischemic mice. p66shc KO cells migrated faster than wt fibroblasts in normoxic 5 mM glucose (0.05±0.02 vs 0.22±0.05,

% of initial area after 12 hours, p=0.04) and when exposed to high glucose (25mM) in both normoxia (0.12±0.03 vs 0.36±0.11 % of initial area after 12 hours, p=0.03) and hypoxia (0.31±0.04 vs 0.47±0.02 % of initial area after 12 hours, p=0.04) (fig 17). Collectively, these data suggest that deletion of p66Shc partly mitigate the detrimental effects of hypoxia and high glucose on the migration properties of dermal fibroblasts.

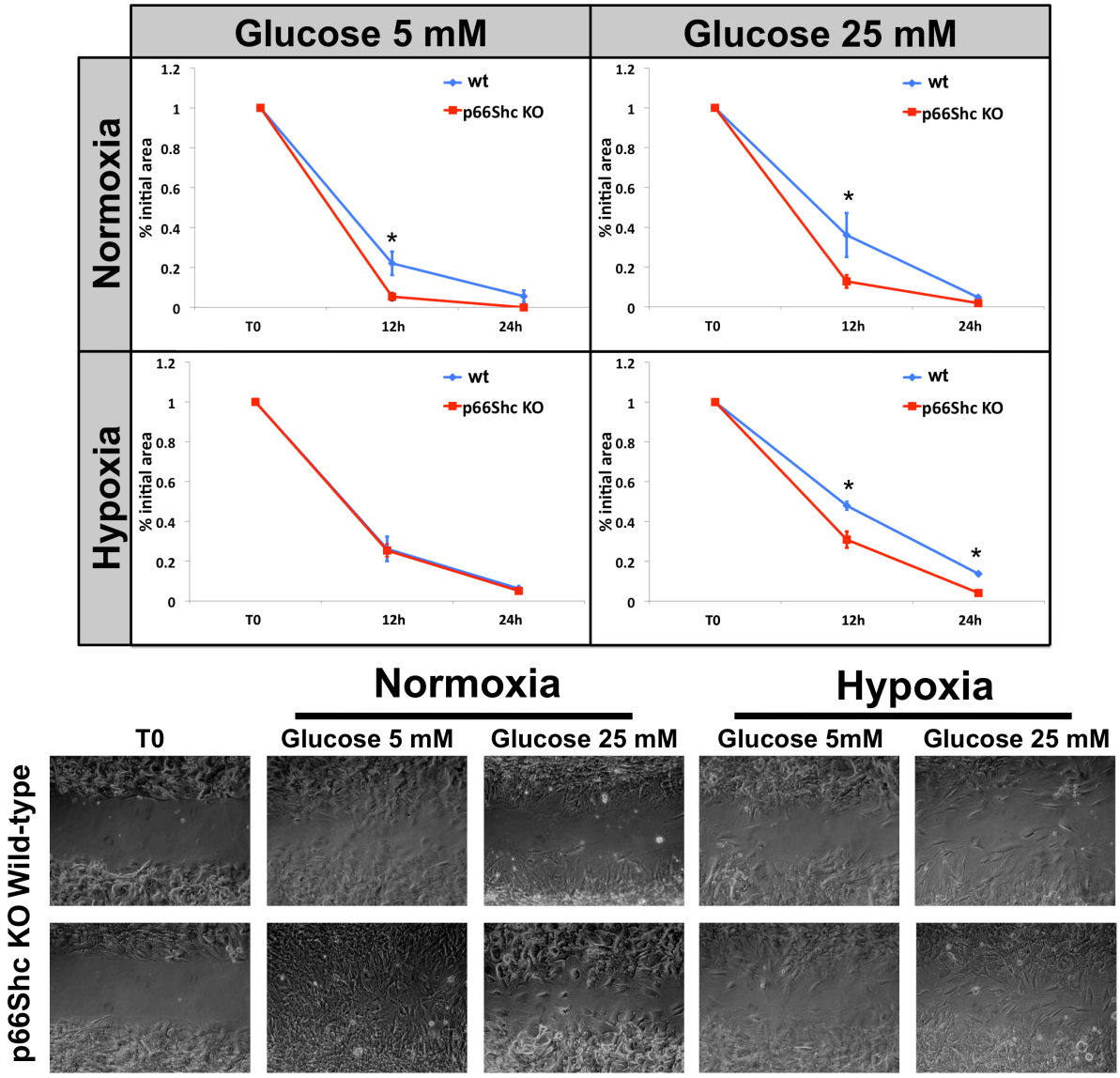


Figure 17: Fibroblasts migration in vitro. Fibroblasts were isolated from skin explants of wild-type (wt) and p66Shc knockout (KO) non-diabetic non-ischemic mice to asses migration properties. p66Shc KO fibroblasts migrated faster than wt fibroblasts when cultivated in low (5mM) and high glucose (25 mM) in both hypoxia and normoxia. Migration experiments were repeated at least in triplicate, * p<0.05 in p66Shc KO vs wt.

6. Diabetes increases p66Shc expression in the skin and in dermal fibroblasts

So far the knockout of p66Shc seemed to improve the healing of diabetic and ischemic wounds, therefore, we tested whether diabetes increases the expression of p66Shc. In skin lysates we saw that 4-weeks diabetes increased the expression of both ser36 phosphorilated-p66Shc (1.28±0.12 vs 2.07±0.13 ser36p66Shc/action ratio, p=0.01) and p66Shc (0.81±0.08 vs 1.29±0.19 p66Shc/action ratio, p=0.049; fig. 18A) . Moreover, we measured the expression of p66Shc in fibroblasts exposed to normoxia or hypoxia in 5mM and 25 mM glucose. We found that high glucose increased p66Shc expression (0.77±0.02 vs 0.97±0.12 p66Shc/action ratio, p=0.05) especially in combination with hypoxia (0.72±0.07vs 1.08±0.02 p66Shc/action ratio, p=0.045; fig. 18B)

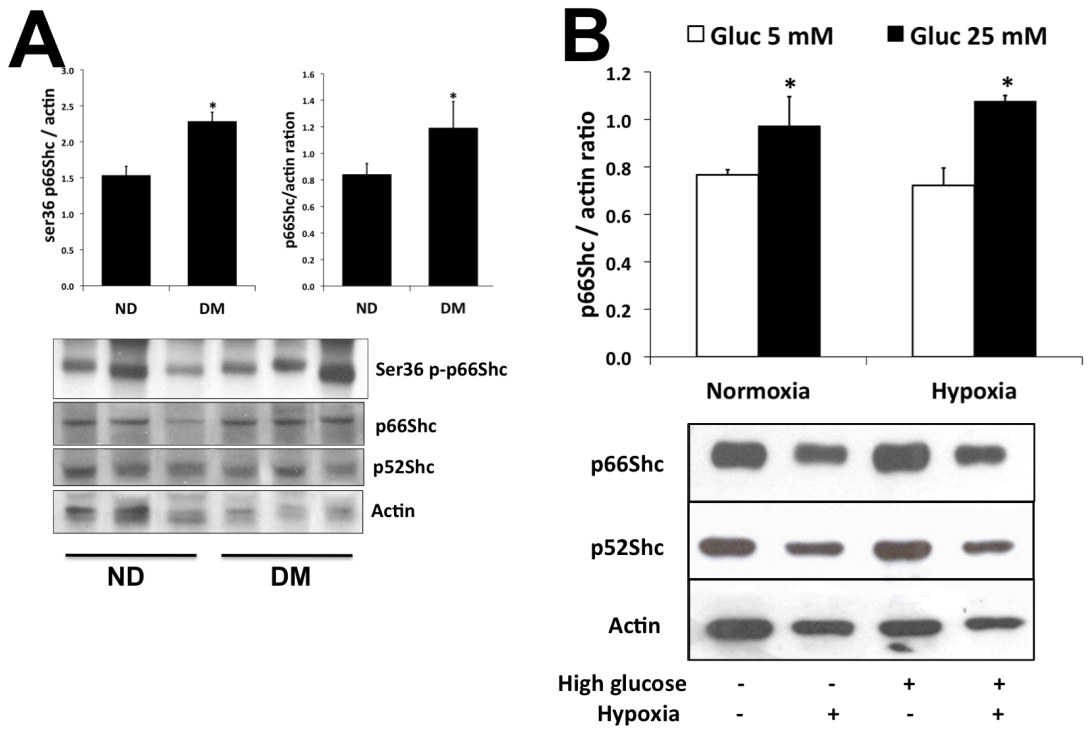


Figure 18: Expression of p66Shc and ser36 p66Shc in skin and fibroblasts. Diabetes increases both p66Shc and serine 36-phosphorilated p66Shc (ser36 p66Shc) expression in skin lysates from wt mice (fig. 18A). By consent, p66Shc expression was increased in fibroblast when exposed to high glucose (25 mM) and hypoxia (fig. 18A). Experiments was repeated in triplicate. * p≤0.05 in DM vs ND or glucose 5 mM vs 25 mM, respectively.

7. p66Shc deletion protects skeletal muscles from ischemic damage

Peripheral ischemia is a critical predisposing factor for wound development in diabetes and strongly impairs healing itself. We assessed the effects of ischemia on adductor muscles from wt e p66Shc mice, diabetic and non-diabetic. We found that in wt animals diabetes strongly empower the negative effects of ischemia on skeletal muscles in the means of increased inflammation (385±72 vs 210±51 arbitrary units; p=0.023) and apoptosis (48.1±2.2 vs 2.6±0.9 cells/hpf; p<0.0001) compared to non-diabetic wt mice (fig. 19 and 20).

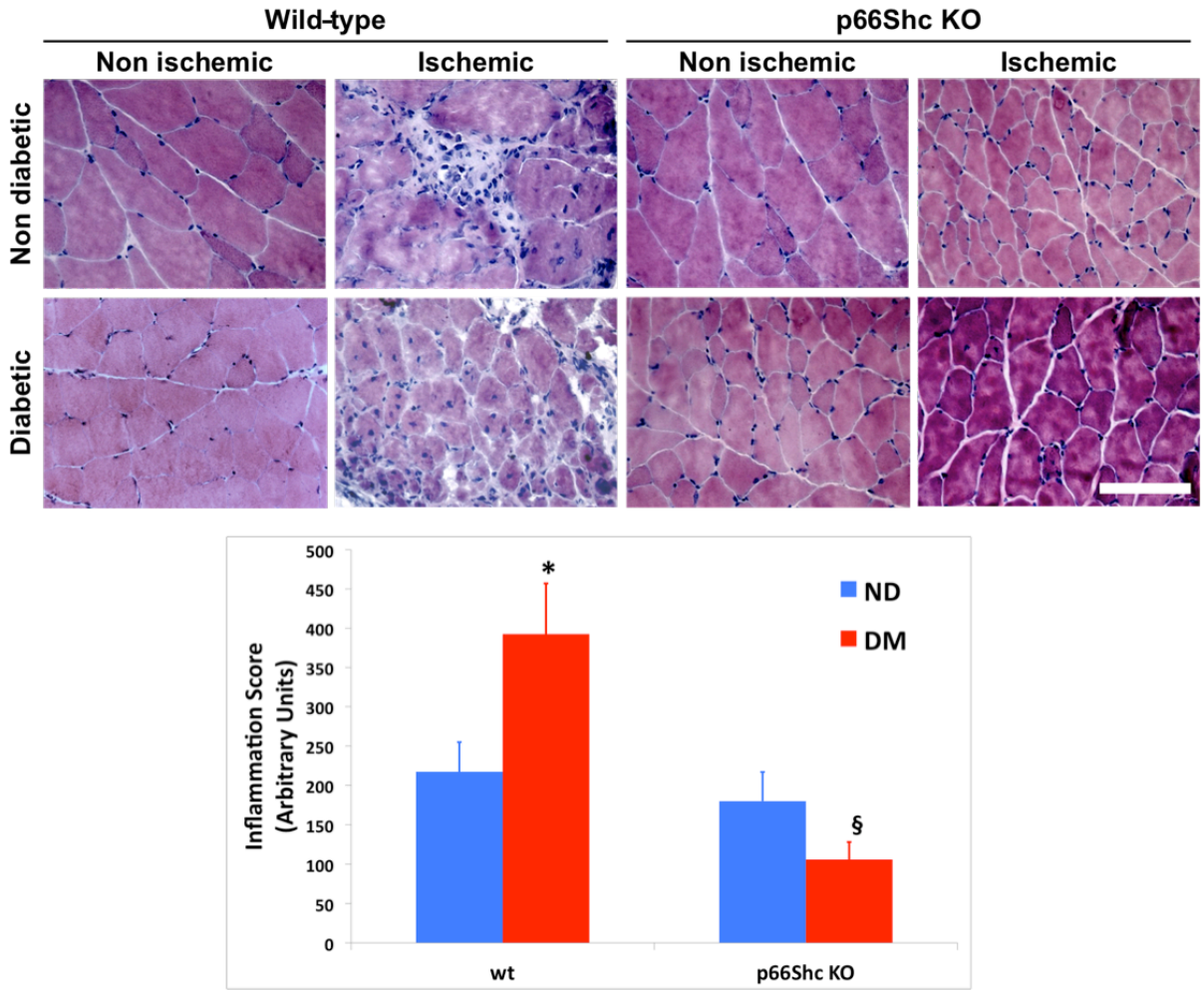


Figure 19: p66Shc KO protects skeletal muscle from ischemia in diabetes. p66Shc KO reduced tissue injury and inflammation in diabetic skeletal muscle after ischemia. Representative hematoxylin-eosin stainings are shown. Scale bar 100 µm. N≥3 mice for each group; quantification were performed in 10 random hpf. * p<0.05 in diabetic vs the corresponding group non-diabetic. § p<0.05 in p66Shc KO vs the corresponding wt.

Moreover, diabetes inhibits post-ischemic angiogenic responses reducing both capillary density (0.83±0.07 vs 1.05±0.07 capillaries/fiber; p=0.047, fig. 21A) and muscle perfusion in wt mice (0.48±0.06 vs 0.54±0.05 ischemic/non-ischemic perfusion ratio; p=0.05, fig. 21B). The

deletion of p66Shc was associated with a significant improvement in all these parameters: in p66Shc KO diabetic mice apoptosis and inflammation is not increased after ischemia in comparison to non-diabetic p66Shc KO mice (fig. 19 and 20). Moreover, p66Shc KO mice in the settings of diabetes and ischemia show reduced apoptosis and inflammation in comparison to corresponding wt mice (fig. 19 and 20). Notably, in non-diabetic animals, the knockout of p66Shc reduced post-ischemic perfusion recovery as compared to wt (0.81±0.03 vs 1.05±0.07 capillaries/fibers; p=0.05; ischemic/non-ischemic perfusion ratio 0.30±0.03 vs 0.54±0.05; p=0.02, fig. 21A and B). However, p66shc knockout improved perfusion recovery in diabetic as compared to non-diabetic mice (1.15±0.11 vs 0.81±0.03 capillaries/fiber; p=0.04; 0.40±0.05 vs 0.30±0.03 ischemic/non-ischemic perfusion ratio; p=0.04, fig. 21A and B), suggesting that p66shc deletion counteracts the negative impact of diabetes on blood flow recovery after ischemia.

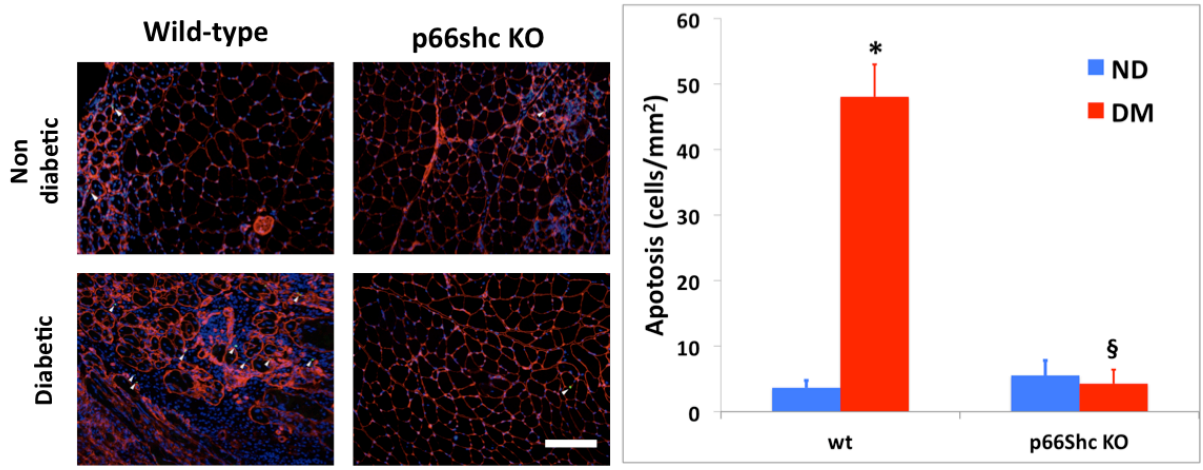


Figure 20: Apoptosis in skeletal muscle after ischemia. p66Shc KO diabetic mice showed reduced apoptosis in ischemic skeletal muscle compared to wt mice. Apoptotic cells are showed in green (arrows), fibers are stained in red with with α-laminin antibody. Nuclei in blue, stained with Hoechst. Scale bar 200 µm. N≥3 mice for each group. counts were performed in 10 random hpf per sample. * $p<0.05$ in diabetic vs the corresponding group non-diabetic. § $p<0.05$ in p66Shc KO vs the corresponding wt.

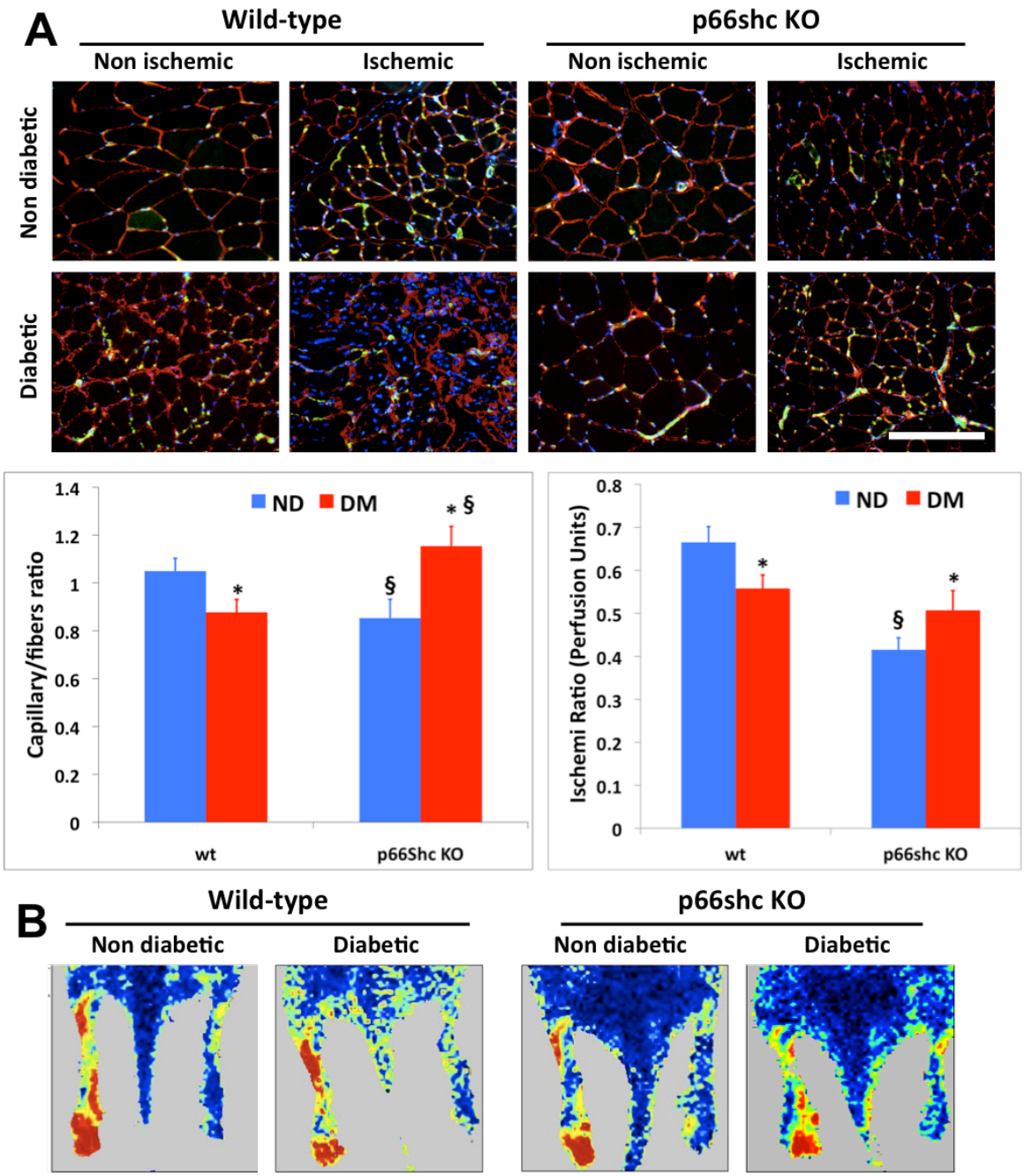


Figure 21: Post-ischemic angiogenesis and perfusion in skeletal muscle. Post-ischemic angiogenesis in skeletal muscle was evaluated quantifying capillary density and expressed as capillary/fibers ratio. Representative images are shown (fig. 21A) Blood vessels are stained in green with BS4-lectin, fibers are stained in red with α-laminin antibody. Nuclei in blue, stained with Hoechst. Scale bar 200 μm. Perfusion was measured with laser-doppler and expressed as ischemic ratio (ischemic vs non-ischemic). Representative images of hind limbs perfusion are shown (fig. 21B). On the left of each picture the perfused limb (reddish coloration); on the right the ischemic limb. N≥3 mice for each group. Capillary density was quantified in 10 random hpf per sample. Perfusion was quantified in 5 measurement per animal. * $p<0.05$ in diabetic vs the corresponding group non-diabetic. § $p<0.05$ in p66Shc KO vs the corresponding wt.

8. p66Shc KO mice don't mobilize endothelial progenitor upon ischemia

Endothelial progenitor cells (EPCs) are mobilized upon ischemia and are involved in compensatory angiogenic responses [122]. Therefore, we tried to find out whether p66Shc was somehow involved in the mobilization of EPCs in response to ischemia. We quantified $CD34^+$/ $Flk\text{-}1^+$ cells four days after ischemia in wt and p66Shc KO mice, diabetic and non-diabetic. Wild-type non-diabetic mice potently mobilized EPCs after ischemia (12.0±1.15 vs 42.67±2.67 cells/millions events, p=0.0004) while diabetes completely blunted bone marrow release of EPCs after ischemia (15.5±4.77 vs 14.0±8.94 cells/millions events, p=0.87). On the other hand, neither in absence of diabetes (15.5±4.77 vs 14.0±8.94 cells/millions events, p=0.15) nor in the setting of diabetes (4.33±4.33 vs 8.67±4.67 cells/millions events, p=0.53) did p66Shc mice mobilize EPCs after ischemia (fig. 22).

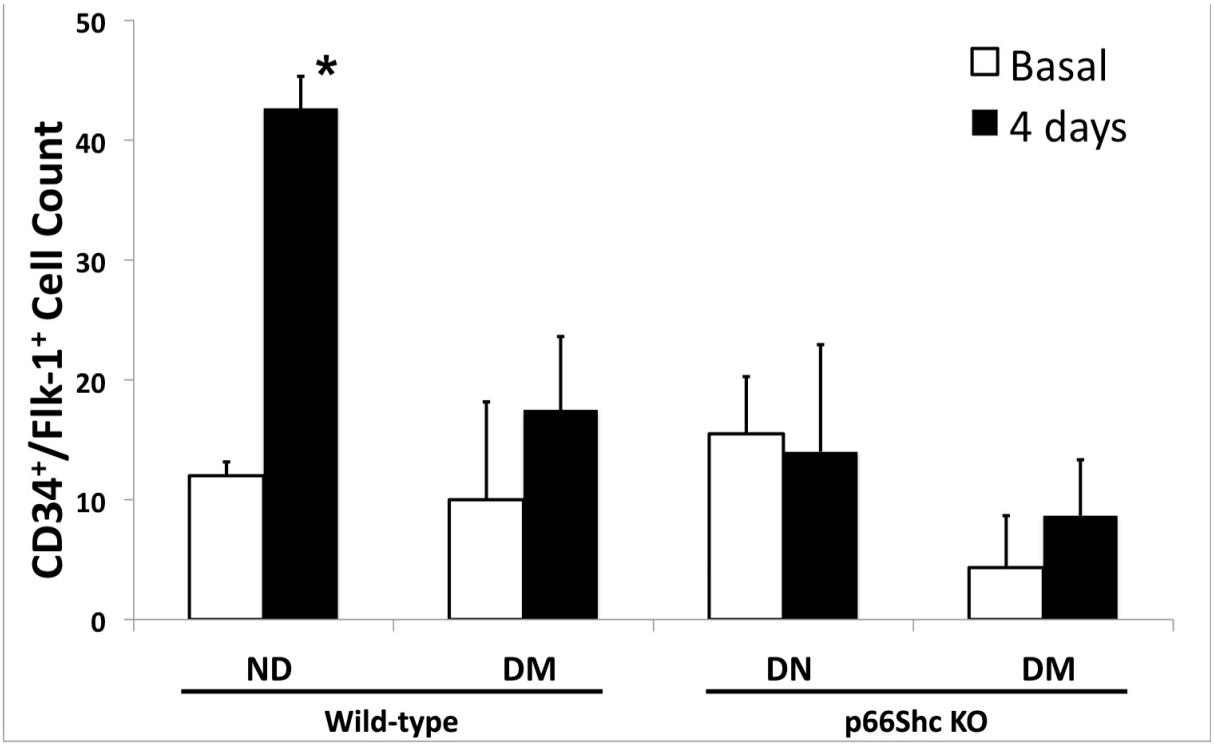


Figure 22: Progenitor cells count. CD34+/Flk-1+ cells were counted in wt e p66Shc knockout (KO) mice at basal and four days after ischemia. Ischemia triggers mobilization of EPCs in wt non-diabetic (ND) animals but does not mobilize EPCs in wt diabetic (DM) mice. On the contrary, in p66Shc KO mice, we have mobilization neither in DM animals nor in ND ones. Cell counts are expressed as cells on millions events. N≥3 mice for each experiment. * p<0.05 in basal vs 4 days.

VII. Discussion

In this work, we have demonstrated for the first time that genetic ablation of p66Shc improves wound healing in the presence of diabetes and hind limb ischemia, by reducing oxidative stress, AGE formation, and by improving fibroblast migration and granulation tissue maturation. Moreover, diabetes increases p66Shc expression and activation, thus depicting a causative role for p66Shc in diabetes-induced delayed wound healing.

Diabetes mellitus affects 150 millions people worldwide and by 2025 about 300 millions people are expected be diagnosed with diabetes [123]. These numbers are so overwhelming that we are entitled to speak about diabetic pandemy. Among all the complications, peripheral ischemia and impaired wound healing consistently threaten diabetic patients' life [40]. p66Shc, is a pivotal regulator of redox status and lifespan in mammals [82]. Upon stress stimuli, such as hyperglycemia, ischemia and oxidative stress, p66Shc translocates to the intermembrane space of mitochondria where it oxidizes cytocrome c, and generates H_2O_2, which in turn triggers PTP opening and apoptosis [83, 84]. Notably, p66Shc seems to account for about 30% of the total pool of intracellular H_2O_2. The leading role of oxidative stress in the development of diabetic complications and CVDs had prompted several groups to investigate the putative role of p66Shc in this setting. Indeed, p66Shcwas shown to be involved in the development complications such as diabetic-glomerulopathy, diabetic heart failure and hyperglycemia-induced endothelial dysfunction [99, 103, 106]. This background allowed us to speculate that p66Shc might be also involved in diabetic-induced delayed wound healing.

The main finding of this work, indeed, confirmed this hypothesis, showing that p66Shc knockout protected diabetic animals from impaired wound healing. Moreover, when diabetes was combined with peripheral ischemia, the differences between wt and p66Shc KO animals were amplified and outstanding (Results, fig. 8). Data from literature showed that most, if not all, of the positive effects of p66Shc knockout comes from dampened oxidative stress, which, consequently, reduces tissue injury [96, 98, 103]. By consent, we showed that p66Shc KO reduced nitrotyrosine (a marker of oxidative stress) and pentosidine (a marker of advanded glycation) in skin, which are both known to be hallmarks of impaired diabetic wound healing (Results, fig. 13 and 14) [69, 121, 124]. Glycated haemoglobin and plasma glucose levels did not differed between wt and p66Shc KO diabetic animals; since erythrocytes do not have

mitochondria, which are, so far, the known sites of p66Shc action, indirectly we could warmly state that reduced AGE and nitrotyrosine accumulation in diabetic skin are mediated by p66Shc KO. Nevertheless, a recent paper from Tomilov and colleagues showed that p66Shc KO mice had decreased superoxide production in macrophages due to reduced activation of NADPH oxidase, arguing of a role of p66Shc also in cytoplasmactic ROS generation [116].

Diabetic ulcers typically display impaired granulation tissue formation, collagen deposition, decreased reepithelialization and new vessels formation [69, 124, 125] which are mostly due to increased AGE deposition and increased oxidative stress [69, 124, 126]. This scenario is consistent with our data, showing that skin from diabetic mice have increase nitrotyrosine and pentosidine accumulation, while the analysis of the granulation tissue showed that diabetes and ischemia-induced delayed wound healing is sustained by defective development of granulation tissue: reduced thickness and collagen area, impaired vascularization and reepithelialization (Results, fig. 9). Moreover, diabetes and ischemia augmented apoptosis both in wound edges and in granulation tissue (Results, fig. 10). In p66Shc knockout animals, along with better wound healing, all these features were ameliorated: granulation tissue thickness, collagen area, vascular network and reepitheliliazation were improved as compared to diabetic and diabetic-ischemic wt animals, while apoptosis was greatly reduced (Results, fig. 9 and 10). To complete the study of granulation tissue cells, we analyzed fibroblast migration, which is a critical component for wound healing. Fibroblasts, indeed, once migrated into the healing ulcers, start secreting growth factors and synthesizing collagen to replace the injured extracellular matrix [22, 23]. Moreover, the fibroblast-myofibroblast transition is essential to wound contraction [32]. Diabetic fibroblasts showed decreased migratory capacity and decreased proliferation [127, 128]. We isolated fibroblasts from dermal explants of wt and p66Shc KO non-diabetic mice to asses *in vitro* migratory capacity under hyperglycemia and hypoxia, to mimic diabetes and ischemia. Wt fibroblasts exposed to high glucose (25 mM) and hypoxia showed impaired migration compared to normoxic-low glucose (5 mM) cultivated fibroblasts; p66Shc KO fibroblasts, instead, showed improved migration both in high glucose and high glucose/hypoxia, compared to wt (Results, fig. 17).

According to Stojadinovic *et al*., chronic-non healing ulcers display a molecular fingerprint which included β-catenin and c-*myc* activation in keratinocytes at wound edges. They showed that β-catenin inhibited keratinocyte migration by causing activation of c-*myc*, which induces cell cycle of epidermal stem cells, exhausting cells at wound site. Thus, keratinocytes are

trapped in a proliferative pathway but they could not migrate [120]. Therefore we investigate in our model whether diabetic ulcers shared these molecular hallmarks. Diabetes and ischemia actually increased c-*myc* expression and β-catenin nuclear localization at wound edges; conversely p66Shc KO mice showed reduced activation of both pathways, which is compatible with improved wound healing (Results, fig. 11 and 12).

Data displayed so far clearly show a positive association between p66Shc KO and improved wound healing in diabetes and ischemia. To provide a putative mechanism, we investigated the expression of p66Shc. Diabetes increased p66Shc expression and ser36 phosphorylation in the skin; high glucose and hypoxia exerted similar effects in cultured fibroblasts. Collectively, we pooled a large amount of data to characterize several features of the healing process and understand the role of p66Shc KO to formulate an hypothetical mechanism to enlighten the role of p66Shc in diabetes/ischemia-delayed wound healing (fig. 23).

Hyperglycemia-induced ROS production is believed to be the keystone to activate all the major pathway of diabetic complications [41, 42, 53]. p66Shc is imported into the mitochondria upon phosphorylation by PKC, which is activated by several stress stimuli, including hyperglycemia, oxidative stress and ischemia [83]. These stimuli also release p66Shc from the TIM-TOM complex in the mitochondria, boosting H_2O_2 production, which increases oxidative stress, triggers apoptosis and seems to contribute to ROS-dependent AGE formation [84]. PKC also activates NAPDH oxidase thus augmenting the oxidative stress burden. Hyperglycemia and oxidative stress are crucial to the formation of AGE, which propagate oxidative stress after interacting with their receptor (RAGE) by downstream activation of PKC and NADPH oxidase [48, 69]. Thereafter, as in other diabetic complications, the establishment of a vicious circle amplifies and propagates damage [102].

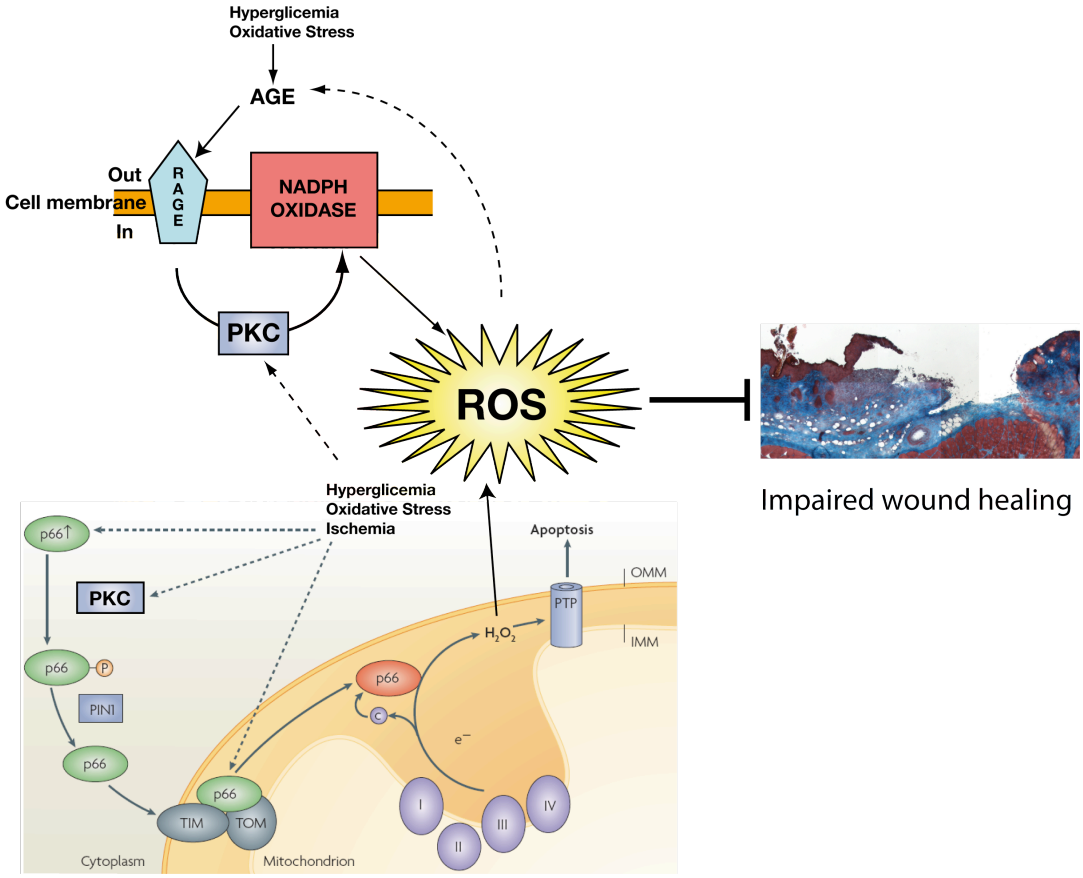
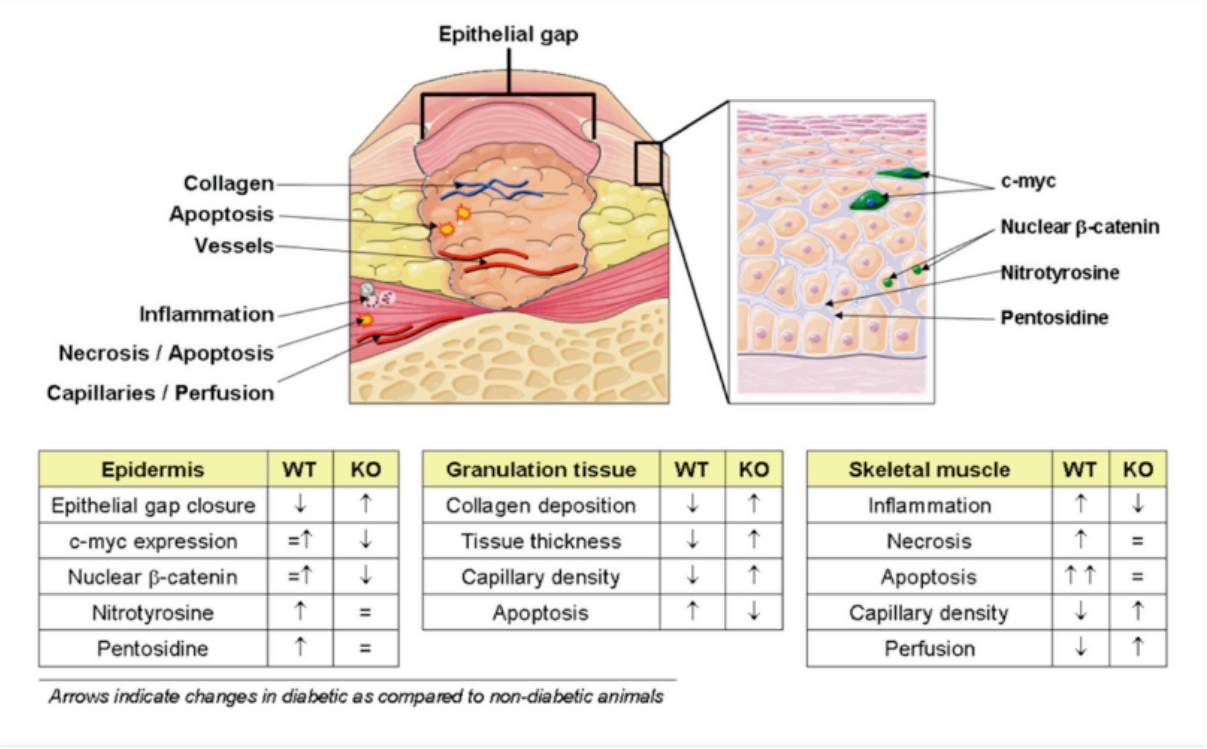


Figure 23: Proposed mechanism of p66Shc-induced delayed wound healing. Stress stimuli activate p66Shc, which translocates into the mitochondria and increases intracellular ROS. ROS increase also AGE deposition, which further augment ROS concentration. p66Shc is the core of this vicious circle, which eventually results in impaired wound healing. (Adapted from Giorgio *et al*., 2007 and from Niedowicz and Daleke, 2005).

AGE are critical determinants of impaired wound healing in diabetes, by reducing wound closure and collagen deposition [126]. Moreover, advanced glycation could negatively affects virtually all the phases of wound healing, including the pathogenesis of predisposing factors such as micro- and macroangiopathy and neuropathy [69]. H_2O_2, the most stable of all ROS with the highest intracellular concentration, is actively involved in wound healing [129, 130]. Therefore dysregulation of H_2O_2 production leads to impaired wound healing [131] and tissue damage. p66Shc is the core of all the pathway because its knockout is sufficient to reduced AGE and oxidative stress markers in the skin from diabetic mice; furthermore diabetes-increased p66Shc expression is associated with AGE and oxidative stress markers in the skin, confirming, also, the existence of a vicious circle linking ROS and AGE [102].

Herein, we provide a characterization of the wound healing process in some key features, to dissect the role of p66Shc in the setting of diabetes. p66Shc increased oxidative stress un-

leashing a cascade of cellular and biochemical modifications that finally lead to impaired wound healing (fig. 24).



Epidermis	WT	KO
Epithelial gap closure	↓	↑
c-myc expression	=↑	↓
Nuclear β-catenin	=↑	↓
Nitrotyrosine	↑	=
Pentosidine	↑	=

Granulation tissue	WT	KO
Collagen deposition	↓	↑
Tissue thickness	↓	↑
Capillary density	↓	↑
Apoptosis	↑	↓

Skeletal muscle	WT	KO
Inflammation	↑	↓
Necrosis	↑	=
Apoptosis	↑↑	=
Capillary density	↓	↑
Perfusion	↓	↑

Arrows indicate changes in diabetic as compared to non-diabetic animals

Figure 24: Wound healing in p66Shc KO vs wt. This cartoon resumes the many differences in wound healing features between p66Shc KO and wt. Arrows indicate changes in diabetic as compared to non-diabetic.

The second part of this study focused on the effects of p66Shc during ischemia in skeletal muscle. p66Shc knockout protected skeletal muscles from ischemic injury, reducing apoptosis, inflammation and increasing capillary density and perfusion compared to wild-type animals. Moreover, p66Shc KO mice are protected also in the setting of diabetes, which exacerbated ischemic insults in wild-type animals. These data are consistent with a previous work from Zaccagnini *et al.*, extended to the setting of diabetes [104]. Notably, we concordantly found that p66Shc KO adductor muscles in absence of ischemia had reduced capillary density compared to wt (Results, fig. 21). This observation was confirmed also by perfusion data. Interestingly, while diabetes dampened capillary density and perfusion in ischemic muscle from wt mice, in p66Shc KO mice we observed an inverted trend; diabetic ischemic muscles were more perfused and had higher capillary density as compared to diabetic non-ischemic muscles. This strange pattern was conserved also in granulation tissue capillary network, suggesting that p66Shc may have a role in angiogenesis. The study of

angiogenic signal conveyed by p66Shc was not among the specific aims of this thesis, but we propose a mechanisms to explain these data. ROS are currently reconsidered as important signalling molecules, and vascularization appears to be tightly regulated by the oxidative status [132]. The expression of many angiogenic factors is regulated by ROS; specifically, VEGF, the most potent angiogenic gene, is induced by micromolar concentration of H_2O_2 [133]. While physiological amount of ROS could improve vascularization, both excess or paucity of ROS could results in impaired vascular response: increased ROS concentration leads to apoptosis and tissue damage while reduced ROS could be rapidly detoxified by scavenging enzymes.

Therefore, p66Shc KO animals in physiological conditions may have reduced ROS levels, below the threshold to exert vasculogenic effects after ischemia. With the induction of diabetes, wt animals are exposed to pathological levels of oxidative stress with apoptotic and antiangiogenic effects. On the contrary, the knockout of p66Shc may counteract the negative effects of diabetes, lowering the ROS levels to a concentration which lead to pro-angiogenic effects (fig. 25).

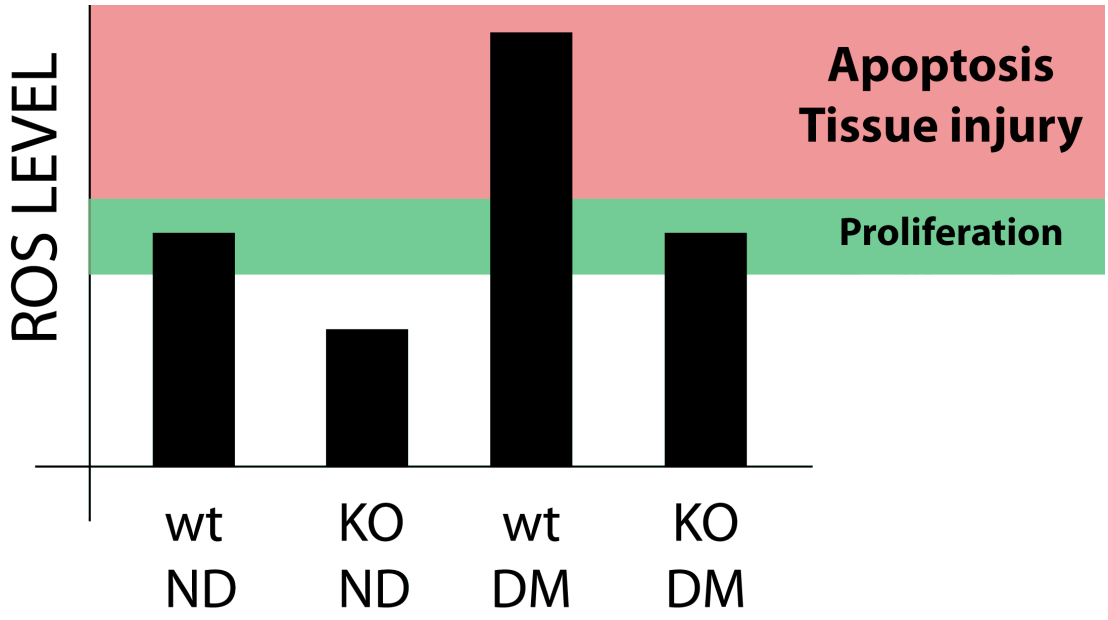


Figure 25: p66Shc and ROS levels. Hypothetical relation between p66Shc and redox status. In non-diabetic (ND) wild-type animals (wt) ROS levels reach physiological concentration, which in response to ischemia and wound healing triggers proliferation and stimulates angiogenesis. In p66Shc knockout animals (KO), the reduced levels of ROS do not reach the threshold to induce angiogenesis upon ischemia. In the settings of diabetes, however, wt animals had increased ROS concentration, which leads to tissue injury. The knockout of p66Shc, on the other hand, counteract diabetes-induced ROS production, lowering ROS concentration to a pro-angiogenic concentration.

EPCs are bone marrow-derived cells which may contributes to postnatal vasculogenesis [64]. Ischemia is one most powerful stimuli that triggers EPCs mobilization, but we have previously shown that diabetes dampens ischemia-induced mobillization in rats [74, 75, 134]. In a preliminary batch of experiments, we investigated in this model the role of p66Shc in EPCs mobilization. We confirmed that diabetes impairs ischemia-induced mobilization of EPCs (defined as $CD34^+/Flk\text{-}1^+$ cells). p66Shc KO mice, however, failed to mobilize EPCs after ischemia both with and without diabetes. Ischemia triggers mobilization by inhibiting the degradation of transcription factor hypoxia-inducible factor-1α (HIF-1α), which in turn activates transcription of target genes as VEGF, nitric oxide synthase [130]. Importantly it is not the absolute oxygen tension (*p*O2) that activates response to ischemia but actually delta *p*O2. In p66Shc KO animals, according to our data and to Zaccagnini *et al*. as well, the little differences in perfusion between ischemic and non-ischemic muscle could be insufficient to stimulate mobilization. Moreover, p66Shc KO mice had improved recovery after ischemia, suggesting that ischemic muscle may be able to face injury without the support of EPCs. A recent paper from Di Stefano *et al*., demonstrated that p66Shc exert protective effects also in EPCs exposed to high glucose [109]; therefore, even if we did not see mobilization of EPCs, p66Shc KO mice may have more functionally active EPCs to face ischemic injury. The role of p66Shc in angiogenic signaling and EPCs biology surely deserve further investigations.

A final consideration should be done. As underlined in the introduction, the model of genetic ablation of p66Shc is a very powerful tool to study aging and pathological mechanisms linked to oxidative stress, but we have to consider that, in this model, animals did not express p66Shc since the beginning of their development and the knockout affect all tissues. In the light of devising a pharmacologic approach to target p66Shc, it is reasonable to ask whether the effects of an entire life without p66Shc could be mirrored by pharmacological inhibition. Since recent papers have discovered possible side effects of p66Shc knockout,and that human regulation of p66Shc appeares to be more complex than previously thought, further studies are needed to clarify the biology of p66Shc [110, 111, 115]

VIII. Bibliography

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