***Fadini et al.* "The redox enzyme p66Shc contributes to diabetes and ischemia-induced delay in cutaneous wound healing".**

DATA SUPPLEMENT

MATERIAL AND METHODS

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 authorised by our local institutions. All experiments were performed in SV129 mice, while part of the histopathological characterization was replicated in C57Bl/6 mice, once the p66Shc$^{-/-}$ backcross was completed. Wild type SV129 and C57Bl/6 animals were purchased from Harlan Italy. p66Shc$^{-/-}$ mice were on matched SV129 or C57Bl/6 genetic backgrounds. Generation of p66Shc$^{-/-}$ mice was described previously (1). Animals were divided into 4 groups: wild-type non-diabetic, wild type diabetic, p66Shc$^{-/-}$ non-diabetic and p66Shc$^{-/-}$ diabetic. All animals were subjected to hind kimb ischemia on the right hind limb while the left hind limb served as non-ischemic control.
Diabetes was induced with a single intraperitoneal injection of 150 mg/kg streptozotcin (Sigma Aldrich, St. Louis, MO, USA) in citrate buffer 50 mM, ph 4.5. Non diabetic animals were injected with the vehicle. Blood glucose was measured with a commercially available kit (Glucocard Gmeter, Menarini, Florence, Italy) on days 2, 7 and 14. Animals with blood glucose ≥300 mg/dl in at least two measurements within the first two weeks were considered diabetic, while animals with glucose lower than 300 mg/dl were not used further. Diabetic mice were allowed feeding and drinking ad libitum and caged in controlled conditions. Animals were then stabulated for 4 or 12 weeks before experiments in order to stabilize diabetes and allow establishing of hyperglycaemic damage.

Hindlimb ischemia. Hind limb ischemia was induced by surgical resection of femoral artery and vein. Animals were sedated with 10 mg/kg tilethamine hydrochloride–zolazepam hydrochloride (Zoletil, Laboratories Virbac, Nice, France) and 7 mg/kg xylazine (Xilor, Laboratories Carlier, Spain). Femoral artery and vein were surgically dissected from femoral nerve and then cauterized with low temperature cautery (FIAB, Florence, Italy) and excised between inguinal ligament and hackle. Fifteen days after surgery, we performed Laser Doppler imaging on ischemic animals to measure microvascular perfusion of both hind limbs. Briefly, 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; adductor and semimembranous muscles were harvested and frozen in liquid nitrogen cooled isopentane.

Nitrotyrosine, pentosidine and HbA1c quantification. Nitrotyrosine concentration in mouse skin was measured with an ELISA assay. Skin was shaved and cleaned from fat and cellular debris and then processed according to Fallon et al. (2). For the ELISA assay, we used a mouse α-nitrotyrosine monoclonal Ab (Millipore, Tecumela, CA, USA). Pentosidine was quantified using HPLC (High Performance Liquid Cromatography) on a Prostar 363 HPLC detector (Varian, Palo Alto, CA, USA) equipped with C-18 columns. Pentosidine peak was eluted after 20 minutes. At least 3 samples per group of animals were quantified. The operator was blind to the status of animals. Red cell haemoglobin A1c (HbA1c) was measured with an automated DCA2000+ analyzer (Bayer, Milan Italy).

Skin wounds. To create cutaneous wounds, animal was sedated with inhaled sodium isofluorane, skin of dorsal surface of the hind limb was shaved with Veet cream and disinfected with alcohol/ether; a 4 mm 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 commercially available image processing software (ImageJ, NIH, Bethesda, MD, 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.

Progenitor cell counts. Blood samples were collected from the orbital sinus at basal condition and 4 days after induction of hind limb ischemia for progenitor cells count. There is no consensus as to the phenotype to identify endothelial progenitor both in humans and experimental studies. We chose CD34 and Flk-1 to identify endothelial progenitor cells, as in other studies (3). This phenotype is reminiscent of the CD34+KDR+ cell population, which is considered the best human EPC phenotype at flow cytometry (4). 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 the Macintosh CELLQuest software program (Beckton Dickinson)

Culture of dermal fibroblast. Fibroblasts were isolated from skin explants of wild type and $p66Shc^{-/-}$ 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 in a sterile Petri dish and cut in about 1 mm^2 pieces that were placed with dermis adherent to bottom of the Petri 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 it. We used cells from passage 3 to 7 and every experiment was repeated at least in triplicate. For the migration assay we used a commercially available device (Ibidi, Munich, Germany) according to the 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 maintained in high glucose for the whole experiments lasting. Hypoxia was achieved with a commercial available device (GasPack EZ, Beckton Dickinson) according to the manufacture's instructions; cells were cultivated in normoxia and then placed in hypoxic condition for the whole migration assay. To test resistance to oxidative stress, fibroblasts from w/t and $p66Shc^{-/-}$ mice were exposed to hydrogen peroxide (H_2O_2) for 24 hours. Viability was assessed by trypan blue exclusion and expressed as percentage of cells (supplemental figure II).

Histology and immunohistology. All stainings were performed on 7 µm thick cryosections. Hematoxilyn & Eosin and Masson Trichrome stainings were performed using commercially available kits (both by Bio-Optica, Milan, Italy) according to manufacture's protocols. Counts and quantifications were performed on whole sections 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 TRITC conjugated α-rabbit Ab (1:150 DakoCytomation, Denmark). Counts ware performed in 10 random high-power fields 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 α c-myc monoclonal Ab (1:100, Roche Diagnostic, Basel, Switzerland) and mouse α-β-catenin monoclonal Ab (1:50, Beckton Dickinson). Secondary antibody was goat α-rabbit-TRITC (1:150, Jackson Labs). Count was performed in random high-power fields repeated for at least 3 animals per group. Images were acquired with Leica TCS SP5 confocal microscope. 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 high power random fields. Histological quantifications were performed using a commercially available image processing software (ImageJ).

Western Blot. Tissue (skin or muscle) extracts were prepared by 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 protease inhibitor (Roche Diagnostic) and phosphatase inhibitor (Sigma Aldrich). Protein concentration was quantified with standard Bradford reagent (Sigma Aldrich). Proteins were separated by SDS-PAGE (10% polyacrilamide gel). After proteins transferring on nitrocellulose membrane, the membranes were incubated overnight in phosphate buffered saline-0,1% Tween-20 buffer (PBSt) containing 5% non-fat dry milk and with primary polyclonal antibody anti-Shc (Upstate, Lake Placid, USA) or anti-Shc/p66, phospho-specific(Ser36) (Calbiochem, Nottingham, UK) for 3h at room temperature. HRP-conjugated antibody were used as secondary antibody. Immunoreactive proteins were visualized with chemiluminescence (Pierce, Rockford, USA) and captured on X-ray film.

Gene expression analysis. Total RNA was extracted from heart, skeletal muscle (gastrocnemius), kidney and white adipose tissue of control and STZ diabetic mice by the guanidine thiocyanate-phenol-chloroform method using TRIzol Reagent (Invitrogen Italia, San Giuliano Milanese, Italy). p66Shc transcript was quantified by competitive RT-PCR, as previously described (5). Briefly, 1 μg total RNA was reverse transcribed using the Retroscript kit (Ambion, Austin, TX), and the resulting cDNA was amplified using the following primers (sense: 5'ACTACCCTGTGTTCCTTCTTTC 3'; antisense: 5'TCGGTGGATTCCTGAGATACTGT 3') and conditions (annealing temp 57°C, n° of cylces 32; expected size 795 bp). Competitive PCR was performed by using increasing amounts of mutants made by creating a deletion in the original PCR product. After electrophoresis of PCR products, the unknown cDNA/mutant ratio was quantified by scanning densitometry using ImageJ software and results expressed as the ratio of each target to β-actin mRNA level.

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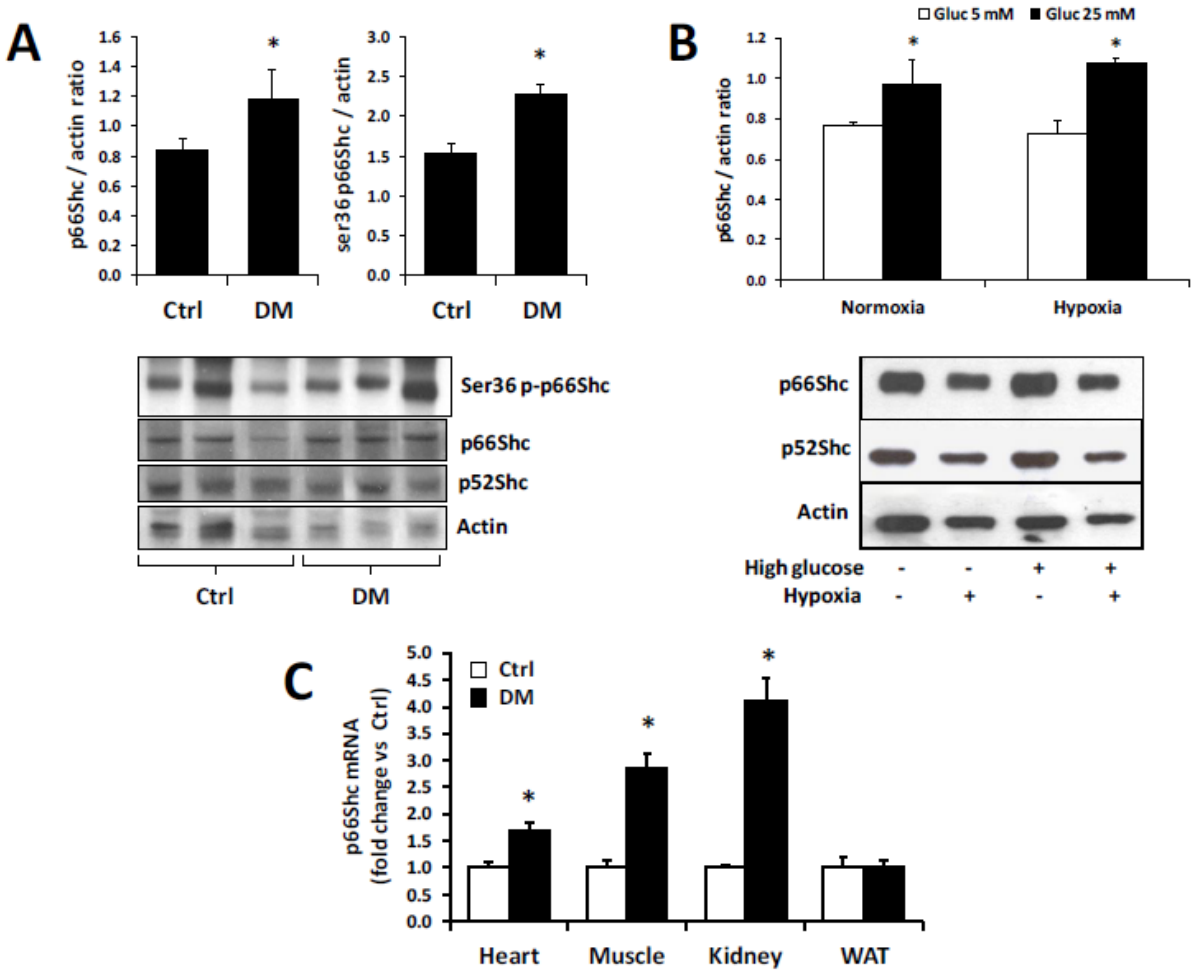
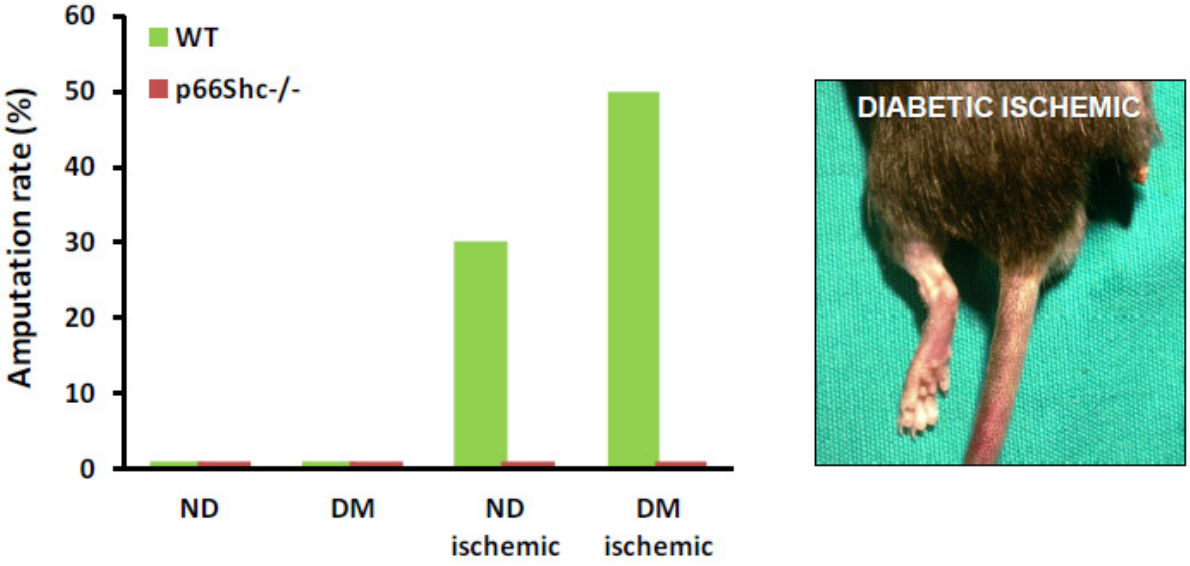
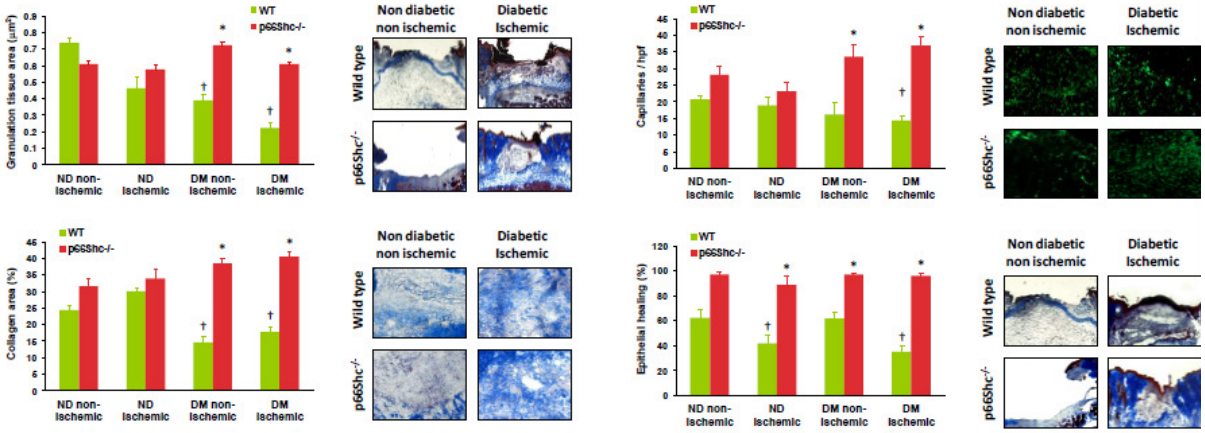
Supplemental figure I. p66Shc expression and activation in skin samples, dermal fibroblasts and other tissues. A) p66Shc protein expression in skin samples of non-diabetic (CTRL) and diabetic (DM) mice was determined by Western Blot analyses, normalized for the housekeeping actin. p66Shc activation was determined as phosphor-serine 36 p66Shc protein expression normalized by actin expression. *p<0.05 vs CTRL. **B**) p66Shc protein expression was determined in dermal fibroblasts exposed to normal (5 mM) or high (25 mM) glucose under normoxia or hypoxia with Western Blot, normalized for actin expression. *p<0.05 vs 5 mM glucose. C) p66Shc gene expression was assessed in distant tissues by quantitative PCR relative to non-diabetic mice. * p<0.05 vs non-diabetic (Ctrl). WAT, white adipose tissue.



Supplemental Figure II. Amputation rate in diabetic and non diabetic C57Bl/6 mice with or without ischemia. 30% to 50% of ischemic WT mice developed necrosis and amputation, while no p66Shc$^{-/-}$ mice underwent amputation in any condition. Photo on the right shows auto-amputation after ischemia in a C57Bl/6 mouse with 3-month diabetes.



Supplemental figure III. Characteristics of the granulation tissue in C57Bl/6 mice. Morphological characteristics of the granulation tissue were evaluated in wild type (wt) and p66Shc knock-out (KO) diabetic (4 week, DM) and non diabetic (ND) mice with or without hind limb ischemia. Masson's trichrome staining allowed determination of collagen area and granulation tissue thickness. Re-epithelialization was quantified in histological sections by measuring the percentage of re-covered epithelial gap. Capillary density within the granulation tissue was quantified by B4 isolectin immunofluorescence. Representative images are shown for control (non diabetic non ischemic) and diabetic ischemic wt and KO mice. N≥3 mice for each group; N≥3 sections for tissue sample. * p<0.05 in KO vs the corresponding wt group. † p<0.05 in diabetic vs the corresponding non-diabetic group.



Supplemental figure IV. Dermal fibroblasts from wilt type or p66Shc$^{-/-}$ mice were exposed to control conditions of 100 nM H_2O_2 for 24 hours. Cell viability was study with trypan blue exclusion and expressed as percentage. Representative light microscopy images are also shown. *p<0.01 versus basal condition.