New approaches to gene therapy of wound healing

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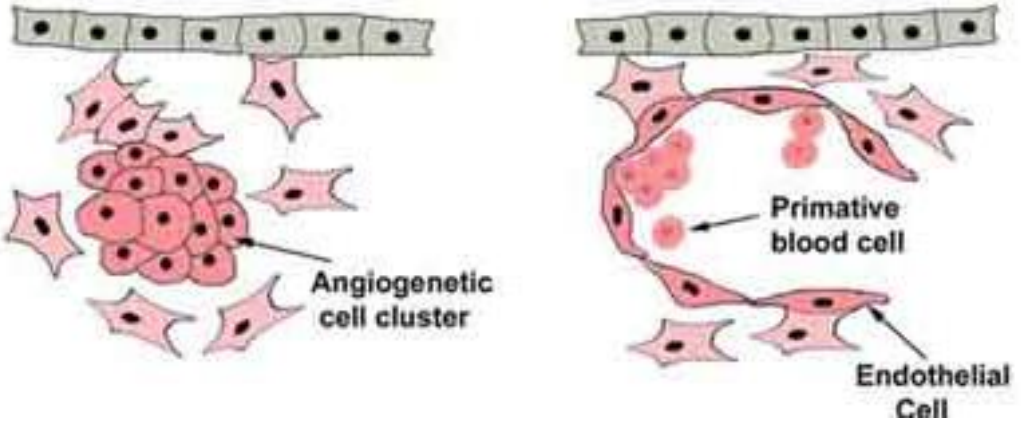
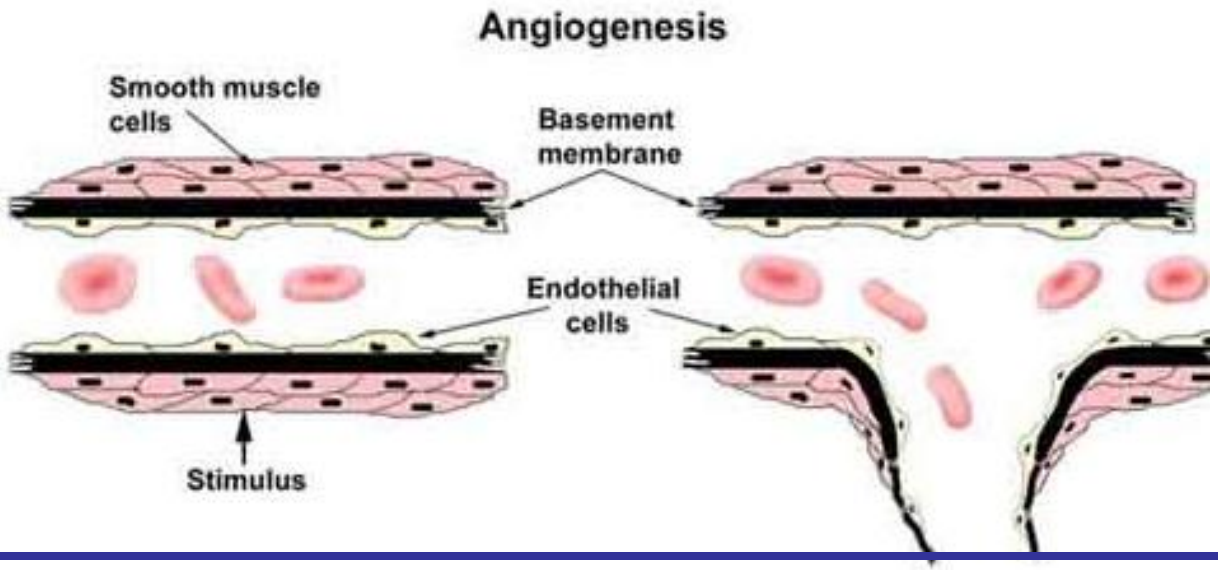
Lecture 11

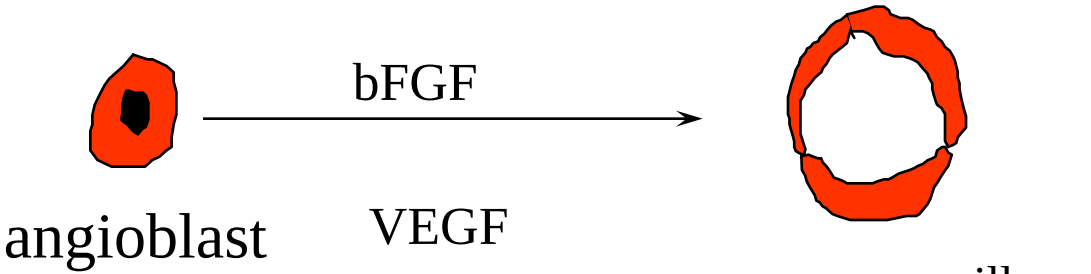
Blood vessel formation

Vasculogenesis in embryo



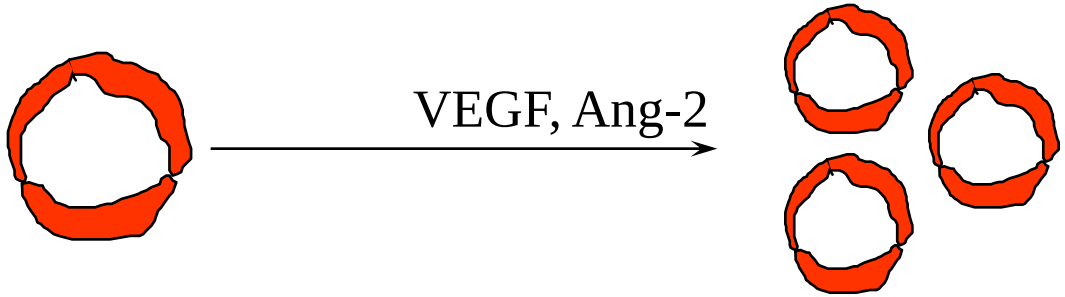


Three ways of formation of blood vessels



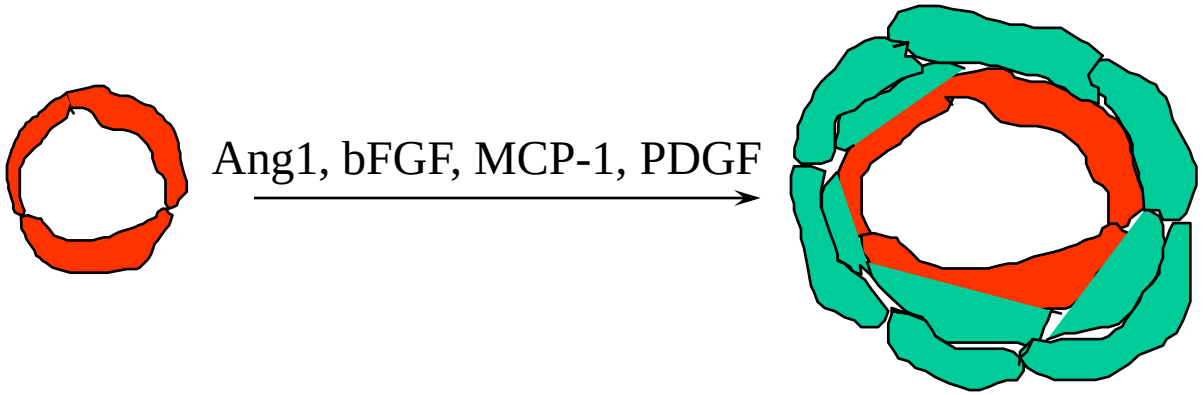
Vasculogenesis

capillaries are formed from vascular progenitor cells



Angiogenesis

formation of new blood vessels from pre-existing vessels

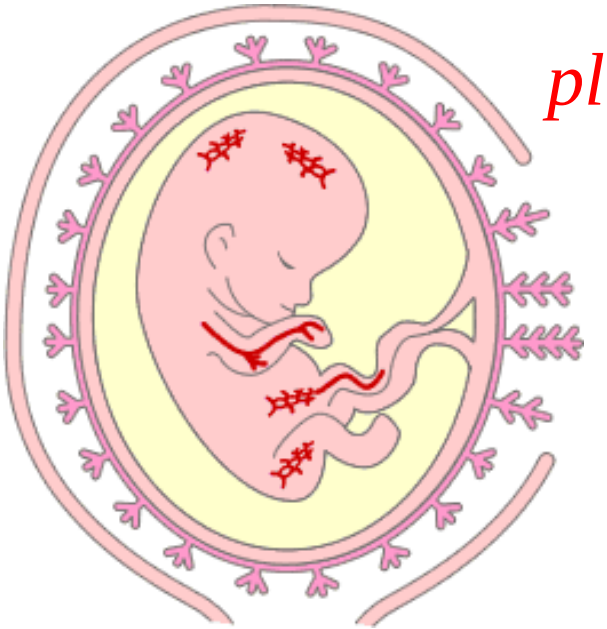


Arteriogenesis

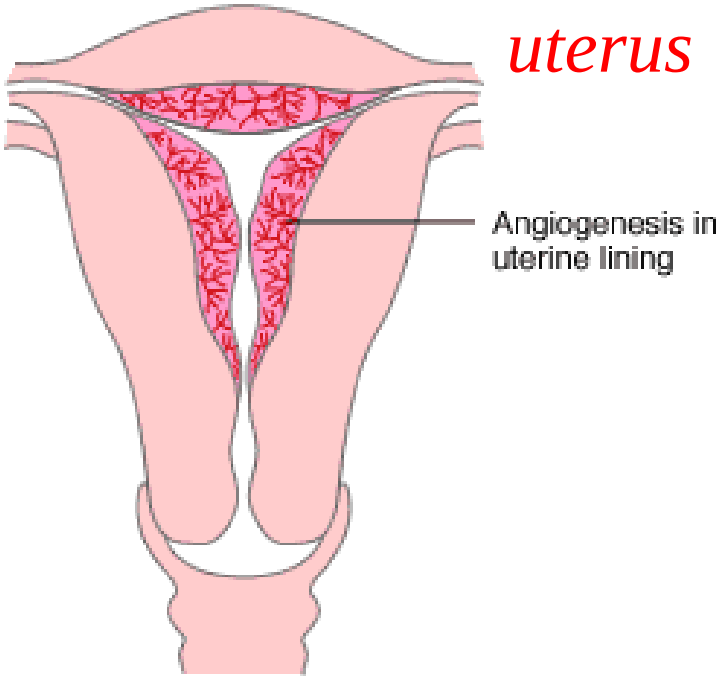
formation of mature blood vessels; differentiation into veins and arteries

Physiological angiogenesis in adults

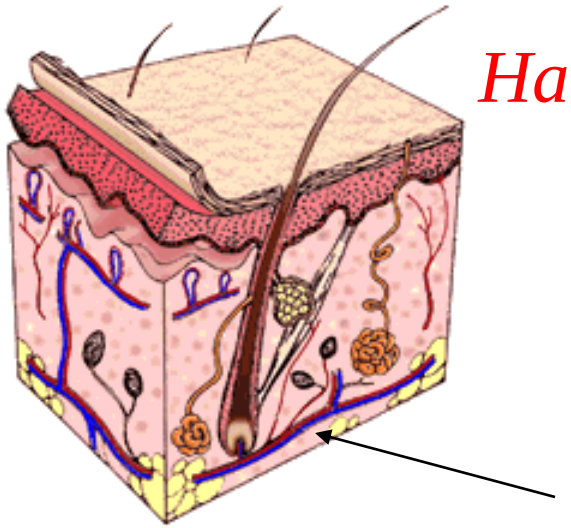
placenta



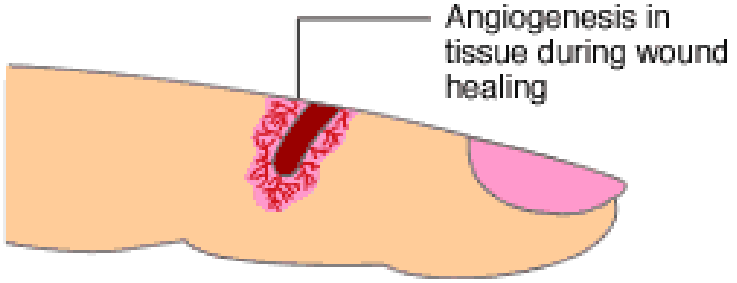
uterus

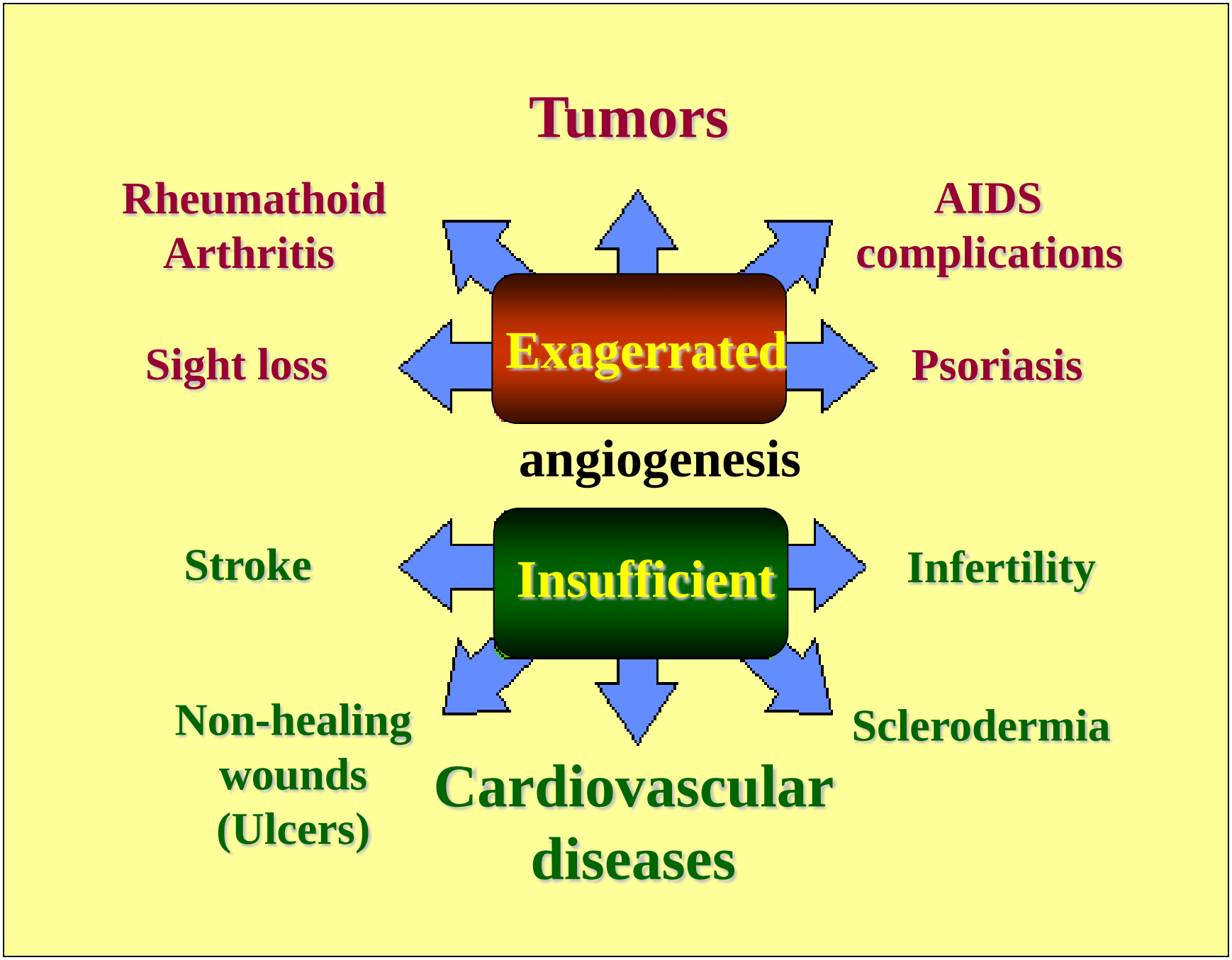


Hair growth

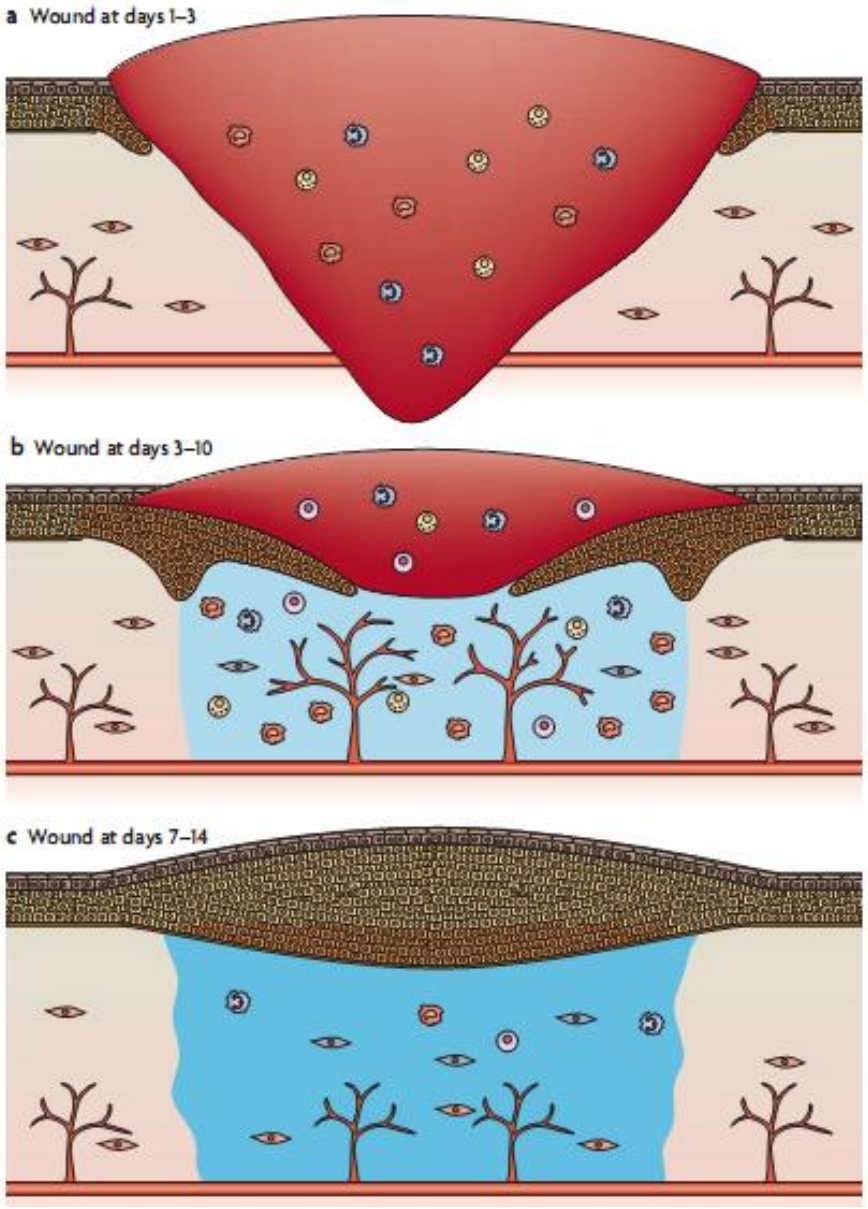


Wound healing




Tumors
Rheumathoid Arthritis
AIDS complications
Sight loss
Exagerrated
Psoriasis
angiogenesis
Stroke
Insufficient
Infertility
Non-healing wounds (Ulcers)
Sclerodermia
Cardiovascular diseases

Different phases of skin wound repair



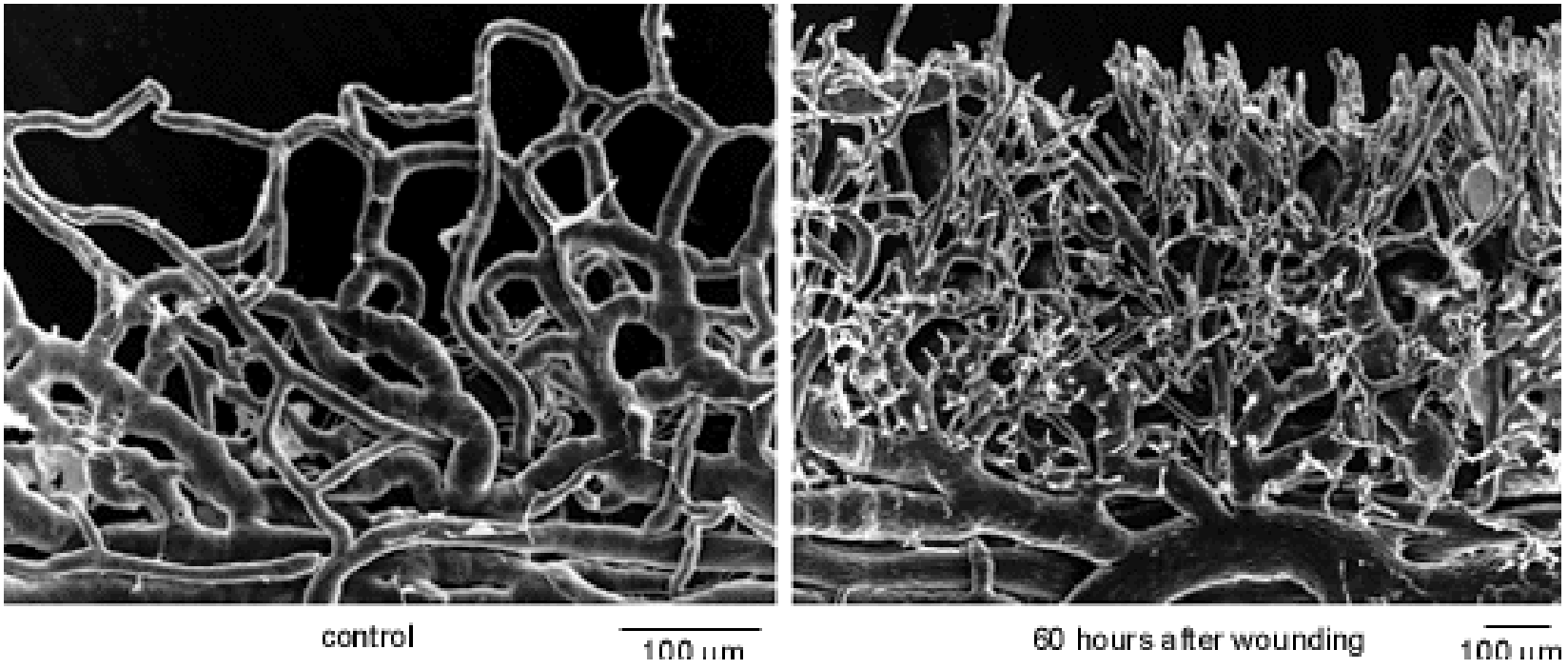
Inflammatory phase

New tissue formation

Remodelling phase

Schafer & Werner, Nature Review Mol Cell Biol 2008

New capillary formation in response to wounding

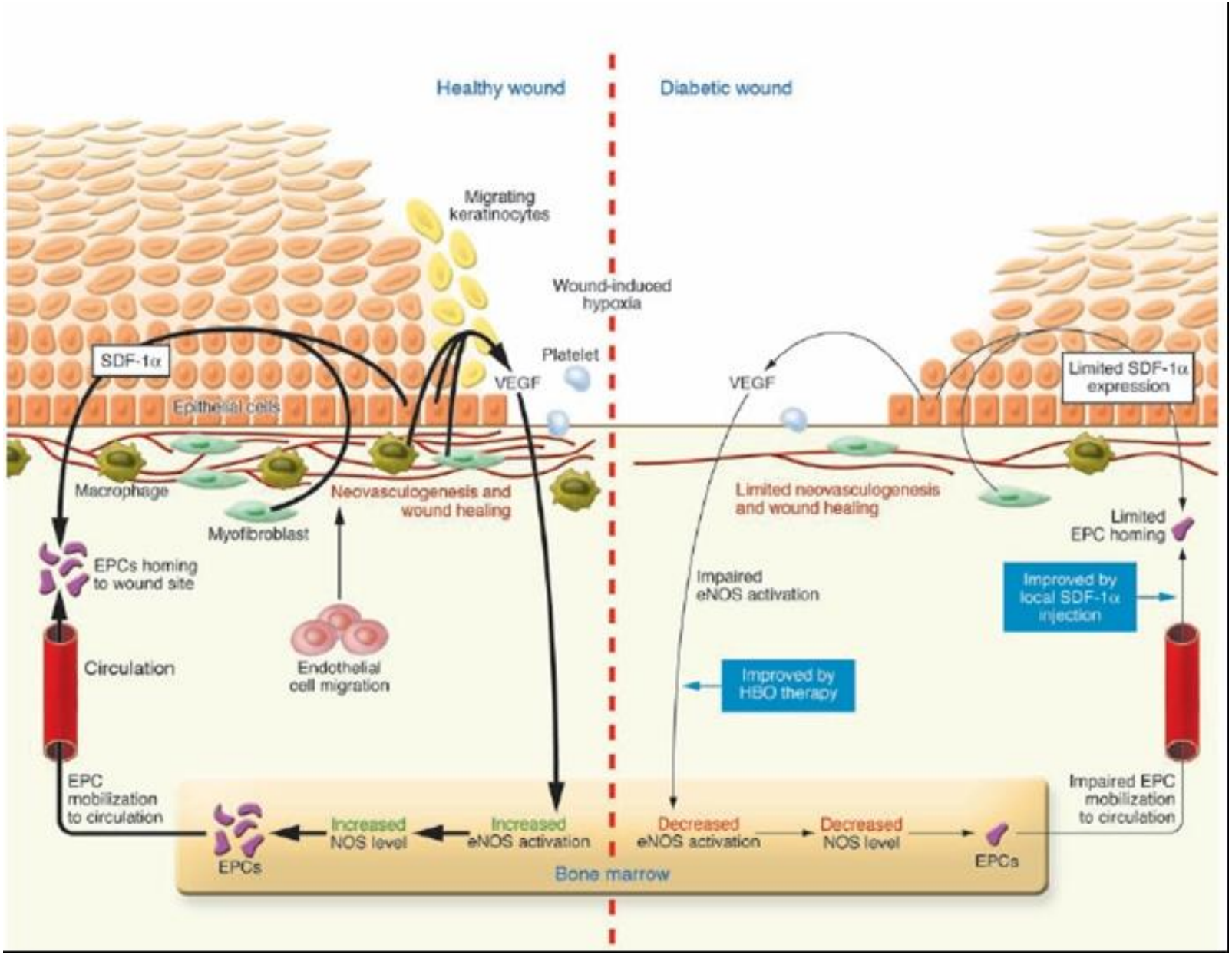


Impaired wound healing in diabetes

One consequence of diabetes mellitus is the disruption of the normal process of wound healing, which is related to the decreased production of different growth factors.

Healing impairment in diabetes is characterized by delayed cellular infiltration and granulation tissue formation, reduced angiogenesis and decreased collagen organization.

Wound healing is impaired in diabetes mellitus



Brem & Tomic-Canic, J Clin Invest, 2007

Animal models of diabetes mellitus

- **Goto-Kakizaki (GK) rats** - hyperglycemia, hypoinsulinemia (impaired pancreatic secretion), and impaired insulin sensitivity in the liver, skeletal muscle and adipose tissues

- **Zucker diabetic fatty (ZDF) rats** - resistance to leptin (two defective leptin receptor alleles; problems with leptin binding), hyperphagia, hyperlipidemia, obesity, hyperglycemia and insulin resistance

- **db/db mouse** - a defect in both alleles of the leptin receptor gene (problems with signal transduction), hyperphagia, hyperlipidemia, obesity, hyperglycemia and insulin resistance

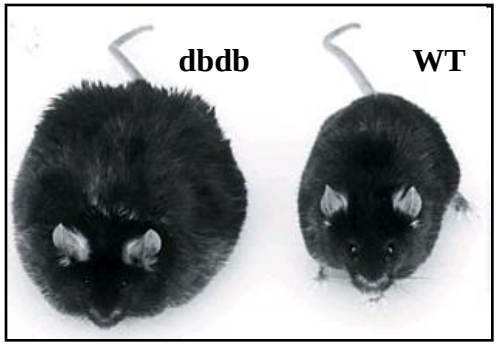
- **Streptozotocin(STZ)-induced diabetes Type 1** - STZ is a naturally occurring chemical that is particularly toxic to the insulin-producing beta cells of the pancreas in mammals. It is used in medicine for treating certain cancers of the islets of Langerhans

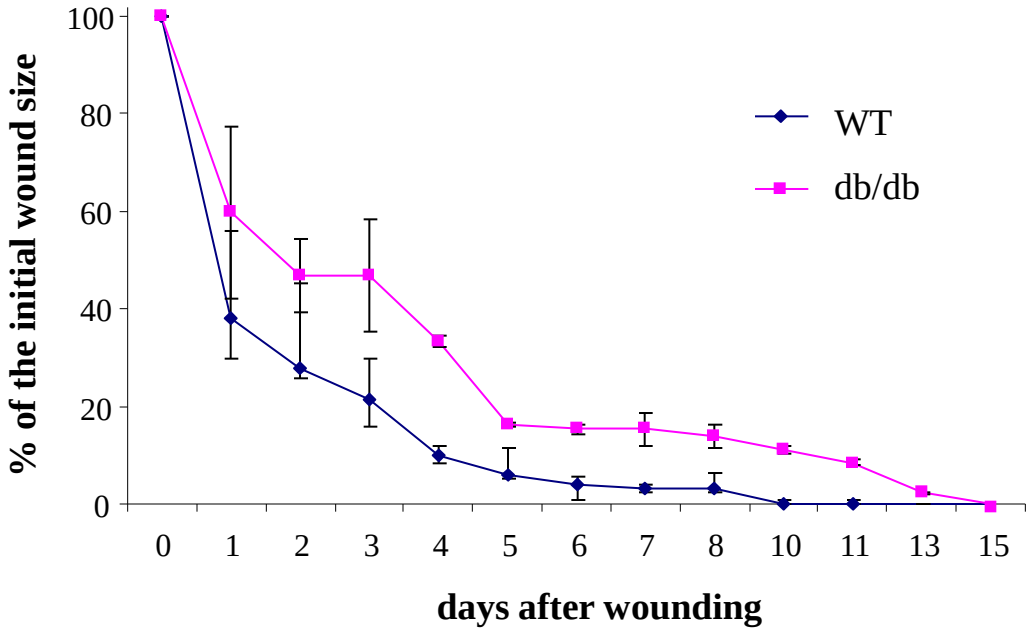
Clinically relevant healing impairment in genetically diabetic mice leptin receptor deficient (db/db)

- obesity
- insulin resistance
- severe hyperglycemia

(that resembles human adult onset diabetes)

- markedly delayed wound healing





Wound closure in diabetic (dbdb) and normoglycemic (WT) mice

Treatment of impaired wound healing in diabetic animals

Application of different recombinant proteins or their genes e.g.:

- platelet-derived growth factor (PDGF)
- vascular endothelial growth factor (VEGF)
- insulin-like growth factor (IGF-I)
- transforming growth factor (TGF)-β
- epidermal growth factor (EGF)
- fibroblast growth factor (FGF)

has been reported to accelerate formation of various components in healing wounds.

Sonoporation of the Minicircle-VEGF165 for Wound Healing of Diabetic Mice

Adventagas of minicircle DNA

- Minicircle is a new form of supercoiled DNA molecule for the non-viral gene transfer that has neither bacterial origin of replication nor antibiotic resistance marker.
- It is thus smaller and potentially safer than the standard plasmids currently used in the non-viral gene therapy.
- Minicircle DNAs have been demonstrated to show more robust and prolonged transgene expression due to its small size and the absence of un-methylated CpG motifs which causes immune responses.

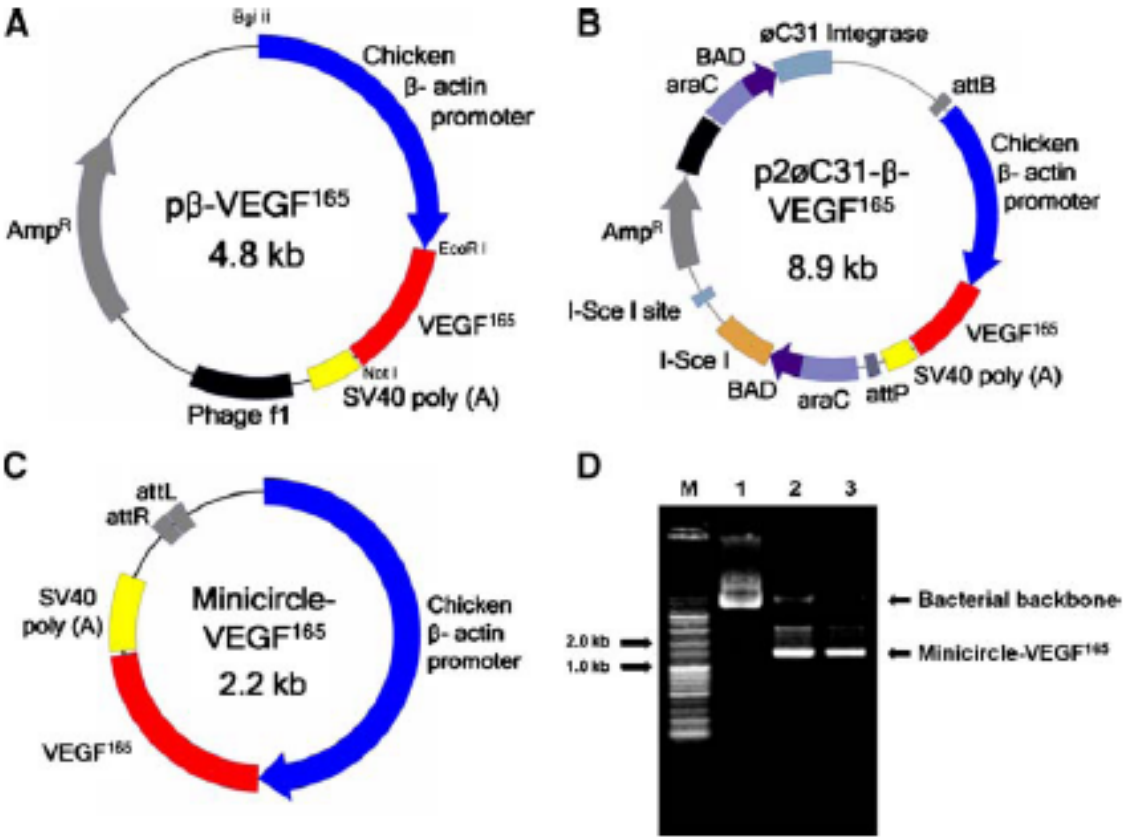
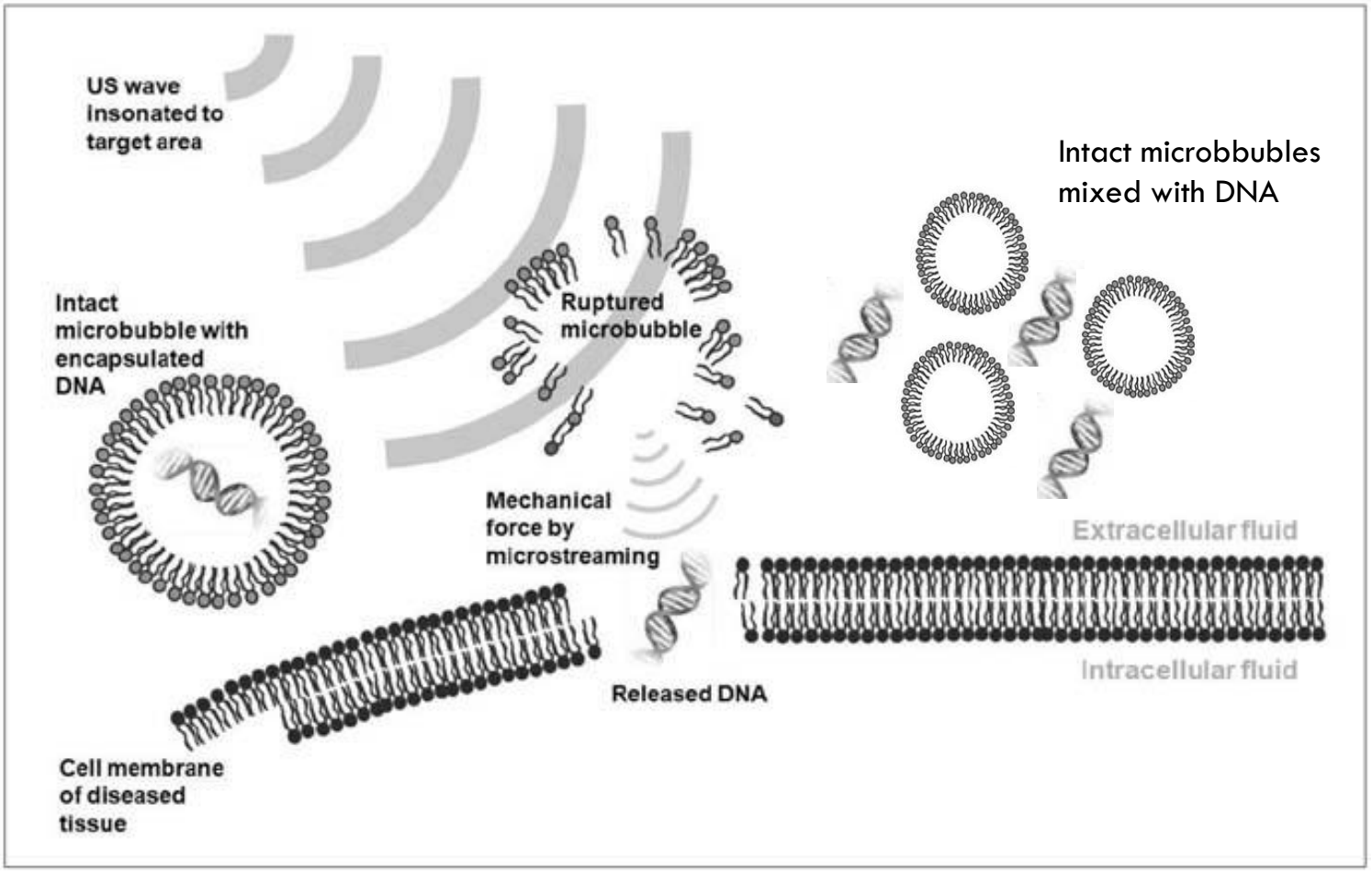


Fig. 1. Physical maps of the constructed vectors. **A** pβ VEGF^{165}; a conventional plasmid containing bacterially originated backbone. The cDNA sequence coding VEGF^{165} was inserted under a chicken β actin promoter. **B** p2øC31 β VEGF^{165}; the expression cassette from pβ VEGF^{165} was excised by restriction enzymes and bluntly ligated between attB and attP site of p2øC31 vector which contains phi C31 integrase and I SceI homing endonuclease. **C** Minicircle VEGF^{165}. **D** Process of minicircle VEGF^{165} production. By adding 1% L arabinose to the bacterial culture media, the att sites of p2øC31 β VEGF^{165} (*lane 1*) were recombined to generate the minicircle DNA (*lane 2*). The remaining circular bacterial backbone plasmids were linearized by I SceI homing endonuclease and were removed by bacterial exonucleases at 37°C (*lane 3*).

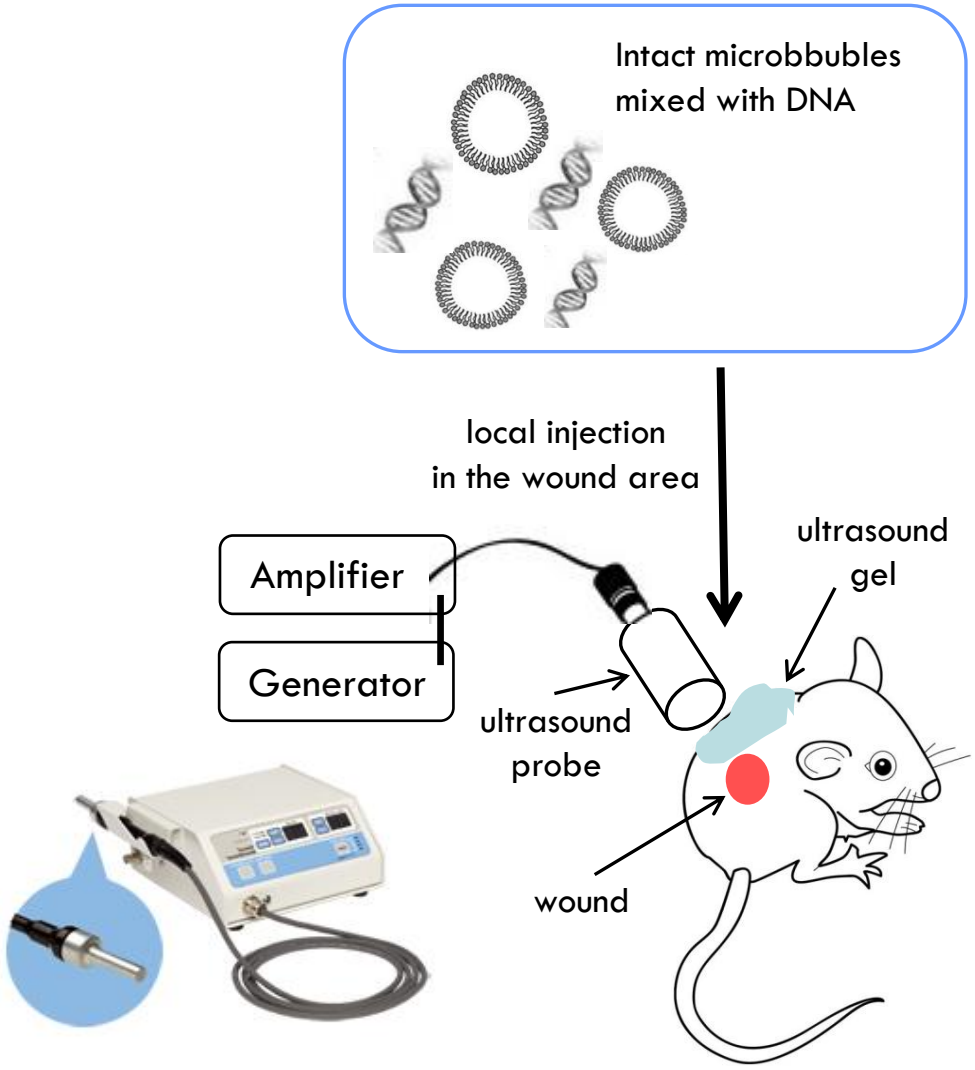
Yoon C.S. et al, Pharm Res, 2009

Sonoporation - ultrasound-induced gene therapy



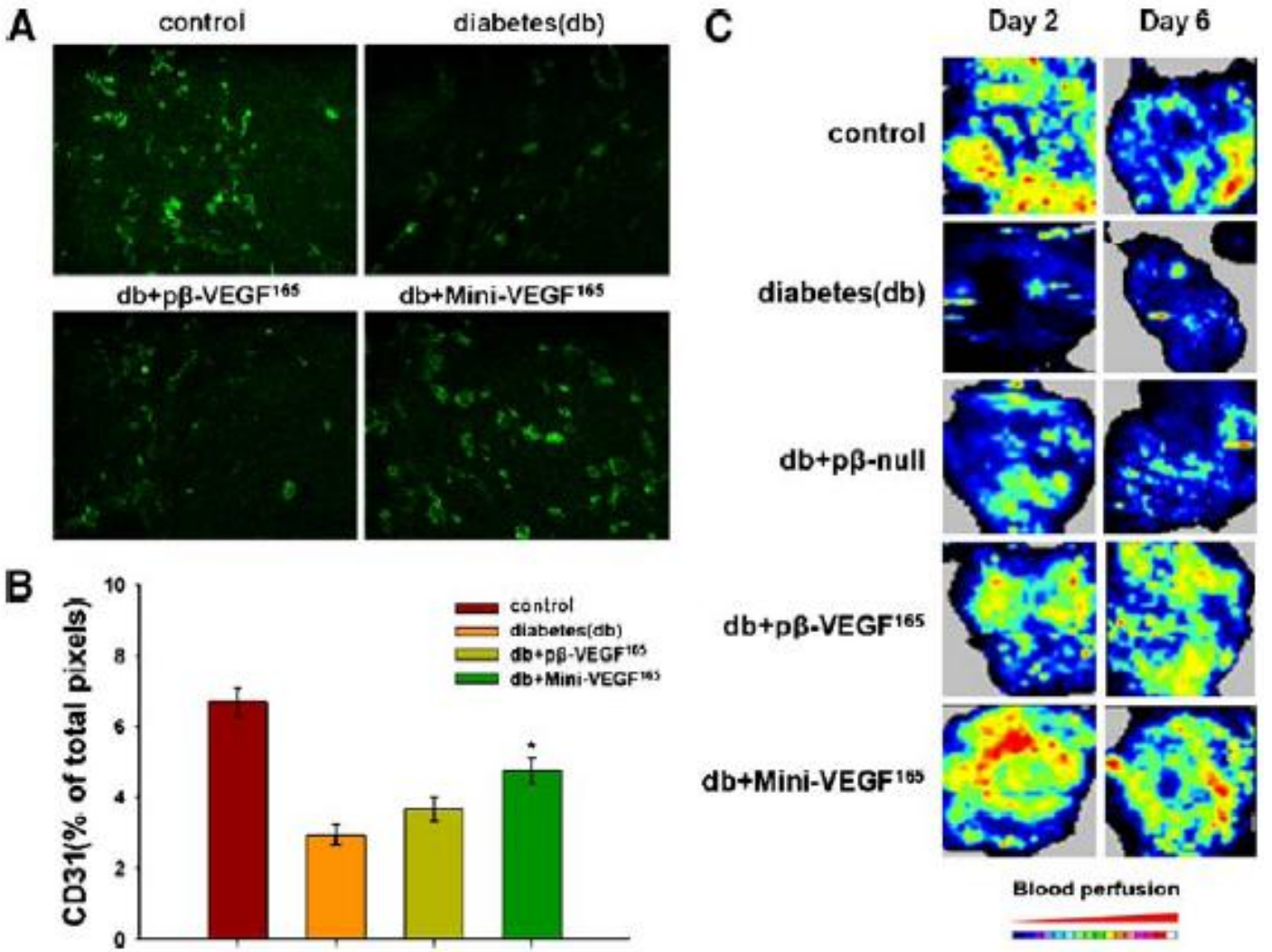
Young-sun Kim et al, Korean J Radiol. 2008 (modified)

Sonoporation - ultrasound-induced gene therapy



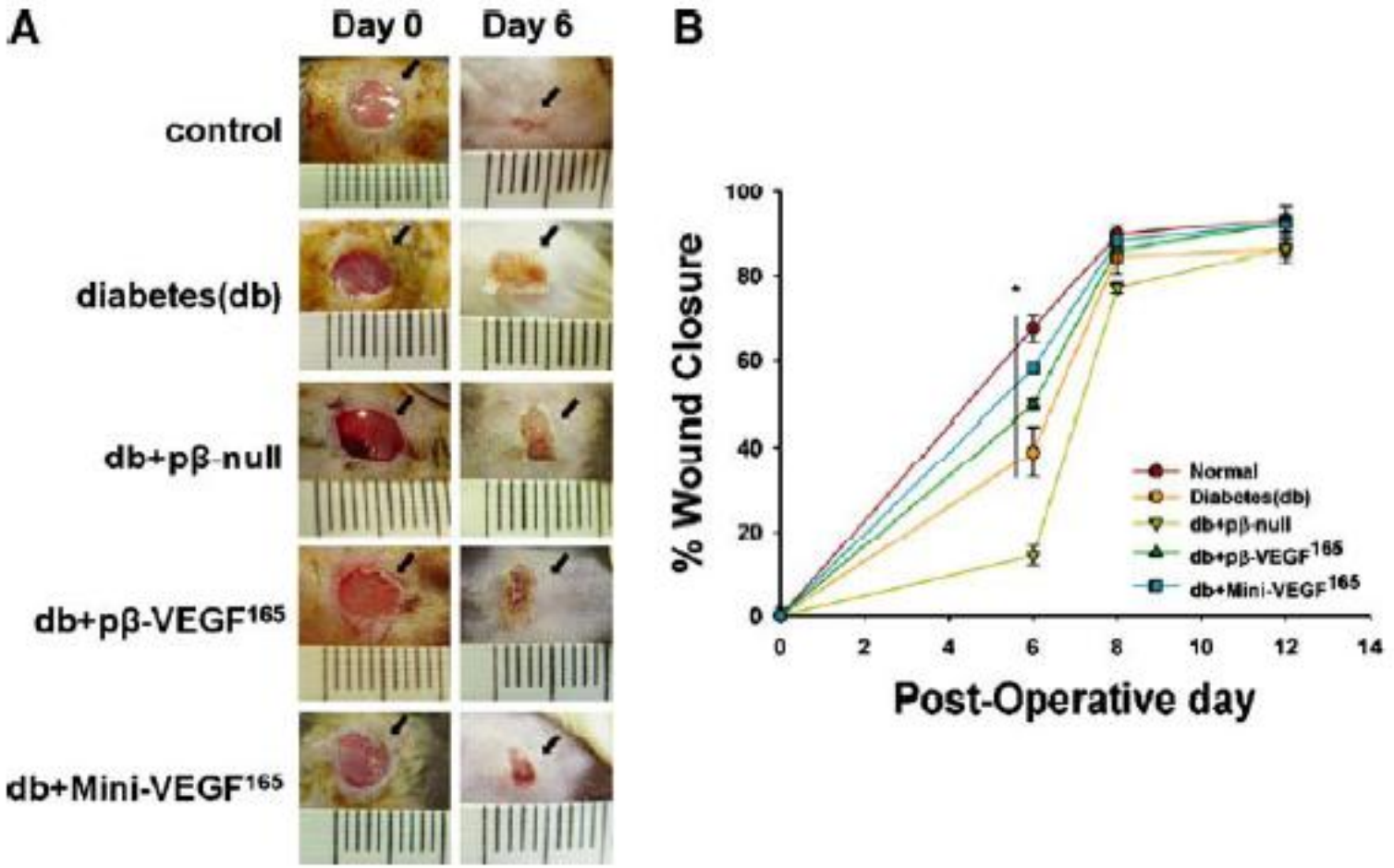
Vevo Soni Gene (Visual Sonics)

Minicircle-VEGF165 increased capillary density and blood perfusion in the wound tissue of treated diabetic mice



Yoon C.S. et al, Pharm Res, 2009

Minicircle-VEGF165 gene delivery via sonoporation enhanced wound closure



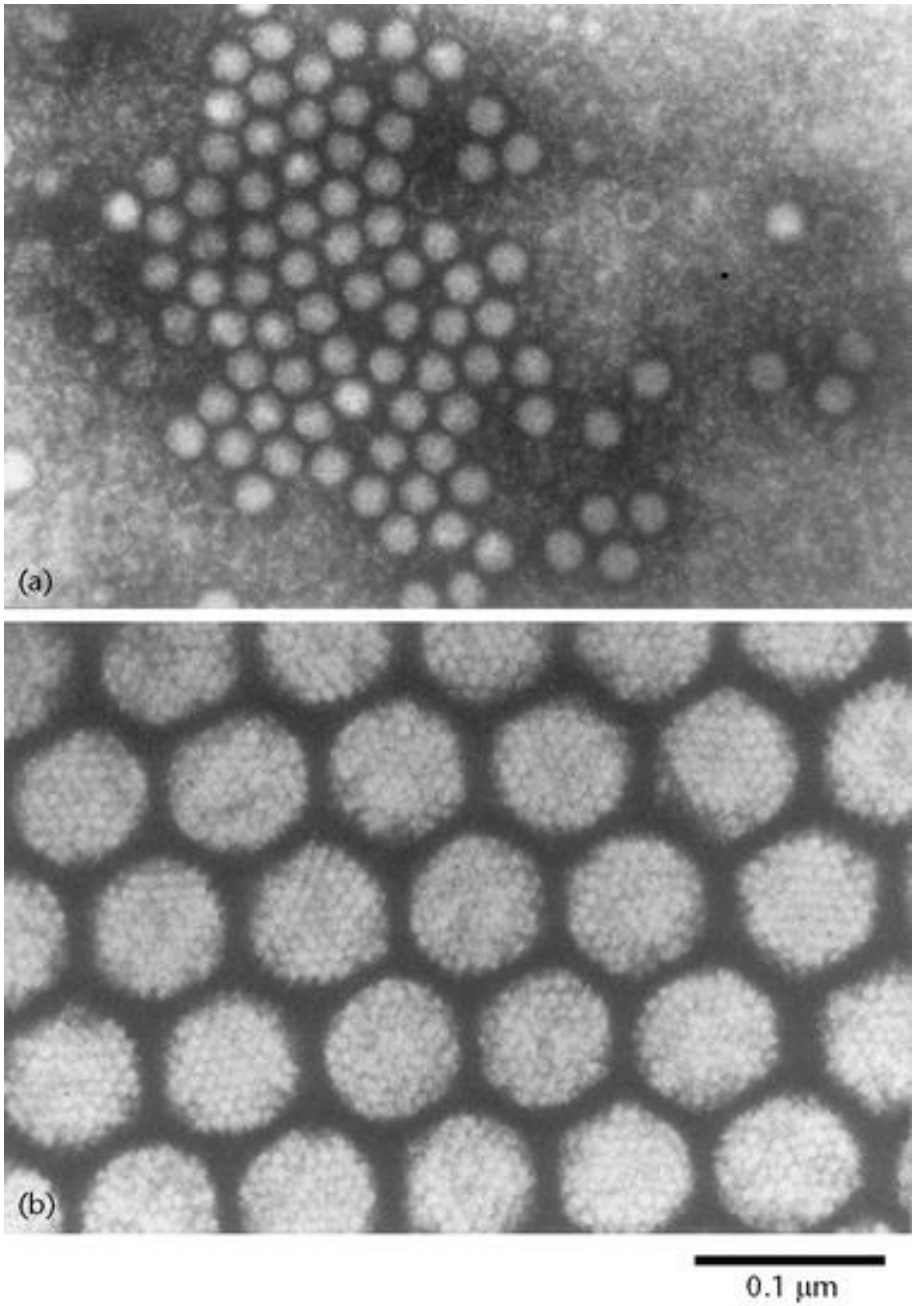
Yoon C.S. et al, Pharm Res, 2009

AAV vectors

removal of rap and cap genes
transgene insertion

ITR

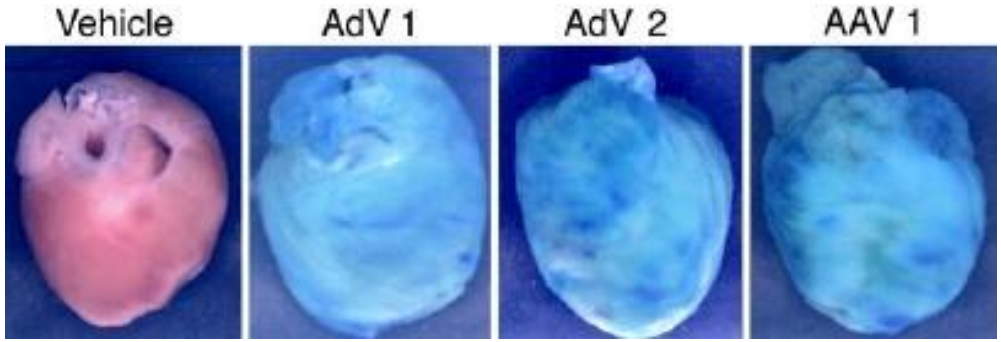
ITR



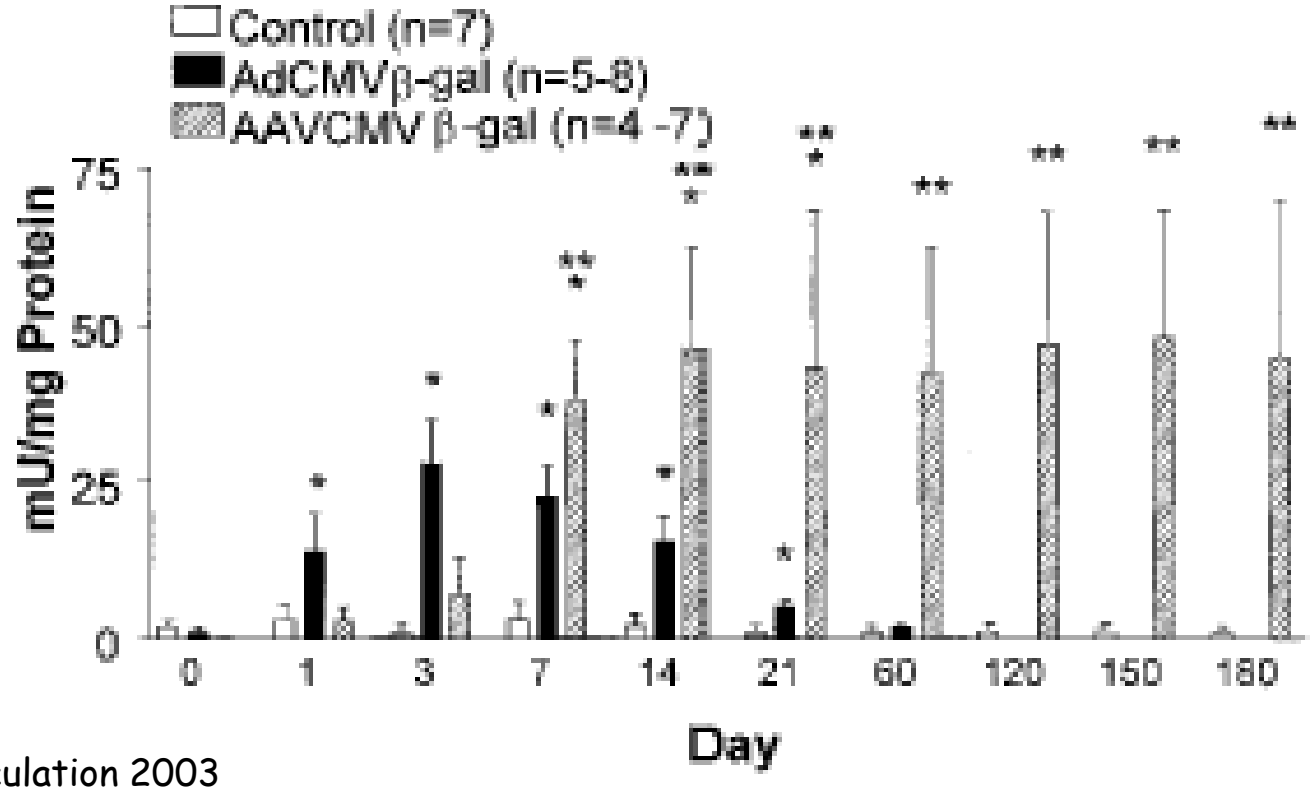
AAV

adenovirus

AAV vectors, in contrast to adenoviral, can provide long-term expression

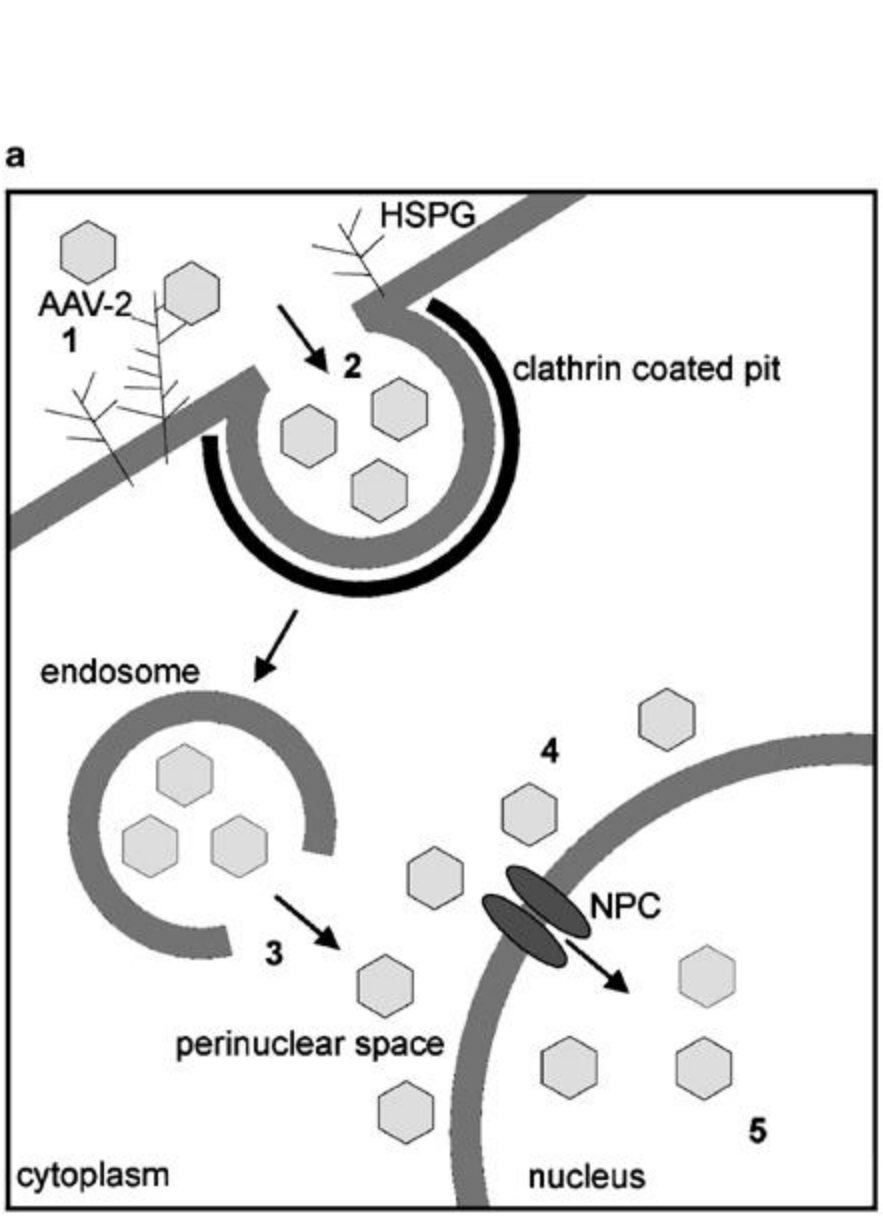


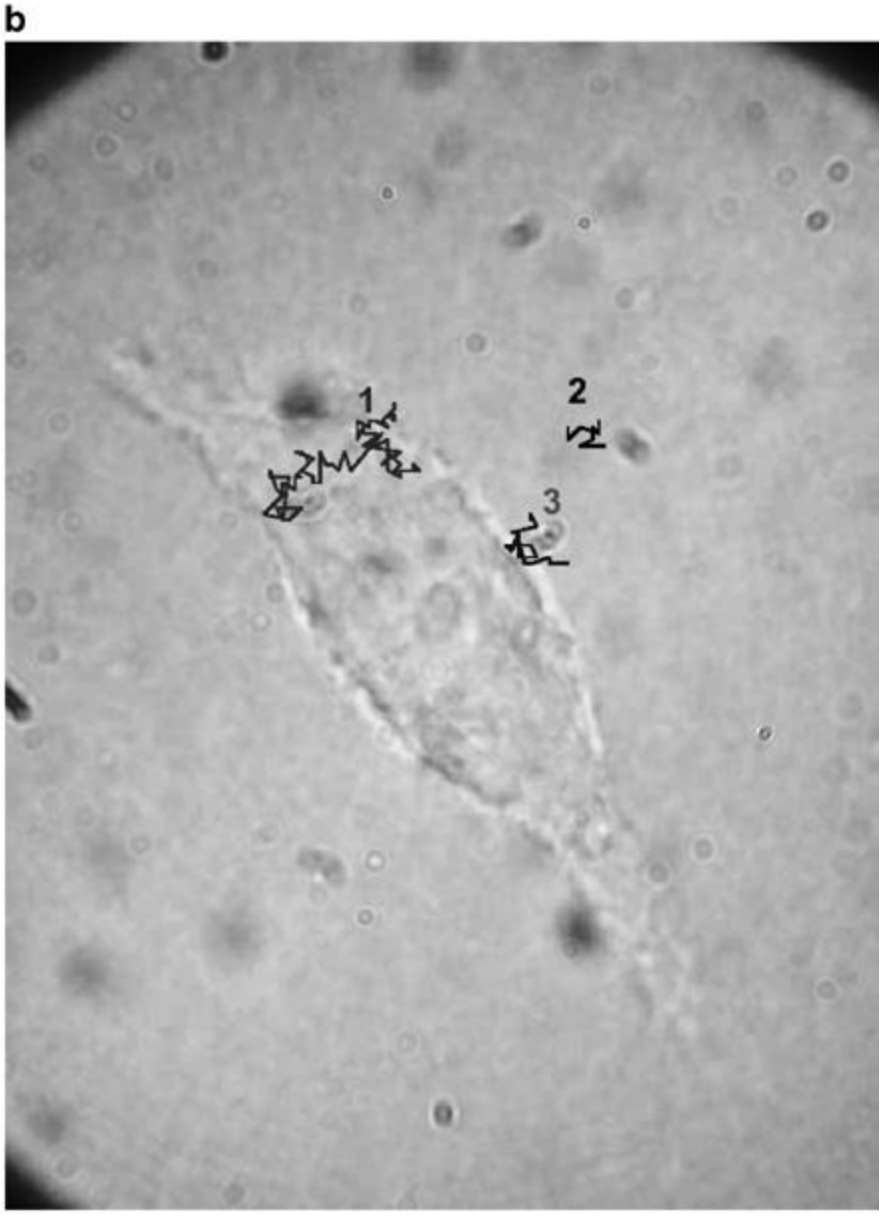
Mouse heart



Champion et al., Circulation 2003

AAV2 binds to heparan sulphate

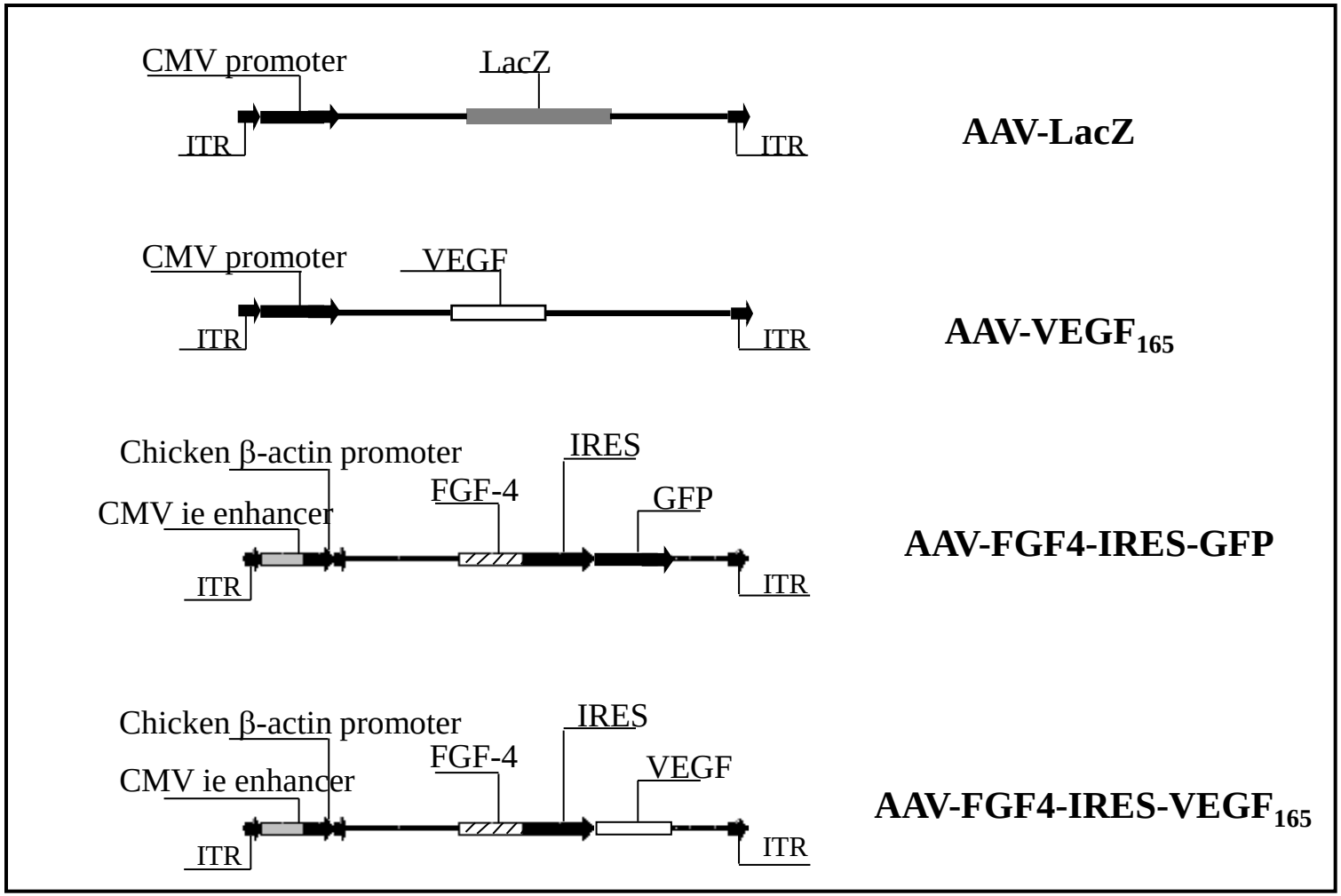




Synergistic administration of growth factors to the wound area was demonstrated to improve the wound healing process in comparison to the single agent delivery

Materials and Methods

Expression cassettes in Adeno-Associated Viral vectors serotype 2 (AAV-2)



IRES - internal ribosome entry site

Jazwa et al., Genet Vaccines Ther. 2010 Aug 30;8:6.

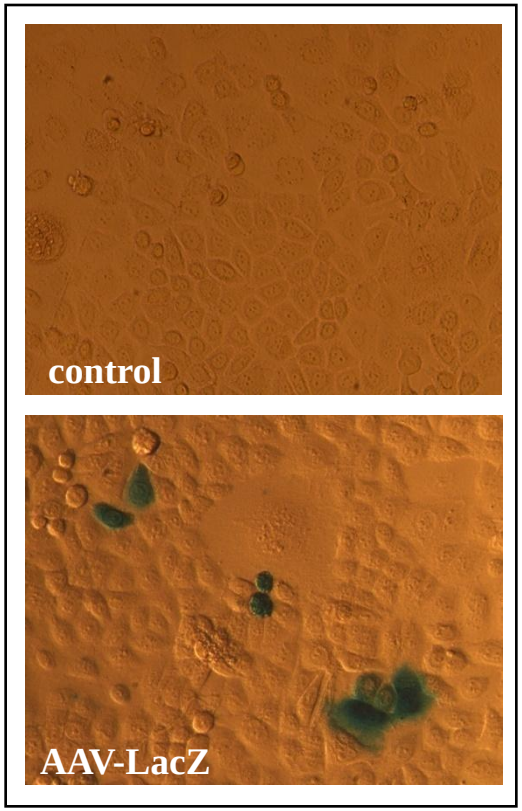
VEGF and FGF-4

1. VEGF is a major angiogenic growth factor
2. VEGF has been used in numerous studies and demonstrated its effectiveness as a stimulator of blood vessel formation
3. FGF-4 can induce angiogenesis, and has been tested in clinical trials
4. FGF-4 can potentially stimulate formation of mature vessels
5. FGF-4 is generally not produced in adults

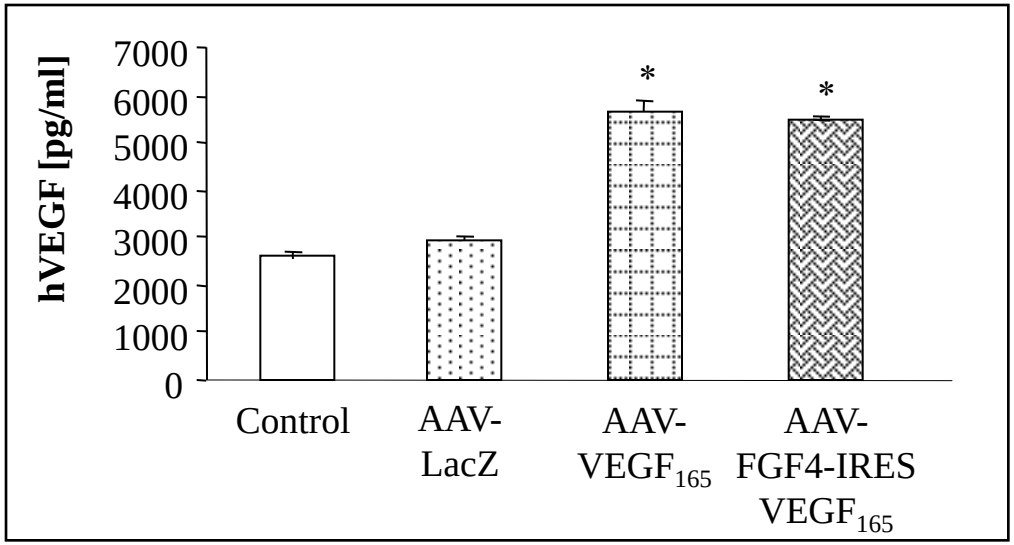
Gene expression in transduced HeLa cells

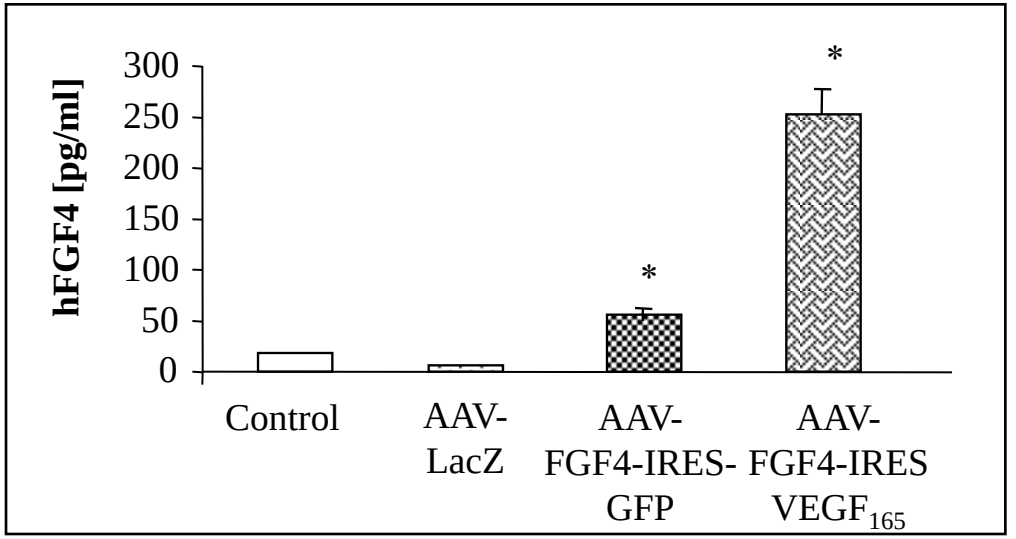
(1000 MOI of AAV-2 vectors)

β-galactosidase activity-



ELISA

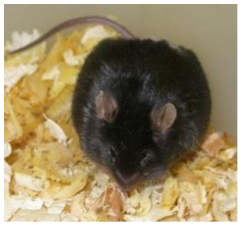




* p<0,05 vs kontrola i AAV-LacZ

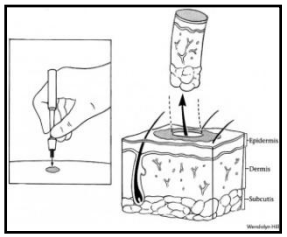
Jazwa et al., Genet Vaccines Ther. 2010 Aug 30;8:6.

Materials and Methods



1. Animals

12 week-old leptin receptor-deficient diabetic (dbdb) mice

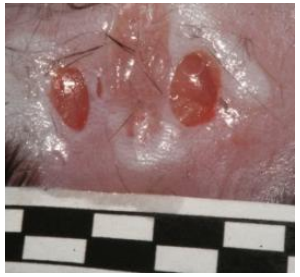


2. Animal wounding model

Two wounds were generated on dorsum of each mouse using a 4-mm-diameter punch biopsy

3. Gene transfer (GT) – 4 local intradermal wound injections of 3x10^10 vp of AAV-2 in a final volume 100 μl / 2 wounds (5 groups, n=5 per each group)

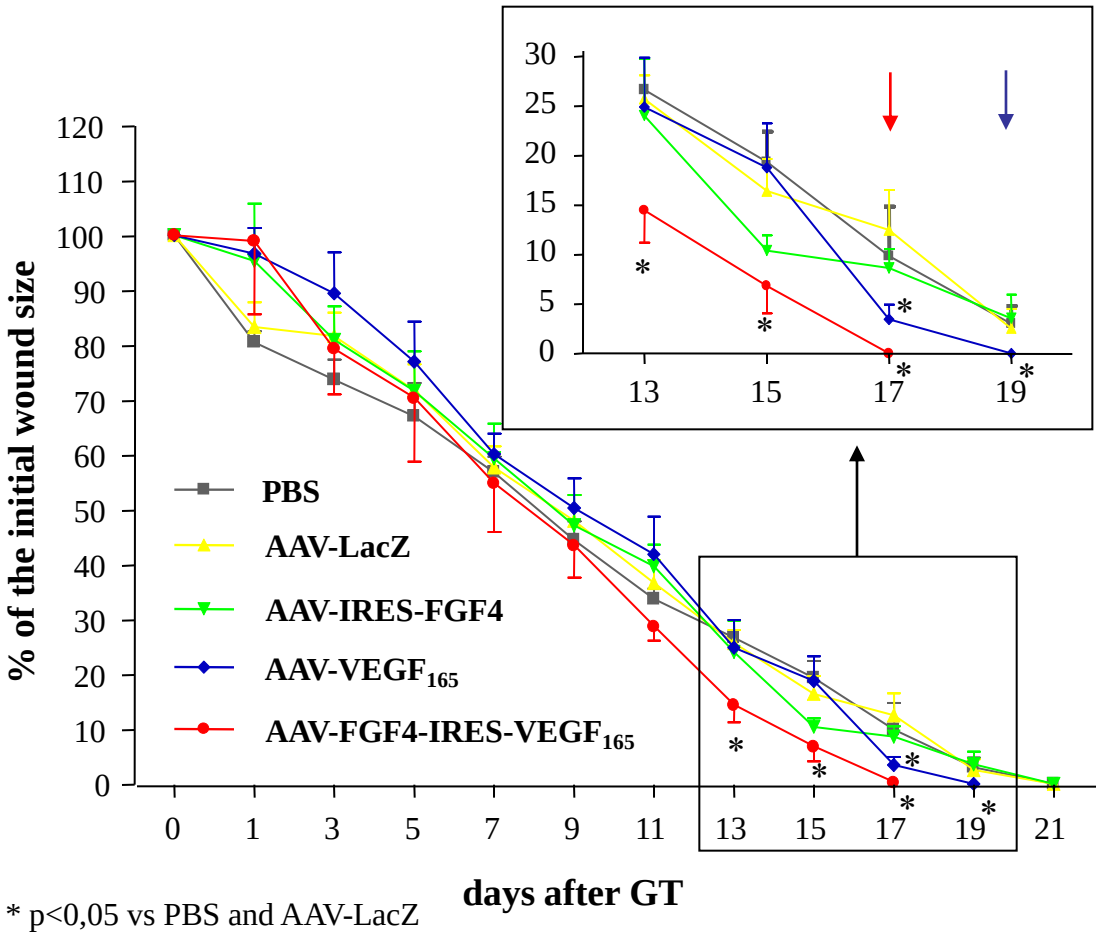
- **PBS**
- **AAV-LacZ**
- **AAV-FGF4-IRES-GFP**
- **AAV-VEGF$_{165}$**
- **AAV-FGF4-IRES-VEGF$_{165}$**



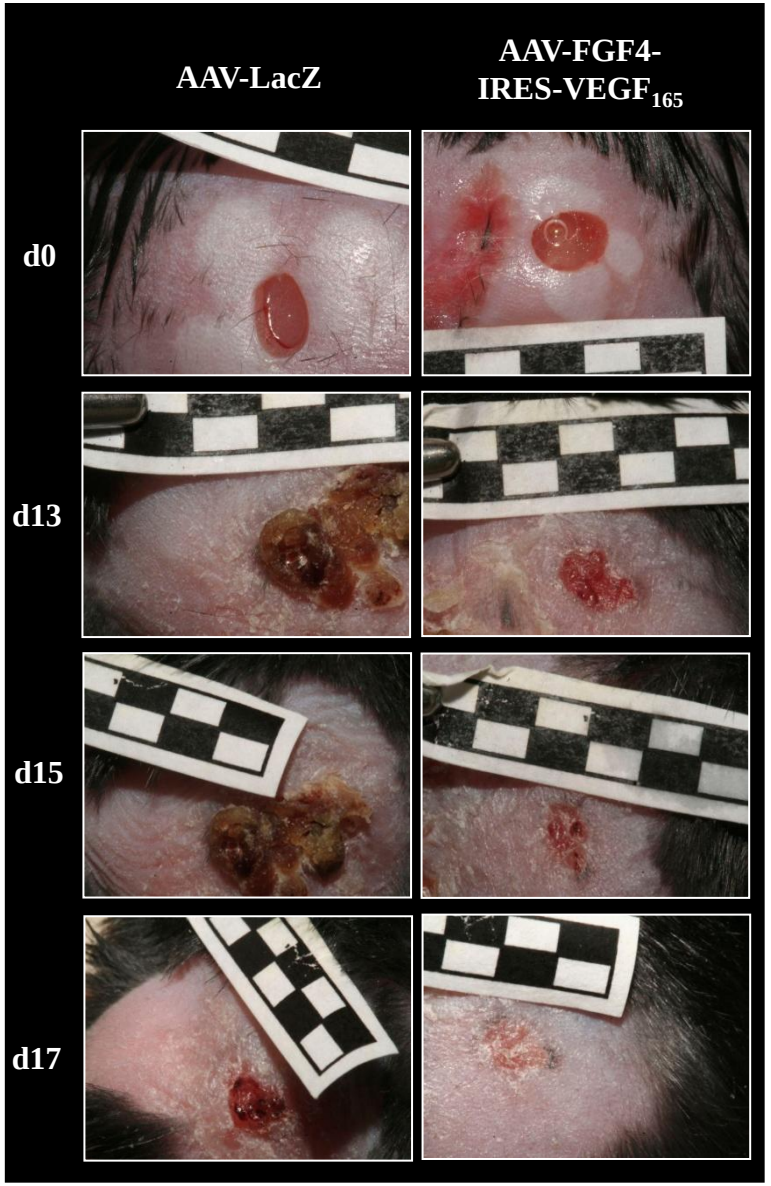
4. Measurements of the wound area – day 0, 1 and every second day till the end of the experiment (day 21)

5. Histological analysis 21 days after wounding and gene transfer

Wound closure is significantly accelerated after AAV-FGF4-IRES-VEGF$_{165}$ delivery

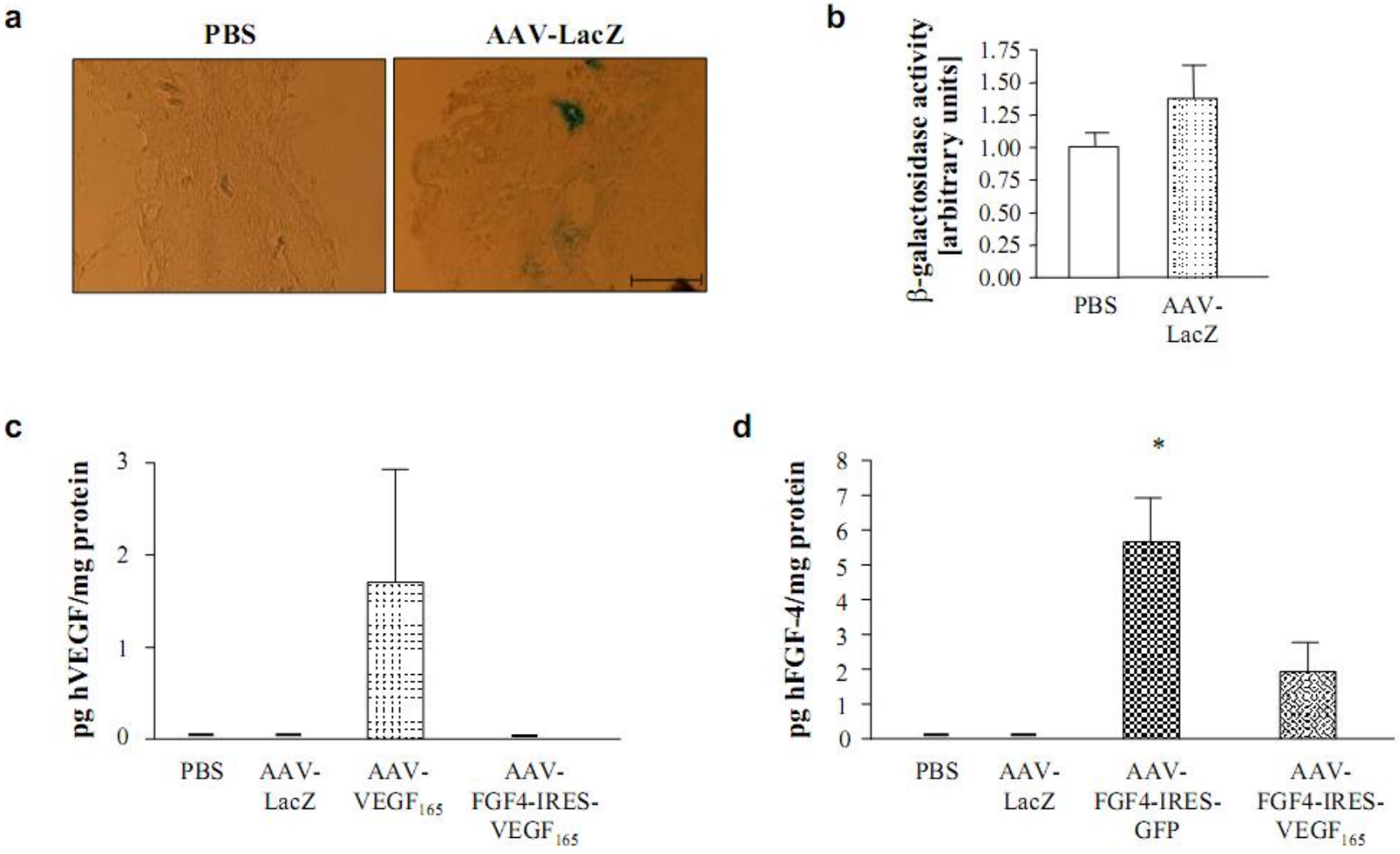


* p<0,05 vs PBS and AAV-LacZ

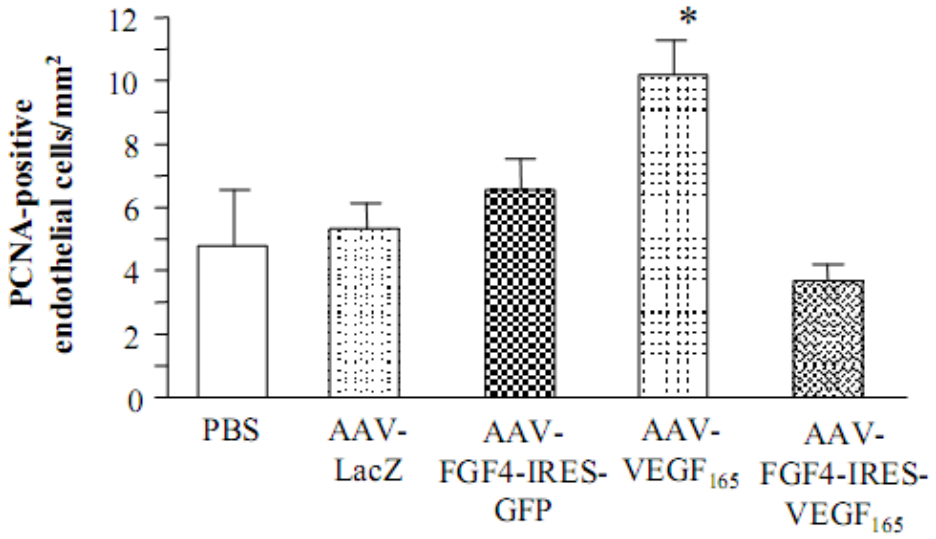


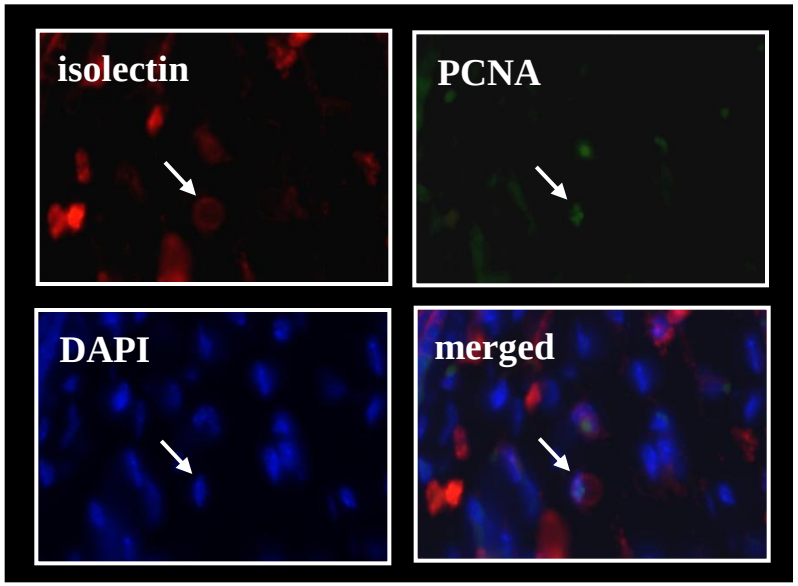
Jazwa et al., Genet Vaccines Ther. 2010 Aug 30;8:6.

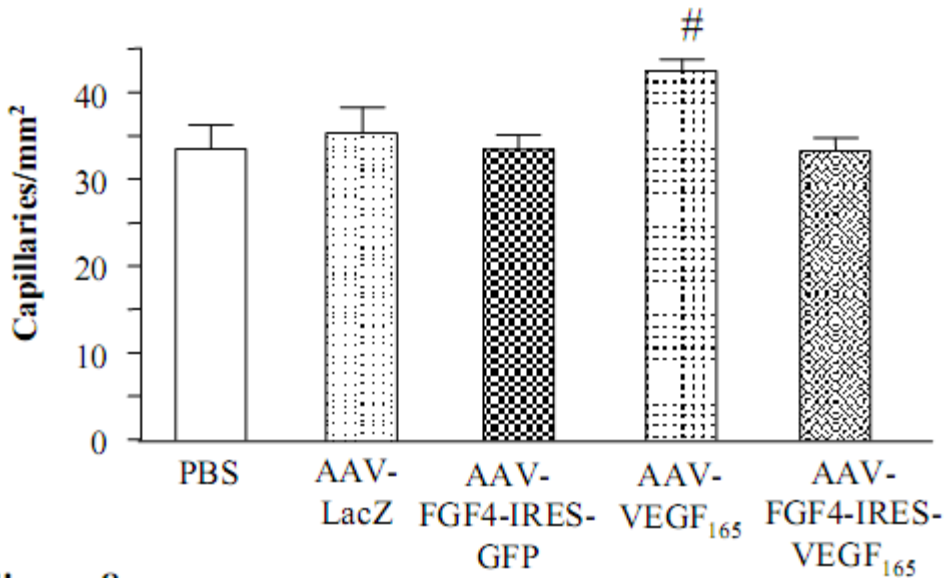
Gene expression in the skin of db/db mice – 21 days after injury



Neovascularization of the skin after AAV-VEGF$_{165}$ administration



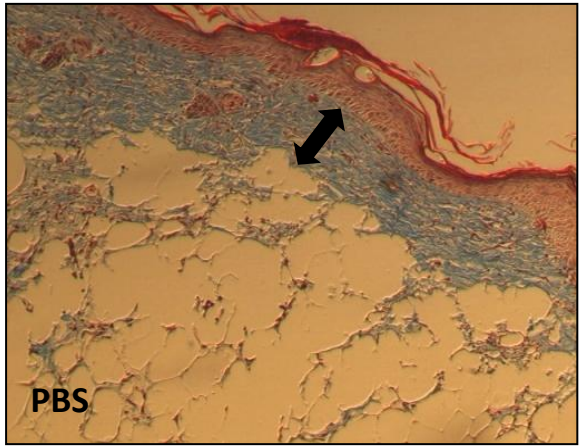




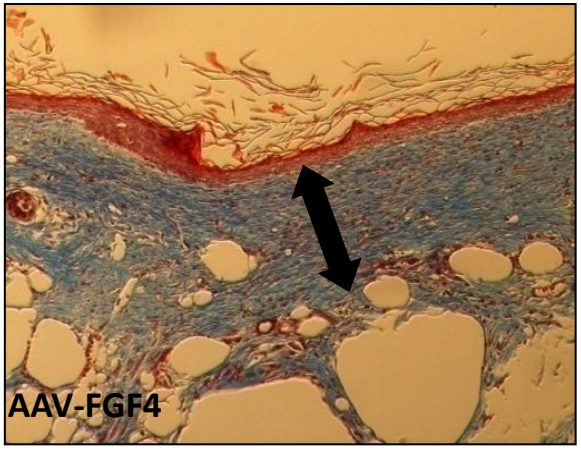
* p<0,05 vs PBS and AAV-LacZ

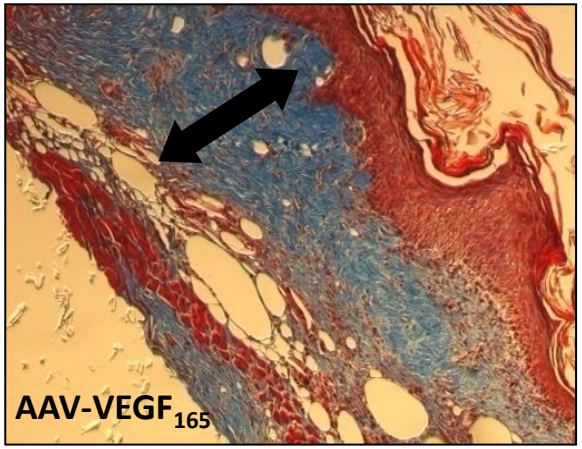
Both $VEGF_{165}$ and FGF-4 increase the thickness of the granulation tissue and collagen deposition

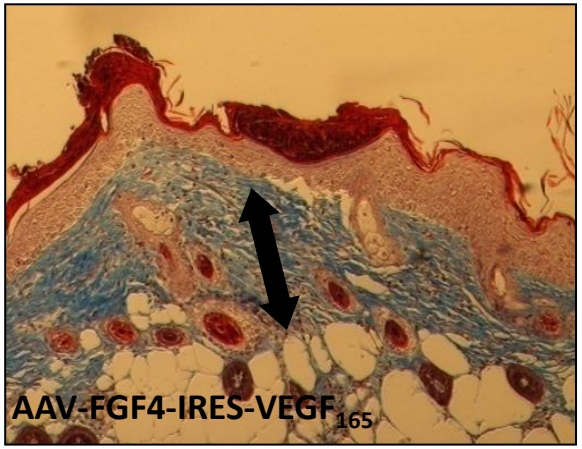
Masson's trichrome staining



Thicker, more organized collagen fibers which are hallmarks of mature granulation







FGF-4 improves vascularity and granulation tissue formation

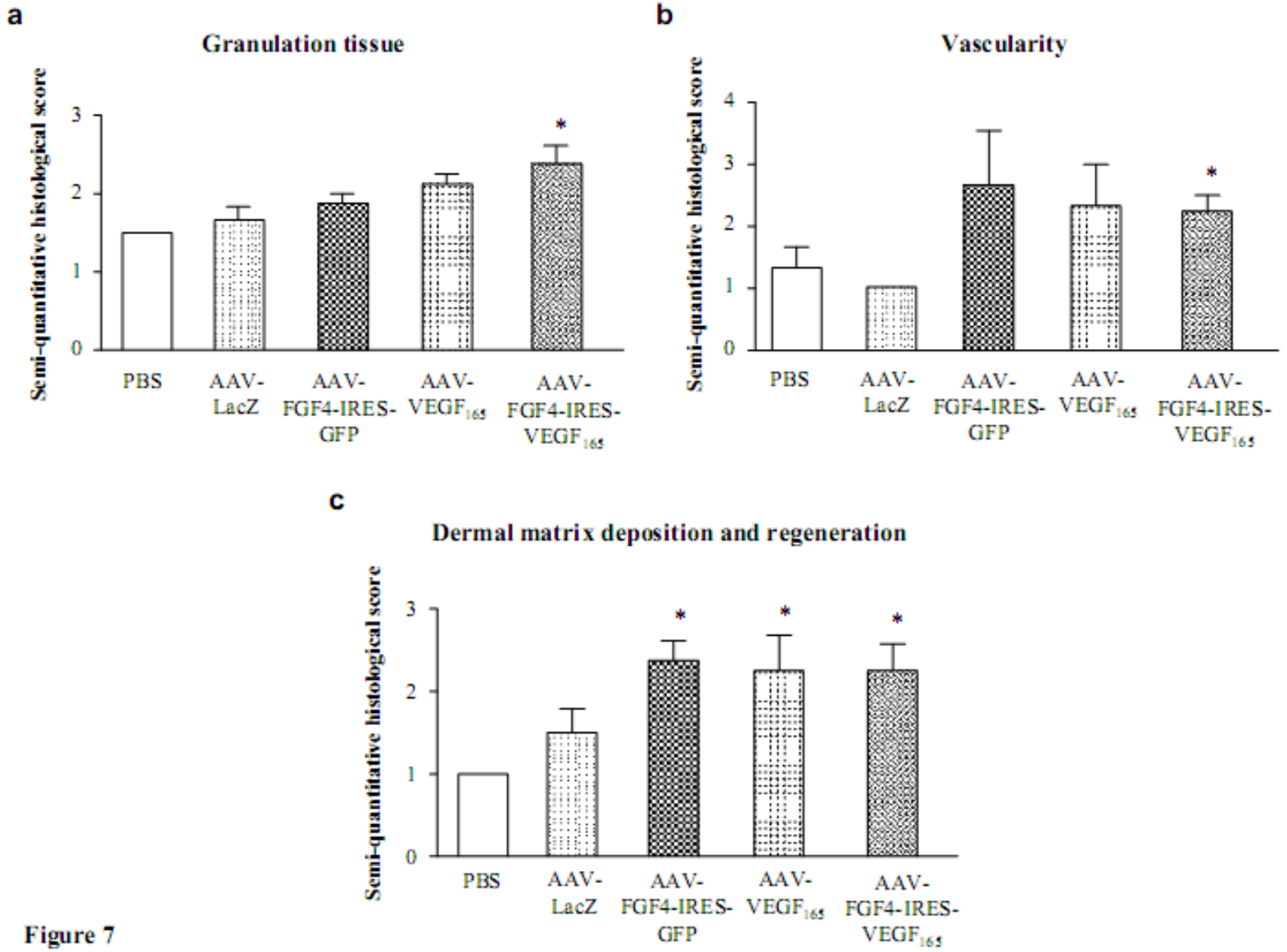
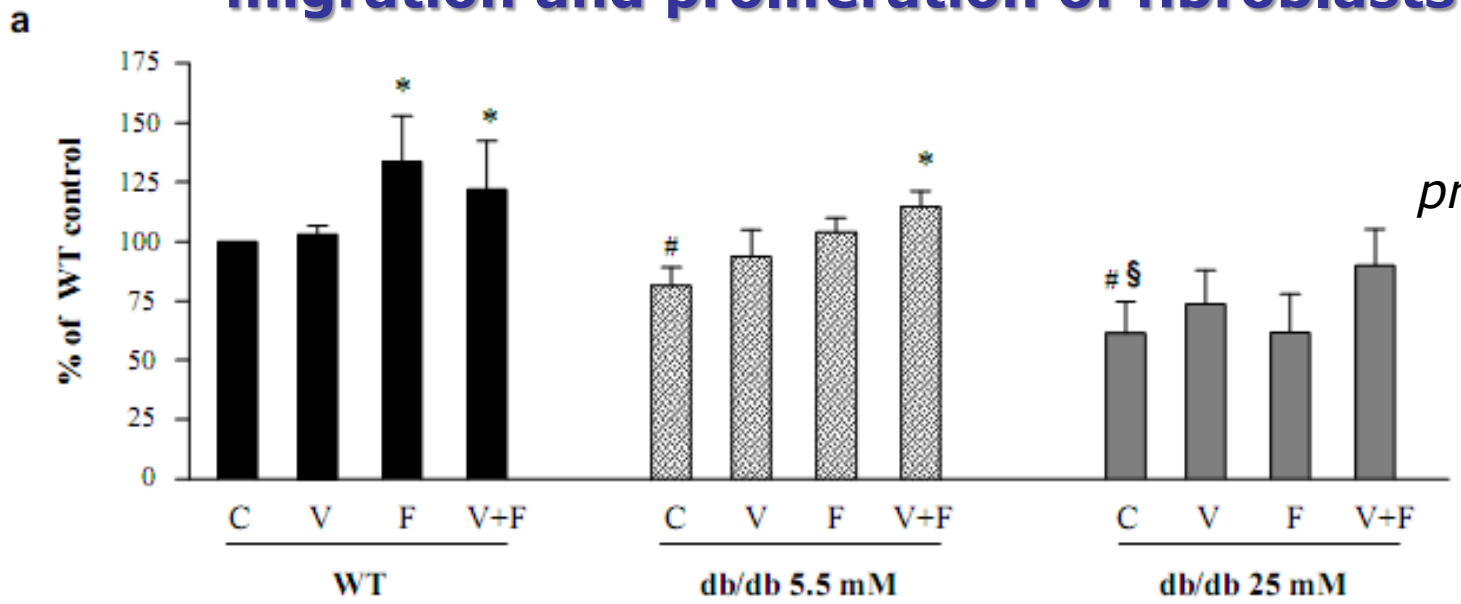


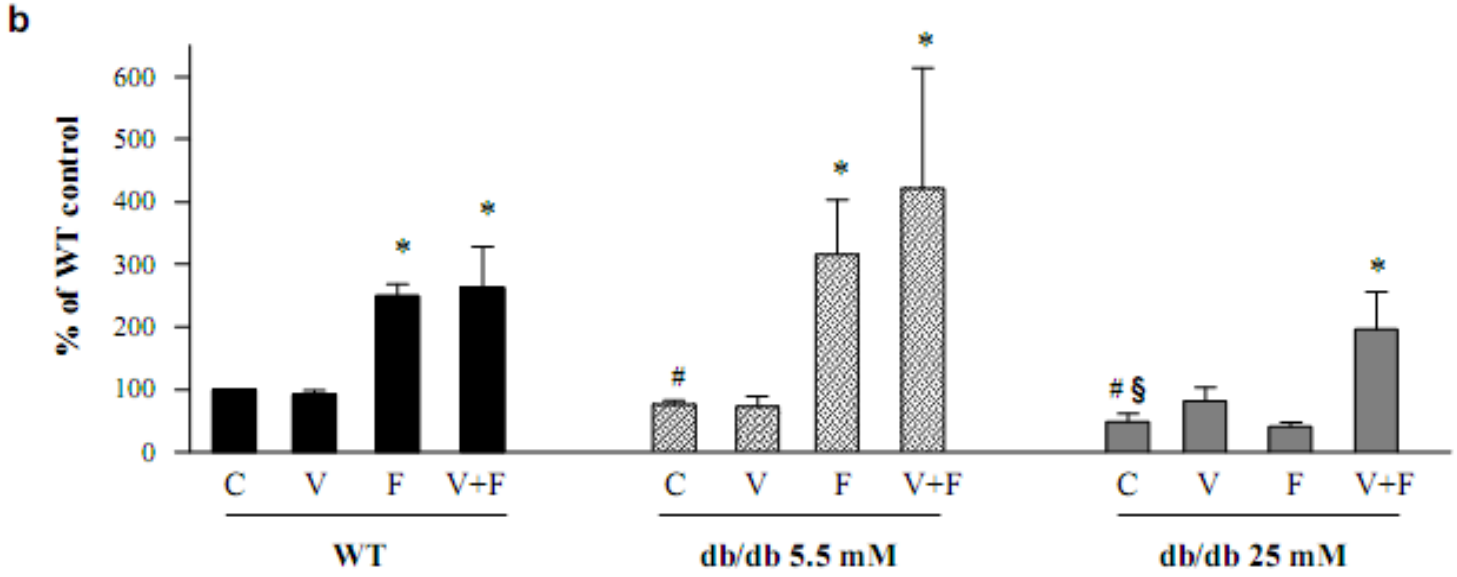
Figure 7

Jazwa et al., Genet Vaccines Ther. 2010 Aug 30;8:6.

Combined recombinant VEGF and FGF-4 treatment improves migration and proliferation of fibroblasts



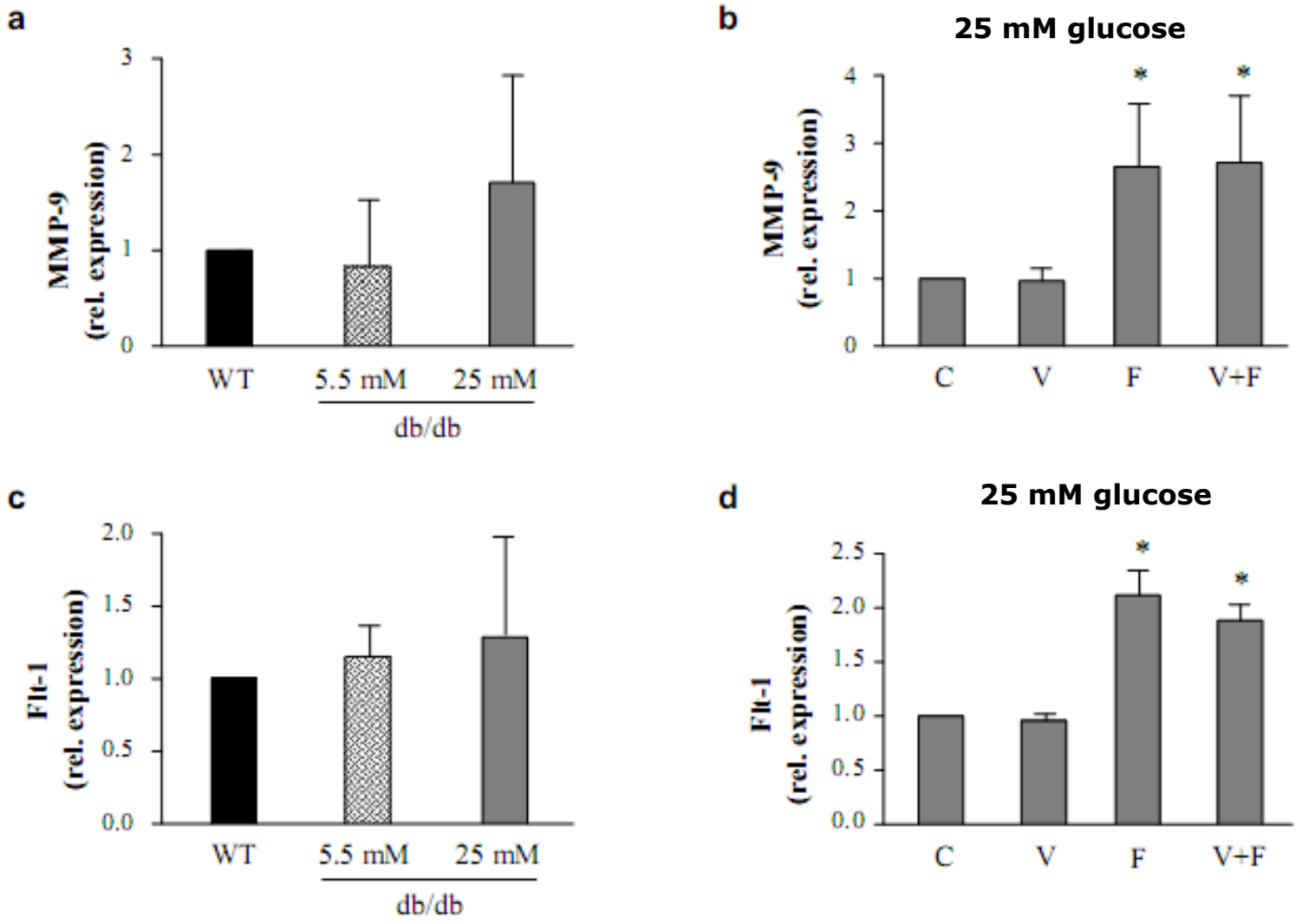
proliferation



migration

Jazwa et al., Genet Vaccines Ther. 2010 Aug 30;8:6.

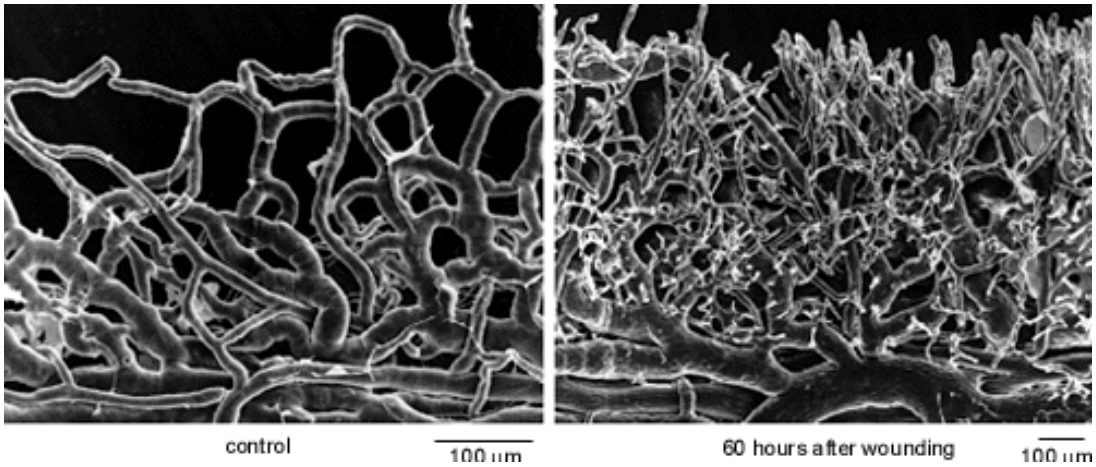
FGF-4 enhances MMP-9 and Flt-1 expression in fibroblasts



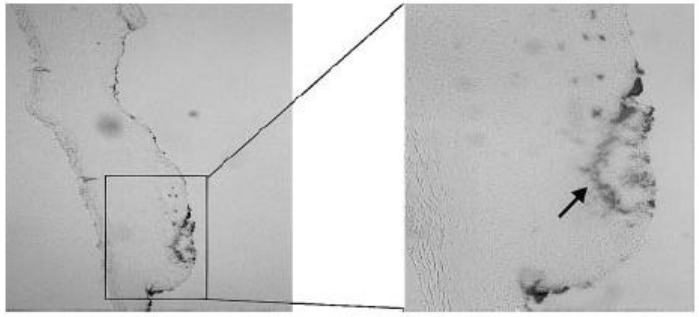
Jazwa et al., Genet Vaccines Ther. 2010 Aug 30;8:6.

Combination of two (or more…) growth factor might be better than single therapy

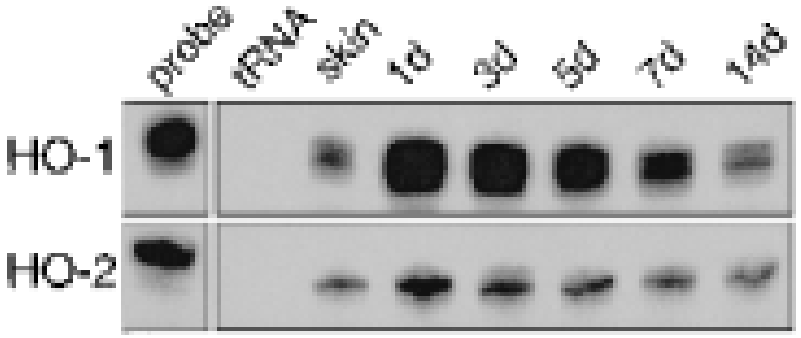
New capillary formation in response to wounding



Heme is released during skin injury and induces HO-1 expression

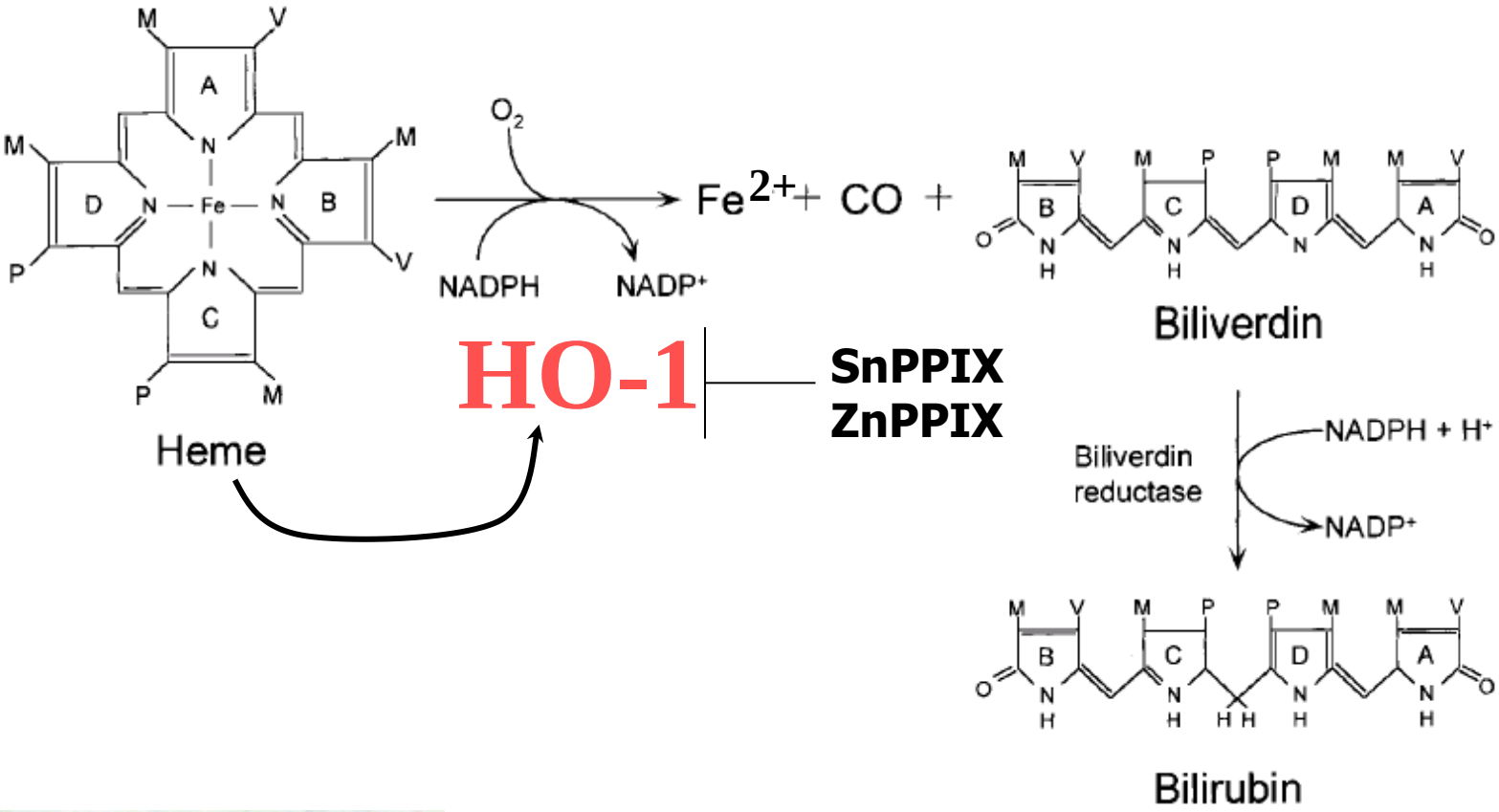


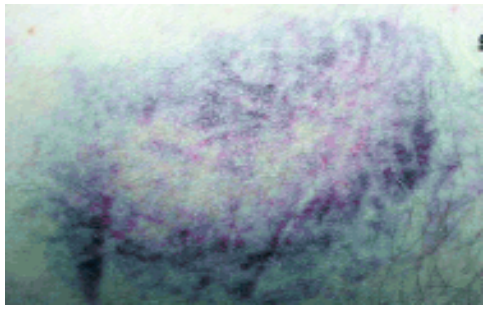
Wagener et al, Blood, 2003

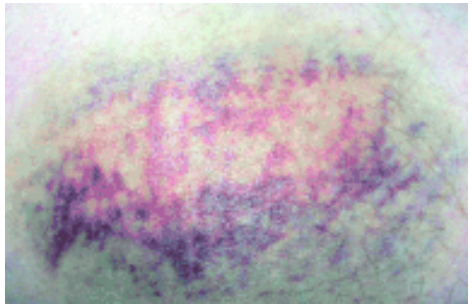


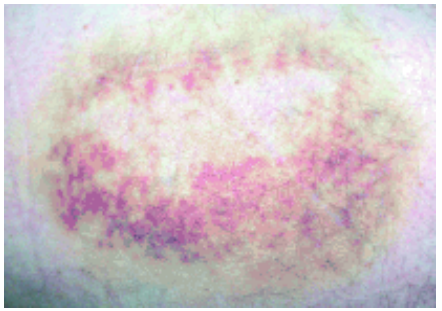
Hanselmann et al.., 2001

Heme oxygenase activity

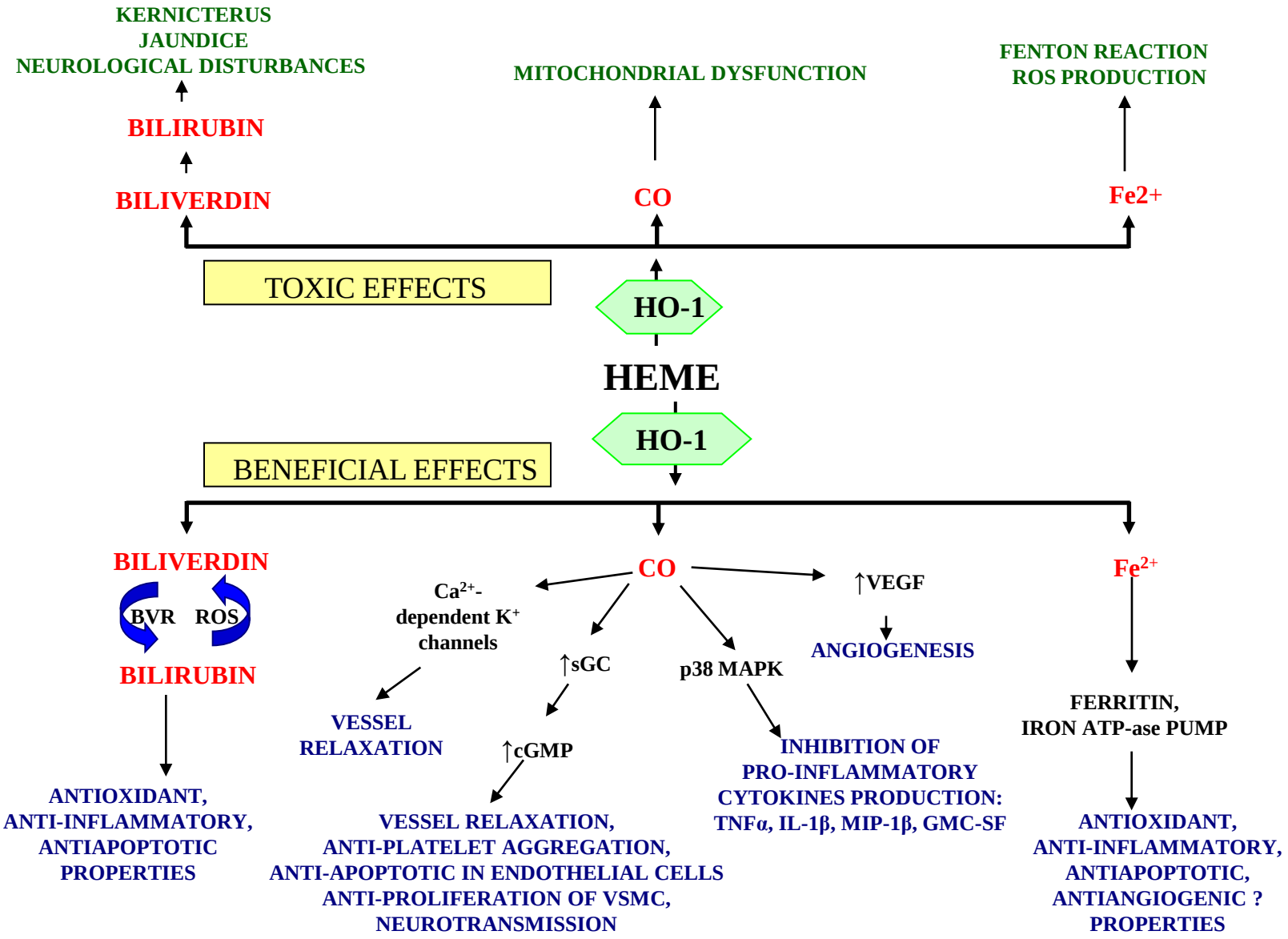






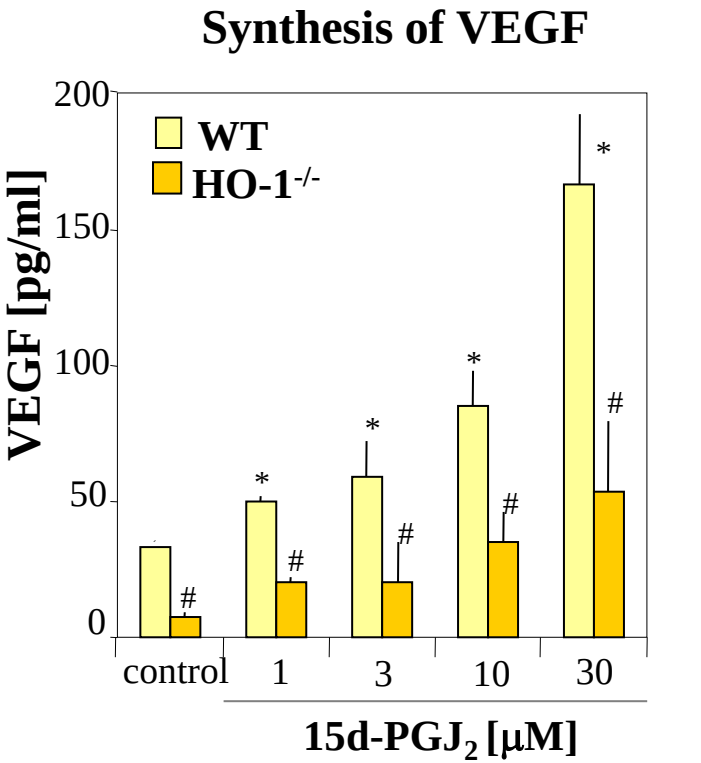


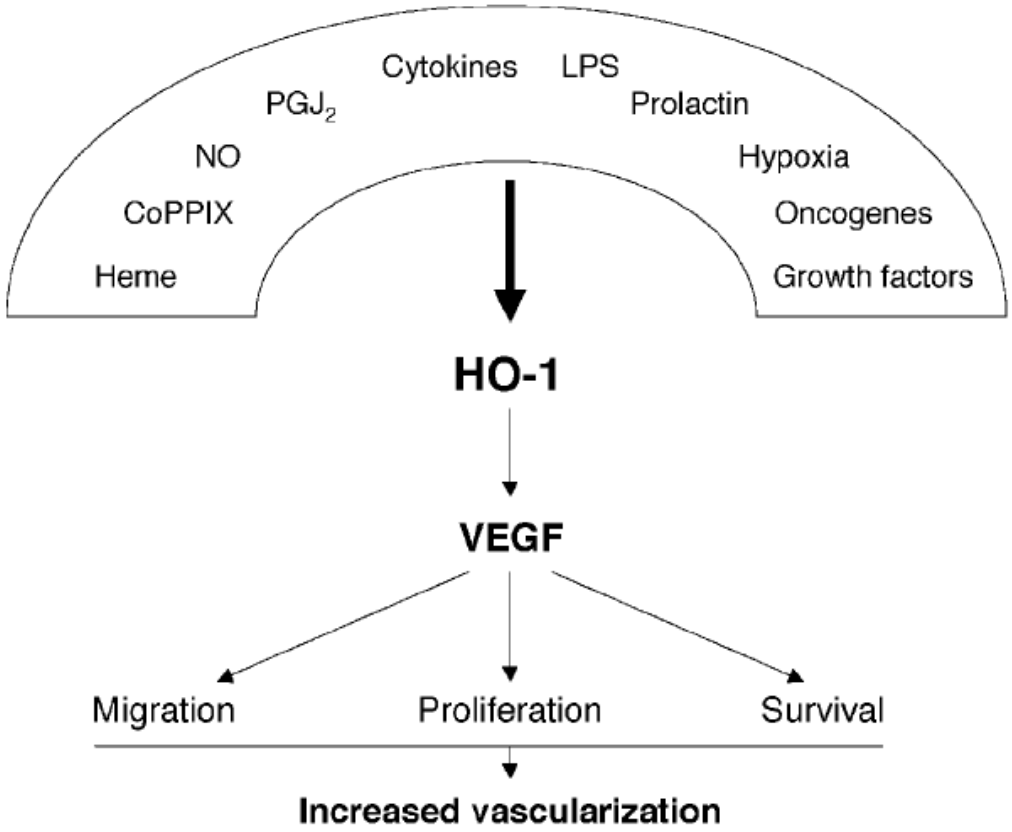
Multiple functions of HO-1 in cellular metabolism



Loboda et al., Antioxid Redox Signal, 2008

HO-1 regulates VEGF synthesis in response to different stimuli

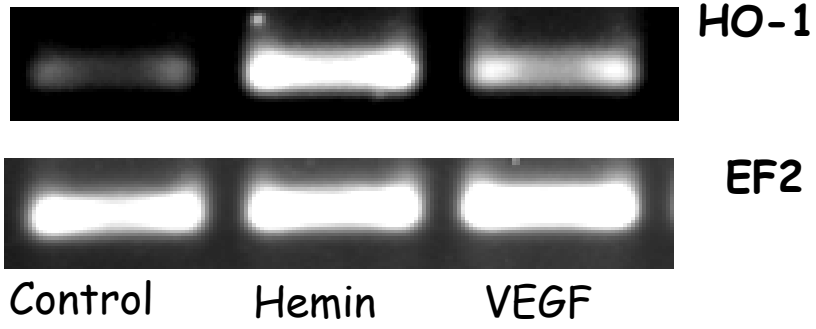


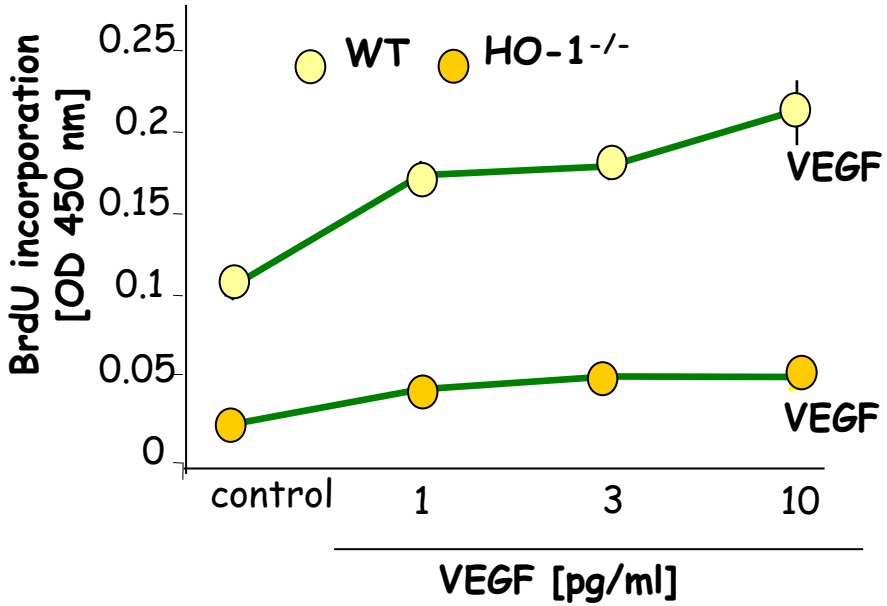


Cisowski et al., BBRC 2005

Dulak et al., Circulation, 2008: 117: 231-241

VEGF induces HO-1 expression in endothelial cells and HO-1 is required for VEGF-induced proliferation

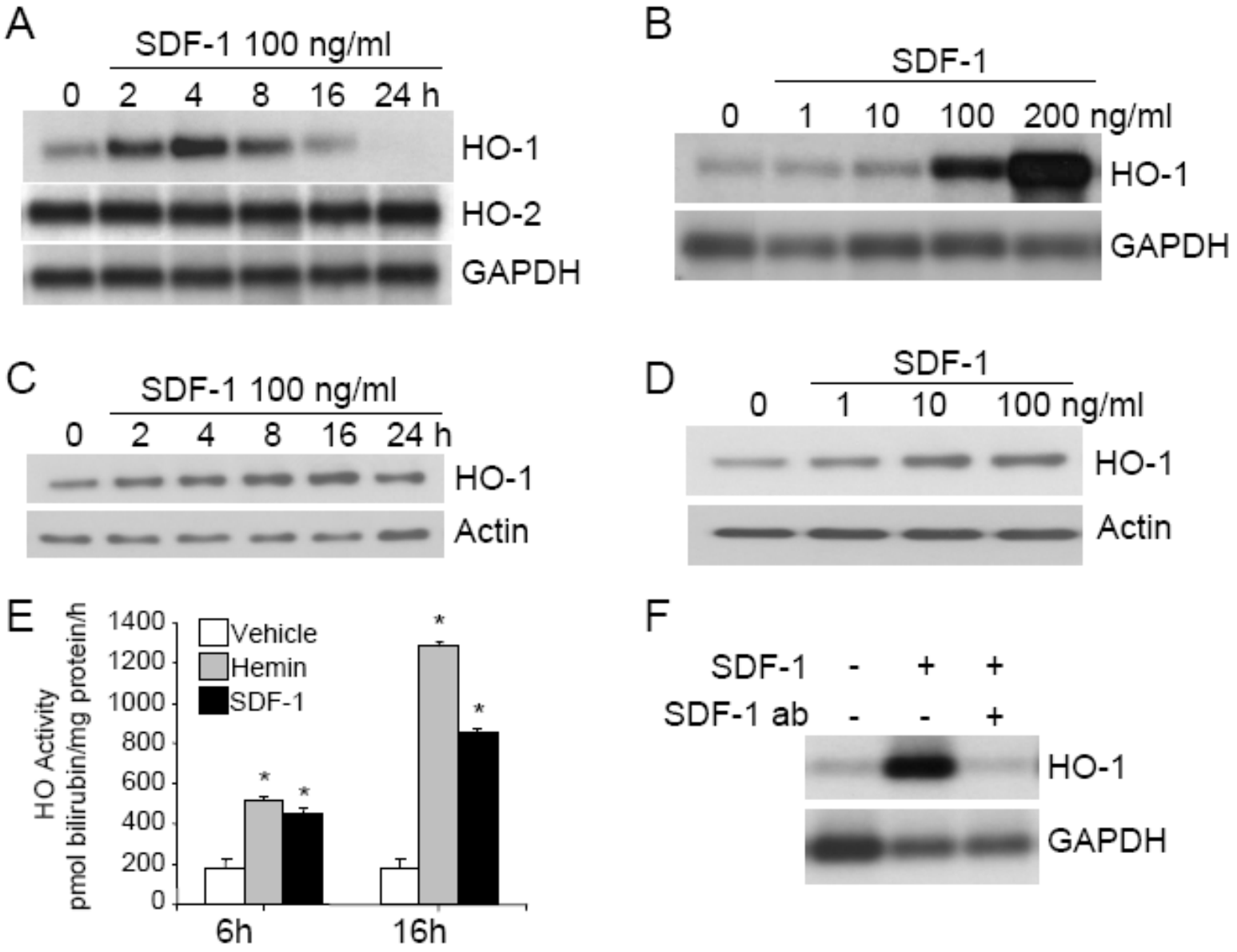




Dulak et al. Antioxid Redox Signal, 2004

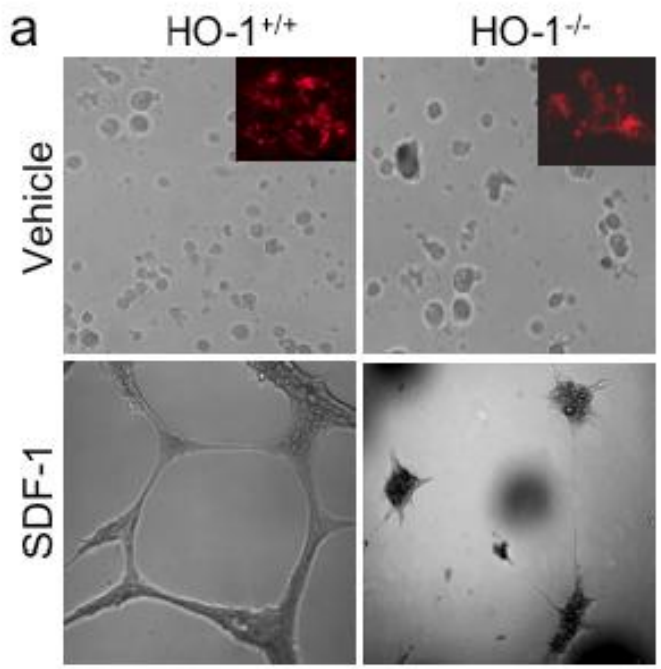
Dulak et al. In: Heme Oxygenase in Health and Diseases, Nova Publishers, New Yoirk, 2005

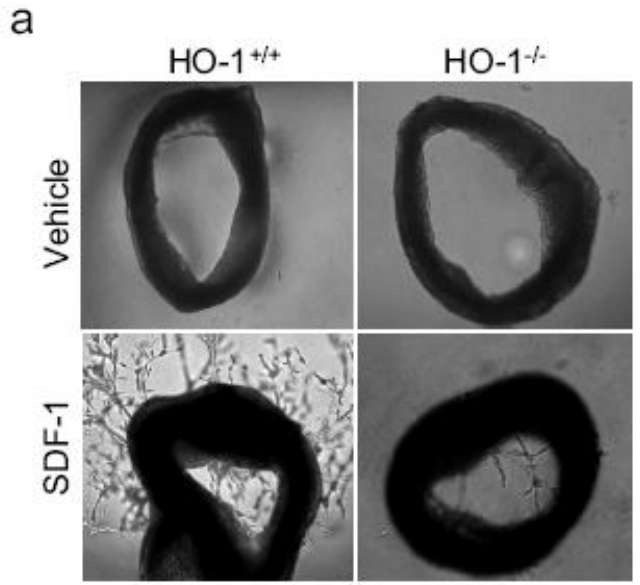
SDF-1 induces HO-1 in endothelial cells

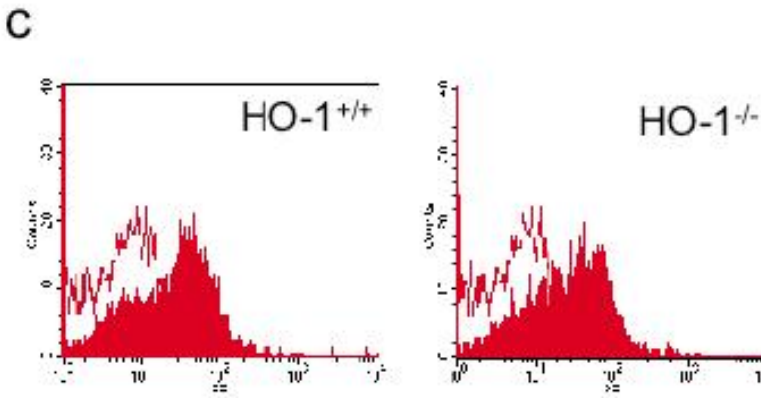


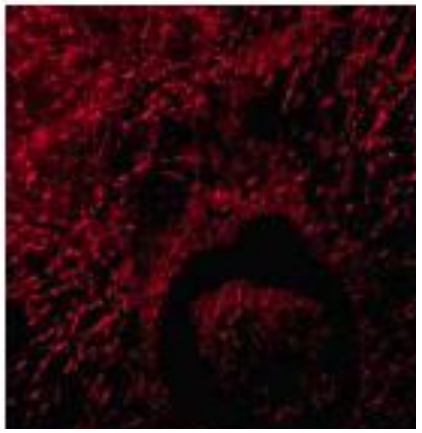
Deshane et al., J Exp Med., 2007; 204(3):605-18.

Lack of HO-1 impairs angiogenic effect of SDF-1





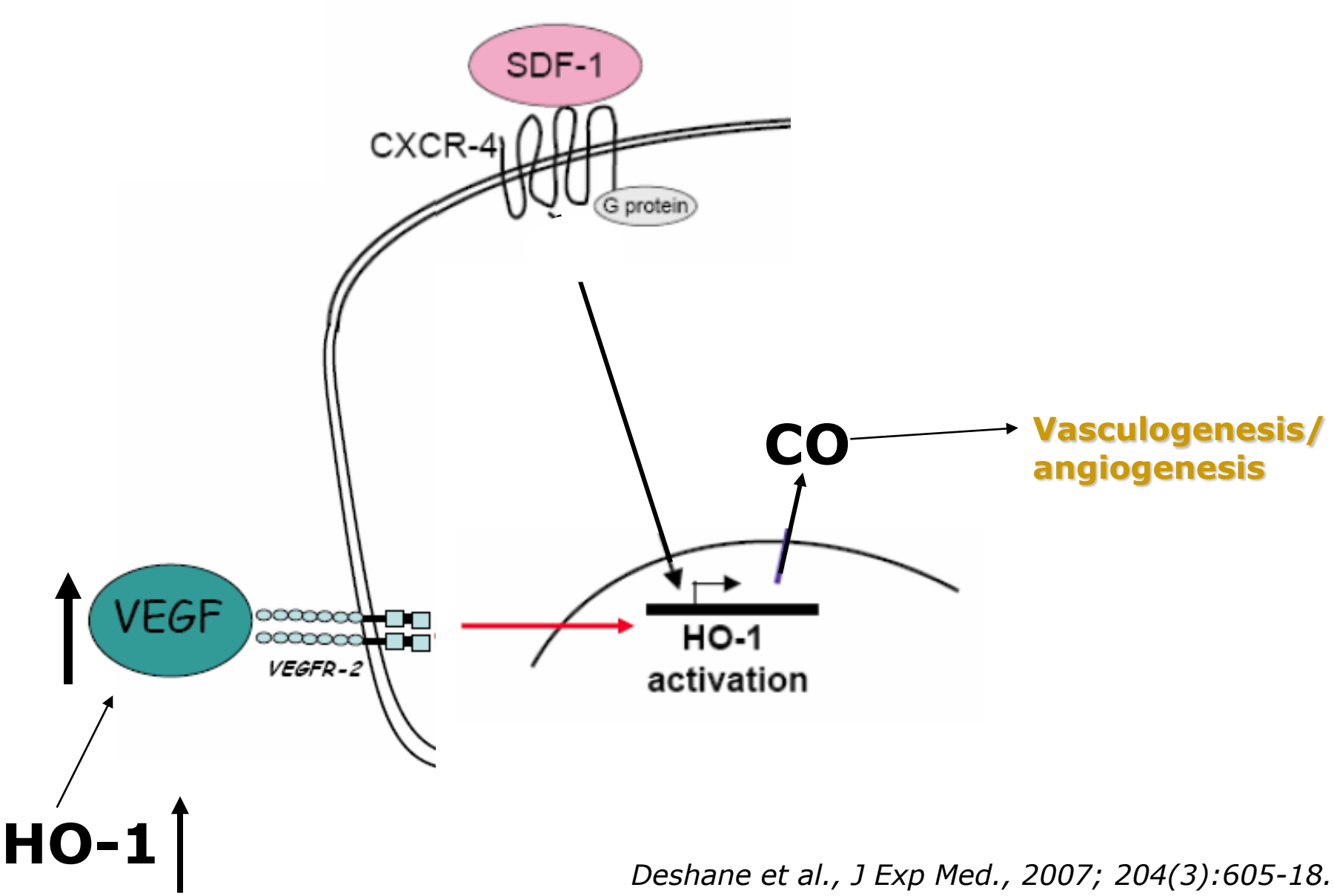




Level of CXCR4

Deshane et al., J Exp Med., 2007; 204(3):605-18.

HO-1 is required for the effect of SDF-1 and VEGF in endothelial cells



Deshane et al., J Exp Med., 2007; 204(3):605-18.

ANTIOXIDANTS & REDOX SIGNALING
Volume 10, Number 10, 2008

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Comprehensive Invited Review

Heme Oxygenase-1 and the Vascular Bed: From Molecular Mechanisms to Therapeutic Opportunities

Agnieszka Loboda,[1] Agnieszka Jazwa,[1] Anna Grochot-Przeczek,[1] Andrzej J. Rutkowski,[1] Jaroslaw Cisowski,[1] Anupam Agarwal,[2] Alicja Jozkowicz,[1] and Jozef Dulak[1]

Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION
American Heart Association. Learn and Live.

Heme Oxygenase-1 and Carbon Monoxide in Vascular Pathobiology

Focus on Angiogenesis

Jozef Dulak, PhD; Jessy Deshane, PhD; Alicja Jozkowicz, PhD; Anupam Agarwal, MD

(*Circulation*. 2008;117:231-241.)

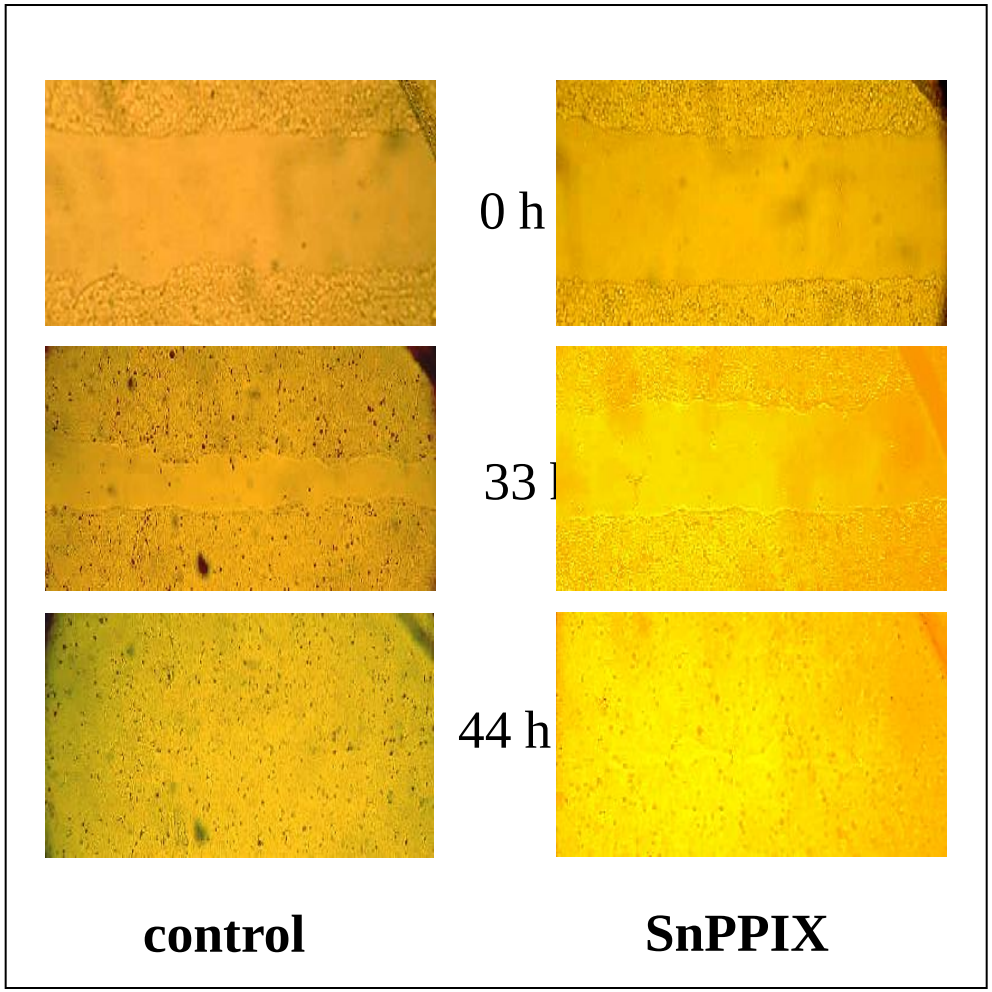
Effect of heme oxygenase-1 on vascular function and disease

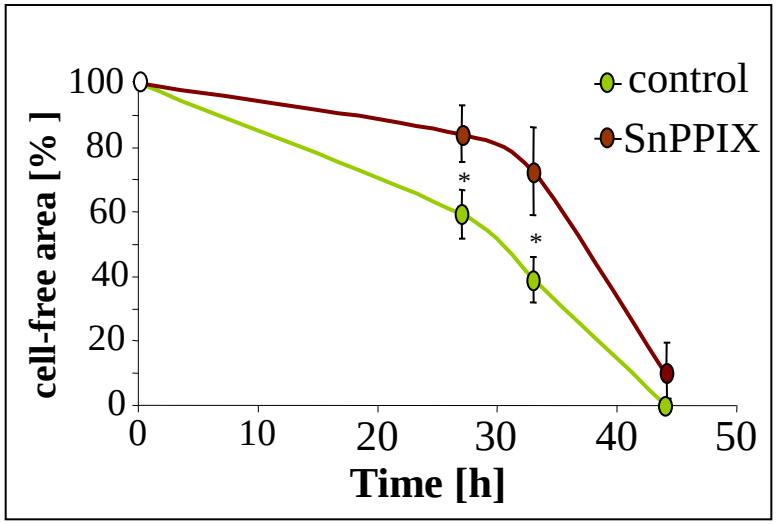
Jozef Dulak, Agnieszka Loboda and Alicja Jozkowicz

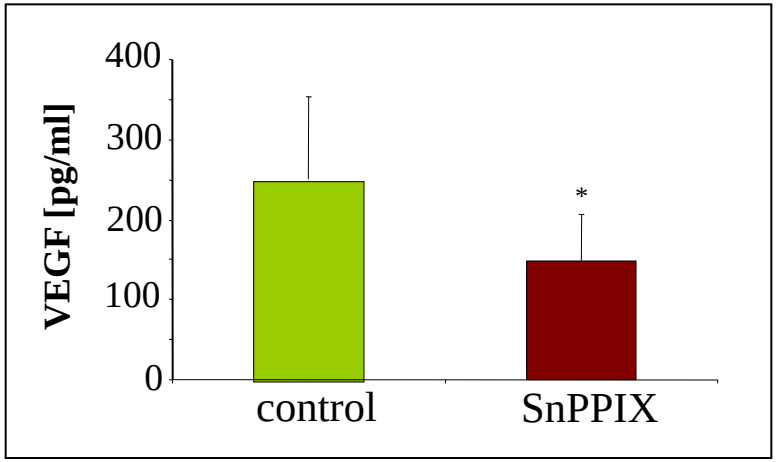
Current Opinion in Lipidology 2008, 19:505–512

HO-1 promotes keratinocytes migration

(scratch assay – HaCaT keratinocytes)

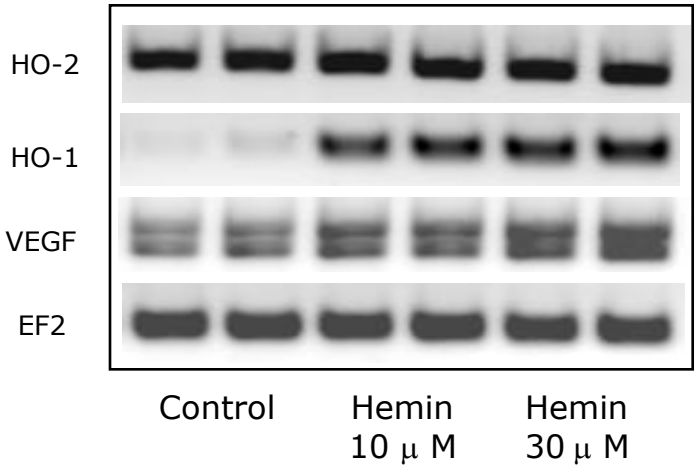


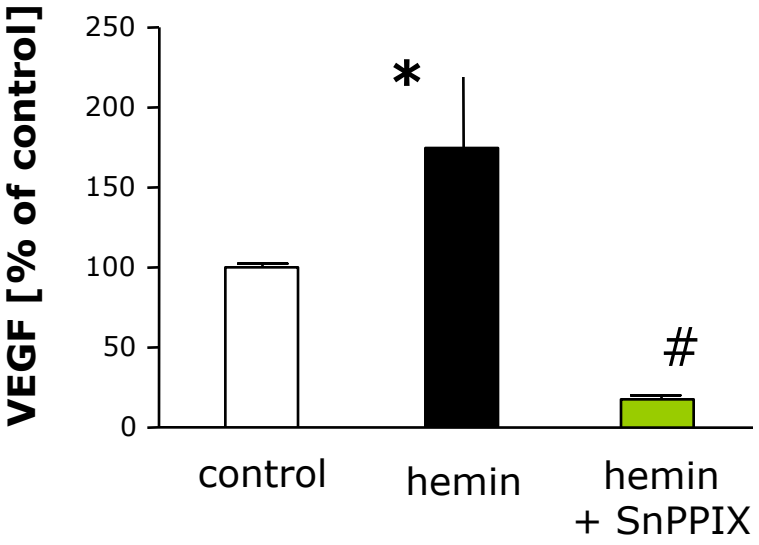


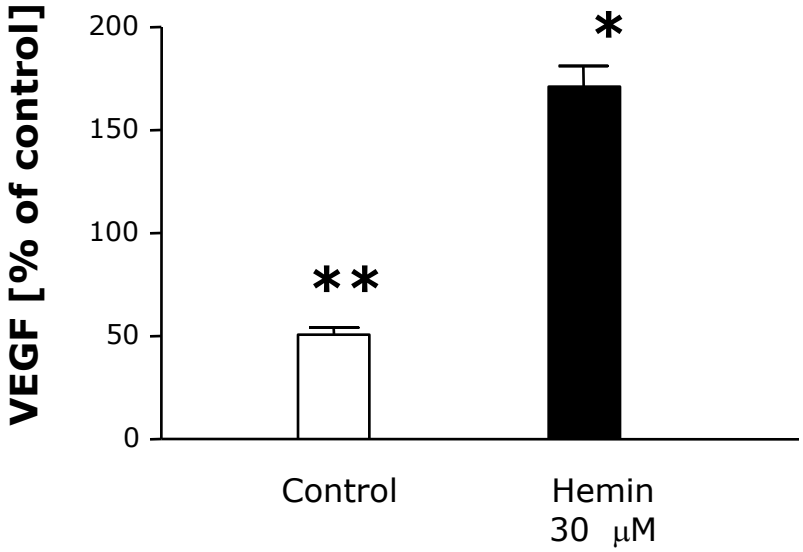


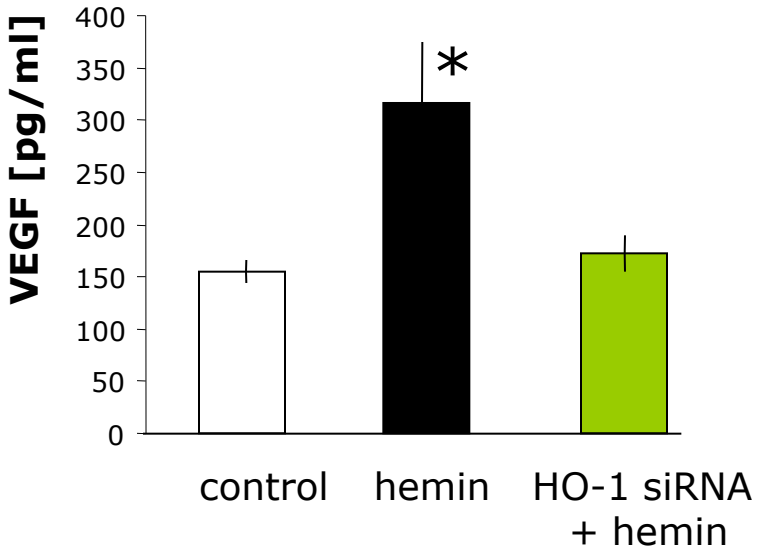
Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

HO-1 induction in HaCaT keratinocytes enhances VEGF synthesis



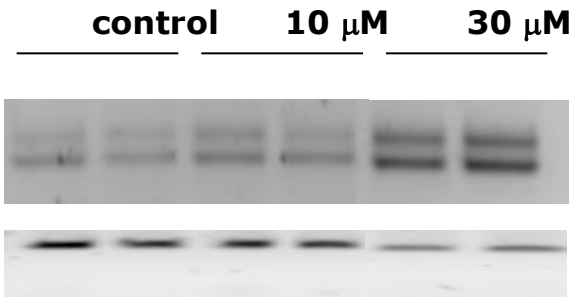


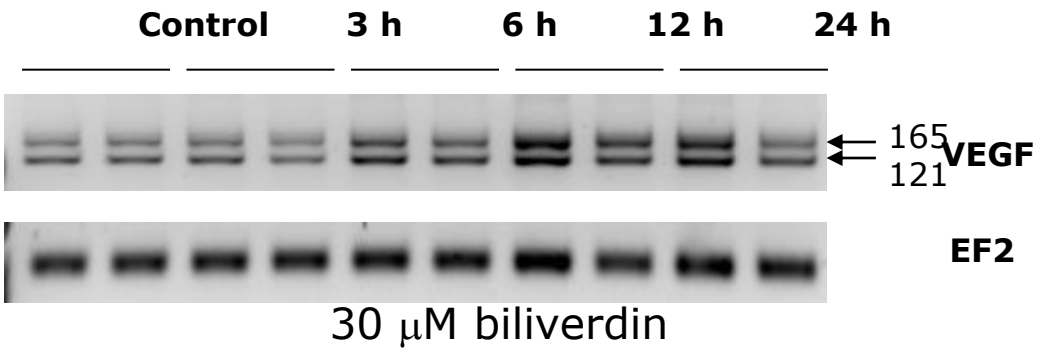




Jazwa et al. Free Radical Biol Med., 2006: 40: 1250-1263

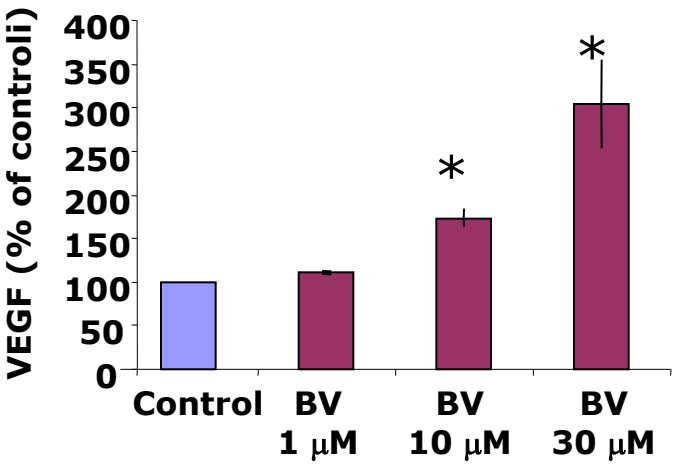
Biliverdin dose- and time-dependently enhances VEGF gene expression in HaCaT



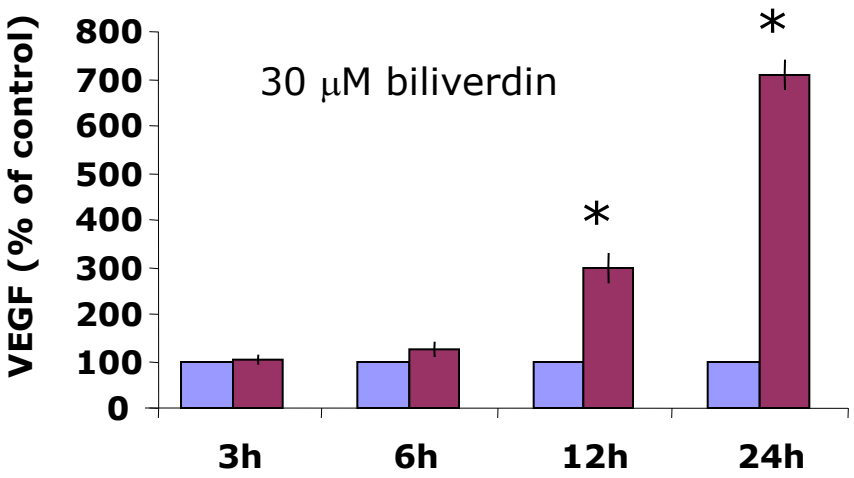


30 μM biliverdin

dose-dependent



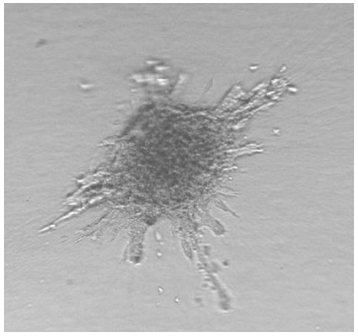
time-dependent



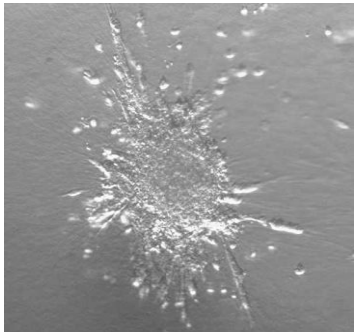
 # - p<0,05 vs control

Loboda et al., Antioxid Redox Signal 2008

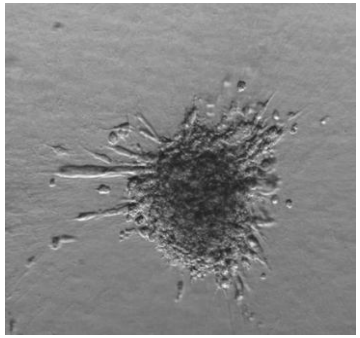
Conditioned media from HaCaT expressing HO-1 and producing more VEGF enhance angiogenesis



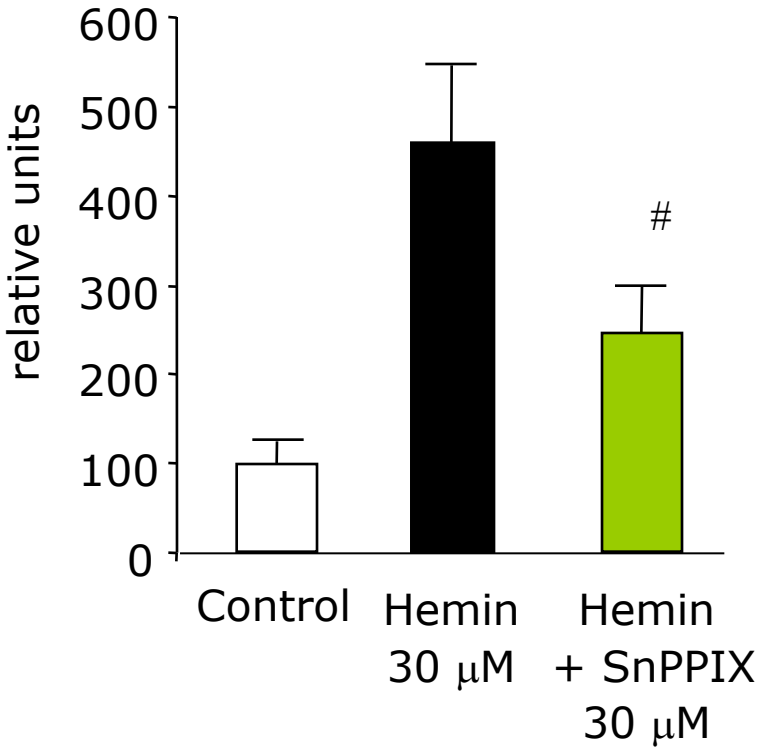
Control



Hemin 30 μM



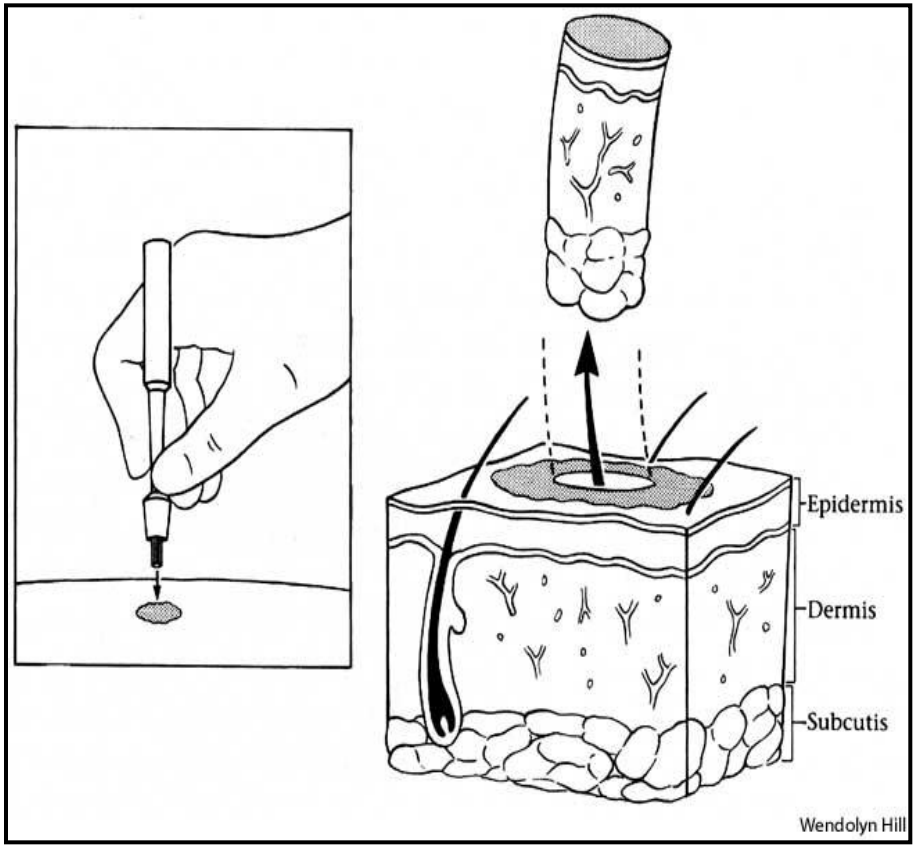
Hemin
+ SnPPIX 30 μM



Jazwa et al. Free Radical Biol Med., 2006: 40: 1250-1263

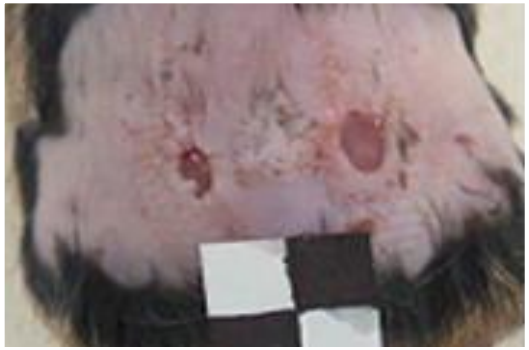
HO-1 enhances angiogenic potential of keratinocytes and is required for their migration

Cutaneous wound healing model



C57Bl

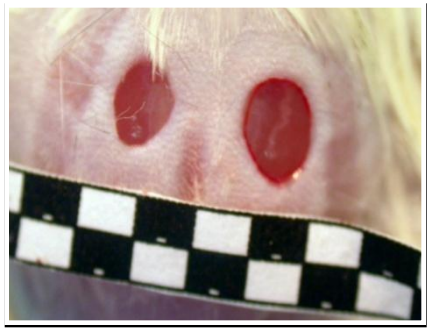
- Two wounds, both 4 mm in diameter, generated with a disposable biopsy punch tool (Stiefel).



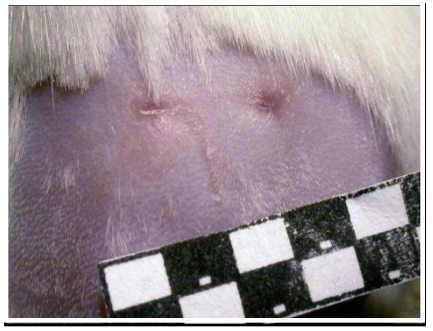
Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

Expression of HO-1 in wounded skin

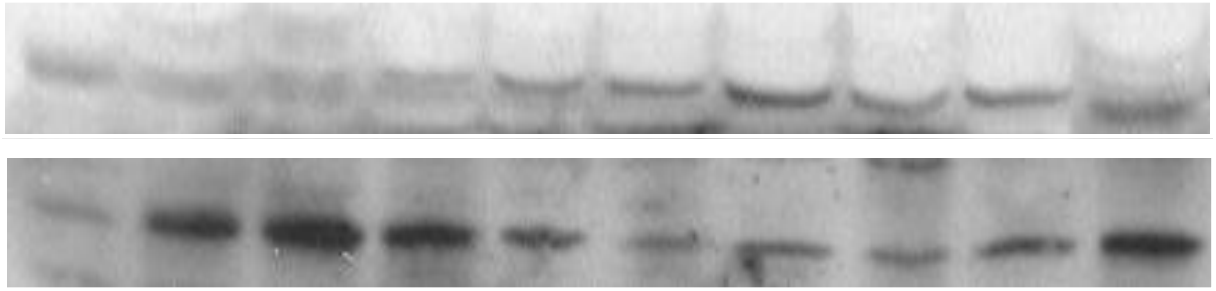
Day 0



Day 10



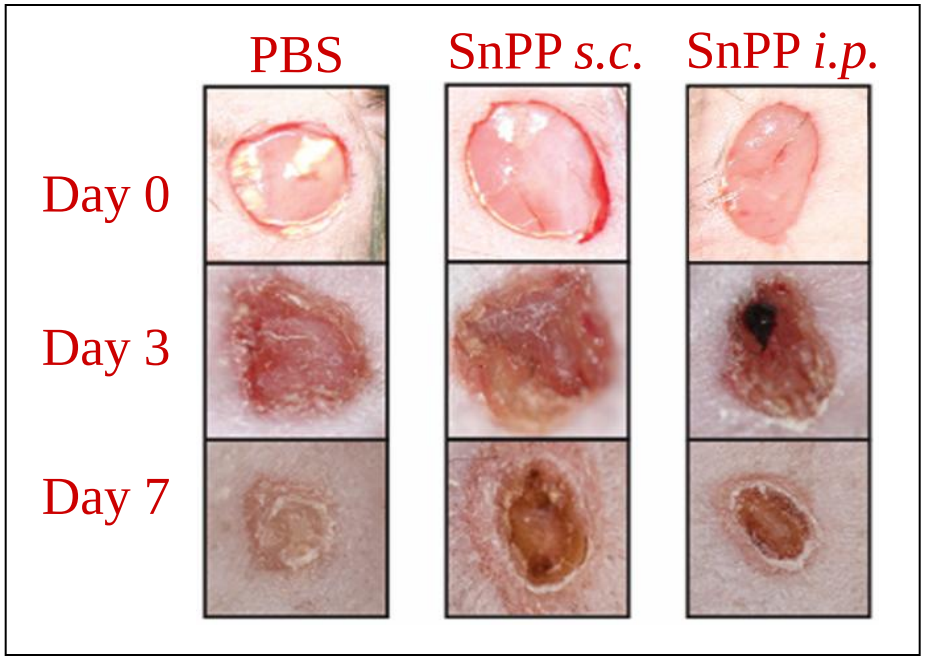
Western blot

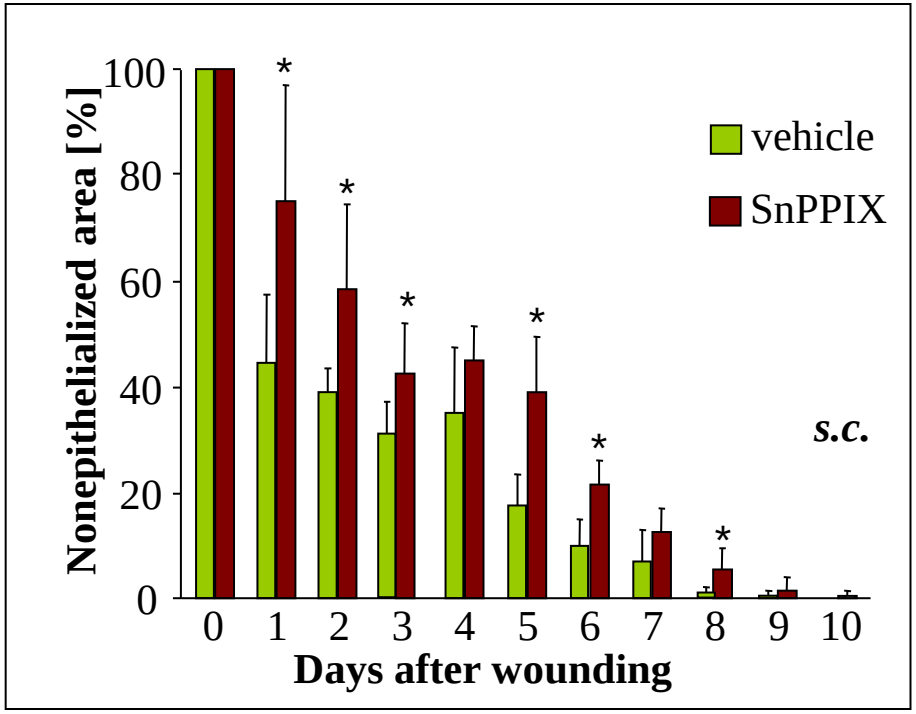


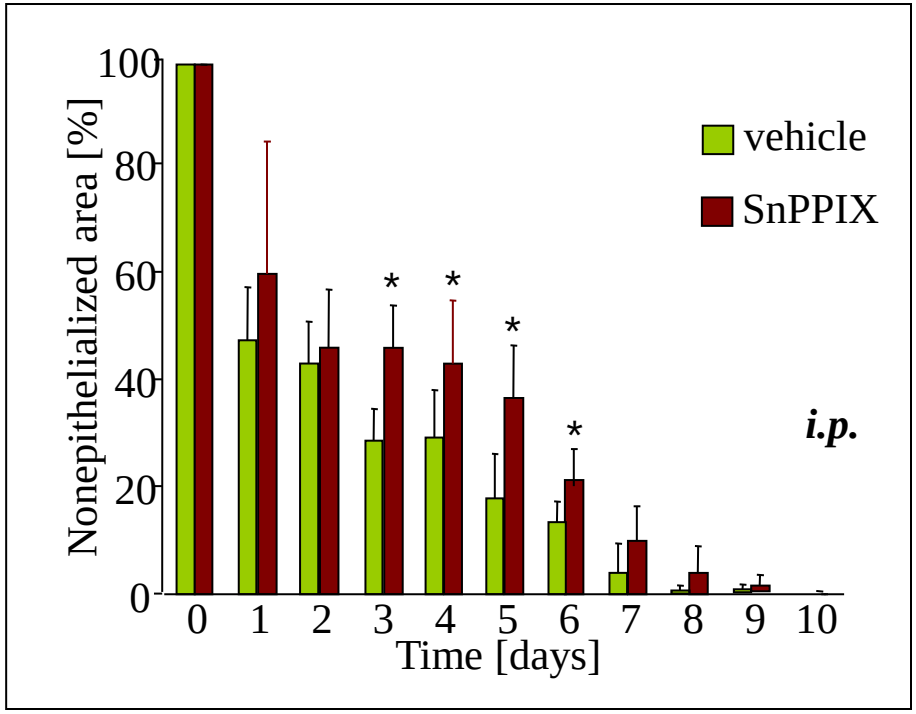
Days after wounding

Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

Inhibition of HO activity delays wound healing



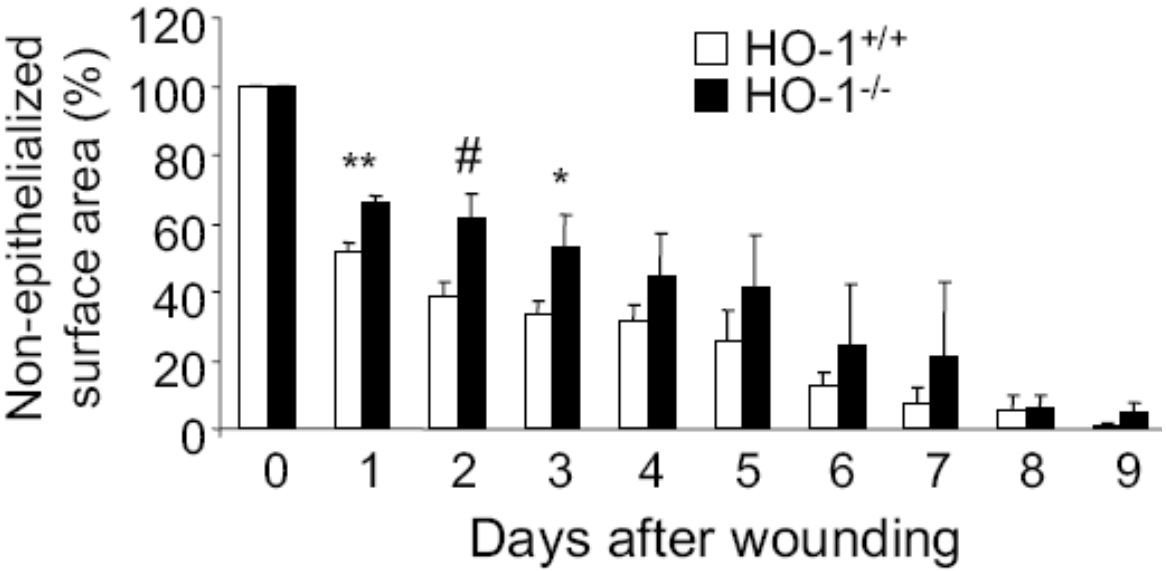


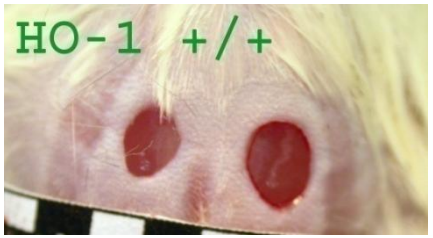


Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

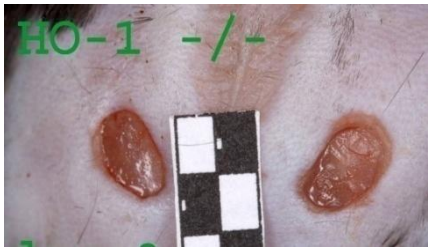
Wound healing in HO-1 knockout mice

Lack of HO-1 impairs epithelialization and wound healing angiogenesis



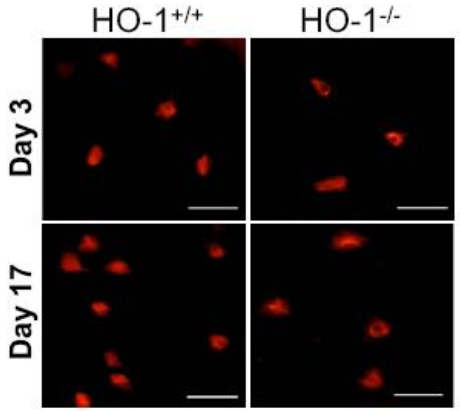


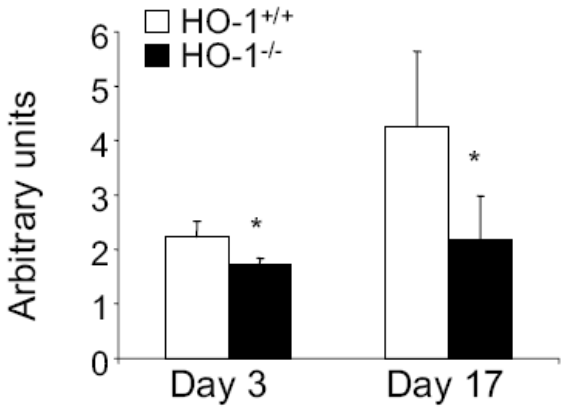
HO-1$^{+/+}$

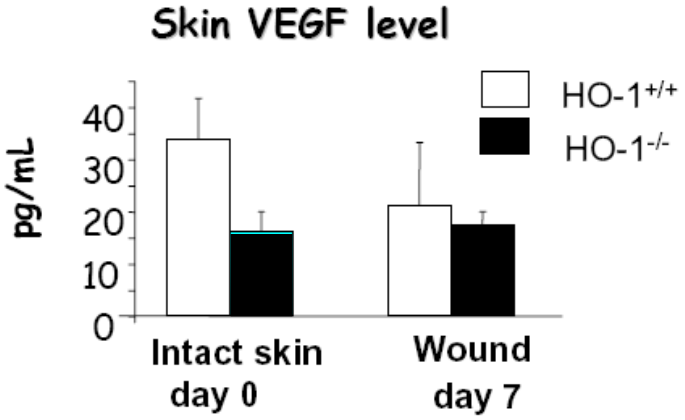


HO-1$^{-/-}$

CD31 staining

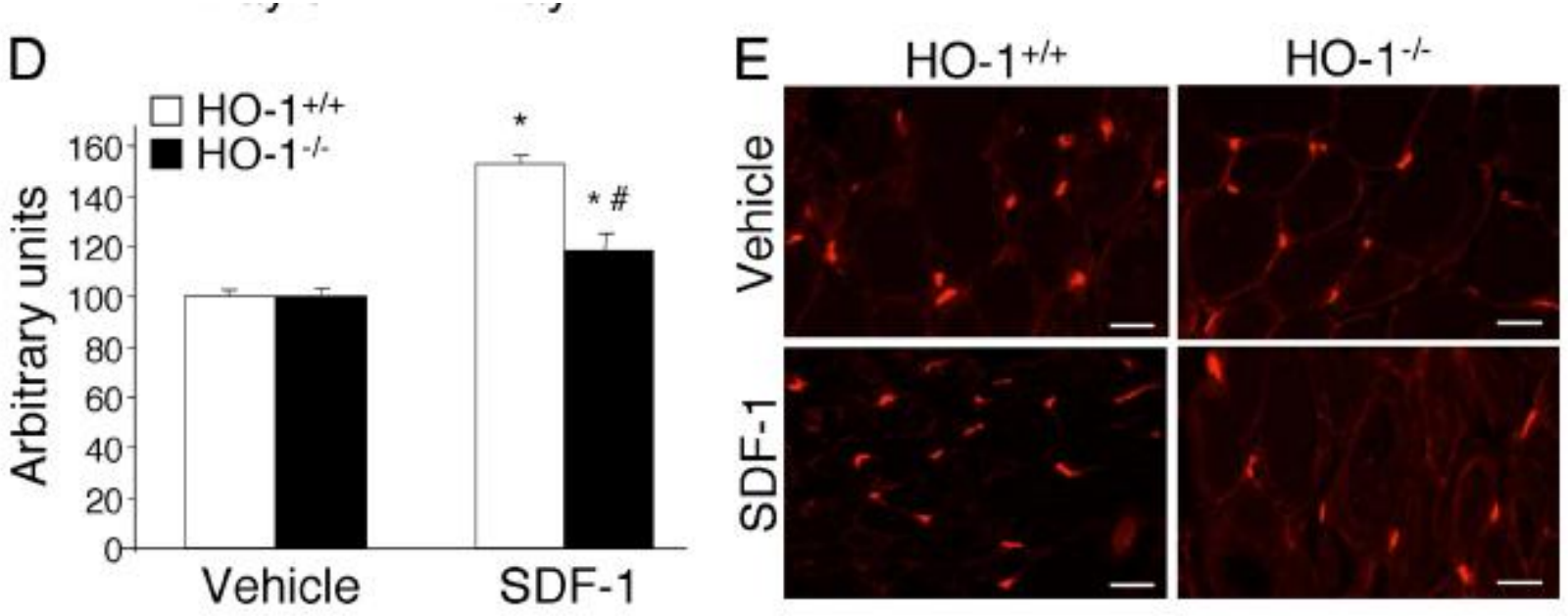






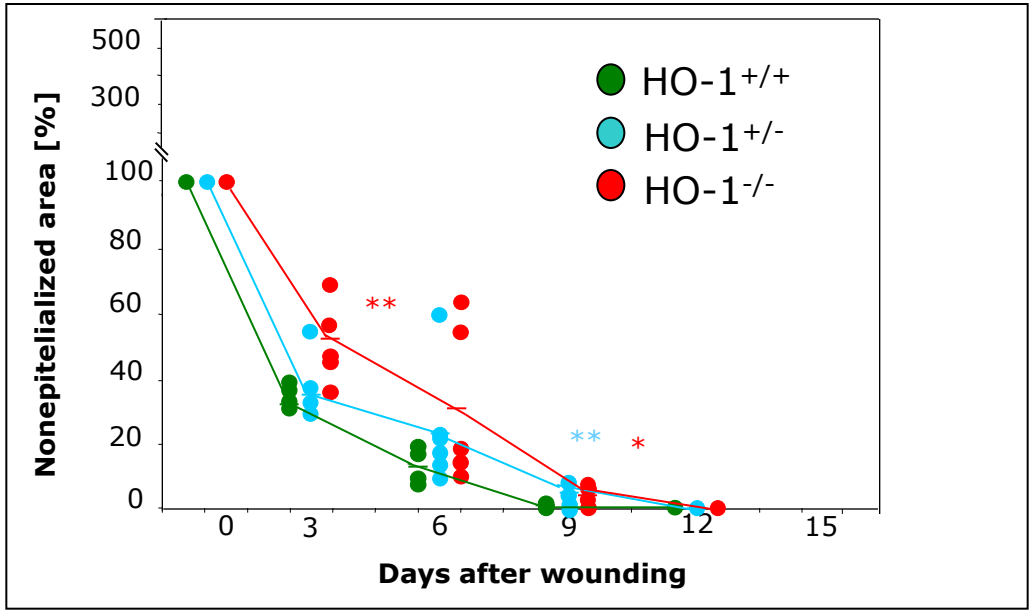
Deshane et al., J Exp Med., 2007,

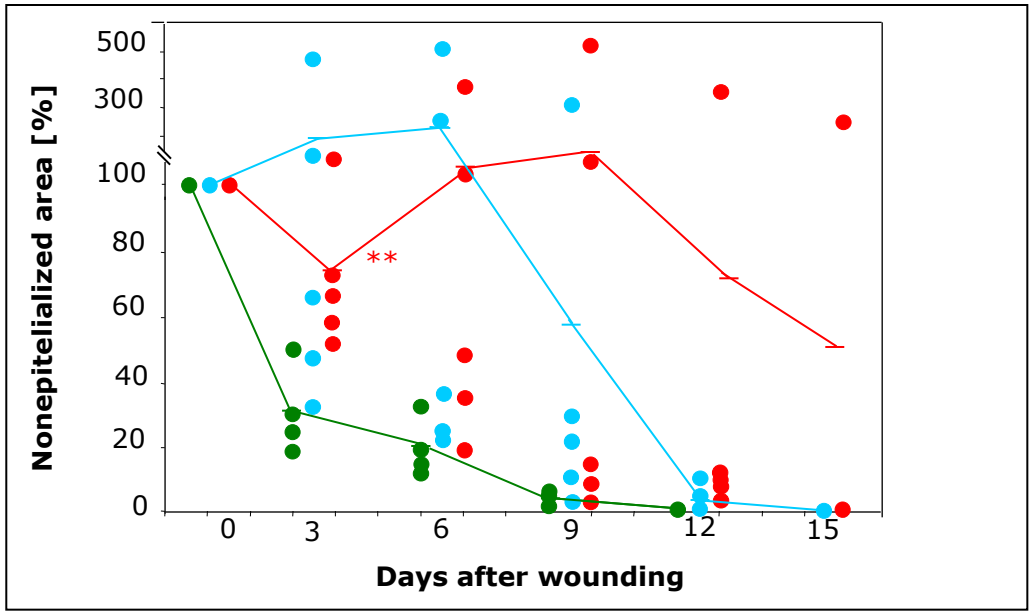
Lack of HO-1 impairs SDF-1 induced angiogenesis in wounds



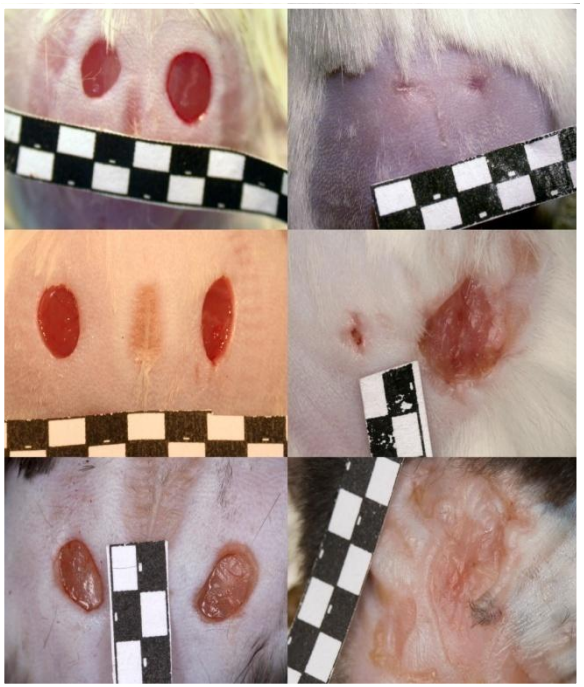
Deshane et al., J Exp Med., 2007; 204(3):605-18.

Effect of HO-1 deficiency on wound healing





3 months



HO-1+/+

HO-1+/-

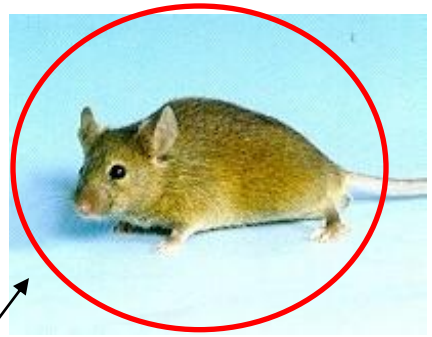
HO-1-/-

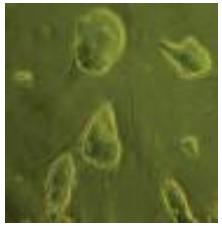
Day 0 Day 10

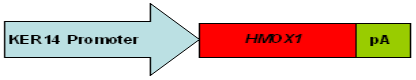
6 months

Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

Transgenic mice overexpressing HO-1 in the skin







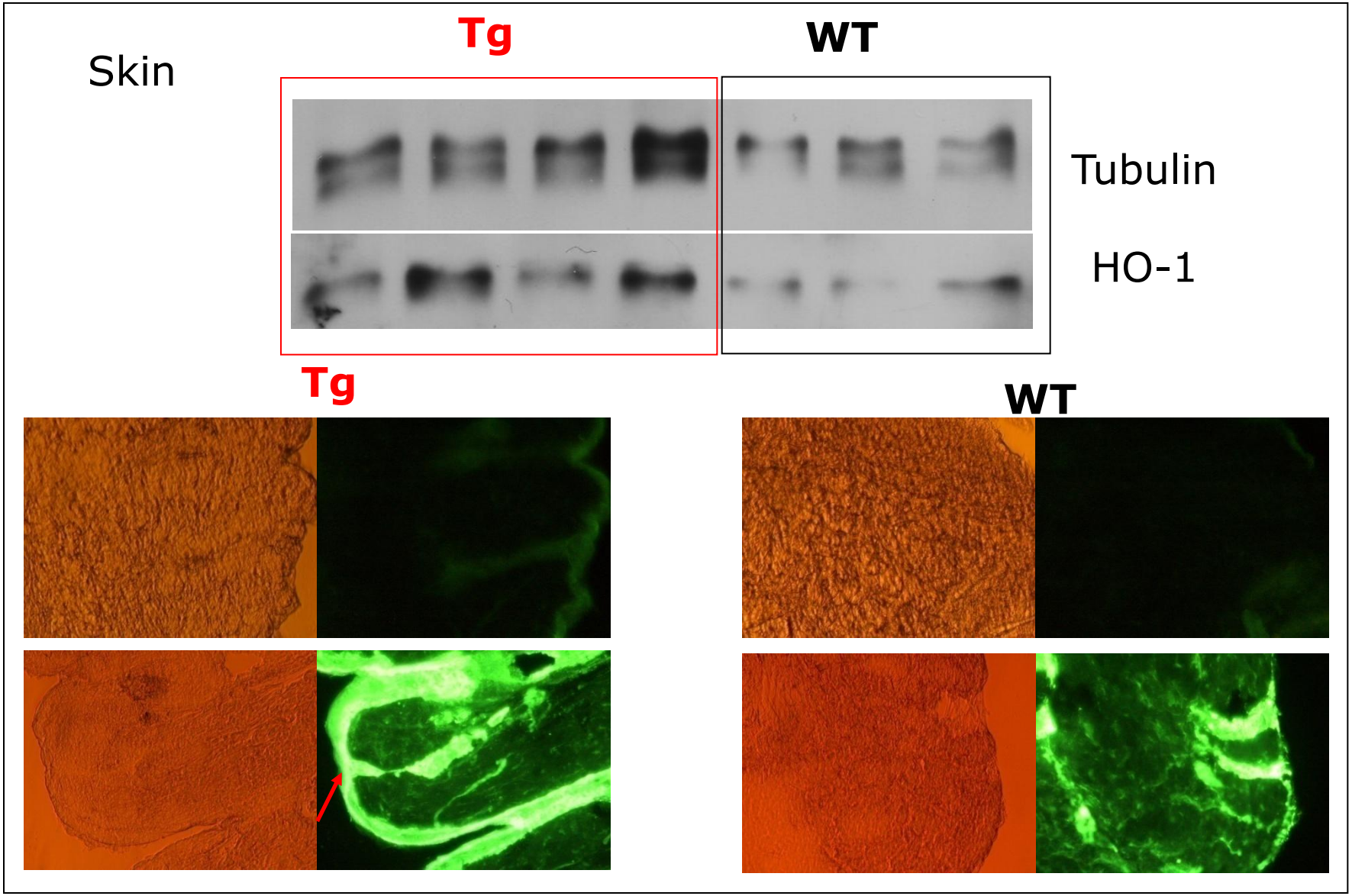
3 lines of transgenic mice:

- K14HO-1 clone 9
- K14 HO-1 clone 78
- K14 HO-1 clone 94

Germline transmission

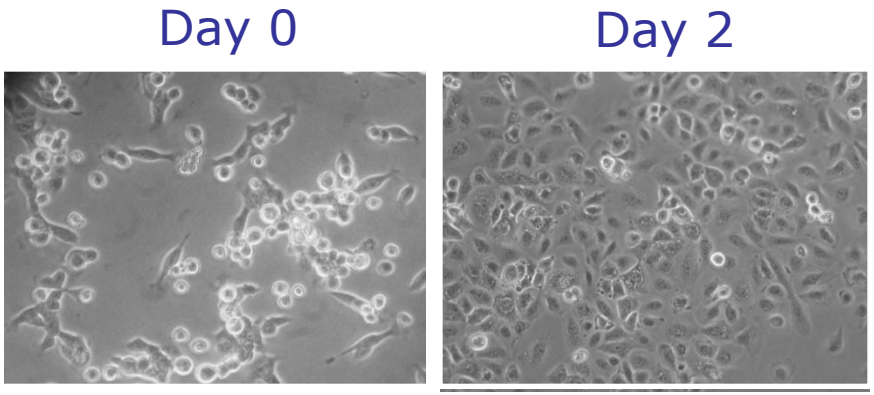
Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

Higher expression of HO-1 in epidermis of KER14-HO1 mice

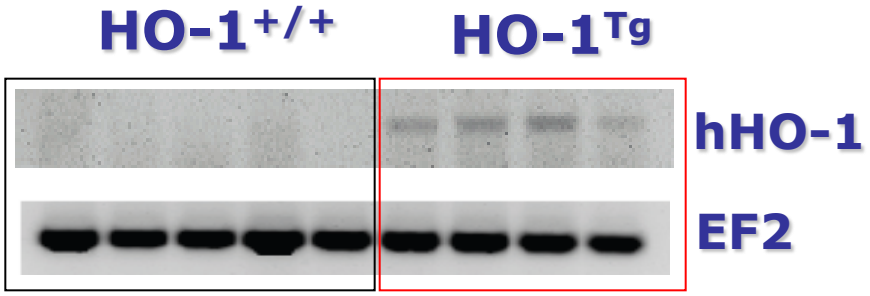


Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

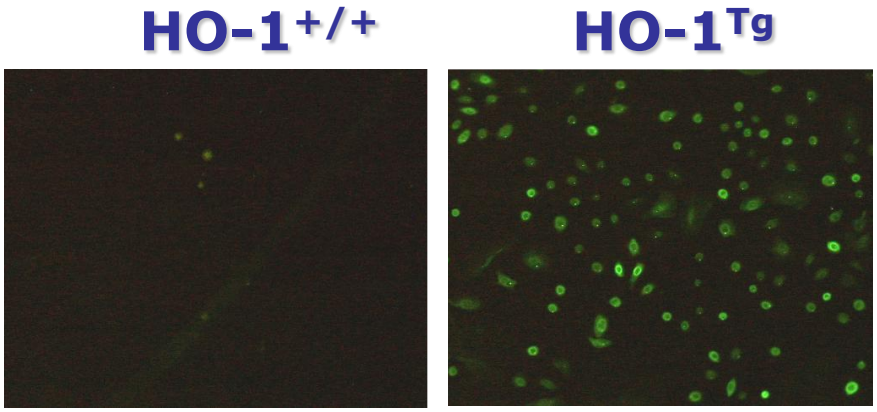
Overexpression of human HO-1 in keratinocytes of KER14-HO-1 mice



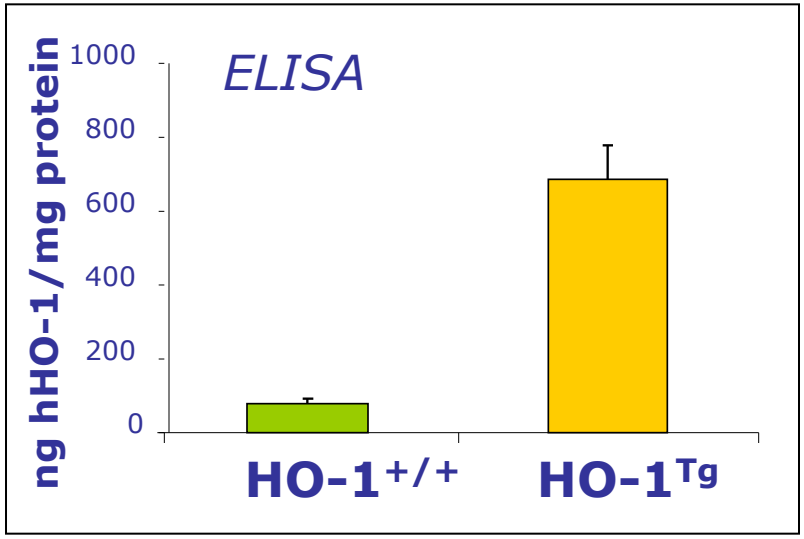
Primary HO-1^{Tg} keratinocytes



RT-PCR

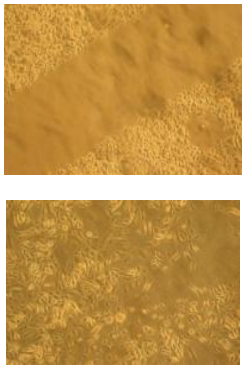


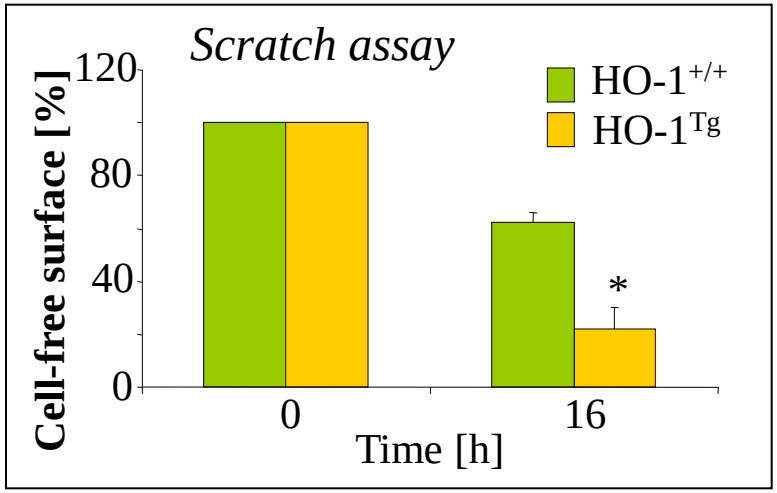
Primary keratinocytes (staining for hHO-1)

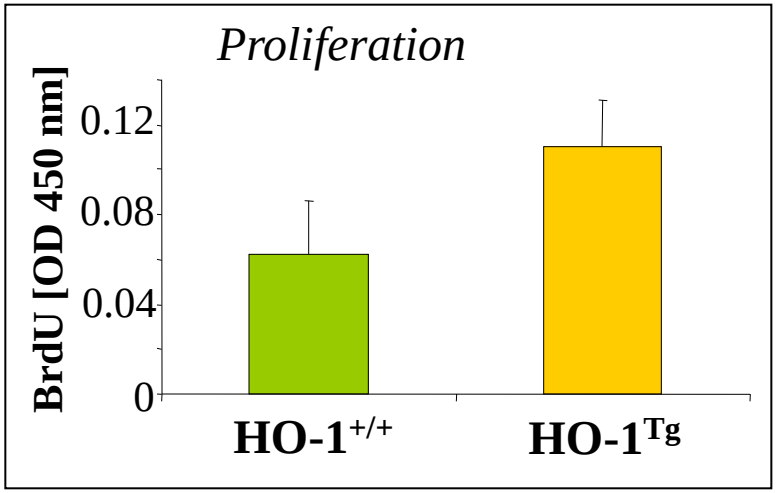


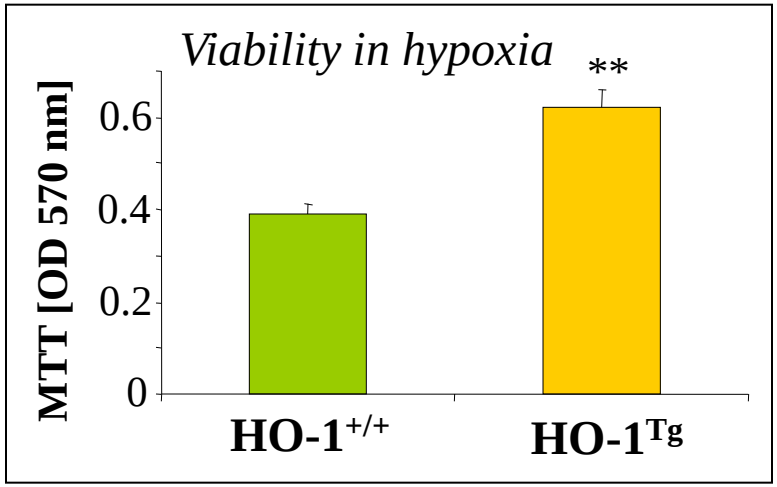
Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

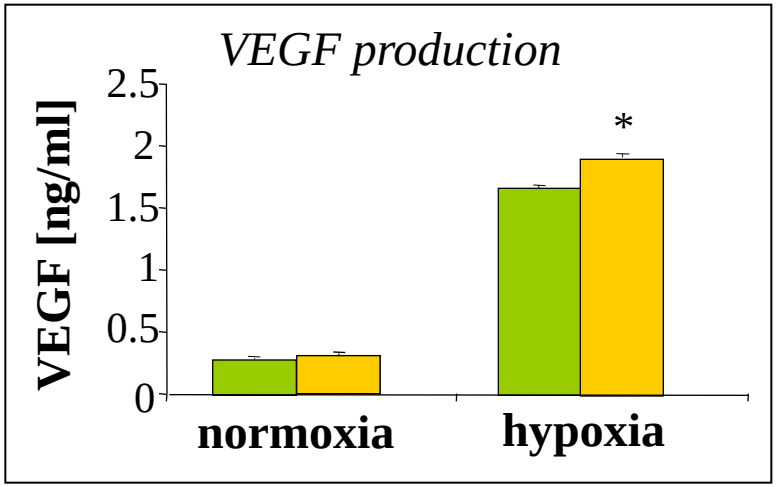
Effect of HO-1 overexpression on primary murine keratinocytes





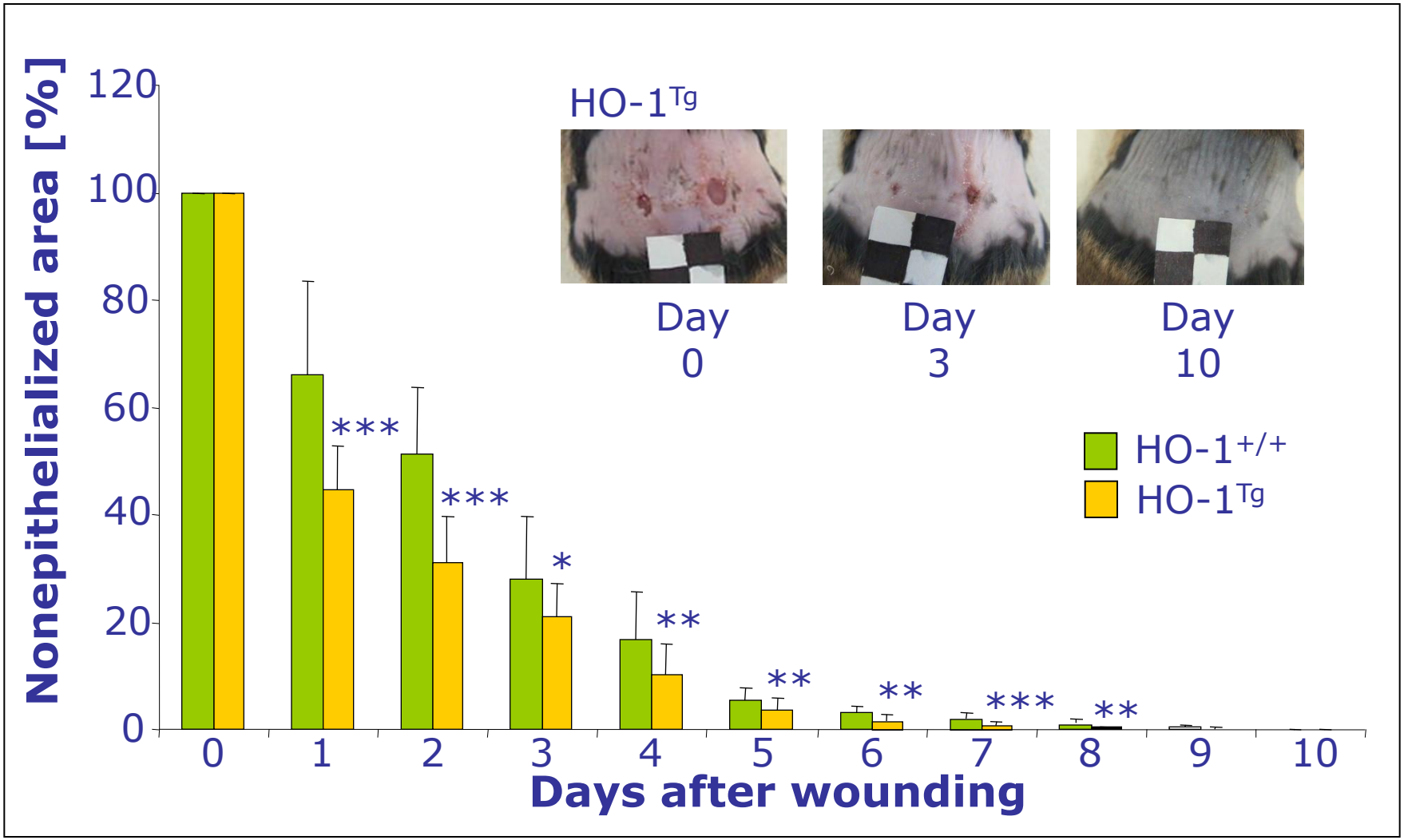






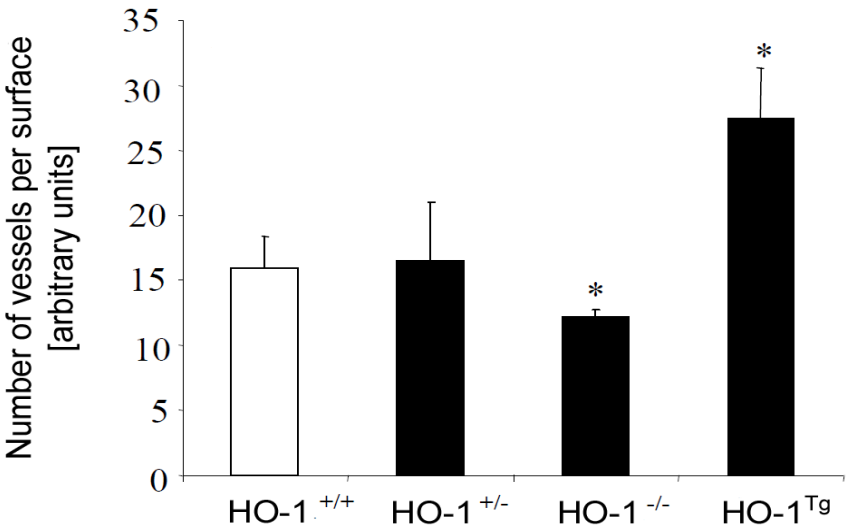
Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

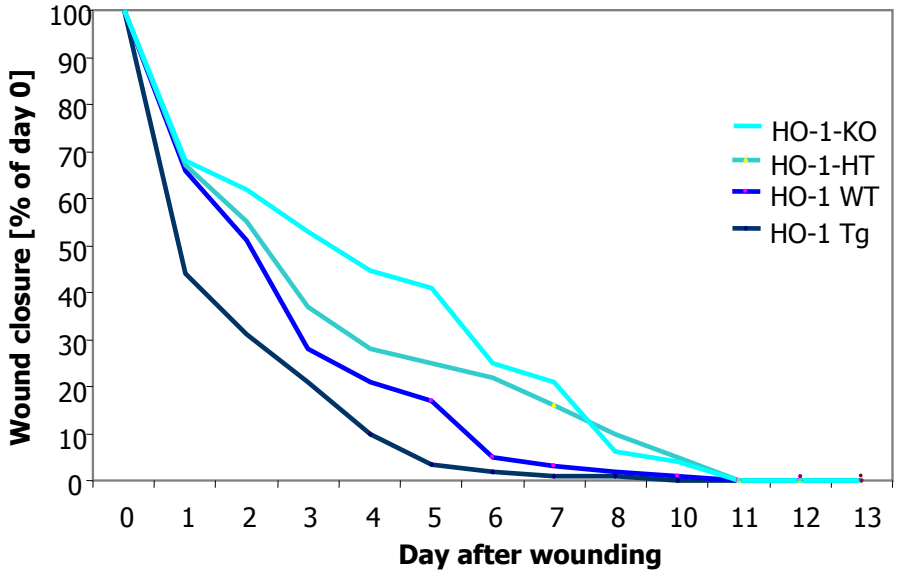
Effect of HO-1 overexpression in keratinocytes on wound healing

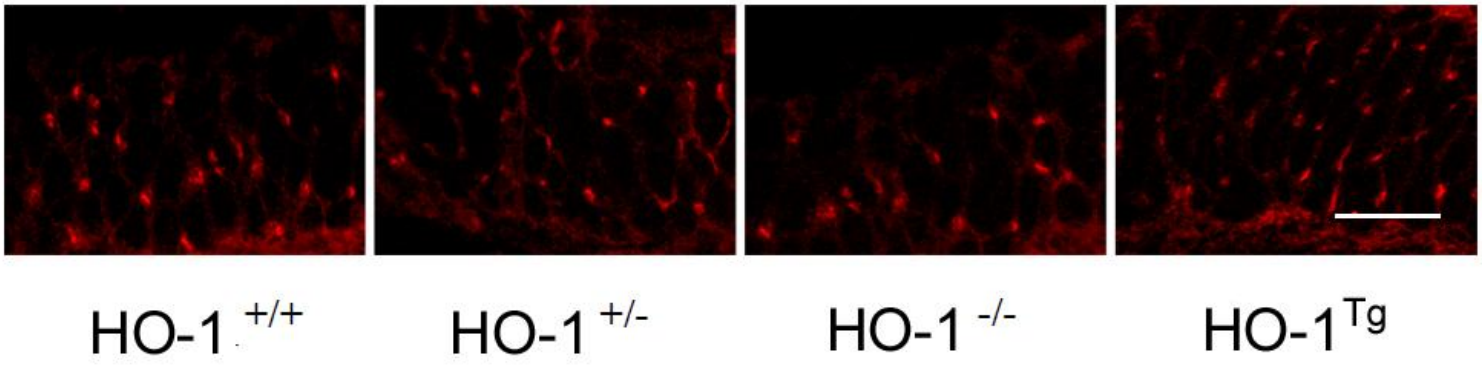


Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

HO-1 is required for blood vessel formation during wound healing







Grochot-Przeczek et al. , PLoS ONE 4(6): e5803; 2009

Diabetic mice

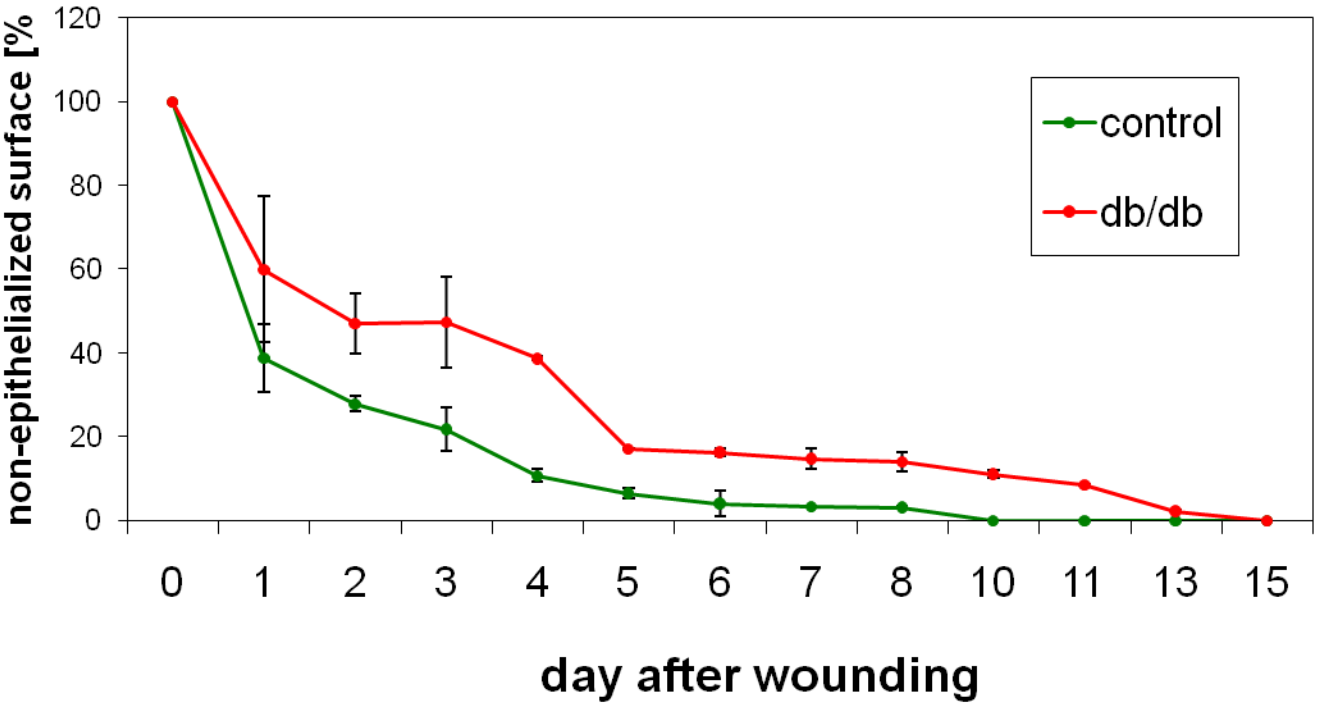
WT

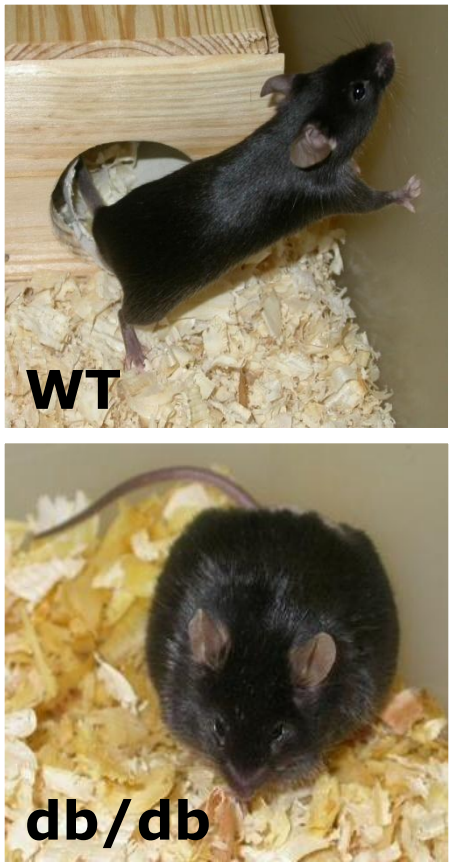
db/db

db/db mice:

- Leptin receptor deficien
- Obesity
- Insulin resistance and diabetes type 2
- Hyperinsulinenia
- Hyperleptinemia
- Hyperglycemia
- Dyslipidemia

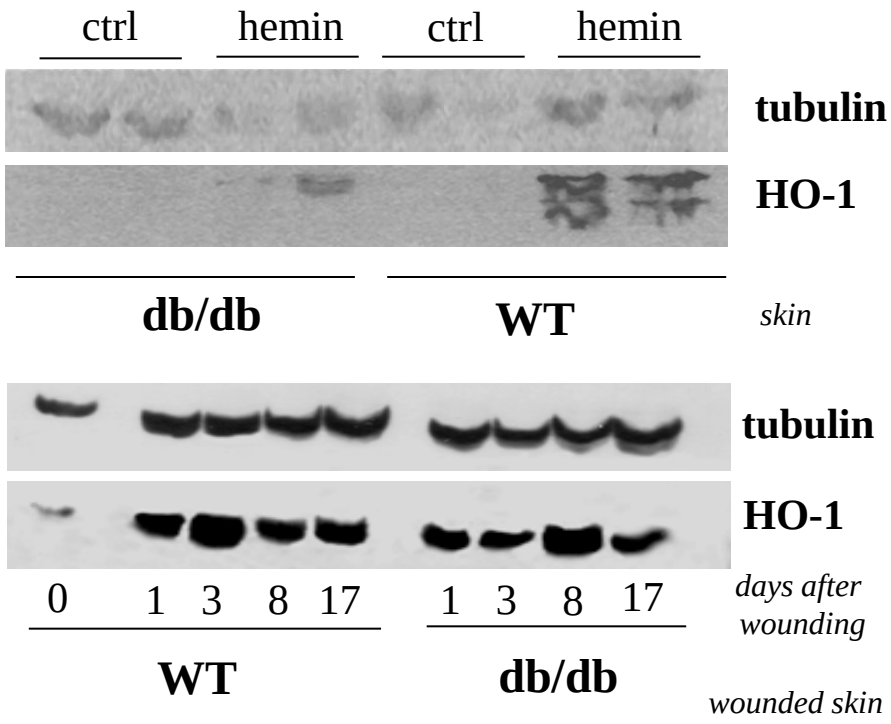
Impaired wound healing in diabetic mice





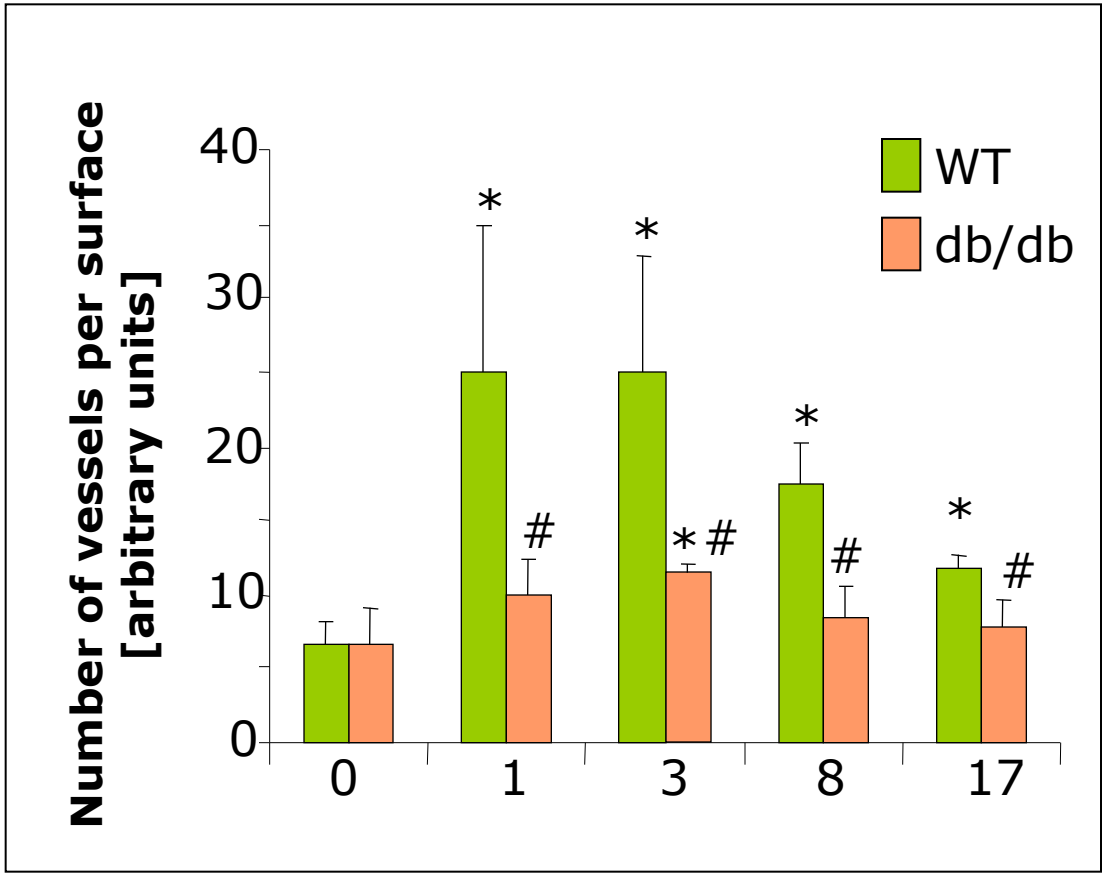
Grochot-Przeczek et al. , PLoS ONE 4(6): e5803; 2009

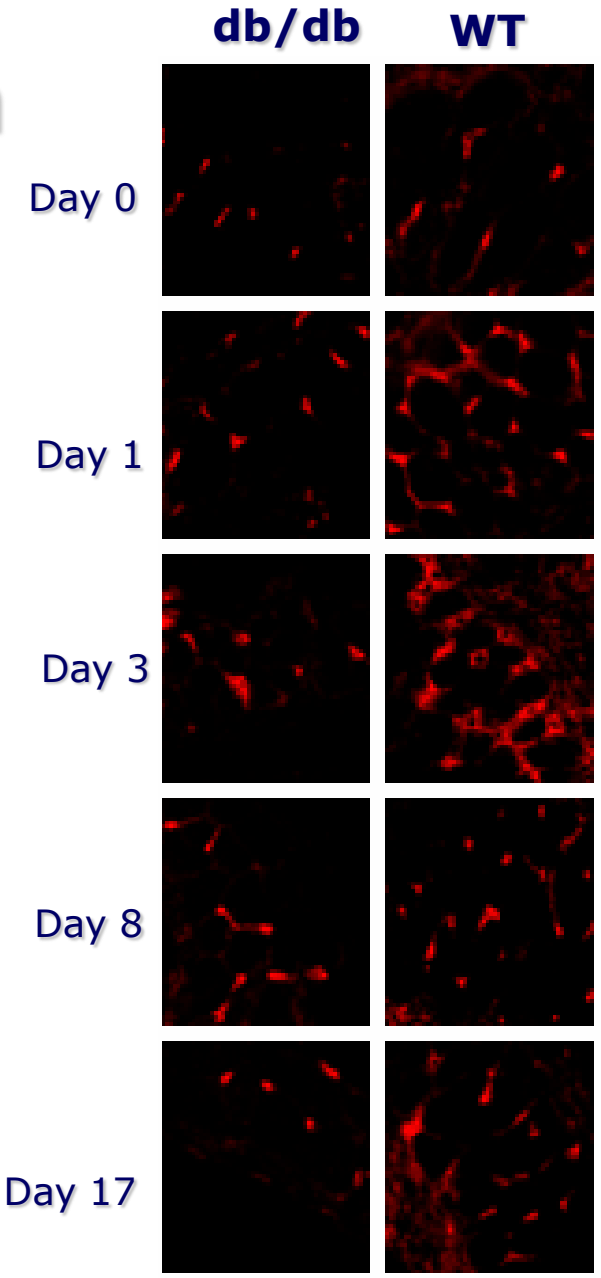
Expression of HO-1 in diabetic mice



Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

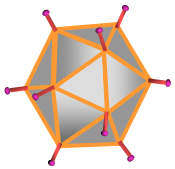
Neovascularization of the wounded skin in diabetic mice



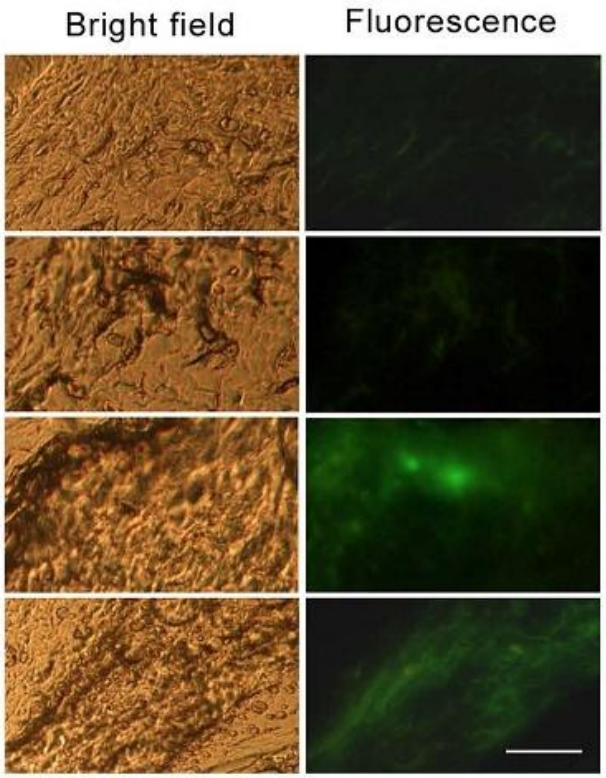


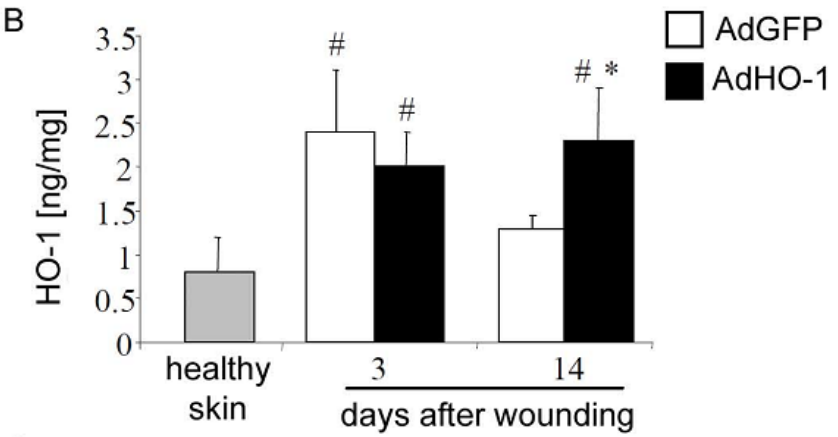
Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

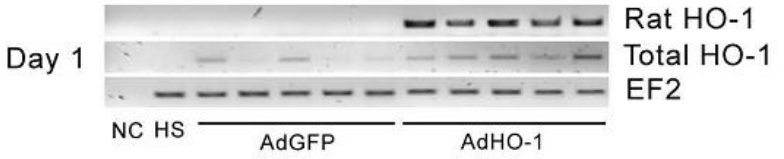
Expression of HO-1 and GFP transgenes in the skin

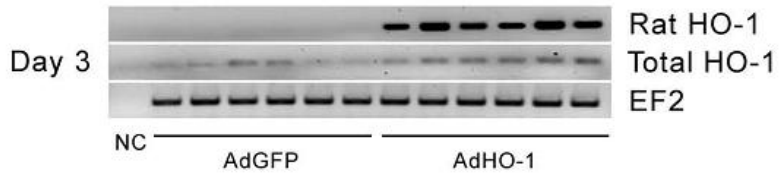


AdGFP
AdHO-1



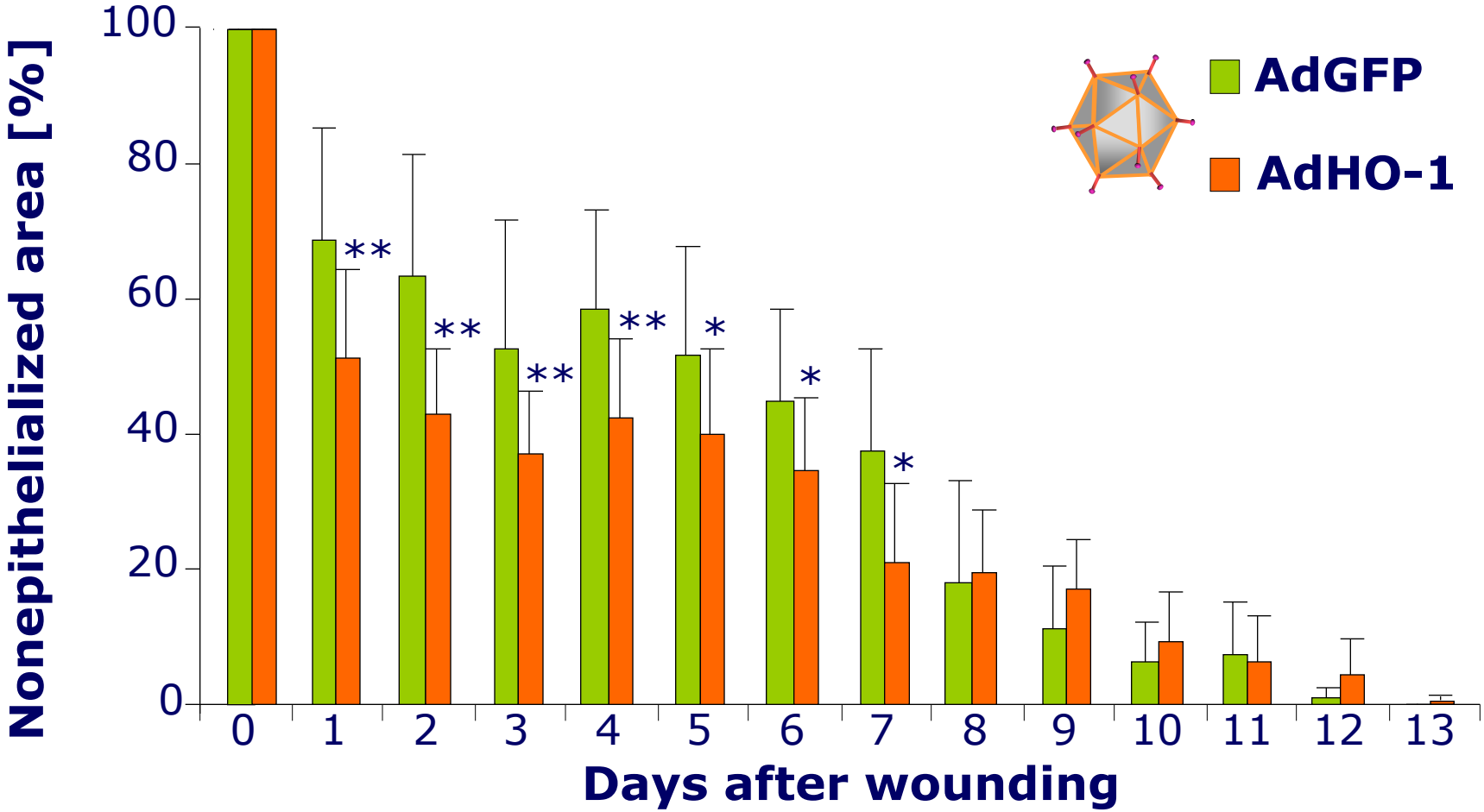






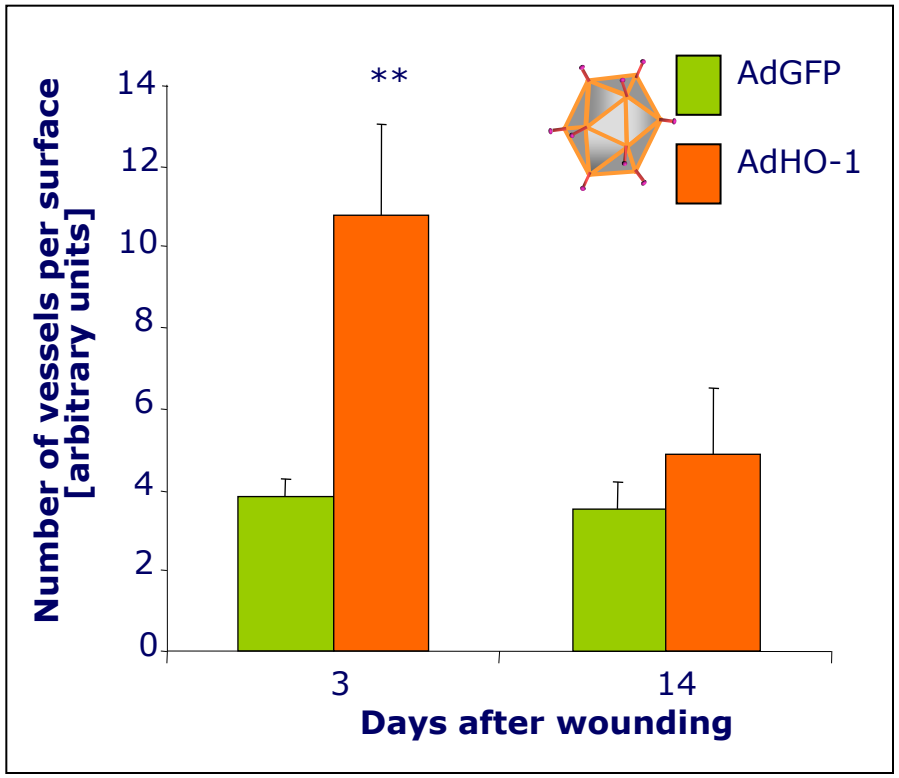
Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

Effect of AdHO-1 injection on reepithelialization of wounds in diabetic mice

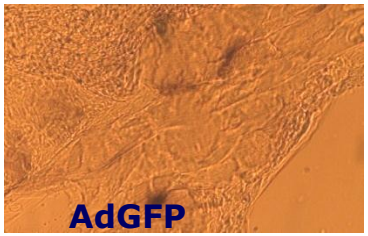


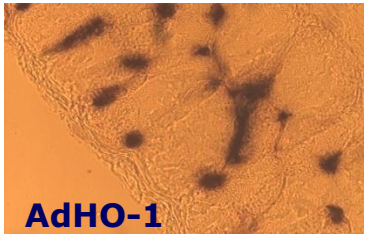
Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

Neovascularization of the wounds in diabetic mice injected with AdHO-1

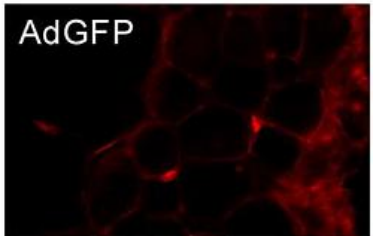


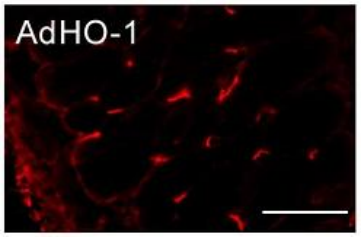
Alkaline phosphatase, 3 days after wounding





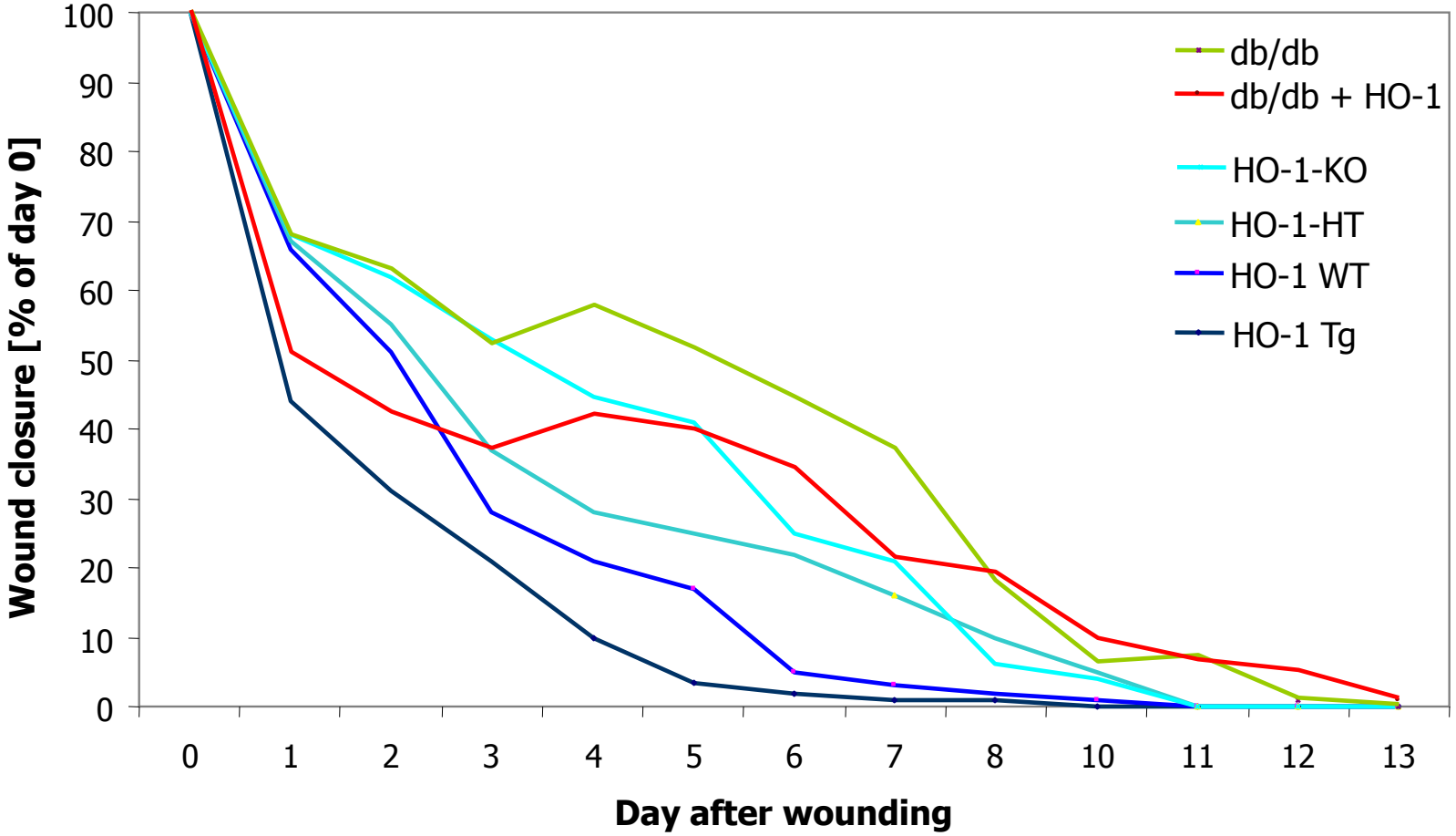
CD31 expression, 3 days after wounding





Grochot-Przeczek et al., PLoS One. 2009;4(6):e5803

Gene therapy with HO-1 accelerates wound healing



Grochot-Przeczek et al. , PLoS ONE 4(6): e5803; 2009

Conclusions

- Proangiogenic factors, such as VEGF or HO-1, improve cutaneous wound healing.

- Impaired induction of HO-1 in wounded skin appears to contribute to the delayed wound healing in diabetic mice.

- Increased expression of HO-1 in keratinocytes may significantly augment reepithelialization and neovascularization.

- Overexpression of HO-1 by gene transfer may facilitate wound healing in diabetic mice.