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# Vascular endothelial insulin/IGF-1 signaling controls skin wound vascularization

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ABSTRACT

Type 2 diabetes mellitus affects 6% of western populations and represents a major risk factor for the development of skin complications, of which impaired wound healing, manifested in e.g. "diabetic foot ulcer", is most prominent. Impaired angiogenesis is considered a major contributing factor to these non-healing wounds. At present it is still unclear whether diabetes-associated wound healing and skin vascular dysfunction are direct consequences of impaired insulin/IGF-1 signaling, or secondary due to e.g. hyperglycemia. To directly test the role of vascular endothelial insulin signaling in the development of diabetes-associated skin complications and vascular function, we inactivated the insulin receptor and its highly related receptor, the IGF-1 receptor, specifically in the endothelial compartment of postnatal mice, using the inducible Tie-2CreERT ($DKO^{IVE}$) deleter. Impaired endothelial insulin/IGF-1 signaling did not have a significant impact on endothelial homeostasis in the skin, as judged by number of vessels, vessel basement membrane staining intensity and barrier function. In contrast, challenging the skin through wounding strongly reduced neo-angiogenesis in $DKO^{IVE}$ mice, accompanied by reduced granulation tissue formation reduced. These results show that endothelial insulin/IGF signaling is essential for neo-angiogenesis upon wounding, and imply that reduced endothelial insulin/IGF signaling directly contributes to diabetes-associated impaired healing.

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## 1. Introduction

Type 2 diabetes mellitus represents the most frequent endocrine disease, affecting more than 6% of western populations [1]. Thereby, it represents a major socio-economical health burden. Lethality results largely from diabetic complications including micro- and macrovascular diseases [1]. Moreover, diabetes is associated with a plethora of skin diseases. One hallmark of diabetes is impaired wound healing [2]. It is now well established that ulcerations and subsequent amputation events represent serious complications of diabetes mellitus and are associated with significant mortality as well as socio-economical costs [3–5]. The lifetime risk for any diabetic patient to develop such a complication ranges around 15% [6]. It is not resolved yet whether diabetic skin complications arise in response to metabolic alterations such as hyperglycemia or directly from impaired insulin action in skin [7]. A severe impairment of insulin signaling in wounds of diabetic mouse models has been described [8], suggesting a potential direct role for altered insulin signaling in impaired healing.

A range of physiological changes can contribute to diabetes-associated wound healing deficiencies, such as impaired responsiveness to growth factors, macrophage function, collagen accumulation, and altered granulation tissue formation [2,9]. Vascular insufficiency is thought to be one major driver of impaired healing [9–12]. Vascular endothelial cells express functional insulin receptors and even higher levels of the highly related IGF-1 receptors [13,14]. Importantly, endothelial dysfunction is observed early upon systemic insulin resistance [15,16] and is associated with a blunted insulin/IGF signaling response in endothelial cells [17,18]. A direct function for cell-autonomous insulin/IGF signaling in endothelial dysfunction associated with diabetes was further

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confirmed using transgenic mice. Vascular endothelial specific deletion of the insulin receptor (IR) reduced basal expression of the vasoactive mediators eNOS and ET-1 [19], whereas endothelial expression of a dominant-negative IR mutant resulted in reduced phosphorylation of eNOS accompanied by a blunted vasodilation response upon insulin stimulation [20]. In both cases global glucose homeostasis was unaltered, suggesting that dysfunctional vascular endothelial IR signaling does not directly contribute to overall insulin resistance. Perhaps more importantly in the light of diabetic wound healing, either IR or IGF-1 receptor inactivation reduced neo-angiogenesis upon hypoxia-induced retinopathy, although the effect was less dramatic than loss of IR [21].

To address the role of vascular endothelial insulin/IGF-1 signaling in the homeostasis of the skin vasculature and in neovascularization in the skin in wound healing, we inactivated both the IR as well as the IGF-1R in adult vascular endothelia using inducible Cre-LoxP technology. We show that endothelial insulin/IGF-1 signaling upon wounding regulates angiogenesis and granulation tissue formation.

## 2. Material and methods

### 2.1. Mice

Floxed IR and IGF-1R alleles and tamoxifen inducible Tie2-CreER$^{T2}$ mouse lines and their genotyping have been described previously [22,28]. All mice used in experiments were derived from at least 5th generation C57BL/6 crossings. For induction of Cre activity, adult 4–5 weeks old mice carrying the Tie2-CreER$^{T2}$ transgene and their Cre-negative littermates were fed a tamoxifen-containing diet (400 mg/kg tamoxifen citrate, 5% sucrose, 95% Teklad Global, 16% Rodent Diet) from Harlan Teklad[22,23]. All animal procedures were conducted in accordance with European, national, and institutional guidelines, and protocols and were approved by local government authorities. PCR was used to detect deletion efficiency for *Insr* or *Igf1*r using the following primers: *Igf-1r*. 5′-TTATGCCTCCTCTCTTCATC-3′ (sense) and 5′-CTTCAGCTTTGC AGGTGCACG-3′ (antisense) resulting in products of 1165 bp for wt, 1350 bp for floxed and 491 bp for deleted alleles. *Insr*. 5′-ACGCCTACACATCACATGCATATG-3′ (sense) and 5′-CCTCCTGAATA GCTGAGACCACAG-3′ (antisense) resulting in products of 2048 bp for wt, 2188 bp for floxed and 249 bp for deleted alleles on DNA isolated from lung, heart and on occasion endothelial cells.

### 2.2. Skin irritant response, wounding and preparation of wound tissues

Irritant response was elicited and measured as previously described [30]. Wounding and preparation of wound tissue for histology was performed as recently described [29]. Briefly, mice were anesthetized by i.p injection of Ketanest/Rompun (Ketanest S: Park Davis GmbH; Rompun 2%: Bayer), the back was shaved and four full thickness punch biopsies were created. For histological analysis wounds were excised at indicated time points post injury, bisected in caudocranial direction and the tissue was either fixed overnight in 4% formaldehyde or embedded in OCT compound (Tissue Tek). Histological analysis was performed on serial sections from the central portion of the wound.

### 2.3. Histology and immunohistochemistry

For histology paraffin skin or wound sections were stained for hematoxilin/eosin (H&E) as described [28]. Immunohistochemistry was performed on either cryosections (10 μm) or paraffin sections fixed with 4% PFA or acetone, blocked (10% NGS, 1& BSA, 0.02% Tween-20), and incubated with the appropriate primary antibodies followed by Alexa Fluor 488- or 594- conjugated (Invitrogen) secondary antibodies. Nuclei were counterstaining with DAPI (Invitrogen). Primary antibodies used were rabbit antibodies to collagen IV (Progen), laminin411 (a kind gift of Lydia Sorokin, University of Munster, Germany) and mouse mAbs, Desmin (DakoCytomation), rat mAbs to CD31 (BD pharmingen), VE-cadherin (BD Biosciences) as well as the appropriate isotype-matched negative control antibodies. Histological images were generated using a LEICA microscope equipped with a digital DZM1200 camera. Fluorescent analysis and images were done either with a Nikon Microscope Eclipse 800E or with an Olympus IX81 microscope with a digital cool snapTM HQ2 camera (photometrics) or an Olympus Fluoview 1000 laser scanning microscope. Autoquant software was used for the high magnification vessel images.

### 2.4. Morphometric analysis

Morphometric analysis was performed on digital images using the Imaging Software Lucia G 4.80 (Laboratory Imaging Ltd., Prague, Czech Republic). The extent of epithelization and granulation tissue formation was determined on hematoxylin/eosin (H&E)-stained paraffin tissue sections as previously described [29]. The length of the epithelial tongue was determined as the distance between the epithelial tip and the margin of the wound as defined by the presence of hair follicles in non-wounded skin. This parameter reflects the formation of neo-epithelium. In addition, the width of the gap between the epithelial tips reflects wound closure. Granulation tissue was defined as the cellular and vascular tissue that formed underneath the neo-epithelium and between the wound margins and above the subcutaneous fat tissue. Images were processed with Adobe Photoshop 7.0. Staining for CD31, desmin, collagen IV and VE-cadherin was quantified in high magnification fields ($7000 \times 5500\ \mu m^2$) using ImageJ software. Histomorphometric analyses were performed in a blinded manner by two independent investigators. Statistical analyses were performed using GraphPad Prism5 (GraphPad Software). Significance of difference was analyzed using Student's unpaired two-tailed *t*-test. All data presented as mean ± SD, a $p$ value of $<0.05$ was considered significant.

## 3. Results

### 3.1. Vascular homeostasis is not obviously affected by loss of endothelial IR/IGF-1R

To examine the role of Insulin and its closely related growth factors IGFs in the adult vasculature in the skin, mice floxed for both the IR and IGF-1R were crossed with mice carrying Tie2-CreER$^{T2}$, which induces Cre activity specifically in vascular endothelial cells (IVE) upon feeding mice tamoxifen for 5 weeks [22,23]. In general, after 5 weeks of tamoxifen feeding, efficient deletion of the insulin and IGF-1R alleles was induced (Fig. 1A) in mice carrying the Tie2-CreER$^{T2}$ allele (hereafter referred to as DKO$^{IVE}$). As controls we used mice carrying either floxed alleles only or the Tie2-Cre$^{ERT2}$ allele with wt IGF-1R and IR alleles. DKO$^{IVE}$ mice were viable and showed no obvious defects, even upon prolonged deletion (not shown). Histological analysis of the skin did not reveal any major alterations (Fig. 1B, upper panel) and vessels appeared microscopically similar when compared to controls (Fig. 1B lower panel).

To address if loss of endothelial insulin/IGF signaling affected vessel density in the skin, sections were stained for VE-cadherin (Fig. 2A) and CD31 (not shown). No obvious changes in localization were observed between control and DKO$^{IVE}$ for either endothelial specific marker. Further quantification did not reveal any obvious changes in VE-cadherin in the area stained positive for VE-cadherin

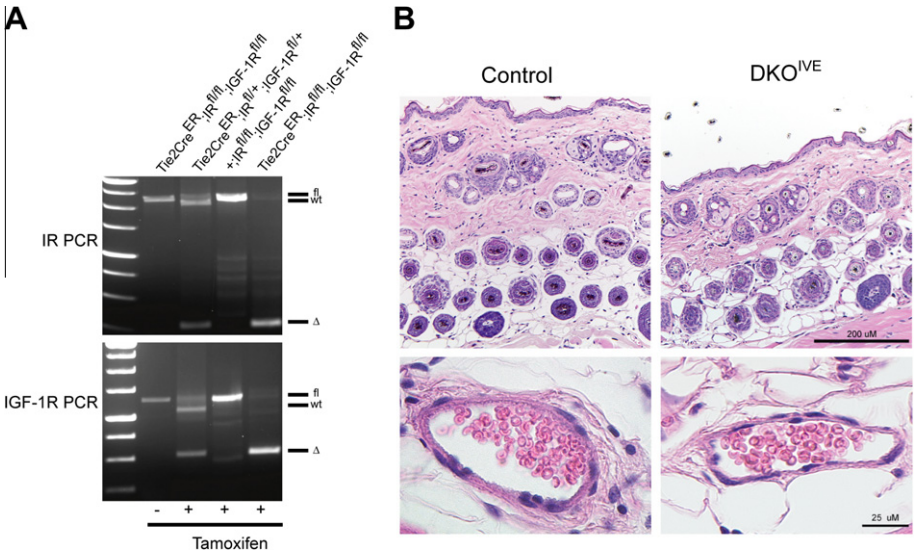


**Fig. 1.** Adult endothelial inactivation of insulin/IGF-1 receptors does not affect vascular and skin morphology. (A) Deletion PCR for the Igf1r or Insr gene in vascular endothelial cells. Genomic DNA was isolated from heart tissues of mice with the indicated phenotypes that were either not fed or fed for 5 weeks with tamoxifen to activate Cre by promoting its translocation into the nucleus. The Tie2 promoter was used since this is specific for endothelial cells. The deletion PCR detects the floxed, wt and deleted alleles, as indicated. (B) H&E staining of skin sections from control and DKO$^{IVE}$ showing overall skin morphology in the upper panel and a high magnification of one vessel in the lower panel. No obvious changes in skin as well as in vessel morphology could be observed between control and DKO$^{IVE}$ mice.

(Fig. 2C), indicating that loss of endothelial insulin/IGF-1 did not alter skin vessel density.

A thickening of capillary basement membranes and increased deposition of basement membrane components, such as e.g. collagen IV, is one hallmark of diabetes [24,25]. We therefore asked if loss of vascular IR/IGF-1R signaling had an impact on the composition of the vascular basement membrane, by staining for the basement membrane marker collagen IV. VE-cadherin was used as a marker to identify the skin vasculature, because collagen IV is also an essential component of the dermal-epidermal basement membrane and therefore shows staining below the epidermis and around hair follicles. No obvious differences in vessel staining pattern were observed (Fig. 2A) and quantification of intensity of collagen staining around vessels did not reveal any significant change upon loss of endothelial insulin/IGF receptors (Fig. 2D). We next examined localization of laminin411, a more specific marker for vascular endothelial basement membranes[26]. Similar to collagen IV, no obvious difference could be observed for laminin411 in DKO$^{IVE}$ mice, either in staining pattern (Fig. 2B) or in staining intensity/vessel (Fig. 2E). Similar results were found for the basement membrane proteins nidogen 1 and 2 (not shown). Together, these data indicate that functional insulin/IGF-1 receptor signaling in vascular endothelial cells in the skin is not essential for the maintenance of skin vasculature.

### 3.2. Barrier function is similar in control and DKO$^{IVE}$ mice

Constitutive endothelial loss of either the IR or IGF-1R insulin or IGF-1 receptor does not affect the blood brain barrier [27]. Since the other receptor is still present in vascular endothelia, this result might be explained by compensation by the IR for the IGF-1R and vice versa. To directly test this, we examined the vascular barrier function in skin by injecting biotin and examining leakage into the tissue. Very little staining for biotin was detected in the dermis and no obvious difference was found between control and DKO$^{IVE}$ mice (not shown), in agreement with the observation that inactivation of IR and IGF-1R in the adult vasculature did not result in histological or basement membrane component changes.

Next we asked if loss of endothelial insulin/IGF-1 signaling would impair the irritant response in the skin, which depends on the partial loss of vascular barrier function, to allow extravasation of immune cells into the tissue. To examine whether inflammation-mediated vascular leakage with subsequent edema formation is regulated by vascular IR/IGF-1R signaling, ears of control and DKO$^{IVE}$ mice were subjected to a skin irritation assay using croton oil as the irritant. Ear thickness peaked at 24 h after croton oil treatment and no difference was observed for control and DKO$^{IVE}$ mice either at 24 h or 48 h after treatment (Fig. 2F), suggesting that IR/IGF-1R does not control inflammation mediated vascular leakage. Together, our results indicate that endothelial insulin/IGF-1 signaling does not directly regulate vascular endothelial barrier function.

### 3.3. Decreased angiogenesis and granulation tissue formation

To assess the functional impact of endothelial cell-restricted deletion of IR/IGF-1R on the wound healing response, control and DKO$^{IVE}$ mice were subjected to full-thickness wounds and then allowed to heal for varying times. No significant differences were observed in wound closure rate at any time point, as assessed either macroscopically (not shown), microscopically (Fig. 3A), and measured by the distance between the epithelial tips (Fig. 3B). Histological analysis at day seven revealed a highly cellular and vascularized granulation tissue beneath the neo-epidermis in wounds of control mice, whereas a minimal and scarcely vascularized granulation tissue was found in DKO$^{IVE}$ mice at this time (Fig. 3A and C). At day 10 post injury, wounds in DKO$^{IVE}$ mice had a similar area of early scar tissue to controls (Fig. 3C). Morphometric quantification of the endothelial cell marker CD31 and desmin, a marker of perivascular cells, within the area of granulation tissue was used to assess the impact of endothelial insulin/IGF signaling on wound-induced neovascular processes. Whereas in controls the number of blood vessel and perivascular cells within the granulation tissue increased during the healing response, peaking at day seven post wounding, in DKO$^{IVE}$ mice, the number of both vascular structures and desmin-positive perivascular cells was strongly reduced at day seven (Fig. 4), while this difference was no longer apparent at healing stage day 10 (Fig. 4B and C). The ratio of desmin/CD31-positive stained cells was similar in DKO$^{IVE}$ and control mice (Fig. 4D), suggesting that recruitment of perivascular cells and blood vessel maturation is not affected in DKO$^{IVE}$ mice. Thus, loss of endothelial

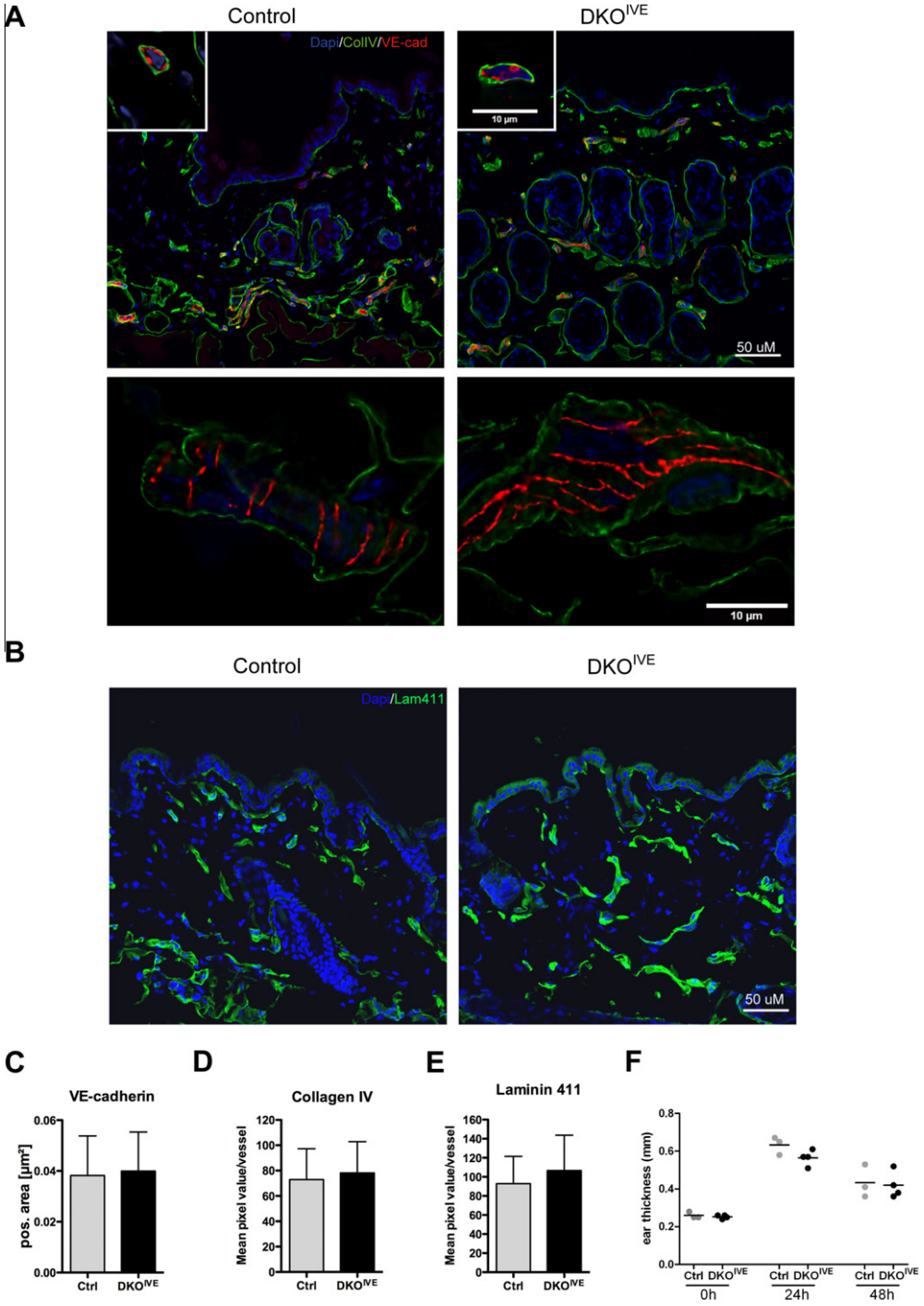


**Fig. 2.** Endothelial IR/IGF-1R signaling does not control skin vascular homeostasis. (A) Immunofluorescence analysis of cryosections of the skin of control and DKO$^{iVE}$ mice stained for VE-cadherin (red), collagen IV (green) and DAPI (blue). High magnifications of microvessels (insets) and vessels of larger size (lower panels) are shown. (B) Immunofluorescence analysis of the endothelial basement membrane marker laminin411 (green) skin paraffin sections of control and DKO$^{IVE}$ mice. Nuclei were counterstained with DAPI (blue). (C) Morphometric quantification of areas in the skin positively stained for VE-cadherin, as in indirect indication of vessel density in the skin. (D) Morphometric quantification of collagen IV staining intensity per vessel. In total $N = 26$ (control) and $N = 21$ vessels in DKO$^{IVE}$ were quantified in 2 mice/genotype. (E) Quantification of laminin411 staining intensity per vessel. $N = 205$ in control and $N = 234$ vessels in DKO$^{IVE}$ in 5 mice/genotype control or DKO$^{iVE}$ mice. (F) Irritant response in control (Ctrl) and DKO$^{IVE}$ mice. The percent increase in ear thickness was measured 24 and 48 h after application of croton oil.

insulin/IGF signaling does directly impair wound induced neo-angiogenesis and granulation tissue formation.

## 4. Discussion

Here we show that loss of cell autonomous insulin/IGF-1 signaling in vascular endothelia, through inactivation of both insulin and IGF-1 receptors in these cells, inhibits the angiogenic response and affects the kinetics of granulation tissue formation upon wounding in mice. Perhaps surprisingly, these observed differences did not result in an obvious delay in wound closure. This may be explained by the fact that endothelial-specific impairment of insulin/IGF signaling and its associated reduction in angiogenesis is not sufficient to impair wound closure when all other parameters are normal. It

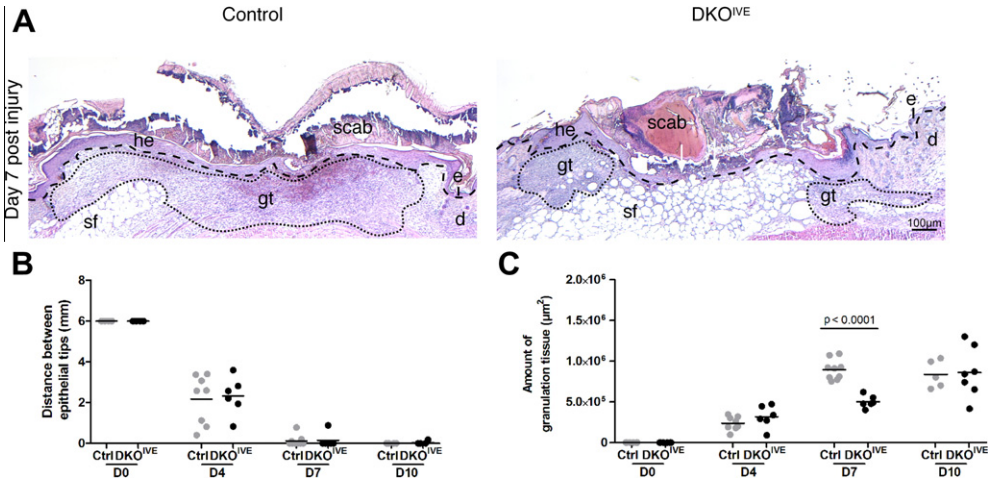


**Fig. 3.** Vascular IR/IGF-1R controls granulation tissue development. (A) H&E staining of wound sections in control and DKO$^{IVE}$ mice at day seven post injury. Whereas in control mice the wound is filled with a highly cellular and vascularized granulation tissue in DKO$^{IVE}$ mice only scarce granulation tissue has formed at the wound edges. (B) Morphometric analysis of wound tissue at different time points post injury: distance between the epithelial tips and amount of granulation tissue. Each dot represents one wound; three independent experiments were performed. Dotted line: outlines granulation tissue; hatched line: underlines hyperproliferative epithelial tongue; arrows: indicate tip of epithelial tongue; d, dermis; gt, granulation tissue; he, hyperproliferative epithelium; sf, subcutaneous fat tissue; bar = 100 µm.

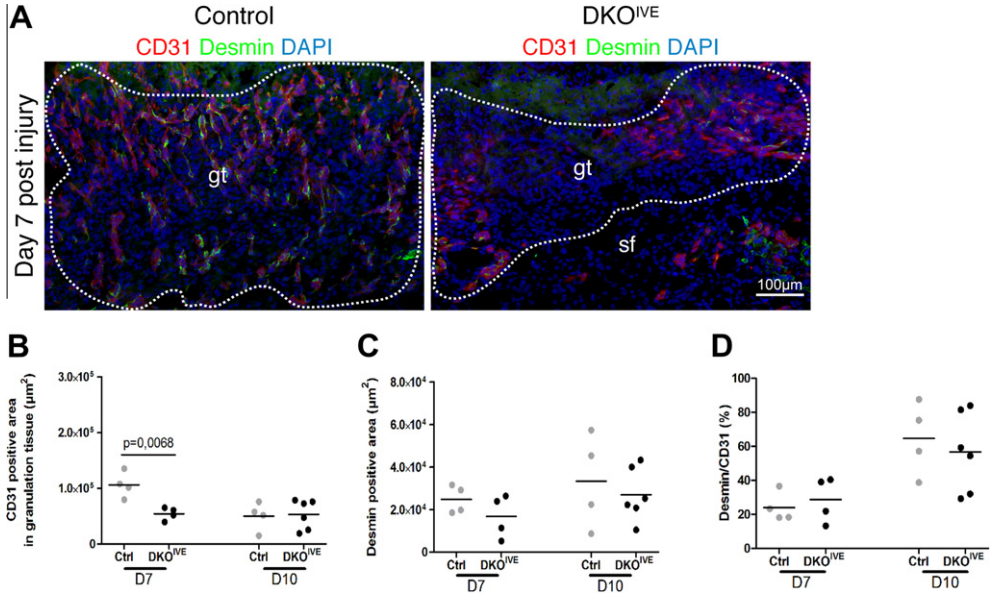


**Fig. 4.** Wound angiogenesis is attenuated in DKO$^{IVE}$ mice. (A) Immunofluorescence analysis for CD31 (red) and desmin (green) of day seven wound tissue in control and DKO mice. Nuclei were counterstained by DAPI (blue). Dotted line outlines granulation tissue. (B, C, D) Morphometric quantification of the area within the granulation tissue that stained positive for (B) CD31 and (C) desmin at indicated time points after injury. (D) Ratio of VE-cadherin positive vessels also positive for desmin; each dot represents one wound; gt, granulation tissue; sf, subcutaneous fat tissue, bar = 100 µm.

may also be possible that even though closure appears normal the reduction in angiogenesis may reduce the overall quality of tissue repair leading to increased susceptibility to subsequent damage. Indeed, diabetic foot ulcers usually develop in areas exposed to repeated external injury [3,4]. Moreover, these ulcers are not only associated with reduced angiogenesis but also with a range of other changes, such as e.g. altered growth factor production or impaired macrophage or keratinocyte function. It will therefore be of interest to assess the contribution of cell autonomous loss of insulin/IGF function to impaired healing in these and other skin cell types. It has also been shown previously in other mouse models that inhibition of angiogenesis and granulation tissue formation during the early stage of repair does not necessarily lead to delayed epithelial closure [29]. In the wounds inflicted in this study, closure is mostly likely driven by myofibroblast contraction and might not be so affected by the increase in hypoxia and/or reduced growth factors/cytokines as a result of the reduced neo-vasculature. Since endothelial dysfunction is already observed early in insulin resistance, our results nevertheless suggest a critical contribution of impaired endothelial insulin/IGF-1 signaling to impaired wound healing associated with type II diabetes when other conditions are no longer optimal.

Deletion of IR and IGF-1R in adult mice did not obviously impair vascular homeostasis in the skin as judged by histology, ECM localization and barrier function even after prolonged deletion (4 months, results not shown). Similarly, blood brain barrier function was also not impaired in mice with a constitutive deletion of either the IR or IGF-1R [27]. Although previous reports had impli-

cated insulin/IGF signaling in the regulation of tight junctional proteins [31,32] crucial for vascular barrier function, the Kondo data [27] together with our data indicate that endothelial insulin/IGF receptor signaling is not crucial to regulate vascular barrier function. In addition, no obvious changes in intensity and localization were observed for several vascular basement membrane proteins. This suggest that, at least in the skin, microvasculature impaired insulin/IGF-1 signaling is not directly responsible for the vascular basement membrane thickening observed in diabetes [24,25].

The impaired neo-angiogenesis in the skin is in agreement with previous results in mice with individual vascular deletion of either insulin or IGF-1 receptors. Hypoxic stress reduced retinal neovascularization in both mutant mice, although loss of IR reduced it more [21]. Together, these results show that vascular endothelial insulin/IGF-1 signaling cooperatively regulates neo-angiogenesis in different tissues upon different challenges, thus suggesting a general role for these receptors in the regulation of neo-angiogenesis. In conclusion, our results strongly suggest that in the skin endothelial insulin resistance can directly impair angiogenesis and therefore is likely to be an important contributing factor to impaired healing and the occurrence of diabetic foot ulcers.

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