Overexpressing IRS1 in Endothelial Cells Enhances Angioblast Differentiation and Wound Healing in Diabetes and Insulin Resistance

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Running Title: IRS1 overexpression and wound healing
Abstract

The effect of enhancing insulin’s actions in endothelial cells (EC) to improve angiogenesis and wound healing was studied in obesity and diabetes. Insulin Receptor Substrate 1 (IRS1) was overexpressed in EC using VE-cadherin promoter to create ECIRS1 TG mice, which elevated pAkt activation and expressions of vascular endothelial growth factor (VEGF), FLK1 and VE-cadherin in EC and granulation tissues (GT) of full thickness wounds. Open wound and epithelialization rates and angiogenesis significantly improved in normal mice and high fat (HF) diet induced diabetic mice with hyperinsulinemia in ECIRS1 TG vs. wild type (WT), but not in insulin deficient diabetic mice. Increased angioblasts and EC numbers in GT of ECIRS1 mice were due to proliferation in situ rather than uptake. GT in HF-fed diabetic mice exhibited parallel decreases in insulin and VEGF induced pAkt and EC numbers by >50% without changes in angioblasts vs. WT mice which were improved in ECIRS1 TG mice on NC or HF diet. Thus HF induced diabetes impaired angiogenesis by inhibiting insulin signaling in GT to decrease the differentiation of angioblasts to EC which was normalized by enhancing insulin’s action targeted to EC, a potential target to improve wound healing in diabetes and obesity.

Introduction

Every step of the complex process of wound healing has been reported to be
defective including impairments of neutrophil activation and responses, fibroblast migration
and proliferation, and angiogenesis (1-5). Poor glycemic control, neuropathy, presence of
micro- and macro-vascular complications and insulin resistance are associated with impaired
wound healing (6). Most strategies devised to improve chronic wound healing in diabetic
patients have not exhibited clear efficacy, possibly due to a lack of full understanding of the
mechanisms induced by diabetes to impair the wound healing process (5-8).

One major factor which contributes to impaired wound healing in diabetic and
insulin resistant states is reduced angiogenesis in the granulation tissue (GT) which could be
the result of decreased VEGF expression or its actions in response to hypoxia (4; 9; 10).

Multiple metabolic abnormalities can affect VEGF expression and actions including
hyperglycemia related oxidative stress, glycation products and activation of protein kinase C
(PKC) (10-13). Systemic insulin resistance could also affect angiogenesis since insulin’s
signaling can regulate VEGF expression which has been reported to be inhibited in diabetes
(14; 15). Thus, we postulate that insulin resistance may exist in the GT to impair angiogenesis,
by inhibiting insulin signaling to enhance VEGF expression and actions.

Insulin receptors are present in many cells of the GT, including: keratinocytes,
fibroblasts, endothelial cells and inflammatory cells (16-21). Mice with deletion of insulin
receptors in the fibroblasts and myocardium exhibited decreases in VEGF expression, and
capillary density in response to hypoxia (14). Insulin can induce VEGF expression mostly
through the IRS1/PI3K/Akt pathway which is selectively inhibited in insulin resistance and
diabetes (14; 15; 22-24). Activation of pAkt affected VEGF secretion in keratinocytes, and
angiogenesis in cutaneous wound healing (25-27). The finding of insulin resistance may
impair wound healing also suggests differential pathogenic mechanisms may exist for
defective wound healing associated with diabetes due to insulin deficiency or resistance with
hyperinsulinemia. This study investigated the regulation of insulin signaling pathway in GT
and on the differentiation of angioblasts to endothelial cells in the GT using rodent diabetic
models of insulin deficiency or hyperinsulinemia and insulin resistance.

Materials and Methods

Wild type (WT) C57/BL6J mice were purchased from Jackson Laboratory (Bar
Harbor, Maine). Recombinant human VEGF (R&D Systems, Minneapolis, MN) and
antibodies to pAkt, Akt, pErk, Erk1/2, Fibronectin, IRS1 (Cell signaling, Danvers, MA),
VCAM1 (Millipore, Billerica, MA), Flk1, eNOS (BD Biosciences, San Jose, CA) and,
β-Actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were obtained commercially.

Animals

All protocols for animal use and euthanasia were approved by the Animal Care
Committee of the Joslin Diabetes Center and are in accordance with NIH guidelines. Mice
with endothelial specific overexpression of IRS1 (ECIRS1 TG) with VE-cadherin promoter
were described previously (28) (Suppl. Fig. 1a). Following primers were used for IRS1
genotyping: 5’-ATCTGCAGGCAGCTCACAAAG-3’ and 5’-CGAAGAAGCGTTTGTGCA
TGC-3’. Two mice models of diabetes were used: 1) For hyperinsulinemia with insulin
resistance and hyperglycemia, male mice at age four weeks were fed with normal chow (NC),
(3.3% fat) or high fat (HF 60% fat) for ten weeks(28). Insulin deficient diabetic mice, was
produced by 5 consecutive days of intraperitoneal injection of 50 mg/kg streptozotocin (STZ)
(Sigma, St. Louis, MO) and fasting blood glucose concentrations of >400 mg/dl were
documented. Insulin treatment was performed using insulin pellet (LinShin Canada, Inc.,
Canada) placed subcutaneously and with a release rate of 0.1u/day/insulin which were
adjusted to maintain plasma glucose at 150mg/dL range (at steady state) in STZ-induced
diabetic mice for 2 weeks. Intraperitoneal glucose tolerance tests (IPGTT) were performed as
described (29).

Wound Healing Model

Mice dorsal hair was depilated on the day before surgery (Nair; Church & Dwight,
Co., Inc., Ewing, NJ), and anesthetized by isoflurane inhalation (Isoflurane, USP; Novaplus,
LakeForest, Ill.). Their dorsum was disinfected with 70% alcohol and marked with standard
square template (1.0 cm²). Skin plus panniculus carnosus was excised to create a
full-thickness dorsal excisional wound, which was covered with transparent, semi-occlusive
adhesive polyurethane dressings (Tegaderm; 3M, St. Paul, Minn) and changed 2 x per week
(30-32).
**Tissue morphometric analysis**

Digital macroscopic images of the wound were analyzed using NIH ImageJ software v1.40 (Bethesda, MD). Standardized photographs were taken on the day of surgery and 3rd, 7th, 10th and 14th days for 14 days, from a standard height. Re-epithelialization and open wound surface were measured as a percentage of the initial wound area as published (30-32).

Wound kinetics were quantified as follow: open wound rate (OWR) = open wound / initial area of wound size, contraction rate (CR) = contraction / initial area of wound size, and epithelialization rate (ER) = epithelialization / initial area of wound size – contraction (30; 31). On days 3, 7 and 10 post-surgery, wounds from 7-8 animals in each group were harvested as described (32).

For signaling studies, insulin (1U/kg body weight) was administrated intravenously and GT were collected 10 minutes afterward. For histological studies, excised tissues were fixed in 4% formalin at 4°C. Panoramic cross-sectional digital images of each wound were examined using FSX100 microscope (Olympus, Center Valley, CA).

**Immunofluorescence (IF) studies**

Immunohistochemistry studies were performed using paraffin sections, incubated with 0.5% BSA, and rabbit mAbs to CD31 (Dianova GmbH, Hamburg, CA) as primary antibody to identify EC, followed by Alexa Flour 488-conjugated secondary antibodies (Invitrogen, Gland Island, NY). Nuclei were counterstained with DAPI (Invitrogen).
Quantitative fluorescent analyses were performed using FSX100 microscope and Adobe Photoshop CS Software (San Jose, CA).

**Immunoblot and Immunoprecipitation Studies**

GT, frozen in dry ice, was homogenized in ice-cold tissue lysis buffer, as described (33). Protein concentration was determined by Bradford assay and processed as described (33).

**Real-time Polymerase Chain Reaction (RT-PCR)**

RT-PCR procedures were used to assess mRNA levels (Applied Biosystems, Grand Island, NY) and normalized to 36B4 or 18S rRNA. PCR primers used in the study are listed in Supplemental Table 1.

**Culture of endothelial cells:**

Lung tissues from c57/BL6J mice were used to obtain primary endothelial cells and used between passages 3-5 (33; 34). EC was incubated for 16 hours in DMEM containing 0.1% BSA and stimulated with 100nM insulin for 24 hours. Expression of VEGF165 in media was measured by ELISA kit (R&D Systems).

**Bone marrow cell transplantation**

Bone marrow (BM) cells from green fluorescent protein (GFP) expressing mice (Jackson Lab, Bar Harbor, Maine) were harvested from tibias and femurs by flushing with Dulbecco’s PBS+ 5% FBS, resuspended and filtered through a 70um cell strainer (35).
Erythrocytes were depleted using ACK lysis buffer (Lonza, Basel, Switzerland). BM cells were stained with lineage marker mix (Lin: Anti-CD3e (17-A2), Anti-CD4 (L3T4), anti-CD8 (53-6.72), anti-B220 (RA3-6B2), anti-TER-119, anti-Gr-1 (RB6-8C5), anti-Mac-1 (M1/70) from eBioscience, San Diego, CA). Flow cytometry sorted lineage negative cells were transplanted intravenously (2x10^6 cells / mouse) into congenic recipient mice and analyzed 7 days after transplantation (36).

**Evaluating proliferation in vivo**

At four and six days post wounding, bromodeoxy uridine (BrdU, 100ul) was injected intraperitoneally and BrdU in angioblasts or EC from blood and GT was detected by flow cytometry after 7 days (FITC BrdU Flow Kits, BD) (37).

**Flow Cytometry of cells in GT**

Dissected GT were incubated with collagenase I, II, DNAse and hyaluronidase in Hepes buffer for 30 minutes at 37°C, and then filtered through 70um cell strainer with erythrocytes depleted by ACK lysis buffer. Cells were incubated with insulin (100nM), anti-CD16/32 (BioLegend, San Diego, CA), fixed in 2% formaldehyde and permeabilized using the Perm/Wash buffer (BD Bioscience, San Diego, CA) followed by incubation with monoclonal antibodies: anti-CD45, for leukocyte, anti-CD31 for EC or anti-Flk1, marker for angioblast and PI for viability. Rabbit-anti-Akt and rabbit-anti-phospho-Akt (Ser473) were used to detect Akt activation, which were followed with Alexa Fluor ®647 goat anti-rabbit
IgG. All populations were routinely k-gated to verify purity and gating. One million cells from BM or 200,000 cells from GT were analyzed using FlowJo software version 10.0 (Tree Star, Inc., Ashland, OR).

Statistical Analysis

Values are expressed as mean and standard error (SE) of the mean. Student t tests were performed for comparison of two groups. One-way analysis of variance, followed with Tukey-Kramer was performed for comparisons of multiple groups using SPSS 22.0 (SPSS, Inc., Chicago, Ill.). A value of $p < 0.05$ was considered significant.

Results

Characterization of ECIRS1 transgenic mice and insulin signaling in GT.

To enhance insulin’s actions, IRS1 was overexpressed in the EC using VE-cadherin promoter to produce ECIRS1 TG mice which elevated IRS1 expression in the EC by 170% and in the retina, but not in PBMC (Fig. 1a & Suppl. Fig. 1b and c). Metabolically, WT and ECIRS1 TG mice did not differ by glucose tolerance as measured by IPGTT and fasting plasma insulin levels (Suppl. Fig. 1d and e).

Insulin increased VEGFB mRNA expression by 240% at 1 and 3 hours and protein levels in the media by 200% after 24 hrs in EC from ECIRS1 TG mice vs WT mice (Fig. 1b and c). IRS1 protein expression was increased in GT from ECIRS1 TG mice by 72% as compared to WT mice (Fig. 1d, $p<0.01$). Further, Akt phosphorylation (pAkt) in the GT of
ECIRS1 TG mice was significantly increased at basal (261%, p < 0.05) and after the addition of insulin (480% <0.01) compared to WT (Fig. 1e), without difference in insulin’s activation of Erk (p-Erk) (Fig. 1g). Expressions of eNOS, a marker of capillary density, in the GT also increased significantly by 133% comparing ECIRS1 TG to WT mice. (Fig. 1f)

**Insulin’s actions on wound healing in control and STZ-induced diabetic mice**

The effect of increasing IRS1 expression and insulin’s actions in EC on wound healing was evaluated by comparing OWR, CR, and ER in WT and ECRIS1 TG mice. The results indicated that OWR decreased more rapidly in ECRS1 TG mice vs WT mice with improvements at 3 and 7 days (p < 0.01) (fig. 2a – e). CR was significantly improved on day 14 and ER was increased in ECRIS1 TG mice by 96% and 48% on 3 and 7 days, respectively. VEGF mRNA and FLK1 protein expressions in the GT were also increased significantly on day 7 and VE-cadherin expression was increased on day 10. No changes in fibronectin were observed comparing ECIRS1 TG to WT mice (Figs. 3a-d).

STZ induced diabetic ECIRS1 TG and WT mice had comparable weights and fasting blood glucose of more than 500 mg/dl during the study (Suppl. Figs. 2a and b), with comparable plasma insulin levels of < 0.2 ng/ml which were 4 times lower than non-diabetic mice (Suppl. Fig. 2c). STZ diabetes delayed OWR, CR and ER significantly, compared to non-diabetic WT and ECIRS1 TG mice at the 3 and 7 days (Figs. 2a - e) although no differences were observed between diabetic STZWT and STZECIRSI TG mice. VEGF
mRNA expression in the GT after 7 days of wound initiation was decreased by 38% in STZ WT vs. WT mice (p< 0.05) and in STZECIRS1 vs. ECIRS1 mice (p< 0.01) (Suppl. Fig. 2d).

We also evaluated wound healing between STZWT mice treated with insulin (STZWTins) and STZECIRS1 mice treated with insulin (STZECIRS1ins). The results showed that OWR was decreased (p< 0.05), and ER was increased (p< 0.01) in STZECIRS1ins mice compared to STZWTins mice at 7 days after injecting (Suppl. Figs. 3a-d).

Assessment of HF diet induced diabetes and hyperinsulinemia on wound healing.

To determine whether diabetes and hyperinsulinemia affect wound healing, we studied HF feeding in ECIRS1 TG (ECIRS1HF) and WT (WTHF) mice. After 10 weeks of HF feeding, body weights were significantly increased in both WTHF and ECIRS1HF mice compared to NC. Fasting blood glucose levels were elevated equally in both WTHF and ECIRS1HF mice to above 200 mg/dl after of HF feeding (Suppl. Figs. 4a and b). IP-GTT were similarly elevated fasting and post–infusion glucose levels at 15 minutes >500 mg/dl (Suppl. Fig. 4c). Fasting plasma insulin levels increased significantly from 0.5 to 2.5 and 3.2ng/dl in WTHF and ECIRS1HF mice (Suppl. Fig 4d). Fasting plasma IGF1 level also increased in ECIRS1HF and WTHF mice (Suppl. Fig 4e). Interestingly, VEGF mRNA expressions were increased in WTHF vs WT mice (p< 0.05), and in ECIRS1HF vs ECIRS1 TG mice by 276% and to 341% respectively (Fig. 3e). Analysis of the wound showed HF feeding delayed OWR and ER at 3 and 7 days. Further, ECIRS1HF mice had significant
greater reduction in OWR, and increased ER than WTHF mice at 3 and 7 days (Figs. 4a - d).

Thickness of GT measured at seven days after surgery was greater in ECIRS1 TG mice vs. WT mice (p < 0.01). HF feeding decreased GT thickness in both WTHF and ECIRS1HF mice (p<0.05, Figs. 5 a and b). Immunohistological analysis of the GT showed the number of CD31 positive cells, a marker for EC and capillaries, were increased in ECIRS1 TG vs. WT mice (p < 0.01). HF feeding for 10 weeks decreased CD31\(^+\) cells by 84% in WTHF vs. WT mice (Figs. 5c and d), and decreased by 63% in ECIRS1HF vs ECIRS1 TG mice. However, a 290% elevation of CD31\(^+\) cell numbers was observed in ECIRS1HF vs. WTHF mice (p < 0.05, Figs. 5c and d).

Assessing HF diet induced insulin resistance and diabetes on angioblast and endothelial cell distributions in GT.

To characterize the potential mechanism for the reduction of EC in the GT associated with HF diet, the distribution of angioblasts and EC in the GT of WT and ECIRS1 TG mice on NC and HF were characterized. Angioblasts were identified by FLK1\(^+\), PI\(^-\), CD45\(^-\) and CD31\(^-\), and EC by CD45\(^-\), PI\(^-\) and CD31\(^+\) cell staining. Angioblast numbers increased significantly by 50% in ECIRS1 TG compared to WT mice and by 65% in ECIRS1HF vs WTHF mice (Figs. 6a and b). Interestingly, the elevations of angioblast levels were similar between NC and HF fed WT and ECIRS1 TG mice. EC numbers in GT increased by 95% in ECIRS1 TG vs. WT mice on NC. However, HF feeding decreased EC levels by 72% in
WTHF mice compared to WT mice (p < 0.01). Similarly, ECIRS1 HF fed mice also had 64% less EC than ECIRS1 TG mice (Figs. 6a and b). However, EC levels in the GT were significantly increased by 95% in ECIRS1 TG mice vs. WT mice (Fig. 6b). IRS1 overexpression in the EC only partially restored its reduction induced by HF feeding and diabetes with elevation of EC by 150% in ECIRS1 HF vs. WTHF mice (Fig. 6b).

DNA synthesis of EC and angioblast were measured by BrdU incorporation in GT and bone marrow. Figure 7a showed proliferation of angioblasts in the GT was increased by 47% in ECIRS1 TG vs. WT mice (p<0.05), but no differences in BrdU incorporation were noted in these cells in the bone marrow (Fig. 7b). To determine whether the elevation of angioblasts and EC in the GT of ECIRS1 TG mice could also be due to uptake from circulation rather than proliferation in situ, the uptake of circulating Lin- and GFP+ cells, which were negative for PI, B220, CD4, CD8, Ter119, CD11b and Ly6G cells and isolated from bone marrow of GFP+ mice, were infused into WT and ECIRS1 TG mice. Figs. 7c and d showed that no increases of GFP+ cells in the GT of ECIRS1 TG or WT mice were observed.

**Analysis of insulin’s signaling in isolated angioblasts and EC from granulation tissue.**

To support the findings that insulin actions could be inhibited by HF diet and positively enhanced by IRS1 overexpression in EC of ECIRS1 TG mice, IRS1 expressions and signal changes in pAkt were studied in angioblasts and EC isolate from the GT. The results showed that IRS1 expression was increased specifically in the EC of ECIRS1 TG
mice by 291% (p< 0.01), but not in the angioblasts (Figs. 8a and b). IRS1 expression was associated with the expression of VE-cadherin since its promoter was used in the transgene to make ECIRS1 TG mice (Suppl. Fig. 1a). VE-cadherin expressions were only observed in EC but not in angioblasts (Fig. 8c). The levels of pAkt stimulated by insulin (100 nM) were similar in angioblasts from TG and WT mice (Fig. 8d) as assessed by FACS using antibodies to pAkt. Similarly, HF feeding did not affect pAkt levels in angioblasts from WT or ECIRS1TG mice. Unlike angioblasts, insulin induced pAkt levels in EC from GT were significant higher by 196% in ECIRS1TG mice compared to WT mice (Fig. 8e). In contrast, insulin induced pAkt levels only increased by 127% in EC isolated from HF fed ECIRS1HF compared to WTHF mice (Fig. 8e). Unlike the angioblasts, the levels of pAkt were significantly lower by 50% in the EC of GT from ECIRS1 HF mice vs. from or WT mice on NC (Fig. 8e). Similarly, pAkt levels in the EC from ECIRS1 HF were decreased by 64% vs ECIRS1 TG mice. Nevertheless, insulin induced pAkt levels in EC from ECIRS1 HF mice were still significantly higher than those from WTHF mice by 107% (Fig. 8e). The levels of pAkt stimulated by VEGF (2.5ng/ml) in angioblasts from GT were significantly higher in WT mice compared to WTHF mice (Fig. 8f).

Discussion

This study demonstrated that enhancing insulin actions in the EC can improve wound healing in non-diabetic, diabetic and insulin resistant states when insulin is present. Further we have
identified a novel defect in the differentiation of angioblast to EC with a parallel reduction of insulin induced pAkt as a potential mechanism for the deficiency in angiogenesis in the GT induced by diabetes and insulin resistance.

Our study confirmed that diabetes whether due to insulin deficiency or insulin resistance by HF diet can impair both angiogenesis and the wound healing process (6; 38; 39). However, mechanisms causing the impaired wound healing process may be different when diabetes is the due to insulin deficiency or obesity and insulin resistance. In the insulin deficiency model, enhancement of insulin action in the ECs did not have any beneficial effects on wound healing which support the conclusion that the improvement in ECIRS1 TG mice was likely due to enhancing of insulin’s actions in the EC of the granulation tissues. Evaluation of the GT in ECIRS1 TG mice showed a sequential elevation of VEGF, FLK1 and VE-cadherin expressions after the initial injury compared to WT mice. These findings strongly supported the idea that improving insulin’s actions in EC enhanced VEGF expression and its signaling cascades to improve angiogenesis and wound healing. It is also possible that insulin may have perivascular effects due to its specific action on the ECs, such as the activation eNOS, to elevate NO and blood flow from the increases in angiogenesis to enrich the cells of the GT and improve wound healing (20; 33; 34; 40). Further, increased blood flow to GT has been reported to improve the influx of inflammatory cells which can enhance angiogenesis (21). Clearly, the elevated VEGF expression in the GT observed in ECIRS1 TG mice is likely due
to the enhancement of insulin signaling through the IRS1/PI3K/Akt pathway, which has been shown to regulate VEGF expression in EC (14; 40; 41).

The reduced in VEGF expression in the GT from STZ induced diabetic mice, which has been reported, is likely due to insulin deficiency (3; 6; 42). This conclusion is supported by the findings of the paradoxical increases of VEGF expression in GT from mice with HF induced diabetes, which exhibited hyperinsulinemia rather than insulin deficiency. Hyperinsulinemia may induce VEGF not only in ECs, but from fibroblasts and inflammatory cells (14; 42). The paradoxical findings of VEGF expression in GT indicate that the impaired angiogenesis in wound healing may have different pathogenic mechanisms for insulin resistant and deficient induced diabetes. This study provided the first direct comparative analysis of GT and wound healing between insulin deficient and hyperinsulinemic and insulin resistant models of diabetes. A previous report showed that VEGF expression in whole wound from HF fed mice was similar to control diet, but higher than ob/ob diabetic mice (39).

Interestingly, HF induced obesity clearly caused defects in GT formation and angiogenesis with decreased capillary density, even in the presence of hyperinsulinemia, and elevated VEGF levels suggesting the possibility of resistance to insulin or VEGF actions in the GT of WTHF or ECIRS1HF mice. Analysis of GT cells indicated that enhancing EC’s insulin action increased both angioblasts and ECs. Interestingly, diabetes and insulin resistance inhibited only EC and not angioblasts in the GT numbers. This provided the first identification that
defect in capillary formation in GT induced by diabetes or insulin resistance is partly due to a
selective inhibition in the differentiation of angioblasts to ECs. Further, the reduction of ECs
could only be partially normalized by improving insulin’s signaling in the ECs, even though
VEGF levels were elevated in HF conditions. Our results also provide evidence that insulin
can have actions on angioblasts as shown by increasing DNA synthesis of angioblasts in the
GT of ECIRS1 TG mice may not be inhibited by obesity or diabetes since angioblast numbers
were not changed in these states. The effect of insulin to increase angioblasts and ECs in the
GT was the results of elevating in situ cellular proliferation since there were no increases in
the uptake from infused GFP labeled angioblasts or EC in WT or ECIRS1 TG mice.
Signaling studies indicated that HF diet induced selective inhibition of insulin induced pAkt
in the ECs but not in the angioblasts which is consistent with the finding that HF diet did not
decrease the number of angioblasts but only the ECs. These findings confirmed the idea that
diabetes and insulin resistance caused a selective defect in the differentiation of angioblasts to
EC. By using VE-cadherin promotor, IRS1 overexpression is limited to EC and not in
angioblasts which do not express VE-cadherin (43). This approach may not enhance
angioblasts differentiation to EC which appears to be abnormal also in diabetes. This study
suggests that angioblasts are sensitive to insulin. Previously, Schatteman et al., reported that
circulating angioblasts responded initially to insulin at 6-12 µg/ml but they become resistant
after prolonged exposure, which the authors suggested is the reason the hyperinsulinemia of
type 2 diabetes could be causing poor angiogenesis in wound healing. However, the level of
insulin used by Schattaman et al., was \( \approx 1\)uM, which is much higher than physiological levels
(<10nM), which makes the finding difficult to interpret for in vivo studies (44).

These studies provide documentation that diabetes caused by insulin deficiency or diet
induced obesity can induce a selective abnormality in the differentiation of angioblasts to ECs
in the GT which is related to resistance of pAkt activation induced by insulin and possibly
VEGF. Detailed studies to understand the mechanisms that are causing the inhibition of
insulin’s or VEGF activation of pAkt in the GT in diabetes will need to be performed in the
future. Further, we also observed that enhancing insulin action specifically in the ECs through
the IRS1/PI3K/pAkt pathway could improve wound healing both in non-diabetic and
obesity-induced diabetes, suggesting a new therapeutic target for wound healing.

Author contributions

S.K. performed most of the experiments and wrote the 1st draft of the manuscript.
K.P., Y.M., M.K., Q.L. and H.Y. assisted in some studies and reviewed the manuscript. L.L.
and D.P.O. provided expertise on the wound healing model. T. N. and A.W. provided advice
on the analysis of the angioblasts and EC from GT and BM. A.M. made the ECIRS1 TG
mouse. G.L.K. supervised all the studies and the writing of the manuscript. We want to thank
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Figure legends

Figure 1: Characterization of insulin signaling and VEGF expression in lung EC and GT of WT and ECIRS1 TG mice. (a) IRS1 protein expression in lung ECs, (b) VEGF mRNA expressions in cultured ECs from WT and ECIRS1 TG mice with insulin stimulation (100nM) at 1, 3 and 8 hours (c) secreted VEGF protein levels in the media of cultured ECs from WT and ECIRS1 TG mice with insulin stimulation (100nM) at 0, 12 and 24 hours (d) IRS1 protein expressions in granulation tissue in WT and ECIRS1 TG mice at 7 days post-surgery. (e) Total Akt and pAkt expressions at 7 days in granulation tissue (f) eNOS expression in granulation tissue (g) Total Erk and pErk expressions in granulation tissue *: p<0.05, **: p<0.01, (n=4 in Fig. 1a, n=5 in Fig. 1b – g).

Figure 2: Comparison of wound healing among WT, ECIRS1 TG, STZWT and STZECIRS1 TG mice (a) Photographs of wound at 0, 3, 7, 10 and 14 days in WT and ECIRS1 TG mice. Black bar = 1 cm. (b) in STZWT and STZECIRS1 mice; (c) Open wound rate, (d) Contraction rate and (e) Epithelialization rate (n=5) **: p<0.01.

Figure 3: Analysis of genes for vessels in granulation tissue in (a) VEGF mRNA expressions; (b) Ve-cadherin mRNA expressions; (c) Flk1 expression; (d) fibronectin mRNA at 3, 7 and 10 days post-surgery in the granulation tissue from WT and ECIRS1 TG mice (n=7 at each time point); (e) VEGF mRNA levels in the granulation tissues from WT, ECIRS1, WTHF and ECIRS1HF mice at 7 days post-surgery *: p<0.05, **: p<0.01 (n=7)
Figure 4: Comparison of wound healing among WT, ECIRS1, WTHF and ECIRS1HF mice

*: p< 0.05, **: p<0.01 (a) Photographs of wound at 0, 3, 7, 10 and 14 days after surgery. Black bar = 1 cm. (b) Open wound rate (%). (c) Contraction rate (%). (d) Epithelialization rate (%) (n=5 for each type of mice and at each time point).

Figure 5: Histological analysis of the wound 7 days post-surgery in WT, ECIRS1, WTHF and ECIRS1HF mice. *
*: p< 0.05, **: p<0.01 (a) HE stained photograph. Black bar = 1 cm. G: granulation tissue, E: epidermis, D: dermis, F: fatty tissue (b) Thickness of granulation tissue. (n=5) (c) IHC of granulation tissue. Blue: DAPI. Green: CD31. (d) Percentage of CD31+ cells isolated from GT (n=5).

Figure 6: Characterization of angioblasts and ECs in granulation tissue by flow cytometry at 7 days post-surgery in WT, ECIRS1, WTHF and ECIRS1HF mice. (a) angioblasts: PI(-)CD45(-)Flk1(+)CD31(-), ECs: PI(-)CD45(-)Flk1(-/+CD31(+). (b) Percentage of angioblasts and ECs in PI(-)CD45(-) cells. n=5 *: p< 0.05, **: p<0.01

Figure 7: Analysis of angioblasts and ECs proliferation and uptake in granulation tissue (GT) 7 days after surgery in WT and ECIRS1 TG mice. *
*: p< 0.05 (a) BrdU positive cells in bone marrow (BM). (n=7) (b) Percentage of angioblasts and ECs in PI(-)CD45(-) cells in BM. (c) Flow cytometry analysis of uptake of lineage negative cells in granulation tissue. (n=6), (d) GFP positive cells (%) in granulation tissue and blood at 7 days after cells transplantation from GFP mice to WT and ECIRS1 TG mice.
Figure 8: Expression and insulin/VEGF activation of signaling molecules in angioblasts and endothelial cells in granulation tissue from WT, ECIRS1, WTHF and ECIRS1HF mice. (a) IRS1 mRNA expression in angioblasts. (b) IRS1 mRNA expression in ECs of WT and ECIRS1 TG mice. (c) Ve-cadherin mRNA expression in angioblasts and ECs; (d) analysis for insulin induced (100nM) Akt phosphorylation in angioblasts and (e) ECs (f) analysis for VEGF induced (2.5ng/ml) Akt phosphorylation in angioblasts by using flow cytometry measuring the mean of the peak of fluorescent intensity. *: p< 0.05, **: p<0.01 (n=5).
**Fig 1**

(a) IRS1 and β actin protein expression in endothelial cells. IRS1/actin (fold of WT)

(b) VEGF mRNA expression in endothelial cells

(c) Secreted VEGF levels (ng/mL) over time (0h, 12h, 24h)

(d) IRS1 expressions in granulation tissue

(e) Akt Phosphorylation in granulation tissue

(f) eNOS expressions in granulation tissue

(g) Erk phosphorylation in granulation tissue

*+: with insulin
-: without insulin

WT ECIRS1
Fig. 2 (a) Comparison of wound healing between WT and ECIRS1 mice at different time points.

(b) Comparison of wound healing between STZWT and STZECIRS1 mice at different time points.

(c) Open wound rate (%)

(d) Contraction rate (%)

(e) Epithelialization rate (%)

Diabetes

WT

ECIRS1

STZWT

STZECIRS1

Normalized open wound area (% of initial wound area)

Contraction rate (% of initial wound)

Epithelialization rate (% of total wound area)
(a) VEGF mRNA expressions

(b) Vc-cadherin mRNA expressions

(c) Fibronectin mRNA expressions

(d) Flk1 protein expressions

(e) VEGF mRNA expressions at 7 days
Fig. 4 (a) Open wound rate

(b) Contraction rate

(c) Epithelialization rate
(a) Granulation tissue (HE staining)

![Granulation tissue (HE staining)](image)

(b) Thickness of granulation tissue (mm)

![Thickness of granulation tissue](image)

(c) IHC (green: CD31, blue:Dapi)

![IHC (green: CD31, blue:Dapi)](image)

(d) Percentage of CD31 (+) cells

![Percentage of CD31 (+) cells](image)
(a) Flow cytometry in granulation tissue

(b) Percentage of angioblasts/endothelial cells in PI(-)CD45(-) cells from granulation tissue
(a) BrdU positive cells in granulation tissue (GT) and bone marrow (BM)

(b) Percentage of angioblasts/endothelial cells in PI(-)CD45(-) cells from bone marrow

(c) Flow cytometry after transplantation of Lineage(-) cells from GFP positive mice to WT/ECIRS1 mice

(d) GFP positive cells (%)
Fig. 8

(a) IRS expression in angioblasts

(b) IRS1 expression in ECs

(c) Ve-cadherin

(d) p-Akt in angioblasts (stimulated with insulin)

(e) p-Akt in endothelial cells (stimulated with insulin)

(f) p-AKT in angioblasts (stimulated with VEGF)
| Gene       | Sense (5’-3’)       | Anti-sense (5’-3’)            |
|------------|---------------------|-------------------------------|
| IRS1       | TCTTCTGTTACACCTCAAGGA | GAGTAGGTGCTGAGAAAGG           |
| vegf       | CTCGCAGTCCGAGCCGGGA  | GGTGCAGCCTGGGACCACCTTG        |
| ve-cadgerin| CAGCAACTTCACCCTCATAAC | TCCCGATTAACCTGCCCATAC           |
| fibronectin| CTTTGGCAGTGTCATTTCAG  | ATTCCTCCCTTTCCATTTCCG          |
| 36B4       | GCTCCAAGCAGATGCAGCA  | CCGGATGTGAGGCAGCACG           |
| 18s        | GTAACCCGTTGAACCCCATT | CCATCCAATCGGTAGTAGCG           |
Supplemental Figure 1: Characterization of WT and ECIRS1 TG mice (a) construct of promoter VE-cadherin –IRS1 (DNA) (b) IRS1 protein in retina (c) IRS1 protein in PBMC (d) IP-GTT in WT and ECIRS1 mice (e) Fasting plasma insulin concentration in WT and ECIRS1 mice
Supplemental Figure 2: Physiologic studies in STZWT and STZECIRS1 mice. (a) Body weights at 4 and 8 weeks in mice (b) Fasting blood glucose at 8 weeks in mice (c) Fasting plasma insulin concentration between among WT, ECIRS1, STZWT and STZECIRS1 mice (n=5) **: p< 0.01 (d) VEGF mRNA expression in GT at 7 days from WT, ECIRS1, STZWT and STZECIRS1 mice (n=7, *:p < 0.05, **: p< 0.01)
Supplemental Figure 3: Comparison of wound healing among STZWT, STZECIRS1, STZWTins and STZECIRS1ins mice *: p< 0.05, **: p<0.01 (a) Photographs of wound at 0, 3, 7, 10 and 14 days after surgery. Black bar = 1 cm. (b) Open wound rate (%). (c) Contraction rate (%). (d) Epithelialization rate (%) (n=5 for each type of mice and at each time point).
Supplemental Figure 4: Physiological studies in WT, ECIRS1, WTHF and ECIRS1HF mice. (n=5) **: p<0.01 (a) Body weights at 8 and 14 weeks in mice. (b) Fasting blood glucose at 8 and 14 weeks in WTHF and ECIRS1HF mice. (c) Blood glucose levels in IP-GTT at 14 weeks in WT, ECIRS1, WTHF and ECIRS1HF mice (d) Fasting plasma insulin concentrations. (e) Plasma IGF1 concentration (n=5).