Original Article Clinical application of a FOXO1 inhibitor improves connective tissue healing in a diabetic minipig model

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Abstract: The forkhead box O1 (FOXO1) transcription factor plays a key role in wound healing process. Recently it has been reported that lineage-specific genetic ablation of FOXO1 significantly improves diabetic wound healing in a mouse model. To investigate the clinical usefulness of these findings, translational preclinical studies with a large animal model are needed. We report for the first time that the local application of a FOXO1 inhibitor (AS1842856) significantly improves connective tissue healing in a preclinical T2DM minipig model, reflected by increased collagen matrix formation, increased myofibroblast numbers, improved angiogenesis, and a shift in cell populations from pro-inflammatory (IL-1 β ⁺, TNF- α ⁺ and iNOS⁺) to pro-healing (CD163⁺). Our results set up the basis for the clinical application of a FOXO1 antagonist in early diabetic wounds where there is impaired connective tissue healing.

Keywords: Wound healing, forkhead box 01 (FOX01), diabetes, hyperglycemia, minipig, inflammation skin

Introduction

According to the 2020 National Diabetes Statistics Report, 34.2 million people, 10.5% of the US population had diabetes in 2018 and the total estimated cost of diagnosed diabetes in the US in 2017 was \$327 billion [1]. One of the biggest diabetes-related complications is the delayed wound healing which increases the risk of infections and other serious complications [2]. The initial cause of impaired healing is due to complex factors such as an altered host response, increased and prolonged inflammation, a reduced rate of re-epithelialization, and a failure to form sufficient extracellular matrix [2]. Transcription factors coordinate the complex series of events needed for wound healing. The forkhead box O1 (FOXO1) transcription factor plays a key role in activating keratinocytes to participate in the wound healing process.

Lineage-specific FOXO1 deletion in keratinocytes interferes with keratinocyte migration, angiogenesis, and connective tissue formation in normal skin and mucosal wounds [3, 4] whereas FOXO1 haploinsufficiency accelerates the healing at normal skin wounds [5]. Interestingly, FOXO1 deletion in keratinocytes in diabetic wounds has the opposite effect, significantly improving the healing response [4, 6]. Despite the important roles of FOXO1 in the treatment of diabetic wounds, translational preclinical trials using a large animal model have not been carried out. Furthermore, no studies on the effect of FOXO1 in a T2DM model have been performed.

The minipig is frequently used as a preclinical large animal model for cutaneous wound healing and diabetes research [7, 8]. In both porcine and human skin, the relative thickness of the

epidermis and dermis, the epidermis' turnover time, and the immune cells in the skin are similar and the porcine wound healing model closely approximates the healing process in humans. In contrast to humans, rodents have a thin epidermis and a higher density of epidermal appendages and their healing occurs largely by contraction [9]. Furthermore, T2DM minipig models show similar metabolic abnormalities to humans including hyperglycemia, hyperlipidemia, insulin resistance, and a pro-inflammatory state [8]. In this study we demonstrate for the first time that the local application of a FOX01 inhibitor significantly improved several parameters of connective tissue healing in a preclinical T2DM female minipig model, reflected by increased collagen formation, increased myofibroblast numbers, and improved angiogenesis. Mechanistically, FOXO1 inhibitor treatment reversed an anti-wound healing, proinflammatory environment as shown by reduced numbers of cells that express IL-1 β , TNF- α , and iNOS and a shift to increased numbers of CD163⁺ cells.

Materials and methods

T2DM minipig model

The following study was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC #: #17PCDI-001). Ten female Göttingen minipigs (3.0±0.6 years of age) were acquired and allowed to acclimate for 7 days. To induce T2DM the pigs were fed (2 times/day) a high-fat diet and subsequent low-dosage administration of streptozotocin (STZ) as previously described [10]. The cafeteria diet consisted of highly saturated hydrogenated fats, cholesterol, and sugar while the control pigs were fed with a "normal" low-fat standard diet (MH500, Test Diet, NJ, USA). The cohort of minipigs was randomly assigned to the DM group (n=5) and injected with a streptozotocin (STZ; Enzo Life Sciences, Raamsdonksveer, Netherlands) (20 mg/kg in 0.1-mol/L sodium citrate, pH 4.5) to induce the diabetic condition [10]. The DM group minipigs were treated with 25 g of glucose during STZ treatment to prevent hypoglycemia. Animal weight (initial weight, 8 weeks, 14 weeks and subsequently every 2 weeks until termination), complete blood count (CBC), and HOMA2-IR value (calculated using fasting glucose and fasting insulin by HOMA calculator) were recorded. Fasting glucose was monitored weekly using Glucomen LX (A. Menarini Diagnostics, Berlin, Germany). The plasma cortisol and insulin levels were evaluated with the Porcine Insulin ELISA kit (Mercodia, Uppsala, Sweden) and Radioimmunoassay Coat-a-Count Cortisol (Siemens Healthcare Diagnostics, Los Angeles, CA). Blood assays were performed 6 weeks after STZ treatment.

Porcine skin wounding procedure

Skin wound procedures were performed as previously described [11]. Under general anesthesia, the full-thickness dorsal skin wounds were bilaterally approximately 3 cm from the midline starting at the level of the first thoracic vertebra (T1) using a 1-cm punch (depth: 1.2-cm, Acuderm inc., Fort Lauderdale, FL). FOXO1 inhibitor (AS1842856) was purchased from EMD Millipore (Billerica, Massachusetts) [12]. 10 mg of FOX01 inhibitor was dissolved in 1.5 ml DMS0 and diluted 100x in sterile PBS (2.0 mM) just prior to injection and the control was 1% DMSO in PBS. FOX01 inhibitor or vehicle control was injected at six sites around each wound with 10 µl per injection at the time of wounding and every other day. The last injection was performed 2 days before euthanization. Injections were performed at the level of the dermis (three on the upper half and another three on the lower half of the wound). One operator was responsible for all injections and was not blinded to the procedure as it was a split side design. On day 8 and 15 after surgery tissue was collected at the time of euthanasia with the use of a 3 cm biopsy punch to take the wound site and surrounding tissue.

Histology

Specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h. Wounds were bisected at the center of the wound with the use of a dissecting microscope and embedded in paraffin. Five μ m paraffin sections were stained with H&E or Masson's trichrome in one batch and histomorphometric analysis was performed with NIS-elements image analysis software (Nikon, Melville, NY) at the center/edges of each lesion. Total 30-40

Table 1. T2DM minipig models

		Normal	Diabetes	P value
Weight (kg)		31.9±0.9	73.6±3.4	< 0.001
Glucose (mg/dL)		70.4±3.0	153.8±13.9	0.001
Cortisol (ug/dL)		5.41±1.3	11.1±1.6	0.01
Insulin (uIU/mL)		5.41±1.3	9.2±1.2	0.03
HOMA2-IR	% B	120±15.6	49±12.2	0.006
	% S	182.6±33.4	72.4±17.2	0.025
	IR	0.67±0.2	1.6±0.2	0.0008

images were taken with a 20× objective from the middle (10) and the two edges of the wound (10-15 per each) and combined using the Image Composite Editor (Microsoft, Redmond, WA). Images were examined by a double-blinded examiner and the results were confirmed with a second examiner.

Immunohistochemistry in histological sections

Immunofluorescence was performed as previously described [11] with the following primary antibodies: α-SMA (ab21027; Abcam, Cambridge, MA), CD31 (ab28364; Abcam), TNF-α (ab6671; Abcam), IL-1β (sc-7884; Santa Cruz, Dallas, TX), FOXO1 (ab70382; Abcam), iNOS (ABIN 615202, antibodies-online Inc., Limerick, PA) and CD163 (ABIN 2478731; antibodiesonline Inc.). Image analysis was performed using NIS-Element software (Nikon). The number of immunopositive cells divided by the area was compared among different groups for each measured antibody. Blood vessels were described as small or moderate vessels depending on the number of endothelial cells associated with the vessel as previously described [13]. Images were examined by a double-blinded examiner and the results were confirmed with a second examiner.

Statistics

Statistical analysis with multiple groups was performed using ANOVA with Scheffe's posthoc test or non-parametric Kruskal-Wallis test with Dunn test. Statistical analysis between diabetic vehicle and diabetic FOXO1 inhibitor groups was performed using a 2-tailed paired t-test or non-parametric Wilcoxon matched-pairs signed-rank test. Results were expressed as the mean \pm SEM. P < 0.05 was considered statistically significant.

Results

Establishment of T2DM minipig models

Eight months of full high-fat high-energy feeding and subsequent STZ injection resulted in an increase in body weight of 230% compared to control normal minipigs with the abnormalities in glucose tolerance and insulin sensitivity, characterized by hyperglycemia, compensatory hyperinsulinemia, and increased Homeostatic Model

Assessment 2 of Insulin Resistance (HOMA2-IR) value (P < 0.05, **Table 1**) [10].

FOXO1 inhibitor increases the collagen formation and the number of myofibroblasts in diabetic minipig wounds but does not have a significant effect on epithelial healing

Histologic examination of the H&E and Masson's trichrome-stained tissue sections showed open wounds at a mid-stage of healing with granulation tissue in the center of the wound and more mature healing at the wound edges. The edges and center of the wounds were examined separately. On day 8 we found that the epithelial gap and mean epithelial thickness were similar in both diabetic vehicle and FOXO1 inhibitor groups (P > 0.05, Figure 1A-C). Therefore, we focused on the connective tissue healing next. The production of collagen assessed with Masson's trichrome stain was decreased by 28-60% in diabetic wounds compared to wounds in normoglycemic minipigs. Local application of a FOXO1 inhibitor reversed the effect of T2DM, increasing the amount of collagen by 150-230% (P < 0.05, Figure 1D, 1E).

To probe the potential mechanisms, we examined the formation of myofibroblasts, which are crucial for extracellular matrix production and wound maturation [14]. Diabetes reduced the number of myofibroblasts by 43% compared with normal wounds. This reduction was rescued by treatment with FOXO1 inhibitor (P < 0.05, **Figure 2A-C**) so that the number of myofibroblasts rose to normal levels. In addition, we examined the wound samples at day 15 and found both FOXO1 inhibitor- and vehicle-treated group showed the complete re-epithelialization and similar collagen percentage at day 15 (data not shown).



Figure 1. Local application of a FOXO1 inhibitor improves connective tissue by not epithelial healing in a preclinical T2DM minipig model. A. H&E stained sections (2×), bar =1000 μ m. B. Epithelial gap (μ m). C. Mean epithelial thickness (μ m). D, E. Quantitative analyses of newly formed collagen at the wound edges and center measured in Masson's trichrome stained sections. Each *in vivo* value is the mean ± SEM for n=5 minipigs per group. *, P < 0.05 versus diabetic vehicle group; *, P < 0.05 versus normal vehicle group. Bar, 50 μ m.

FOXO1 inhibitor improves the wound angiogenesis in diabetic minipig wounds

We examined the wound angiogenesis using an endothelial cell marker, CD31, which is expressed in immature microvascular endothelial cells as well as mature endothelial cells in small to moderate-sized vessels [13]. Diabetes reduced the number of CD31-immunopositive single cells and moderate-sized vessels by 34-54% compared with normal wounds, which was rescued by 157-198% by FOXO1 inhibitor at the wound edge (P < 0.05, **Figure 3A-C**). We did not observe the statistically significant difference in small-sized vessels and wound centers (P > 0.05, data not shown).

FOXO1 inhibitor causes a shift in cell populations

Since inflammation has been shown to impair diabetic wound healing [15], we examined IL-1 β and TNF- α levels in diabetic wounds. A patholo-

gist (FA, co-author) determined that the cell types which express IL-1 β and TNF- α were largely leukocytes, endothelial cells, and fibroblastic cells as supported by other studies [16]. Immunopositive cells were counted at both the wound edges and wound center. IL-1ß immunopositive cells increased by ~10 fold and TNF- α levels increased by 4-22 fold in diabetic wounds compared with normoglycemic wounds (P < 0.05, Figure 4A-F). Treatment with a FOXO1 inhibitor rescued the high numbers of IL-1B+ and TNF- α^+ caused by diabetes (P < 0.05. Figure 4A-F). We also investigated inducible nitric oxide synthase (iNOS) and CD163 expressing cells. The number of iNOS⁺ cells increased by 5-11 folds in diabetic wounds compared with normoglycemic wounds, whereas FOXO1 inhibition blocked this increase (P < 0.05, Figure 4G). In contrast, the number of CD163⁺ cells, positively associated with enhanced wound healing, decreased by 76-83% in diabetic wounds compared with normoglycemic



Figure 2. Local application of a FOXO1 inhibitor significantly improves myofibroblast numbers in a preclinical T2DM minipig model. A-C. α -SMA immunofluorescence analyses (40×). EPI, epithelium; CT, connective tissue. Each *in vivo* value is the mean ± SEM for n=5 minipigs per group. *, P < 0.05 versus diabetic vehicle group; +, P < 0.05 versus normal vehicle group. Bar, 50 μ m.

wounds, and treatment of diabetic wounds with a FOXO1 inhibitor reversed this decline, increasing CD163⁺ cells by 4-5 fold in diabetic minipigs (P < 0.05, Figure 4H).

Discussion

Data presented here demonstrate that the application of a FOXO1 inhibitor improves connective tissue healing in T2DM minipig dermal wounds. The improved healing was reflected by greater production of connective tissue matrix, enhanced myofibroblast formation, and greater angiogenesis in diabetic wounds. Mechanistic studies indicate that the FOXO1 reduced the formation of a pro-inflammatory environment that is known to interfere with the healing process in diabetic wounds [15]. These studies were carried out in a large animal minipig model, which is needed for a pre-clinical study to expand the validity and translational applicability [17]. Studies in pigs show a much higher concordance with human studies (78%) compared with small laboratory animals (53%) and in vitro results (57%) [18]. In addition, results presented here show for the first time that FOXO1 inhibition can improve connective tissue healing in type-2 diabetic wounds, demonstrating its translational value.

Several aspects of wound healing were improved by FOXO1 inhibition in the type-2 diabetic minipigs. This is reflected by greater production of connective tissue matrix, enhanced myofibroblast formation, and greater angiogenesis in diabetic wounds. A distinctive feature of diabetic wound healing is increased and prolonged inflammation [19]. Pro-inflammatory mediators such as TNF- α and IL-1 β and enzymes that produce reactive oxygen species are increased in diabetes and contribute to diabetic complications, including deficient wound angiogenesis and prolonged inflammation [19-21]. Increased levels of TNF- α may limit the



Figure 3. Local application of a FOXO1 inhibitor significantly improves wound angiogenesis. A. CD31 immunofluorescence (40×). IgG control was negative (data not shown). B, C. Quantitative analyses of CD31 immuno-positive single cells and moderate-sized vessels at the wound edge. EPI, epithelium; CT, connective tissue. Each *in vivo* value is the mean \pm SEM for n=5 minipigs per group. *, P < 0.05 versus diabetic vehicle group; ⁺, P < 0.05 versus normal vehicle group. Bar, 50 µm.

capacity of diabetics to downregulate other inflammatory genes [15, 18, 22]. In this study, we found that FOXO1 inhibition reduced the number of cells that expressed IL-1 β , TNF- α ,

and iNOS. This is important since reducing prolonged inflammation improves the healing of wounds in T2DM animals [15, 22] and consistent with reports that FOXO1 promotes IL-1 β





Figure 4. Local application of a FOXO1 inhibitor reduces the number of inflammatory cells and increases the number of CD163⁺ cells in diabetic wounds. A. IL-1 β immunofluorescence (40×). B, C. Quantitative analyses of IL-1 β immunopositive cells at the wound edge and center area. D. TNF- α immunofluorescence (40×). E, F. Quantitative analyses of TNF- α immunopositive cells at wound edge and center area. G, H. Quantitative analyses of iNOS and CD163 immunopositive cells at wound edge. EPI, epithelium; CT, connective tissue. Each *in vivo* value is the mean ± SEM for n=5 minipigs per group. *, P < 0.05 versus diabetic vehicle group; *, P < 0.05 versus normal vehicle group. Bar, 50 µm.

production [23]. Moreover, previous studies support the effect of the FOXO1 inhibition in diabetic wounds, consistent with our results. IL-6 is an important proinflammatory cytokine during wound healing and its dysregulation is a hallmark of diabetes [24, 25]. FOXO1 activation triggered by TNF-α has been reported to increase the expression of IL-6 through direct binding of FOXO1 to its promoter, while FOXO1 knockdown inhibited IL-6 expression [26]. In addition, high glucose increases FOXO1 interactions with consensus FOXO1 response elements in CCL20, IL-36y, MMP9, and SERPINB2 promoters, which increase transcription in a FOX01-dependent manner [4, 6, 27]. High levels of CCL20, IL-36y, MMP9, and SERPINB2 in diabetic conditions interfere with keratinocyte migration, which leads to the impaired wound healing. Given that high glucose stimulates FOXO1 binding to promoter regions of proinflammatory genes [3, 4, 6], FOXO1 may be a key factor that leads to greater inflammation under hyperglycemic conditions. We also examined CD163, which is associated with a prowound healing environment [28]. Diabetes reduced CD163⁺ cells and the FOXO1 inhibitor largely reversed this effect of diabetes. The improved wound healing environment is also reflected by the increased angiogenesis, which was improved by the application of a FOXO1 inhibitor. The angiogenic balance is modulated by multiple factors, such as hyperglycemiainduced oxidative stress, cytokines, and inflammatory factors [29]. Lim et al. reported that the inflammation plays a major role in diabetesimpaired angiogenesis in fracture healing through its effect on microvascular endothelial cells through a FOXO1 dependent mechanism [13]. High levels of TNF- α , high glucose, and advanced glycation endproducts (AGEs) reduced proliferation and enhanced apoptosis of microvascular endothelial cells mediated by FOX01. Therefore, the reduction of inflammation by a FOXO1 inhibition can be an important factor in the improvement of the diabetic wound healing process as reflected by increased angiogenesis.

Interestingly, we did not observe the clear effect of a FOXO1 inhibition on the epithelial healing in diabetic minipig wounds whereas we previously found that the keratinocyte-specific FOXO1 deletion improved the keratinocyte migration and wound healing accordingly in diabetic mouse models. This discrepancy might be due to the different animal models [9]. Healing in pig wounds is more comparable to humans with similar epidermal/dermal ratios, epidermal turnover time, types of keratinous proteins, the composition of lipids in the skin, blood vessel size, and circulation. An alternative explanation is that the genetic model with lineage specific FOXO1 deletion in keratinocytes may more effectively impact re-epithelialization compared to the use of an inhibitor, due to pharmacokinetics and biodistribution of the inhibitor.

Our results focused on an early phase of wound healing. We also examined a second-time point, day 15, and found no differences between FOXO1 inhibitor- and vehicle-treated groups (data not shown). Thus, we conclude that the primary benefit of FOXO1 inhibition is during the early phases. This is consistent with the major effect of FOXO1 contributing to an inflammatory environment under diabetic conditions that delays healing [4, 15]. Thus, FOXO1 in diabetic wounds may interfere with the resolution of inflammation to delay wound healing. Inhibition of FOXO1 in diabetic wounds may facilitate early healing by reducing inflammation and improving connective tissue formation and angiogenesis.

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Disclosure of conflict of interest

None.

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