FOXO1 differentially regulates both normal and diabetic wound healing

Chenyng Zhang,1,2 Bhaskar Ponugoti,2 Chen Tian,2 Fanxing Xu,2,3 Rohinton Tarapore,2 Angelika Batres,2 Sarah Alsdun,2 Jason Lim,2 Guangyu Dong,2 and Dana T. Graves2

1Department of Preventive Dentistry, Peking University School and Hospital of Stomatology, Beijing 100081, China
2Department of Periodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104
3School of Life Science and Biotechnology, Dalian University of Technology, Dalian 116024, China

Forkhead box O1 (FOXO1) belongs to a large family of forkhead transcription factors characterized by a conserved DNA binding domain. FOXO1 modulates expression of genes involved in apoptosis, cell cycle progression, DNA repair, oxidative stress resistance, cell differentiation, and glucose metabolism (Huang and Tindall, 2007). Because of their importance

Introduction

Wound healing involves a complex series of events characterized by inflammation, migration, proliferation, and remodeling (Reinke and Sorg, 2012). Diabetes impairs wound healing, which leads to considerable morbidity and may result in limb amputation (Moulik et al., 2003). The underlying mechanisms of defective wound repair in diabetic patients are not completely understood (Gary Sibbald and Woo, 2008; Xu et al., 2013). Enhanced levels of advanced glycation end products (AGEs) and TNF are associated with altered diabetic wound healing. Impaired wound healing responses in diabetic animals are improved by blocking TNF, or AGEs, which reduces infiltration by proinflammatory macrophages, reduces fibroblast apoptosis, and improves collagen deposition (Goova et al., 2001; Goren et al., 2007; Siqueira et al., 2010; Ashcroft et al., 2012). Moreover, reduced inflammation by deletion of TLR4 improves the wound healing response (Dasu and Jialal, 2013). Correspondence to Dana T. Graves: dtgraves@dental.upenn.edu

Abbreviations used in this paper: AGE, advanced glycation end product; CCL20, chemokine (C-C motif) ligand 20; ChIP, chromatin immunoprecipitation; CML, carboxymethyllysine; FOXO1, forkhead box O1; NHEK, normal human epidermal keratinocyte; PCNA, proliferating cell nuclear antigen; SERPINB2, serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2), and chemokine (C-C motif) ligand 20 (CCL20). The impact of high glucose on keratinocyte migration was rescued by silencing FOXO1, by reducing SERPINB2 or CCL20, or by insulin treatment. In addition, an advanced glycation end product and tumor necrosis factor had a similar regulatory effect on FOXO1 and its downstream targets and inhibited keratinocyte migration in a FOXO1-dependent manner. Thus, FOXO1 expression can positively or negatively modulate keratinocyte migration and wound healing by its differential effect on downstream targets modulated by factors present in diabetic healing.

Reepithelialization is an essential step in the wound healing process and is driven by migration and proliferation of keratinocytes (Coulombe, 2003). Migration appears to be the most important cellular activity for reepithelialization because defects in migration are closely associated with poorly healing wounds (Andriessen et al., 1995; Stojadinovic et al., 2005). In normal wound healing, keratinocyte migration is regulated by growth factors, integrins, extracellular matrix molecules, and metalloproteinases (Raja et al., 2007). Although there have been numerous studies on wound healing in diabetes, relatively little is known regarding the molecular deficits in diabetic impaired reepithelialization.

Forkhead box O1 (FOXO1) belongs to a large family of forkhead transcription factors characterized by a conserved DNA binding domain. FOXO1 modulates expression of genes involved in apoptosis, cell cycle progression, DNA repair, oxidative stress resistance, cell differentiation, and glucose metabolism (Huang and Tindall, 2007). Because of their importance

Supplemental Material can be found at: http://jcb.rupress.org/content/suppl/2015/04/23/jcb.201409032.DC1.html
in maintaining cellular homeostasis, FOXO1 is tightly regulated by a sophisticated signaling network that includes inactivation by insulin and growth factors that exclude FOXO1 from the nucleus (Barthel et al., 2005). It is striking that the impact of FOXO1 is often evident under stressed conditions such as diabetes as a result of the responsiveness of FOXO1 to environmental changes (Eijkelenboom and Burgering, 2013). Factors that are increased by diabetes, such as TNF and AGEs, stimulate FOXO1 activation (Alkhani et al., 2007; Behl et al., 2009; Ponugoti et al., 2012). FOXO1 is up-regulated by wound healing, particularly in diabetic animals (Siqueira et al., 2010). Moreover, Foxo1 deletion in keratinocytes impairs the healing response, indicating that FOXO1 promotes reepithelialization in normoglycemic conditions (Ponugoti et al., 2013). One of the primary mechanisms through which FOXO1 was shown to enhance healing was up-regulation of TGFβ1. Because its functional role in diabetic healing has not been investigated we performed experiments to address this issue. Surprisingly, lineage-specific deletion of Foxo1 led to enhanced wound healing behavior of keratinocytes. Thus, in diabetic mice, FOXO1 has the opposite effect on keratinocyte wound healing behavior as it does under normal conditions. The differential effect of FOXO1 on normal and diabetic healing was caused by changes in its regulation of downstream targets, which was modulated by factors that are elevated in diabetes. In normal conditions, FOXO1 binds to the TGFβ1 promoter and up-regulates TGFβ1 expression, which is crucial for keratinocyte migration. When stimulated with high glucose, AGEs, or TNF, FOXO1 fails to bind to the TGFβ1 promoter and does not up-regulate TGFβ1 expression. Instead, FOXO1 enhances the expression of factors that lead to reduced keratinocyte migration. Moreover, insulin rescued the negative effect of these factors on keratinocyte through migration through Akt, which blocks FOXO1. This provides a mechanistic explanation for impaired reepithelialization in situations where the levels of glucose, AGEs, and TNF are elevated, such as diabetes.

Results

Keratinocyte-specific Foxo1 deletion delays wound healing in normoglycemic mice while improving wound repair in diabetic mice

Small excisional skin wounds were created in experimental transgenic mice (K14.Cre<sup>−</sup>.Foxo1<sup>L/L</sup>) with keratinocyte-specific deletion of Foxo1 driven by keratin-14 Cre recombinase and littermate control (K14.Cre<sup>−</sup>.Foxo1<sup>L/−</sup>) mice without Foxo1 deletion. Deletion of Foxo1 in keratinocytes of nondiabetic mice delayed wound healing (Fig. 1 A and Fig. S1 A). In contrast, lineage-specific deletion of Foxo1 in diabetic mice had the opposite effect, accelerating wound closure. Normoglycemic mice with Foxo1 deletion had up to twofold larger wounds compared with matched littermate controls (P < 0.05; Fig. 1 A). In contrast, wounds in diabetic experimental Foxo1-deficient mice were reduced by 30–60% compared with diabetic control mice (Fig. 1 A). At the histological level, keratinocyte-specific Foxo1 deletion in normoglycemic mice resulted in up to twofold larger wounds, whereas Foxo1 deletion in diabetic mice reduced wound size by 40–80% compared with matched control littermates (P < 0.05; Fig. S1 A and Table 1). Lineage-specific deletion of Foxo1 in diabetic mice improved healing by increasing epithelial thickness and epithelial area of healing wounds so they reached levels similar to normal mice (P < 0.05; Table 1). In contrast, Foxo1 deletion in normoglycemic mice had the opposite effect on the epithelial area and thickness in healing wounds (Table 1).

Because it was possible that differential expression of FOXO1 was responsible for the variable effect of Foxo1 deletion on normal and diabetic wound healing, FOXO1 nuclear translocation and expression was assessed. Diabetic control mice had higher levels of FOXO1 nuclear localization compared with normoglycemic control mice (Fig. 1 B and Fig. S1 B). The overall level of FOXO1 expression at the protein level and the mRNA level was increased in wounded compared with nonwounded epithelium in both normal and diabetic mice (Fig. S1, C and D). As expected, FOXO1 nuclear localization and expression were significantly reduced by 80–90% in keratinocytes of experimental versus control mice (Fig. 1 B; and Fig. S1, C and D). Thus, the different effect of FOXO1 on wound healing behavior in normal and diabetic mice was not caused by a failure to induce FOXO1 in keratinocytes of diabetic animals. This was also demonstrated in vitro as high glucose significantly increased FOXO1 nuclear localization in a time-dependent manner in primary cultures of murine keratinocytes isolated from K14.Cre<sup>−</sup>.Foxo1<sup>L/L</sup> mice (P < 0.05; Fig. 1 C). In addition, high glucose also apparently increased FOXO1 DNA binding in primary cultures of normal human epidermal keratinocytes (NHEKs) compared with low glucose (P < 0.05; Fig. 1 D).

FOXO1 silencing reduces the closure of in vitro scratch wounds in low glucose conditions, whereas it improves in vitro wound closure in high glucose conditions

To determine whether the effect of FOXO1 on wound healing was directly related to glucose levels, an in vitro scratch assay was performed under defined conditions and reepithelialization of the denuded area was measured (Fig. S2). Transfection of NHEK cells with FOXO1 siRNA significantly reduced FOXO1 mRNA levels by 80–90% compared with scrambled siRNA (P < 0.05; Fig. S1 E) and significantly reduced FOXO1 DNA binding activity (Fig. 1 D). Under low glucose conditions, knockdown of FOXO1 significantly reduced the reepithelialization of the in vitro scratch wound by 35–50% (P < 0.05), whereas knockdown of FOXO3 had no effect compared with scrambled control siRNA (P > 0.05; Fig. 1 E). In contrast, under high glucose conditions, FOXO1 knockdown significantly increased reepithelialization in vitro by 55–70% (P < 0.05), whereas FOXO3 knockdown had no effect (P > 0.05; Fig. 1 F). Thus, in vitro experiments in high glucose mimicked the effect of diabetes, and the positive impact of FOXO1 deletion on both was consistent. In contrast, overexpression of constitutively active FOXO1 (FOXO1-AAA) significantly increased reepithelialization of the scratch wound in low glucose but in high glucose FOXO1 overexpression had the opposite effect, reducing reepithelialization (P < 0.05; Fig. 1, G and H).
FOXO1 deficiency reduces migration in low glucose conditions, whereas it has the opposite effect in high glucose conditions

To investigate mechanisms through which FOXO1 affected wound healing, keratinocyte migration and proliferation were assessed in vivo. Wounding increased keratinocyte migration as determined by expression of urokinase-type plasminogen activator receptor (uPAR; Fig. 2 A), a marker of keratinocyte migration (Lund et al., 2006). Diabetes had a significant negative effect on migration of keratinocytes (P < 0.05; Fig. 2 A and Fig. S3 A). FOXO1 ablation in diabetic mice increased migration twofold compared with diabetic control littermates (P < 0.05). The effect of Foxo1 deletion increased keratinocyte migration to a level that was similar to normoglycemic mice (Fig. 2 A).

Table 1. Histological change of the mice scalp wound

<table>
<thead>
<tr>
<th>Wound parameter</th>
<th>Day</th>
<th>Normoglycemic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial gap (mm)</td>
<td>4</td>
<td>0.59 ± 0.27</td>
<td>1.76 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.35 ± 0.28</td>
<td>0.56 ± 0.36</td>
</tr>
<tr>
<td>Epithelial thickness (mm)</td>
<td>4</td>
<td>0.09 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.08 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Epithelial area (mm²)</td>
<td>4</td>
<td>0.21 ± 0.02</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.18 ± 0.01</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Epithelial gap (mm)</td>
<td>4</td>
<td>1.66 ± 0.08</td>
<td>1.01 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.52 ± 0.13</td>
<td>0.92 ± 0.18</td>
</tr>
<tr>
<td>Epithelial thickness (mm)</td>
<td>4</td>
<td>0.07 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.06 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Epithelial area (mm²)</td>
<td>4</td>
<td>0.17 ± 0.01</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.10 ± 0.01</td>
<td>0.16 ± 0.01</td>
</tr>
</tbody>
</table>

All numbers are means ± SEM.

*Significant change compared to normoglycemic K14.Cre+Foxo1+/− mice (P < 0.05).

**Significant change compared to diabetic K14.Cre−Foxo1+/− mice (P < 0.05).
In contrast, Foxo1 deletion in normoglycemic mice decreased the number of migrating keratinocytes by 60% compared with matched controls (P < 0.05; Fig. 2 A).

Foxo1 deletion in normoglycemic mice caused a small but significant 27% reduction in the number of proliferating cell nuclear antigen (PCNA)–positive proliferating keratinocytes in wound epithelium (P < 0.05; Fig. 2 B). In contrast, Foxo1 deletion in diabetic mice increased PCNA-positive keratinocytes by 20% (P < 0.05; Fig. 2 B). Notably, the magnitude of the positive effect of Foxo1 deletion in vivo on keratinocyte proliferation in diabetic mice was not as great as the effect on migration: 1.2-fold versus twofold.

The effect of FOXO1 on keratinocyte migration was investigated further in vitro in a transwell migration assay. Similar to in vivo results, keratinocyte migration in high glucose in vitro was reduced 64% compared with standard media (P < 0.05; Fig. 2 C). In contrast, knockdown of FOXO1 had little effect on keratinocyte proliferation both in low glucose and high glucose (Fig. S3 C). The effect of different glucose concentrations in modulating keratinocyte migration in a FOXO1-dependent manner was tested (Fig. 2 D). Glucose reduced keratinocyte migration in control cells but had the opposite effect when FOXO1 was deleted. The tipping point where FOXO1 deletion had no effect was ~19 mM glucose.

These studies examined the effect of FOXO1 knockdown in primary cultures of human keratinocytes. The differential impact of FOXO1 was also examined in primary cultures of murine keratinocytes isolated from K14.Cre Foxo1L/L and K14.Cre+.Foxo1L/L mice. FOXO1 protein levels were not increased by high glucose (Fig. 2, E and F) but FOXO1 nuclear localization was increased 5.6-fold in keratinocytes from K14.Cre−.Foxo1L/L control mice in high glucose condition (P < 0.05; Fig. 2 G). Moreover, FOXO1 expression was reduced by 70–90% and FOXO1 nuclear localization by >90% in keratinocytes from experimental mice compared with keratinocytes from control mice (P < 0.05; Fig. 2, E–G). In agreement with immunofluorescent studies, Western blot analysis also showed that high glucose
increased FOXO1 nuclear localization in keratinocytes from control mice, whereas it had little effect on overall FOXO1 expression levels (Fig. 2 H), consistent with results from diabetic mice in vivo.

The effect of glucose on migration of primary murine keratinocytes isolated from normal and experimental mice was also examined. In high glucose media, Foxo1 ablation increased keratinocyte migration twofold (P < 0.05; Fig. 2 I). In contrast, deletion of Foxo1 reduced murine keratinocyte migration by 54% when cells were incubated in standard glucose media (P < 0.05; Fig. 2 I). Results examining migration of murine keratinocytes from mice with Foxo1 deleted by Cre recombinase agreed well with experiments with human NHEK cells that had FOXO1 knockdown by siRNA. Similarly, Foxo1 deletion in murine keratinocytes largely reversed the small but significant negative effect of high glucose on murine keratinocyte proliferation (P < 0.05; Fig. 2 J). Thus, the large glucose-dependent effect of Foxo1 deletion on murine keratinocyte migration and small effect on proliferation was similar to that observed for human keratinocytes and consistent with results obtained in vivo with diabetic mice. Collectively, these results point to the dramatic positive effect of Foxo1 deletion under high glucose conditions and, combined with in vivo data, suggest that the effect on keratinocyte migration represents an important mechanism by which FOXO1 affects reepithelialization and wound healing.

FOXO1 regulation of TGFβ1 is dependent on glucose levels in vitro and diabetic condition in vivo

Foxo1 deletion in vitro reduced TGFβ1 levels by 45% in standard glucose media (P < 0.05; Fig. 3 A). High glucose in vitro also reduced TGFβ1 expression. However, Foxo1 deletion in high glucose had no effect on TGFβ1 expression, indicating that in high glucose media FOXO1 does not regulate TGFβ1 (P > 0.05; Fig. 3 A). Foxo1 deletion in vivo also significantly reduced TGFβ1 mRNA levels in wounds from normoglycemic mice but Foxo1 deletion had little effect on its mRNA levels in wounds from diabetic mice (P > 0.05; Fig. 3 B).
diabetic mice (P > 0.05; Fig. 3 B). The differential regulation of TGFβ1 by FOXO1 in normoglycemic and diabetic mice was further confirmed at the protein level. When Foxo1 was deleted in normoglycemic mice the number of TGFβ1-positive keratinocytes was reduced by 50% compared with littermate controls (Fig. 3 C). However, Foxo1 ablation in diabetic mice did not affect the number of keratinocytes that expressed TGFβ1 (P > 0.05; Fig. 3 C). This is consistent with the concept that FOXO1 plays a role in TGFβ1 expression under normal conditions but not in diabetic or high glucose conditions.

Chromatin immunoprecipitation (ChIP) assays showed that FOXO1 interacted with the TGFβ1 promoter under low glucose conditions (Fig. 3 D). High glucose blocked FOXO1–TGFβ1 promoter interactions (Fig. 3 D). We then determined whether glucose levels modulated the capacity of FOXO1 to transcriptionally regulate TGFβ1 using a TGFβ1 reporter. Overexpression of FOXO1 increased TGFβ1 promoter activity in low glucose, but in high glucose FOXO1 overexpression had no effect on TGFβ1 promoter activity (Fig. 3 E). Thus, glucose levels determine whether FOXO1 binds to the TGFβ1 promoter and regulates TGFβ1 transcriptional activity.

SMAD2/3 phosphorylation is downstream of TGFβ1 and represents a functional test of the degree of TGFβ1 signaling. Lineage-specific Foxo1 ablation reduced SMAD2/3 phosphorylation by 75% in keratinocytes in wounds of normoglycemic mice in vivo (P < 0.05; Fig. 3 F). However, deletion of Foxo1 in diabetic mice had no effect on phospho-SMAD2/3 levels compared with diabetic control mice (P > 0.05; Fig. 3 F). The lack of an effect of Foxo1 ablation on this functional assay in diabetic mice is consistent with the failure of FOXO1 to regulate TGFβ1 under these conditions. In contrast, deletion of Foxo1 in normoglycemic mice has a significant effect on TGFβ1 signaling.

One of the principal mechanisms by which TGFβ1 affects wound healing is regulation of keratinocyte migration (Gailit, et al., 1994). We determined functionally whether treatment with TGFβ1 could rescue deficits in keratinocyte migration. Notably, the reduced migration caused by Foxo1 knockout was rescued by TGFβ1 (Fig. 3 G). This suggests that in low glucose conditions the TGFβ1 deficit caused by FOXO1 deletion is problematic.

FOXO1 regulation of SERPINB2 and CCL20 is dependent on glucose levels in vitro and presence of diabetes in vivo

Microarray analysis was performed to further investigate potential inhibitory factors regulated by FOXO1 (Table S1). Two of these, serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2), and chemokine (C-C motif) ligand 20 (CCL20) were chosen for further analysis because they have previously been shown to be involved in wound healing (Yamazaki et al., 2008; Tarcic et al., 2012). High glucose induced a 6.4-fold increase in SERPINB2 mRNA levels in NHEK cells in vitro (P < 0.05), and knockdown of FOXO1 reduced SERPINB2 mRNA levels by 94% (P < 0.05; Fig. 4 A). At the protein level, high glucose increased SERPINB2 expression three- to fourfold (P < 0.05), and this increase was blocked by Foxo1 ablation in keratinocytes (P < 0.05; Fig. 4, B and C). Whether or not FOXO1 regulated SERPINB2 in vivo was investigated in diabetic wounds (Fig. S4 A). Keratinocytes in diabetic wounds had a twofold increase in SERPINB2 compared with matched normoglycemic mice (P < 0.05; Fig. 4 D and Fig. S4 B). Deletion of Foxo1 in vivo by Cre recombinase blocked diabetes-enhanced SERPINB2 expression (P < 0.05; Fig. 4 D and Fig. S4 B), demonstrating that the impact of diabetes on SERPINB2 is FOXO1 dependent. ChIP assays were then conducted and determined that FOXO1 interacted with the SERPINB2 promoter (Fig. 4 E). Notably, high glucose induced a significantly higher level of FOXO1 binding to the SERPINB2 promoter compared with low glucose (P < 0.05; Fig. 4 E). Consistent with ChIP results, the increase in SERPINB2 promoter activity in keratinocytes stimulated by high glucose did not occur when Foxo1 was deleted, also demonstrating FOXO1 dependence (P < 0.05; Fig. 4 F).

Transwell assays were then used to examine the functional impact of SERPINB2 on migration of primary murine keratinocytes from control (K14.Cre–/–Foxol/+/+) mice. Knockdown of SERPINB2 with siRNA increased migration of keratinocytes from control mice by 2.8-fold in high glucose (P < 0.05; Fig. 4 G), demonstrating that SERPINB2 expression in high glucose limited migration. The result indicates that the negative effect of high glucose on keratinocyte migration is caused in part by high levels of SERPINB2 mediated by FOXO1 in high glucose.

CCL20 was examined for the aforementioned reasons. CCL20 mRNA levels in vitro increased 9.3-fold in human keratinocytes in high glucose media (P < 0.05) and knockdown of FOXO1 blocked this increase (P < 0.05; Fig. 5 A). At the protein level, high glucose induced an almost twofold increase in CCL20 expression in murine keratinocytes (P < 0.05), which was blocked by Foxo1 deletion (P < 0.05; Fig. 5, B and C). The impact of FOXO1 on CCL20 expression in vivo was then examined in wounds of experimental and control mice. CCL20 levels were increased approximately twofold in diabetic wounds compared with normoglycemic wounds in K14.Cre–/–Foxol/+/+ control mice on both day 4 and 7 after wounding (P < 0.05; Fig. 5 D and Fig. S4 C). Keratinocyte-specific deletion of Foxo1 blocked the diabetes-induced increase in CCL20 expression in vivo (Fig. 5 D and Fig. S4 C). Thus, hyperglycemia in vivo and high glucose in vitro induced CCL20 expression in a FOXO1-dependent manner. Whether FOXO1 regulates CCL20 transcription was further explored. ChIP results showed that high glucose stimulates FOXO1 binding to the CCL20 promoter (Fig. 5 E). A reporter assay demonstrated that high glucose induced a significant increase in CCL20 promoter activity in keratinocytes, which was largely blocked by Foxo1 ablation (P < 0.05; Fig. 5 F). The results collectively indicate that high glucose regulates CCL20 expression and transcription in a FOXO1-dependent manner. Whether or not FOXO1 regulation of CCL20 represents a mechanism by which FOXO1 affects keratinocyte migration was then tested. CCL20-neutralizing antibody increased keratinocyte migration twofold in high glucose (Fig. 5 G). When combined with the aforementioned results that data suggest that glucose stimulates production of CCL20 in a FOXO1-dependent manner and CCL20 interferes with keratinocyte migration.
Insulin regulates FOXO1 nuclear localization and reverses high glucose-induced expression of FOXO1 and downstream targets SERPINB2 and CCL20

Diabetes involves several factors including low levels of insulin or insulin resistance. The effect of insulin on FOXO1 nuclear localization in keratinocytes in vitro was examined. FOXO1 nuclear localization increased 2.3-fold under stimulation of high glucose when insulin was absent (P < 0.05; Fig. 6 A), whereas mannitol had no effect compared with low glucose (P > 0.05; Fig. S5 A). High glucose-induced FOXO1 nuclear localization was significantly reduced by insulin (P < 0.05; Fig. 6 A). In contrast, FOXO1 nuclear localization was barely detected in keratinocytes from K14.Cre⁻Foxo1L/L mice under all conditions (P > 0.05; Fig. 6 A). These results indicate that the maximum induction of FOXO1 nuclear localization under high glucose conditions is dependent on low levels of insulin signaling.

The effect of insulin on FOXO1 targets SERPINB2 and CCL20 was further investigated. High glucose stimulated the expression of SERPINB2 in keratinocytes from K14.Cre⁻Foxo1L/L control mice but not by mannitol osmotic control or in keratinocytes from Foxo1-deleted mice (Fig. 6 B and Fig. S5 B). Increased SERPINB2 expression stimulated by high glucose was significantly reduced by insulin (P < 0.05; Fig. 6 B). Similarly, high glucose but not mannitol osmotic control stimulated the expression of CCL20 in K14.Cre⁻Foxo1L/L keratinocytes after transfection with scrambled or Serpinb2 siRNA. Each in vivo value is the mean ± SEM for n = 5–8 mice per group. In vitro values represent the mean ± SEM of three independent experiments. *, P < 0.05 versus matched scrambled siRNA or IgG control; †, P < 0.05 versus matched low glucose group; ‡, P < 0.05 versus Cre group; **, P < 0.05 versus normal epithelium.
The capacity of insulin to rescue keratinocyte migration in high glucose was blocked by inhibition of AKT (P < 0.05; Fig. 6 D). Akt mediates the inhibitory effect of insulin on FOXO1 (Tsuchiya et al., 2012).

**AGE regulates FOXO1 and interferes with keratinocyte migration through FOXO1.** AGEs are elevated in diabetic and aging skin (Bos et al., 2011; Gkogkolou and Böhm, 2012). The effect of an AGE, carboxymethyllysine (CML)-modified BSA (CML-BSA), was tested on FOXO1 and regulation of its target genes, TGF\(\beta\)1, SERPINB2, and CCL20. AGE stimulated a time-dependent increase in FOXO1 nuclear localization (P < 0.05; Fig. 7 A). In contrast, incubation with AGE reduced TGF\(\beta\)1 expression at the protein level almost in half in a time-dependent manner (P < 0.05; Fig. 7 A). ChIP experiments determined that the interaction of FOXO1 with TGF\(\beta\)1 promoter was largely blocked by AGE treatment (P < 0.05; Fig. 7 C). Consistently with the inhibitory effect of AGE on FOXO1 binding to the TGF\(\beta\)1 promoter, AGE also blocked the capacity of FOXO1 overexpression to stimulate TGF\(\beta\)1 promoter activity (Fig. 7 D).

In contrast to the effect on TGF\(\beta\)1, CML-BSA induced a threefold increase in SERPINB2 and CCL20 protein levels (P < 0.05; Fig. 7, E and F). The capacity of the AGE to induce SERPINB2 and CCL20 was blocked by FOXO1 deletion (P > 0.05). CML-BSA stimulated FOXO1 binding to both SERPINB2 and CCL20 promoters compared with control BSA (P < 0.05; Fig. 7, G and H). Cotransfection of a FOXO1 expression vector and SERPINB2 or CCL20 reporter demonstrated that AGE further enhanced SERPINB2 and CCL20 transcriptional activity 65–100% (P < 0.05; Fig. 7, I and J).

The effect of AGE on keratinocyte migration was then investigated. Similar to the effect of high glucose, CML-BSA reduced keratinocyte migration by 65% in wild-type keratinocytes (K14.Cre:\(^{lox/lox}\); P < 0.05; Fig. 7 K). Foxo1 ablation reversed the inhibitory effect of AGE on keratinocyte migration (P < 0.05; Fig. 7 K). The role of insulin signaling in keratinocyte migration was then explored. The negative impact of AGE...
on keratinocyte migration was reduced by insulin (P < 0.05; Fig. 7 L). Furthermore, the rescue by insulin was blocked by an AKT inhibitor (P < 0.05; Fig. 7 L).

**Discussion**

Results presented here demonstrate that one of the primary differences between normal and diabetic reepithelialization is the role of FOXO1 in the wound healing behavior of keratinocytes. In normal mice, FOXO1 has a positive impact on healing whereas in diabetic mice FOXO1 has the opposite effect and impedes reepithelialization. In normal conditions, deletion of Foxo1 in keratinocytes reduced wound closure examined grossly or histologically. In contrast, Foxo1 deletion in diabetic mice enhanced gross wound closure and histological reepithelialization. The effect is caused by the specific role of FOXO1 in keratinocytes because the in vivo model used keratinocyte-specific deletion of Foxo1 mediated by keratin-14–driven Cre recombinase. Similarly, in standard glucose media, Foxo1 deletion impaired reepithelialization in vitro, whereas Foxo1 overexpression enhanced it. Foxo1 deletion or overexpression in keratinocytes had the opposite effect in high glucose.

The primary difference in FOXO1’s effect on reepithelialization of normal and diabetic wounds in vivo and in scratch wounds in vitro can be attributed to its effect on keratinocyte migration. Reduced keratinocyte migration is an important factor in impaired reepithelialization of chronic and diabetic healing (Stojadinovic et al., 2005; Lan et al., 2008; Usui et al., 2008; Jacobsen et al., 2010). We identified two distinct mechanisms through which FOXO1 affects keratinocyte migration, both of which are affected by diabetes in vivo and by high levels of glucose, an AGE, or TNF in vitro. Under normal conditions,
loss of one of the driving forces of TGFβ1 expression, FOXO1 binding to its promoter to induce transcription. Consistent with this finding is that Foxo1 ablation in normal mice had significantly reduced phosphorylation of SMAD2/3 (p-SMAD2/3), an immediately downstream target of TGFβ1 receptor signaling. In contrast, deletion of Foxo1 in diabetic mice did not affect p-SMAD2/3 levels. Moreover, the loss of TGFβ1 signaling caused by FOXO1 has a significant effect because the negative effect of FOXO1 knockdown on keratinocyte migration FOXO1 binds to the TGFβ1 promoter and up-regulates TGFβ1 expression. This is significant because TGFβ1 is a potent inducer of keratinocyte migration (Gailit et al., 1994; Decline and Rousselle, 2001; Tredget et al., 2005) and plays an important role in reepithelialization (Sun et al., 2009; Zhang et al., 2012). In contrast, FOXO1 has significantly reduced binding to the TGFβ1 promoter or induced TGFβ1 transcription in keratinocytes incubated in high glucose, an AGE, or TNF despite enhanced FOXO1 nuclear translocation. Thus, factors present in diabetes cause the loss of one of the driving forces of TGFβ1 expression, FOXO1 binding to its promoter to induce transcription. Consistent with this finding is that Foxo1 ablation in normal mice had significantly reduced phosphorylation of SMAD2/3 (p-SMAD2/3), an immediately downstream target of TGFβ1 receptor signaling. In contrast, deletion of Foxo1 in diabetic mice did not affect p-SMAD2/3 levels. Moreover, the loss of TGFβ1 signaling caused by FOXO1 has a significant effect because the negative effect of FOXO1 knockdown on keratinocyte migration...
is rescued by addition of TGFβ1. The results are also consistent with findings that impaired TGFβ1 signaling is involved in delayed diabetic wound healing and provides a mechanistic basis for this previous observation (Al-Mulla et al., 2011).

Although the capacity of FOXO1 to induce TGFβ1 transcription is reduced in diabetic conditions the opposite occurs with negative regulators of keratinocyte migration. Diabetic wounds expressed higher levels of SERPINB2 and CCL20 than normoglycemic wounds in vivo, which were restored to normal levels by Foxo1 deletion. Thus, factors present in diabetes such as high levels of glucose, AGEs, or TNF increase FOXO1 binding to SERPINB2 and CCL20 promoters and stimulate increased transcription. Increased expression of SERPINB2 and CCL20 is problematic because it interferes with keratinocyte migration.

Figure 8. TNF stimulates FOXO1 nuclear localization, modulates FOXO1 downstream targets, and reduces keratinocyte migration in a FOXO1-dependent manner. NHEK cells (A, C, D, and G–J) or primary murine keratinocytes (B, E, F, K, and L) from K14.Cre–Foxo1L/L and K14.Cre+Foxo1L/L mice were incubated with TNF for the indicated time points (A, B, E, and F) or for 24 h (C, D, and G–L). (A) FOXO1 nuclear localization was determined by FOXO1 immunofluorescence and DAPI nuclear staining. (B) TGFβ1 immunofluorescence analyses for murine keratinocytes. (C) ChIP assay to assess FOXO1 binding to the TGFβ1 promoter. (D) TGFβ1 luciferase reporter gene analyses after cotransfection with control or FOXO1 plasmid. SERPINB2 (E) and CCL20 (F) immunofluorescence analyses for primary murine keratinocytes. (G and H) ChIP assays for the binding of FOXO1 to the SERPINB2 (G) or CCL20 (H) promoters. SERPINB2 (I) and CCL20 (J) luciferase reporter gene analyses after cotransfection with control or FOXO1 plasmid. (K) Keratinocyte migration was measured by transwell migration assay in K14.Cre–Foxo1L/L keratinocytes with the addition of insulin with or without AKT inhibitor as indicated. *P < 0.05 versus control group; #, P < 0.05 versus IgG control group; +, P < 0.05 versus pcDNA3.1 control group; **, P < 0.05 versus Cre– group; #, P < 0.05 versus vehicle group; ***, P < 0.05 versus AGE alone treatment group; &, P < 0.05 versus AGE plus insulin treatment group.
This is supported by evidence that reduced migration of keratinocytes in high glucose is rescued by knockdown of SERPINB2 or inhibition of CCL20. That SERPINB2 can inhibit cell migration was previously reported (Praus et al., 1999; Shimizu et al., 2003). High levels of SERPINB2 reduce human prostate cancer cell and fibrosarcoma cell migration (Praus et al., 1999; Shimizu et al., 2003). The inhibitory effect of high CCL20 levels on cell migration has not been previously reported.

Diabetes is characterized by a deficit in insulin signaling caused by a relative absence of insulin or resistance to insulin stimulation. The assays were performed with low insulin or none to reflect this condition. We determined whether insulin affected the impact of elevated levels of glucose, an AGE, or TNF. Insulin reversed the negative effect of each factor on keratinocyte migration. Moreover, this was mediated by Akt because the effect of insulin was blocked by an Akt inhibitor. This finding is significant because Akt blocks FOXO1 activation (Tsuchiya et al., 2012). Insulin also blocked stimulation of SERPINB2 and CCL20 expression by high glucose, an AGE, and TNF. That reduced insulin is important is shown by diminished FOXO1 nuclear localization when keratinocytes are exposed to insulin. Collectively, these results point out the permissive effect of low insulin in facilitating the negative impact of FOXO1 on the wound healing behavior of keratinocytes. This result may explain previous findings that topical administration of insulin promotes wound healing in diabetic animals mediated by the Akt pathway (Lima et al., 2012) and that topical treatment with insulin improves diabetic wound healing in humans (Paul, 1966; Glasser and Barth, 1982).

In summary, we found that FOXO1 has opposite effects on normal and diabetic wound healing. In normal conditions, FOXO1 promotes wound healing by up-regulating TGFβ1 signaling pathway but fails to do this in diabetic wounds. However, in diabetic wounds or when keratinocytes are exposed to high levels of glucose, AGEs, or TNF, FOXO1 impedes wound healing by inducing overexpression of SERPINB2 and CCL20, which interferes with keratinocyte migration. Thus, the relative change (reduced TGFβ1 and increased SERPINB2/CCL20) caused by the impact of the “diabetic condition” on FOXO1–promoter interactions plays an important role in wound healing. The results suggest that FOXO1 may be a useful therapeutic target to treat diabetic wounds and potentially other complications caused by hyperglycemia and insulin insufficiency/resistance. Accumulating evidence suggests that high levels of activated FOXO1 are linked to several diabetic complications. For example, the early loss of endothelial cells and pericytes that occurs in diabetic retinopathy is significantly reduced by knockdown of FOXO1 with siRNA in vivo (Behl et al., 2009). In diabetic cardiomyopathy, deletion of Foxo1 in cardiomyocytes rescues high-fat diet–induced declines in cardiac function, which is associated with improved survival (Battiprolu et al., 2012). Moreover, some of the negative effects of diabetes on fracture healing have been linked to FOXO1 (Kayal et al., 2010). FOXO1 is hyperactivated in diabetes because of the stimulatory effects of high glucose and reduced insulin signaling that lead to insufficient inactivation of FOXO1 (Ni et al., 2007). Thus, in diabetic wound healing FOXO1 interferes with keratinocyte migration by enhancing the expression of SERPINB2 and CCL20. In addition, in diabetic conditions, high levels of glucose, AGEs, or TNF, FOXO1 fails to bind to the TGFβ1 promoter, thus failing to induce a critical wound healing factor. These results point to a previously unrecognized mechanism for delayed diabetic wound healing and the role of FOXO1. In addition, AGEs and TNF are elevated in aging skin (Schnider and Kohn, 1980, 1981; Yarosh et al., 2000; Rittie and Fisher, 2002; Lohwasser et al., 2006) and associated with impaired healing during aging (Goova et al., 2001; Ashcroft et al., 2002; Hardman et al., 2008; Peppa et al., 2009). Thus, the impact of AGEs and TNF on FOXO1-mediated expression of TGFβ1, CCL20, and SERPINB2 may also negatively affect wound healing in the aged.

Materials and methods

Animals and induction of diabetes

Animal experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Mice with floxed Foxo1 were provided by R.A. DePinho (MD Anderson Cancer Center, Houston, TX) as previously described (Park et al., 2007). Mice expressing Cre recombinase under the control of keratin 14 promoter (K14.Cre; strain Tg(KRT14-cre)1Amc/J) were obtained from the Jackson Laboratory. Lineage-specific Foxo1 deletion was obtained by crossing these mice to generate experimental (K14.Cre;Foxo1lo/lo) and control (K14.Cre; Foxo1+/+; Foxo1−/−) mice. Two to five mice were housed per cage under standard conditions with a 14-h light/10-h dark cycle. All the experiments were performed with adult mice 16–20 wk old. Type 1 diabetes was induced by multiple low dose i.p. injections of streptozotocin (40 mg/kg; Sigma-Aldrich) in 10 mM citrate buffer daily for 5 d. Control mice were treated identically with vehicle alone. The glucose levels were monitored after completion of multiple low dose streptozotocin or citrate buffer injections (Table S2). Mice were considered to be hyperglycemic when serum glucose levels were >220 mg/dl. Experiments were performed when mice had been hyperglycemic for at least 3 wk.

Skin wounding experiment

Mice were anaesthetized by i.p. administration of ketamine (80 mg/kg) and xylazine (5 mg/kg). The scalp hair was shaved and cleansed, and two excisional wounds were made with a 2-mm sterile biopsy punch in the scalp at the midline as described previously (Siqueira et al., 2010). Wound healing was grossly monitored with calibrated digital photographs at the indicated time points. A ruler was placed next to the specimens and images were captured. NIS-elements software (Nikon) was used to measure wound area at each time point. The mean area of two wounds per animal was measured to establish the wound size for each animal, which was the unit of measurement. Wound size was presented as the percentage of wound area compared with day 0. Animals were euthanized to collect the wound tissue at the indicated time points.

Histology

Excised skin specimens with scalp and attached calvarial bone were fixed in 4% paraformaldehyde for 24 h, decalcified in 10% EDTA solution, and embedded in paraffin. 5-µm paraffin sections were stained with hematoxylin and eosin and histomorphometric analysis was performed with NIH-elements D image analysis software at the center of each lesion.

Immunohistochemistry in histological sections

Paraffin-embedded, formalin-fixed skin sections were processed for immuno-fluorescence analyses. Antigen retrieval was performed in 10 mM of citric acid, pH 6.0, at 120°C except for CCL20, for which EDTA solution (1 mM; pH 9.0) was used at 120°C. Sections were then incubated with primary antibody to FOXO1 (rabbit; Santa Cruz Biotechnology, Inc.), PCNA (rabbit; Santa Cruz Biotechnology, Inc.), uPAR (rabbit; Santa Cruz Biotechnology, Inc.), TGFβ1 (rabbit; Abcam), phospho-Smad2/3 (rabbit; Santa Cruz Biotechnology, Inc.), SMA1/2/3 (rabbit; Santa Cruz Biotechnology, Inc.), CCL20 (rabbit; Abcam), and SERPINB2 (goat; Santa Cruz Biotechnology, Inc.) overnight at 4°C as well as the appropriate isotype-matched negative control IgG. Biotinylated secondary antibody (Vector Laboratories) and ABC reagent (Vector Laboratories) were then used. Tyramide signal amplification (PerkinElmer) was also used to enhance the chromogenic signal. Finally, Alexa Fluor 546–conjugated streptavidin (Invitrogen) and DAPI-containing mounting media were used to visualize the staining (Sigma-Aldrich).
Cell culture, treatment, and transfection

Primary cultures of NHEK cells were purchased from Lonza and maintained in KGM-2 growth medium supplemented with human keratinocyte growth supplements (Lonza). Primary mouse epidermal keratinocytes were isolated from the neonates (0–2 d old) of experimental (K14.Cre:\:Foxo1L/L) and control (K14.Cre:\:Foxo1L/L) mice. In brief, mouse skin was collected and digested with 2.5 U/ml Dispase II (Roche) overnight at 4°C. The dermis was then separated from the epidermis by digesting with 0.1% trypsin and 0.02% EDTA in PBS for 1.5 min at 37°C. Keratinocytes from the epidermis were cultured in KGM-2 growth medium containing antibiotics. All cell cultures were maintained in a 5% CO₂ humidified incubator at 37°C. Keratinocytes were passaged in KGM-2 growth media with supplements including standard insulin (8.6 × 10⁻⁷ M). For assays, cells were transferred to KGM-2 media with supplements except the amount of insulin was reduced 100-fold unless otherwise stated, i.e., low insulin represents 1% of the amount of insulin in standard keratinocyte growth media. In some experiments no insulin was added. ON-TARGETplus SMARTpool siRNAs against human FOXO1 and FOXO3 and control siRNA (ON-TARGETplus Non-Targeting Control Pool) were obtained from GE Healthcare and transfection was performed using GenMute siRNA Transfection Reagent (SignaGen Laboratories). In most transfection experiments, cells were incubated for 6 h with siRNA and transfection reagent 2 d before assay. Cells were then rinsed and transfected back to the indicated culture media for the remaining incubation period. Plasmid DNA transient transfections were performed using Lipofectin (Invitrogen) according to the manufacturer’s instructions. Transfection occurred 2 d before assay.

Immunofluorescence analysis in vitro

Primary keratinocytes isolated from K14.Cre:\:Foxo1L/L and K14.Cre:\:Foxo1L/L mice were grown on 8-well chamber slides (Thermo Fisher Scientific) and incubated in low glucose (5 mM d-glucose), high glucose (25 mM d-glucose), or osmotic control medium (25 mM mannitol) for 5 d. In some experiments, cells were incubated with CML-BSA (200 µg/ml), which was prepared by chemical modification of BSA (Sigma-Aldrich) as previously described (Alikhani et al., 2010), and compared with control cells incubated with unmodified BSA in low insulin. In brief, 50 µg BSA was dissolved in 25 ml HCl (1 M) freshly made in sterile water and incubated at 37°C with occasional mixing. Sterile PBS, pH 7.8 (25 ml), was added, followed by sodium cyanoborohydride (1.42 g) and sodium glyoxyliate acid (0.715 g). Control BSA was prepared at the same time except that no glyoxyliate acid was added. In some cases, cells were incubated with 10 ng/ml TNF (PrepTech) in low insulin. In experiments measuring the response to insulin, cells were incubated with defined amounts of insulin (Santa Cruz Biotechnology, Inc.) for 24 h before assay and compared with incubation in the same media without insulin. For immunofluorescence, keratinocytes were fixed in 3.7% formaldehyde for 10 min, permeabilized in 0.5% Triton X-100 for 5 min, blocked in 2% BSA, and stained with primary antibody anti-FOXO1 (Santa Cruz Biotechnology, Inc.), TGFB1 (Abcam), SERPINB2 (Santa Cruz Biotechnology, Inc.), or CCL20 (Abcam). Primary antibody was localized with biotinylated secondary antibody and Alexa Fluor 546-conjugated streptavidin and mounted with DAPI-containing mounting media. Images were captured at a magnification of 200 at a fluorescent microscope with the same exposure time for experimental and negative control groups. Image analysis was performed using NIS Elements AR image analysis software. The percentage of immunopositive cells and mean fluorescence intensity were measured.

In vitro scratch wound assay

NHEK cells were treated with low (5 mM d-glucose) or high (25 mM d-glucose) glucose for 5 d and transfected with FOXO1 or scrambled siRNA. Confluent cells were “scratched” using a 200-µl pipette tip and rinsed with PBS to remove scratched cells as described previously (Liang et al., 2007). Images were captured as indicated to assess the number of keratinocytes that had migrated into the wounded area using NIS-elements D image analysis software.

Keratinocytes transwell migration assay

NHEK cells were incubated in indicated doses of d-glucose (5, 12.17, and 25 mM) or osmotic control (25 mM mannitol) medium for 5 d and transfected with FOXO1 or scrambled siRNA. In some cases, cells were treated with 2 ng/ml TGFβ1 for 24 h after transfection. Migration was then assessed in a transwell assay with a polycarbonate membrane filter (6.5-µm diameter and 8-µm pore size; Corning). In brief, 105 cells were placed in the upper chamber of a transwell plate. After 6 h, cells remaining in the upper surface of the membrane were removed with cotton swabs and migrated cells on the lower surface of the membrane were stained with DAPI and counted by fluorescence microscopy. Assays were performed as triplicates.

In some experiments, conditioned medium in high glucose conditions was first collected from primary keratinocytes isolated from K14.Cre:\:Foxo1L/L mice. The cells were preincubated with 0.4 µg/ml CCL20 blocking antibody (R&D Systems) or matched control IgG and incubated in conditioned medium plus antibody to CCL20 or matched control IgG, which were added to the upper chamber of a transwell plate. In some cases, murine keratinocytes were first transfected with 5 nM Serpinb2 siRNA (Santa Cruz Biotechnology, Inc.), and then the cells were incubated in conditioned medium and added to the upper chamber to measure the effect on keratinocyte migration.

In some experiments, murine keratinocytes isolated from K14.Cre:\:Foxo1L/L and K14.Cre:\:Foxo1L/L mice were first incubated with high glucose (25 mM d-glucose) for 5 d, CML-BSA (200 µg/ml) for 3 d, or TNF (10 ng/ml) for 1 d before migration assay. In other experiments, keratinocytes were also incubated in high glucose, CML-BSA, or TNF with the addition of insulin (10⁻⁸ or 10⁻⁷ M) with or without 5 mM AKT inhibitor tricribine (Abcam) for the final 24 h. After incubation, keratinocyte migration was measured in a transwell migration assay.

DNA binding capacity of FKHR (FOXO1)

The effect of high glucose on FOXO1 binding to DNA was measured by ELISA using TransAM FKHR (FOXO1) Transcription Factor ELISA kit (Active Motif). In brief, nuclear extract of NHEK cells was prepared and DNA binding of FOXO1 was performed following the manufacturer’s instructions.

BrdU incorporation assay

DNA synthesis was measured by ELISA using a BrdU Cell Proliferation Assay kit (Cell Signaling Technology). NHEK cells and primary murine keratinocytes isolated from K14.Cre:\:Foxo1L/L and K14.Cre:\:Foxo1L/L mice were treated in low (5 mM d-glucose) or high (25 mM d-glucose) glucose conditions for 5 d, during which NHEK cells were transfected with FOXO1 or scrambled siRNA. Then, 2 × 10⁵ cells were seeded in 96-well plates and incubated with 10 µM BrdU for 6 h. ELISA for BrdU-incorporated keratinocytes was then performed according to the manufacturer’s instruction. Experiments were performed three times with similar results.

Western blotting

Primary keratinocytes isolated from K14.Cre:\:Foxo1L/L mice were incubated in low (5 mM d-glucose) or high (25 mM d-glucose) glucose medium without insulin for 5 d, and then were lysed with lysis buffer (Thermo Fisher Scientific). The NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Thermo Fisher Scientific) was used to separately isolate cytoplasmic and nuclear protein fractions. Proteins (50 µg of cell lysate) were resolved by 4–20% SDS-PAGE (Bio-Rad Laboratories) and transferred onto PVDF membrane (Bio-Rad Laboratories) and then the cells were incubated in conditioned medium and incubated with 10 µM BrdU for 6 h. ELISA for BrdU-incorporated keratinocytes was then performed according to the manufacturer’s instruction. Western blotting reagents (Thermo Fisher Scientific).

ChIP

ChIP assays were performed using ChIP-IT kit (Active Motif) following the manufacturer’s instructions. NHEK cells were incubated in high glucose for 5 d, CML-BSA (200 µg/ml) or unmodified BSA (200 µg/ml) for 24 h, or TNF (10 ng/ml) for 1 h in culture media without insulin before lysing cells. To precipitate FOXO1, anti-FOXO1 antibody (Santa Cruz Biotechnol- ogy, Inc.) was used. Quantitative real-time PCR for TGFB1, SERPINB2, and CCL20 promoters were performed, respectively, using immunoprecipitated chromatin with probes (Roche) and oligonucleotide primers (Integrated DNA Technologies). The following primers were used: TGFB1 (forward, 5’-CCAT GTTGACAGACCTCTCTT3’; and reverse, 5’-TAAATCCGGGATGACAC3’).

Published April 27, 2015 jcb.rupress.org Downloaded from job.rupress.org on November 19, 2015

301
SERPINC2 (forward, 5′-GAACGACAGAAAGCAGAAAGAAG-3′; and reverse, 5′-ACTGCCCAAGGAAAGGATAAC-3′); and CCL20 (forward, 5′-TCTGATAGGCATCAACCCT-3′; and reverse, 5′-GAATCTCCTCAATGACCAATAATG-3′).

Luciferase reporter assay

Transient transfection with luciferase reporter constructs was performed using Lipofectamine (Invitrogen) in 24-well plates. In brief, NIH/3T3 cells were incubated in culture media without insulin for 5 d with 5 or 25 mM glucose or with CMLB5A (200 μg/ml) for 3 d or TNF (10 ng/ml) for 24 h before assay. Cells were cotransfected with TGFβ1 luciferase reporter (provided by D. Sinnett, University of Montreal, Montreal, Quebec, Canada), SERPINC2 luciferase reporter (provided by T.M. Antalis, University of Maryland School of Medicine, Baltimore, MD), or CCL20 luciferase reporter (provided by J.-M. Wang, National Cheng Kung University, Tainan, Taiwan) together with pRL-TK luciferase control vector, FOXO1-AAA plasmid that is constitutively transported to the nucleus, or pcDNA3.1 control plasmid. 2 d after transfection, cells were lysed, and Firefly and Renilla luciferase activities were measured using Dual Luciferase Reporter Assay kit (Promega) according to the manufacturer’s instructions. Firefly luciferase activities were divided by Renilla activities to normalize for transfection efficiency. Experiments were performed three times with similar results. In some experiments, primary murine keratinocytes from K14.Cre(+/-) and K14.Cre(-/-); Foxo1(−/−) and Foxo1(+/-) mice in low (5 mM glucose) or high (25 mM glucose) glucose medium or media supplemented with mannitol osmotic control. Table S1 presents a list of genes that were up-regulated in high glucose conditions media or media supplemented with mannitol osmotic control. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201409032/DC1.

Glucose levels of the mice after multiple low dose streptozotocin or citrate buffer injections. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201409032/DC1.

We thank Dr. R.A. DePinho for Foxo1 mice, Dr. D. Sinnett for TGFβ1 reporter, Dr. Toni M. Antalis for SERPINC2 reporter, and Dr. Ju-Ming Wang for CCL20 reporter. The authors would like to thank Sunitha Batchu for help in preparing this manuscript. The authors would also like to thank Deepthi Kunchey and Bharath V.K. Seekantam for assistance with genotyping.

The authors declare no competing financial interests.

Submitted: 5 September 2014
Accepted: 19 March 2015

References


We thank Dr. R.A. DePinho for Foxed FOXO1 mice, Dr. D. Sinnett for TGFβ1 reporter, Dr. Toni M. Antalis for SERPINC2 reporter, and Dr. Ju-Ming Wang for CCL20 reporter. The authors would like to thank Sunitha Batchu for help in preparing this manuscript. The authors would also like to thank Deepthi Kunchey and Bharath V.K. Seekantam for assistance with genotyping.

The authors declare no competing financial interests.

Published April 27, 2015