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Foxo1 Inhibits Diabetic Mucosal Wound Healing but Enhances Healing of Normoglycemic Wounds

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Re-epithelialization is an important part in mucosal wound healing. Surprisingly little is known about the impact of diabetes on the molecular events of mucosal healing. We examined the role of the transcription factor forkhead box O1 (Foxo1) in oral wounds of diabetic and normoglycemic mice with keratinocyte-specific Foxo1 deletion. Diabetic mucosal wounds had significantly delayed healing with reduced cell migration and proliferation. Foxo1 deletion rescued the negative impact of diabetes on healing but had the opposite effect in normoglycemic mice. Diabetes in vivo and in high glucose conditions in vitro enhanced expression of chemokine (C-C motif) ligand 20 (CCL20) and interleukin-36γ (IL-36γ) in a Foxo1-dependent manner. High glucose–stimulated Foxo1 binding to CCL20 and IL-36γ promoters and CCL20 and IL-36γ significantly inhibited migration of these cells in high glucose conditions. In normal healing, Foxo1 was needed for transforming growth factor-β1 (TGF-β1) expression, and in standard glucose conditions, TGF-β1 rescued the negative effect of Foxo1 silencing on migration in vitro. We propose that Foxo1 under diabetic or high glucose conditions impairs healing by promoting high levels of CCL20 and IL-36γ expression but under normal conditions, enhances it by inducing TGF-β1. This finding provides mechanistic insight into how Foxo1 mediates the impact of diabetes on mucosal wound healing.

Mucosal surfaces are subjected to frequent trauma. Wounding of mucosal surfaces involves disruption of the epithelium, which is an interface between the external environment and underlying connective tissue and serves as an important barrier against pathogenic microbes (1,2). Mucosal and cutaneous wound healing proceeds through similar processes, including hemostasis, inflammation, repair, and remodeling (3), whereas mucosal wounds demonstrate accelerated healing and less scar formation compared with cutaneous wounds (4). Re-epithelialization by mucosal epithelial cells is important for normal healing and is driven by migration and proliferation (5).

Diabetes has been reported to decrease production of growth factors, such as epidermal growth factor, transforming growth factor-β1 (TGF-β1), and insulin-like growth factor 1, and to increase the levels of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6, during mucosal wound healing (5). High glucose levels in vitro lead to the production of proinflammatory cytokines, such as TNF-α, and increased expression of receptor for advanced glycation end products (6), which are linked to impaired mucosal re-epithelialization (7). Previous reports have shown that diabetes and high glucose conditions negatively affect

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cutaneous healing primarily by decreasing keratinocyte migration and proliferation (8–12).

Forkhead box O1 (Foxo1), which belongs to a large family of forkhead transcription factors, participates in a wide range of cellular processes, including cell cycle arrest, DNA repair, apoptosis, oxidative stress resistance, and glucose metabolism (13–15). Compared with skin wounds in normoglycemic mice, Foxo1 DNA binding is increased in diabetic skin wounds through an TNF-α–mediated mechanism (16). Keratinocyte-specific Foxo1 deletion delays skin wound closure in vivo in normoglycemic mice and re-epithelialization in vitro in standard glucose media (17). Chemokine (C-C motif) ligand 20 (CCL20) is another proinflammatory cytokine shown to be upregulated during cutaneous wound healing (18). Interleukin-36γ (IL-36γ) is a cytokine that has been linked to inflammation (19,20). Despite that CCL20 and IL-36γ are expressed in inflamed skin (20,21), their role in regulating re-epithelialization has not been investigated.

To study the impact of Foxo1 on mucosal re-epithelialization, we examined normal and diabetic tongue wounds and determined whether Foxo1 deletion plays an important role in the healing process. The results indicate that Foxo1 plays an important but different role in re-epithelialization of normal and diabetic mucosal wounds. In normal conditions, Foxo1 promotes mucosal repair, whereas it inhibits repair under diabetic conditions.

RESEARCH DESIGN AND METHODS

Mice

All the animal experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Lineage-specific Foxo1 deletion was conducted according the methods described in a previous study to generate experimental (K14.Cre^{+}.*Foxo1*$^{L/L}$) and control (K14.Cre^{-}.*Foxo1*$^{L/L}$) mice (17). Experiments were performed with adult mice aged 16–20 weeks. Type 1 diabetes was induced by multiple low-dose (40 mg/kg) intraperitoneal injections of streptozotocin (Sigma, St. Louis, MO) dissolved in 10 mmol/L citrate buffer daily for 5 days. Control mice were treated identically with vehicle alone. Mice were considered to be hyperglycemic when blood glucose levels were >220 mg/dL. The blood glucose levels of mice are shown in Supplementary Table 1. Diabetic mice did not exhibit signs of distress and had normal behavior with no sign of lethargy after streptozotocin injection. Wound healing experiments were started when mice had been hyperglycemic for at least 3 weeks. All mice were maintained in a regular 12-h day-night cycle.

Mucosal Healing In Vivo

Mice were anesthetized by intraperitoneal injection of ketamine 80 mg/kg and xylazine 5 mg/kg. An excisional wound was made with a 1.5-mm sterile biopsy punch on the dorsal surface of the tongue as previously described (22). Mice were killed to collect the wounded tissue 1 or 2 days after wounding. Six mice per group were examined at each time point for all in vivo experiments.

Analysis of In Vivo Specimens

Excised specimens were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Five-micrometer paraffin sections were stained with hematoxylin-eosin, and histomorphometric analysis was performed with Nikon NIS-Elements microscope image analysis software (Nikon Instruments Inc., Melville, NY) at the center of each lesion (23,24). The degree of wound healing, defined as slight (wound diameter <50% of the original wound gap), moderate (wound diameter 50–80%), or high (wound diameter >80%) as modified from a previously published scale (23), was analyzed. The number of neutrophils per field was quantified in hematoxylin-eosin–stained sections 1 or 2 days after healing. Immunofluorescent analysis with histologic sections was carried out following the method described previously (17) with primary antibodies specific for Foxo1 (Santa Cruz Biotechnology, Santa Cruz, CA), Ki-67 (Leica Biosystems, Newcastle, U.K.), urokinase-type plasminogen activator receptor (uPAR) (Santa Cruz Biotechnology), CCL20 (Abcam, Cambridge, MA), and IL-36γ (Santa Cruz Biotechnology). Images were captured using a fluorescence microscope (Eclipse 90i; Nikon Instruments Inc.) and a CoolSNAP EZ camera (Photometrics, Tucson, AZ). Image analysis was performed using NIS-Elements AR image analysis software (Nikon Instruments Inc.).

Cell Culture and Transfection

Primary human mucosal epithelial (PHME) cells from the oral cavity were purchased from CELLnTEC Advanced Cell Systems (Bern, Switzerland) and maintained in KGM-2 growth medium (Lonza, Walkersville, MD) supplemented with human keratinocyte growth supplements (Lonza) and antibiotics (Life Technologies, Gaithersburg, MD). Cells were grown in a humidified atmosphere with 5% CO_2 at 37°C. For some experiments, cells were cultured for 5 days in media containing 25 mmol/L D-glucose (high glucose media) and compared with 5 mmol/L D-glucose (standard glucose media) or 25 mmol/L mannitol osmotic control. Small interfering RNA (siRNA) against human Foxo1 and control scrambled siRNA were purchased from Dharmacon (Chicago, IL) with GenMute siRNA Transfection Reagent (SignaGen, Rockville, MD). Experiments were performed 2 days after transfection.

Transwell Migration Assay

PHME cells were incubated in standard or high glucose media for 5 days and transfected with Foxo1 or scrambled siRNA. In some cases, cells were treated with 2 ng/mL TGF-β1 or insulin (Santa Cruz Biotechnology) for 24 h before transwell migration assay. A concentration of 10^5 PHME cells was added to the upper transwell chamber (Corning Costar; Thermo Fisher Scientific, Waltham, MA) with a polycarbonate membrane filter (6.5-mm diameter, 8-μm pore size). After 6–8 h, cells remaining in the upper surface of the membrane were removed, and

migrated cells on the lower surface of the membrane were counted by fluorescence microscopy after staining with DAPI. For migration assays with conditioned media, cells were incubated in standard (5 mmol/L) or high glucose (25 mmol/L) media for a total of 5 days, with media changed for the last 48 h and collected. In some cases, neutralizing antibodies or cytokines were added to the standard or high glucose–conditioned media and then tested in migration assays. Before testing, assay cells were preincubated in either high or standard glucose media for 5 days.

BrdU Cell Proliferation Assay

Proliferation was measured by BrdU incorporation with a BrdU Cell Proliferation Assay kit (Cell Signaling Technology, Beverly, MA). PHME cells were seeded into 96-well plates (2×10^4 cells/well) and incubated for 24 h in test media with the addition of 10 μmol/L BrdU added for the final 6 h. Cells were fixed with cold methanol and incubated with anti-BrdU antibody. BrdU incorporation was determined by measuring absorbance at 450 nm.

IL-36γ ELISA

PHME cells were incubated in standard or high glucose media for a total of 5 days and transfected with Foxo1 or scrambled siRNA after the second day. Two days after transfection, standard or high glucose–conditioned media were collected, and the concentration of IL-36γ was measured by ELISA according to the manufacturer's instructions (Aviscera Bioscience Inc., Santa Clara, CA).

Immunofluorescence In Vitro

PHME cells were grown on 8-well chamber slides (Thermo Fisher Scientific) and incubated in standard or high glucose media for 5 days. In some cases, cells were treated with insulin for 24 h before immunofluorescence. In other cases, cells were transfected with Foxo1 or scrambled siRNA and cultured for another 2 days. Cells were fixed with cold methanol and incubated with anti-Foxo1 (Santa Cruz Biotechnology), anti-CCL20 (Abcam), anti-IL-36γ (Santa Cruz Biotechnology), or matched control (R&D Systems, Minneapolis, MN) antibodies overnight at 4°C. Primary antibody was localized with biotinylated secondary antibody and Alexa 546–conjugated streptavidin. Slides were mounted with DAPI-containing mounting medium. Mean fluorescence intensity was measured by NIS-Elements AR image analysis software, with a maximum fluorescence intensity set at 3,000 arbitrary units to obtain results in the linear response range.

Microarray Analysis and Real-Time PCR

Normal human epidermal keratinocytes were incubated in standard or high glucose media for 5 days followed by transfection with Foxo1 or scrambled siRNA. RNA was isolated using an RNeasy kit (QIAGEN, Valencia, CA), and mRNA profiling was performed using an Affymetrix GeneChip Human Gene 1.0 ST array (Affymetrix, Santa Clara, CA). Genes that exhibited a 1.3-fold upregulation by high glucose and mRNA levels reduced >0.7-fold by Foxo1 knockdown were of interest.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed using ChIP-IT Kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions. To precipitate Foxo1, anti-Foxo1 antibody was used, and the quantitative real-time PCR of CCL20 and IL-36γ promoters was performed.

Statistics

Statistical analysis between two groups was performed using two-tailed Student *t* test. In experiments with multiple time points or treatments, significant differences were determined by ANOVA with Scheffé post hoc test. Results are expressed as the mean ± SEM. $P < 0.05$ was considered statistically significant.

RESULTS

Keratinocyte-Specific Foxo1 Deletion Improves Mucosal Wound Healing in Diabetic Mice but Impairs Healing in Normoglycemic Mice

Excisional tongue wounds were created in experimental transgenic mice with keratinocyte-specific Foxo1 deletion (K14.Cre$^+$.*Foxo1*$^{L/L}$) and in littermate control mice (K14.Cre$^-$.*Foxo1*$^{L/L}$) to examine mucosal wound healing. Quantitative immunofluorescence with Foxo1-specific antibody indicated that the mean fluorescence intensity of Foxo1 in the healing epithelium of experimental mice with lineage-specific Foxo1 deletion was decreased by a minimum of 75% compared with control mice ($P < 0.05$) (Fig. 1*A* and *B*). Wounding stimulated a 3.4-fold increase in Foxo1 nuclear localization in epithelium of normoglycemic control mice ($P < 0.05$). Nuclear localization of Foxo1 was increased an additional 2.6-fold in wounded diabetic mice ($P < 0.05$) (Fig. 1*C*). In vitro, Foxo1 siRNA reduced Foxo1 mRNA levels by 74%, demonstrating efficient knockdown in PHME cells (Fig. 1*D*). High glucose media increased Foxo1 nuclear localization by 89% ($P < 0.05$) as measured by colocalization of Foxo1 immunofluorescent images with DAPI nuclear staining (Fig. 1*E*) or by deconvolution immunofluorescence microscopy (Supplementary Fig. 1). High glucose levels did not increase the overall level of Foxo1 expression in vitro (Fig. 1*E*), consistent with in vivo results. The increase in Foxo1 nuclear localization stimulated by high glucose was blocked by insulin in a dose-dependent relationship, almost completely blocking the effect of high glucose at 10 nmol/L without affecting the level of Foxo1 expression (Fig. 1*F* and *G*).

One day after wounding, mucosal wounds of diabetic control mice were 71% larger than corresponding wounds in matched normoglycemic mice ($P < 0.05$) (Fig. 2*A* and *B*). Foxo1 deletion in diabetic experimental mice improved mucosal healing by 63% compared with diabetic control mice ($P < 0.05$). In contrast, Foxo1 deletion in keratinocytes of experimental normoglycemic mice had the opposite effect, with wound gaps that were 37% larger than matched control mice ($P < 0.05$) (Fig. 2*A* and *B*). By day 2, 100% of the normoglycemic control mice had

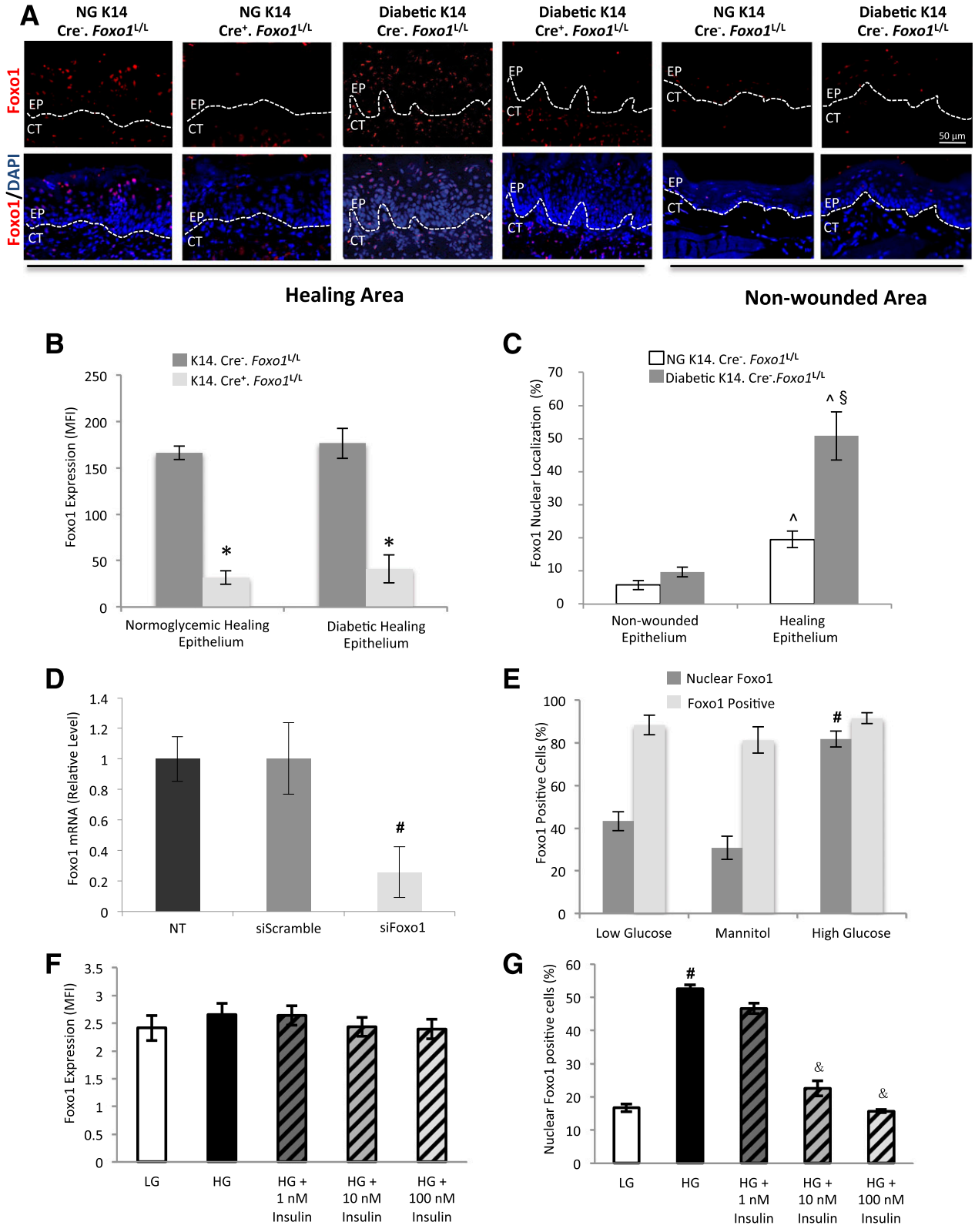

Figure 1—Diabetes and high glucose media increase Foxo1 nuclear localization in mucosal epithelial cells both in vivo and in vitro. Small mucosal wounds were created on the dorsal tongue surface. Diabetes was induced in mice by multiple low-dose streptozotocin injections. *A*: Immunofluorescent images of Foxo1 in the wounded and nonwounded mucosal epithelium of diabetic and normoglycemic experimental (K14.Cre^{+}.*Foxo1*$^{L/L}$) and control (K14.Cre^{-}.*Foxo1*$^{L/L}$) mice 1 day after wounding. No positive immunostaining was observed with matched control antibody (data not shown). Dashed lines demarcate the epithelial surface from the dermis. *B*: Expression of Foxo1 was measured by immunofluorescence expressed as mean fluorescence intensity. *C*: Foxo1 nuclear localization was determined by colocalization of Foxo1 and DAPI nuclear staining. *D*: Foxo1 mRNA level in PHME cells with or without transfection of Foxo1 siRNA or scrambled siRNA. *E*: Quantitation of Foxo1 nuclear localization in PHME cells cultured in standard or high glucose media. *F*: PHME cells were incubated in

a high degree of healing (>80% healing), whereas only 17% of the normoglycemic experimental mice had this degree of re-epithelialization (Fig. 2*C*). In contrast, 17% of the diabetic control mice had a high degree of healing, which was increased to 83% in the experimental diabetic group (Fig. 2*C*).

Foxo1 Deletion Partially Rescues the Negative Effect of Diabetes on Mucosal Epithelial Cell Migration In Vivo and the Inhibitory Effect of High Glucose on Migration In Vitro

To investigate whether Foxo1 affects cell migration in vivo, we examined the number of mucosal epithelial cells that expressed uPAR (Supplementary Fig. 2*A*), a marker of migrating epithelial cells in vivo (25). Diabetes significantly reduced by 83% the number of migrating mucosal epithelial cells in K14.Cre^{-}.*Foxo1*$^{L/L}$ mice ($P < 0.05$). However, Foxo1 deletion in diabetic mice partly reversed the negative impact of diabetes on mucosal epithelial cell migration ($P < 0.05$) (Fig. 3*A*). In contrast, Foxo1 deletion in normoglycemic mice reduced mucosal epithelial cell migration by 64% ($P < 0.05$). For in vitro migration assays, PHME cell migration in high glucose media was reduced 54% compared with standard media ($P < 0.05$). Foxo1 knockdown reversed most of the inhibition caused by high glucose ($P < 0.05$). In contrast, Foxo1 silencing decreased migration by 63% in standard media ($P < 0.05$) (Fig. 3*B*). Thus, there was a high degree of consistency regarding the impact of Foxo1 on migration of mucosal epithelial cells in hyperglycemic or high glucose conditions compared with normal conditions. Moreover, with an increased concentration of insulin, cell migration increased significantly in a dose-dependent manner in high glucose conditions in vitro ($P < 0.05$) (Fig. 3*C*).

Foxo1 Deletion Rescues the Negative Impact of Diabetes on Mucosal Epithelial Cell Proliferation In Vivo and Inhibitory Effect of High Glucose on Proliferation In Vitro

Immunofluorescence with antibody to Ki-67 was used to determine the impact of Foxo1 on mucosal epithelial cell proliferation in vivo (Supplementary Fig. 2*B*). Diabetes significantly reduced the number of proliferating mucosal epithelial cells by 54% compared with matched normoglycemic mice ($P < 0.05$). Foxo1 deletion in diabetic mice increased by 98% the number of proliferating mucosal epithelial cells ($P < 0.05$), whereas it did not significantly change cell proliferation in normoglycemic mice ($P > 0.05$) (Fig. 3*D*). For in vitro assays, high glucose media reduced by 33% PHME cell proliferation compared with standard media ($P < 0.05$), and this decrease was reversed by Foxo1 knockdown ($P < 0.05$) (Fig. 3*E*). Foxo1 silencing did not affect cell proliferation in standard media ($P > 0.05$) (Fig. 3*E*). Thus, Foxo1 deletion or knockdown reverses the effect of hyperglycemia in vivo or high glucose in vitro on mucosal epithelial cell proliferation but has little effect in normal conditions.

TGF-β1 Rescues the Negative Effect of Foxo1 Silencing on PHME Cell Migration

To determine how Foxo1 deletion may affect normoglycemic mucosal wound healing (Fig. 2*A*–*C*), TGF-β1 was evaluated as we previously reported for cutaneous healing (17). Foxo1 deletion in normoglycemic mice decreased TGF-β1 expression by 61% in mucosal wounds ($P < 0.05$) (Fig. 4*A*); however, it had the opposite effect in diabetic wounds ($P < 0.05$) (Fig. 4*A*). In vitro, TGF-β1 had a small, but significant effect on stimulating PHME cell migration in standard media, whereas Foxo1 silencing reduced it by almost 60% ($P < 0.05$). However, TGF-β1 had a much greater effect on PHME cell migration when Foxo1 was silenced, increasing it by 2.3-fold ($P < 0.05$). In contrast, TGF-β1 had little effect when cells were incubated in high glucose media ($P > 0.05$) (Fig. 4*B*). Thus, TGF-β1 rescued the deficit in PHME migration caused by Foxo1 knockdown in standard glucose media. Foxo1 silencing in high glucose conditions significantly enhanced PHME migration, suggesting that Foxo1 in high glucose media regulated the expression of factors that inhibit migration.

CCL20 and IL-36γ Expression Is Increased by Diabetes, and Both Inhibit Mucosal Epithelial Cell Migration in High Glucose

CCL20 is a chemokine expressed by keratinocytes (18) and regulated by Foxo1 under high glucose conditions (Supplementary Table 2). Diabetes increased the number of CCL20-positive mucosal epithelial cells in wounded tissue by 74% ($P < 0.05$), whereas Foxo1 deletion reduced CCL20-positive cells by ~75% in both diabetic and normoglycemic mice ($P < 0.05$) (Fig. 5*A* and Supplementary Fig. 3). High glucose media in vitro stimulated an ~65% increase in CCL20 mRNA and protein levels ($P < 0.05$) (Fig. 5*B* and *C*). This increase was completely blocked by Foxo1 siRNA ($P < 0.05$) (Fig. 5*B* and *C*). ChIP assays demonstrated that Foxo1 binds to the CCL20 promoter and that high glucose conditions increase this binding by

standard or high glucose media followed by treatment of indicated doses of insulin for 24 h; expression of Foxo1 in cells was measured by immunofluorescence, with data expressed as mean fluorescence intensity. *G*: Cells were prepared as in *F*, and Foxo1 nuclear localization was quantified. In vivo data are mean ± SEM ($n = 6$ mice per group). In vitro data are mean ± SEM of three independent experiments. *$P <$ 0.05 vs. normoglycemic or diabetic K14.Cre^{-}.*Foxo1*$^{L/L}$ mice; ^$P < 0.05$ vs. matched normoglycemic or diabetic nonwounded epithelium; §$P < 0.05$ vs. matched normoglycemic or diabetic healing epithelium; #$P < 0.05$ vs. scrambled siRNA transfection group or standard media control group; &$P < 0.05$ vs. high glucose media control group. CT, connective tissue; EP, epithelial surface; HG, high glucose; LG, low glucose; MFI, mean fluorescence intensity; NG, normoglycemic; NT, nontransfected; siFoxo1, Foxo1 siRNA; siScramble, scrambled siRNA.

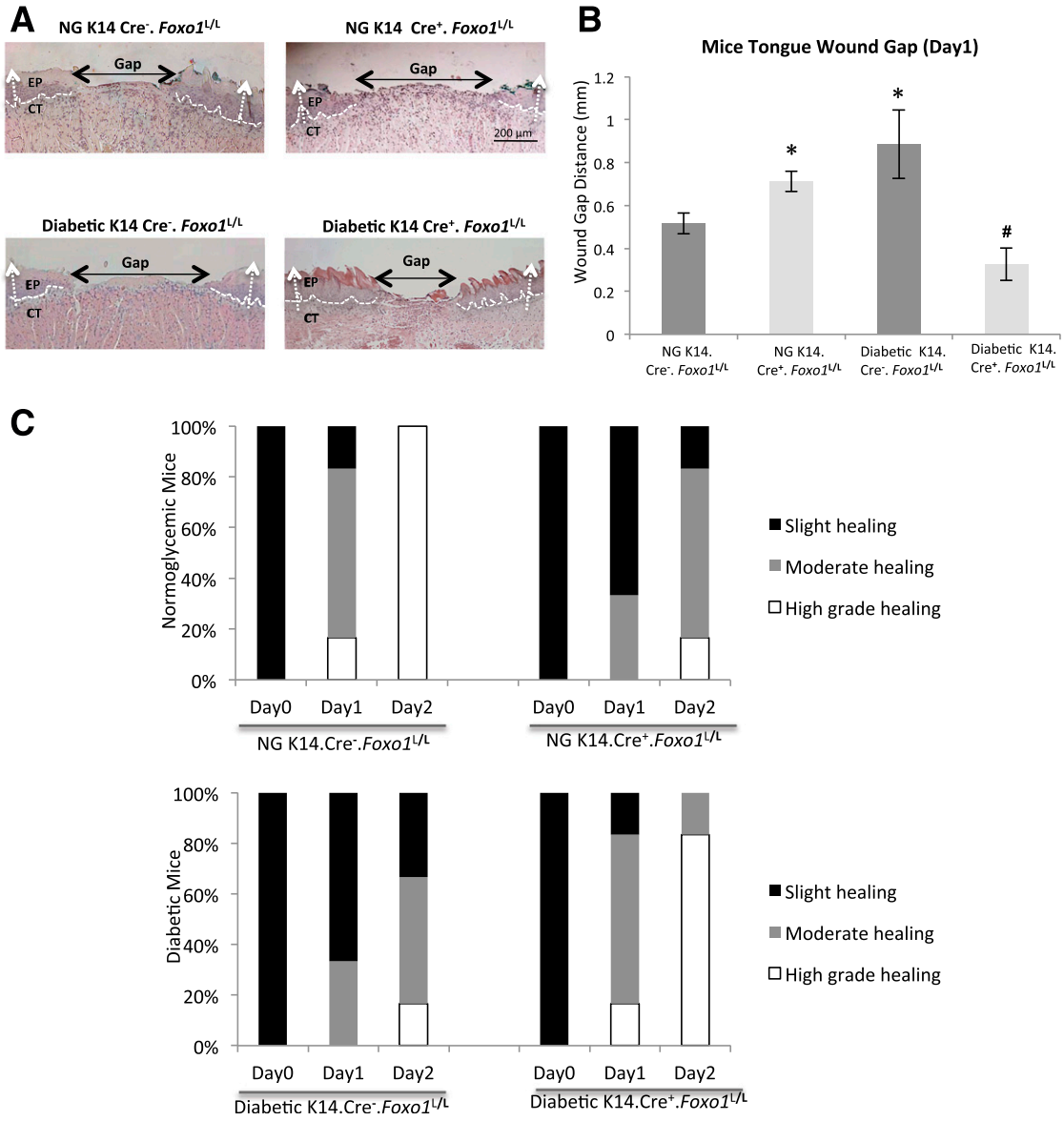

Figure 2—Keratinocyte-specific Foxo1 deletion impairs mucosal healing in normoglycemic mice, whereas it accelerates healing in diabetic mice. Wounds were created in normal and diabetic mice as described in Fig. 1. *A*: Representative photomicrographs of mucosal wounds of diabetic and normoglycemic experimental (K14.Cre^{+}.*Foxo1*$^{L/L}$) and control (K14.Cre^{-}.*Foxo1*$^{L/L}$) mice 1 day after wounding. Dashed lines demarcate the epithelium from the dermis; white arrows indicate demarcation between normal and wounded epithelia. *B*: The distance between the wound edges was measured in histologic sections. *C*: The degree of healing was stratified into three groups: slight healing (wound diameter <50% of the original wound gap); moderate healing (wound diameter 50–80%); and high-grade healing (wound diameter >80%). Data are mean ± SEM (n = 6 mice per group). *P < 0.05 vs. normoglycemic K14.Cre^{-}.*Foxo1*$^{L/L}$ mice; #P < 0.05 vs. diabetic K14.Cre^{-}.*Foxo1*$^{L/L}$ mice. CT, connective tissue; EP, epithelial surface; NG, normoglycemic.

>2.5-fold (Fig. 5*D*). Thus, hyperglycemia and high glucose increase CCL20 expression in a Foxo1-dependent manner in mucosal epithelial cells in vivo and in vitro. PHME cell migration was tested in vitro. High glucose–conditioned media was 50% less effective than low glucose–conditioned media in stimulating migration ($P < 0.05$) (Fig. 5*E* and *F*). The potential impact of CCL20 was assessed with CCL20 blocking antibody. CCL20 antibody increased by 1.6-fold the stimulation of high glucose–conditioned media on PHME migration ($P < 0.05$) (Fig. 5*E*). In contrast, the addition of CCL20 ligand to standard glucose–conditioned media reduced cell migration by up to 54% (Fig. 5*F*), indicating that CCL20 at high levels can impair migration.

IL-36γ, also known as IL-1F9, is a proinflammatory cytokine that induces neutrophil influx (19,26) and is regulated by Foxo1 (Supplementary Table 2). Diabetes increased fourfold the number of IL-36γ–positive mucosal epithelial cells; IL-36γ levels were reduced 80% by Foxo1 deletion in diabetic experimental mice compared with

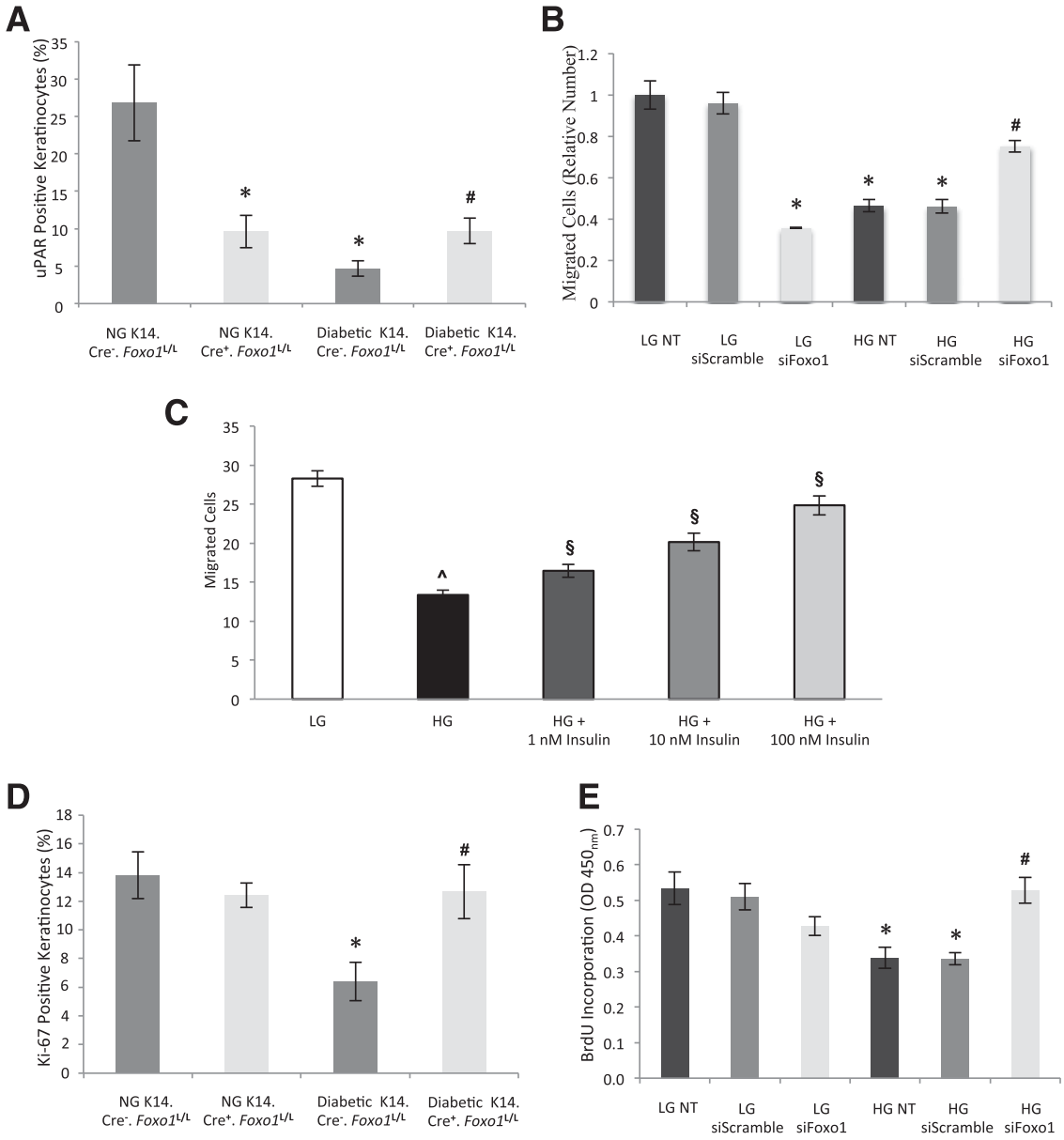

Figure 3—Foxo1 deletion reduces the number of migrating mucosal epithelial cells under normal conditions but improves migration and proliferation in diabetic mice in vivo or in high glucose media in vitro. Wounds were created in normal and diabetic mice as described in Fig. 1. *A*: The percentage of migrating epithelial cells was determined by immunofluorescence with antibody specific for the migration marker uPAR in healing mucosal wounds of diabetic and normoglycemic experimental (K14.Cre^{+}.*Foxo1*$^{L/L}$) and control (K14.Cre^{-}.*Foxo1*$^{L/L}$) mice 1 day after wounding, as measured by immunofluorescence. *B*: PHME cells were incubated in standard or high glucose media for 5 days followed by transfection with Foxo1 siRNA or control siRNA. Cell migration was assessed by transwell migration assay. *C:* Cells were incubated in standard or high glucose media followed by treatment with indicated doses of insulin for 24 h, then cell migration was assessed by transwell migration assay. *D*: Quantitation of Ki-67 expression in healing mucosal wounds measured by immunofluorescence. *E*: PHME cell proliferation was determined by BrdU incorporation after transfection with siRNA. In vivo data are mean ± SEM (n = 6 mice per group). In vitro data are mean ± SEM of three independent experiments. *P < 0.05 vs. matched normoglycemic K14.Cre^{-}.*Foxo1*$^{L/L}$ mice or cells incubated in standard media and transfected with scrambled siRNA; #P < 0.05 vs. diabetic K14.Cre^{-}.*Foxo1*$^{L/L}$ mice or cells incubated in high glucose media and transfected with scrambled siRNA; ^P < 0.05 vs. cells incubated in standard media without insulin treatment; §P < 0.05 vs. cells incubated in high glucose media without insulin treatment. HG, high glucose; LG, low glucose; NG, normoglycemic; NT, nontransfected; siFoxo1, Foxo1 siRNA; siScramble, scrambled siRNA.

diabetic control mice ($P < 0.05$) (Fig. 6*A* and Supplementary Fig. 4). High glucose in vitro increased mRNA levels of IL-36γ by 33% compared with standard media ($P < 0.05$), and this increase was blocked by Foxo1 knockdown ($P < 0.05$) (Fig. 6*B*). High glucose stimulated IL-36γ at the protein level by a similar extent in a Foxo1-dependent manner (Fig. 6*C*). Moreover, high glucose conditions increased recruitment of Foxo1 to the IL-36γ promoter fourfold, as determined by ChIP assay (Fig. 6*D*). When high glucose–conditioned media were incubated with

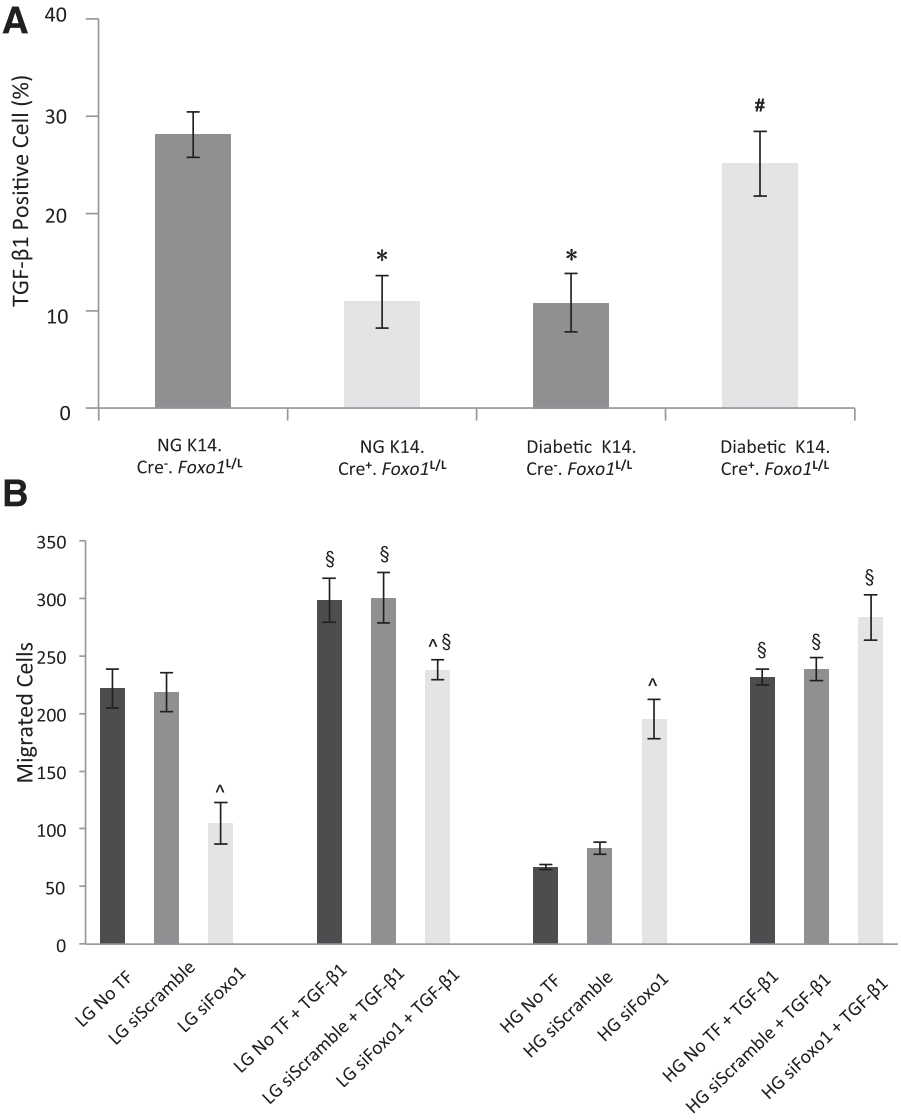

Figure 4—Foxo1 modulates normoglycemic wound healing through TGF-β1 pathway. *A*: TGF-β1 immunofluorescence of wounded epithelium of normoglycemic and diabetic experimental (K14.Cre^{+}.*Foxo1*$^{L/L}$) and control (K14.Cre^{-}.*Foxo1*$^{L/L}$) mice 1 day after wounding. *B*: PHME cells were incubated in standard or high glucose media for 5 days followed by transfection with Foxo1 siRNA or control siRNA and were treated with or without TGF-β1. Cell migration was assessed by transwell migration assay. In vivo data are mean ± SEM (n = 6 mice per group). In vitro data are mean ± SEM of three independent experiments. *P < 0.05 vs. normoglycemic K14.Cre^{-}.*Foxo1*$^{L/L}$ mice; #P < 0.05 vs. diabetic K14.Cre^{-}.*Foxo1*$^{L/L}$ mice; ^P < 0.05 vs. cells treated with or without TGF-β1 after scrambled siRNA transfection; §P < 0.05 vs. matched cells without TGF-β1 treatment. HG, high glucose; LG, low glucose; NG, normoglycemic; siFoxo1, Foxo1 siRNA; siScramble, scrambled siRNA; TF, transfected.

IL-36γ neutralizing antibody, cell migration increased 2.1-fold compared with control IgG ($P < 0.05$) (Fig. 6*E*). The addition of IL-36γ to standard glucose–conditioned media significantly inhibited PHME cell migration ($P < 0.05$) (Fig. 6*F*).

CCL20 and IL-36γ had an additive effect on inhibiting mucosal epithelial cell migration. Incubating high glucose–conditioned media with anti-CCL20 plus anti-IL-36γ–neutralizing antibodies increased PHME cell migration up to 4.4-fold ($P < 0.05$), which was significantly greater than antibody to CCL20 or IL-36γ alone (Fig. 7*A*). Of note, the blocking antibodies did not improve migration in high glucose when Foxo1 was silenced, suggesting that deletion of Foxo1 had a similar effect as inhibiting CCL20/IL-36γ (Fig. 7*B*). The relationship among glucose levels, CCL20/IL-36γ, and impaired PHME cells was further linked by the failure of inhibition of CCL20/IL-36γ to improve migration under low glucose conditions (Fig. 7*C*). In fact, CCL20/IL-36γ antibodies reduced keratinocyte migration, suggesting that a minimal level was needed for migration to occur normally. The concept that high levels of CCL20/IL-36γ interfere with migration was supported by findings that the addition of CCL20 plus IL-36γ ligand to standard glucose–conditioned media reduced

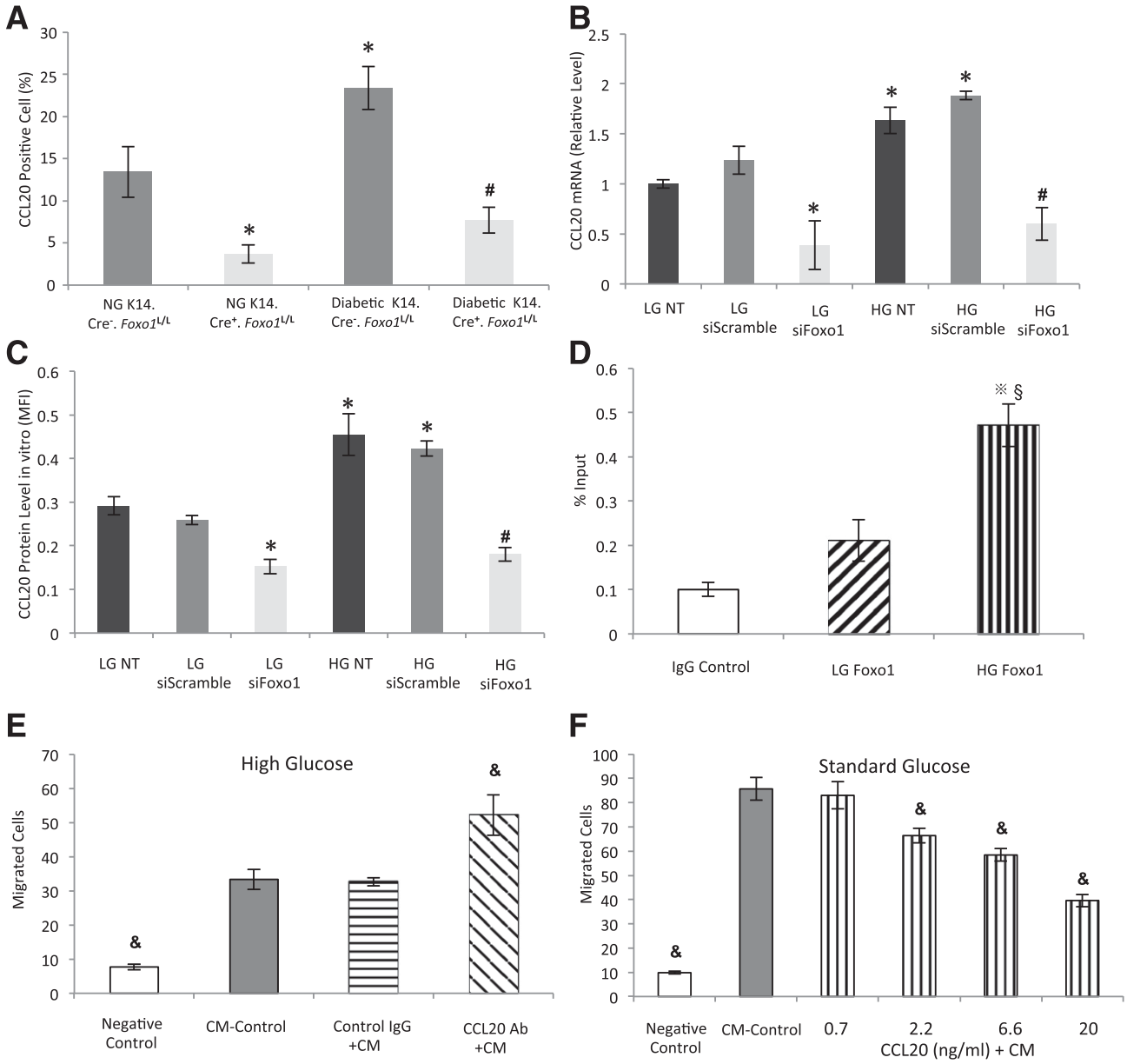

Figure 5—Foxo1 mediates CCL20 expression, and blocking CCL20 rescues keratinocyte migration impaired by high glucose. Wounds were created in normal and diabetic mice as described in Fig. 1. *A*: Quantitation of CCL20 expression in the healing mucosal epithelium of wounded tongues of diabetic and normoglycemic experimental (K14.Cre^{+}.*Foxo1*$^{L/L}$) and control (K14.Cre^{-}.*Foxo1*$^{L/L}$) mice 1 day after wounding. *B*: PHME cells were incubated in standard or high glucose media followed by transfection with Foxo1 siRNA or scrambled siRNA. Real-time PCR analysis was performed to measure CCL20 mRNA levels. *C*: Cells were prepared as in *B*, and CCL20 expression was measured by immunofluorescence, with data expressed as mean fluorescence intensity. *D*: ChIP assay for binding of Foxo1 to CCL20 promoter was performed, ChIP-enriched DNA was quantified by quantitative real-time PCR, and values are expressed as percentage of input DNA. *E*: Migration of PHME cells stimulated with conditioned media from PHME cells with or without 0.4 μg/mL CCL20 blocking antibody or normal goat IgG. Migration was examined by transwell assay. *F*: Conditioned media by PHME cells were tested for stimulation of PHME cell migration with or without addition of CCL20 ligand. In vivo data are mean ± SEM (n = 6 mice per group). In vitro data are mean ± SEM of three independent experiments. *P < 0.05 vs. normoglycemic K14.Cre^{-}.*Foxo1*$^{L/L}$ mice or cells incubated in standard glucose media and transfected with scrambled siRNA; #P < 0.05 vs. diabetic K14.Cre^{-}.*Foxo1*$^{L/L}$ mice or cells incubated in high glucose media and transfected with scrambled siRNA; ※P < 0.05 vs. IgG control group; §P < 0.05 vs. standard glucose experimental group; &P < 0.05 vs. conditioned media control group. Ab, antibody; CM, conditioned media; HG, high glucose; LG, low glucose; MFI, mean fluorescence intensity; NG, normoglycemic; NT, nontransfected; siFoxo1, Foxo1 siRNA; siScramble, scrambled siRNA.

mucosal epithelial cell migration by 67%, which was greater than the effect of each ligand alone (Fig. 7*D*). Moreover, when Foxo1 was silenced under high glucose conditions, the addition of CCL20 and IL-36γ ligand reversed the positive effect of Foxo1 silencing on PHME migration ($P < 0.05$) (Fig. 7*E*), agreeing with the results in Fig. 7*B* that the effect of Foxo1 mimics that of high levels of CCL20/IL-36γ.

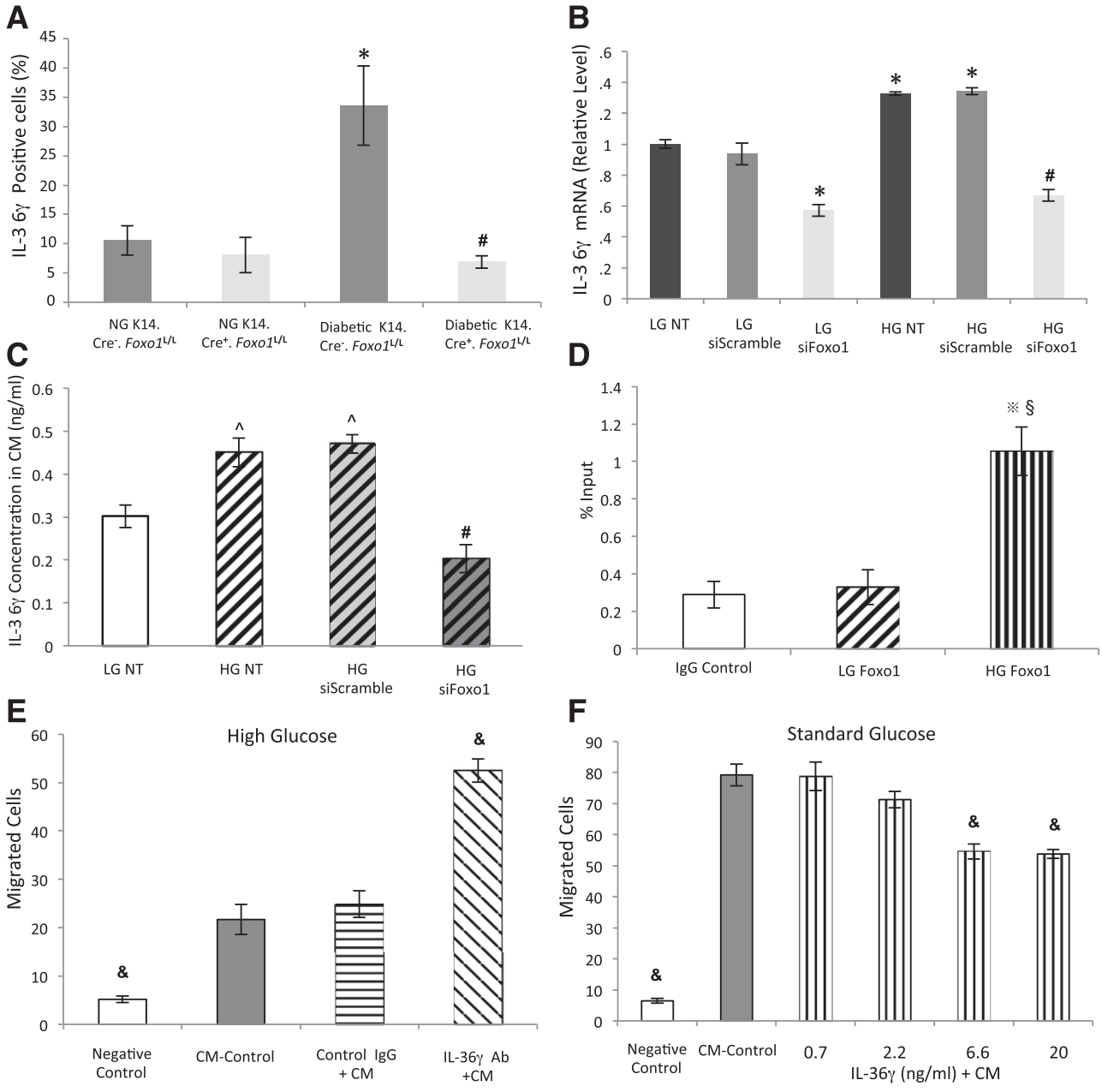

Figure 6—Diabetes enhances IL-36γ expression in a Foxo1-dependent manner, and blocking IL-36γ rescues keratinocyte migration impaired by high glucose conditions. Wounds were created in normal and diabetic mice as described in Fig. 1. *A*: Quantification of IL-36γ in the healing mucosa of wounded tongues of diabetic and normoglycemic experimental (K14.Cre^{+}.*Foxo1*$^{L/L}$) and control (K14.Cre^{-}.*Foxo1*$^{L/L}$) mice 1 day after wounding. *B*: PHME cells were incubated in standard or high glucose media followed by transfection with Foxo1 siRNA or control siRNA, then real-time PCR analysis was performed to measure IL-36γ mRNA levels. *C*: Two days after transfection with Foxo1 siRNA or control siRNA, standard or high glucose–conditioned media were collected, and the concentration of IL-36γ was measured by ELISA. *D*: ChIP assay for binding Foxo1 to IL-36γ promoter was performed, ChIP-enriched DNA was quantified by quantitative real-time PCR, and values are expressed as percentage of input DNA. *E:* Migration of PHME cells stimulated with conditioned media from PHME cells with or without 0.3 μg/mL IL-36γ blocking antibody or normal rat IgG was examined by transwell assay. *F*: Conditioned media by PHME cells were tested for stimulation of PHME cell migration with or without addition of IL-36γ. In vivo data are mean ± SEM (n = 6 mice per group). In vitro data are mean ± SEM of three independent experiments. *P < 0.05 vs. normoglycemic K14.Cre^{-}.*Foxo1*$^{L/L}$ group or cells incubated in standard glucose media and transfected with scrambled siRNA; #P < 0.05 vs. diabetic K14.Cre^{-}.*Foxo1*$^{L/L}$ mice or cells incubated in high glucose media and transfected with scrambled siRNA; ^P < 0.05 vs. standard glucose media without cells being transfected with siRNA; ※P < 0.05 vs. IgG control group; §P < 0.05 vs. standard glucose experimental group; &P < 0.05 vs. conditioned media control group. Ab, antibody; CM, conditioned media; HG, high glucose; LG, low glucose; NG, normoglycemic; NT, nontransfected; siFoxo1, Foxo1 siRNA; siScramble, scrambled siRNA.

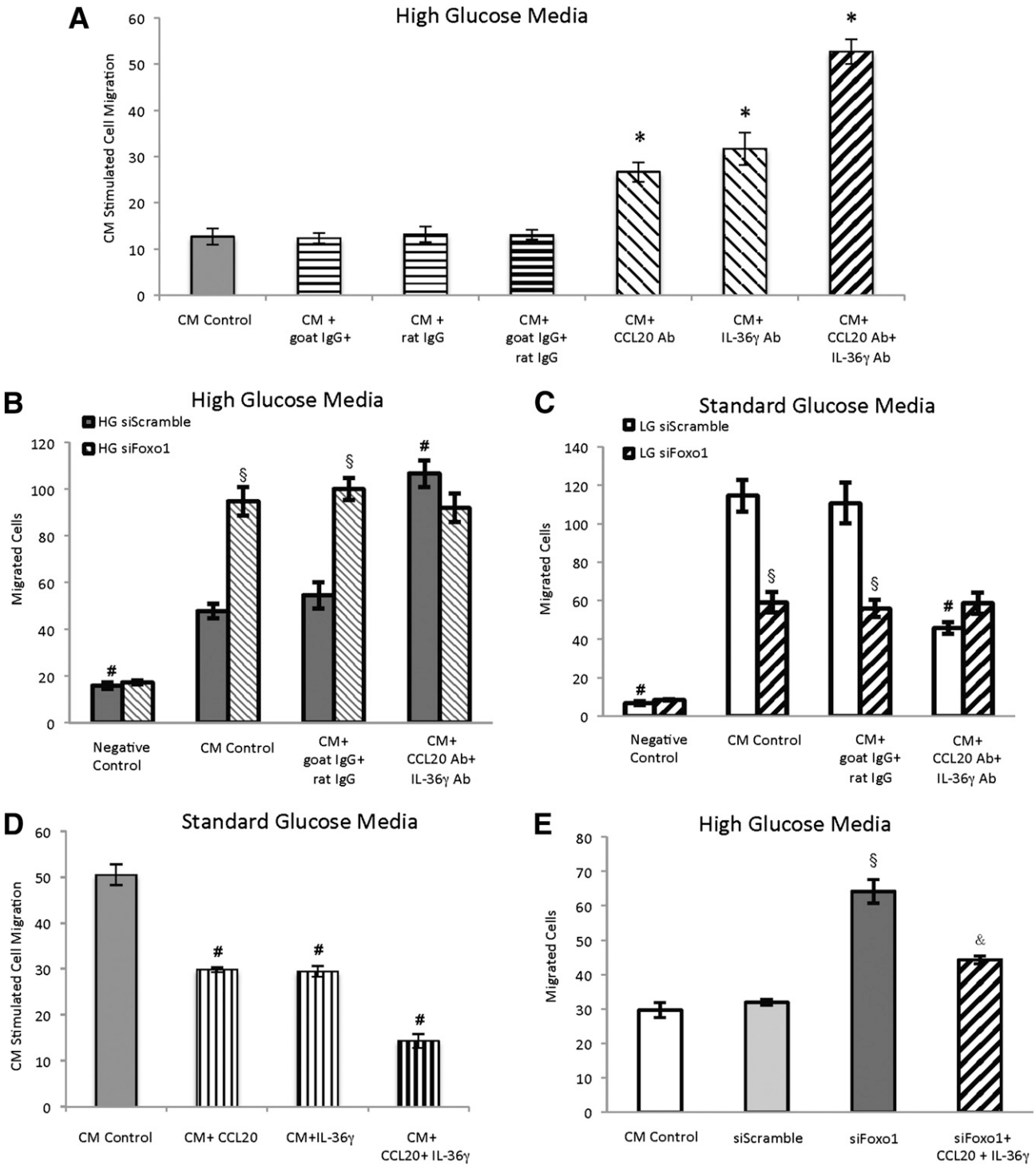

Figure 7—Foxo1 inhibits mucosal epithelial cell migration through CCL20 and IL-36γ pathways in high glucose conditions. PHME cells were incubated in standard or high glucose media for a total of 5 days. Conditioned media were collected for the last 48 h. *A*: High glucose–conditioned media were incubated with or without 0.4 μg/mL CCL20 and/or 0.3 μg/mL IL-36γ blocking antibody or normal goat and/or rat IgG. Cells were then tested for migration in a transwell assay. *B*: High glucose–conditioned media were incubated with or without 0.4 μg/mL CCL20 and 0.3 μg/mL IL-36γ blocking antibodies or normal goat and rat IgG after Foxo1 was deleted in PHME cells. Cells were then tested for migration. *C*: Standard glucose–conditioned media were incubated with or without 0.4 μg/mL CCL20 and 0.3 μg/mL IL-36γ blocking antibodies or normal goat and rat IgG after Foxo1 was deleted in PHME cells. Cells were then tested for migration. *D*: Standard glucose–conditioned media were incubated with or without 2.2 ng/mL CCL20 and/or 6.6 ng/mL IL-36γ ligand. Cells were then tested for migration. *E*: High glucose–conditioned media were incubated with or without 2.2 ng/mL CCL20 and 6.6 ng/mL IL-36γ after Foxo1 was deleted in PHME cells. Cells were then tested for migration. *$P < 0.05$ vs. matched group with control antibody; #$P < 0.05$ vs. conditioned media control group; §$P < 0.05$ vs. cells transfected with scrambled siRNA in high glucose or standard low glucose conditions; &$P < 0.05$ vs. cells without CCL20 or IL-36γ treatment after transfected with Foxo1 siRNA. Data are mean ± SEM of three independent experiments. Ab, antibody; CM, conditioned media; HG, high glucose; LG, low glucose, siFoxo1, Foxo1 siRNA; siScramble, scrambled siRNA.

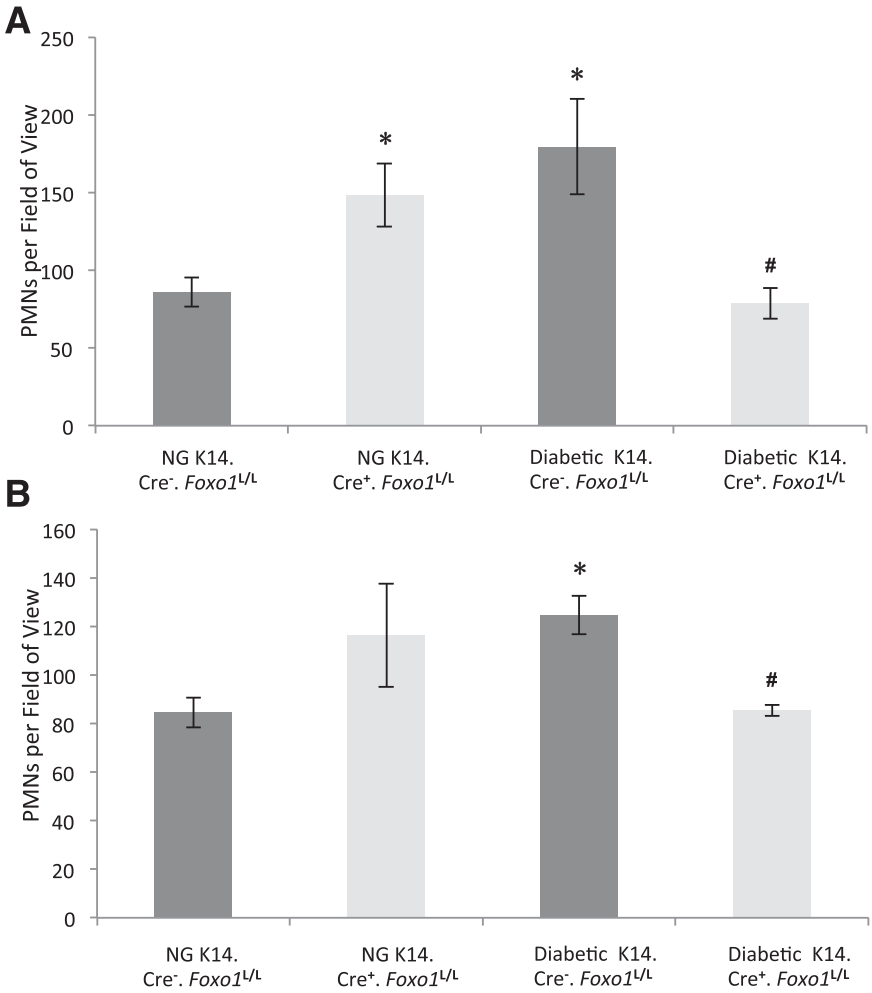

Figure 8—Keratinocyte-specific Foxo1 deletion decreases neutrophil infiltration in diabetic mice, whereas it increases neutrophil infiltration in normoglycemic mice. Number of neutrophils per field of view was quantified in the healing area of diabetic and normoglycemic experimental (K14.Cre^{+}.*Foxo1*$^{L/L}$) and control (K14.Cre^{-}.*Foxo1*$^{L/L}$) mice 1 day (*A*) or 2 days (*B*) after wounding. *$P < 0.05$ vs. normoglycemic K14.Cre^{-}.*Foxo1*$^{L/L}$ mice; #$P < 0.05$ vs. diabetic K14.Cre^{-}.*Foxo1*$^{L/L}$ mice. NG, normoglycemic; PMN, polymorphonuclear leukocyte.

Keratinocyte-Specific Foxo1 Deletion Decreases Neutrophil Infiltration in Diabetic Mice but Increases It in Normoglycemic Mice

To further examine how Foxo1 may affect wound healing through CCL20 and IL-36γ, we examined neutrophil infiltration. Diabetes increased neutrophil infiltration 2.1-fold 1 day after wounding ($P < 0.05$), whereas Foxo1 deletion in keratinocytes in diabetic mice reversed this increase ($P < 0.05$). In contrast, Foxo1 deletion in keratinocytes of normoglycemic mice increased neutrophil infiltration by 73% ($P < 0.05$) (Fig. 8*A*). Lineage-specific deletion of Foxo1 also had a significant effect on modulating neutrophil infiltration on day 2 (Fig. 8*B*).

DISCUSSION

In the current study, diabetic mice exhibited delayed mucosal wound healing compared with normoglycemic mice when small mucosal wounds were created on the dorsal tongue surface. One day after wounding, diabetic wound gaps were 71% larger than corresponding wounds in normoglycemic mice. All the wounds in normoglycemic mice had a high degree of healing after 2 days, whereas only 17% of the diabetic mice exhibited this degree of healing. Mechanistically, the impaired healing in diabetic animals can be explained by a large decrease in epithelial migration (83%) and a moderate decrease in proliferation (54%). The in vivo results were supported by in vitro experiments, which demonstrated that high glucose significantly reduced migration of oral epithelial cells by 54% and proliferation by 33%. Thus, diabetes affects healing of mucosal wounds by negatively affecting migration and proliferation. The result contrasts with cutaneous wound healing in which hyperglycemia has a profound effect on migration but relatively little effect on proliferation (27).

To identify the mechanism for altered healing, we examined mice with keratinocyte-specific deletion of Foxo1, which was found to be elevated in diabetic skin wounds in a previous study (16). Foxo1 nuclear

localization increased 2.6-fold in wounded mucosal epithelial cells in diabetic compared with normoglycemic mice. Foxo1 nuclear localization is indicative of its activation status (28). It is striking that deletion of Foxo1 rescued the impaired healing behavior of mucosal epithelial cells in vivo in diabetic mice and in vitro in high glucose media by improving migration and proliferation. Furthermore, insulin decreased Foxo1 nuclear localization and reversed the negative effect of high glucose on migration, suggesting that the combination of high glucose and insulin deficiency limits migration.

To understand how Foxo1 affects migration of mucosal epithelial cells, we examined CCL20 and IL-36γ, which are upregulated by wounding (18) and linked to inflammation (18–20). These in vivo studies established that CCL20 and IL-36γ expression was elevated in mucosal epithelial wounds of diabetic mice compared with normoglycemic mice. Keratin 14–driven Foxo1 deletion in vivo reduced both CCL20 and IL-36γ in diabetic animals, suggesting that their expression is driven by Foxo1. In vitro Foxo1 knockdown blocked high glucose–increased CCL20 and IL-36γ levels in PHME cells. ChIP assay results demonstrated that high glucose stimulated increased recruitment of Foxo1 to the promoters of CCL20 and IL-36γ. The result that Foxo1 enhances expression of CCL20 is consistent with previous reports that Foxo1 regulates CCL20 in hepatoma cells (29) and that expression of CCL20 during facture healing is increased by diabetes and regulated by Foxo1 (30). However, the regulation of IL-36γ by Foxo1 has not been previously reported.

We propose that diabetes-enhanced Foxo1 activation leads to high levels of CCL20 and IL-36γ and provides a mechanistic basis for impaired mucosal epithelial cell migration. This is supported by findings that silencing Foxo1 and the addition of CCL20 or IL-36γ antibody both enhanced PHME cell migration in high glucose media. The addition of both antibodies had a strong additive effect, increasing migration by more than fourfold. Moreover, when Foxo1 was silenced, the addition of CCL20 and IL-36γ antibodies had no effect on migration, suggesting that they have overlapping function. Gain-of-function experiments demonstrated that the addition of CCL20 or IL-36γ ligand significantly reduced with the combination of CCL20 and IL-36γ, reducing PHME migration by 67%. Thus, Foxo1-regulated CCL20 and IL-36γ expression in diabetic mice may significantly hinder the healing of mucosal wounds by inhibiting epithelial cell migration. In addition, CCL20 and IL-36γ can induce neutrophil infiltration and contribute to inflammation, which is elevated in diabetic wounds (26,31,32). This is consistent with the current findings that diabetes increased neutrophil infiltration in the wound area, whereas lineage-specific Foxo1 deletion in keratinocytes in diabetic mice reversed it.

Contrary to its effect in diabetic wounds, lineage-specific Foxo1 deletion impaired normal healing. Foxo1 deletion reduced TGF-β1 expression in mucosal epithelial cells. To investigate a potential mechanism, we showed that Foxo1 silencing in vitro significantly reduced PHME migration, which was completely reversed by TGF-β1 stimulation. Thus, we propose that under normal conditions, Foxo1 expression is needed in oral mucosal epithelial cells to facilitate migration. Foxo1 deletion in normoglycemic mice significantly delayed mucosal wound healing compared with improving it in diabetic mice. The detrimental effect of Foxo1 in mucosal healing may be due to the enhanced production of two mediators, CCL20 and IL-36γ, that have the capacity to inhibit mucosal epithelial cell migration and enhance inflammation.

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Author Contributions. F.X. planned and performed the in vivo and in vitro experiments, reviewed data, and contributed to writing the manuscript. B.O., J.L., A.B., B.P., C.Z., L.Y., J.L., C.T., A.H., S.A., and R.T. performed the in vivo and in vitro experiments. D.T.G. supervised all aspects of the study, designed the experiments, and contributed to writing the manuscript. D.T.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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