

FOXO1 promotes wound healing through the up-regulation of TGF- β 1 and prevention of oxidative stress

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Keratinocyte mobilization is a critical aspect of wound re-epithelialization, but the mechanisms that control its precise regulation remain poorly understood. We set out to test the hypothesis that forkhead box O1 (FOXO1) has a negative effect on healing because of its capacity to inhibit proliferation and promote apoptosis. Contrary to expectations, FOXO1 is required for keratinocyte transition to a wound-healing phenotype that involves increased migration and up-regulation of transforming growth factor β 1 (TGF- β 1) and its downstream targets,

integrin- α 3 and - β 6 and MMP-3 and -9. Furthermore, we show that FOXO1 functions in keratinocytes to reduce oxidative stress, which is necessary to maintain cell migration and prevent cell death in a TGF- β 1-independent manner. Thus, our studies identify a novel function for FOXO1 in coordinating the response of keratinocytes to wounding through up-regulation of TGF- β 1 and other factors needed for keratinocyte migration and protection against oxidative stress, which together promote migration and decrease apoptosis.

Introduction

Cutaneous wound healing is a highly coordinated multistep process that involves inflammation, re-epithelialization, granulation tissue formation, remodeling of basement membrane and extracellular matrix, and dermal and epidermal maturation. The initial inflammatory response stimulates the release of growth factors and chemokines that initiate the later phases of wound repair (Singer and Clark, 1999; Martin and Leibovich, 2005; Li et al., 2007; Raja et al., 2007). The process of wound re-epithelialization requires efficient coordination of multiple events, including the formation of a provisional wound bed matrix, the migration of epidermal keratinocytes into the wound, the proliferation of keratinocytes, and the differentiation of new epithelium into stratified epidermis (Raja et al., 2007).

Keratinocyte migration is critical for re-epithelialization and is tightly regulated by various signaling pathways (Ridley

et al., 2003). After an injury, the basal and suprabasal keratinocytes migrate from the wound edge to re-epithelialize the wounded tissue (Martin, 1997). However, the transcription factors that activate and initiate keratinocyte mobilization in response to wound healing have not been well established. Keratinocyte migration during wound healing is influenced by several factors (Martin, 1997). An impaired keratinocyte migration, but not defects in proliferation or differentiation, is associated with chronic healing defects and is a limiting factor in wound healing (Raja et al., 2007). Keratinocyte migration is initiated by various growth factors including transforming growth factor β 1 (TGF- β 1). TGF- β 1 is one of the most extensively studied molecules and its role in promoting wound healing is well established (Tsuboi et al., 1992; Bennett and Schultz, 1993; Werner and Grose, 2003). In contrast, some reports suggest that TGF- β 1 signaling in epidermal keratinocytes negatively affected wound healing (Ashcroft et al., 1999; Guasch et al., 2007). Activation of TGF- β 1 leads to phosphorylation and nuclear translocation of its downstream

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Abbreviations used in this paper: 8-OHdG, 8-hydroxydeoxyguanosine; ChIP, chromatin immunoprecipitation; CM-H2DCFDA, chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester; FOXO1, forkhead box O1; GADD45 α , DNA damage-inducible gene 45 α ; NAC, N-acetyl-cysteine; NHEK, normal human epithelial keratinocyte; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; TGF- β 1, transforming growth factor β 1; uPAR, urokinase-type plasminogen activator receptor.

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signaling molecules SMAD2/3, which in turn induces target genes involved in cell proliferation and migration (Klass et al., 2009). TGF- β 1 promotes keratinocyte migration by stimulating the expression of various integrins including integrin β 6 (Gailit et al., 1994; Zambruno et al., 1995; Margadant and Sonnenberg, 2010).

Accumulating evidence suggests that reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) are important regulators of wound healing (Schäfer and Werner, 2008). Wound healing induces a small increase in ROS, which are removed by protective antioxidant defense mechanisms. Low levels of ROS are essential to stimulate wound healing (Nishio and Watanabe, 1997; Rodriguez et al., 2008; Guo and Dipietro, 2010), whereas high levels result in epithelial cell damage and impaired wound repair (Xu et al., 2009).

The forkhead box O1 (FOXO1) transcription factor belongs to a large family of forkhead transcription factors that share a highly conserved forkhead DNA-binding domain. FOXO1 participates in a wide range of cellular processes including cell cycle arrest, DNA repair, apoptosis, and oxidative stress resistance (Myatt and Lam, 2007; Ho et al., 2008). FOXO1 controls cell cycle progression by regulating target genes such as p27Kip1 and p21 (Medema et al., 2000), apoptosis by regulating target genes such as Fas ligand and Bim (Dijkers et al., 2000), and protects against oxidative stress by up-regulating oxygen radical scavengers, such as mnSOD and glutathione peroxidase (van der Horst and Burgering, 2007; Storz, 2011). In addition, FOXO1 protects cells against stress by regulating DNA damage-inducible gene 45 α (GADD45 α ; Brunet et al., 2004). Loss of FOXO1 can increase oxidative stress (Ponugoti et al., 2012). Although FOXO1 has been shown to be up-regulated in wounds (Siqueira et al., 2010), its role in wound healing has not been examined.

We investigated the functional impact of FOXO1 deletion in normal wound repair by generating keratinocyte-specific FOXO1-deficient mice in vivo and by *RNAi* in primary cultures of dermal keratinocytes in vitro. Because FOXO1 inhibits proliferation and promotes apoptosis we hypothesized that FOXO1 would negatively regulate wound healing. Contrary to our expectations, our results show that FOXO1 is needed to orchestrate the cellular events critical to wound healing by promoting keratinocyte migration and wound healing primarily through up-regulation of TGF- β 1, but also by protection against oxidative stress through a TGF- β 1-independent mechanism. Thus, a FOXO1 agonist may be an important therapeutic means to enhance the wound-healing response.

Results

Keratinocyte-specific FOXO1 deletion delays wound closure

To determine the role of FOXO1 in wound healing, we generated mice with deletion of FOXO1 in keratinocytes by breeding floxed FOXO1 mice with keratin 14 (K14) Cre-expressing mice. Small excisional wounds of 2.0 mm were made in the scalp of 16-wk-old K14.Cre⁺.FOXO1^{FL/FL} mice and K14.Cre⁻.FOXO1^{FL/FL} mice. Real-time PCR analysis of wounded mouse skin demonstrates an 80% loss of FOXO1 expression in the skin of K14.Cre⁺.FOXO1^{FL/FL} mice compared with control

K14.Cre⁻.FOXO1^{FL/FL} mice ($P < 0.05$; Fig. 1A). Immunofluorescence with FOXO1-specific antibody with histological sections from K14.Cre⁻.FOXO1^{FL/FL} and K14.Cre⁺.FOXO1^{FL/FL} mice establish an 80% reduction of FOXO1 expression specifically in the epidermis of K14.Cre⁺.FOXO1^{FL/FL} compared with littermate control mice (Fig. 1, B and C). In contrast, there was little difference observed in the connective tissue (Fig. 1 B), demonstrating lineage-specific deletion in keratinocytes. FOXO1 activation in vivo was assessed by nuclear translocation. In keratinocytes from normal unwounded tissue, FOXO1 was in the inactivate state with $\sim 10\%$ of keratinocytes exhibiting FOXO1 nuclear localization. Upon wounding, FOXO1 nuclear localization was increased over threefold (Fig. 1 D). In contrast, deletion of FOXO1 by keratin 14-driven Cre-recombinase reduced its nuclear localization by 85% and 82% in keratinocytes of wounded and normal epithelium, respectively (Fig. 1 D). For most wounds, thickened epithelium and FOXO1 expression returned to basal levels within 2.0 mm of the wound edge.

Deletion of FOXO1 in keratinocytes markedly reduced the rate of wound healing (Fig. 1, E and F). Wound area was threefold larger in K14.Cre⁺.FOXO1^{FL/FL} mice compared with K14.Cre⁻.FOXO1^{FL/FL} mice on day 3 after wounding ($P < 0.05$). By day 7 all the wounds in control mice were healed, but were still open in K14.Cre⁺.FOXO1^{FL/FL} experimental mice. Moreover, most of the control mice showed a high degree of healing on day 4 whereas most of the K14.Cre⁺.FOXO1^{FL/FL} experimental mice did not show the same degree of healing until day 7, demonstrating considerably delayed healing (Fig. 1 G).

Loss of FOXO1 leads to reduced keratinocyte migration but not proliferation

We performed experiments using an in vitro wound-healing assay as described in Liang et al. (2007) to assess the direct effect of FOXO1 on keratinocyte wound-healing behavior using primary normal human epithelial keratinocytes (NHEKs). FOXO1 knockdown by FOXO1 siRNA was verified by real-time PCR (Fig. 2 A) and Western blot analysis (Fig. 2 B). The closure of the scratch wound was significantly slower in FOXO1-silenced keratinocytes (Fig. S1 A). At each time point examined, FOXO1 knockdown caused a 42–70% reduction in the number of keratinocytes filling the denuded wound gap compared with cells transfected with scrambled siRNA, which was statistically significant ($P < 0.05$; Fig. 2 E and Fig. S1 A). In contrast, knockdown of a related FOXO transcription factor, FOXO3, had no effect ($P > 0.05$; Fig. 2, C–E and Fig. S1 A) at any time point under the same conditions, nor did scrambled siRNA. Thus, FOXO1 reduction in in vitro “wounds” exhibited the same deficit as in vivo wounds with FOXO1 deletion. In addition, gain of function by overexpression of FOXO1 improved the number of keratinocytes filling the wound gap significantly compared with keratinocytes transfected with empty vector alone (Fig. 2 F).

Epithelial closure of wounds is facilitated by keratinocyte proliferation and migration and reduced by apoptosis. Each parameter was examined separately. Silencing FOXO1 resulted in an 80% decrease in keratinocyte migration in a transwell assay ($P < 0.05$; Fig. 2 G and Fig. S1 B). Quantitative real-time PCR revealed that FOXO1 knockdown significantly decreased mRNA

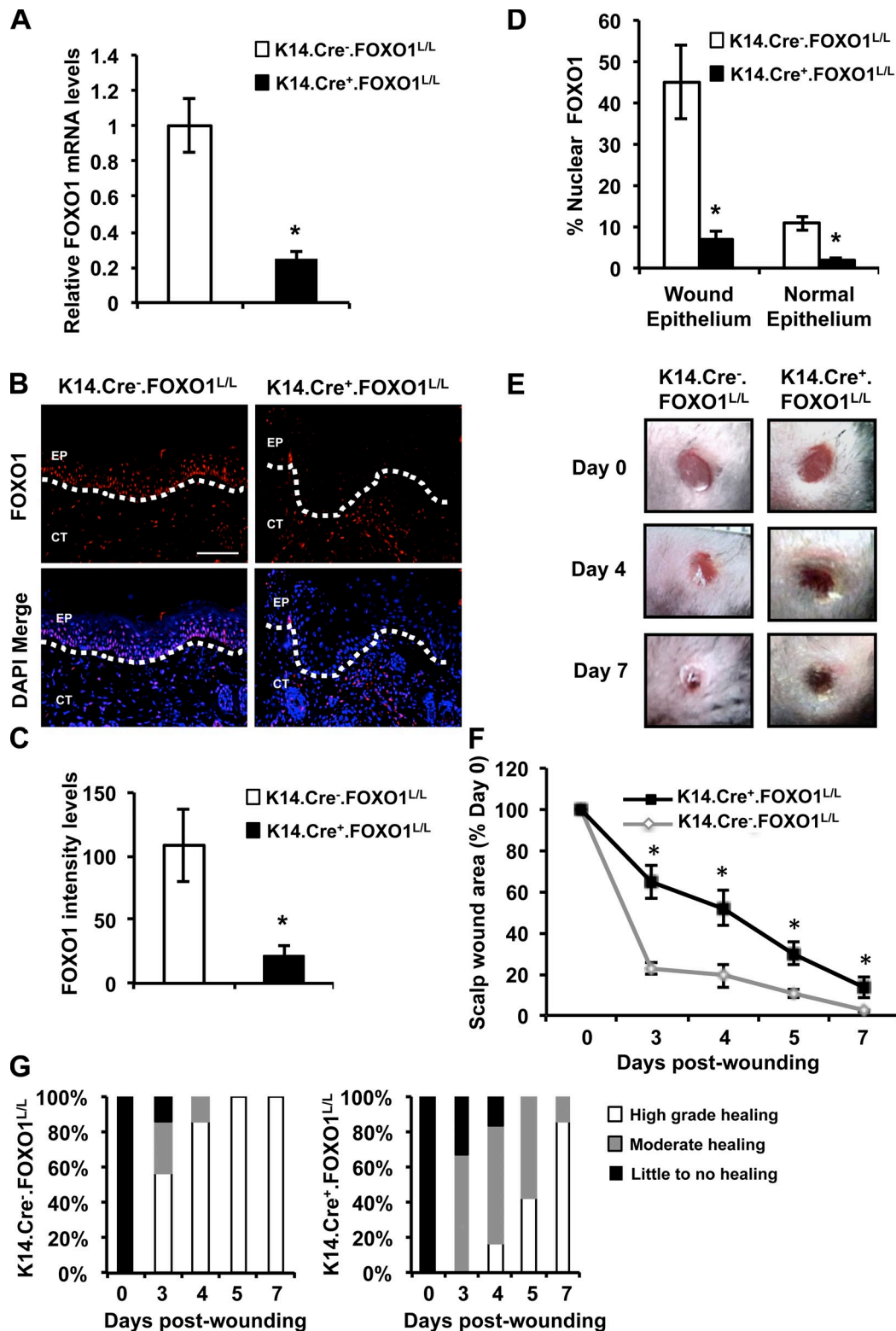


Figure 1. Keratinocyte-specific FOXO1 deletion delays wound closure. (A) Relative fold change of FOXO1 mRNA expression levels normalized to ribosomal protein L32 mRNA expression levels in the scalp wounds of control K14.Cre⁻.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice as measured by qRT-PCR. (B) FOXO1-immunostained sections (red) were counterstained with DAPI (blue). Bar, 100 μ m. (C) Relative intensity measurements of FOXO1 immunostaining in the epithelium. (D) Quantification of FOXO1 nuclear localization in the wounded epithelium and normal epithelium on FOXO1-immunostained sections. (E) Representative photographs of scalp wounds on day 4 and 7 from K14.Cre⁻.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice. (F) Wound-healing process was analyzed at different time points after scalp wounding. Average wound area was presented as percentage of the wound area on day 0 (100%). (G) K14.Cre⁻.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice were stratified into three groups: (1) high degree of healing (covering >70% of the original wound surface), light bars; (2) moderate (covering 30–70%), gray bars; or (3) little to no healing (covering >30%), dark bars. EP, epidermis; CT, connective tissues; White dashed lines demarcate the epidermis from the dermis. Asterisks indicate significant increase or decrease compared with control mice ($P < 0.05$). Error bars represent mean \pm SEM, $n = 6$ –9 per group.

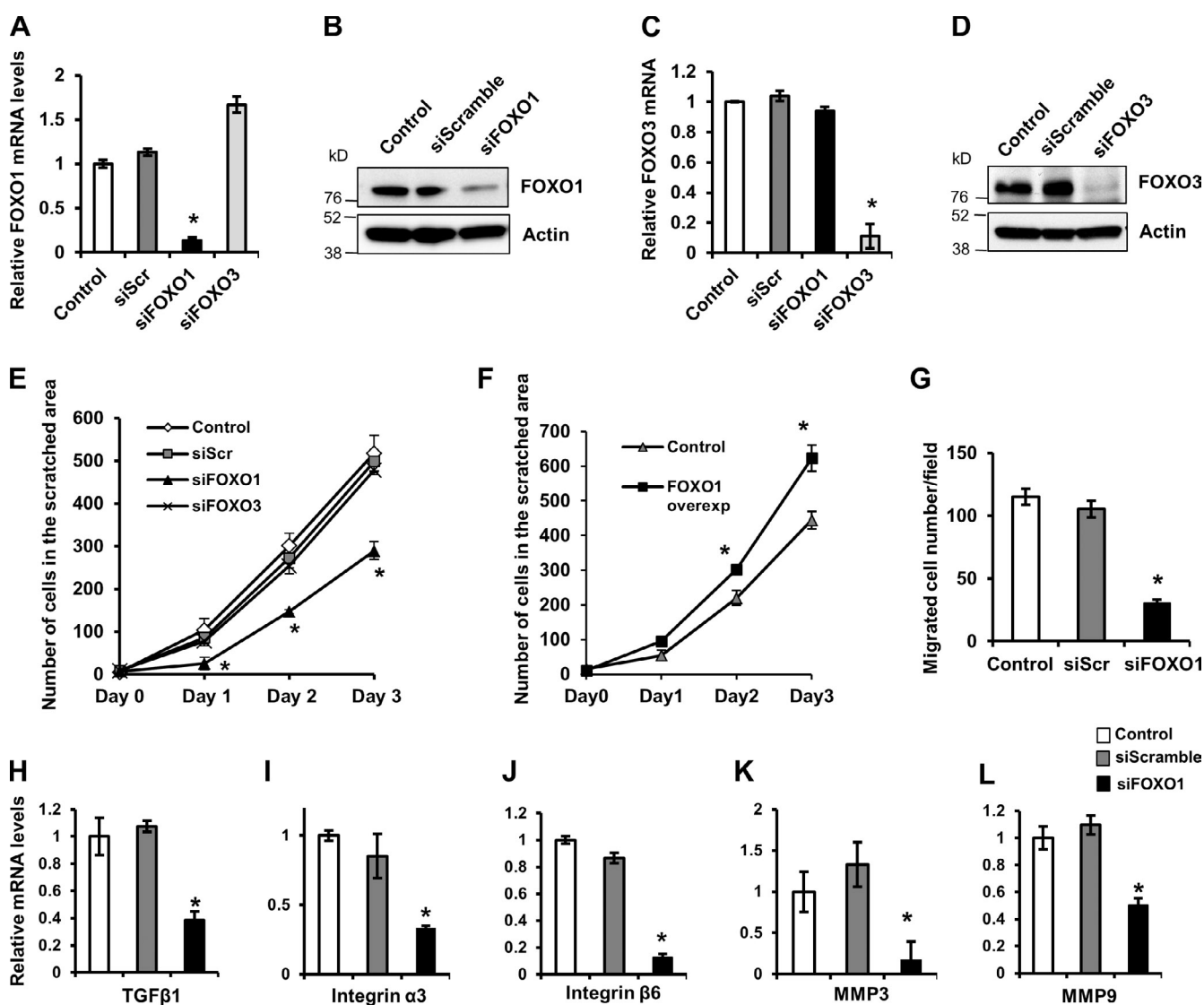


Figure 2. FOXO1 silencing reduces closure of an in vitro scratch wound and keratinocyte migration. NHEK cells were transfected with control siRNA or siRNA specific for FOXO1 or FOXO3 for 48 h. QRT-PCR and Western blot analysis of FOXO1 (A and B) and FOXO3 (C and D) expression. (E) Quantitative analysis of scratch wound closure at the indicated time points in FOXO1/FOXO3 knockdown cells. (F) Quantitative analysis of scratch wound closure at the indicated time points in FOXO1-overexpressed cells. (G) Transwell migration assay of cells transfected with control or FOXO1 siRNA; the migrated cells were photographed and counted. Real-time PCR analysis of TGF- β 1 (H), integrin α 3 (I), integrin β 6 (J), MMP-3 (K), and MMP-9 (L) mRNA levels in FOXO1 knockdown keratinocytes. Data show mean \pm SEM of at least three independent measurements per time point. Asterisks indicate significant increase or decrease compared with cells transfected with control siRNA ($P < 0.05$).

levels of genes involved in cell migration, including TGF- β 1 by 61%, integrin α 3 by 60%, integrin β 6 by 84%, MMP-3 by 82%, and MMP-9 by 50% ($P < 0.05$; Fig. 2, H–L). We also noticed a 33% decrease in ECM remodeling protein collagen IV (Fig. S5 A). In contrast, FOXO1 knockdown in human keratinocytes had no effect on proliferation ($P > 0.05$; Fig. S2, A–C).

TGF- β 1 treatment rescues the migration defect of keratinocytes with FOXO1 knockdown

To establish whether FOXO1 regulation of TGF- β 1 played a critical role in keratinocyte migration, a transwell migration assay was performed under conditions where FOXO1 was knocked down and TGF- β 1 rescue was performed. TGF- β 1 treatment completely reversed the migration defect when FOXO1 was

knocked down ($P > 0.05$; Fig. 3 A and Fig. S1 C). Furthermore, we determined whether TGF- β 1 could rescue the effect of silencing FOXO1 in the in vitro wound-healing assay. At each time point TGF- β 1 treatment rescued the wound closure defect in FOXO1-silenced keratinocytes ($P < 0.05$; Fig. 3 B and Fig. S1 D), demonstrating that one of the principle mechanisms through which FOXO1 affects re-epithelialization in vitro is due to its impact on keratinocyte migration mediated by TGF- β 1.

Because of the central importance of the above mechanism we then determined whether treatment with TGF- β 1 reversed the effect of FOXO1 knockdown on integrins that participate in migration. When FOXO1 was suppressed by RNAi, TGF- β 1 stimulation led to a 130% increase in integrin α 3 and 316% increase in integrin β 6 expression in FOXO1-silenced keratinocytes ($P < 0.05$; Fig. 3, C and D). Taken together, these results

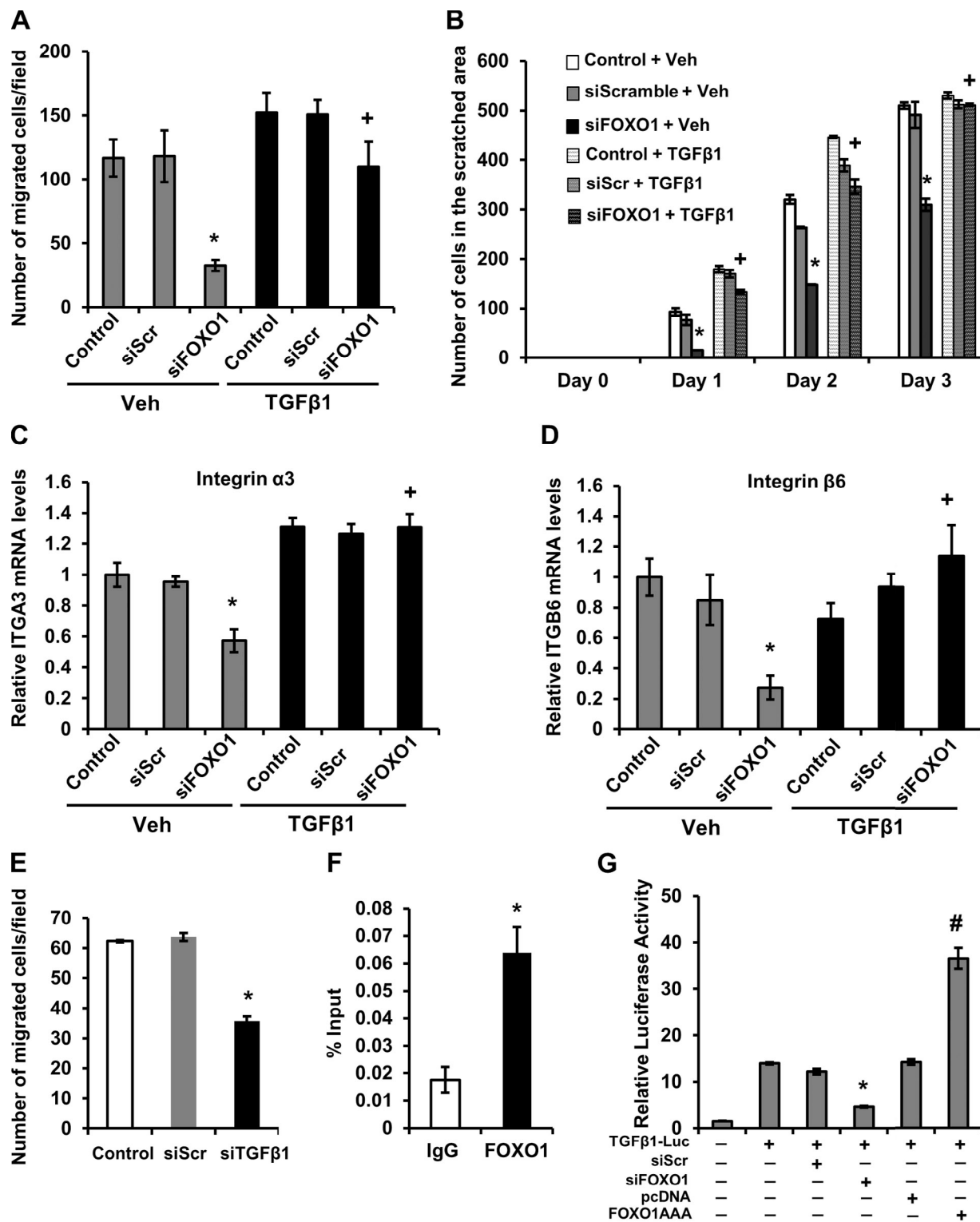


Figure 3. TGF- β 1 treatment rescues the migration defect of keratinocytes with FOXO1 knockdown. NHEK cells were transfected with control or FOXO1 siRNAs for 48 h and then treated with vehicle or TGF- β 1. (A) Transwell migration assay of cells transfected with control or FOXO1 siRNA; the migrated cells were stained with DAPI and photographed and counted. (B) Quantitative analysis of scratch-wound closure at the indicated time points in FOXO1 knockdown cells without or with TGF- β 1. The mRNA levels of integrin α 3 (C) and integrin β 6 (D) were analyzed by real-time PCR. (E) Cell migration was assessed by transwell migration assay and the cells that have migrated across were stained with DAPI and photographed and counted. (F) ChIP assays for binding FOXO1 to the TGF- β 1 promoter were performed and ChIP-enriched DNA was quantified by qPCR with the indicated primers, and values are expressed as percentage of input DNA. (G) NHEK cells were cotransfected with a control pcDNA vector or a vector that expresses constitutively active FOXO1 (FOXO1AAA), control siRNA, or siRNA specific to FOXO1, together with TGF- β 1 reporter plasmid. Renilla luciferase reporter was used as an internal control. Luciferase activity was measured 36 h after transfection. Data show mean \pm SEM of at least three independent experiments. *, $P < 0.05$ vs. scrambled siRNA; +, $P < 0.05$ vs. siFOXO1; #, $P < 0.05$ vs. control pcDNA vector.

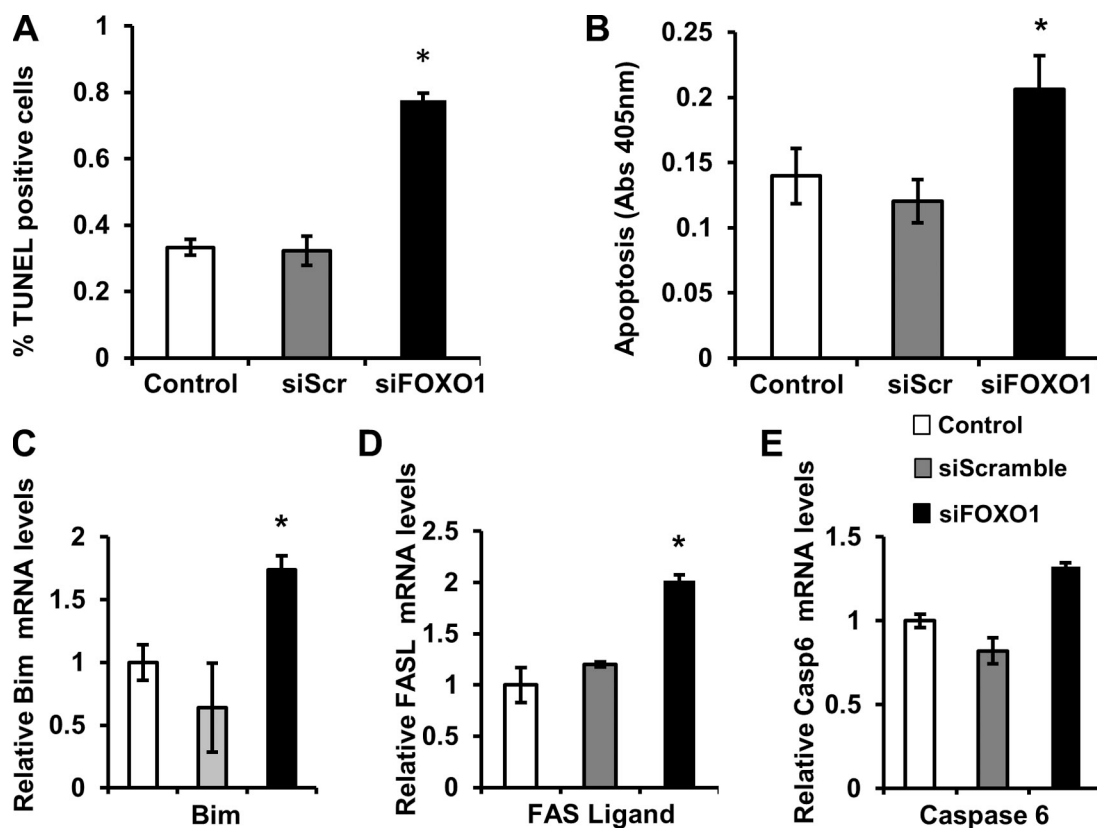


Figure 4. **FOXO1 knockdown results in increased keratinocyte apoptosis.** NHEK cells were transfected with control or FOXO1 siRNAs for 48 h. (A) Apoptotic keratinocytes were measured by TUNEL staining. Quantitative analysis of TUNEL-positive NHEK cells. The percentage of TUNEL-positive nuclei is reported as mean \pm SD. (B) Apoptotic keratinocytes were confirmed by cell death detection ELISA. Real-time PCR analysis of Bim (C), FAS ligand (D), and caspase-6 (E) mRNA levels in FOXO1 knockdown keratinocytes. Data show mean \pm SEM of at least three independent measurements. Asterisks indicate significant increase compared with cells transfected with siRNA control ($P < 0.05$).

indicate that mRNA levels of integrins $\alpha 3$ and $\beta 6$, which are important in keratinocyte migration, are regulated through FOXO1 in a TGF- $\beta 1$ -dependent manner and that delayed migration observed in FOXO1 knockdown keratinocytes is due to attenuated TGF- $\beta 1$ signaling. To confirm that TGF- $\beta 1$ can stimulate keratinocyte migration in an autocrine manner we silenced TGF- $\beta 1$ by siRNA, which was verified by real-time PCR (Fig. S1 E). TGF- $\beta 1$ knockdown reduced keratinocyte migration by 45% in a transwell assay ($P < 0.05$; Fig. 3 E). This is consistent with previous reports that TGF- $\beta 1$ stimulates keratinocyte migration (Hebda, 1988; Kane et al., 1991; Gailit et al., 1994).

FOXO1 is recruited to the TGF- $\beta 1$ promoter and transactivates its expression

The human TGF- $\beta 1$ promoter has two consensus FOXO1 binding elements at positions -192 bp (TTATTTCC) and -1263 bp (TTGTTTCC) upstream of the start codon in the TGF- $\beta 1$ promoter (Furuyama et al., 2000). Chromatin immunoprecipitation (ChIP) assays showed that endogenous FOXO1 is recruited to the TGF- $\beta 1$ promoter (Fig. 3 F). To determine whether FOXO1 can transactivate the TGF- $\beta 1$, we examined the luciferase activity of the TGF- $\beta 1$ reporter in primary keratinocytes. The luciferase reporter analysis revealed that overexpression of constitutively active FOXO1 (FOXO1AAA) results in a 2.5-fold increase in TGF- $\beta 1$ promoter activity, whereas FOXO1 silencing produced

a 2.6-fold decrease in TGF- $\beta 1$ promoter activity ($P < 0.05$; Fig. 3 G). Taken together, these results indicate that FOXO1 directly binds and transactivates TGF- $\beta 1$ promoter activity and confirms that TGF- $\beta 1$ is a downstream target of FOXO1.

FOXO1 knockdown results in increased keratinocyte apoptosis

To investigate whether FOXO1 knockdown affects keratinocyte apoptosis in vitro, we performed TUNEL assays (Fig. 4). Apoptosis was 2.4-fold higher in FOXO1 knockdown keratinocytes compared with the controls, whereas scrambled siRNA had no effect ($P < 0.05$; Fig. 4 A and Fig. S4). Results were confirmed using cell death detection ELISA (Fig. 4 B). Given that FOXO1 silencing enhanced keratinocyte apoptosis, we tested whether increased apoptosis was linked with increased expression of apoptotic genes. FOXO1 silencing significantly increased genes that promote apoptosis such as Bim by 73%, FAS ligand by 68%, and caspase 6 by 32% ($P < 0.05$; Fig. 4, C-E), indicating that constitutive FOXO1 expression protected against apoptosis and that FOXO1 in these cells functioned to decrease mRNA levels of pro-apoptotic genes. This is somewhat surprising because FOXO1 expression is associated with pro-apoptosis in most cell types (Ho et al., 2008), but in some cells, as demonstrated here with keratinocytes, FOXO1 is needed to prevent apoptosis (Calnan and Brunet, 2008).

FOXO1 silencing enhances the impact of oxidative stress to reduce keratinocyte migration and enhance apoptosis

FOXO1 has been reported to protect cells against ROS such as H₂O₂ by activating various ROS detoxifying enzymes (Storz, 2011). As shown in Fig. 5 A, loss of FOXO1 induced a 38% increase in ROS levels compared with controls. Incubation of keratinocytes with H₂O₂ enhanced ROS levels by 187% and silencing FOXO1 further enhanced H₂O₂-treated keratinocyte ROS levels by another 65%, whereas, treatment with *N*-acetyl-cysteine (NAC), a ROS inhibitor, markedly suppressed keratinocyte ROS levels (Fig. 5 A).

To determine the functional importance of FOXO1 in the response to oxidative stress, *in vitro* scratch-wound assays were performed. Silencing FOXO1 caused a 51% decrease in the number of keratinocytes that filled the “wound” gap at day 1 (Fig. S3), a 54% decrease at day 2 (Fig. 5 B), and 39% reduction at day 3 (Fig. S3). Incubation of keratinocytes with H₂O₂ further decreased filling of the wound gap by an additional 24% at day 1 (Fig. S3), 56% at day 2 (Fig. 5 B), and 123% at day 3 (Fig. S3). Furthermore, incubation of keratinocytes with the ROS inhibitor NAC rescued 75% of the wound closure defect observed in the FOXO1-silenced keratinocytes at day 2 (Fig. 5 B). In contrast, TGF-β1 treatment had no effect on H₂O₂-suppressed scratch-wound closure with or without FOXO1 silencing ($P > 0.05$; Fig. 5 B) and did not provide additional protection afforded by the antioxidant NAC ($P > 0.05$; Fig. 5 B). We further examined the effect of oxidative stress on keratinocyte migration by transwell migration assay. As shown in Fig. 2 D, silencing FOXO1 significantly impaired keratinocyte migration. Incubation with NAC rescued the negative impact of FOXO1 knockdown on keratinocyte migration (Fig. 5 C). Incubation of keratinocytes with low amounts of H₂O₂ resulted in a 71% reduced migration of keratinocytes and silencing FOXO1 further decreased keratinocyte migration by another 18% (Fig. 5 C). Treatment of NAC reversed the inhibitory effect of H₂O₂ partially but significantly (Fig. 5 C). Consistent with the scratch-wound assay, TGF-β1 treatment had no effect on H₂O₂-suppressed keratinocyte migration (Fig. 5 C).

The effect of FOXO1 silencing and oxidative stress on keratinocyte apoptosis was examined. The antioxidant NAC rescued the pro-apoptotic effect of FOXO1 knockdown ($P < 0.05$; Fig. 5 D). H₂O₂ increased keratinocyte apoptosis twofold relative to controls ($P < 0.05$), which was enhanced another 3.7-fold by silencing FOXO1 (Fig. 5 D). TGF-β1 had relatively little effect on keratinocyte apoptosis (Fig. 5 D). Taken together, these results indicate that FOXO1 has an important antioxidant role that protects keratinocytes against ROS, which is critical for maintaining keratinocyte migration as well as protection against apoptosis. Moreover, this protection is largely independent of TGF-β1.

FOXO1 protects cells by stimulating antioxidant defense mechanisms such as glutathione peroxidases or inducing DNA repair enzymes such as GADD45α. Silencing FOXO1 in keratinocytes significantly decreased mRNA levels of antioxidant genes such as glutathione peroxidase 2 (GPX-2) by 49% and cytoglobulin by 39%, as well as DNA damage repair gene GADD45α by 64% ($P < 0.05$; Fig. 5, E–G). Thus, the loss of

FOXO1 expression in keratinocytes results in decreased expression of antioxidative genes and GADD45α, which repairs oxidative damage.

As an alternative mechanism, we examined whether the delayed scratch-wound closure in FOXO1-deficient keratinocytes is due to defects in glucose uptake or ATP production. However, we did not observe any changes in glucose uptake or ATP production in FOXO1-silenced keratinocytes compared with the controls (Fig. S2, D and E).

Decreased migration and proliferation, and increased apoptosis in keratinocytes isolated from FOXO1-deleted mice

To further establish the role of FOXO1 in regulating keratinocytes, experiments were performed with primary cells isolated from experimental K14.Cre⁺.FOXO1^{L/L} and control K14.Cre⁻.FOXO1^{L/L} mice. Western blot analysis showed that Cre recombinase reduced FOXO1 expression in experimental compared with control keratinocytes (Fig. 6 A). Furthermore, Western blot analysis for TGF-β1 demonstrates the loss of TGF-β1 expression at the protein level when FOXO1 is deleted (Fig. 6 A). Similarly, TGF-β1 signaling measured by phosphorylation of SMAD2/3 was found to be decreased in K14.Cre⁺.FOXO1^{L/L} keratinocytes, while the total SMAD2/3 protein levels remained the same (Fig. 6 A), indicating that the delayed wound-healing phenotype observed in FOXO1-deleted mice was due to decreased TGF-β1 expression.

The effect of FOXO1 deletion on cell migration, proliferation, and apoptosis *in vitro* was examined using primary dermal keratinocytes isolated from K14.Cre⁻.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice. As shown in Fig. 6 B, deletion of FOXO1 resulted in a 50% decrease in cell migration compared with control keratinocytes. Deletion of FOXO1 also caused a twofold increase in apoptosis in keratinocytes isolated from experimental mice compared with control keratinocytes (Fig. 6 C). When proliferation was examined, FOXO1 deletion resulted in a small but significant 22% decrease in proliferation compared with wild-type keratinocytes ($P < 0.05$; Fig. 6 D). These results are very similar to those obtained by FOXO1 knockdown with specific siRNA.

Keratinocyte-specific FOXO1 deletion results in decreased keratinocyte migration and proliferation and increased apoptosis *in vivo*

Further investigation of the role of FOXO1 in delayed wound healing was performed by histological analysis of specimens obtained from small excisional wounds created in K14.Cre⁻.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice. Wound closure was significantly reduced by FOXO1 deletion in keratinocytes *in vivo* (Fig. 7 A). The gap between the edges of the epithelium was ~2.5-fold larger in day-4 wounds of experimental K14.Cre⁺.FOXO1^{L/L} mice compared with matched K14.Cre⁻.FOXO1^{L/L} control mice ($P < 0.05$; Fig. 7 B). This difference was fivefold on day 7 ($P < 0.05$; Fig. 7 F). Keratinocyte number was ~1.5-fold higher in the wounds of control mice compared with experimental FOXO1-deleted mice ($P < 0.05$; Fig. 7,

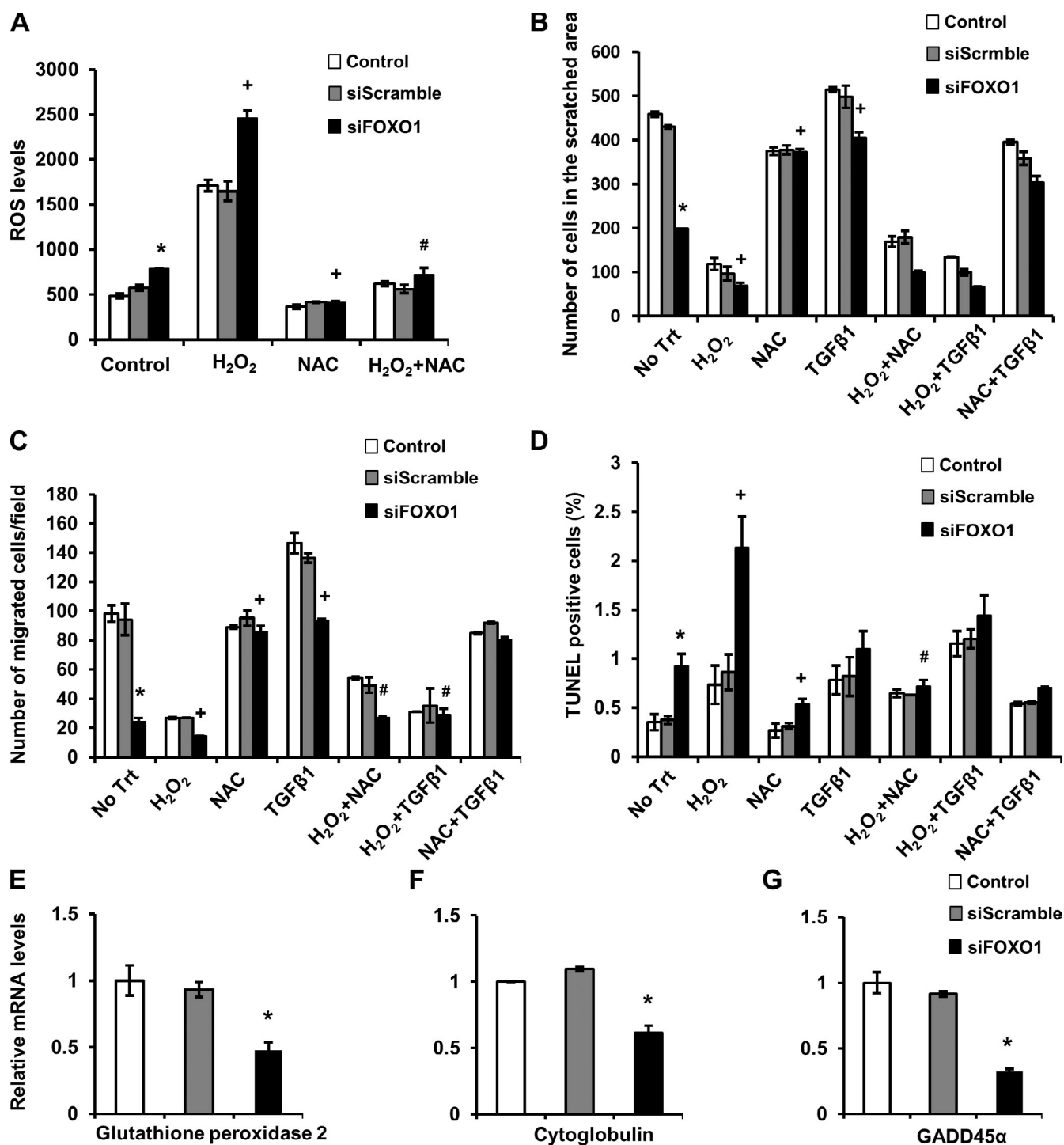


Figure 5. FOXO1 silencing enhances the impact of oxidative stress to reduce keratinocyte migration and increase apoptosis. NHEK cells were transfected with control or FOXO1 siRNAs for 48 h and then treated with 150 μ M H₂O₂, 1 mM NAC, 2 ng/ml TGF- β 1, or various combinations of these agents. (A) Intracellular ROS were determined using the fluorescent probe CM-H₂DCFDA staining. (B) Quantitative analysis of migrated cells after scratch wounds. (C) Transwell migration assay was carried as described in Materials and methods; the cells that have migrated across the transwells were stained with DAPI and photographed and counted. (D) Apoptotic keratinocytes were measured by TUNEL staining. Quantitative analysis of the percentage of TUNEL-positive nuclei is reported as mean \pm SD. The mRNA levels of GPX2 (E), cytoglobulin (F), and GADD45 α (G) were analyzed by real-time PCR. Data show mean \pm SEM of at least three independent experiments. *, Significantly changed compared with scrambled siRNA; +, significantly changed compared with FOXO1 siRNA in the no treatment group; #, significantly changed compared with the H₂O₂ plus FOXO1 siRNA. Significance set at $P < 0.05$.

C and G), which increased the area of healing epithelium (Fig. 7, D and H). We also observed a decrease in epithelial thickness by 25% ($P < 0.05$) in wounds of the FOXO1-deleted mice on day 4 (Fig. 7, E and I).

To determine whether the effect of FOXO1 deletion in keratinocytes was due to altered migration, immunofluorescence was performed to measure the urokinase plasminogen activator receptor (uPAR), which is expressed in migrating

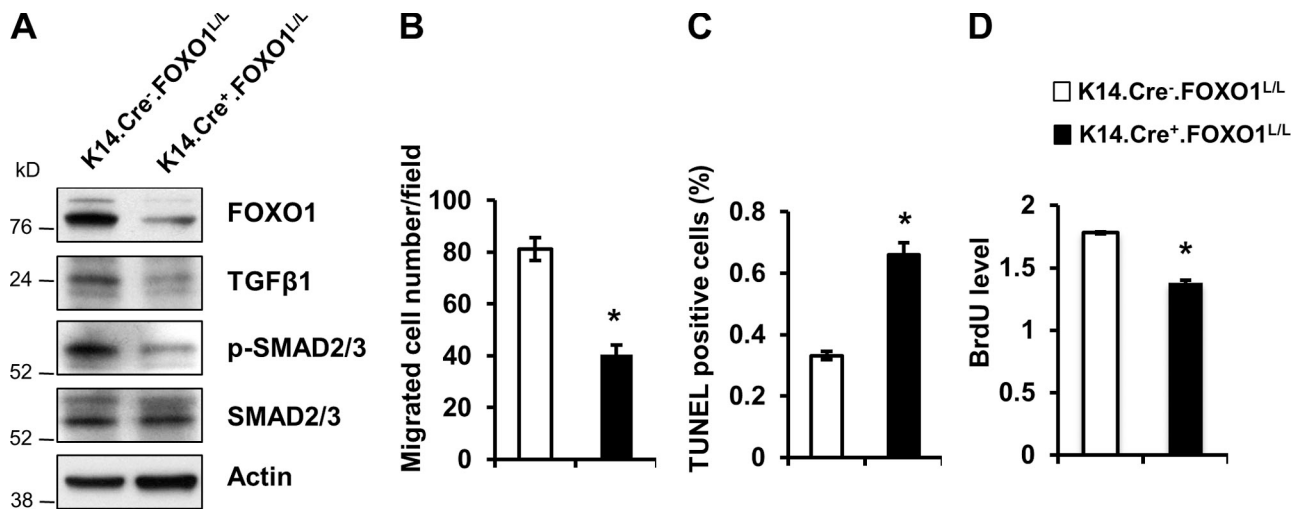


Figure 6. **Reduced migration and proliferation, and increased apoptosis in keratinocytes isolated from FOXO1-deleted mice.** (A) Western blot analysis for FOXO1, TGF- β 1, phospho-SMAD2/3, SMAD2/3, and actin as loading control in K14.Cre⁻.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} keratinocytes. (B) Cell migration of K14.Cre⁻.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} keratinocytes was assessed by transwell migration assay; the migrated cells were stained with DAPI and photographed and counted. (C) Apoptotic keratinocytes were measured by TUNEL staining. (D) BrdU level of K14.Cre⁻.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} keratinocytes. BrdU incorporation was determined by measuring absorbance at 450 nm. Data show mean \pm SEM of at least three independent experiments. *, $P < 0.05$ vs. K14.Cre⁻.FOXO1^{L/L} keratinocytes.

keratinocytes and is a marker of keratinocytes that are in the process of migrating (Lund et al., 2006). The expression of uPAR in keratinocytes was reduced $\sim 47\%$ in the wounds of experimental K14.Cre⁺.FOXO1^{L/L} mice compared with matched K14.Cre⁻.FOXO1^{L/L} control mice ($P < 0.05$; Fig. 8, A and B). The impact of FOXO1 on migrating keratinocytes during wound healing in vivo was similar to that observed in vitro. Previous studies suggest that hair follicles play important roles in normal wound healing (Lavker et al., 1993; Ito et al., 2005). Therefore, we examined the effect of keratinocyte-specific FOXO1 deletion on indicators of cell migration in hair follicles. The number of uPAR-positive cells in hair follicles was reduced 54% in FOXO1-deleted mice compared with the control mice, indicating that deletion of FOXO1 in the keratinocytes affects the number of migrating hair follicle cells (Fig. 8 B).

We next investigated the effect of keratinocyte-specific FOXO1 deletion on keratinocyte apoptosis and proliferation in vivo. The percentage of apoptotic keratinocytes increased by 1.6-fold in FOXO1-deficient wounds compared with control mice ($P < 0.05$; Fig. 8, C and D). In addition, we observed a 21% decrease in keratinocyte proliferation in the wound epithelium of FOXO1-deleted mice measured by the number of proliferating cell nuclear antigen (PCNA)-positive cells (Fig. 8, E and F; $P < 0.05$). We also observed a 36% decrease in the number of PCNA-positive hair follicle cells in FOXO1-deleted mice (Fig. 8 F).

Loss of FOXO1 expression in keratinocytes leads to enhanced oxidative stress in vivo

To examine whether there were changes in oxidative stress caused by FOXO1 deletion in keratinocytes in vivo we analyzed the expression of 8-hydroxy-2-deoxyguanosine (8-OHdG), a widely used marker of oxidative damage (Shigenaga et al., 1989). The number of 8-OHdG-positive keratinocytes increased 2.4-fold in healing wounds of K14.Cre⁺.FOXO1^{L/L} mice compared with the control K14.Cre⁻.FOXO1^{L/L} mice ($P < 0.05$;

Fig. 8, G and H). These results indicate that deletion of FOXO1 in keratinocytes in vivo enhances damage caused by oxidative stress. It is consistent with the in vitro studies (Fig. 5) supporting the notion that FOXO1 protection against oxidative stress is important in the cellular events of wound healing.

Loss of FOXO1 expression in keratinocytes leads to down-regulation of TGF- β 1 and reduced SMAD signaling

TGF- β 1 plays a critical role in re-epithelialization and wound repair (Werner and Grose, 2003). Experiments were performed to determine whether loss of FOXO1 in keratinocytes had an effect on the expression of TGF- β 1 in healing wounds of K14.Cre⁻.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice. Control mice showed strong TGF- β 1 immunostaining in both basal and suprabasal cells in the wounded epithelium (Fig. 9 A). Deletion of FOXO1 in keratinocytes resulted in a 71% decrease in the number of keratinocytes that expressed TGF- β 1 in the wounds of experimental mice compared with control mice ($P < 0.05$; Fig. 9 B). These results showing FOXO1 regulation of TGF- β 1 in vivo are consistent with results above that FOXO1 regulates TGF- β 1 production.

TGF- β 1 regulates its target gene expression through phosphorylation of its downstream signaling molecules, SMAD2/3. The number of keratinocytes with nuclear phospho-SMAD2/3 decreased 42% in wounds of K14.Cre⁺.FOXO1^{L/L} mice compared with control mice ($P < 0.05$; Fig. 9, C and D), indicating that loss of FOXO1 expression in keratinocytes results in decreased TGF- β 1 signaling. However, total SMAD2/3 levels were not altered significantly (Fig. 9, E and F).

TGF- β 1 treatment rescues wound closure in FOXO1-deleted mice

To further investigate the role of TGF- β 1 in FOXO1-mediated wound healing, excisional wounds were treated topically with

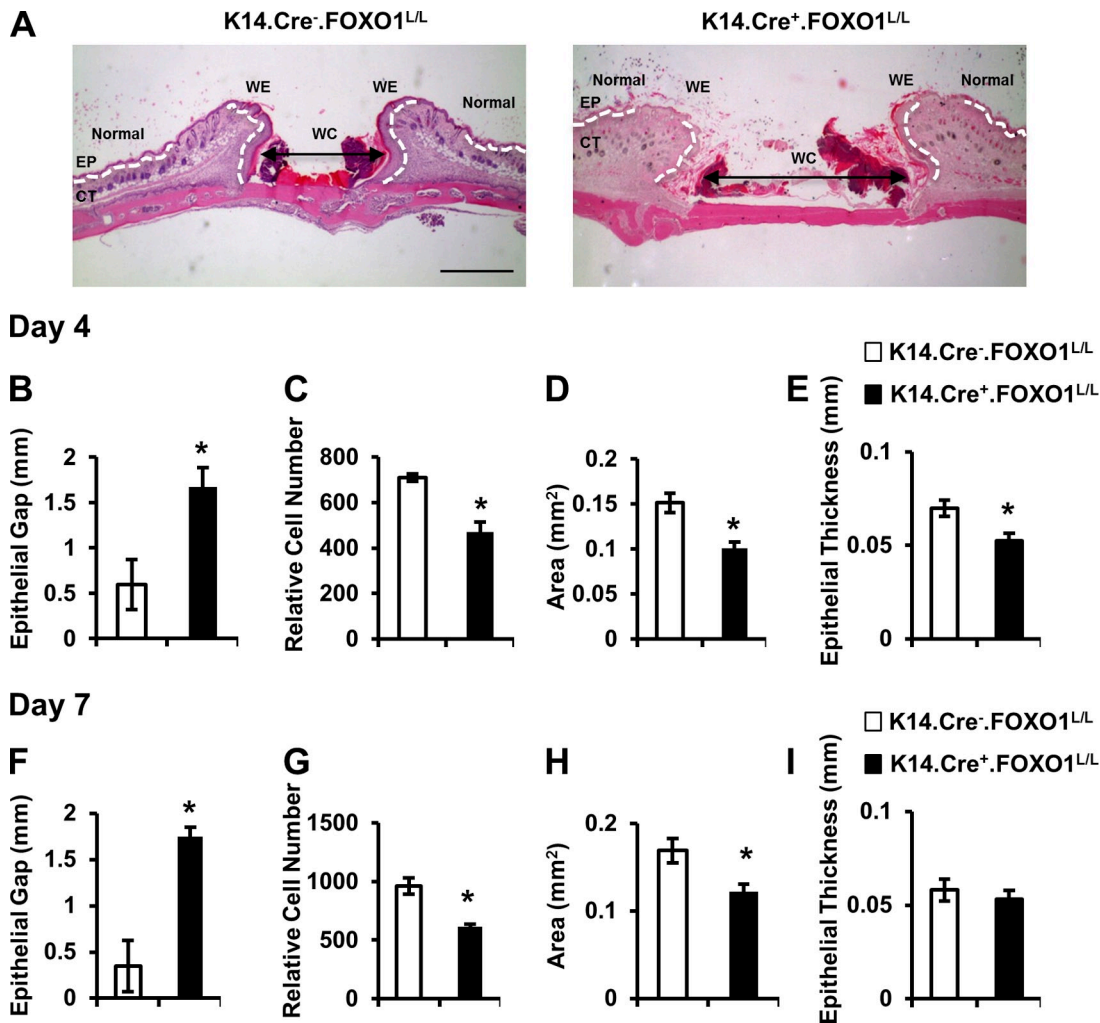


Figure 7. **Delayed wound healing in keratinocyte-specific FOXO1-deleted mice.** (A) Hematoxylin and eosin (H&E) staining of K14.Cre⁻.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice wound biopsies on day 4 after wounding. Bar, 500 μ m. WC, wound center; WE, wound epithelium; Normal, normal epithelium. Quantification of epithelial migration and re-epithelialization in the day-4 and day-7 scalp wounds. The gap between epithelium (B and F), number of cells in the wound epithelium (C and G), epithelial area (D and H), and epithelial thickness (E and I) was assessed by image analysis of H&E-stained sections at the center of each wound. EP, epidermis; CT, connective tissues. Each value is the mean \pm SEM for $n = 6-9$ mice per group. Asterisks indicate significant increase or decrease compared with control mice ($P < 0.05$).

either recombinant TGF- β 1 or vehicle. TGF- β 1 treatment enhanced wound closure. On day 4, wounds were 58% larger in the vehicle-treated compared with the TGF- β 1-treated in K14.Cre⁺.FOXO1^{L/L} mice ($P < 0.05$; Fig. 10, A and B). On a histological level TGF- β 1 treatment rescued the wound-healing defect in FOXO1-deleted mice by decreasing the size of the epithelial gap by 75% and enhancing the size of the healing epithelium measured by epithelial length and thickness (Fig. 10, C–G). FOXO1-deleted mice treated with TGF- β 1 also had a 2.4-fold increase in the number of migrating cells in the wounded epithelium and a twofold increase in the number of migrating cells in hair follicles (Fig. 10, H and I). However, there was no significant difference in proliferating keratinocytes when TGF- β 1-treated wounds were compared with vehicle-treated wounds in FOXO1-deleted mice ($P > 0.05$; Fig. 10 J). Thus, the negative effect of FOXO1 deletion was rescued by TGF- β 1 treatment in vivo as was also noted in vitro. This further links the functional importance of FOXO1 regulation of TGF- β 1 as a mechanism by which FOXO1

controls the cellular events of wound healing. A summary of the cellular events regulated by FOXO1 in keratinocytes in vitro and in vivo are shown in Table 1.

Discussion

FOXO1 has been shown to play an important role in cellular processes that are linked to repair, particularly inhibiting proliferation, increasing apoptosis, and reducing oxidative stress. However, its role in regulating keratinocytes and keratinocyte behavior in wound healing has not been investigated. Based upon the effects of FOXO1 we anticipated that deletion of FOXO1 would enhance wound healing by increasing proliferation and reducing apoptosis. To test this we performed wound-healing experiments both in vivo using a conditional deletion of FOXO1 in a keratinocyte lineage as well as in vitro. Unexpectedly, mice with FOXO1 deletion in keratinocytes exhibited impaired wound closure in vivo and also displayed very similar

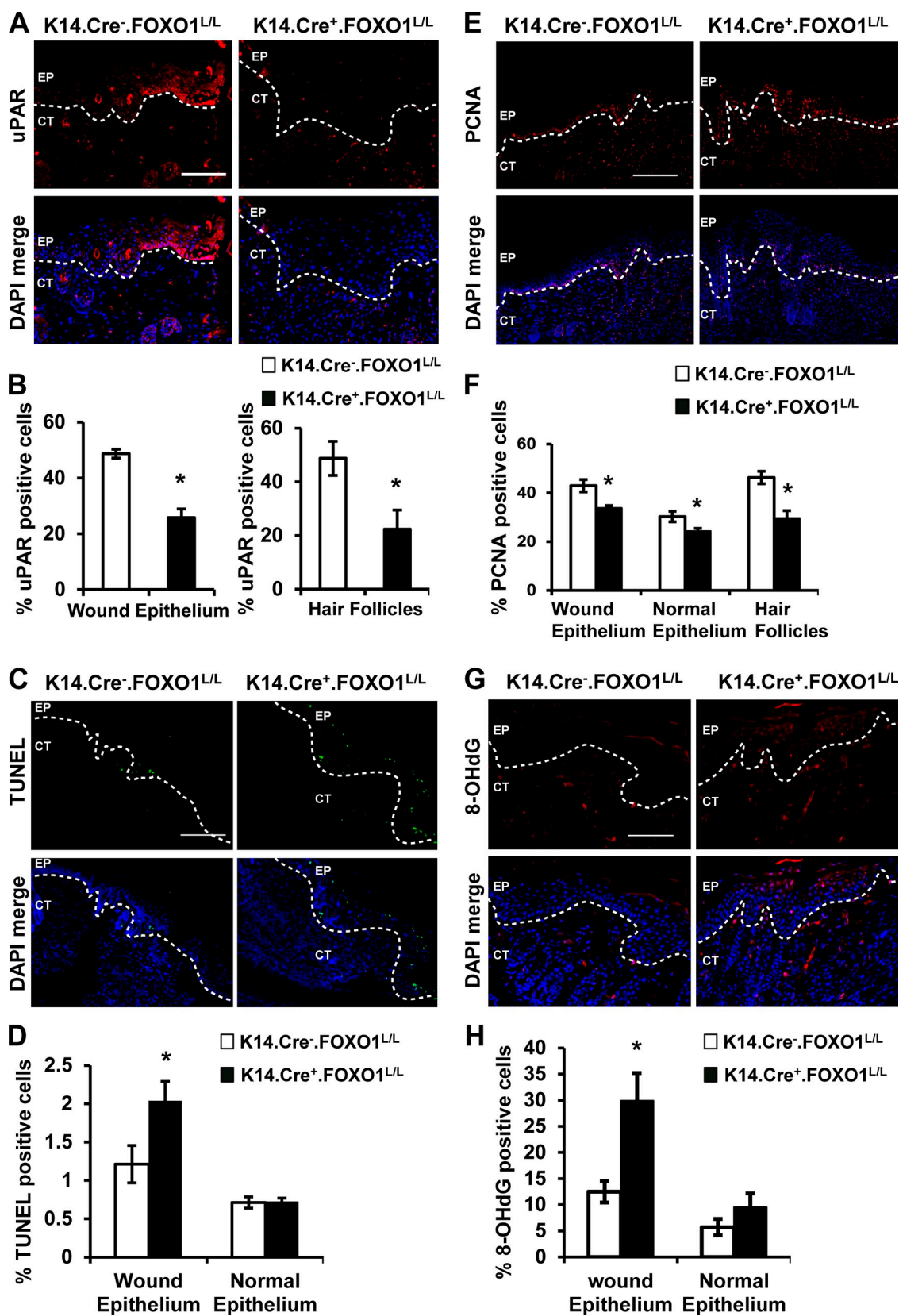


Figure 8. Loss of FOXO1 expression in keratinocytes results in decreased keratinocyte migration and proliferation and increased apoptosis. Paraffin-embedded cross sections from K14.Cre⁻.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice were analyzed by immunofluorescence using an anti-uPAR antibody (A), anti-PCNA antibody (E), or anti-8-OHdG antibody (G), and by TUNEL assay (C). Quantification of uPAR-positive migrating keratinocytes (B), TUNEL-positive apoptotic keratinocytes (D), PCNA-positive proliferative keratinocytes (F), and 8-OHdG-positive keratinocytes (H). Bars: (A and G) 100 μ m; (C and E) 200 μ m. EP, epidermis; CT, connective tissues. White dashed lines demarcate the epidermis from the dermis. Each value is the mean \pm SEM for $n = 6-9$ mice per group. Asterisks indicate significant changes compared with control mice ($P < 0.05$).

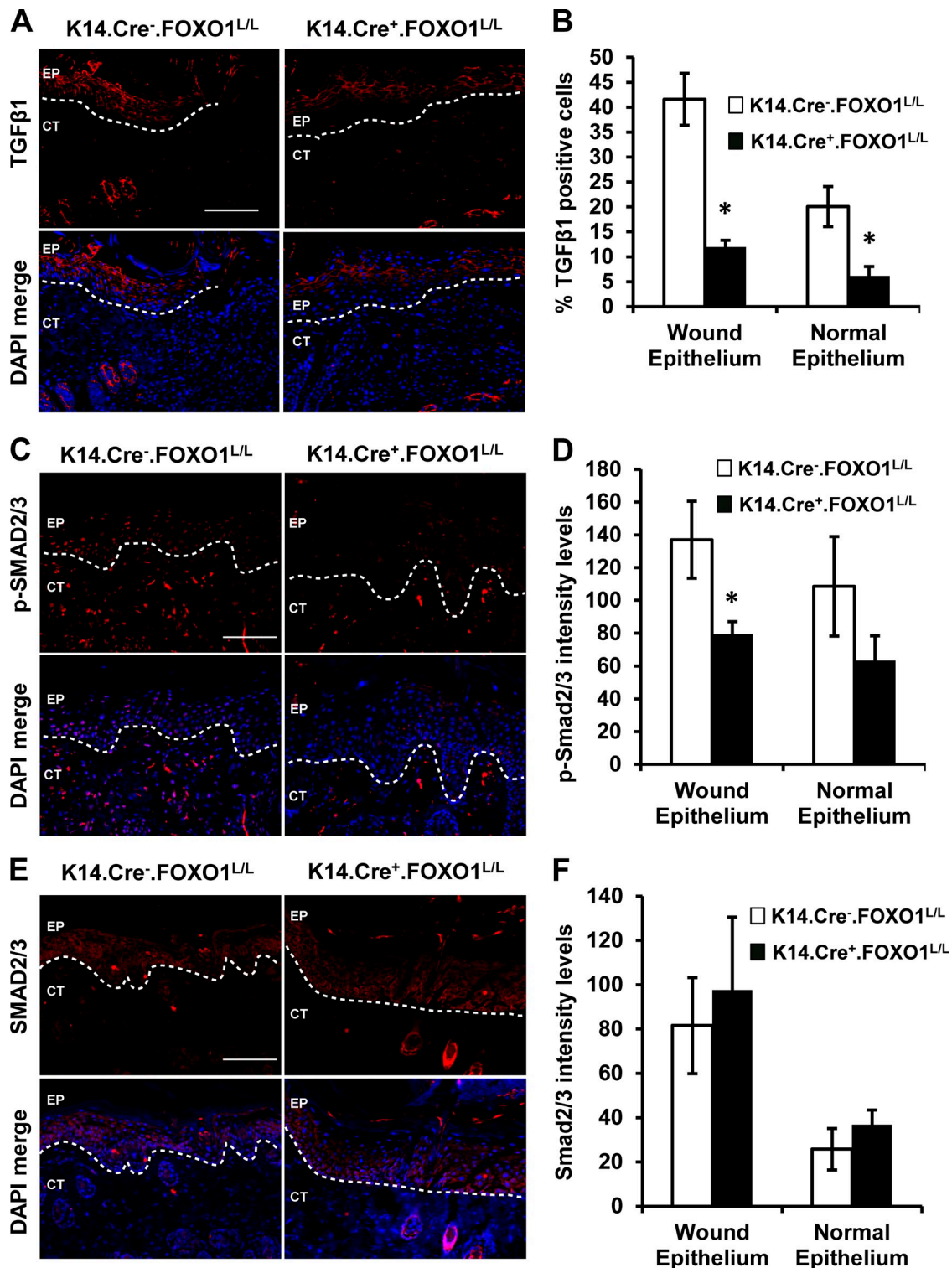


Figure 9. Reduced TGF-β1 and phospho-SMAD2/3 expression in the FOXO1-deleted mice keratinocytes. Paraffin-embedded cross sections from K14.Cre⁻.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice were analyzed by immunofluorescence using (A) anti-TGF-β1 antibody, (C) phospho-SMAD2/3 antibody, and (E) anti-SMAD2/3 antibody. Quantitative analysis of (B) TGF-β1-positive keratinocytes, (D) phospho-SMAD2/3-positive keratinocytes, and (F) SMAD2/3-positive keratinocytes. Bar, 100 μm. EP, epidermis; CT, connective tissues. White dashed lines demarcate the epidermis from the dermis. Each value is the mean ± SEM for *n* = 6–9 mice per group. Asterisks indicate significant changes compared with control mice (*P* < 0.05).

results in an in vitro model of wound healing. Delayed wound healing caused by FOXO1 deletion was primarily caused by a decrease in migrating keratinocytes both in the wounded epithelium and in hair follicles. We determined that a primary

downstream event regulated by FOXO1 was TGF-β1 expression and that wound healing impaired by FOXO1 deletion could be rescued by treatment with TGF-β1 in vivo and in vitro. Thus, FOXO1 is required for the up-regulation of TGF-β1 in wound

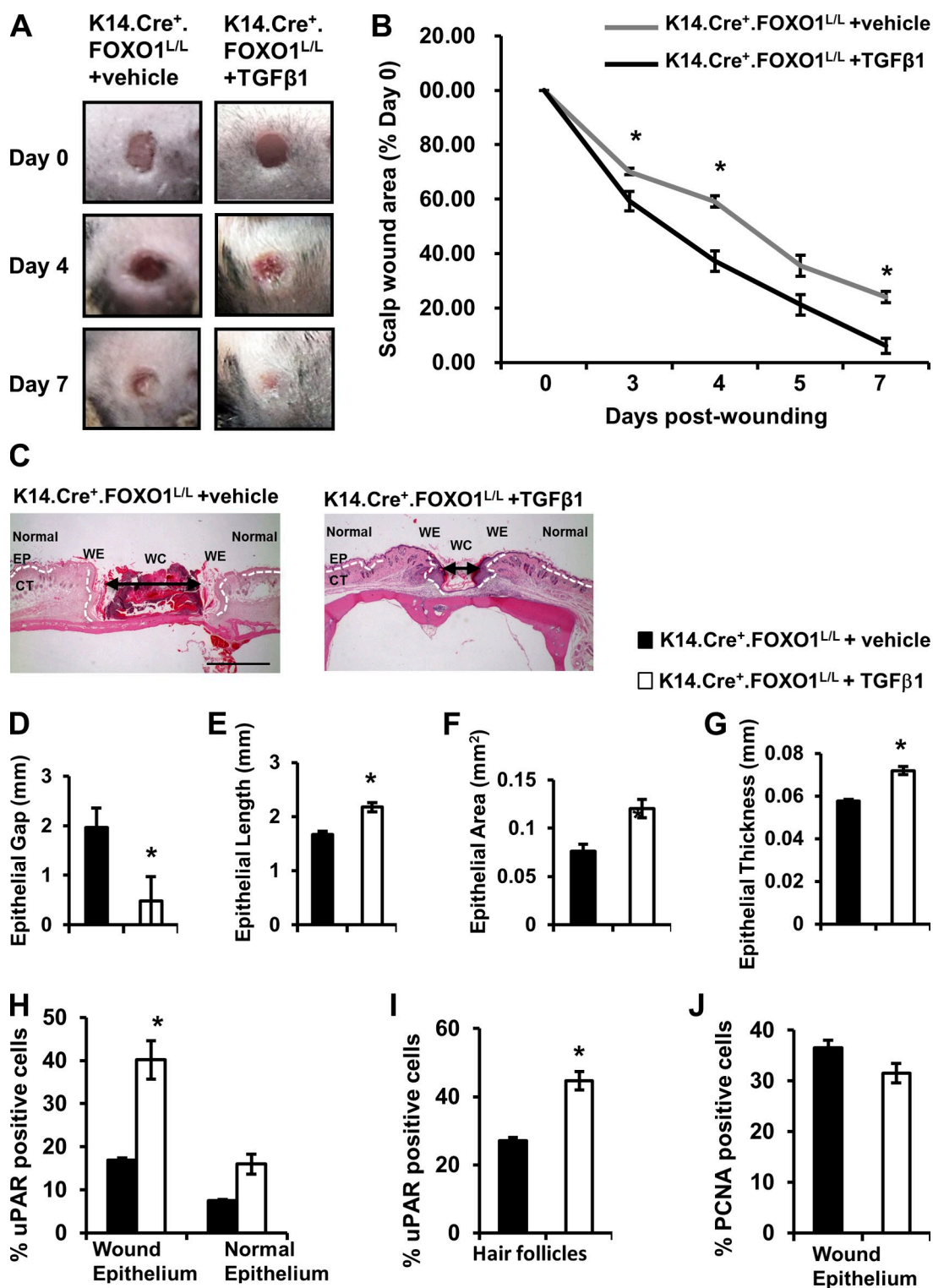


Figure 10. TGF-β1 treatment rescues impaired wound closure in FOXO1-deleted mice. (A) Representative photographs of scalp wounds on day 4 and day 7 from K14.Cre⁺.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice treated with vehicle or TGF-β1. (B) Wound-healing process was analyzed at different time points after wounding. Average wound area was presented as percentage of the wound area on day 0 (100%). (C) Hematoxylin and eosin staining of K14.Cre⁺.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice wound biopsies on day 7 after wounding. Bar, 500 μm. WC, wound center; WE, wound epithelium; Normal, normal epithelium. The quantification of epithelial migration and re-epithelialization in the day-7 scalp wounds of K14.Cre⁺.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice. The gap between epithelium (D), epithelial length (E), epithelial area (F), and epithelial thickness (G) was assessed by image analysis of hematoxylin and eosin-stained sections at the center of each wound. Paraffin-embedded cross sections from K14.Cre⁺.FOXO1^{L/L} and K14.Cre⁺.FOXO1^{L/L} mice were treated with either vehicle or TGF-β1 and were analyzed by immunofluorescence using an anti-uPAR antibody and anti-PCNA antibody. Percentage of uPAR-positive migrating keratinocytes was quantified in normal and wound epithelium (H) as well as in hair follicles (I). (J) PCNA-positive proliferative keratinocytes in wound epithelium. EP, epidermis; CT, connective tissues; *, *P* < 0.05. Error bars represent mean ± SEM, *n* = 4–6 mice per group.

Table 1. Effect of FOXO1 deletion or knockdown

Phenotype	In vivo	In vitro
Wound healing	Decrease	Decrease
Migration	Decrease	Decrease
Apoptosis	Increase	Increase
ROS or Ox damage	Increase	Increase
Proliferation	Decrease	Decrease or No change
TGF- β 1 expression	Decrease	Decrease

healing. That this is a critical event is supported by findings that TGF- β 1 is needed for normal wound healing, and when absent, wound healing is significantly retarded (Werner and Grose, 2003).

Several lines of evidence indicate that FOXO1 regulates TGF- β 1 in keratinocytes. Cre recombinase deletion of FOXO1 in keratinocytes in vivo substantially reduced the expression of TGF- β 1 with a corresponding decrease in phosphorylation of its downstream effectors SMAD2/3. The latter provides evidence for the biological activity of FOXO1-regulated TGF- β 1. In vitro knockdown of FOXO1 by *RNAi* substantially reduced TGF- β 1 mRNA levels and decreased TGF- β 1 promoter activity, and Cre recombinase deletion of FOXO1 in mouse primary keratinocytes reduced TGF- β 1 protein levels. Conversely, overexpression of constitutively active FOXO1 significantly increased TGF- β 1 promoter activity in luciferase reporter assays. ChIP assays indicate that FOXO1 directly binds to the TGF- β 1 promoter. Silencing TGF- β 1 reduced keratinocyte migration, indicating that TGF- β 1 acts as an autocrine factor to enhance migration of these cells. This is consistent with previous reports that TGF- β 1 stimulates keratinocyte migration (Hebda, 1988; Kane et al., 1991; Gailit et al., 1994). Moreover, TGF- β 1 treatment rescued impaired migration of keratinocytes with FOXO1 knockdown in a transwell assay and in an in vitro scratch assay. TGF- β 1 treatment also reversed impaired keratinocyte migration but not proliferation in vivo and rescued impaired healing in mice with conditional deletion of FOXO1 in vivo. These results agree with previous studies that TGF- β 1 regulates keratinocyte migration and wound re-epithelialization (Hebda, 1988; Zambruno et al., 1995). Thus, FOXO1 is needed for adequate TGF- β 1 expression and normal keratinocyte migration and wound healing.

Keratinocyte migration requires the up-regulation of integrins and MMPs (Gailit et al., 1994; Zambruno et al., 1995; Hsieh et al., 2010). Our data demonstrate that FOXO1 deficiency in keratinocytes significantly attenuated the expression of integrins- β 6 and - α 3 needed for wound re-epithelialization (Huang et al., 1998). In addition, silencing of FOXO1 decreased mRNA levels of matrix metalloproteinases MMP-3 and MMP-9 in keratinocytes that facilitate keratinocyte migration and wound healing (Agren, 1999). Thus, FOXO1 is needed to maintain expression of integrins and MMPs that are required for optimal keratinocyte migration and wound healing. In addition, silencing FOXO1 reduced basement membrane protein collagen IV, indicating that deletion of FOXO1 could have direct or indirect effects on wound healing that are due to altered basement membrane protein production.

We examined alternative mechanisms by which FOXO1 could affect wound healing. The formation of reactive oxygen species and the level of oxidative stress are increased in healing wounds (Schäfer and Werner, 2008; Guo and Dipietro, 2010). When normal levels of ROS are exceeded, such as in diabetic wound healing, excessive oxidative stress occurs and causes a decrease in keratinocyte proliferation and migration and increased apoptosis (Nishio and Watanabe, 1997; Wenk et al., 1999; Deveci et al., 2005). We found that the loss of FOXO1 expression in keratinocytes in vitro increased ROS levels and deletion of FOXO1 in keratinocytes in vivo resulted in increased oxidative damage in healing wounds as measured by a marker of oxidative damage, 8-OHdG (Shigenaga et al., 1989). We showed two additional mechanisms through which elevated ROS levels caused by reduced expression of FOXO1 limits the wound-healing behavior of keratinocytes. The increased susceptibility to oxidative stress caused by FOXO1 deletion is directly linked to impaired cell migration because it can be rescued by treatment with an antioxidant, NAC. Under mild oxidative stress caused by deletion of FOXO1, TGF- β 1 was also able to rescue impaired migration, but under more severe oxidative stress such as H₂O₂ combined with FOXO1 deletion it did not. Thus, FOXO1 affects keratinocyte migration through two mechanisms, regulation of TGF- β 1 and protection against the anti-migratory effect of elevated ROS. In addition, FOXO1 protection against ROS is needed to reduce oxidative damage and reduce keratinocyte apoptosis. An increase in both is linked to impaired wound healing (Soneja et al., 2005; Sen and Roy, 2008).

FOXO1 activity protects cells against oxidative stress by activating antioxidant defense mechanisms such as MnSOD and glutathione peroxidases (van der Horst and Burgering, 2007; Storz, 2011). The expression of antioxidant genes such as glutathione peroxidase 2 and cytoglobin were down-regulated in FOXO1-silenced keratinocytes in vitro. Another mechanism by which FOXO1 is protective is by modulating the expression of the DNA repair enzyme, GADD45 α , which repairs DNA damaged by oxidative stress (Furukawa-Hibi et al., 2002; Tran et al., 2002). GADD45 α had reduced expression in FOXO1-silenced keratinocytes. Collectively, these data indicate that loss of FOXO1 expression in keratinocytes results in increased ROS levels and decreased antioxidant defense mechanisms, leading to reduced keratinocyte migration and increased apoptosis. Although in a number of cell types FOXO1 promotes apoptosis, several lines of evidence suggest that FOXO1 plays a central role in cell survival during conditions of oxidative stress through up-regulation of antioxidant enzymes and regulation of cell survival pathways (Burgering and Medema, 2003; Tothova et al., 2007; Sengupta et al., 2011). Our results are in agreement with previous results that FOXO1 can protect against cellular oxidative stress (Ponugoti et al., 2012).

FOXO1 is also known to affect progression through the cell cycle. We determined that in vitro FOXO1 deletion had only a small effect by slightly reducing proliferation of primary mouse keratinocytes, and in primary human keratinocytes FOXO1 deletion had no effect. In addition, FOXO1 silencing had little effect on mRNA levels of cell cycle regulators tested such as cyclin D1 and D2, and CDK1, 2, and 4. In vivo FOXO1

deletion caused a small reduction in keratinocyte proliferation *in vivo*. This suggests that FOXO1 has only a minor effect on regulating the cell cycle of keratinocytes in wound healing.

In summary, we unexpectedly found that FOXO1 promotes healing because it is needed for keratinocyte transition to a wound-healing phenotype that involves migration and up-regulation of TGF- β 1 and its downstream targets, integrin- α 3 and - β 6 and matrix metalloproteinase-3 and -9. The negative effect of FOXO1 deletion both *in vitro* and *in vivo* was rescued by treatment with TGF- β 1. The impact of FOXO1 deletion on keratinocyte migration *in vitro* was also reversed by an antioxidant in a TGF- β 1-independent manner. Lastly, FOXO1 deletion increased susceptibility to ROS-mediated apoptosis. This change was linked to the up-regulation of genes that protect against oxidative damage and prevent apoptosis. Thus, treatment with FOXO1 agonists may represent a potential therapeutic target for enhancing tissue repair by mobilizing keratinocytes for rapid wound epithelialization and protecting them from oxidative stress.

Materials and methods

Mice

Mice expressing a floxed allele of FOXO1 were provided by R.A. DePinto (MD Anderson Cancer Center, Houston, TX) and have been previously described (Paik et al., 2007). The second major coding exon (encoding the C-terminal half of FOXO1) was targeted by the insertion of loxP sites and mice were backcrossed three times onto the FVBn background. Mice expressing keratin 14-Cre (strain Tg(KRT14-cre)1Amc/J) were obtained from the Jackson Laboratory. These mice were bred to generate experimental (K14.Cre⁺.FOXO1^{fl/fl}) and control (K14.Cre⁻.FOXO1^{fl/fl}) mice and experiments were performed using mice 16–20 wk of age. Mice with floxed allele of FOXO1, K14.Cre⁻.FOXO1^{fl/fl}, and K14.Cre⁺.FOXO1^{fl/fl} developed normally and did not show any sign of disease. All mice were maintained in a regular 12-h day-night cycle. All the animal experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

In vivo wound-healing experiments

Mice were anesthetized by i.p. administration of ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg); the scalp hair was shaved, cleansed, and two excisional wounds of 2.0 mm were created in the scalp at the midline with a sterile dermal punch as described previously (Siqueira et al., 2010). Wounds were photographed and animals euthanized to collect the wounded tissue at the indicated time points. For TGF- β 1 treatment, mice were treated topically with 100 ng of recombinant TGF- β 1 (PeproTech) in 3% methylcellulose or vehicle alone at the time of wounding and every second day thereafter. In all *in vivo* experiments 6–9 mice per group were examined at each time point and the value per animal was the unit of measurement.

Histological analysis

The scalp and attached calvarial bone were fixed in 4% paraformaldehyde for 24 h, decalcified in 10% EDTA solution, embedded in paraffin, and sectioned at 5 μ m. Sections were stained with hematoxylin and eosin (H&E) and histomorphometric analysis was performed with computer-assisted image analysis at the center of each lesion. We measured parameters such as epithelial gap, epithelial cell number, epithelial area, and epithelial thickness at the wound site.

Immunohistochemistry, immunofluorescence, and apoptosis in histological sections

Antigen retrieval was performed by heating slides in 10 mM sodium citrate (pH 6.0) for 5 min at 95°C. Sections were then incubated with primary antibody against FOXO1 (Santa Cruz Biotechnology, Inc.), proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Inc.), urokinase-type plasminogen activator receptor (uPAR; Santa Cruz Biotechnology, Inc.), 8-hydroxydeoxyguanosine (8-OHdG; EMD Millipore), TGF- β 1 (Abcam), phospho-SMAD2/3 (Santa Cruz Biotechnology, Inc.), and SMAD 2/3 (Santa Cruz Biotechnology, Inc.) or matched nonimmune primary antibody overnight at 4°C. For immunofluorescence, antibody was localized by a biotinylated secondary antibody and visualized using Alexa 546-conjugated

streptavidin, and counterstained with DAPI. For immunohistochemistry, antibody was localized by incubation with avidin-biotin horseradish peroxidase complex using a biotinylated secondary antibody. To enhance the signal, tyramide signal amplification was used that enhances the chromogenic signal (PerkinElmer). Apoptosis was assessed by the DeadEnd Fluorometric TUNEL System (Promega).

Microscopy

Sections were observed with 4x and 20x objectives and images were acquired using a fluorescence microscope (Eclipse 90i; Nikon) and a Cool-Snap EZ camera (Photometrics). All images were taken at room temperature with a TXRED or FITC filter. Image analysis was performed using Nikon AR image analysis software.

Cell culture and siRNA transfections

Primary cultures of human epidermal keratinocytes (NHEK) were purchased and maintained in KGM-2 growth medium (Lonza) supplemented with 0.1 ng/ml hEGF, 5.0 μ g/ml insulin, 0.5 μ g/ml hydrocortisone, 0.15 mM calcium, 5 ng/ml epinephrine, 10 μ g/ml transferrin, and 2 ml bovine pituitary extract. All cells were grown in a humidified atmosphere with a 5% CO₂ concentration. ON-TARGETplus SMARTpool siRNAs against FOXO1, FOXO3, TGF- β 1, and control siRNA were obtained from Thermo Fisher Scientific. DNA transfections were performed using Lipofectin (Invitrogen) and siRNA transfections were performed using GeneMute siRNA transfection reagent (Signagen).

In vitro wound-healing and transwell migration assay

NHEK cells were transfected with FOXO1 or scrambled siRNA 48 h before assay. For *in vitro* wounds, confluent cells were “scratched” using a 200- μ l pipette tip as described previously (Liang et al., 2007) and photographs were taken as indicated to assess the number of keratinocytes that had migrated into the wounded area. For the migration assay, 2 \times 10⁵ cells were incubated in the upper chamber of a transwell (Corning Costar; Thermo Fisher Scientific) containing a polycarbonate membrane filter (6.5-mm diameter, 8- μ m pore size). After 6 h, cells remaining in the upper surface of the membrane were removed and migrated cells on the lower surface of the membrane were counted after staining with DAPI. In some cases, cells were treated with 150 μ M H₂O₂, 1 mM N-acetyl-cysteine (NAC), 2 ng/ml TGF- β 1, or various combinations of these agents. Experiments were performed at least three times.

Real-time PCR

Real-time PCR was performed essentially as described previously (Siqueira et al., 2010). Total RNA was isolated from confluent NHEK cells following the manufacturer’s instructions (RNeasy; QIAGEN). 1 μ g of total RNA was reverse transcribed using High Capacity RNA-to-cDNA kit (Applied Biosystems), and real-time PCR was performed using the Universal Probe Library System (Roche). All primers were designed using the Universal Probe Library Assay Design Center (Roche). Results were normalized with mitochondrial ribosomal protein L32. Each experiment was performed 2–4 times with similar results.

Western blot

Epithelial cells were lysed with lysis buffer (Thermo Fisher Scientific) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Protein concentration was measured using a protein assay with BSA as a standard (Bio-Rad Laboratories). 50 μ g cell lysate was resolved in 4–20% SDS-PAGE (Bio-Rad Laboratories) and transferred onto PVDF membrane (Thermo Fisher Scientific). The membranes were incubated with primary antibodies against FOXO1 (Santa Cruz Biotechnology, Inc.), FOXO3 (Cell Signaling Technology), TGF- β 1 (Promega), p-SMAD2/3 (Santa Cruz Biotechnology, Inc.), SMAD2/3 (Santa Cruz Biotechnology, Inc.), and β -actin (Sigma-Aldrich) after blocking with 5% BSA. The samples were then incubated with horseradish peroxidase-labeled anti-rabbit IgG or anti-mouse IgG, and immunoreactive bands were detected with ECL Western blotting reagents (Thermo Fisher Scientific).

ChIP assays

ChIP assays were performed using ChIP-IT kit (Active Motif) following the manufacturer’s instructions, as described previously (Alblowi et al., 2009). Isolated DNA was subjected to quantitative real-time PCR by using primers specific for the TGF- β 1 promoter with a Universal Probe Library Probe (Roche). The TGF- β 1 promoter primer sequences used were: set 1 (left, 5'-CCATGTTGACAGACCCTCT-3'; right, 5'-TTAATCCGGGGGATGAGAC-3'); set 2 (left, 5'-TAAGACGGTGGGAGCCTAGA-3'; right, 5'-GGCCAGTCTTTCTCTCT-3'). ChIP experiments were repeated three times with reproducible results.

Luciferase reporter assay

Luciferase activity was measured using the Dual Luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions. In brief, NHEK cells were cotransfected with plasmid DNA of TGF- β 1-Luc, Renilla-Luc, pcDNA-FOXO1AAA, scramble, or FOXO1 siRNA. Empty vector DNA was added as needed so that the same amounts of expression vector were present in each transfection. Transfection was performed using Lipofectin (Invitrogen) in 24-well plates. 24 h after transfection, cells were lysed, and luciferase and Renilla activities were measured using a luminometer (Infinite M200; Tecan). Firefly luciferase activities were divided by Renilla activities to normalize for transfection efficiency. Experiments were performed three times with similar results.

ROS assay

The cellular ROS levels were measured by using chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA) following the manufacturer's instructions (Invitrogen). NHEK cells were transfected with FOXO1 or scrambled siRNA for 48 h in 96-well plates and then treated with 150 μ M H₂O₂, 1 mM NAC, 2 ng/ml TGF- β 1, or various combinations of these agents. After 16 h, cells were incubated with 10 μ M CM-H₂DCFDA for 30 min at 37°C and fluorescent intensity was recorded by excitation at 492 nm and emission at 527 nm using a plate reader (Infinite M200; Tecan). Experiments were performed three times with similar results.

BrdU incorporation assay

Detection of BrdU incorporation was performed by ELISA using the BrdU Cell Proliferation Assay kit (Cell Signaling Technology) according to the manufacturer's instructions. In brief, 2.5×10^3 cells were seeded onto 96-well plates and incubated with 10 μ M BrdU. The cells were then fixed and incubated with anti-BrdU antibody. BrdU incorporation was determined by measuring absorbance at 450 nm. Experiments were performed three times with similar results.

Apoptosis in vitro

Apoptosis were measured by ELISA for cytoplasmic histone-associated DNA (Roche) according to the manufacturer's instructions. In brief, NHEK cells were transfected with FOXO1 or scrambled siRNA for 48 h and seeded onto 96-well plates and cell lysates were analyzed in the ELISA. Apoptotic cells were also identified by the DeadEnd Fluorometric TUNEL System (Promega) following the manufacturer's instructions. Experiments were performed three times with similar results.

Glucose uptake assay

To measure glucose uptake, NHEK cells transfected with scrambled siRNA or FOXO1 siRNA were incubated with 200 μ M 2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose [2-NBDG; Invitrogen] for 30 min, washed three times with cold PBS, followed by flow cytometric detection of fluorescence produced by the cells. For each measurement, a minimum of 10,000 events were collected using a FACStar^{PLUS} flow cytometer (BD). Data analysis was performed using Modfit software (Verity Software House). Experiments were performed three times with similar results.

ATP determination assay

The intracellular ATP levels were measured using the ATP Determination kit (Molecular Probes; Invitrogen) in a luminometer (Infinite M200; Tecan), following the manufacturer's instructions. The protein concentration was measured using the BCA Protein Assay kit (Thermo Fisher Scientific). The relative ATP levels were normalized with the protein concentration. Experiments were performed three times with similar results.

Statistics

Statistical analysis between two groups was performed using two-tailed Student's *t* test. In experiments with multiple time points or treatments, differences between the wild-type and experimental groups were determined by one-way ANOVA with Scheffe's post-hoc test. Results are expressed as the mean \pm SEM. *P* < 0.05 was considered statistically significant.

Online supplemental material

Fig. S1 shows the images from scratch-wound and transwell migration assays of FOXO1 knockdown cells. Fig. S2 shows the effect of FOXO1 knockdown on proliferation, glucose uptake, or ATP production in keratinocytes. Fig. S3 shows that increased oxidative stress impairs keratinocyte migration of in vitro scratch wound. Fig. S4 contains images of TUNEL assay and shows that silencing FOXO1 results in increased apoptosis. Fig. S5 shows that silencing FOXO1 in keratinocytes results in decreased collagen IV α 1 mRNA levels. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201305074/DC1>.

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