GSK-3β in mouse fibroblasts controls wound healing and fibrosis through an endothelin-1–dependent mechanism

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Glycogen synthase kinase–3 (GSK-3) is a widely expressed and highly conserved serine/threonine protein kinase encoded by 2 genes, GSK3A and GSK3B. GSK-3 is thought to be involved in tissue repair and fibrogenesis, but its role in these processes is currently unknown. To investigate the function of GSK-3β in fibroblasts, we generated mice harboring a fibroblast-specific deletion of Gsk3b and evaluated their wound-healing and fibrogenic responses. We have shown that Gsk3b-conditional-KO mice (Gsk3b-CKO mice) exhibited accelerated wound closure, increased fibrogenesis, and excessive scarring compared with control mice. In addition, Gsk3b-CKO mice showed elevated collagen production, decreased cell apoptosis, elevated levels of profibrotic α-SMA, and increased myofibroblast formation during wound healing. In cultured Gsk3b-CKO fibroblasts, adhesion, spreading, migration, and contraction were enhanced. Both Gsk3b-CKO mice and fibroblasts showed elevated expression and production of endothelin-1 (ET-1) compared with control mice and cells. Antagonizing ET-1 reversed the phenotype of Gsk3b-CKO fibroblasts and mice. Thus, GSK-3β appears to control the progression of wound healing and fibrosis by modulating ET-1 levels. These results suggest that targeting the GSK-3β pathway or ET-1 may be of benefit in controlling tissue repair and fibrogenic responses in vivo.

Introduction

During tissue repair, which requires the reconstitution of the epithelial barrier and the underlying connective tissue (1), mesenchymal cells such as fibroblasts migrate into the wound area, where they create a new collagen- and cellular fibronectin–rich ECM (2). The specialized fibroblasts that perform this function are termed myofibroblasts, so called as they express the highly contractile protein α-SMA. Myofibroblasts generate the adhesive and tensile forces required for wound closure (3). During normal tissue repair, myofibroblasts disappear from the wound; persistence of the myofibroblast characterizes fibroproliferative diseases (3, 4), which affect the skin and internal organs, often resulting in organ failure and death. There is no therapy for fibrotic disease, as the fundamental mechanism underlying the progression from normal tissue repair to fibrogenesis is largely unknown.

Glycogen synthase kinase–3 (GSK-3), a widely expressed and highly conserved serine/threonine protein kinase, is encoded by 2 genes in mammals, generating GSK-3α and GSK-3β (5). GSK-3 was first isolated based on its activity on glycogen synthase but has broader roles in many cellular processes (6). The protein kinase is unusual, as it is generally highly active in resting cells but inhibited in response to cellular signals. These include signals activating receptor tyrosine kinases that activate the PI3K/PKB (Akt) pathway (7). GSK-3 also is involved with the canonical Wnt pathway, which relies on β-catenin (8). When Wnts bind the frizzled (Fz) receptor and coreceptor lipoprotein receptor–related protein 5 or 6 (LRP5/6), a cascade of signaling events causes the phosphorylation and inactivation of GSK-3β. GSK-3β normally phosphorylates β-catenin on serine and threonine residues, causing β-catenin to be targeted for degradation (9, 10). β-Catenin then migrates into the nucleus to activate transcription (11). β-Catenin expression is elevated in mesenchymal cells during the proliferative phase of wound healing, and it is overexpressed in conditions of fibroblast hyperproliferation, such as fibromatosis and hyperplastic scars (12, 13). Overexpression of β-catenin in mice results in fibrotic tumors in vivo, and wounding of these mice results in hyperplastic scarring (13). It has been hypothesized that inactivity of GSK-3β may play a role in these processes (14); however, this hypothesis has yet to be tested. Moreover, the transcriptional targets of the GSK-3β/β-catenin cascade mediating its effects on tissue repair and fibrogenesis have yet to be identified.

Mice lacking GSK-3β die late in gestation due to liver apoptosis (15). To evaluate whether GSK-3β plays a role in particular tissues or in adults, such as in wound healing or fibrogenesis, conditional ablation of GSK-3β in vivo is required. To investigate the mechanism of GSK-3β action in fibroblasts in vivo, we conditionally deleted Gsk3b in fibroblasts and assessed the response of these mice and fibroblasts derived from them to wound healing and fibrogenic responses in vivo and in vitro. Our results show that GSK-3β normally acts as a brake to terminate tissue repair through its ability to block ET-1 production and are consistent with the notion that a failure to properly terminate tissue repair results in fibrosis.

Results

Generation of Gsk3b-conditional-KO mice. To generate mice with deletion of Gsk3b in fibroblasts, we crossed mice harboring a Gsk3b allele with a floxed (i.e., flanked by loxP sites) exon 2 (Gsk3bβ) with mice containing a CreER(T) recombinase gene located down-
Conditional deletion of Gsk3b results in increased cutaneous wound repair, enhanced collagen synthesis, and decreased apoptosis, resulting in excessive scarring and fibrogenesis in vivo. Immunofluorescence using GSK-3β antibody in day 0 unwounded skin of Gsk3b-C and -CKO mice. Representative data from n = 8 cell lines from 8 mice. Scale bar: 50 μm. (E) Loss of GSK-3β protein expression in fibroblasts isolated from Gsk3b-CKO mice. n = 8 cell lines from 8 mice is shown. *P < 0.05.
Migration, spreading, adhesion, and contraction are elevated in GSK-3β-deficient fibroblasts. To further investigate the basis for the wound-healing phenotype of the Gsk3b-CKO animals, we explanted dermal fibroblasts from Gsk3b- and -CKO mice and subjected them to the scratch wound assay of in vitro repair. Gsk3b-CKO cells migrated faster than their counterparts (Figure 5, A and B). Cell spreading was monitored after adhesion microscopically, using rhodamine-phalloidin and anti-vinculin antibodies. We found that loss of GSK-3β also resulted in enhanced spreading of fibroblasts on fibronectin, as revealed with anti-vinculin antibody (green) and rhodamine-phalloidin (red) staining to detect actin (Figure 5C). To further investigate the contribution of GSK-3β to the function of fibroblasts, we assessed whether Gsk3b-CKO cells showed enhanced ability to adhere to fibronectin. We performed a standard quantitative adhesion assay, which showed increased Gsk3b-CKO cell adhesion to fibronectin (Figure 5D). Finally, Gsk3b-CKO cells had an elevated ability to contract a floating collagen gel matrix (Figure 5E).

α-SMA, collagen, and ET-1 expression is elevated in Gsk3b-CKO fibroblasts. The α-SMA–expressing myofibroblast is the cell type responsible for ECM remodeling in wound healing. Western blot analysis showed a significant increase (P < 0.05) in α-SMA protein expression in Gsk3b-CKO cells (Figure 6A). To investigate whether loss of GSK-3β showed any effect on myofibroblast formation, we used indirect immunofluorescence analysis with an anti–α-SMA antibody to show that Gsk3b-CKO cells had increased α-SMA expression and α-SMA-containing stress fibers (Figure 6B). Real-time PCR analysis also indicated elevated α-SMA and Col1a2 mRNA expression in Gsk3b-CKO cells (Figure 6C).

The vasoconstrictive protein endothelin-1 (ET-1) is elevated in fibrotic diseases such as scleroderma and is responsible for the persistent fibrotic phenotype of fibroblasts isolated from lesions of these patients (17–19). To investigate whether ET-1 may be playing a role in the phenotype of Gsk3b-CKO mice, we first showed elevated ET-1 protein expression in day 7 Gsk3b-CKO compared with Gsk3b-C wounds (Figure 7A). Immunohistochemistry showed enhanced ET-1–positive staining in day 7 wound tissues of Gsk3b-CKO compared with Gsk3b-C mice (Figure 7B). In isolated Gsk3b-CKO compared with Gsk3b-C fibroblasts, ET-1 mRNA and protein levels were elevated, as revealed by real-time PCR and a specific ET-1 ELISA, which detected secreted ET-1 in cell culture media (Figure 7, C and D).

ET-1 signaling is responsible for the Gsk3b-CKO phenotype. Based on these data, we hypothesized that ET-1 may be directly responsible for the phenotype of Gsk3b-CKO fibroblasts. We used the dual
ETA/B receptor antagonist bosentan to assess whether the in vitro phenotype of Gsk3b-CKO fibroblasts was ET-1 dependent. We found that bosentan blocked the excess α-SMA stress fiber formation and protein expression (Figure 8, A and B), the overexpression of α-SMA and type I collagen mRNA (Figure 8, C and D), and the enhanced cell migration (Figure 8E), adhesion (Figure 8F), and ECM contraction of Gsk3b-CKO fibroblasts (Figure 8G). These results indicate that the in vitro fibrotic phenotype of Gsk3b-CKO fibroblasts was ET-1 dependent.

ETA/B receptor antagonism reverses the wound-healing and fibrotic phenotype of Gsk3b-CKO mice. Given that our previous analyses had uncovered that the in vitro fibrotic phenotype of Gsk3b-CKO fibroblasts was due to ET-1, we sought to assess whether the in vivo phenotype of Gsk3b-CKO mice was also due to ET-1. To investigate this question, mice were fed bosentan every day for 21 days. Bosentan had no effect on wound closure kinetics of normal control mice, indicating that ET-1 signaling did not play a role in normal wound healing. However, bosentan reversed the accelerated wound healing phenotype of Gsk3b-CKO mice (Figure 9, A and B). Moreover, bosentan reduced the collagen deposition and overexpression phenotype of Gsk3b-CKO mice (Figure 9, C and D) and reduced numbers of α-SMA-expressing myofibroblasts (Figure 9, E and F).

In addition, bosentan rescued the loss of apoptosis in Gsk3b-CKO mice (Figure 9, G and H). These results indicate that ET-1 signaling contributes substantially to the altered wound repair responses resulting from the loss of GSK-3β but not appreciably to normal tissue repair. These results extend our in vitro analyses and suggest that the wound-healing and fibrotic phenotype of GSK-3β-deficient mice is due to elevated ET-1 production.

Overexpression of ET-1 in Gsk3b-CKO fibroblasts is due to elevated β-catenin expression. GSK-3β normally phosphorylates β-catenin on serine and threonine residues, causing β-catenin to be targeted for degradation. In the absence of GSK-3β, β-catenin escapes degradation and is translocated into the nucleus. We investigated whether loss of GSK-3β showed any effect on the expression of β-catenin in day 7 wounds of Gsk3b-CKO mice versus Gsk3b-C mice and in dermal fibroblasts isolated from Gsk3b-CKO mice versus Gsk3b-C mice. Results showed that loss of GSK-3β in fibro-
blasts resulted in enhanced protein expression of β-catenin in day 7 wounds of Gsk3b-CKO mice versus Gsk3b-C mice (Figure 10A). In addition, we observed enhanced β-catenin protein expression in Gsk3b-CKO versus Gsk3b-C fibroblasts (Figure 10B). Transfection with siRNA directed toward β-catenin, compared with control siRNA, showed that reduction in β-catenin expression decreased endothelin production in Gsk3b-CKO fibroblasts (Figure 10, C–E). These results indicate that loss of GSK-3β results in increased ET-1 production via elevated β-catenin expression, culminating in fibrogenesis (Figure 10F).

Discussion

In this study, we tested the effect of loss of GSK-3β in fibroblasts on tissue repair in vivo and in vitro. We performed this analysis using mice homozygous for a Gsk3b gene flanked by loxP sites and hemizygous for a transgene encoding a tamoxifen-dependent CreER(T) recombinase driven by a fibroblast-specific proα2(1) collagen promoter/enhancer (16). The tissue specificity of this promoter/enhancer construct has been confirmed in vivo (20). Gsk3b-CKO mice showed accelerated wound closure and enhanced collagen deposition. Gsk3b-CKO mice displayed decreased apoptosis and ECM contraction. The fibrogenic protein ET-1 (17–19) was overexpressed in Gsk3b-CKO mice in vivo and by Gsk3b-CKO fibroblasts in vitro. The phenotype of Gsk3b-CKO fibroblasts in vitro and Gsk3b-CKO mice in vivo was rescued by the dual ETA/B receptor antagonist bosentan. Of significance, ET receptor antagonism did not impede tissue repair in Gsk3b-C mice yet reverted the phenotype of Gsk3b-CKO mice to normal. These results suggest for the first time to our knowledge that loss of GSK-3β results in enhanced tissue repair and fibrogenesis in vivo, due to the elevated ET-1 levels.

Fibrotic diseases are characterized by the failure to terminate normal tissue repair and the persistence of myofibroblasts within lesions (4, 17, 19). A specific role for GSK-3β in wound healing is suggested by our finding that wound repair is severely enhanced.

### Table 1

<table>
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<tr>
<th>Epidermal thickness (time course of wound healing)</th>
<th>Day 7</th>
<th>Day 21</th>
<th>Day 28</th>
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<tbody>
<tr>
<td>Gsk3b-C (n=6)</td>
<td>69.81 ± 0.80</td>
<td>54.26 ± 5.29</td>
<td>44.43 ± 6.81</td>
</tr>
<tr>
<td>Gsk3b-CKO (n=5)</td>
<td>79.22 ± 9.33</td>
<td>63.29 ± 11.01</td>
<td>48.99 ± 7.16</td>
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Epidermal thickness (measured from the basement membrane to the stratum corneum) of day 7, 21, and 28 wounds of Gsk3b-C and -CKO mice were measured using Northern Eclipse software. No significant changes in the epidermal thickness were observed on days 7, 21, and 28 after wounding in Gsk3b-CKO mice. Data represent epidermal thickness in micrometers. n, number of animals used at each time point/group.
in mice carrying a dermis-specific deletion of Gsk3b. Moreover, our results indicate that loss of GSK-3β results in decreased cell apoptosis and excessive tissue repair, resulting in fibrogenesis through an ET-1-dependent mechanism. We have previously shown that overproduction of ET-1 by fibrotic fibroblasts is a key phenotypic feature of the disease systemic sclerosis, being responsible for the elevated α-SMA expression, adhesion, contraction, and migration of this cell type (17–19). Blockade of ET-1 action in these models did not appreciably affect normal cell physiology. Results presented here indicating that ET receptor antagonism suppressed the profibrotic effects of Gsk3b deletion without affecting the kinetics of the normal wound healing response emphasize that ET-1 appears to contribute to pathological wound-healing responses. Moreover, our data suggest that mutations or loss of function of GSK-3β may result in fibrotic conditions in vivo and that this effect may be mediated by ET-1. Our results showing that GSK-3β may function in vivo to suppress excessive tissue repair, and hence myofibroblast activity and fibrogenesis, provide a useful step in identifying the overall process underlying normal tissue repair and its progression to fibrogenesis. Finally, our results also strongly suggest that targeting GSK-3β or ET-1 may be an appropriate strategy for antifibrotic drug intervention.

ET-1 was identified more than 15 years ago as a small bioactive peptide derived from a larger peptide secreted from endothelial

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Figure 5
Loss of GSK-3β results in an enhanced ability of fibroblasts to migrate, spread, adhere, and contract on ECM in vitro. (A and B) Migration. Scratch assay was performed to assess the rate of migration of Gsk3b-C and Gsk3b-CKO dermal fibroblasts at 0, 24, and 36 hours after scratch injury. A significant (P < 0.05) increase in the migration rate was observed in Gsk3b-CKO compared with Gsk3b-C fibroblasts at 36 hours after scratch injury (n = 4 cell lines from 4 mice). Scale bar: 200 μm. (C) Cell spreading over fibronectin. Dermal fibroblasts were plated on fibronectin-coated plates for 30, 60, 90, and 120 minutes and stained with F-actin and vinculin. Gsk3b-CKO fibroblasts showed greater spreading over fibronectin compared with Gsk3b-C fibroblasts (n = 4 cell lines from 4 mice). Scale bar: 50 μm. (D) Percent adhesion to fibronectin. Dermal fibroblasts were plated on a 96-well plate coated with fibronectin and assessed for percent adhesion to fibronectin at 30, 60, 90, and 120 minutes by MTT assay. Gsk3b-CKO fibroblasts showed significantly greater adhesion to fibronectin compared with Gsk3b-C fibroblasts (n = 4 cell lines from 4 mice). (E) Contraction of collagen gel matrices. Loss of GSK-3β results in an enhanced ability of fibroblasts to contract a collagen gel matrix (n = 4 cell lines from 4 mice; mean ± SEM is indicated). *P < 0.05.
cells (21). ET-1 has a potent vasoconstrictive role and, more recently, has been proposed to be a key contributor to pathological processes, including cancer and fibrosis (21, 22). Our data support a previously proposed notion that ET-1 is essential for driving the excessive scarring observed in fibrotic disease and, in particular, the lung fibrosis observed in scleroderma (17–19, 21, 22). Loss of GSK-3β results in activation of the β-catenin/Wnt signaling cascade, and previous data revealing that ET-1 is a direct target of Wnt signaling in NIH 3T3 fibroblasts (23) and that β-catenin induces ET-1 in colon cancer cells (24) are therefore consistent with the observations presented in this report. Nonetheless, our data indicate, we believe for the first time, (a) that loss of GSK-3β in fibroblasts results in excessive tissue repair and fibrosis and (b) that this is due to an elevation of ET-1. To our knowledge, ET-1 expression has not been previously demonstrated to impact GSK-3β.

In summary, our studies examining the involvement in GSK-3β in dermal function may have profound implications for both homeostatic and pathological wound-healing processes by contributing to our understanding of basic mechanisms regarding wound healing and its progression to fibrogenesis. Our results may therefore have long-term therapeutic implications for the treatment of nonhealing or chronic skin wounds and of fibrosis.

**Methods**

**Generation of Gsk3b-CKO mice.** The generation of mice expressing a conditional allele of GSK-3β was previously described (25). Briefly, R1 embryonic stem cells were electroporated with a modified GSK-3β targeting vector in which loxP sites were introduced in flanking exon 2 of GSK-3β. ES cell clones that had undergone correct homologous recombination were identified by Southern blot analysis and microinjected into C57BL/6J

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**Figure 6**

Loss of GSK-3β in fibroblasts results in increased α-SMA and type I collagen expression. (A–C) α-SMA and Col1a2 (type I collagen) expression. (A) α-SMA protein expression was assessed in Gsk3b-CKO versus Gsk3b-C dermal fibroblasts by Western blotting. Gsk3b-CKO fibroblasts showed significantly (P < 0.05) higher expression of α-SMA protein versus Gsk3b-C fibroblasts. Representative data from n = 4 cell lines from 4 mice are shown. (B) Immunofluorescence for α-SMA showed greater stress fiber formation and higher expression of α-SMA in Gsk3b-CKO versus Gsk3b-C fibroblasts (n = 4 cell lines from 4 mice). Arrows indicate α-SMA-positive stress fibers. Scale bar: 50 μm. (C) α-SMA and type I collagen mRNA expression was assessed in Gsk3b-CKO versus Gsk3b-C fibroblasts by real-time PCR. Gsk3b-CKO fibroblasts showed significantly (P < 0.05) higher expression of both α-SMA and type I collagen (Type I Col) protein versus Gsk3b-C fibroblasts (n = 5 cell lines from 5 mice). *P < 0.05.

**Figure 7**

Loss of GSK-3β results in increased ET-1 expression. (A) ET-1 protein expression. Western blot analysis showed a significant (P < 0.05) increase in the protein expression of ET-1 in day 7 wounds of Gsk3b-CKO compared with Gsk3b-C mice. Representative data for n = 8 animals per group are shown. (B) ET-1 immunohistochemistry. Higher expression levels of ET-1 were detected in day 7 wounds of Gsk3b-CKO compared with Gsk3b-C mice. Representative data for n = 4 animals per group are shown. Arrows indicate ET-1-positive staining. Scale bar: 100 μm. (C) ET-1 mRNA expression was assessed in Gsk3b-C/CKO versus Gsk3b-C fibroblasts by real-time PCR. Gsk3b-CKO fibroblasts showed significantly (P < 0.05) higher expression of ET-1 mRNA versus Gsk3b-C fibroblasts (n = 4 cell lines from 4 mice). (D) ET-1 ELISA. ET-1 levels were assessed in the cell culture supernatants of Gsk3b-CKO versus Gsk3b-C fibroblasts by ELISA. Gsk3b-CKO fibroblasts showed significantly (P < 0.05) higher levels of ET-1 versus Gsk3b-C fibroblasts (n = 6 cell lines from 6 mice). *P < 0.05.
blastocysts. The resultant chimeric mice were crossed to C57BL/6J (The Jackson Laboratory). Germline transmission of the Gsk3b floxed allele was verified by PCR. Interbreeding of these mice yielded Gsk3b floxed (Gsk3b<sup>F/F</sup>) mice that were viable, healthy, born at the expected Mendelian frequency, and expressed GSK-3β at levels indistinguishable from those of wild-type animals.

C57BL/6J mice that carry a tamoxifen-inducible Cre-recombinase [CreER(T)] under the control of a fibroblast-specific regulatory sequence

**Figure 8**

The dual ETA/B receptor antagonist bosentan alleviates the phenotype of GSK-3β–deficient fibroblasts (in vitro). (A) α-SMA immunofluorescence. Gsk3b-C and Gsk3b-CKO fibroblasts were treated with bosentan for 48 hours, and α-SMA immunofluorescence was performed. Representative data from n = 4 cell lines from 4 mice are shown. Arrows indicate α-SMA–positive stress fibers. Scale bar: 50 μm. (B) α-SMA protein expression. Bosentan treatment significantly (P < 0.05) reversed the enhanced protein expression of α-SMA in Gsk3b-CKO fibroblasts. Representative data from n = 4 cell lines from 4 mice are shown. (C and D) α-SMA and Col1a2 mRNA expression. Bosentan treatment significantly (P < 0.05) reduced the expression of both α-SMA and type I collagen in Gsk3b-CKO fibroblasts (n = 5 cell lines from 5 mice). (E) Migration scratch assay. Bosentan treatment significantly (P < 0.05) decreased migration of Gsk3b-CKO fibroblasts (n = 4 cell lines from 4 mice). Scale bar: 200 μm. (F) Percent adhesion to fibronectin. Bosentan treatment significantly (P < 0.05) reversed the enhanced adhesion of Gsk3b-CKO dermal fibroblasts to fibronectin (n = 4 cell lines from 4 mice). (G) Gel contraction assay. Bosentan treatment significantly (P < 0.05) reversed the enhanced ability of Gsk3b-CKO fibroblasts to contract the collagen gel matrix (n = 3; mean ± SEM is indicated). *P < 0.05.
Figure 9
The dual ETA/B receptor antagonist bosentan alleviates the phenotype of GSK-3β-deficient mice (in vivo). (A and B) Kinetics of wound closure was determined in Gsk3b-C and Gsk3b-CKO mice treated with bosentan (100 mg/kg) or gum arabic (vehicle) on days 0, 7, and 10 after wounding. Bosentan treatment significantly (P < 0.05) reversed the enhanced rate of wound closure in Gsk3b-CKO mice without affecting the wound closure of Gsk3b-C mice (n = 4 wounds from 4 mice). Scale bar: 1 mm. (C) Effect of bosentan treatment on collagen synthesis was assessed using van Gieson staining in day 21 wounds. Note the reduction in the intensity of pink/red staining in Gsk3b-CKO animals in the presence of bosentan. Representative data from n = 4 wounds from 4 mice are shown. Scale bar: 100 μm. (D) Hydroxyproline levels. Bosentan (P < 0.05) reversed elevated wound collagen (hydroxyproline) synthesis in Gsk3b-CKO mice on day 21 after wounding (n = 4 wounds from 4 mice). (E) Bosentan (P < 0.05) reduced and reversed elevated α-SMA expression in Gsk3b-CKO mice on day 21 after wounding (n = 4 wounds from 4 mice). Scale bar: 100 μm. (F) Bosentan significantly (P < 0.05) reduced and reversed elevated α-SMA expression in Gsk3b-CKO mice on day 21 after wounding (n = 3). *P < 0.05. (G and H) Bosentan (P < 0.05) rescued loss of apoptosis in Gsk3b-CKO mice on day 7 after wounding (n = 4 wounds from 4 mice). Arrows indicate apoptotic cells. Scale bar: 100 μm. *P < 0.05.
from the proα2(I) collagen gene [C57BL/6J-Tg(COL1a2-CreER(T))] (16) were crossed with Gsk3β<sup>F/F</sup> mice to generate mice heterozygous for both alleles. The second cross between Gsk3β<sup>F/F</sup> mice and heterozygous mice from the first cross produced Col1a2-CreER(T)/0; Gsk3β<sup>F/F</sup> mice, which were used for further experiments. Animals used for experiments were genotyped by PCR to detect GSK-3β and CreER(T) as described previously (5, 25). All animal protocols were approved by the regulatory authority of the Animal Use Subcommittee of the Council on Animal Care of the University of Western Ontario. To delete Gsk3β, a stock solution of tamoxifen (4-hydroxitamoxifen; Sigma-Aldrich) in ethanol (100 mg/ml) was diluted in corn oil to 10 mg/ml. Adult mice (age, 3 weeks) were given intraperitoneal injections of the tamoxifen suspension (0.1 ml of 10 mg/ml) over 10 days. Deletion of Gsk3β was tested by PCR genotyping using the following primers: forward, 5′-GGGGCAACCTTAATTTCATT-3′ and reverse, 5′-TCTGGGCTATAGCTATCTAGTAACG-3′. For CreER(T), the following primers were used: forward, 5′-ATCCGAAAAGAAAACGTTGA-3′ and reverse, 5′-ATCCAGGT-TACGGATATAGT-3′. Deletion of Gsk3β was further confirmed by both immunofluorescence (both in vitro and in vivo) and Western blotting using an anti–GSK-3β antibody (Cell Signaling Technology).

**Wound surgery.** Experiments were performed on littermate mice homozygous for the GSK-3β<sup>Flox–Flox</sup> (loxP–GSK-3β) allele and hemizygous for Col1a2-creER(T) [Col1a2-creER(T)/0; Gsk3β<sup>F/F</sup>] that were treated with either tamoxifen (conditional knockout Gsk3β [Gsk3β-CKO]) or corn oil (control Gsk3β [Gsk3β-C]) alone. Wounding experiments were carried out 2 weeks after the last injection of tamoxifen. Mice were anesthetized by intraperitoneal injection of 90 μg ketamine plus 10 μg xylazine/g, and their back skin was shaved, depilated with Nair hair removal spray (Church & Dwight Co.), and cleaned with alcohol. Using a sterile 4-mm biopsy punch, 4 bilateral full-thickness skin wounds were created on the dorsorostral back skin without injuring the underlying muscle. Wounds were separated by a minimum of 6 mm of uninjured skin. Wounds were photographed at 0, 3, 7, and 10 days after wounding using a Sony D-9 digital camera. Wound area was measured using Northern Eclipse (Empix) software, and percentage wound closure at each time point was derived by following formula: (1 – current wound area/initial wound area) × 100%.

**Figure 10** Loss of GSK-3β results in increased β-catenin expression, which is responsible for ET-1 overexpression. (A and B) β-Catenin protein expression. Western blot analysis showed a significant (P < 0.05) increase in the expression of β-catenin in day 7 wounds and dermal fibroblasts of Gsk3β-CKO compared with GSK-3β C mice. Representative data for n = 4 animals per group are shown. (C) siRNA recognizing β-catenin reduces β-catenin protein expression in Gsk3β-CKO fibroblasts. Transfection of Gsk3β-CKO fibroblasts with siRNA recognizing β-catenin, compared with control siRNA, reduced the expression of β-catenin as revealed by Western blot analysis with anti-β-catenin antibody. Hours after transfection are indicated. (D and E) siRNA recognizing β-catenin reduces ET-1 protein and mRNA expression in Gsk3β-CKO fibroblasts. Forty-eight hours after transfection with control or β-catenin siRNA, Gsk3β-CKO fibroblasts were examined for ET-1 protein production by Western blot analysis with anti–ET-1 antibody or by real-time PCR to detect ET-1 mRNA. Fibroblasts from 6 mice were used. *P < 0.05. (F) Model showing effect of loss of GSK-3β expression on tissue repair in vivo.
wound size/initial wound size) × 100. Mice were sacrificed by CO2 euthanasia after 3, 7, 21, and 28 days after wounding. Wound tissue biopsies were collected for histology, immunohistochemistry, immunofluorescence, hydroxyproline assay, and biochemical analysis.

**Cell culture, immunofluorescence, and Western blot analysis.** Dermal fibroblasts were isolated from explants (4 to 6-week-old animals) as previously described (4). Cells were subjected to indirect immunofluorescence analysis as previously described (6, 12) using anti–α-SMA, rhodamine-phalloidin (Sigma–Aldrich), and anti-vinculin (Sigma–Aldrich) antibodies, followed by an appropriate secondary antibody (Jackson Immunoresearch Laboratories Inc.), and photography (Zeiss Axiopt) using a digital camera (Empix). Alternatively, cells were lysed in 2% SDS, and proteins quantified (Pierce) and subjected to Western blot analysis as previously described (4, 17, 26). The following primary antibodies were used for Western blotting: anti–GSK-3β (Cell Signaling Technology), anti–PCNA (Abcam), anti–α-SMA (Sigma–Aldrich), anti–β-actin (Sigma–Aldrich), phalloidin (Cyto–skeleton), anti–vinculin (Sigma–Aldrich), ET-1 (Peninsula Laboratories), and anti–β-catenin (Cell Signaling Technology).

**Apoptosis assay.** Wound tissue sections (0.5 mm) were cut using a microtome (Leica) and collected on Superfrost Plus slides (Fisher Scientific). Sections were then dewaxed in xylene and rehydrated by successive immersion in descending concentrations of alcohol. Morphological evaluation of cells undergoing apoptosis during wound healing in Gsk3b-C and -CKO mice was performed using Fluorescein FragEL DNA Fragmentation Detection Kit (Calbiochem). Assay was performed according to the manufacturer’s recommendations. Detection and analysis of labeling was performed by fluorescence microscopy (Zeiss Axiopt).

**Real-time PCR.** Real-time PCR was performed essentially as previously described (26, 27). Cells were cultured until 80% confluence and serum starved for 24 hours. Total RNA was isolated (RNeasy; QIAGEN). The integrity of the RNA was verified by gel electrophoresis or Agilent bioanalyzer. Total RNA (25 ng) was reverse transcribed and amplified using TaqMan Assays-on-Demand (Applied Biosystems) in a 15-μl reaction volume containing 2 unlabeled primers and 6-carboxyfluorescein–labeled TaqMan MGB probe. Samples were combined with One-Step MasterMix (Eurogen technologies). Amplified sequences were detected using the ABI Prism 7900 HT sequence detector (Applied Biosystems) according to the manufacturer’s instructions. Triplicate samples were run, and expression values were standardized to values obtained with control 28S RNA primers using the ΔΔCt method. Statistical analysis was performed using Student’s paired t test.

**Adhesion, contraction, and migration assays.** Adhesion and contraction experiments were performed as previously described (4, 17). For in vitro wound healing (migration) experiments, cultured fibroblasts obtained from Gsk3b-C and -CKO mice were grown in 12-well plates. Medium was removed, and cells were rinsed once with serum-free medium plus 0.1% BSA and were cultured for 24 hours in serum-free medium plus 0.1% BSA. The monolayer was artificially injured by scratching across the plate with a blue pipette tip (approximately 1.3 mm in width). The wells were washed 2 times to remove detached cells or cell debris. The cells were then cultured in serum-free medium. Mitomycin C (10 μg/ml; Sigma–Aldrich) was always included in the media to prevent cell proliferation. After 24 and 48 hours, images of the scratched areas under each condition were photographed. Scratch wound area was measured using Northern Eclipse (Empix) software, and percentage of wound closure at each time point was derived by following formula: (1 – [current wound size/initial wound size]) × 100.

**Semiquantitative histological assessment of quality of scarring.** Wound tissue sections (0.5 mm) were cut using a microtome (Leica) and collected on Superfrost Plus slides (Fisher Scientific). Sections were then dewaxed in xylene and rehydrated by successive immersion in descending concentrations of alcohol. To assess the effects of Gsk3b conditional deletion on wound collagen synthesis and quality of scarring, van Gieson collagen stain was used. Van Gieson stain results in a deep red color for mature collagen fibers and a pink color for immature collagen fibers. Muscle and fibrin appear yellow with black nuclei. Maturity of collagen fibers was assessed in a blinded manner using the following assessment criteria: 0 signifies pale pink color of collagen fibers; 1, pink color of collagen fibers; 2, pink and red color of collagen fibers; 3, deep red color of collagen fibers (similar to the surrounding un wounded dermis fibers).

**Immunohistochemistry.** Sections were cut and processed as described above. Immunolabeling of PCNA, α-SMA, and ET-1 was performed using the DakoCyto Consortium LSAB+ System–HRP kit. Immunohistochemical procedures were performed according to the manufacturer’s recommendations. Briefly, endogenous peroxide was blocked using 0.5% H2O2 in methanol for 5 minutes. Sections were incubated with BSA (1%) in PBS for 1 hour to block nonspecific IgG binding, then incubated with primary antibody against PCNA (1:50), α-SMA (1:1,000; Sigma–Aldrich), and ET-1 (1:100; Peninsula Laboratories) in a humidified chamber, and left overnight at 4°C. Next, sections were incubated with biotinylated link for 30 minutes, followed by incubation with streptavidin for 30 minutes. The chromogen diaminobenzidine tetrahydrochloride (DAB) was then added, and after color had developed sufficiently, sections were counterstained with Harris’s hematoxylin. For PCNA, α-SMA, and ET-1 immunostaining, the number of PCNA-, α-SMA-, and ET-1–positive cells was calculated, divided by total number of cells, and the results expressed as percentage positive cells.

**Hydroxyproline assay.** Hydroxyproline assay was performed as a marker of collagen synthesis in wound tissues using the method previously described (28). Wound tissues were homogenized in saline and hydrolyzed with 2N NaOH for 30 minutes at 120°C, which was followed by the determination of hydroxyproline by modification of the Neumann and Logan’s reaction (29) using chloramine T and Ehrlich’s reagent, using a hydroxyproline standard curve and measuring at 550 nm. Values were expressed as micrograms of hydroxyproline per milligram of protein.

**Bosentan treatment (in vitro).** To inhibit the effects of ET-1, Gsk3b-C and -CKO dermal fibroblasts were grown to confluence and serum starved for 24 hours and then treated with bosentan (an endothelin receptor antagonist; 10 μM) for 48 hours. The effect of bosentan on migration, adhesion, and the expression of α-SMA and Coll1a2 in Gsk3b-C and -CKO fibroblasts was determined as described above in Methods.

**Bosentan treatment (in vivo).** Gsk3b-C and -CKO mice were treated with 100 mg/kg/d bosentan monohydrate (a kind gift from Actelion; dosage recommended by the manufacturer) or vehicle (gum arabic; Sigma-Aldrich) for 21 days after wounding via oral gavage. The effect of bosentan treatment on wound closure was determined by photographing wounds at 0, 3, 7, and 10 days after wounding using a Sony D-9 digital camera. Wound area was analyzed using Northern Eclipse (Empix) software, and wound closure was expressed as percentage of initial wound size as described above in Methods. At the end of the 21-day time course, mice were killed by CO2 asphyxiation. Wound tissue biopsies were collected for histology, immunohistochemistry, hydroxyproline assay, and biochemical analysis.

**ET-1 enzyme immunoassay.** The levels of ET-1 in cell culture supernatants of dermal fibroblasts extracted from Gsk3b-C and -CKO mice were determined by Endothelin ELISA Kit (Biomedica). The assay was performed according to the manufacturer’s recommendation.

**β-Catenin siRNA transfection.** To silence β-catenin, Gsk3b-C and -CKO dermal fibroblasts were transfected with β-catenin siRNA (20 nM; Dharmacon, USA) or control siRNA SiGLO (20 nM; Dharmacon) using Alogen fibroblast transfection reagent (Alogen Biosystems), and the manufacturer’s recommended fibroblast transfection protocol was followed. The cells were grown for 48 hours after siRNA transfection, and efficacy of
β-catenin siRNA was assessed by Western blotting. The effect of β-catenin siRNA on the expression of ET-1 was then determined by real-time PCR and Western blotting.

Statistics. Statistical analysis was performed using 2-tailed Student’s t test. Results are expressed as the mean ± SEM. P < 0.05 was considered statistically significant.

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