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Targeting *O*-Glycosyltransferase (OGT) to Promote Healing of Diabetic Skin Wounds*

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Kasper Runager[‡], Meryem Bektas[‡], Paula Berkowitz[‡], and David S. Rubenstein[‡§¶1]

From the [‡]Departments of Dermatology and [§]Pharmacology and the [¶]Lineberger Comprehensive Cancer Center, The University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

Background: Increased intracellular protein *O*-GlcNAc modification may contribute to delayed wound healing in diabetes.
Results: Hyperglycemia increases intracellular protein *O*-GlcNAc modification and delays wound healing in keratinocytes. Targeted knockdown of OGT altered rates of wound closure.
Conclusion: OGT knockdown accelerates wound healing under both normal and hyperglycemic culture conditions.
Significance: OGT may represent a novel druggable target for promoting healing of diabetic wounds.

Non-healing wounds are a significant source of morbidity. This is particularly true for diabetic patients, who tend to develop chronic skin wounds. *O*-GlcNAc modification of serine and threonine residues is a common regulatory post-translational modification analogous to protein phosphorylation; increased intracellular protein *O*-GlcNAc modification has been observed in diabetic and hyperglycemic states. Two intracellular enzymes, UDP-*N*-acetylglucosamine-polypeptide β-*N*-acetylglucosaminyl transferase (OGT) and *O*-GlcNAc-selective *N*-acetyl-β-D-glucosaminidase (OGA), mediate addition and removal, respectively, of *N*-acetylglucosamine (GlcNAc) from intracellular protein substrates. Alterations in *O*-GlcNAc modification of intracellular proteins is linked to diabetes, and the increased levels of protein *O*-GlcNAc modification observed in diabetic tissues may in part explain some of the observed underlying pathophysiology that contributes to delayed wound healing. We have previously shown that increasing protein *O*-GlcNAc modification by overexpression of OGT in murine keratinocytes results in elevated protein *O*-GlcNAc modification and a hyperadhesive phenotype. This study was undertaken to explore the hypothesis that increased *O*-GlcNAc modification of cellular proteins in diabetic skin could contribute to the delayed wound healing observed in patients with diabetic skin ulcers. In the present study, we show that human keratinocytes cultured under hyperglycemic conditions display increased levels of *O*-GlcNAc modification as well as a delay in the rate of wound closure *in vitro*. We further show that specific knockdown of OGT by RNA interference (RNAi) reverses this effect, thereby opening up the opportunity for OGT-targeted therapies to promote wound healing in diabetic patients.

Non-healing wounds are a significant source of morbidity affecting 6.5 million patients in the United States and costing approximately $25 billion annually to treat (1). Patients with diabetes are at increased risk for developing non-healing wounds. A variety of factors likely contribute to the predisposition of diabetic patients to develop non-healing wounds including neuropathy, vasculopathy, as well as the underlying endocrine dysfunction that results in elevated glucose levels.

Like phosphorylation, intracellular protein *O*-GlcNAc modification is a common, dynamic post-translational modification that regulates many intracellular proteins including enzymes, transcription factors, structural, and cell adhesion proteins. *N*-Acetylglucosamine (GlcNAc) modification of serine and threonine is catalyzed by the enzyme UDP-*N*-acetylglucosamine-polypeptide β-*N*-acetylglucosaminyl transferase (*O*-GlcNAc transferase, OGT),[2] whereas GlcNAc is removed by *O*-GlcNAc-selective *N*-acetyl-β-D-glucosaminidase (GlcNAcase, OGA) (reviewed in Ref. 2).

Hyperglycemia, excess glucose, feeds into the glucosamine pathway to provide excess UDP-GlcNAc for OGT to modify intracellular proteins (3). Excess glucose is converted to glucosamine, which is ultimately converted to UDP-*N*-acetylglucosamine (UDP-GlcNAc), the donor substrate for OGT modification of intracellular proteins. Consequently, hyperglycemia is associated with increased *O*-GlcNAc modification of a variety of proteins (3–7). The increased *O*-GlcNAc modification of intracellular proteins observed in hyperglycemic states including diabetes is thought to contribute to some of the pathology associated with diabetes. For example, (i) pancreatic β-cells have high levels of OGT and are sensitive to alterations in intracellular *O*-GlcNAc modification and (ii) overexpression of OGT in muscle and adipose tissue causes diabetes in transgenic mouse models (8). Increased *O*-GlcNAc modification of intracellular proteins is observed in diabetic tissue, including human diabetic tissue (9) and hyperglycemic animal models (4). Further support for a pathologic role for intracellular *O*-GlcNAc modification in diabetes comes from studies demonstrating a genetic association of diabetes and mutations causing increased

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[1] To whom correspondence should be addressed: Dept. of Dermatology, The University of North Carolina School of Medicine, Ste. 405 Mary Ellen Jones Bldg. CB 7287, Chapel Hill, North Carolina 27599-7287. Tel.: 919-843-7092; Fax: 919-966-3898; E-mail: druben@med.unc.edu.

[2] The abbreviations used are: OGT, *O*-GlcNAc transferase; OGA, GlcNAcase.

intracellular protein *O*-GlcNAc modification; a mutation that results in early termination in the gene encoding OGA has been associated with a genetic predisposition to adult onset type II diabetes in a Mexican American population (10). OGA removes *O*-GlcNAc from intracellular proteins, and the identified OGA mutations result in increased levels of protein *O*-GlcNAc modification.

During wound healing, keratinocytes at the wound margin must down-regulate adhesion to adjacent cells to permit movement away from the edge and into the wound (11). Previous data from our group demonstrated that increased *O*-GlcNAc modification stabilizes cell-cell adhesion in part by increasing the post-translational stability of desmosome components including plakoglobin (12). By increasing cell-cell adhesion, we hypothesize that increased intracellular protein *O*-GlcNAc modification retards wound healing. This study was undertaken to explore the hypothesis that increased *O*-GlcNAc modification of cellular proteins in diabetic skin could contribute to the delayed wound healing observed in patients with chronic diabetic skin ulcers. In the present study we modeled hyperglycemia by culturing human keratinocytes in elevated glucose. Under hyperglycemic conditions, we observed (i) increased levels of *O*-GlcNAc modification of keratinocyte proteins and importantly (ii) delays in wound closure. Hyperglycemia-induced delays in wound closure were reversed by shRNA and siRNA knockdown of *OGT*, the gene responsible for adding the GlcNAc moiety to proteins. These observations suggest that targeting OGT may be beneficial for treating non-healing diabetic wounds.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media were obtained from Invitrogen. shRNA plasmids were purchased from Open Biosystems (Thermo Fisher Scientific) and packaged into inactivated lentivirus particles at The University of North Carolina at Chapel Hill Lenti-shRNA core facility. The sequences for the mature sense strands in the hairpins were: shOGT (TRCN0000035064), 5′-GCCCTAAGTTTGAGTCCAAAT-3′, and shOGA (TRCN0000134040), 5′-CCAGAAACTTTCCTTGCTAAT-3′. The RNAi Consortium (TRC) lentiviral enhanced GFP shRNA was used as a positive control for transduction (Open Biosystems catalog number RHS4459). Mouse monoclonal *O*-GlcNAc-specific antibodies (clone RL2) were from Thermo Scientific. Rabbit monoclonal antibodies to GAPDH were from Cell Signaling (Danvers, MA). Mouse monoclonal antibodies to β-actin were from Sigma. Rabbit polyclonal OGT antibodies were from Abcam (Cambridge, MA). Mouse and rabbit anti-sheep horseradish peroxidase-conjugated secondary antibodies were from GE Healthcare. Control siRNA (sense strand: GCAGUUAUAAUGACUAGAU) and OGT siRNA (sense strand: GCACAAUCCUGAUAAAUUU) with 3′UU overhangs were purchased from Sigma-Aldrich.

Cell Culture and Scratch Wounding—Untransfected and shRNA-transfected HaCaT cells were cultured in normal or high glucose Dulbecco's modified Eagle's medium (DMEM) (5.5 or 25 mM glucose, respectively) (13), 1% fetal bovine serum (FBS), 1,000 units penicillin/ml, 100 μg streptomycin/ml. Media were supplemented with the amounts of glucose or inhibitor specified in the figure legends. shRNA-transfected cells were selected using 1 μg of puromycin/ml of medium. Puromycin-containing media were replaced 6 h prior to scratching. Cells were grown for 60 h (until confluent) before scratch assays were performed. Scratch wounds were performed by making a linear scratch across monolayers of confluent cells in 24-well culture plates followed by one wash with 1× PBS and the addition of fresh culture medium. Pictures were taken on a Nikon TE2000-U spinning disk microscope using a 10× magnification immediately after scratching and incubated at 37 °C for the amount of time stated in the figure legends before another set of pictures was taken. Wounds were subsequently analyzed using the TScratch software package (14). Only wounds of the same initial wound-size were evaluated and compared.

Statistical Analyses—Error bars reflect the S.E. Student's *t* tests were performed as two-sided tests with unequal variance as described in the TScratch software manual.

Stable Transduction of Keratinocytes with shRNAs—HaCaT cells were cultured in DMEM, 10% FBS to 50–60% confluency and incubated with 10 μg/ml Polybrene and shRNA (shGFP, shOGT, or shOGA) using a multiplicity of infection of two, 5 h after which the medium was changed to fresh DMEM. The following day, medium containing 1 μg/ml puromycin was added to the cells to select for successfully transduced cells. Cell cultures were passaged 6–8 times under puromycin selection before they were used for experiments.

Quantification of Immunoblot Signals—Samples were equally loaded on and separated by SDS-PAGE as described previously (12). Immunoblotting was performed according to established protocols and developed by enhanced chemiluminescence (ECL) reaction (Amersham Biosciences). Protein bands from immunoblots were quantified using the GeneSnap software (SynGENE, Frederick, MD). For RL2 staining, the three most prominent bands were analyzed using GeneSnap software.

siRNA Transfection of Keratinocytes—siRNA against OGT (3′-GCACAAUCCUGAUAAAUUU-5′) and a scrambled control siRNA (3′-GCAGUUAUAAUGACUAGAU-5′) were synthesized with 3′-UU overhands and were diluted to 20 mM in water (working stock), and each well in a 24-well plate with 40% confluent keratinocytes was transfected using Oligofectamine (Invitrogen) according to the protocol. Briefly, 3 μl of Oligofectamine (Invitrogen) was diluted in 12 μl of Opti-MEM I (Invitrogen) and incubated for 8 min. In the meantime 3 μl of siRNA was mixed with 50 μl of Opti-MEM I, and this was added to the Oligofectamine dilution and left to form complexes for 20 min. 32 μl of Opti-MEM was then added to the mix and added to the cells (in 500 μl of high glucose DMEM). After 48 h the medium was changed to high glucose DMEM, and at 60 h the cells were used for scratch assay. Pictures were taken at the time points described in the figure legends.

RESULTS

Hyperglycemic Conditions Result in Elevated Protein O-GlcNAc Levels in Human Keratinocytes—To investigate whether increased levels of *O*-GlcNAc modification in diabetic skin may be linked to elevated tissue glucose levels, these conditions were mimicked in cell culture by growing human kera-

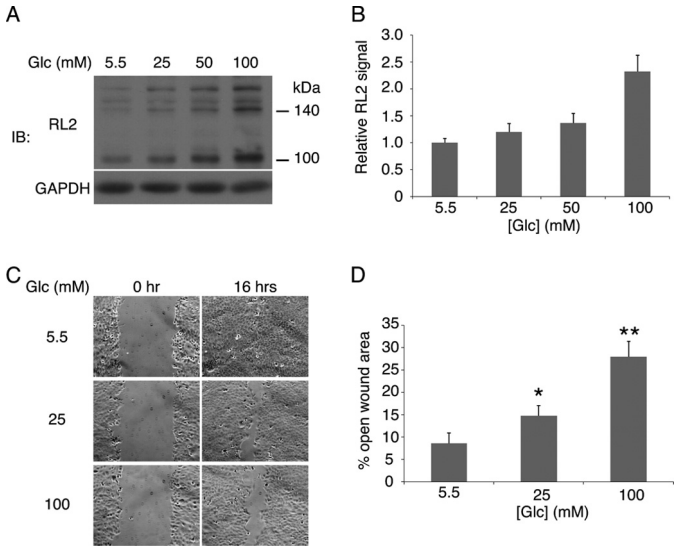

FIGURE 1. **Hyperglycemia increases *O*-GlcNAc modification and retards wound healing in human keratinocytes.** HaCaT cells were cultured in media supplemented with glucose to the final concentrations indicated. *A*, representative immunoblot (*IB*) of cell lysates separated by SDS-PAGE and immunoprobed with antibodies to (i) RL2, which recognizes the *O*-GlcNAc modification, and (ii) GAPDH as a loading control. *B*, the RL2 signal was quantified relative to the GAPDH loading control ($n = 3$). *C* and *D*, scratch assays. Human keratinocyte monolayers were incubated in DMEM with the indicated glucose concentrations. Cells were scratched with a pipette tip, and micrographs were made at 0 and 16 h. Wound sizes were then measured using image analysis software. Representative micrographs are shown in *C* and quantified in *D*. $n = 11$ for all conditions. *Error bars* reflect the S.E. * indicates p value <0.07 and ** indicates p value < 0.0005 as compared with normal glucose levels (5.5 mM). The p value between 25 and 100 mM is <0.01.

tinocytes (HaCaT) for 48 h with various glucose concentrations (Fig. 1). Immunoblot of cell lysates shows that increased levels of glucose indeed resulted in more *O*-GlcNAc modification in keratinocyte lysates, as detected by the *O*-GlcNAc-specific antibody RL2 (Fig. 1, *A* and *B*). This dose-dependent increase in *O*-GlcNAcylation emphasizes the link between increased glucose concentrations and intracellular protein *O*-GlcNAc modification in keratinocytes.

Human Keratinocytes Exhibit Delayed Wound Healing under Hyperglycemic Conditions—We then utilized the *in vitro* keratinocyte scratch assay model of wound healing to test the hypothesis that hyperglycemic conditions decrease the rate of wound closure (Fig. 1, *C* and *D*). The assay was performed by preincubating HaCaT cells with different amounts of glucose for 48 h, after which a wound was introduced in the confluent layer of cells. Elevated levels of glucose in the culture media decreased the rate of wound closure in a dose-dependent manner (Fig. 1, *C* and *D*).

Gene Knockdown of Key Enzymes for the O-GlcNAc Pathway by RNA Interference Affects the Rate of Wound Closure in Human Keratinocyte Culture—The apparent link between delayed wound closure and elevated levels of *O*-GlcNAc modification in HaCaT cells led us to further investigate the role of the enzymes responsible for the addition and removal of *O*-GlcNAc protein modification (OGT and OGA, respectively) in more detail. To do this, we stably transduced HaCaT cells with shRNAs against either enzyme and analyzed cell lysates by immunoblot analysis (Fig. 2, *A* and *B*). Immunoblot analysis of the cell lysates confirmed the impact of RNAi on *O*-GlcNAc levels, with shOGT displaying significantly reduced levels of *O*-GlcNAc modification. The shOGA transduced cells displayed levels of *O*-GlcNAc modification similar to both the untransduced cells and the shGFP controls (Fig. 2*B*). Scratch-wounding assays of shRNA-transduced cells show that knocking down OGT significantly increases the rate of wound closure (Fig. 2, *C* and *D*). shOGA transduced cells were not significantly different from shGFP transduced controls despite evidence that targeting OGA with shRNA reduced total OGA protein levels (data not shown). Collectively, these data strongly suggest that decreasing the amount of *O*-GlcNAc (via shOGT) accelerates wound healing, providing additional support for the relationship between *O*-GlcNAc protein modification and wound healing.

siRNA Knockdown of OGT Decreases Keratinocyte O-GlcNAc Modification and Accelerates Wound Closure in Hyperglycemic Conditions—To investigate the therapeutic potential of siRNA knockdown to down-regulate OGT expression, we tested OGT-specific siRNAs as a means to knock down OGT and accelerate wound healing in the keratinocyte scratch model (Fig. 3). A 19-mer siRNA directed against the OGT mRNA sequence was synthesized, and HaCaT cells were transfected with the OGT-specific silencing RNA as well as a control siRNA with a scrambled sequence. 2 days after transfection, cell lysates were probed for RL2 and OGT immunoreactivity (Fig. 3, *A* and *B*). The results show that siRNA against OGT results in a marked knockdown in both OGT levels and RL2 immunoreactivity as quantified from immunoblots.

Next, siRNA-transfected cells were tested in a scratch-wounding assay to examine the effect of this form of OGT RNAi on wound closure *in vitro*. Fig. 3*C* shows that wound healing at the 26-h time point is significantly more progressed with OGT siRNA as compared with both control siRNA and untreated cells (Fig. 3*D*). These results further support the hypothesis that the level of intracellular *O*-GlcNAc modification in human keratinocytes is linked to wound closure rate and that this may be manipulated using OGT knockdown.

DISCUSSION

Increasing evidence from the literature suggests that alterations in the hexosamine pathway play a key role in the pathophysiology of diabetes. For example, overexpression of OGT in mice results in a diabetic phenotype (8), and increased levels of *O*-GlcNAc modification have been observed in cells and tissue from type 2 diabetes patients relative to healthy controls (9, 15). Previously, we had reported that overexpression of OGT in keratinocytes (i) increases GlcNAc modification of cellular proteins and (ii) markedly enhances cell-cell adhesion (12). Consistent with these observations, we observed a dose-dependent increase in protein *O*-GlcNAc modification in human keratinocyte cultures grown in increasing concentrations of glucose. Furthermore, increasing concentrations of glucose and *O*-GlcNAc protein modification were associated with delayed wound closure in a dose-dependent fashion. Significantly, silencing OGT activity with either OGT-specific shRNA or OGT-specific siRNA decreases GlcNAc modification of keratinocyte proteins and promotes wound healing in a scratch model assay, even in the presence of elevated glucose concen-

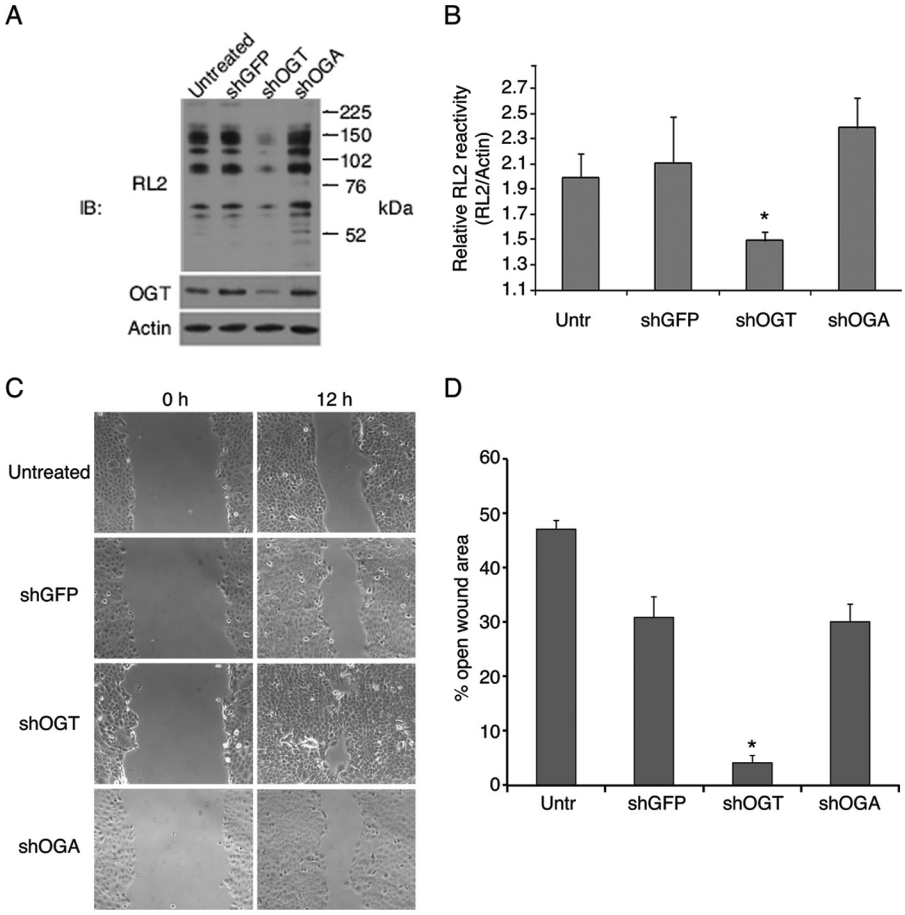

FIGURE 2. **OGT knockdown using shRNA accelerates wound healing.** HaCaT cells were stably transduced with shRNA targeting OGT, OGA, and GFP (control) and grown to confluency. *A*, OGT knockdown decreases protein *O*-GlcNAcylation. Cell lysates were analyzed by immunoblot (*IB*) with antibodies to (i) *O*-GlcNAc (RL2), (ii) OGT, and (iii) actin (loading control). *B*, RL2 reactivity was quantified as compared with actin. *Untr*, untreated cells. *C* and *D*, OGT knockdown promotes wound healing in hyperglycemic cultures. Transduced cells were grown to confluency in growth medium with 25 mM glucose and scratched to introduce wounds. Representative micrographs obtained at 0 and 12 h are shown in *panel C*, whereas the quantification of open wound areas is shown in *panel D*. In the scratch-wounding assay, $n = 18$ for untreated cells, $n = 10$ for shGFP, $n = 8$ for shOGT, and $n = 14$ for shOGA. *Error bars* reflect the S.E. * indicates p value < 0.05.

trations. Collectively, these observations suggest that increased intracellular *O*-GlcNAc modification, mediated by the enzyme OGT, likely contributes to delayed wound healing in non-healing diabetic skin wounds.

The effects of increased OGT activity on promoting cell adhesion and delaying wound healing may in part be due to regulation of keratinocyte cell adhesion components, including desmosomes, adherens junctions, and cytoskeletal elements as we have previously reported (12). In this context, we previously showed that plakoglobin, a component of both adherens junction and desmosome cell-cell adhesion complexes, is post-translationally stabilized by increased *O*-GlcNAc modification in OGT-overexpressing keratinocytes. This increased plakoglobin protein level drove formation of desmosomes and plakoglobin-based adherens junctions and markedly enhanced cell-cell adhesion (12). These observations indicate that in keratinocytes, *O*-GlcNAc modification functions in part to regulate the post-translational stability of plakoglobin, and significantly, to regulate keratinocyte cell-cell adhesion. During wound healing, keratinocytes migrate into the wound to promote re-epithelialization. Keratinocytes at the wound margin must down-regulate adhesion to adjacent cells at the trailing margin to permit movement away from the edge and into the wound. By increasing cell-cell adhesion, we suggest that increased intracellular protein *O*-GlcNAc modification retards wound healing, whereas down-regulation of intracellular protein *O*-GlcNAc modification promotes wound healing.

It is worth noting that *O*-GlcNAc is a ubiquitous intracellular modification. In addition to modifying cell adhesion and structural proteins, transcription factors and regulatory enzymes are also modified by OGT-catalyzed addition of GlcNAc to serine and threonine residues. Thus, the effects of OGT activity are likely to be pleiotropic. In addition to its effects on adhesion, altering levels of intracellular protein *O*-GlcNAc modification may also impact cell proliferation and chemotaxis, and it may be the combination of these effects that contributes to the observed delayed wound healing.

Diabetic wounds represent a significant health care burden. The incidence and social and financial cost of treating these wounds are likely to increase as the incidence of diabetes rises due to the obesity epidemic and aging populations. We have demonstrated that decreasing the global level of *O*-GlcNAc modification through knockdown of OGT using RNAi accelerates wound healing in a hyperglycemic keratinocyte culture model. Collectively, these data show that locally targeting OGT

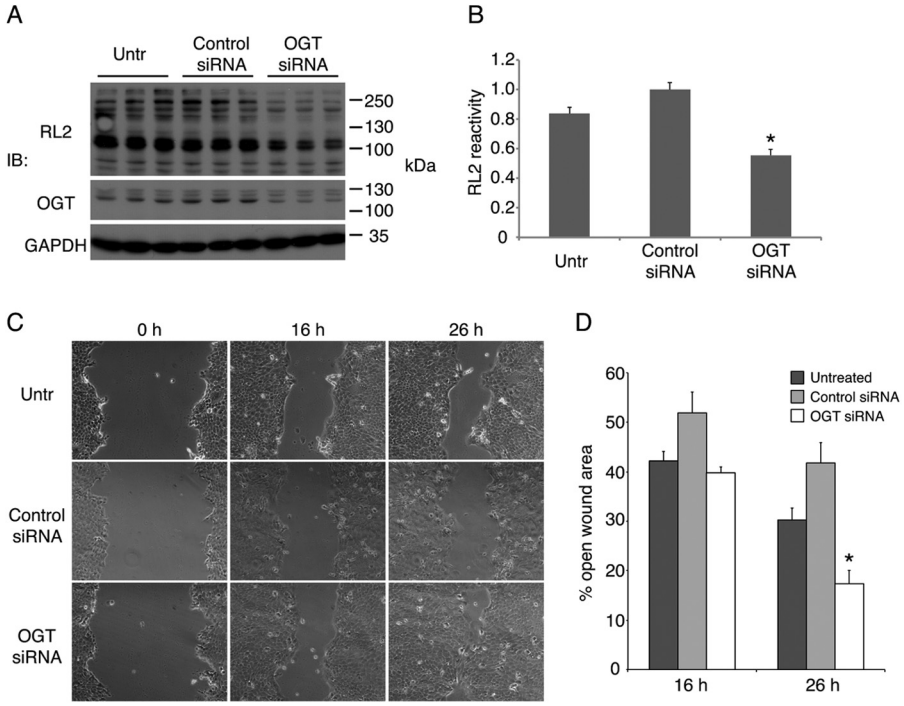

FIGURE 3. **OGT knockdown using siRNA accelerates wound healing.** HaCaT cells were transfected with 100 nM siRNA against OGT or a scrambled control siRNA. *A* and *B*, cell lysates of confluent cultures were subjected to immunoblot (*IB*) with antibodies to (i) *O*-GlcNAc (RL2), (ii) OGT, and (iii) GAPDH (control) (*A*) and quantified (*B*). *Untr*, untreated cells. *C* and *D*, 60 h after transfection with siRNAs, the confluent cultures were scratched, and micrographs were obtained at 0, 16, and 26 h (*C*). The open wound area was quantified using image analysis software (*D*). $n = 7$ for the untransfected cells, $n = 7$ for control siRNA, and $n = 6$ for OGT siRNA. *Error bars* reflect the S.E. * indicates p value < 0.05 as compared with controls.

may prove an effective approach to promote healing in diabetic ulcers. As it has previously been demonstrated that the impaired barrier function in wounds allows for transfection with oligonucleotides (16), we suggest that topical administration of siRNAs or antisense oligodeoxynucleotides against OGT may be an effective treatment to promote healing of diabetic skin wounds.

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