Supplementary Data

Supplementary Methods

Preparation of short synthetic hairpin RNAs and antimiR oligonucleotides

Short synthetic hairpin RNAs (sshRNAs) were chemically synthesized and high-performance liquid chromatography purified either by Integrated DNA Technologies (Coralville, IA) or TriLink Biotechnologies (San Diego, CA). sshRNAs were resuspended and annealed to monomeric hairpin conformations, and analyzed by denaturing polyacrylamide gel electrophoresis as described in Dallas *et al.*[1] AntimiR oligonucleotides were resuspended in RNase-free H_2O. The sequences of sshRNA SG404 and antimiR SG608 are as follows. SG404: 5′- CUGAAUUG GGCUUGAGUUCUUGAACUCAAGCCCAAUUCAG-3′; SG608: 5′- UCAGCCGCUGUCACACGCACA-3′.

Cell culture

Human kidney 293FT (Invitrogen, Carlsbad, CA) and human keratinocyte cell line HaCaT (Robyn Hickerson, TransDerm) were cultured in Dulbecco's modified Eagle's medium (DMEM; Cambrex) with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, and 1 mM sodium pyruvate. Human primary keratinocytes (Normal Human Epidermal Keratinocytes; ATCC) were cultured in Dermal Cell Basal Medium (ATCC) supplemented with Keratinocyte Growth Kit (ATCC). Mouse fibroblast cell line NIH-3T3 (ATCC) was cultured in DMEM (Cambrex) with 10% bovine calf serum (Hyclone), 2 mM L-glutamine, and 1 mM sodium pyruvate. Human fetal lung fibroblast line MRC-5 was cultured in MEM and 10% fetal calf serum.

Real-time quantitative polymerase chain reaction of messenger RNA and microRNA

Complementary DNA (cDNA) for messenger RNA (mRNA) analysis was synthesized from 10 μL of total RNA samples using the High-Capacity cDNA Kit (Applied Biosystems). cDNA for microRNA (miRNA) quantification was reverse transcribed using the TaqMan-MicroRNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative polymerase chain reaction (RT-qPCR) amplification was performed using 5×HOT FIREPol Probe qPCR Mix Plus (ROX; Solis BioDyne), and the appropriate TaqMan probes (Applied Biosystems): human prolyl hydroxylase domain-containing protein 2 (PHD2; Hs00254392_m1), human GAPDH (Hs99999905_m1), mouse PHD2 (Mm00459770_m1), mouse GAPDH (Mm99999915_g1), miR-210 (00512), and sno-234 (001234).

Western blots

One day before transfection, 293FT cells were seeded at 90,000 cells/well in 48-well plates. sshRNAs (final concentrations 1 or 10 nM) along with 300 ng carrier plasmid pUC19 were transfected with Lipo2K. Cells treated with 500 μM $CoCl_2$ (final concentration) served as a positive control. Forty-eight hours post-transfection, the cells were lysed in 100 μL of 1×LDS lysis buffer (Novex) supplemented with 100 mM DTT and incubated at 95°C for 10 min. Twenty microliters of lysate was loaded on NuPage 4–12% Bis Tris gel (Invitrogen) and resolved at 60 V for 20 min, followed by 1.5 h at 100 V. Resolved proteins were transferred to a nitrocellulose membrane at 30 V for 3 h. The membrane was blocked with 5% nonfat dry milk in TBST overnight at room temperature. The membrane was then stained with hypoxia-inducible factor-1α (HIF-1α) antibody (H1alpha67; Novus Biologicals), followed by staining with goat anti-mouse IgG-AP (Santa Cruz Biotechnologies). Proteins were visualized using an NBT/BCIP system (Promega) and quantified using ImageJ software.

Construction of miR-210 biosensor plasmid (pSG247)

The miR-210 biosensor reporter (pSG247) was prepared by subcloning double-stranded oligonucleotides containing four tandem complementary miR-210-3p binding sites (5′-TCAGCCGCTGTCACACGCACAG-3′), each separated by a short unrelated 8 bp sequence, as *Xho*I-Not1-sticky fragments into the 3′-untranslated region of the Renilla luciferase (rLuc) gene of the psiCHECK-2 dual luciferase reporter vector (Promega). This vector also contains constitutively expressed firefly luciferase (fLuc) for transfection normalization.

Supplemental biosensor assay

Triplicate transfections were performed in HaCaT cells with 0.2 ng of pSG247, 50 ng of carrier plasmid pUC19, and miR-210-specific antimiRs (0.3–10 nM) or nonspecific control (NSC) antimiRs over the same concentration range using Lipo2K (Invitrogen). Twenty-four hours post-transfection, $CoCl_2$ was added to a final concentration of 500 μM to stimulate expression of miR-210. At 48 h, cells were lysed in 150 μL of passive lysis buffer (Promega) and 10 μL of lysate was used to measure fLuc and rLuc levels with the Dual-Glo Luciferase Assay (Promega) on a TR 717 Microplate Luminometer (Applied Biosystems). miR-210 antimiR activity was measured by the derepression of rLuc expression relative to cells not transfected with antimiRs, normalizing to fLuc.

Serum stability assay and electrophoresis

sshRNAs (3.35 μg) were incubated with 10% human serum (Sigma, St Louis, MO) in phosphate-buffered saline at 37°C. An aliquot was taken out at different time points, immediately mixed with 2×gel loading buffer (Ambion, Austin, TX), and stored at −80°C. Gel electrophoresis was performed under denaturing conditions (12% polyacrylamide, 20% formamide, and 8 M urea) and stained with SYBR Gold (Invitrogen).

Cytotoxicity assay

One day before transfection, HaCaT cells were seeded at 15,000 cells/well in a 96-well plate. Transfections were performed in triplicate, using HiPerFect (Qiagen). sshRNAs and antimiRs were transfected at 10, 30, and100 nM alongside mock transfection controls. One micromolar (in dimethyl

sulfoxide [DMSO]) staurosporine was used as a positive control. Cell viability was measured 48 h post-transfection using the Vybrant MTT Cell Proliferation Assay Kit (Invitrogen) following the manufacturer's protocol. Absorbance at 570 nm was measured on a Thermomax microplate reader (Molecular Devices), and percent viability was calculated relative to wild-type cells.

Mapping of RNA target cleavage sites by 5′-rapid amplification of cDNA ends analysis

NIH-3T3 cells were transfected as described in the main text with SG404 and NSC sshRNA (SG221c). Untransfected cells were an additional negative control. Total RNA was extracted 18 h post-transfection. The mRNA was then subjected to 5′-RACE (rapid amplification of cDNA ends) analysis by first ligating an adaptor of sequence 5′-CGAC UGGAGCACGAGGACACUGACAUGGACUGAAGGAG UAGAAA-3′ to mRNAs at their 5′ ends. Ligated RNAs were reverse transcribed using the PHD2-specific primer 5′-CTGGCAACAATCATCGCAGGAGG-3′ and amplified by PCR using primers 5′- ACTGAAGGAGTAGAAAGCCCA-3′ and 5′-AGCAGCCAAGAGCAGTCACA-3′. PCR was started with 1 cycle of 94°C for 2 min, 5 cycles of 94°C for 30 s, and 70°C for 30 s, followed by 25 cycles of 94°C for 30 s and 60°C for 30 s and 70°C for 30 s. The PCR products were analyzed on a 2% agarose gel and the band with the predicted length (251 bp) of the cleavage product was excised and sequenced (Retrogen).

Fluorescence uptake of PHD2-sshRNA

SG405, an sshRNA of identical sequence and modification pattern as SG404 but additionally containing an AlexaFluor-594 label, was formulated by layer-by-layer (LbL-SG405) and applied to full-thickness excisional wounds on db/db mice ($n = 6$ per time-point). At days 2 and 6, wounds were harvested and imaged by fluorescence microscopy with a Leica DM5000B automated upright microscope and a Leica DFC550 digital color camera. Mean fluorescence was quantified by ImageJ.

Immunofluorescence analysis of von Willebrand factor

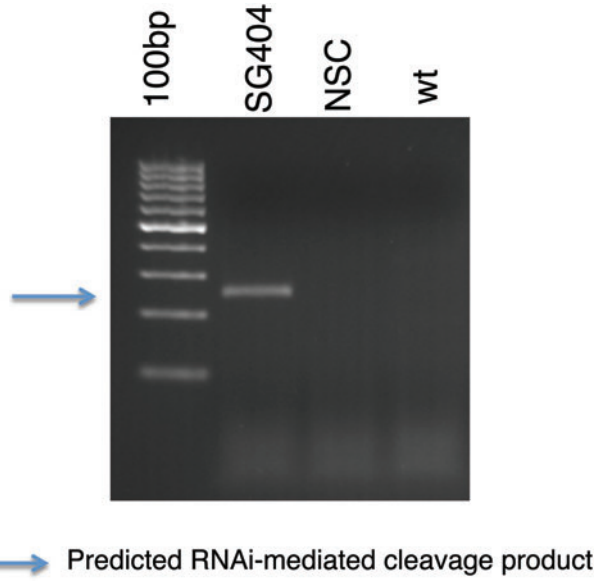
LbL-SG404 and LbL-NSC (NSC = SG221c) were applied to full-thickness excisional wounds in db/db mice. An additional untreated control group was included for comparison. In all groups, $n = 6$. On day 7 post-treatment application, animals were sacrificed and sections were prepared as described in the main text. Ten histology slides were prepared per wound. Immunofluorescence was monitored with von Willebrand factor (vWF) antibody (No. 7356; EMD Millipore, St. Louis, MO). Three images were captured per slide. Quantification was performed by blinded analysis using two independent observers, measuring fluorescence intensity by ImageJ.

RNA analysis

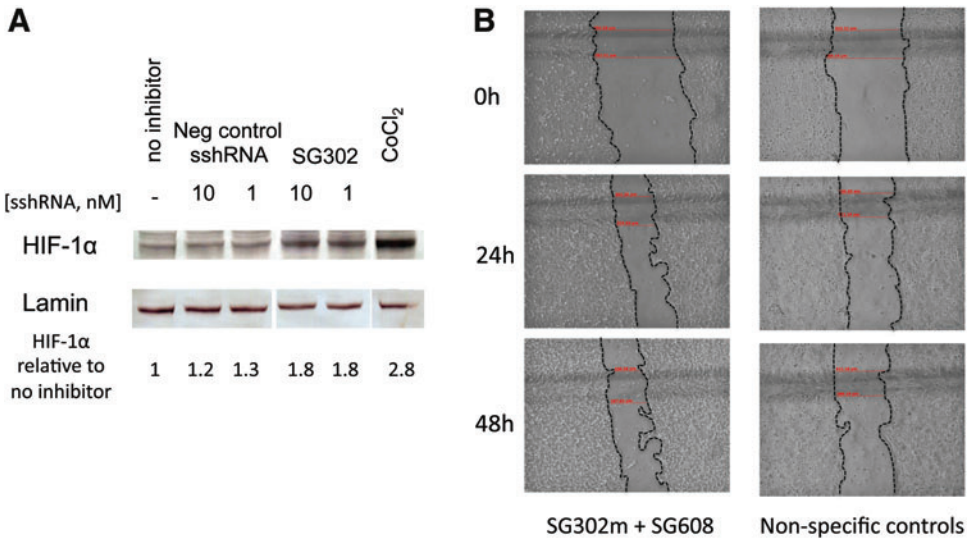
Tissue sections designated for RNA analysis were immediately immersed in RNAlater solution (Invitrogen), incubated overnight at 4°C, and then stored at −80°C until testing. Lysing Matrix ''D'' beads (1.2 g) (MP Biomedicals, Solon, OH) and 1 mL of QIAZOL (Qiagen) were added and the samples were processed with a FastPrep-24 homogenizer (MP Biomedicals) by four 60-s cycles ($6\ m \cdot s^{-1}$) followed by cooling on ice. Cellular debris was removed by centrifugation at 12,000 *g* for 2 min. The homogenate was extracted with chloroform and the aqueous layer was used to isolate total RNA using an RNeasy Plus Universal Mini Kit (Qiagen). RT-qPCR for quantification of miRNA was performed as described above.

Supplementary Reference

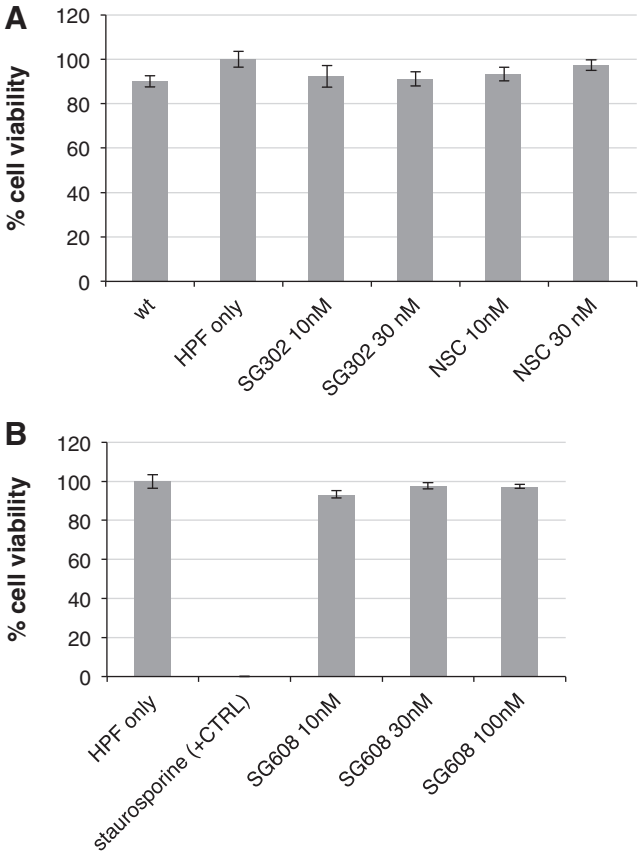
S1. Dallas, A., Ilves, H., Shorenstein, J., *et al.* Minimal-length synthetic shRNAs formulated with lipid nanoparticles are potent inhibitors of hepatitis C virus IRES-linked gene expression in mice. Mol Ther Nucleic Acids **2,** e123, 2013.



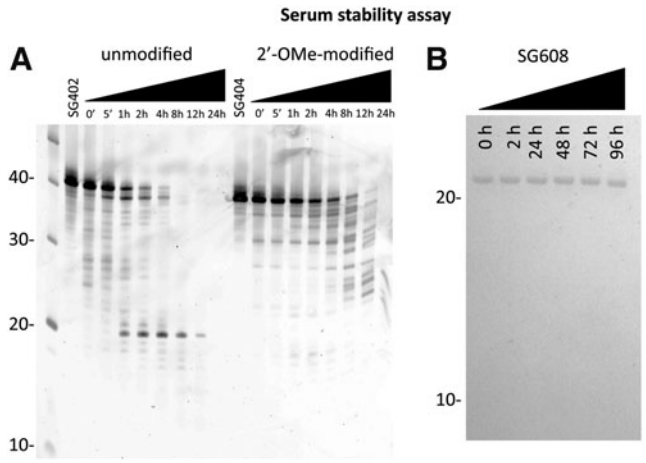
SUPPLEMENTARY FIG. S1. 5′-RACE analysis shows predicted RNA interference (RNAi)-mediated cleavage product of PHD2 mRNA. Two percent agarose/TBE gel showing 5′-RACE products from amplification of total RNA isolated from NIH-3T3 cells transfected with SG404, NSC sshRNA, or wt cells. The predicted band (251 bp) is only detected in SG404-transfected cells and was confirmed by sequencing. cDNA, complementary DNA; mRNA, messenger RNA; NSC, nonspecific control; PHD2, prolyl hydroxylase domain-containing protein 2; RACE, rapid amplification of cDNA ends; sshRNA, short synthetic hairpin RNA; wt, wild type.



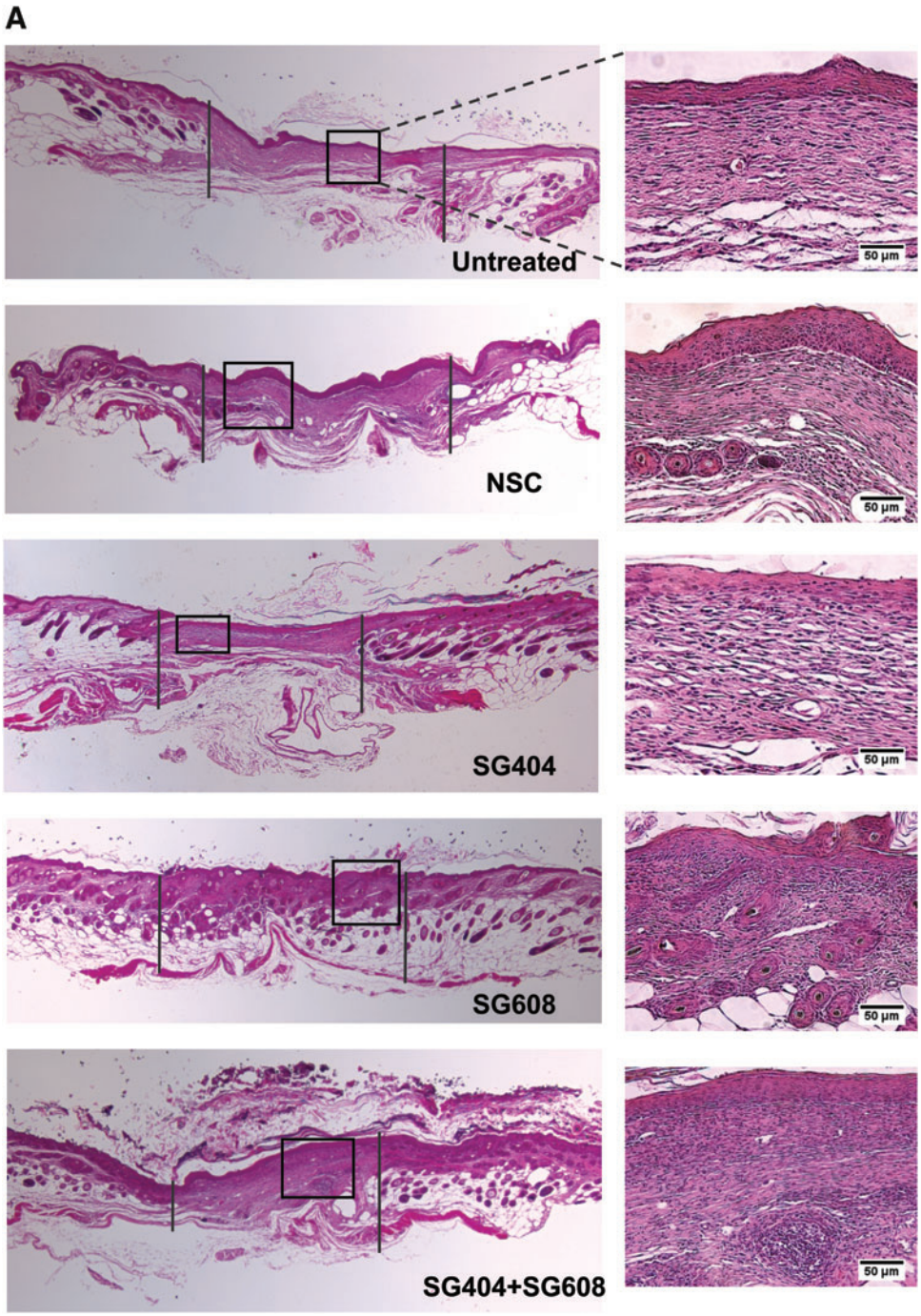
SUPPLEMENTARY FIG. S2. **(A)** Western blot analysis of induction of HIF-1α by PHD2-specific sshRNA SG302 compared with cells not transfected with inhibitor (no inhibitor) or cells transfected with NSC sshRNA. Cells treated with $CoCl_2$ serve as a positive control analyzed by Western blot. Lamin (loading control). **(B)** Representative images of the scratch wounds at 0, 24, and 48 h for combination SG302m PHD2-sshRNA+SG608 miR-210 antimiR versus NSC sshRNA and antimiR. HIF-1α, hypoxia-inducible factor-1α.



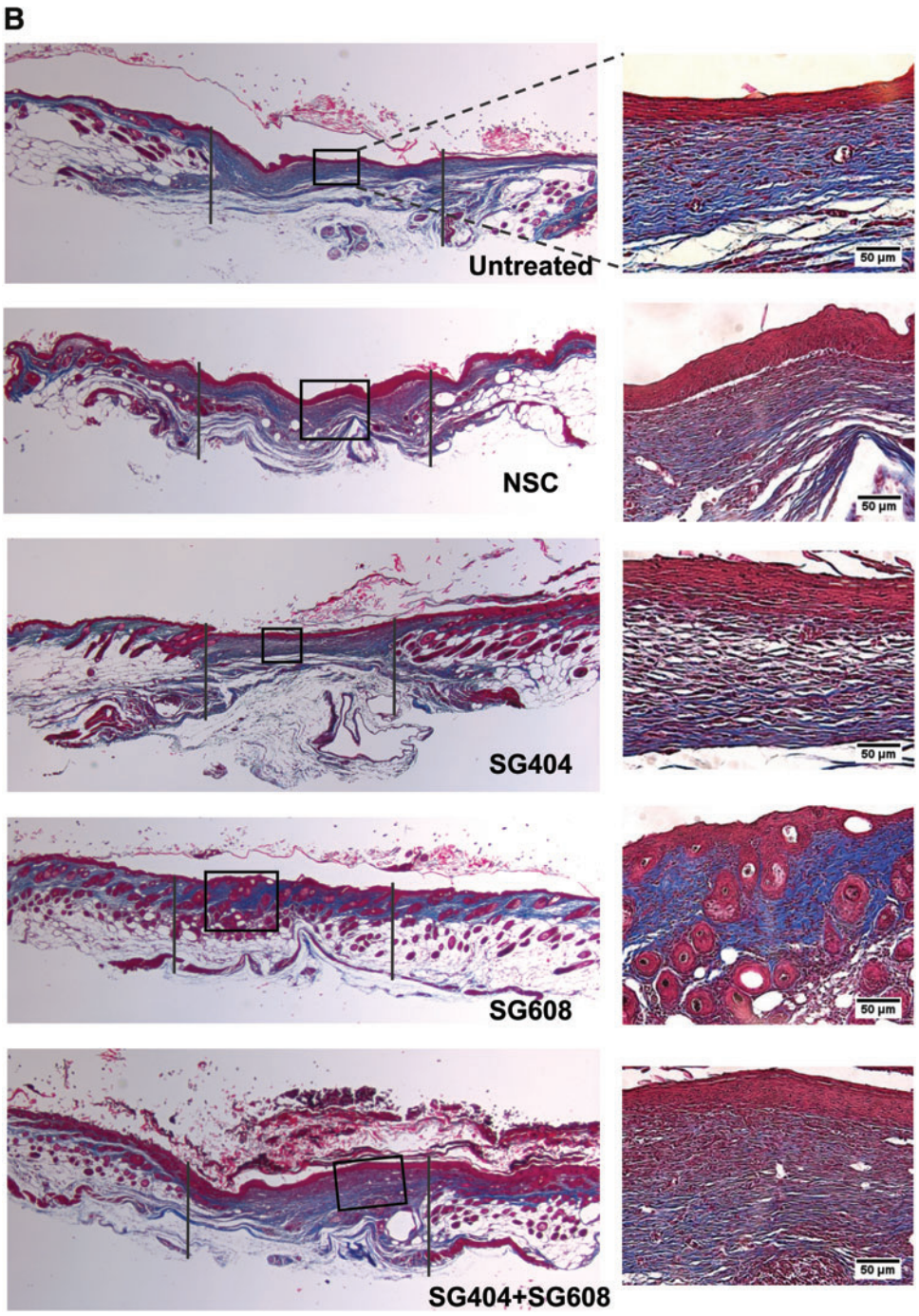
SUPPLEMENTARY FIG. S3. MTT cell toxicity assay to measure cell viability 48 h after transfection of HaCaT cells with sshRNAs **(A)** and antimiR-210 **(B)** at the indicated final concentrations. In **(A)**, percent cell viability is normalized to mock transfection with HiPerFect reagent (HPF only). In **(B)**, percent cell viability is normalized to mock transfection with HiPerFect (no inhibitor). Staurosporine (1 μM) is a positive control for cell toxicity. SG302, PHD2-targeting sshRNA; SG608, N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenyl amine (ZEN)-modified antimiR-210.



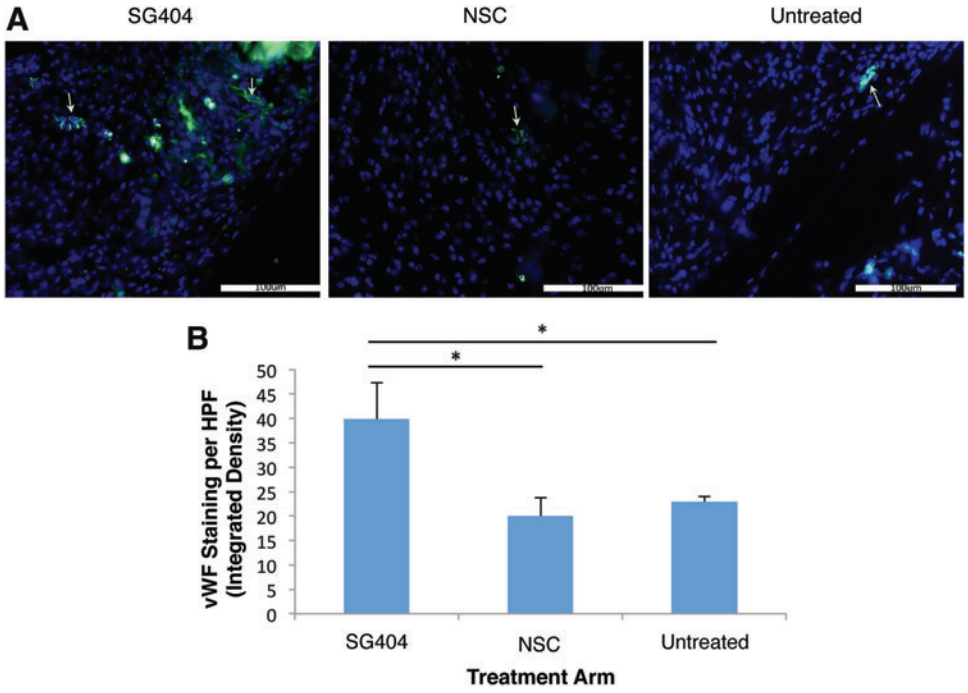
SUPPLEMENTARY FIG. S4. Stability of oligonucleotides in human serum. Oligonucleotides were incubated with 10% human serum for various times in PBS at 37°C. At each time point, an aliquot was removed, mixed with 2 × gel loading buffer, and immediately stored at −80°C. **(A)** Analysis of unmodified sshRNA SG402 and 2′-OMe-stabilized sshRNA SG404 by 12% denaturing PAGE (12% polyacrylamide, 20% formamide, and 8 M urea) and staining with SYBR Gold (Invitrogen, Carlsbad, CA). **(B)** Analysis of SG608 antimiR by 12% denaturing PAGE and staining with methylene blue. PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.



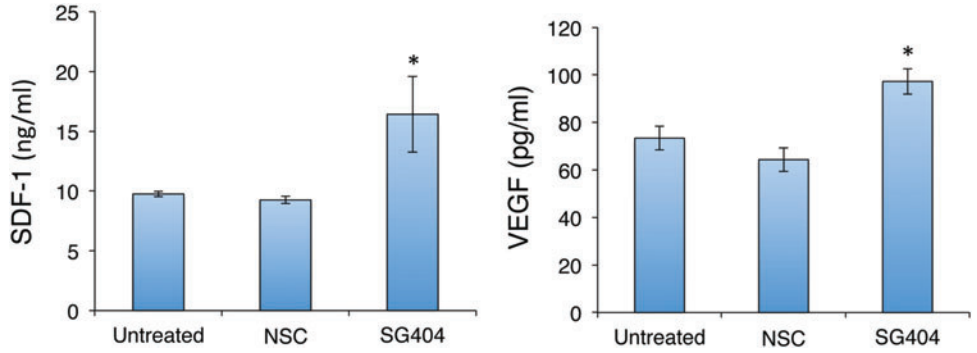
SUPPLEMENTARY FIG. S5. Representative images of **(A)** H&E and **(B)** trichrome staining of fully healed wounds from all treatment groups. Images on the *left* are histology sections magnified at 5×. Images on the *right* are magnifications of the corresponding boxes from the 5× images (scale bar = 50 µm). The area corresponding to the healed wound lies in between the two *vertical lines* in the 5× images. H&E, hematoxylin and eosin.



SUPPLEMENTARY FIG. S5. (*Continued*).



SUPPLEMENTARY FIG. S6. SG404 treatment leads to a sequence-specific increase in neovascularization. **(A)** Representative images of vWF staining at day 7. *Green*, vWF staining; *blue*, DAPI. *Arrows* denote in-focus examples of blood vessels. **(B)** Quantification of vWF fluorescence. $*p < 0.05$. vWF, von Willebrand factor.



SUPPLEMENTARY FIG. S7. Downstream factors that enhance wound healing are induced by day 2 after treatment with LbL-SG404. ELISA measuring protein levels for SDF-1 and VEGF comparing untreated, NSC, and LbL-SG404 ($n=5$ for all groups) from tissue harvested on day 2 after treatment application. *$p<0.05$ versus NSC. ELISA, enzyme-linked immunosorbent assay; LbL, layer-by-layer; SDF-1, stromal cell-derived factor 1; VEGF, vascular endothelial growth factor.