Supporting Information

Local delivery of PHD2 siRNA from ROS-degradable scaffolds to promote diabetic wound healing

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1. Materials and Methods
    a. Materials

    All chemical reagents were obtained from Sigma-Aldrich (Milwaukee, WI) and were used as received unless otherwise indicated. 2-mercaptoethyl ether (MEE) was purchased from Tokyo Chemical Industry Co (Tokyo, Japan). Glycolide and D,L-lactide were obtained from Polysciences (Warrington, PA). Lysine triisocyanate (LTI) was obtained from Kyowa Hakko USA (New York, NY). The tertiary amine catalyst (TEGOAMIN33), composed of 33 wt% triethylene diamine (TEDA) in dipropylene glycol, was obtained from Goldschmidt (Hopewell, VA). Tegaderm was obtained from 3M (St. Paul, MN) and the hydrogel dressing was obtained from ReliaMed (Fort Worth, TX). Lipofectamine 2000 was obtained from Life Technologies (Grand Island, NY), and all cell culture reagents, including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were supplied by Gibco Cell Culture (Carlsbad, CA). Dicer substrate siRNAs were obtained from Integrated DNA technologies (IDT, Coralville, IA).

    b. siNP Synthesis and Characterization

    A diblock copolymer composed of 2-(dimethylamino) ethyl methacrylate (DMAEMA), 2-propylacrylic acid (PAA), and butyl methacrylate (BMA) was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization as previously described.[1] The final polymer, DMAEMA-*block*-(DMAEMA-*co*-BMA *co*-PAA), was characterized by gel permeation chromatography (GPC, Agilent Technologies, Santa Clara, CA) with an inline Wyatt mini-DAWNTREOS light scattering detector (Wyatt Technology Corp., Santa Barbara, CA) and ^{1}H nuclear magnetic resonance spectroscopy (NMR, Bruker 400 MHz Spectrometer) for molecular weight, polydispersity, and polymer composition. For the formation of NPs, the polymer was dissolved in a minimal volume of ethanol in a 2 mL RNAse free polypropylene tube followed by slow addition of deionized water to trigger spontaneous micelle formation (final polymer concentration 1 mg in 1 mL). Micellar size and polydispersity was characterized by Dynamic Light Scattering (DLS, Zetasizer

nano-ZS Malvern Instruments Ltd, Worcestershire, U.K.). 5 nmol (0.08 mg) siRNA was added to the NP solution and allowed to electrostatically complex for 30 min to form siNPs. 5 mg of trehalose (60:1 weight ratio to siRNA), a lyophilization excipient, was added to the siNP solution and allowed to dissolve for 30 min. The siNP solutions were then frozen and lyophilized.

c. Polyester Polyol and PTK Diol Synthesis and Characterization
A trifunctional polyester polyol (PE) was synthesized as previously described.[2] Briefly, glycerol was vacuum dried for 48h at 80°C and then added to a 100mL three neck flask. By molar amount, 60% ε-caprolactone, 30% glycolide, and 10% D,L-lactide were added to the glycerol starter with a stannous octoate catalyst to yield a 900 g mol^{-1} triol. The PTK diol was synthesized as previously documented,[3] as adapted from Wilson et al.[4] P-toluenesulphonic acid monohydrate was dried by azeotropic distillation with toluene, dissolved in 60°C hydrochloric acid, and then recrystallized at -20°C. Crystals were extracted, rinsed with cold HCl, and dried under vacuum for 1 h. The catalyst was added to a tri-neck boiling flask with an attached addition funnel. Both vessels were put under positive nitrogen pressure and charged with anhydrous acetonitrile. MEE (1*x* molar eq) was added to the boiling flask, while 1*x* molar eq of 2,2 dimethoxypropane (DMP) was added to the addition funnel. The solutions in both the addition funnel and boiling flask were purged with flowing nitrogen for 30 min before submerging the boiling flask into an oil bath at 80°C. After 15 min of temperature equilibration, the acetonitrile-DMP solution was added drop-wise into the continuously–stirring, boiling flask for 1 h and allowed to mix for an additional 16 h. Post synthesis, the acetonitrile was removed by rotary evaporation, and the resultant PTK polymer was isolated by precipitation into cold ethanol and dried under vacuum. The resulting dithiol precursor was analyzed by GPC using poly(ethylene glycol) (PEG) standards (400 – 4000 g mol^{-1}) to determine molecular weight. To convert the thiol end-groups to hydroxyl groups, the PTK dithiol polymer was transferred to a boiling flask and put under positive nitrogen pressure, dissolved in anhydrous dichloromethane (DCM), and treated with a 5× molar excess of b-mercaptoethanol to reduce any disulfide bonds and recover the reactive thiol end groups. After 2 h of stirring, the DCM was evaporated and the residue was washed three times in cold ethanol under nitrogen to remove residual b-mercaptoethanol. The reduced PTK polymers were dissolved in anhydrous tetrahydrofuran (THF) before adding a 5× molar excess of cesium carbonate ($CsCO_3$) under nitrogen and stirring for 30 min at room temperature. A 4× molar excess of 2-bromoethanol was next added to the solution and stirred for 18 h under nitrogen at room temperature. After stirring, the solution was added to a separation funnel with an excess of deionized water to effectively separate the PTK-containing THF layer from the water-soluble $CsCO_3$ catalyst. The hydroxyl-functionalized PTKs were extracted in THF before removing the solvent by rotary evaporation, followed by dissolving the product in water-immiscible DCM to remove any residual water. Finally, the PTK diol was analyzed by GPC and NMR before vacuum drying for 24 h.

d. PEUR and PTK-UR Synthesis
100 mg PEUR and PTK-UR scaffolds were prepared using reactive liquid molding with or without lyophilized siNPs. The PE or PTK polyol was added to a micro-centrifuge tube along with water, TEGOAMIN33 catalyst, and calcium stearate pore opener. These components, with or without the lyophilized siNPs, were first mixed for 30 s at 3300 rpm in a Hauschild DAC 150 FVZ-K SpeedMixer (FlackTek, Inc., Landrum, SC). After a homogenous mixture was obtained, the respective isocyanate (HDIt or LTI) was added and mixed for an additional 30 s before allowing the liquid mixture to freely rise and harden for at least 2 h. The targeted index (ratio of isocyanate to hydroxyl equivalents times 100) was 115, where the number of OH equivalents is calculated from the respective polyol's molecular weight. The amounts of each component for the respective scaffold formulations, given as equivalent amounts in parts per hundred parts polyol (PPHP), are given in Table S1.

e. *In Vitro* ROS-Mediated Degradation of PTK-UR Scaffolds
PTK-UR scaffolds were fabricated and weighed pre-degradation. Triplicate 5-10 mg scaffold samples were incubated at 37°C for three days in 1mL of either water, 5 mM hydrogen peroxide (H_2O_2), 5 mM H_2O_2 with 0.08 mM $CoCl_2$, (generates hydroxyl radicals),[5] 5 mM KO_2 (superoxide), or 5 mM 3-Morpholino-sydnonimine (SIN-1, generates peroxynitrite).[6] The media was replaced once after 24h. After three days of incubation, scaffolds were removed, rinsed with water, lyophilized, and then weighed to determine mass loss.

f. *In Vitro* Screen of PHD2 siRNA
Dicer substrate siRNA designed against rat PHD2 messenger RNA (mRNA) was obtained from IDT (sequence modified from well-validated mouse PHD2 siRNA[7]) and screened for mRNA silencing in A7r5 rat smooth muscle cells. Cells were seeded at 24,000 per well in a 12-well plate and allowed to adhere for 24 h in DMEM media supplemented with 1% FBS and 1% penicillin/streptomycin. After 24 h of incubation, cells were treated with fresh media, scrambled siRNA at 50 nM, or PHD2 siRNA at 50 nM. The respective siRNA treatments were pre-complexed with Lipofectamine 2000 in OptiMEM and diluted into culture media following the manufacturer's protocol. After 24 h of incubation with the siRNA, the media was removed and replaced with fresh media. After another 24 h (48 h following original siRNA treatment), cells were washed, lysed, and processed using a QiaShredder kit (Qiagen, Venlo, Netherlands). RNA was extracted using a Qiagen RNeazy column following the manufacturer's protocol and quantified for quality and concentration using a NanoQuant plate on a Tecan microplate reader. cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), and quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Bio-Rad iQ SYBR Green Supermix. Relative PHD2 expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the ΔΔCt method. Primer sequences are given in Table S2.

g. *In Vitro* Nanoparticle Release from PTK-UR Scaffolds
1 mg of the nanoparticle-forming polymer was dissolved in a minimal volume of ethanol in a 2 mL RNAse free polypropylene tube followed by slow addition of deionized water to trigger spontaneous micelle formation (final polymer concentration 1 mg in 1 mL). 5 nmol (0.08 mg) of Cy5-labeled, double-stranded DNA, similar to siRNA molecular weight, was added to the NP solution and allowed to electrostatically complex for 30 min to form fluorescently-labeled DNA-NPs. 5 mg of trehalose (60:1 weight ratio to DNA), a lyophilization excipient, was added to the siNP solution and allowed to dissolve for 30 min. The siNP solutions were then frozen and lyophilized. 100 mg PTK-UR scaffolds were made with the lyophilized DNA-NPs, allowed to harden, and cut into six sections. The individual scaffold sections were incubated in 1 mL of phosphate buffered saline (PBS), with the PBS being removed at designated time points and evaluated for Cy5 fluorescence using a microplate reader (Tecan Infinite F500, Männedorf, Switzerland). The concentration of labeled nanoparticles in solution, and therefore the drug release rate, was determined based on fluorescence measurement of Cy5-labeled DNA-NPs.

h. Diabetic Excisional Wound Healing with PTK-UR and PEUR Scaffolds
To evaluate diabetic wound healing in rats, male Sprague-Dawley rats with STZ-induced diabetes were shaved and sterilized with iodine. Six full-thickness excisional wounds were made on the rats' dorsal skin with an 8-mm biopsy punch. Ethylene oxide-sterilized PEUR or PTK-UR scaffolds (8 mm diameter × 1.5 mm thickness) made with LTI were placed in the wounds, covered with a non-adherent, absorbent ReliaMed hydrogel dressing, and then covered with an adherent Tegaderm outer dressing which was stapled to non-wounded skin to provide additional stability. Finally, each rat was fitted with a velcro-fastened jacket (Lomir Biomedical, Inc., Malone, NY) to prevent self-tampering with the dressings[8] and singly housed in the rodent facility. Rats were euthanized at 4, 7, and 14 days post-surgery and excised scaffold/tissue sections were processed for histology with H&E staining. Stained tissue sections were analyzed for scaffold tissue infiltration (% scaffold wound area containing tissue), thickness of tissue in-growth (distance tissue has infiltrated from bottom of scaffold upwards into the scaffold interior), and macrophage presence in the scaffold tissue (% CD68-positive pixel area per cross-sectional scaffold pixel area stained) with ImageJ 1.48v software (National Institute of Health, Bethesda, MD).

i. *In Vivo* Silencing of PHD2 in Diabetic Excisional Wounds
PEUR and PTK-UR scaffolds were fabricated with or without lyophilized siNPs plus trehalose (1 mg NP polymer, 5 nmol siRNA, and 5 mg trehalose per 100 mg scaffold). Scaffold implants were designated by their siNP payload: no treatment (NT), scrambled siRNA (SCR-siNP), and anti-PHD2 siRNA (PHD2-siNP). siRNA sequences are given in Table S2. Ethylene oxide-sterilized scaffold sections were implanted in excisional wounds in diabetic rats and

covered as described previously. As an initial screen to measure the effects of PHD2-siNP delivery from PEUR vs PTK-UR scaffolds, both scaffold formulations with NT, SCR-siNP, and PHD2-siNPs were implanted into diabetic rats which were euthanized at day 7 post surgery for blood vessel analysis. Vessel area per scaffold was measured from H&E stained tissue sections. A follow-up study was completed using only using the better-performing PTK-UR scaffolds with NT, SCR-siNP, and PHD2-siNP treatments. At days 4 and 7 post-surgery, scaffold/tissue sections were extracted from euthanized rats and processed for histology with H&E and trichrome blue staining. Small pieces of tissue-infiltrated scaffolds (~20mg) from all three time points were also extracted and saved in RNAlater (ThermoFisher Scientific, Grand Island, NY). Tissues were put into 2 mL microcentrifuge tubes with 5 mm stainless steel beads and Qiazol (Qiagen, Venlo, Netherlands) and pulsed at 300 Hz for 2 min in a Qiagen Tissuelyser II to completely disrupt the tissue and extract RNA. Extracted RNA was purified with RNeasy spin columns, including on-column genomic DNA elimination using a Qiagen RNase-free DNase kit, and quantified for quality and concentration using a NanoQuant plate on a Tecan microplate reader. cDNA was synthesized using an iScript cDNA Synthesis Kit, and qRT-PCR was performed using Bio-Rad iQ SYBR Green Supermix. Relative gene expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and cyclophilin B (PPIB) using the ΔΔCt method. Primer sequences are given in Table S2.

j. Western blot analysis of extracted tissues
Western blot analysis was also performed on scaffold/tissue samples extracted from day 4 animals. Frozen samples were extracted with UDC buffer (8 M urea, 10 mM dithiothreitol (DTT), 4% CHAPS containing Phosphatase I and II protease inhibitor cocktail (Sigma, St. Louis, MO)) by vortexing at room temperature overnight and centrifugation at 14,000 rpm for 15 min at 4°C. Soluble protein concentrations were determined using the Bradford assay (Pierce Chemical, Rockfort, IL). Equal amounts (30 μg) of proteins were added to Laemmli sample buffer (Bio-Rad), heated for 5 min at 100°C, and separated on 12% SDS polyacrylamide gels. Proteins from the gels were transferred onto nitrocellulose membranes (Li-COR Biosciences, Lincoln, NE) and blocked with blocking buffer for 1 hour at room temperature (Li-COR Biosciences) prior to incubation overnight at 4°C with antisera against VEGF (1:1000, Santa Cruz Biotechnology), HIF-1α (1:1000, Santa Cruz Biotechnology), and GAPDH (1:1000, Millipore). Membranes were washed three times with TBS containing Tween 20 (0.1%) (TBST) and incubated with 680 nm and 800 nm infrared-labeled secondary antibodies (Li-Cor, Lincoln, NE) for 1h at room temperature. The membranes were subsequently washed with TBST, and protein-antibody complexes were visualized and quantified using the Odyssey direct infrared fluorescence imaging system (Li-Cor Biosciences NE).

k. Immunohistochemical staining of tissue sections

Immunohistochemical staining was performed using commercial antibodies specifically directed against rat CD68 (MCA341GA, Bio-Rad), collagen IV (ColIV) (ab6586, Abcam, Cambridge, MA), α-smooth muscle actin (α-SMA) (RB-9010, Labvision, Freemont, CA), Ki67 (ab16667, Abcam) and S100A4 (ab197896, Abcam). Formalin-fixed paraffin embedded tissues were sectioned at 5 μm, placed on slides and warmed overnight at 60°C. Slides were deparaffinized and rehydrated with graded alcohols ending in Tris buffered saline (TBS-T Wash Buffer, LabVision, Freemont, CA). Heat mediated target retrieval was performed in 1X Target Retrieval Buffer (Citrate, pH 6.0, DAKO, Carpenteria, CA). Endogenous peroxidases and non-specific background were blocked by subsequent incubations in 3% H_2O_2 (Fisher, Suwanee, GA) in TBS-T and protein block (DAKO). For CD68, primary antibody was used at 1:800 for 1 h, followed by secondary incubation in biotinylated rabbit anti-mouse IgG (1:200; Vector, Burlingame, CA) and subsequent tertiary incubation in SA-HRP (RTU, BD Pharmingen, San Jose, CA). For Col IV, primary antibody was used at 1:600 for 1 h, with the Bond Refine Polymer detection system used for visualization. For α-SMA, primary antibody was used at 1:1000 for 1 h, with the Bond Refine Polymer detection system used for visualization. For Ki67, primary antibody was used at 1:100 for 1 h, with the Bond Refine Polymer detection system used for visualization. For S100A4, primary antibody was used at 1:2,000 for 1 h, followed by secondary incubation in anti-rabbit EnVision + labeled polymer (DAKO). Slides were rinsed with TBS-T between each reagent treatment and all steps were carried out at room temperature. Visualization was achieved with DAB+ chromogen (DAKO). Slides were counterstained with Mayer's hematoxylin, dehydrated through a series of alcohols and xylenes, and then coverslipped with Acrytol Mounting Media (Surgipath, Richmond, IL).

l. Histological analysis of wound healing outcomes
Quantitative analysis of healing outcomes between scaffold formulations and siNP treatments was determined histologically from day 7 tissue samples. The excised tissues were fixed in formalin, processed, embedded in paraffin, sectioned, and prepared for staining or immunohistochemistry (IHC). IHC using CD68, Col IV, α-SMA, Ki67, and S100A4 antibodies was performed on scaffold/tissue sections and quantified using ImageJ. Macrophage presence between PEUR and PTK-UR scaffolds was quantified as the CD68-positive pixel area per cross-sectional scaffold pixel area. Overall blood vessel counts per cross-sectional scaffold area were determined from Col IV IHC sections, while more mature vessels were similarly quantified from α-SMA IHC sections. Ki67-positive pixel area per cross-sectional scaffold pixel area was quantified for the presence of proliferating cells, and concurrent S100A4 IHC tissue sections were compared to Ki67 sections to broadly verify if the proliferating cells positively expressed the S100A4 marker. Trichrome blue-stained sections were analyzed for tissue infiltration differences between SCR-siNP and PHD2-siNP as previously described.

m. Statistical Analysis
All data are reported as the mean and standard error of the mean unless otherwise indicated. Statistical analysis was performed using single factor

analysis of variance (ANOVA) and Tukey post-hoc comparison tests for multiple comparisons, and the Student's t-test was used for single comparisons. P-values less than 0.05 were considered statistically significant.

2. Supplemental Results

Table S1. PTK-UR and PEUR scaffold components

Component	***PPHP***	***Mass (mg)***	***Component***	***PPHP***	***Mass (mg)***
PTK Diol (1100 g/mol)	100.0	69.5	PE Triol (900 g/mol)	100.0	62.7
Water	1.5	1.0	Water	1.5	0.9
TEGOAMIN33	2.3	1.6	TEGOAMIN33	2.3	1.4
Calcium stearate	4.0	2.8	Calcium stearate	4.0	2.5
LTI	36.1	25.1	LTI	51.8	32.5

Table S2. Nucleic Acid Sequences

Name	Sequence
Scrambled siRNA	DS Scrambled Neg – From IDT
PHD2 siRNA	S: 5'-GGUACGCAAUAACCGUUUGGUAUTT-3' AS: 5'-AAAUACCAAACGGUUAUUGCGUACCUU-3'
GAPDH Primers	FWD: 5'-CTCACTCAAGATTGTCAGCAATG-3' REV: 5'-GAGGGAGATGCTCAGTGTTGG-3'
PPIB Primers	FWD: 5'-TTCCATCGTGTCATCAAG-3' REV: 5'-GAAGAACTGGGAGCCATT-3'
PHD2 Primers	FWD: 5'-ATCTCACAGGTGAGAAAGGT-3' REV: 5'-ACAGAAGGCAACTGAGAG-3'

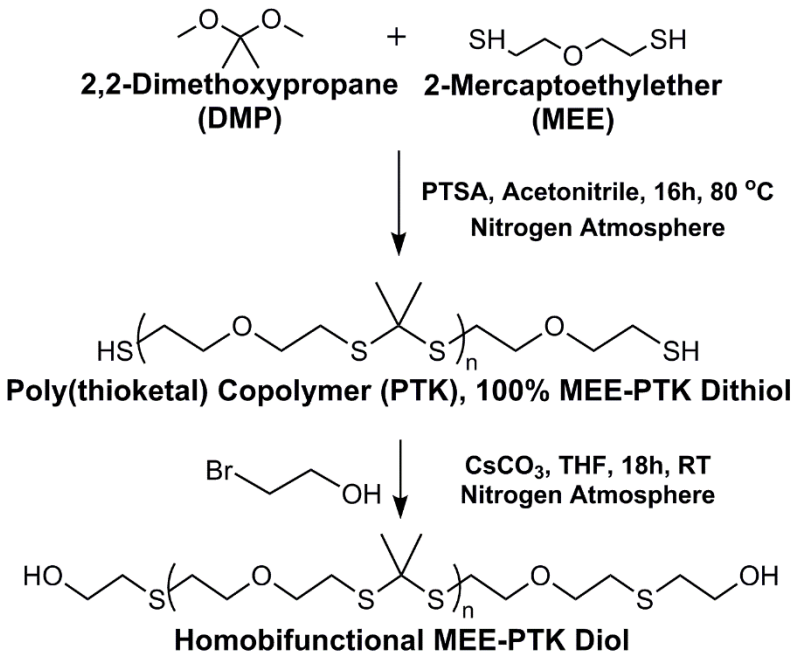

Figure S1. Synthesis scheme for MEE-PTK diol.

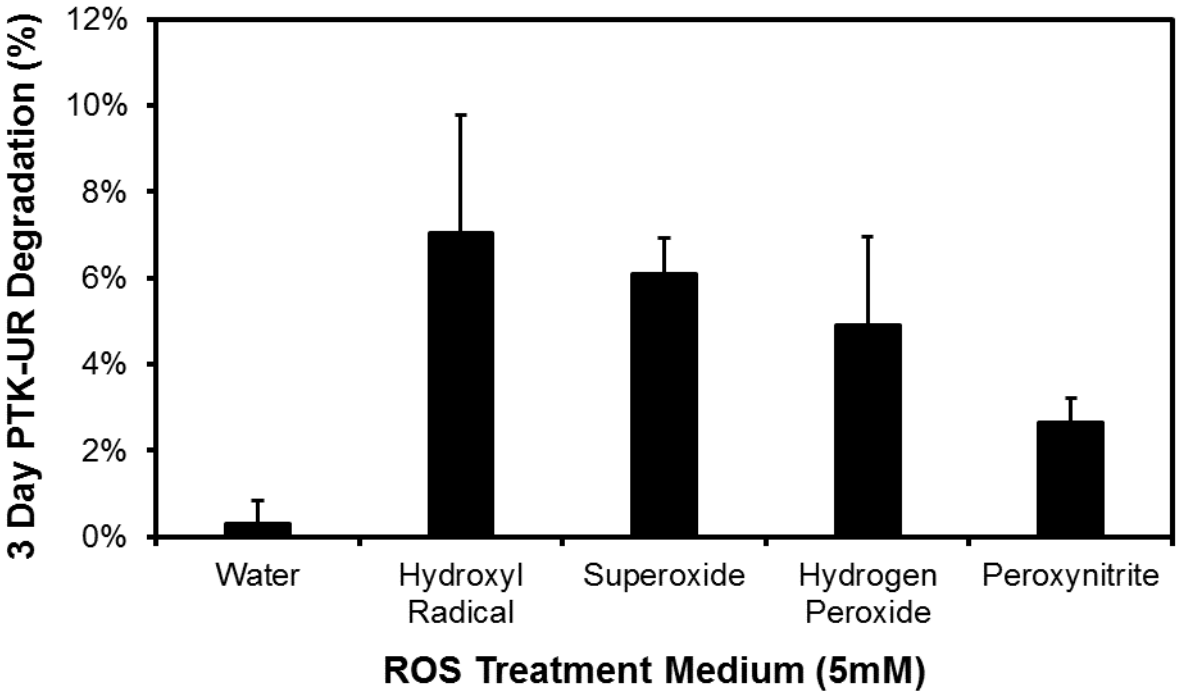

Figure S2. *In vitro* degradation of PTK-UR scaffolds in varying ROS media. Scaffolds were incubated in water or different ROS media containing 5mM of hydrogen peroxide with $CoCl_2$ (produces hydroxyl radicals), 5mM potassium superoxide, 5mM hydrogen peroxide, or 5mM SIN-1 (produces peroxynitrite) for three days and then weighed for mass loss. Scaffolds incubated in the different ROS were all significantly degraded compared to samples incubated in water ($p<0.05$).

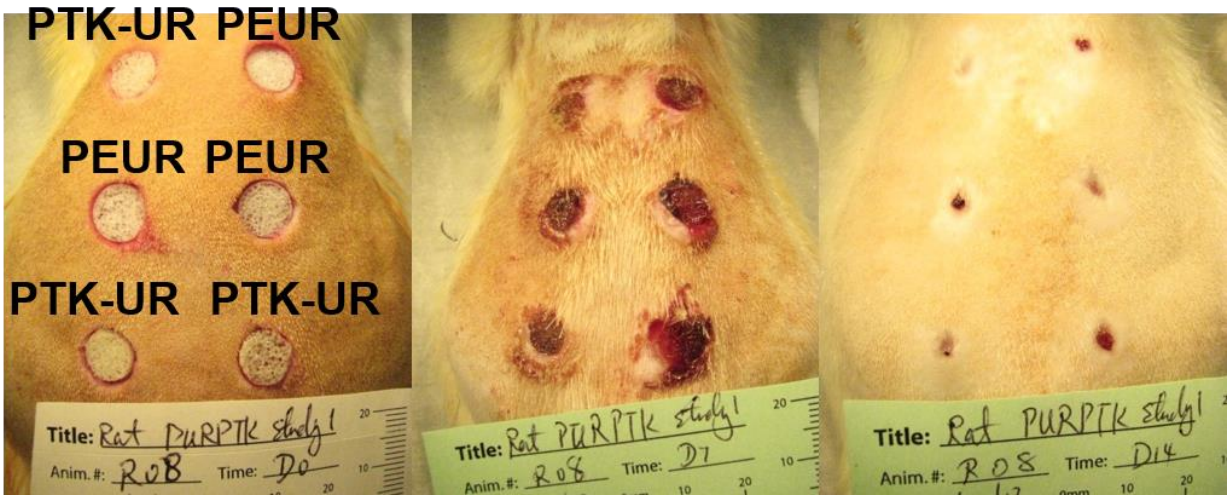

Figure S3. Diabetic rats with excisional wounds and implanted PEUR and PTK-UR scaffolds at day 0, day 7, and day 14 post surgery.

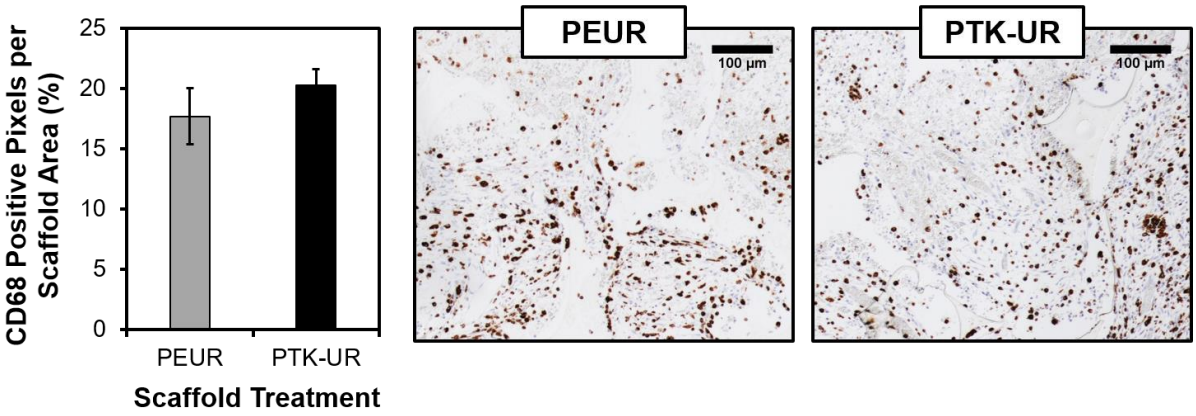

Figure S4. Macrophage presence in PEUR and PTK-UR scaffolds. There was no statistical difference in macrophage number between PEUR and PTK-UR scaffolds at day 7 post implantation in diabetic rats as quantitatively determined from CD68 IHC tissue sections.

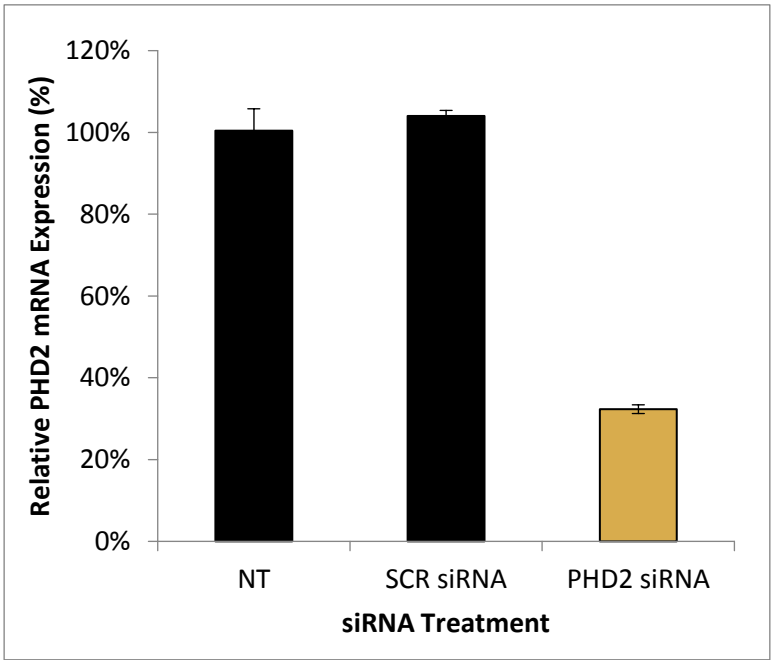

Figure S5. *In vitro* screen of PHD2 siRNA in A7r5 rat smooth muscle cells by qRT-PCR.

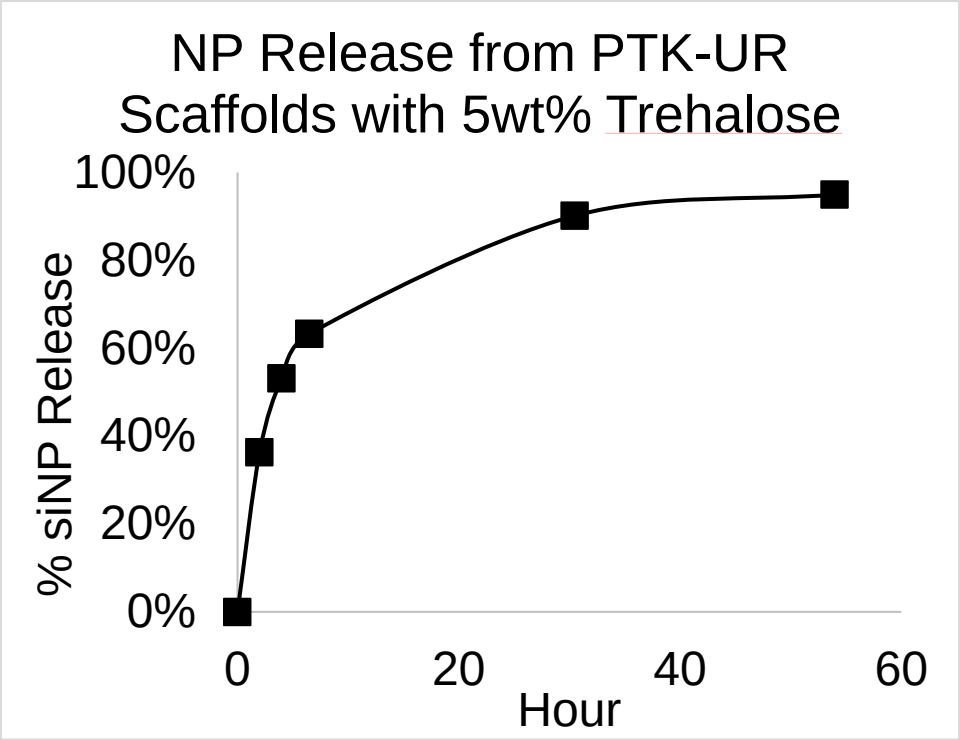

Figure S6. *In vitro* release of nanoparticles with 5wt% trehalose from PTK-UR scaffolds. Nanoparticles were loaded with fluorescently labeled double-stranded DNA as a model for siRNA.

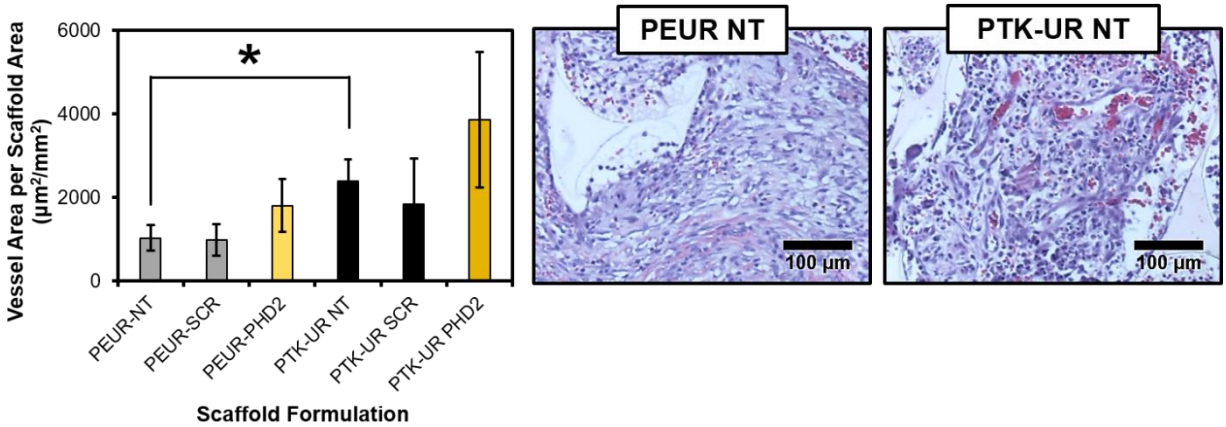

Figure S7. Initial *in vivo* screen of PEUR and PTK-UR scaffolds for both baseline vascularization and stimulation of vascularization through PHD2 siNP delivery (mean ± SEM, n = 4 independent samples, *p<0.05).

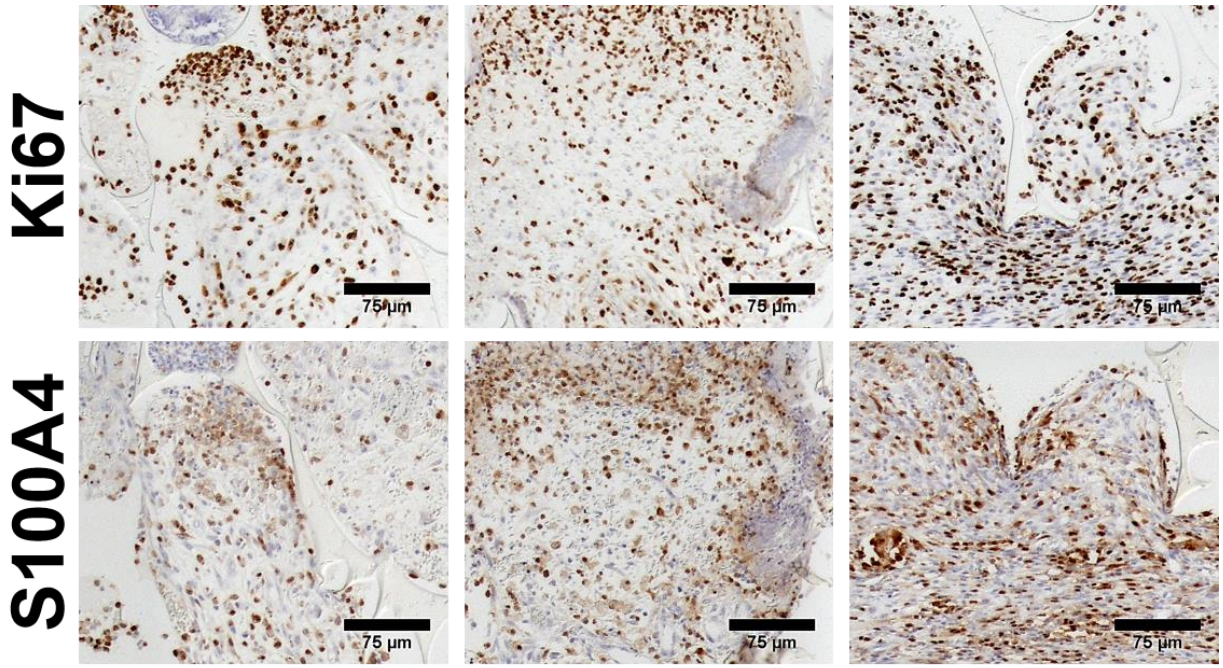

Figure S8. Representative comparison of Ki67 and S100A4 IHC staining. Serial tissue sections taken from day 7 PHD2-siNP scaffold implants, stained for either Ki67 (proliferating cells) or S100A4 (fibroblast marker), indicate that the proliferating cells are fibroblastic in nature.

3. Works Cited

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