www.sciencetranslationalmedicine.org/cgi/content/full/7/290/290ra92/DC1

Supplementary Materials for

Drug-induced regeneration in adult mice

Yong Zhang, Iossif Strehin, Khamilia Bedelbaeva, Dmitri Gourevitch, Lise Clark, John Leferovich, Phillip B. Messersmith, Ellen Heber-Katz*

*Corresponding author. E-mail: heberkatz@limr.org

Published 3 June 2015, *Sci. Transl. Med.* **7**, 290ra92 (2015)
DOI: 10.1126/scitranslmed.3010228

The PDF file includes:

SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Hydrogel and Drug Preparation

Synthesis of 1,4-DPCA (see Supplemental Figure S7).

Diethyl[(quinolin-8-ylamino)methylidene]propanedioate (1). **1** was prepared using a modified protocol described previously (79). 8-aminoquinoline (3.31 g, 22.9 mmol) and diethyl ethoxymethylenemalonate (4.63 mL, 22.9 mmol) were heated to 100℃ for 1 hour and then cooled to 80℃ and 20 mL methanol was added. The crystallized product was washed twice with 20 mL MeOH and dried on high vacuum to afford **1** (5.68g, 18.1 mmol, 79%) as green-brown needles. ^{1}H NMR (500 MHz, Chloroform-d), δ, ppm (J, Hz): 12.37 (1H, d, $^{3}J_{NH,=CH}$ = 14.3, NH); 8.97 (1H, dd, $^{3}J_{2,3}$ = 4.3, $^{4}J_{2,4}$ = 1.7, H-2); 8.80 (1H, d, $^{3}J_{=CH,NH}$ = 14.3, =CH); 8.18 (1H, dd, $^{3}J_{4,3}$= 8.3, $^{4}J_{4,2}$ = 1.7, H-4); 7.55 (3H, m, H-5,6,7); 7.49 (1H, dd, $^{3}J_{3,4}$ = 8.3, $^{3}J_{3,2}$ = 4.2, H-3); 4.42 (2H, q, $^{3}J_{OCH2,CH3}$ = 7.1, (Z)-ester OCH_2); 4.30 (2H, q, $^{3}J_{OCH2,CH3}$ = 7.1, (E)-ester OCH_2); 1.44 (3H, t, $^{3}J_{CH3,OCH2}$ = 7.1, (Z)-ester CH_3); 1.37 (3H, t, $^{3}J_{CH3,OCH2}$ = 7.1, (E)-ester CH_3).

Ethyl 4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylate (2). **2** was prepared using a modified protocol described previously (79). **1** (5.50 g, 17.5 mmol) was added to diphenylether (55 mL) and refluxed (250℃) for 1 hour, then cooled to room temperature and collected through filtration. The precipitate was triturated twice with 25 mL petroleum ether (b.p. 80-110℃) followed by washing with 10 mL of cold Et_2O. The precipitate was dried on high vacuum overnight to afford **2** (2.05 g, 7.65 mmol, 44%) as a beige powder. ^{1}H NMR spectrum (500 MHz, DMSO-d_6), δ, ppm (J, Hz): 12.88 (1H, s, NH); 9.10 (1H, dd, $^{3}J_{2,3}$ = 4.3, $^{4}J_{2,4}$ = 1.6, H-2); 8.57 (1H, dd, $^{3}J_{4,3}$ = 8.3, $^{4}J_{4,2}$ = 1.6, H-4); 8.54 (1H, s, H-8); 8.22 (1H, d, $^{3}J_{6,5}$ = 8.8, H-6); 7.90 (1H, d, $^{3}J_{5,6}$ = 8.8, CH, i); 7.84 (1H, dd, $^{3}J_{3,4}$ = 8.3, $^{3}J_{3.2}$ = 4.3, H-3); 4.25 (2H, q, $^{3}J_{OCH2,CH3}$ = 7.1, OCH_2); 1.30 (3H, t, $^{3}J_{CH3,OCH2}$ = 7.1, CH_3).

1,4-dihydrophenonthroline-4-one-3-carboxylic acid (1,4-DPCA) (3). **3** was prepared using a modified protocol described previously (79). **2** (2.00 g, 7.46 mmol) was combined with 40 mL 10% (w/v) KOH and refluxed (110℃) for 1 hour, allowed to cool to room temperature, and residual diphenyl ether extracted using 28 mL petroleum ether (b.p. 80-110℃). The product was precipitated with 40 mL 10% (w/v) HCl, filtered, washed with dH_2O and dried under high vacuum overnight to afford **3** (1.65 g, 6.87 mmol, 92%) as a beige powder. ^{1}H NMR spectrum (500 MHz, DMSO-d_6), δ, ppm (J, Hz): 15.44 (1H, s, OH); 13.85 (1H, s, NH); 9.16 (1H, dd, $^{3}J_{2,3}$ = 4.3, $^{3}J_{2,4}$ = 1.6, H-2); 8.73 (1H, s, H-8); 8.64 (1H, dd, $^{3}J_{4,3}$ = 8.3, $^{4}J_{4,2}$ = 1.6, H-4); 8.26 (1H, d, $^{3}J_{6,5}$ = 8.8, H-6); 8.04 (1H, d, $^{3}J_{5,6}$ = 8.8, H-5); 7.92 (1H, dd, $^{3}J_{3,4}$ = 8.3, $^{3}J_{3,2}$ = 4.3, CH, k). Mass spectrum, m/z (I_{rel}, %): 241.1 [MH]+ (18), 263.0 (100), 279.0 (30), 503.1 (41). Purity was estimated as 99.8% by HPLC (C_{18}, 10 µm, 4.6x250 mm, 300Å pores, silica; 2-100%, 30 min, acetonitrile gradient, 0.1% TFA; elution time = 18.5 minutes; UV-vis, λ_{max}, nm: 261, 316, 331, 346).

Synthesis of Glutaric Acid Terminated 8 Arm PEG (P8G). Glutaric acid terminated PEG was synthesized as described previously (39). Briefly, 8-arm PEG-OH (19.4 g, 7.74 mmol OH) and glutaric anhydride (4.49 g, 38.7 mmol) were dissolved in chloroform (20 mL). Pyridine (3.12 mL, 38.7 mmol) was added dropwise, and the reaction mixture was refluxed at 82 ℃ for 24 hours under inert air. The product was precipitated with cold diethyl ether (200 mL) and spun down. The supernatant was decanted and the product re-dissolved in MeOH (200 mL). After incubation at -20 ℃ for 1 hour, the precipitate was centrifuged at -5 ℃. The supernatant was discarded, and the MeOH wash procedure was repeated twice more. Following cold diethyl ether precipitations (400 mL), the product was collected and dried under high vacuum overnight to afford a white powder (92% yield, 100% conversion). 1H NMR (500MHz, Chloroform-d), δ, ppm: 4.24 (16H, t, terminal PEG CH2), 3.64 (1823H, m, backbone PEG CH2), 2.43 (16H, t, H-2 of glutaric acid), 2.39 (16H, t, H-4 of glutaric acid), 1.96 (16H, p, H-3 of glutaric acid).

Synthesis of N-Hydroxysuccinimide Terminated 8 Arm PEG (P8NHS). NHS terminated 8 arm PEG was synthesized as described previously (39). P8G (18.6 g, 7.10 mmol COOH), NHS (8.18 g, 71.0 mmol) and

EDC (13.6 g, 71.0 mmol) were dissolved in DMSO (47 mL). The solution was agitated for 30 minutes at room temperature, then it was diluted with MeOH (200 mL), precipitated at -20 ℃ for 1 hour, and spun down at -5 ℃. The supernatant was decanted, and the MeOH wash procedure was repeated twice more with 400 mL MeOH per wash. Following cold diethyl ether precipitations (400 mL), the product was dried under high vacuum to afford a white powder (95% yield, 96% conversion). 1H NMR (500MHz, Chloroform-d), δ, ppm: 4.24 (16H, t, terminal PEG CH2), 3.63 (1823H, m, backbone PEG CH2), 2.84 (32H, m, NHS protons), 2.71 (16H, t, H-4 of glutaric acid), 2.49 (16H, t, H-2 of glutaric acid), 2.06 (16H, p, H-3 of glutaric acid).

Synthesis of Cysteine Terminated 8 Arm PEG (P8Cys). Cysteine terminated 8 arm PEG was synthesized as described previously (39). PEG-NH2 (20 g, 8.12 mmol NH2) was dissolved in DMF (40 mL) after which DIEA was added dropwise (1.41 mL, 8.12 mmol). In a separate reaction vessel, Boc-Cys(Trt)-OH (15.0 g, 32.5 mmol) and BOP (14.4 g, 32.5 mmol) were dissolved in DMF (40 mL) and DIEA (5.65 mL, 32.5 mmol) was added dropwise. Both solutions were combined, and the coupling reaction was allowed to proceed at room temperature for 18 hours. Following precipitation in cold diethyl ether (400 mL), the product was re-dissolved in MeOH (40 mL) and precipitated in cold diethyl ether once more (400 mL). The cysteine was deprotected with TFA:TIS:EDT (300 mL, 95:2.5:2.5) cleavage solution at room temperature for 4 hours. TFA was evaporated under low pressure, and the product was precipitated in cold diethyl ether (400 mL). P8Cys was dissolved in MeOH (200 mL), precipitated at -20 ℃ overnight, and centrifuged at -5 ℃. The supernatant was decanted and the MeOH precipitation was repeated twice more using 100 mL MeOH per wash. Following diethyl ether precipitations (200 mL), the product was dried under high vacuum overnight to afford a white powder (73% yield, 84% endgroup conversion). 1H NMR (500MHz, Acetic Acid-d4), δ, ppm: 4.41 (8H, t, α-C cysteine), 3.68 (1790H, m, backbone PEG CH2), 3.13 (16H, d, CH2 cysteine).

Preparation of 1,4-DPCA Microcrystals. 1.35 g Pluronic F127NF and 100 mg 1,4-DPCA were dissolved in 10 mL DMF. With stirring, the F127NF/1,4-DPCA solution was added dropwise to 500 mL ddH_2O at 60℃ to form microcrystals. The resulting microcrystals were collected by filtration and washed twice with 200 mL 0.27% (w/v) F127NF in ddH_2O. The microcrystals were re-suspended in 0.27% (w/v) F127NF in ddH_2O and lyophilized to afford a white powder of F127NF/1,4-DPCA drug microcrystals. HPLC (C_{18}, 10 µm, 4.6x250 mm, 300Å pores, silica; 2-100%, 30 min, acetonitrile gradient, 0.1% TFA; elution time = 18.5 minutes) was used to quantify the drug content. The amount of 1,4-DPCA in the drug microcrystals showed batch to batch variation within the range of 35-53%.

Filter Sterilization of PEG Polymers. A 10% (w/v) solution of each PEG polymer in MeOH was filtered through a 0.2 µm filter into a sterile receptacle. The product was lyophilized to yield filter sterilized P8NHS or P8Cys.

Formation of Drug-Loaded Hydrogels. Separately, 10% (w/v) solutions of P8NHS and P8Cys were prepared in phosphate buffered saline (PBS) suspension of DCs at the desired drug concentration. The two polymer solutions were then mixed in a 1:1 v/v ratio and left undisturbed for 20 minutes to yield 70 µL cylindrical hydrogels (n = 3).

In-Vitro Drug Release from Hydrogels. Each hydrogel cylinder prepared as described above was immersed in 5 mL PBS and at specified time points transferred into 5 mL of fresh PBS. UV/vis spectrophotometry was used to quantify drug release over time. The standard curve was prepared from stock solutions of known concentration of drug in DMSO. 10 µL of each stock solution was added to 990 µL PBS to yield a standard curve with 100-3000 ng/mL of drug. A PowerWave XS2 microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA) was used to quantify absorbance at 261 nm. Hydrogels without drug were used as negative controls.

Gelation Kinetics

Gelation time was quantified using a previously described protocol (39). Briefly, the drug microcrystals were suspended in PBS and used to prepare 10% (w/v) P8NHS and 10% (w/v) P8Cys. The two polymer solutions were then mixed in a 1:1 (v/v) ratio and pipetted up and down using a standard 0.1-10 µL pipette tip. The time at which the material blocked the pipette tip was designated as the gelation time. Temperature was controlled at 37℃ through the use of a water bath.

Cell Viability

Viability of 3T3 fibroblasts exposed to drug microcrystals was quantified using ISO 10993. Briefly, different dilutions of drug microcrystals in cell culture medium were added to a subconfluent monolayer of 3T3 fibroblasts (n = 3). The cells were cultured for 24 hours at 37℃, 5% CO_2, and >90% RH and then washed with PBS. Neutral red solution (0.4%) in DMEM was added, and the cells were stained for 3 hours. Following removal of the staining solution and washing the cells with PBS, the cells were destained using 50% ethanol, 49% ddH_2O and 1% glacial acetic acid. Following 10 minutes of agitation, absorbance was measured at 540 nm and used to quantify cell viability. SDS was used as a positive control as specified by ISO 10993, and the IC_{50} was found to be in the acceptable range of values hence confirming the validity of the assay. Culture medium was used as a negative control. Cell culture medium consisted of high glucose DMEM substituted with L-glutamine, penicillin/streptomycin, 10% v/v newborn calf serum and 20 mM HEPES.

Tissue Culture

Primary ear dermal fibroblast-like cells were established from MRL and B6 mice and grown in DMEM-10% FBS supplemented with 2 mM L-glutamine, 100 IU/mL penicillin streptomycin and maintained at 37 ℃, 5% CO2, and 21% O2. Cells were split 1:5 as needed to maintain exponential growth and avoid contact inhibition. Passage numbers were documented and cells from early passages (<P20) frozen in liquid nitrogen and used in the described experiments.

Immunohistochemistry

Tissue from normal ears were fixed with Prefer fixative (the active ingredient is glyoxal) (Anatech) overnight and then washed in H20. Tissue was embedded in paraffin and 5-µm thick sections cut. Before staining, slides were dewaxed in xylene and rehydrated. Antigen retrieval was performed by autoclaving for 20 min in 10 mM Sodium Citrate, pH 6.0. Tissue sections were then treated with 3% H2O2 and nonspecific binding was blocked with 4% BSA (A7906; Sigma) for 1 h. The primary antibodies and matched secondary antibodies used for IHC were shown in **Supplemental Table S2**. For immunocytochemistry staining, primary ear skin fibroblasts were grown on coverslips in DMEM with 10% FBS at 37 ℃ in a humidified 5% CO2 incubator. The coverslips were rinsed with 1× PBS, the cells were fixed in cold methanol (−20 ℃) for 10 min, rinsed with 1× PBS, treated with 0.1% Triton-X100, and then incubated with the appropriate primary and secondary antibodies (Table S2). Photomicrographs were produced using the fluorescent microscope (Olympus AX70) and a Spot camera with bounded software .

For histological stains, tissue sections were treated the same as above and then stained with Hematoxylin (Leica Microsystems, # 3801562) and Eosin (Leica Microsystems, #3801602), Picro-Sirius Red (Poly Scientific, cat. # s2365), Alcian blue (1% in 3% acetic acid (Polyscience, Bay Shore, NY, cat # S111A), or toluidine blue O (Allied Chemical, Morristown, NJ, cat. # NA0652), counterstained with Kernechtrot (Polyscience, Bay Shore, NY, cat # S248). The slides were washed, rehydrated, cleared with Xylene and coverslipped with Permount mounting media (Fisher, SP15-500). Staining was visualized using an Olympus (AX70) microscope in bright field for H&E and under polarized light for Picro-Sirius Red.

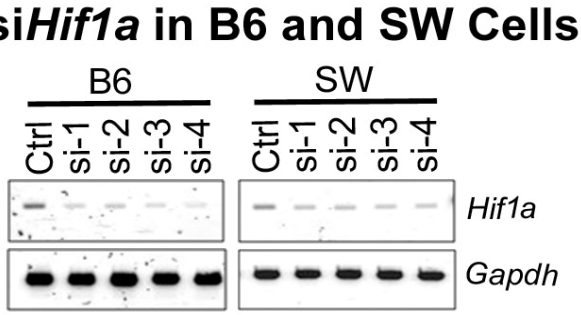
For quantitation of IHC or PSR signal, the method used was previously described (16). Briefly, we used ImagePro v4.0 for image analysis by selecting positive staining from multiple areas in the sections. The number of “positive staining” pixels was determined. The area was expressed in square microns and the final data were expressed as IHC staining signal per square micron. The mean of samples examined were plotted and standard errors calculated.

Western Analysis

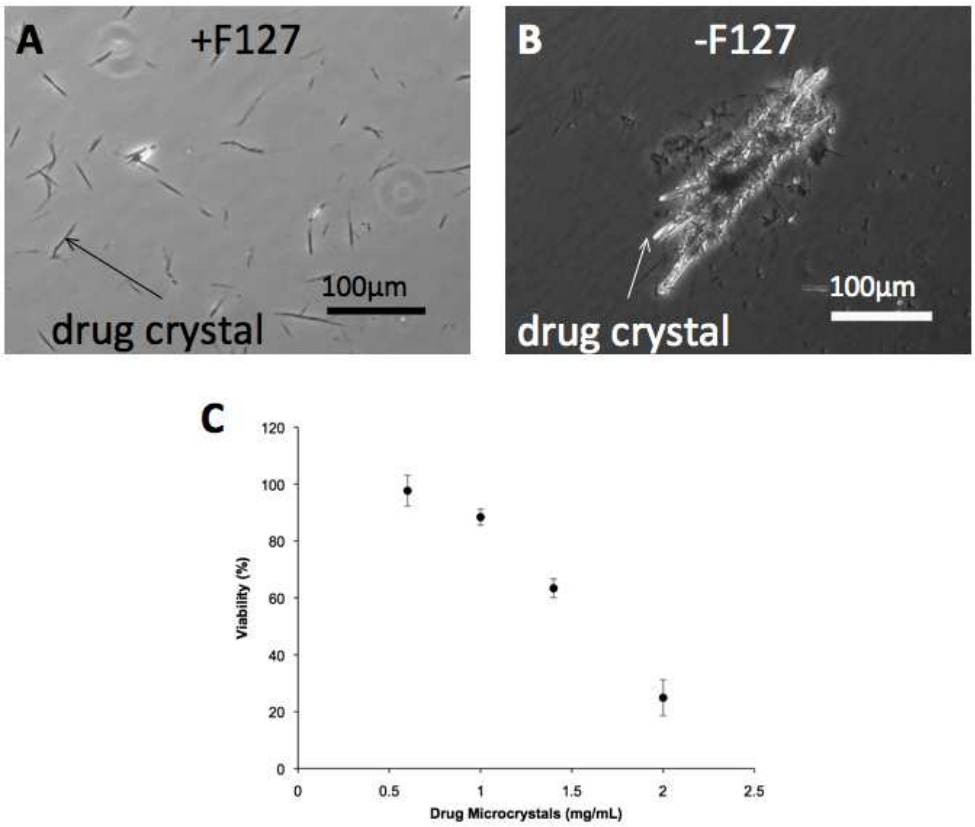
Ear tissue samples (3 ear hole donuts/ear from 3 separate mice) were homogenized in radio-immunoprecipitation assay buffer (50mM Tris-HCl pH 7.6, containing 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA and 0.1% SDS) with 1 mM PMSF and a protease inhibitor cocktail (Sigma). Samples with equal amounts of protein (about 40µg) were loaded into a NuPAGE 4-12%Bis–Tris gradient gel or 8% Bis–Tris gel (Life Technologies, Grand Island, NY), electrophoresed and then electro-transferred onto a PVDF-FL membrane (Immobilon, Billerica, MA). The membrane was subsequently blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE), probed with primary antibodies (HIF-1α (10006421, Cayman Chemical, Ann Arbor, MI), HIF-2α (NB100-132B, Novus, Littleton, CO), Wnt5a (BAF645, R&D System) or α-Tubulin (Sigma) overnight at 4℃, then further inc ubated with Alexa Fluor-labeled secondary antibodies (IRDye 800CW goat-anti rat or IRDye 800CW goat-anti rabbit (LI-COR, Lincoln, NE) for 1 hr and scanned using the Odyssey system (LI-COR, Lincoln, NE).

RNA isolation and Quantitative qPCR.

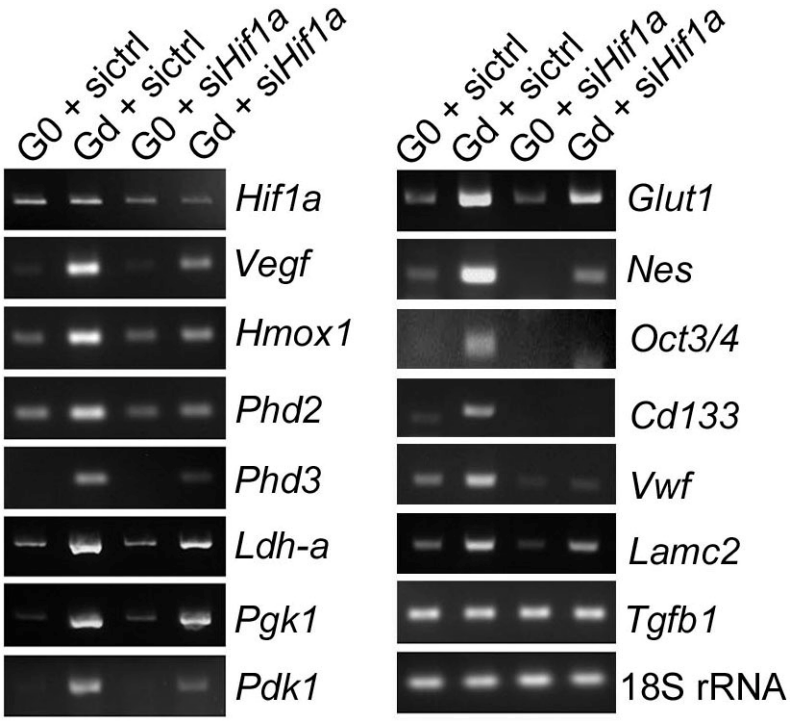
Total RNA from ear fibroblast-like cells or ear hole donuts was prepared with Qiagen RNeasy kit (Qiagen) according to the manufacturer's guidelines. First strand cDNA was synthesized from 1 µg of RNA using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. qPCR was performed with SYBR green PCR Master Mix (Applied Biosystems, Life Technologies). In brief, a 20 µl mixture was used containing 10 µl SYBR Green PCR master mix, 1 µl forward and reverse primer, 6 µl sterile water, and 2 µl of complementary DNA template. A negative control (non-template control) was performed in each run. The real-time PCR was performed using a Quant Studio 6 Flex (Applied Biosystems) according to the manufacturer's instructions. All data were normalized to 18S rRNA and quantitative measurements were obtained using the $\Delta\Delta C_T$ method. All primers used are listed with their sequences in **Supplemental Table S1**.



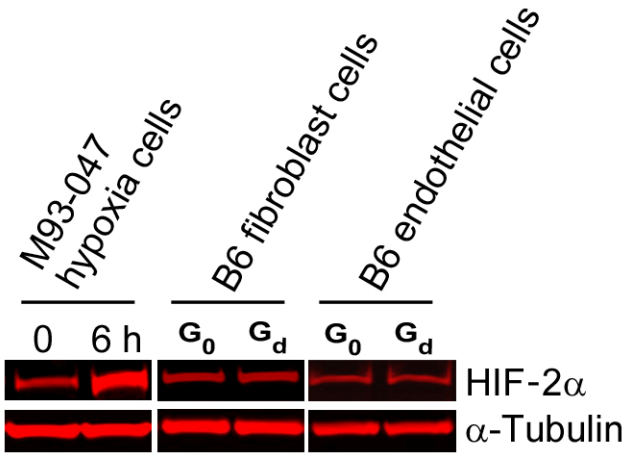
Supplemental Figure S1. Knockdown of *Hif1a* in B6 and Swiss Webster mouse ear fibroblast cells. Four siRNA's specific for *Hif1a* (Qiagen) (si-1, SI00193011; si-2, SI00193018; si-3, SI00193025 and si-4,SI00193032) were tested for blocking *Hif1a* mRNA in vitro on C57BL/6 (B6) or Swiss Webster (SW) ear fibroblast cells. si*Hif1a*_3 was the only effective inhibitor in all three strains tested including MRL (see **Fig. 1G**) with *Gapdh* as a control for all strains.



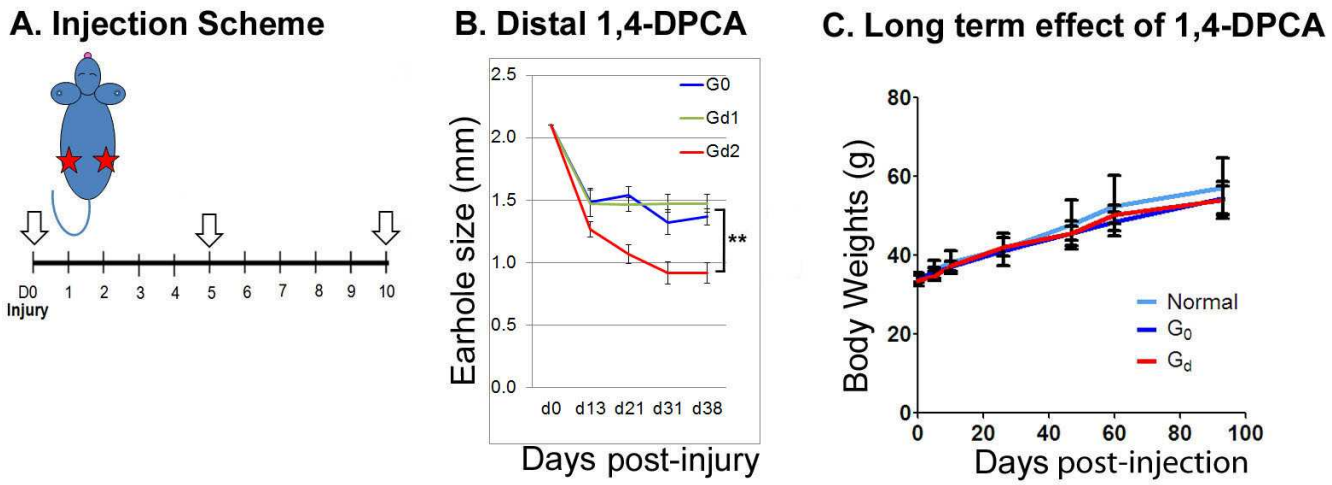
Supplemental Figure S2. Effects of Pluronic F127 on microcrystal growth and stability. (A) Microcrystals formed in the presence of Pluronic were micron sized while in the absence of Pluronic (B) the crystals aggregated and were significantly larger. (C) In the absence of a hydrogel barrier, the drug microcrystals become cytotoxic at concentrations above 1 mg/mL. The direct contact between cells and drug crystal leads to high local drug concentrations that are cytotoxic. The hydrogel barrier serves to disperse the drug crystals and slow down the drug release rate both of which contribute to the lack of cytotoxicity.



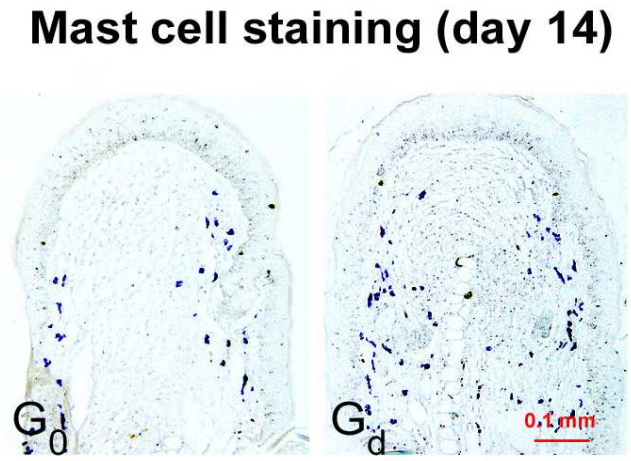
Supplemental Figure S3. HIF-1α target genes and dedifferentiation marker genes up-regulated by 1,4-DPCA/ gel were blocked by knockdown of *Hif1a*. B6 ear fibroblast-like cells were treated with either siRNA control or si*Hif1a* for 48 hours. The transfected cells were cultured with hydrogel but no drug (G_0) and with 2 mg 1,4-DPCA crystal/gel construct (G_d) for another 24 hrs. RT-PCR analysis of mRNA from treated B6 cells revealed that multiple genes, including HIF-1α target genes (*Vegf, Hmox1, Ldh-a, Pgk1, Pdk1* and *Glut1) and de-differentiation marker genes (Nes,Oct3/4, Cd133, Vwf and Lamc2)*, were specifically increased by 1,4-DPCA, but blocked by *Hif1a* interference with si*Hif1a*_3.



Supplemental Figure S4: HIF-2α is not affected by the 1,4-DPCA drug/gel construct in vitro. HIF-2α levels were examined in different cell types including a melanoma cell line M93-047 under hypoxic conditions, and B6 fibroblasts and endothelial cells +/- 1,4-DPCA drug/gel under normoxic conditions. Only hypoxic conditions showed increased levels of HIF-2α, not 1,4-DPCA treatment.

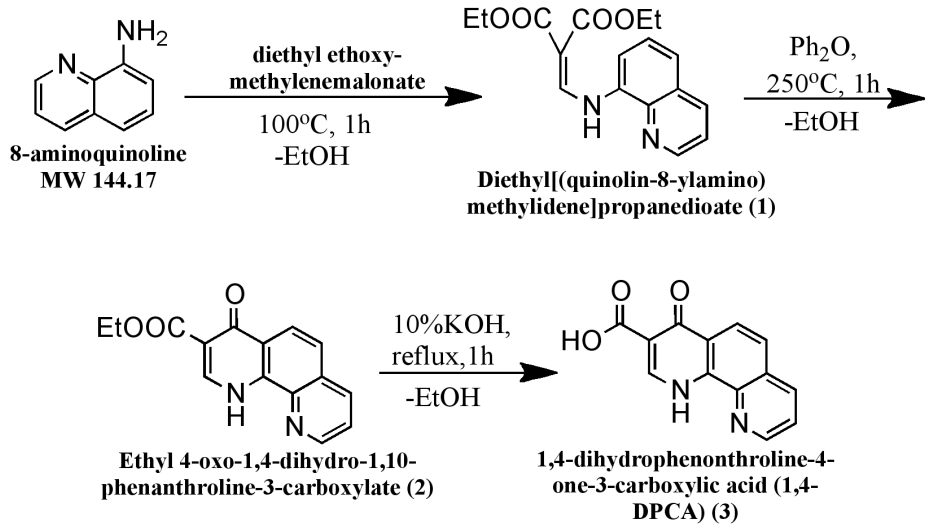


Supplemental Figure S5. Distal and long-term effects and survival of drug/gel-treated Swiss Webster mice. Mice were injected subcutaneously on days 0, 5 and 10 into the right and left flanks (A) using G_0 and two different doses of drug/gel (G_{d1}, 1 mg/ml drug/gel; G_{d2}, 2 mg/ml drug/gel). Ear hole closure was achieved at 2 mg/ml but not 1 mg/ml (B) and results (G_{d2} vs G_{d1}, and G_{d2} vs G_0) were highly significant (**) on days 21, 31, and 38. Normal mice were ear-punched and used as control with results similar to G_0 and G_{d1} and which are presented in Table S8. For long-term effects of the drug/gel, Swiss Webster mice were injected subcutaneously (s.c.) 3 times with G_0 or G_d at the base of the neck. Body weight was measured for 91 days and skin was superficially examined (C). There was no difference between these 3 groups, indicating that the drug/gel has no obvious side-effects in-vivo. ANOVA analysis for experiments in B and C are presented in Table S8.



Supplemental Figure S6. Inflammatory responses are affected by 1,4-DPCA treatment. Ear tissue from G_0 and G_d-treated mice stained with toluidene blue to detect mast cells shows a significant increase in mast cell numbers after Gd treatment. Scale bar=0.1mm. A similar increase in mast cells is seen in MRL mice (17). Also, as seen in Fig 6Cd,e, MPO, a marker of inflammation, and Ly6G, a neutrophil-specific marker, are up-regulated in the non-regenerative Swiss Webster given 1,4-DPCA similar to the untreated MRL mouse (17).

Down-regulation of HIF-1α inhibits inflammation. It has been shown using a *Hif1a* conditional knockout mouse that HIF-1α is required and controls the inflammatory response through regulation of glycolysis, a state necessary for myeloid (including neutrophils and macrophages) survival and function with effects specifically on aggregation, invasion, motility, and cutaneous inflammation (80). NSAIDs such as the COX2 inhibitors indomethacin, meloxicam (81) and ibuprofen (82), which negatively regulate inflammation, also inhibit HIF-1α (81,37) through the up-regulation of pVHL expression (17). Down-regulation of HIF-1α in a *Hif* KO mouse has been shown to heal burn wounds poorly with a concomitant reduction in angiogenesis and SDF1 (38).



Supplemental Figure S7. Synthesis of 1,4-DPCA. Synthesis was accomplished in three steps (see main text for a detailed description). The synthetic scheme and the chemical structures of the three main products (1-3) are shown here.

Supplemental Table S1. Primer sequences used for RT-PCR.

Gene	Forward 5'--3'	Reverse 5'--3'
Vegfa	GAGACCCTGGTGGACATCTTC	ATTTACACGTCTGCGGATCTTG
Hmox1	TCCAGACACCGCTCCTCCAG	GGATTTGGGGCTGCTGGTTTC
Phd2	CTCGTGGACAGGATGGACAG	GCAGCCAAGAGCAGTCACAG
Phd3	CAATGGTGATGGCCGCTGTA	TGTGGATTCCTGCGGTCTGA
Ldh-a	CGTCTCCCTGAAGTCTCTTAACC	CCCACACCATCTCAACACC
Pgk1	CAAACAACCAAAGGATCAAGG	CCCAAGATAGCCAGGAAGG
Pdk1	CGGATCAGAAACCGACACA	ACTGAACATTCTGGCTGGTGA
Glut1	AGAGGTGTCACCTACAGCTC	AACAGGATACACTGTAGCAG
Hif1a	TGCTCATCAGTTGCCACTTC	TGGGCCATTTCTGTGTGTAA
Tgfb1	ATACGCCTGAGTGGCTGTCT	TTCTCTGTGGAGCTGAAGCA
Nes	CCCTGAAGTCGAGGAGCTG	CTGCTGCACCTCTAAGCGA
Oct3/4	CGTGGAGACTTTGCAGCCTG	GCTTGGCAAACTGTTCTAGCTCCT
Cd133	CCTTGTGGTTCTTACGTTTGTTG	CGTTGACGACATTCTCAAGCTG
Vwf	CTTCTGTACGCCTCAGCTATG	GCCGTTGTAATTCCCACACAAG
Nanog	GAACTATTCTTGCTTACAAGGGTCTGC	GCATCTTCTGCTTCCTGGCAA
Pref1	CTGGCGGTCAATATCATCTTCC	GAGGAAGGGGTTCTTAGATAGCG
Cd34	GTTACCTCTGGGATCCCTTCAGGCTC	CTCCAGAGGTGACCAATGCAATAAG
Nefh	AGCCTGCACTACTCGCTGA	GGCCGTTGCTTAGGGTGTC
Pax7	CTCCCGTCAGCTCCGTGTTTCTCA	ATGTCCGGGTAGTGGGTCCTCTCG
Loxl4	AGCGGACAGACCAGAGGAG	CCTTGACCATACTTGGCACTG
Ctgf	GCCCTAGCTGCCTACCGACT	CATAGTTGGGTCTGGGCCAA
Mgp	GGCAACCCTGTGCTACGAAT	CCTGGACTCTCTTTTGGGCTTTA
Itm2a	AGGAGAGCCATACTTTCTGCC	GCCGGATCGCTATCAGAGA
Matn3	TCTCCCGCATCATCGACACT	GTCGGAATAGGTGTTGAGCTG
Mia2	GTGTCTGGAGGGTACAAAGTTG	TCGGGTCCTGTGTAATCTCTC
Col11a1	GACACACTGAAAGCATCCAG	GGCAGCAGTGATTCCTAAAC
Prg4	GAAAATACTTCCCGTCTGCTTGT	ACTCCATGTAGTGCTGACAGTTA
Fmod	CTCCAACCCAAGGAGACCAG	GGATCCACCAGTGAGAGTCTTC
S100a4	CGGCCAGAAAAGGACAGATG	TTGTCCCTGTTGCTGTCCAA
Wif1	CCACCTGAGGAGAGCTTGTACC	TGGCATTCTTTGTTGGGCTTTCC
Dkk3	TCAGGAGGAAGCTACGCTCAATG	TCTCCGTGCTGGTCTCATTGTG
Dcn	CACCCGACACAACCTTGCTAG	GCCTTTCCAACTTCACGAGAGG
Tgfb2	TGGTTCCTCTGTATTGCTCTCTGC	TCCCCCTGGCTTATTTGAGTTC
Lamc2	AGGCGCGGCAAGATATAGAG	GTCCTGCCAAGATGAATGAGAG
Gapdh	CGCAACGACCCCTTCATTGACC	CGATGAGCCCTTCCACAATGCC
18S rRNA	CGGCGACGACCCATTCGAAC	GAATCGAACCCTGATTCCCCGTC

Supplemental Table S2. Antibodies used for Immunostaining.

	1st antibody			2nd antibody			
	Company	**Cat. no.**	**Dilution**	**All From Molecular Probe**	**Company**	**Cat. no.**	**Dilution**
HIF-1α	Abcam	ab2185	1:1000	Alexa Fluor 488 goat anti-rabbit IgG	Molecular Probe	A11008	1:200
Nanog	Calbiochem	SC1000	1:150	Alexa Fluor 568 goat anti-rabbit IgG	Molecular Probe	A11036	1:400
Oct-3/4	Santa Cruz	sc-5279	1:150	Alexa Fluor 568 rabbit anti-mouse IgG	Molecular Probe	A11061	1:400
CD133	Chemicon	MAB4310	1:100	Alexa Fluor 594 goat anti-rat IgG	Molecular Probe	A11007	1:200
CD34	Bioss	bs-0646R	1:200	Alexa Fluor 568 goat anti-rabbit IgG	Molecular Probe	A11036	1:300
Wnt5a	R&D Systems	BAF645	1:150	Alexa Fluor 568 donkey anti-goat IgG	Molecular Probe	A11057	1:200
PAX7	R&D Systems	MAB1675	1:50	Alexa Fluor 568 rabbit anti-mouse IgG	Molecular Probe	A11061	1:400
Pref-1	MBL International	D187-3	1:10	Alexa Fluor 594 goat anti-rat IgG	Molecular Probe	A11007	1:200
Nestin	DHSB		1:50	Alexa Fluor 594 goat anti-mouse IgG	Molecular Probe	A11005	1:200
vWF	Dako	A0082	1:100	Alexa Fluor 488 goat anti-rabbit IgG	Molecular Probe	A11008	1:200
Nefh	Sigma	N0142	1:200	Alexa Fluor 568 rabbit anti-mouse IgG	Molecular Probe	A11061	1:400
Lamc2	Sigma	L-9393	1:50	Alexa Fluor 568 goat anti-rabbit IgG	Molecular Probe	A11036	1:1000
MPO	NeoMarkers	RB-373A1	1:70	Alexa Fluor 488 goat anti-rabbit IgG	Molecular Probe	A11008	1:200
Anti-Neutrophil mAb	Cedarlane	CL8993F	1:40	FITC mouse anti-rat IgG	Jackson ImmunoResearch lab	212096082	1:100
Keratin14	Sigma	C8791	1:200	Alexa Fluor 488 goat anti-mouse IgG	Molecular Probe	A10680	1:100
MMP9	Sigma	M9555	1:200	Alexa Fluor 568 goat anti-rabbit IgG	Molecular Probe	A11036	1:200

Figure 1D. HIF-1α Immunohistochemistry Quantitation

Group/day	day0	day3	day7	day10	day14	day15
MRL-derived	0.9	47.91	261.6	186.1	16	6.2
	17.6	40.06	619.8	191.7	60.1	9.5
	4.2	56.67	422.2	148.1	22.4	6.9
	2.6	31.06	344.4	425.3	108.6	14.7
				345.5		6.1
						41
						67.9
mean	**6.325**	**43.925**	**412.000**	**259.340**	**51.775**	**21.757**
st dev	**7.636**	**10.936**	**153.270**	**119.666**	**42.588**	**23.831**
st error	**4.409**	**6.314**	**88.490**	**59.833**	**24.588**	**9.729**
Group/day	**day0**	**day3**	**day7**	**day10**	**day14**	**day15**
B6-derived	3.3	15	111.7	2.9	0.35	2.6
	3.3	14.45	202.3	7.6	1.48	2.7
	3.7	7.23	172.4	4.8	1.25	1.2
		6.59	185.4	3.4	1.15	4.3
						2.5
						5.5
mean	**3.433**	**10.818**	**167.950**	**4.675**	**1.058**	**3.133**
st dev	**0.231**	**4.525**	**39.447**	**2.109**	**0.491**	**1.521**
st error	**0.163**	**2.613**	**22.775**	**1.218**	**0.284**	**0.680**
p value*	0.55042	0.00139	0.02155	0.00405	0.05465	0.08413

* Based on T test to compare means between group measurements at the same day

Figure 1Ha. Si*Hif1a* in MRL backcross Mice

Group/day	da7	da14	da21	da28
Si*Hif1a*	2.5	1.8	1.4	1.4
	2.1	1.9	1.6	1.3
	2.2	2	2.4	1.7
	2.3	3.2	1.9	2.3
	2.8	3	1.5	1.1
	2.6	2	2.6	1
	2.3	1.9	1.4	1.7
	2.1	2	1.5	2.5
mean	**2.363**	**2.225**	**1.788**	**1.625**
st dev	**0.250**	**0.547**	**0.470**	**0.542**
st error	**0.095**	**0.207**	**0.178**	**0.205**
Group/day	**da7**	**da14**	**da21**	**da28**
PBS	2	1.4	1	0.9
	2.1	1.2	0.8	0.8
	2	1.2	0.7	0.3
	2	1.3	0.7	0.4
	2.1	1.4	0.9	0.4
	2	1.5	0.7	0.6
	2.1	1.3	1	0.7
	2	1.5	0.9	0.5
mean	**2.038**	**1.350**	**0.838**	**0.575**
st dev	**0.052**	**0.120**	**0.130**	**0.212**
st error	**0.020**	**0.045**	**0.049**	**0.080**
p value*	0.00292	0.00058	0.00008	0.00016

Figure 1Hc. HIF-1α Bioluminescence with PBS or SI*Hif* treatment

Treatment	Whole body		Ears
PBS	3.99E+07		1.51E+07
	3.68E+07		1.28E+07
			1.11E+07
			1.10E+07
mean	**3.84E+07**		**1.251E+07**
st dev			**1.912E+06**
st error			**1.104E+06**
Treatment	**Whole body**		**Ears**
si*Hif1a*	2.13E+07		6.37E+06
	2.66E+07		1.06E+07
			6.68E+06
			1.02E+07
mean	**2.40E+07**		**8.467E+06**
st dev			**2.253E+06**
st error			**1.301E+06**
p value*			0.03400

Supplemental Table S3: Raw data and statistical significance testing for Fig. 1.

In Figure 1D, MRL and B6 ear tissue stained with anti-HIF-1α + fluorescent 2^{o} antibodies was analyzed by converting the images to gray scale and measuring the raw integrated density (sum of all intensities from areas of tissue measured) divided by the area of tissue (μm^2) using ImageJ software and finally expressed as fluorescence Integrated density/μm^2 (# ears, n=3-7). **In Figure 1Ha**, ear holes were read on days 7, 14, 21, and 28 from mice treated with PBS or *siHif1a*. Ear holes (n=4 for each group) with read with a reticle and each hole was read twice, first at a longitudinal axis and then a perpendicular axis for any irregular-shaped healing. **In Figure 1Hc,** HIF-luc mice were injected with luciferin on day 28 after ear hole injury and bioluminescence in the ear (n=4) and whole body (n=2) for each group determined using the IVIS 200. Data is expressed as Intensity (p/sec/cm^2/sr) as determined by Living Image (IVIS bounded software) direct data analysis.

Figure 3G. HIF-1α Immunohistochemistry Quantitation

Group/day	day1	day2	day3	day4	day5
Go-treated	13.1868	9.7902	54.612	22.311	21.5
	2.5308	26.0406	22.4442	101.6316	8.658
mean	**7.8588**	**17.9154**	**38.5281**	**61.9713**	**15.079**
Group/day	**day1**	**day2**	**day3**	**day4**	**day5**
Gd-treated	152.2476	232.9002	291.5748	303.363	80.784
	210.3228	188.6112	208.1916	323.0766	44.955
mean	**181.2852**	**210.7557**	**249.8832**	**313.2198**	**62.8695**

Supplemental Table S4: Raw data and statistical significance testing for Fig. 3G.
Ear tissue from G_o and G_d-treated Swiss Webster mice days 1-5 post-injury (n=2 for each group) was treated as described for **Fig 1D**.

Figure 4B: G0 and Gd-Treated Swiss Webster Mice

Group/day	G0-da14	G0-da21	G0-da28	G0-da35
G0-Treated:	1.7	1.8	1.8	1.7
	1.6	1.8	1.7	1.6
	1.6	1.4	1.4	1.5
	1.7	1.3	1.5	1.6
	1.8	1.5	1.4	1.5
	1.9	1.6	1.4	1.5
	1.6	1.5	1	1.2
	1.4	1.4	0.9	1
	1.8	1.5	1.3	1.4
	2	1.4	1.4	1.4
	2.1	1.3	1.2	0.9
	1.8	1.5	1.3	0.9
	1.5	1.7	1.8	1.9
	1.7	1.8	1.9	1.8
	1.4	1.9	1.2	1.3
	1.8	1.8	1.3	1.2
	1.6	1.4	1.6	1.7
	1.4	1.3	1.8	1.7
	1.4	1.5	1.9	1.7
	1.5	1.5	1.6	1.6
mean	1.665	1.545	1.470	1.455
st dev	0.203	0.190	0.289	0.291
st error	0.047	0.044	0.066	0.067

Group/day	Gd-da14	Gd-da21	Gd-da28	Gd-da35
Gd-Treated:	1	0.8	0.7	0.4
	1.3	0.9	0.8	0.3
	0.7	1	0.2	0.2
	0.8	0.8	0.1	0.1
	1.3	1.3	1.3	1.3
	1.4	1.4	1.2	1.2
	0.9	1.2	1.5	1.3
	1.2	1.5	1.1	0.95
	1.5	1	1.3	1.3
	1.1	0.8	1.2	1.2
	0.8	0.4	0	0
	0.9	0.5	0	0
	1.3	0.2	0.7	0.6
	1	0.5	0.6	0.6
	1.4	1.5	0.5	0
	1.3	1.3	0.5	0
	1.5	1	0.9	0.6
	1.5	0.9	0.8	0.5
	1.5	0.5	0	0
	1.8	0.3	0	0
mean	1.210	0.890	0.670	0.528
st dev	0.297	0.399	0.499	0.509
st error	0.068	0.091	0.114	0.117
p value	1.71E-06	7.79E-08	2.92E-07	1.93E-08

Figure 4E: G0, Gd, and Gd/si*Hif1* a-treated Swiss Webster Mice

	day14			day21		
Group/day	Gd	Gd/si*Hif1a*	G0	Gd	Gd/si*Hif1a*	G0
	1.1	1.8	1.3	1	2	1.2
	0.9	1.7	1.7	1	1.7	1.7
	1.1	2	1.7	1.3	1.2	1.7
	1	1.9	1.5	1.2	1.5	1.5
	1.2	1.4	1.1	1	1.8	1.1
	1.3	1.5	1	1	1.6	0.8
	1.5	1.7	1.8	1	1.5	1.5
	1.1	1.7	1.5	1.1	1.4	1.3
	1.2	1.7	1.3	0.6		1.2
	1.3	1.5	1.5	0.7		1
	1.3			1		
	0.9			0.9		
	1.4					
	1.4					
mean	1.193	1.690	1.440	0.983	1.588	1.300
st dev	0.186	0.185	0.263	0.190	0.247	0.298
st error	0.052	0.062	0.088	0.057	0.094	0.099
Anova p value*	1.46E-05			4.44E-05		
Tukey p value**	Gd vs G0	0.02163		0.01484		
	si*Hif1a* vs G0	0.03292		0.051		
	si*Hif1a* vs Gd	8.80E-06		3.07E-05		

* Anova test to compare the mean among the three groups at the corresonding days

** Post-hoc Tukey test to compare between group mean if Anova test is significant

Supplemental Table S5: Raw data and statistical significance testing for Fig. 4.

In Figure 4B, Swiss Webster mice were treated with G_0 (gel, no drug) or G_d (gel +2mg/ml drug) and ear hole diameters read on days 14, 21, 28, and 35. Ear holes were read twice, first at a longitudinal axis and then a perpendicular axis for any irregular-shaped healing. **In Figure 4E**, Swiss Webster mice were treated with G_0, G_d, or G_d + si*Hif1a* and ear hole diameter were read on days 14 and 21. The ANOVA test was performed to indicate if there were a significant difference among the means of the three groups. If the p-value from ANOVA is significant, then the post-hoc Tukey test is applied to compare the mean between each group. P-values less than 0.05 are considered as significant. The day 14 p-value from ANOVA is highly significant (much less than 0.001), which means there is significant difference among the means of the three groups. All p-values from Tukey host-hoc test are significant: the difference of means between group G_d and G_0 is significant (p=0.0216), between group Si*Hif1a* and G_0 is significant (0.0329), and between group Si*Hif1a* and G_d is highly significant (much less than 0.001). The day 21 p-value from ANOVA is highly significant (much less than 0.001), which means there is significant difference among the means of the three groups. Tukey host-hoc test: the difference of means between group G_d and G_0 is significant (p=0.0148), between group Si*Hif1a* and G_0 is not significant (p=0.051), and between group Si*Hif1a* and G_d is highly significant (much less than 0.001).

Figure 5B. Relative gene expression

Group/Gene	G0			Gd			Gd vs G0
	mean	SEM	N	mean	SEM	N	p value
Nanog	1	0.02	3	13.00	1.12	3	0.0004
Oct3/4	1	0.15	3	5.94	0.96	3	0.0071
CD133	1	0.24	3	5.65	1.01	3	0.011
Pref-1	1	0.22	3	9.50	1.89	3	0.0016
Nestin	1	0.12	3	14.63	1.66	3	0.0012
Pax7	1	0.21	3	14.03	1.21	3	0.0004
Vwf	1	0.16	3	18.39	3.78	3	0.0101
CD34	1	0.07	3	15.27	1.72	3	0.0012

Supplemental Table S6: Raw data and statistical significance testing for Fig. 5.
In **Fig. 5B**, relative gene expression is provided. QPCR was carried out using pooled tissue from three ear donuts/sample and each experiment repeated three times. P values are shown comparing amplification in tissue from G_0-treated and G_d-treated Swiss Webster mice.

Figure 6Bg. Relative gene expression

Group/Gene	G0			Gd			Gd vs G0
	mean	SEM	N	mean	SEM	N	p value
Nestin	1	0.13	3	16.11	4.17	3	0.0223
Oct3/4	1	0.3	3	12.96	1.49	3	0.0014
Nf	1	0.16	3	12.55	1.95	3	0.0041
Pax7	1	0.3	3	5.35	0.55	3	0.0023

Figure 6Dd. Collagen Crosslinking

Group	G0	Gd	Gd+Si*Hif1 a*
	171	87	181
	156	93	153
mean	**163**	**90**	**167**

Figure 6De. Relative gene expression

Group/Gene	G0			Gd			Gd vs G0
	mean	SEM	N	mean	SEM	N	p value
Loxl4 (day2)	1	0.14	3	0.02	0.01	3	0.0022
Loxl4 (day3)	1	0.2	3	0.03	0.01	3	0.0084
Ctgf (day2)	1	0.1	3	0.36	0.10	3	0.0024
Ctgf (day3)	1	0.2	3	0.04	0.01	3	0.0087

Figure 6Eb. Soft tissue diameter (day 35)

Group	G0	Gd
	1.7	0.4
	1.6	0.3
	1.5	0.2
	1.6	0.1
	1.5	1.3
	1.5	1.2
	1.2	1.3
	1	0.95
	1.4	1.3
	1.4	1.2
	0.9	0
	0.9	0
	1.9	0.6
	1.8	0.6
	1.3	0
	1.2	0
	1.7	0.6
	1.7	0.5
	1.7	0
	1.6	0
mean	**1.455**	**0.5275**
st dev	**0.2911**	**0.5087**
st error	**0.0668**	**0.1167**
p value	1.9E-08	

Figure 6Ed. Relative gene expression

Group/Gene	G0			Gd			Gd vs G0
	mean	SEM	N	mean	SEM	N	p value
Mgp	1	0.13	3	2.57	0.41	3	0.0218
itm2a	1	0.09	3	19.21	1.04	3	0.00003
matn3	1	0.25	3	7.71	0.39	3	0.0001
Mia2	1	0.15	3	14.02	1.78	3	0.0019
Col11a1	1	0.27	3	8.76	2.7	3	0.0459
Prg4	1	0.02	3	3	0.1	3	0.00007
Fmod	1	0.02	3	2.06	0.31	3	0.027
Chad	1	0.27	3	40.45	1.44	3	0.00001

6Ee. Cartilage diameter (day 35)

Group	G0*	Gd
	2.32	1.76
	2.4	1.68
	2.16	
mean	**2.293**	**1.720**
st dev	**0.122**	
st error	**0.08641**	

Figure 6Ef. Cartilage area (day 35)

Group	G0	Gd
	0.493	2.98
	0	1.705
	0	4.615
	0	0.822
	0	
	0	
mean	**0.082**	**2.531**
st dev	**0.201**	**1.648**
st error	**0.090**	**0.951**
p value		0.005934

Figure 6Ei. Relative gene expression

Group/Gene	G0			Gd			Gd vs G0
	mean	SEM	N	mean	SEM	N	p value
S100a4	1	0.06	3	2.77	0.14	3	0.0003
Wif1	1	0.15	3	4.20	0.25	3	0.0004
Dkk3	1	0.1	3	3.96	0.38	3	0.0017
DCN	1	0.1	3	2.08	0.10	3	0.0016
Tgfb2	1	0.2	3	6.97	0.50	3	0.0004

6Ej. Number of hair follicles (day 35)

Group	G0*	Gd	Normal
	2	7	4
	1	4	4
	0	4	4
	0	3	5
	0	3	5
	0	4	4
	0	2	4
	0		4
mean	**0.375**	**3.857**	**4.250**
st dev	**0.744**	**1.574**	**0.463**
st error	**0.281**	**0.642**	**0.175**
Anova p valu	2.67E-07		
Tukey p valu	G0 vs Gd	4.80E-06	
	G0 vs Nor	6.00E-07	
	Gd vs Nor	0.7343	

* Anova test to compare the mean among the three groups at the corresonding days

** Post-hoc Tukey test to compare between group mean if Anova test is significant

Supplemental Table S7: Raw data and statistical significance testing for Fig. 6.

In Fig.6Dd, data was determined from analysis of ear sections stained with picrosirius red (PSR) from treated mice and examined under polarized light. Level of tissue brightness after PSR staining is directly proportional to degree of

collagen crosslinking. Using ImageJ software, brightness of tissue after gray scale conversion was measured by calculating the raw integrated density related to area of analysed tissue and expressed as Fluorescence Integrated Density per μm^2. **Fig.6Eb** is derived from **Fig.4B**, day 35 and ear hole diameter was determined using mm diameter reads of ear holes using a reticle (n=10 ear sections, 2 reads each). **In Fig.6Ee**, Alcian blue-stained ear sections were examined at 4X so the whole ear punched region could be visualized. The distance between the ends of the cartilage was determined in mm and linear cartilage elongation was assessed by measuring the distance between Alcian blue-positive ends of the cartilage. The smaller the number, the more cartilage had developed. **In Fig.6Ef**, day 35 ear sections were stained with Alcian blue to detect cartilage. The area of newly developed cartilage was measured by determining the area of Alcian blue-positive tissue inwardly from the site of initial puncture. Measurement was done by ImageJ software to determine blue density. **In 6Ej**, day 35 ear sections were immunostained with anti-keratin antibody. The number of hair follicles was then counted in 2 non-consecutive sections per ear (n=4) in a 1 mm section from the center of hole punch, the area that was originally removed. **In Fig.6Bg, 6De, 6Ed,** and **6 Ei,** relative gene expression is shown. QPCR was carried out using pooled tissue from 3 ear donuts and each experiment was repeated 3 times. P values from T-test analysis are provided.

Figure S5B. Distal 1,4-DPCA Injection

	Normal				G0-treated				Gd1-treated				Gd2-treated			
Group/day	da13	da21	da31	da38	da13	da21	da31	da38	da13	da21	da31	da38	da13	da21	da31	da38
	1.7	1.6	1.8	1.7	1.2	1.35	1.1	0.9	1.2	1.65	1.1	1.5	1.1	0.85	1	1.2
			1.4	1.5			0.7	1.1			1.6	1.8			1.2	1.1
	1.2	1.4	1.1	1.2	1.5	1.4	1.1	1.4	1.3	1.4	1.2	1.8	1.2	1.25	0.7	0.7
			1.4	1.3			0.9	1.1			1.7	1.5			0.7	0.8
					1.7	1.7	1.5	1.5	1.2	1.45	1.9	1.5	1.2	1.3	1.3	1.3
							1.4	1.3			1.4	1.5			1.3	1.4
					1.9	1.8	1.6	1.6	1.7	1.35	1.2	1.5	1.2	1.3	1	1.2
							1.2	1.5			1.8	1.5			1.3	1
					1.3	1.5	1.8	1.5	1.3	1.5	1.6	1.6	1.4	0.7	0.7	0.6
							1.4	1.5			1.3	1.6			0.8	0.6
					1.3	1.5	1.8	1.7	1.7	1.45	1.5	1.5	1.5	0.7	0.5	0.8
							1.4	1.5			1.4	1.5			0.5	0.8
mean	1.450	1.500	1.425	1.425	1.483	1.542	1.325	1.383	1.400	1.467	1.475	1.567	1.267	1.017	0.917	0.958
st dev	0.354	0.141	0.287	0.222	0.271	0.174	0.339	0.237	0.237	0.103	0.253	0.115	0.151	0.298	0.307	0.278
st error	0.354	0.141	0.166	0.128	0.121	0.078	0.102	0.071	0.106	0.046	0.076	0.035	0.067	0.133	0.093	0.084

Group/day	da13	da21	da31	da38
Anova p value*	0.458	0.0017	3.7E-04	7.4E-07
Tukey p value**				
G0 vs Gd1		0.9190	0.6166	0.1957
G0 vs Gd2		0.0021	0.0104	0.0002
Gd1 vs Gd2		0.0075	0.0003	4.0E-07

* Anova test to compare the mean among the three groups at the corresonding days

** Post-hoc Tukey test to compare between group mean if Anova test is significant

Figure S5C. Long term Effects of 1,4-DPCA

Normal							
	Da0	Da5	Da10	Da26	Da47	Da60	Da93
	35	39	42	46	54	57	58
	34	36	38	42	49	56	63
	32	37	38	42	49	56	61
	33	33	35	36	39	41	46
mean	33.500	36.250	38.250	41.500	47.750	52.500	57.000
st dev	1.291	2.500	2.872	4.123	6.292	7.681	7.616
st error	0.745	1.443	1.658	2.380	3.632	4.435	4.397
G0-Treated Swiss Webster Mice							
	Da0	Da5	Da10	Da26	Da47	Da60	Da93
	34	34	36	41	47	47	59
	34	36	37	41	47	50	54
	36	37	39	41	44	46	49
	33	35	36	41	44	51	56
mean	34.250	35.500	37.000	41.000	45.500	48.500	54.500
st dev	1.258	1.291	1.414	0.000	1.732	2.380	4.203
st error	0.726	0.745	0.816	0.000	1.000	1.374	2.427
Gd-Treated Swiss Webster Mice							
	Da0	Da5	Da10	Da26	Da47	Da60	Da93
	33	35	37	44	47	52	56
	35	36	39	44	48	52	54
	34	35	36	40	49	50	57
	32	33	37	40	41	47	49
mean	33.500	34.750	37.250	42.000	46.250	50.250	54.000
st dev	1.291	1.258	1.258	2.309	3.594	2.363	3.559
st error	0.745	0.726	0.726	1.333	2.075	1.364	2.055

Anova p value*	0.6467	0.517	0.655	0.876	0.7594	0.5277	0.7132

* Anova test to compare the mean among the three groups at the corresonding days

No need to run the post-hoc test since none of the Anova test results is significant.

Supplemental Table S8: Raw data and statistical significance testing for fig. S5. Fig.S5B shows the effect of distal injection of 1,4-DPCA in hydrogel. Two ears from normal mice were analyzed and data is shown as the values from each ear. Ears from three groups of treated mice (n=6) were measured for changes in ear hole diameter over 38 days. ANOVA analysis shows highly significant differences between G_0 vs G_{d2} and G_{d1} vs G_{d2} and no difference between G_0 and G_{d1}. Figure S5C shows the long-term effects of 1,4-DPCA in hydrogel (n=4, N=2). ANOVA analysis shows no significant differences between groups.