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Supporting Information

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Protein-engineered injectable hydrogel to improve retention of transplanted adipose-derived stem cells

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Supporting Experimental Section

Recombinant synthesis, purification, and sterilization of engineered proteins: C7 and P9 were cloned, synthesized, and purified as previously reported.[13] Briefly, DNA sequences of C7 and P9 proteins were cloned into the pET15b (Novagen) and pJExpress 414 (DNA 2.0) vectors, respectively, and transformed into the BL21(DE3)pLysS *Escherichia coli* expression strain (Invitrogen). Recombinant proteins were expressed following induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified via binding of terminal polyhistidine tags to Ni-nitrilotriacetate resin (Qiagen). Protein identity and purity were confirmed by gel electrophoresis and Western blot. Purified C7 was buffer exchanged, sterile-filtered with a 0.22 μm filter, and concentrated in sterile saline by centrifugation across 10-kDa Amicon Ultracel-10K filter units (Millipore). P9 was dialyzed into water, lyophilized, and reconstituted in sterile saline. Protein solutions were further sterilized by 15 minutes of UV exposure prior to cell encapsulation.

mASC^{Fluc+} harvest and in vitro *culture:* Experiments followed protocols approved by the Stanford Administrative Panel on Laboratory Animal Care. NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed. The subcutaneous inguinal fat pads from five-week-old GFP/firefly luciferase double transgenic mice (Jackson Laboratory) were removed and washed sequentially in serially diluted betadine (Purdue Frederick Co.) and saline. Tissues were diced and digested with 0.075% Type II collagenase (SigmaAldrich) in Hank's Balanced Salt Solution at 37 °C for 30 min in a shaking water bath. Digestion was neutralized with Dulbecco Modified Eagle's α-Glutamax Medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen). Neutralized cells were centrifuged at 4 °C for 10 min to separate mature adipocytes from the stromal-vascular fraction. Floating adipocytes were aspirated, and the pellet was resuspended in medium and filtered through a 100-μm strainer before being plated into a 100-mm dish. Adherent cells were cultured in DMEM supplemented with 10% FBS and 100 IU/ml penicillin/streptomycin (Gibco) at 37 °C and 5% atmospheric CO_2. Cells were expanded, passaged by trypsinization, and used for *in vitro* and *in vivo* assays at passages 2-4.

hASC harvest and in vitro *culture*: ASCs were harvested from human lipoaspirate from the flank and thigh regions by suction assisted liposuction. All subjects donating tissue responded to an Informed Consent approved by the Stanford Institutional Review Board. Specimens were washed in dilute betadine, rinsed twice in saline, and digested with 0.075% Type II collagenase in Hank's Balanced Salt Solution at 37 °C under agitation for 30 min. Collagenase was inactivated by an equal volume of saline with 10% FBS and 100 IU/ml penicillin/streptomycin. The stromal-vascular fraction was then pelleted, resuspended, and filtered through a 100-μm strainer before being plated into a 100-mm dish. Adherent cells were cultured in DMEM supplemented with 10% FBS and 100 IU/ml penicillin/streptomycin at 37 °C and 5% atmospheric CO2. Cells were expanded and passaged by trypsinization for subsequent use in *in vitro* assays.

In vitro *cell encapsulation and analysis:* Cells (12.5 μL, 5000 cells) were added first to P9 solution (12.5 μL, 20% w/v) and then C7 (25 μL, 10% w/v) into a polydimethylsiloxane mold bonded to a glass coverslip. Gels were incubated for 30 min in a hydrated environment before addition of DMEM supplemented with 10% FBS and penicillin/streptomycin (100 IU/ml) at 37 °C and 5% atmospheric CO_2. Cell viability was assessed with a LIVE/DEAD® cytotoxicity kit for mammalian cells (Molecular Probes). Briefly, staining solution containing calcein AM (2.0 μM; ex 488 nm, em 515–540 nm) and ethidium homodimer (4.0 μM ; ex 528 nm, em 560 nm) in saline was incubated at 37 °C for 35 min and then visualized. Other cultures were fixed overnight in 4% paraformaldehyde. These constructs were washed three times in saline and blocked with 10% normal goat serum or FBS containing Triton X-100 (0.1% v/v) in saline for one hour at room temperature. After three rinses in saline, the cells were stained with 6-diamidino-2-phenylindole (DAPI) (0.4 μg/ml, Roche) to visualize cell nuclei and with rhodamine-conjugated phalloidin (1:40 dilution, Invitrogen) to visualize F-actin.

Pre-injection cell encapsulation: $mASCs^{Fluc+}$ were trypsinized, resuspended to a density of 8×10^7 cells/ml in culture medium, and encapsulated in either MITCH, alginate (molecular weight = 75 kDa; NovaMatrix), or rat tail type I collagen (BD Biosciences). For MITCH samples, cell suspension (12.5 μl) was mixed with P9 solution (12.5 μl, 20% w/v in saline). The resulting mixture was mixed with C7 (25 μl, 10% w/v in saline) directly in the barrel of a 1-ml insulin syringe to form a cell-laden gel (10% w/v). For alginate samples, cell suspension (12.5 μl) was mixed with $CaCl_2$ solution (12.5 μl, 2% w/v in saline). The resulting mixture was mixed with alginate solution (25 μl, 2% w/v in saline) in the syringe to form a gel (1% w/v) with 1:4 Ca^{2+} ion to G-subunit stoichiometric ratio. For collagen samples, cell suspension (12.5 μl) was mixed with neutralized collagen solution (37.5 μl) to form a gel (0.25% w/v). As a control group, cell suspension (12.5 μl) at the same concentration was mixed with saline (37.5 μl) in the syringe. All cell-biomaterial samples had a final volume of 50 μl containing 1×10^6 cells (final concentration of 20×10^6 cells/ml).

In vivo *transplantation and bioluminescence imaging:* All experiments followed protocols approved by the Stanford Administrative Panel on Laboratory Animal Care. NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed. For *in vivo* transplantation, athymic nude mice (25-30 g, male,

Charles River Laboratories) were anesthetized with isoflurane, and cell-biomaterial samples (50 µl, prepared as described above) were injected subcutaneously in the dorsum via an insulin syringe with a 28-gauge needle. mASCs^{Fluc+} resuspended at the same concentration in saline (50 µl) were also injected as controls. To monitor cell viability and distribution, bioluminescence imaging (BLI) was performed with an IVIS imaging system (Xenogen Corp.), and data was acquired with LivingImage^{TM} software (Xenogen Corp.) on days 1, 3, 7, 10, and 14. Before imaging, mice were anesthetized with 2% isoflurane/air. Reporter probe D-luciferin was administered via intraperitoneal injection at a dose of 350 mg/kg body weight. Bioluminescent images were acquired at 5-min intervals with an exposure time of 30 sec. For each image acquisition, a gray scale body surface image was collected, followed by an overlay of the pseudo-colored image of photon counts from active luciferase within the mouse. Image acquisition continued until all samples had reached peak intensity (5-30 min). Signal intensity for each sample was quantified as total flux (photons/sec) within a region of interest at peak intensity. All values were normalized to day 1 and reported as mean ± SEM.

Histological analysis: At days 3 and 14 post-injection, samples were explanted from euthanized mice and processed for histological analysis. Samples were fixed in 4% paraformaldehyde for 24 hrs, serially dehydrated in graded ethanol steps, and embedded in paraffin blocks. Day 3 sections of subcutaneous explants were collected at 8-µm thickness and counterstained with hematoxylin. Transplanted GFP/luciferase cells were detected with anti-firefly luciferase (1:1000, Abcam) using Vectastain Elite ABC Kit (Vector Laboratories). Day 14 sections were collected at 4-µm thickness and stained with hematoxylin and eosin to visualize cell nuclei and tissue structures. Blinded histological analysis was preformed by a pathologist.

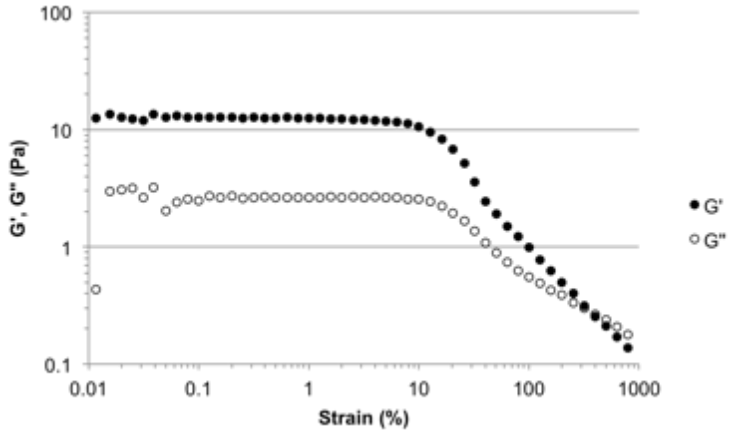

Figure S1: Bulk rheological analysis of rat tail type I collagen at 2.5 mg/mL (0.25% w/v) formulation. Strain sweep showing plateau storage modulus (G') is ~13 Pa.

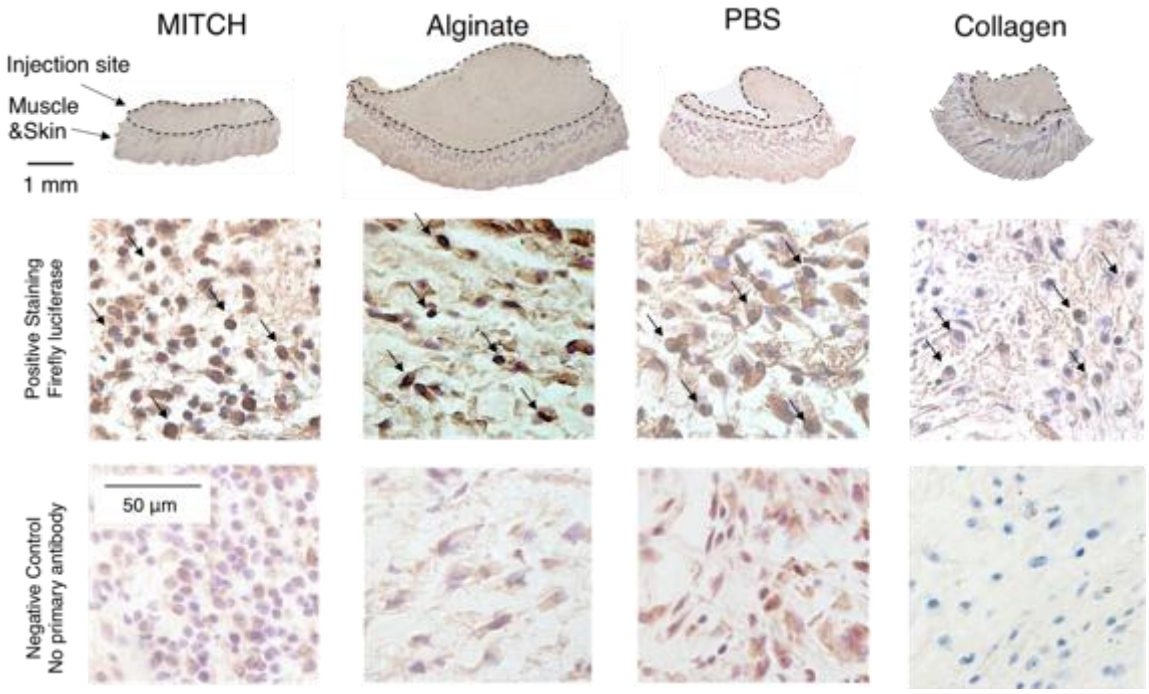

Figure S2: Immunohistochemical analysis of injection sites at day 3. Gross histology of explant cross section, dorsal skin oriented down, dashed line demarcates injection site (*top*). Immunohistochemical analysis of injected mASCs^{Fluc+} (luciferase positive cells, brown); cell nuclei counterstained with hematoxylin (blue); representative positively stained cells indicated by arrows (*middle*). Negative control staining without primary antibody; cell nuclei counterstained with hematoxylin (blue) (*bottom*).

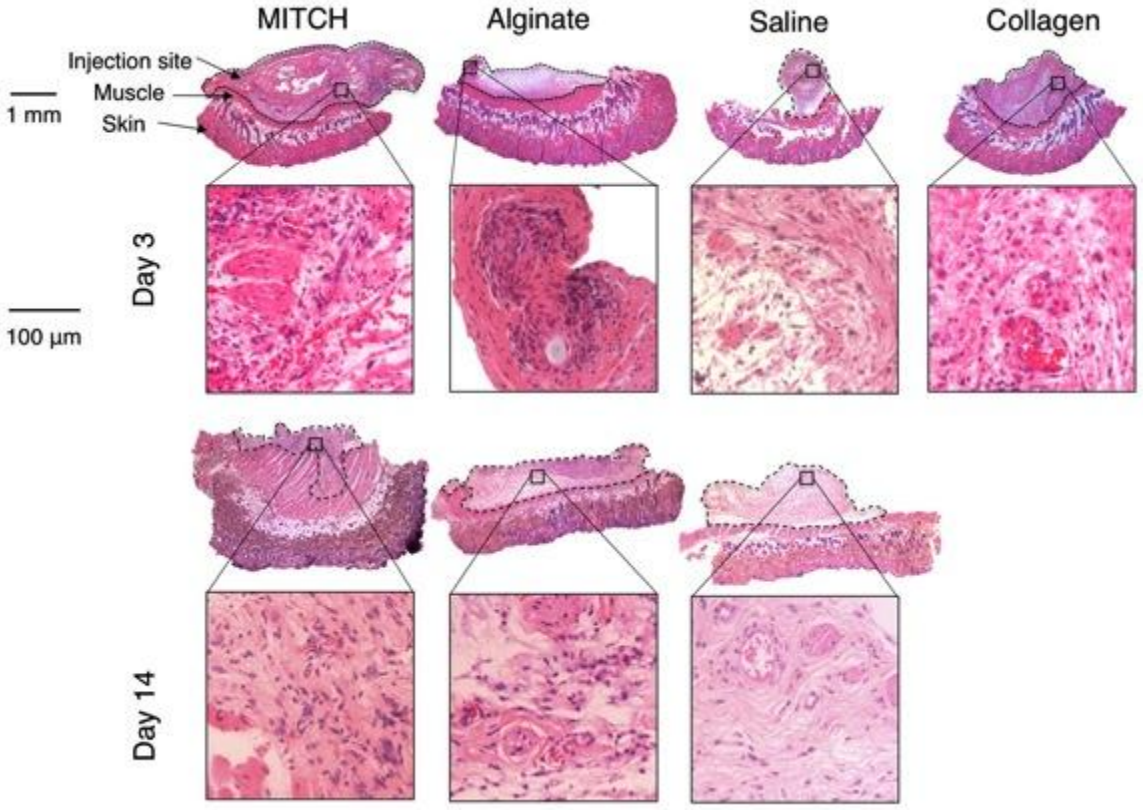

Figure S3: Hemotoxylin and eosin staining of day 3 and 14 explants. Staining colors cell nuclei blue, red blood cells red, and protein and cytoplasm pink. Infiltration of host lymphocytes, macrophages, and small vasculature was seen in all samples. In blinded histological analysis, all samples were scored as having mild acute inflammation consistent with injection injury and not exacerbated by material presence. Stained day 14 collagen explants are not available due to compaction preventing accurate histological sectioning.

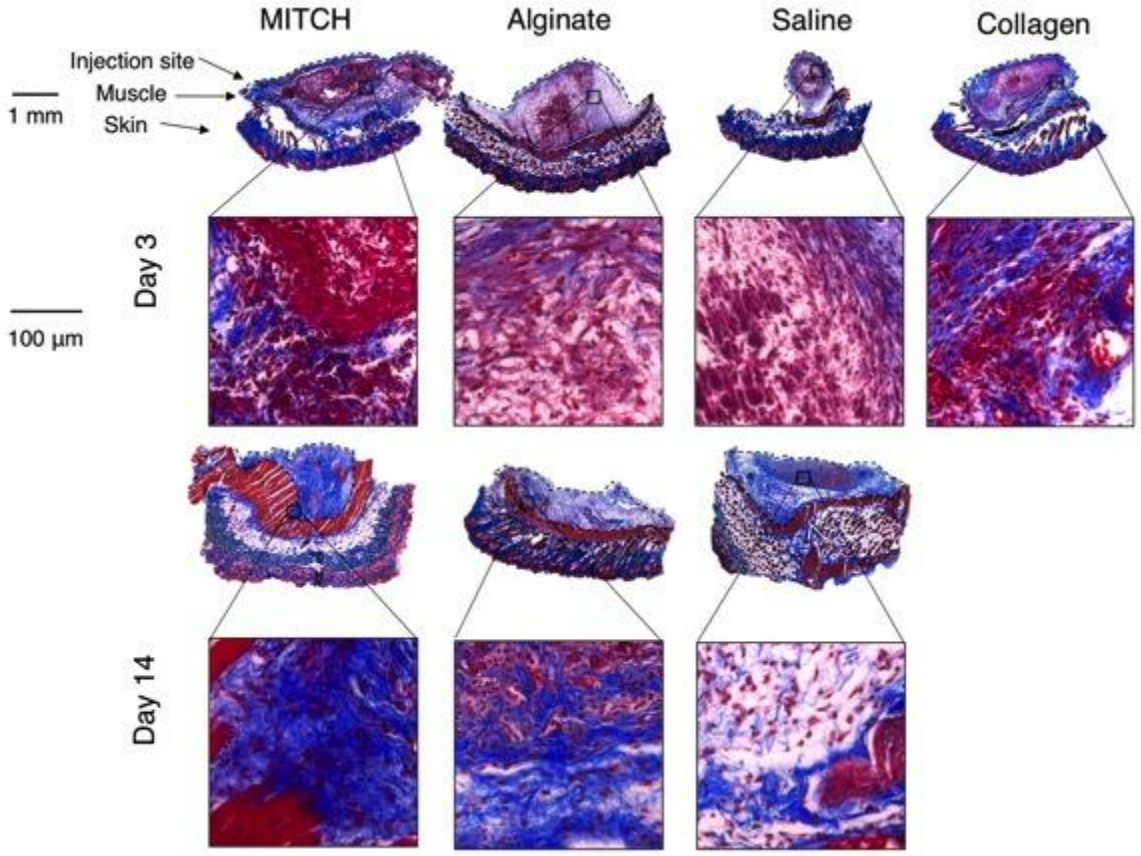

Figure S4: Trichrome staining of day 3 and 14 explants. Staining cell nuclei black, muscle and erythrocytes red, fibrin pink, and collagen blue. Extracellular matrix deposition was apparent within all injection sites with transplanted cells, suggesting possible matrix remodeling by delivered mASCs^{Fluc+} or endogenous cells. Stained day 14 collagen explants are not available due to compaction preventing accurate histological sectioning.