

Biologic-free mechanically induced muscle regeneration

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Severe skeletal muscle injuries are common and can lead to extensive fibrosis, scarring, and loss of function. Clinically, no therapeutic intervention exists that allows for a full functional restoration. As a result, both drug and cellular therapies are being widely investigated for treatment of muscle injury. Because muscle is known to respond to mechanical loading, we investigated instead whether a material system capable of massage-like compressions could promote regeneration. Magnetic actuation of biphasic ferrogel scaffolds implanted at the site of muscle injury resulted in uniform cyclic compressions that led to reduced fibrous capsule formation around the implant, as well as reduced fibrosis and inflammation in the injured muscle. In contrast, no significant effect of ferrogel actuation on muscle vascularization or perfusion was found. Strikingly, ferrogel-driven mechanical compressions led to enhanced muscle regeneration and a ~threefold increase in maximum contractile force of the treated muscle at 2 wk compared with no-treatment controls. Although this study focuses on the repair of severely injured skeletal muscle, magnetically stimulated bioagent-free ferrogels may find broad utility in the field of regenerative medicine.

mechano-therapy | magnetic hydrogel | massage-mimetic | fibrous capsule | immunomodulation

Skeletal muscle comprises a large percentage of the human body mass (40–50%) and plays an essential role in locomotion, postural support, and breathing. In response to minor injuries, skeletal muscle possesses a remarkable capacity for regeneration. Small exercise-induced tears, lacerations, and contusions typically heal without therapeutic intervention (1, 2). However, severe injuries resulting in a muscle mass loss of greater than 20% can lead to extensive fibrosis and loss of muscle function (2). Unfortunately, traumatic injuries resulting from motor vehicle accidents, aggressive tumor ablation, and prolonged denervation are common clinical situations and frequently lead to volumetric muscle loss (2–4). Although surgical reconstruction can lead to improved outcomes, this technique typically does not fully regenerate lost muscle tissue, and often leads to donor site morbidity (5, 6). As a result, the development of therapeutic strategies to treat severe skeletal muscle injuries is an area of active investigation.

Typical current approaches to skeletal muscle repair rely on the delivery of biologics such as growth factors and cells to enhance muscle regeneration. In early clinical trials, the intramuscular injection of cultured myoblasts was proven to be a safe but ineffective cell therapy for human myopathies, likely as a result of rapid death, poor migration, and immune rejection of the donor cells (7, 8). Recently, the identification of several important microenvironmental cues that regulate satellite cell fate has led to the development of cell-instructive biomaterials that improve cell engraftment and muscle regeneration. Biomaterial-based delivery of myogenic [IGF (insulin-like growth factor), FGF-2 (fibroblast growth factor-2), HGF (hepatocyte growth factor)] and angiogenic [VEGF (vascular endothelial growth factor)] factors that appear during the normal regenerative process has proven successful in animal models of severe muscle injury (9–13). Furthermore, the synergistic

presentation of cells and growth factors that mimic normal in vivo presentation has led to improved functional muscle regeneration in mice (13, 14). However, although much progress has been made toward the development of cell and growth factor-based approaches for the treatment of severely injured skeletal muscle in rodents, reliable clinical therapies still do not exist. Cell and drug therapies have likely been limited by rapidly depleted local concentrations, inappropriate gradients, and loss of bioactivity of delivered growth factors (13, 15), as well as the loss of regenerative potential associated with myogenic cell isolation and manipulation (16).

Skeletal muscle and satellite cells are sensitive to biophysical microenvironmental cues (16), and mechanical loading in particular is a strong regulator of muscle biology and function (17, 18). Stretch activation has been shown to play a role in modulating satellite cell activation, glucose uptake, and immune cell recruitment (19–21), while mechanical conditioning has been used extensively in cell culture to replicate the in vivo microenvironment and produce more physiologically relevant skeletal muscle constructs. Specifically, cyclic mechanical stretching of cell-seeded scaffolds has been shown to improve myoblast alignment, myofiber diameter, and skeletal muscle construct hypertrophy (22, 23). Importantly, mechanically loaded tissue-engineered skeletal muscle constructs often more closely mimic the tissue elasticity, structural organization, and force generation capabilities of native skeletal muscle (24–26). In addition, there is some clinical evidence that massage therapy or the physical manipulation of injured skeletal muscle and connective tissue may reduce pain and promote recovery by increasing blood flow (27–29). Because of its wide-ranging benefits on myogenesis, mechanical stimulation may offer a simple, yet effective, approach to skeletal muscle regeneration.

Significance

Much progress has been made toward the development of drug and cell therapies for the treatment of severely injured skeletal muscle, although reliable clinical therapies still do not exist. In contrast, this work demonstrates that biphasic ferrogels with a capacity for large, fatigue-resistant deformations can be used to mechanically stimulate and regenerate injured muscle tissue without the use of growth factors or cells. These biologic-free scaffolds exhibit a potential immunomodulatory role when stimulated and could potentially translate rapidly to the clinic. The therapeutic use of direct mechanical stimulation of injured tissues via externally actuated biomaterials could establish a new paradigm for regenerative medicine broadly.

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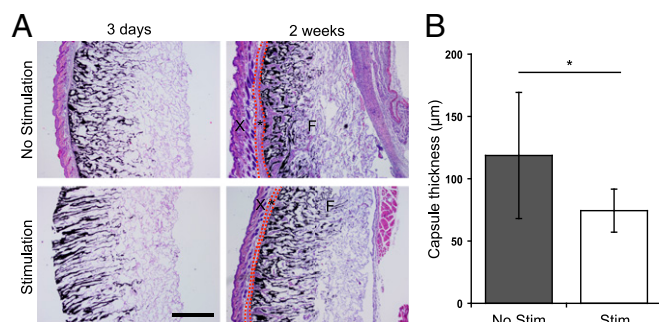


Fig. 2. Magnetic stimulation of ferrogel implants decreases fibrous capsule thickness. (A) Cross-sections of biphasic ferrogels stained with H&E at 3 d and 2 wk after implantation. Skin (X), fibrous capsule (*), and ferrogels (F) are indicated. It is important to note that significant fibrous capsule formation was not observed at 3 d in either ferrogel condition, and surrounding tissues were often lost during processing. (B) Quantified fibrous capsule thickness of nonstimulated and stimulated biphasic ferrogels after 2 wk of implantation. Fibrous capsule boundaries are marked with red dashed lines in A. (Scale bar, 500 μm .) Data were compared using a two-tailed unpaired Student's test with Welch's correction ($n = 9$; $*P < 0.05$). Error bars represent SDs.

perfusion increased to $\sim 40\%$ in all conditions. A difference between the stimulated and nonstimulated biphasic ferrogel conditions appeared at day 9, but did not persist to day 14. Differences in the average capillary density in muscle sections between the various conditions, as measured by immunostaining for the endothelial cell marker CD31, were not observed (Fig. 5B). The oxygen concentration within the muscle remained at a fairly constant baseline level of ~ 20 mm Hg before biphasic ferrogel stimulation (Fig. 5C). Upon stimulation, however, intramuscular oxygen concentration rapidly increased and remained elevated until stimulation ceased. Upon cessation of ferrogel stimulation, the oxygen concentration rapidly returned to the previous baseline levels.

Muscle Function: Contraction Force. To assess the functional quality of muscle regeneration, the contractile force of each injured muscle was measured. At 2 wk, injured muscles treated with stimulated biphasic ferrogels and pressure cuffs showed significant increases in specific peak tetanic force, 2.6- and 2.2-fold over no-treatment controls, respectively (Fig. 6). Stimulated biphasic ferrogels also showed a significant increase in specific peak tetanic force over the magnetic field-only control (1.9-fold). In contrast, no significant difference in specific peak tetanic force was observed between the no-treatment, magnetic field-only, and nonstimulated biphasic ferrogel conditions.

Discussion

Actuation of biologic-free ferrogels resulted in mechanical compressions that affected the host inflammatory response toward the gel and led to a reduction in fibrous capsule thickness after 2 wk of implantation. Strikingly, mechanical stimulation led to a significant reduction in fibrosis and inflammation of the injured muscle, demonstrating a potential immunomodulatory role for ferrogel-driven cyclic compressions. As assessed histologically, severe muscle injury resulting from myotoxin injection and hind limb ischemia led to subsequent active muscle regeneration that was enhanced by stimulated biphasic ferrogels. In addition, mechanical stimulation led to a temporary increase in oxygen concentration at the site of injury. Biologic-free ferrogel and pressure cuff-driven mechanical compressions led to enhanced muscle regeneration and muscle function compared with no-treatment controls, demonstrating the therapeutic potential of these mechanical interventions.

Actuation of both the biphasic ferrogel and the pressure cuff results in uniform cyclic compressions of the severely injured muscle tissue. The pressure cuff was roughly tuned so that the peak pressure values achieved by each system were comparable. However, the peak pressure value achieved by the pressure

cuff remained slightly larger than that achieved by the biphasic ferrogel because of limitations associated with control of the pressure cuff air valves. The kinetics of the pressure profile also varied, as the rate at which the pressure cuff air valves opened and shut was not finely controlled with this system. Nevertheless, both systems were able to apply a similar force (normalized to tibialis anterior wet weight) of ~ 2 N/g, a value similar to that used previously for massage-like compressive loading of rabbit tibialis anterior muscles injured with eccentric exercise (34). Although stimulation parameters were chosen to yield mechanical compressions that approximate those achieved with massage, future studies are needed to optimize the frequency, amplitude, and duration of the stimulations.

Biphasic ferrogel stimulation leads to a reduction in fibrous capsule thickness and inflammatory cells present in the surrounding muscle after 2 wk of implantation, and this may relate to expulsion of inflammatory cells as a result of cyclic compression of the ferrogels. A key event leading to fibrous capsule formation is the adhesion of profibrotic macrophages, foreign body giant cells, and fibroblasts to the implant surface, as these cells secrete proteins that modulate fibrosis and increase collagen deposition (35, 36). Previous *in vitro* experiments have demonstrated significant fibroblast and myoblast cell expulsion from RGD peptide-containing ferrogels *in vitro*, even with stimulation patterns shorter and less frequent than used here (30, 31). It is possible that invading cells near the scaffold edges were expelled from the ferrogel system upon stimulation, because of fluid convection resulting from large gel deformations, leading to an overall diminished cell presence within the scaffold. The decrease in M1 macrophage presence with ferrogel stimulation further suggests a potent immunomodulatory role for cyclic mechanical compressions. These results are perhaps not surprising, as a beneficial role for massage-like compressive loading after exercise-induced muscle damage has recently been demonstrated (34, 37). In addition, massage and massage mimics have been previously hypothesized to moderate the inflammatory

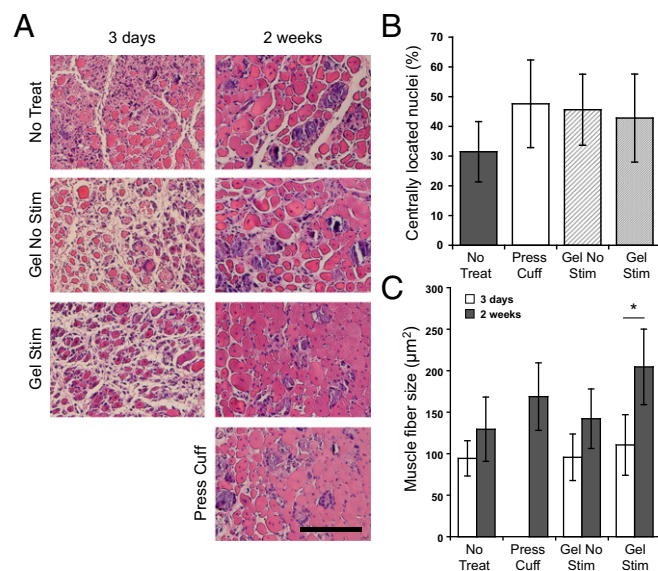
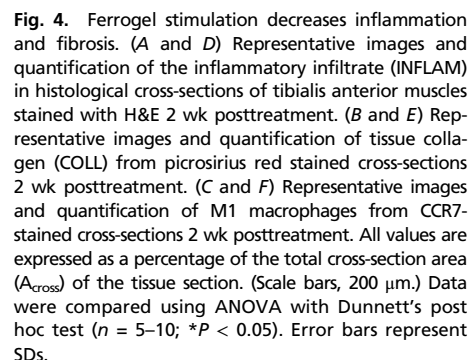
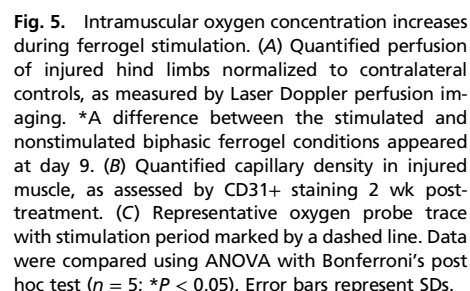


Fig. 3. Ferrogel stimulation leads to improved muscle regeneration. (A) Histological cross-sections of tibialis anterior muscles stained with H&E 3 d and 2 wk after no treatment (No Treat), treatment with a pressure cuff (Press Cuff), treatment with a nonstimulated biphasic ferrogel (Gel No Stim), or treatment with a stimulated biphasic ferrogel (Gel Stim). (Scale bar, 100 μm .) (B) Quantification of myofibers residing in the defect containing centrally located nuclei 2 wk posttreatment. Values are expressed as a percentage of the total number of myofibers in the defect. (C) Quantified mean muscle fiber size in the defect area 3 d and 2 wk posttreatment. Data were compared using ANOVA with Bonferroni's post hoc test ($n = 5$; $*P < 0.05$). Error bars represent SDs.



the regenerative process (13, 39). Although pressure cuff-treated muscles were not harvested at 3 d, mean muscle fiber size values remained remarkably consistent among all treatment groups at this time. At 2 wk, pressure cuff controls exhibited a smaller mean fiber size than stimulated ferrogels, suggesting ferrogel-driven cyclic mechanical compressions provide an additional, likely convection-based benefit. Specifically, enhanced fluid transportation around the implant site may accelerate immune cell and/or metabolic waste product removal. Interestingly, ferrogel-driven mechanical stimulation produces a therapeutic effect on muscle fiber size of the same order of magnitude as that achieved by previous approaches that delivered cells and drugs to severely injured muscle (13, 39).

Cyclic mechanical compressions do not lead to an enhancement in hind limb reperfusion and angiogenesis, but may instead lead to temporary increases in convection through the tissue or blood flow to the injured limb. This is perhaps not surprising, as ferrogels in this study did not deliver any proangiogenic growth factors or cells, such as VEGF and endothelial progenitor cells, that specifically target and support new blood vessel growth (12, 13, 32, 39, 40). Although small differences in perfusion were present 9 d postinjury, they did not persist to the end of the study.



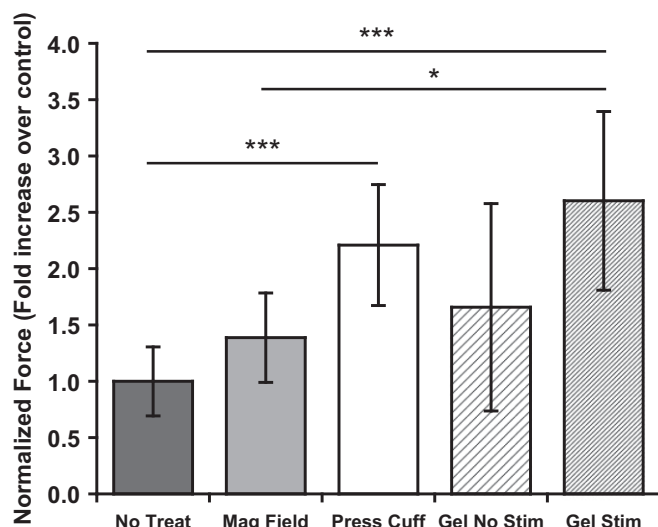


Fig. 6. Cyclic mechanical compressions enhance functional muscle regeneration. Maximum contractile force after tetanic stimulation of injured muscles 2 wk after no treatment (No Treat), treatment with a magnetic field only (Mag Field), treatment with a pressure cuff (Press Cuff), treatment with a nonstimulated biphasic ferrogel (Gel No Stim), or treatment with a stimulated biphasic ferrogel (Gel Stim). Force measurements were normalized to muscle wet weight. Data were compared using ANOVA with Bonferroni's post hoc test ($n = 5-10$; $*P < 0.05$; $***P < 0.001$).

suggesting that growth factor or cell support may be required to maintain any increases in perfusion that appear as a result of cyclic mechanical compressions alone. However, longer-duration studies would be useful to confirm that no differences in angiogenesis or perfusion result from the ferrogel treatment. Strikingly, although steady-state hind limb perfusion and capillary density were not significantly affected, oxygen probe experiments demonstrated an increase in oxygen concentration during the time in which biphasic ferrogel-driven cyclic mechanical compressions were being generated in the muscle. Massage has previously been shown to temporarily increase intramuscular temperature, which may lead to enhanced nutrient transport throughout the injured tissue (41), and a similar phenomenon could have occurred here. More likely, enhanced intramuscular convection driven by tissue compressions may have led to increased oxygen levels and expedited removal of metabolic byproducts that inhibit regeneration. Alternatively, cyclic compressions may increase oxygen concentration by locally and temporarily increasing blood flow to the injured muscle. A parallel to this situation may occur in massage therapy, as controlled clinical trials aimed at identifying a physiological mechanism for massage have often implicated, although not proven, transient increases in blood flow (29, 42, 43).

Strikingly, biphasic ferrogel and pressure cuff-driven cyclic mechanical compressions of injured muscle led to significant functional muscle regeneration. After 2 wk of treatment, biphasic ferrogel and pressure cuff treatment conditions showed 2.6- and 2.2-fold increases, respectively, in peak tetanic force over no-treatment conditions. Previous studies using mouse models of severe muscle injury report comparable enhancements of muscle contractile force at 2 wk after delivery of proregenerative growth factors and/or myogenic cells to the injured muscle. In these studies, scaffold delivery of VEGF and IGF resulted in a 2.4-fold increase in peak tetanic force over blank scaffolds, whereas scaffold delivery of VEGF, IGF, and myoblasts resulted in 1.5–6.0-fold increases in peak tetanic force over control conditions (13, 32, 39). Although additional biomaterial-based approaches have induced similar improvements in muscle function through the codelivery of myogenic bioagents with endothelial cells to enhance vascularization (5.5-fold increase in active stress over blank scaffold) or neural stem cells to promote innervation of muscle constructs (2.0-fold

increase in maximum tetanic force over nerve-deficient controls) (44–47), no bioagents were delivered from the scaffolds in this study. It is possible that the magnetic field directly affected regeneration in the current study, as past studies involving treatment of myoblasts and myotubes with static magnetic fields have demonstrated increased myogenic differentiation and cell alignment (48, 49). However, although a slight increase in peak tetanic force was observed with magnetic field application in the current study, results were not significantly different from the untreated controls, perhaps because of the relatively short duration of magnetic field exposure. Alternatively, cyclic mechanical compressions have been shown to improve muscle regeneration after exercise-induced muscle injury in rabbits and humans (27, 34, 37). Specifically, after 4 d of massage-like compressive loading in rabbits, 3.0–4.1-fold increases in peak torque recovery over untreated controls were observed. However, the current study is the first report, to our knowledge, of functional muscle regeneration resulting from cyclic mechanical compressions in a severe model of muscle damage.

Strikingly, the results of these studies indicate a ferrogel scaffold can be used to mechanically stimulate and regenerate severely injured muscle tissue without the use of growth factors or cells. The demonstration of functional muscle regeneration with a biologic-free material system may offer a simple yet effective alternative to cell-based therapies when treating certain types of muscle injuries. Further, incorporation of cyclic mechanical compressions into existing drug and cell delivery systems could potentially lead to new combinatorial therapies that generate enhanced regenerative outcomes. Although this study focuses on the repair of skeletal muscle, bioagent-free ferrogels and the concept of mechanically driven regeneration are expected to find broad utility and can likely be applied to other tissues and diseases.

Materials and Methods

Materials. Medical-grade, high-molecular weight (~250 kDa) sodium alginate with high guluronate content (Protanal LF 20/40) was purchased from FMC Biopolymers. Alginates were used after covalent RGD peptide modification and dialysis purification, as previously described (50). All other chemicals including adipic acid dihydrazide, 1-ethyl-3-(dimethylaminopropyl) carbodiimide, 2-(N-morpholino)ethanesulfonic acid (MES), 1-hydroxybenzotriazole (HOBt), and Iron(II,III) oxide powder (<5 μm , 310069) were purchased from Sigma-Aldrich.

Animals and Surgical Procedures. All animal work was performed in compliance with NIH and institutional guidelines. For myotxin injuries, tibialis anterior muscles of 6-wk-old female wild-type C57BL/6J mice were injected with 10 μ L of 10 μ g/mL Notexin Np myotoxin. Six days after notexin injection, hind limb ischemia was induced by unilateral external iliac and femoral artery and vein ligation, as described in [SI Materials and Methods](#) (12, 51).

Ischemia and Perfusion Analysis. Blood perfusion measurements of the ischemic and normal limb were performed on anesthetized animals, using a Laser Doppler Perfusion Imaging analyzer, as described in [SI Materials and Methods](#). Perfusion was calculated as the ratio of ischemic to nonischemic limb perfusion for each animal.

Ferrogel Scaffold and Pressure Cuff Stimulation. Biphasic ferrogel scaffolds (7 wt% iron oxide) were fabricated with alginate covalently modified with RGD peptide, as previously described (30). Scaffolds were placed s.c. on the tibialis anterior muscle and stimulated for 5 min at 1 Hz every 12 h by approaching and retracting a permanent magnet with a surface field of 6,510 Gauss. Similarly, the balloon pressure cuff was programmed to inflate and deflate for 5 min at 1 Hz every 12 h, as described in *SI Materials and Methods*.

Histologic Assessment of Skeletal Muscle. Mice were euthanized and hind limb muscle tissues were processed for histologic analyses. Tissue sections were stained with H&E, CCR7, CD31, and picrosirius red for quantification of regenerative markers and the inflammatory infiltrate, as described in *SI Materials and Methods*.

Muscle Function Testing. Intact tibialis anterior muscles were dissected and mounted vertically midway between two cylindrical parallel steel wire electrodes. Muscles were attached by their tendons to microclips connected

to a force transducer and bathed in a physiologic saline solution with continuously bubbled oxygen at 37 °C. Contractions were evoked every 5 min, as described in *SI Materials and Methods*. Specific peak tetanic force was determined as the difference between the maximum force during contraction and the baseline level normalized to muscle wet weight.

Oxygen Probe. An OxyLab pO₂ instrument and implantable optical probe were used to measure rapid temporal changes in intramuscular dissolved oxygen and temperature during biphasic ferrogel stimulation, as described in *SI Materials and Methods*. Oxygen and temperature readings were recorded continuously before, during, and after biphasic ferrogel stimulation at 1-s intervals.

Statistical Analyses. All statistical comparisons were performed using ANOVA with Bonferroni's or Dunnett's post hoc test and a two-tailed unpaired Student's *t* test with Welch's correction and analyzed using INSTAT 3.1a (GraphPad Software, Inc.) software. Differences between conditions were considered significant if *P* < 0.05.

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- Juhas M, Bursac N (2013) Engineering skeletal muscle repair. *Curr Opin Biotechnol* 24(5):880–886.
- Turner NJ, Badylak SF (2012) Regeneration of skeletal muscle. *Cell Tissue Res* 347(3):759–774.
- Järvinen TAHJ, Järvinen TLN, Kääriäinen M, Kalimo H, Järvinen M (2005) Muscle injuries: Biology and treatment. *Am J Sports Med* 33(5):745–764.
- Rossi CA, Pozzobon M, De Coppi P (2010) Advances in musculoskeletal tissue engineering: Moving towards therapy. *Organogenesis* 6(3):167–172.
- Ma CH, et al. (2008) Reconstruction of upper extremity large soft-tissue defects using pedicled latissimus dorsi muscle flaps—technique illustration and clinical outcomes. *Injury* 39(Suppl 4):67–74.
- Tu YK, et al. (2008) Soft-tissue injury management and flap reconstruction for mangled lower extremities. *Injury* 39(Suppl 4):75–95.
- Tedesco FS, Cossu G (2012) Stem cell therapies for muscle disorders. *Curr Opin Neurol* 25(5):597–603.
- Palmieri B, Tremblay JP, Daniele L (2010) Past, present and future of myoblast transplantation in the treatment of Duchenne muscular dystrophy. *Pediatr Transplant* 14(7):813–819.
- Ten Broek RW, Grefte S, Von den Hoff JW (2010) Regulatory factors and cell populations involved in skeletal muscle regeneration. *J Cell Physiol* 224(1):7–16.
- Shansky J, Creswick B, Lee P, Wang X, Vandenburgh H (2006) Paracrine release of insulin-like growth factor 1 from a bioengineered tissue stimulates skeletal muscle growth in vitro. *Tissue Eng* 12(7):1833–1841.
- Pelosi L, et al. (2007) Local expression of IGF-1 accelerates muscle regeneration by rapidly modulating inflammatory cytokines and chemokines. *FASEB J* 21(7):1393–1402.
- Silva EA, Mooney DJ (2007) Spatiotemporal control of vascular endothelial growth factor delivery from injectable hydrogels enhances angiogenesis. *J Thromb Haemost* 5(3):590–598.
- Borselli C, et al. (2010) Functional muscle regeneration with combined delivery of angiogenesis and myogenesis factors. *Proc Natl Acad Sci USA* 107(8):3287–3292.
- Sheehan SM, Allen RE (1999) Skeletal muscle satellite cell proliferation in response to members of the fibroblast growth factor family and hepatocyte growth factor. *J Cell Physiol* 181(3):499–506.
- Hammers DW, et al. (2012) Controlled release of IGF-I from a biodegradable matrix improves functional recovery of skeletal muscle from ischemia/reperfusion. *Biotechnol Bioeng* 109(4):1051–1059.
- Cezar CA, Mooney DJ (2015) Biomaterial-based delivery for skeletal muscle repair. *Adv Drug Deliv Rev* 84:188–197.
- Hornberger TA, Armstrong DD, Koh TJ, Burkholder TJ, Esser KA (2005) Intracellular signaling specificity in response to uniaxial vs. multi-axial stretch: Implications for mechanotransduction. *Am J Physiol Cell Physiol* 288(1):C185–C194.
- Kumar A, Chaudhry I, Reid MB, Boriek AM (2002) Distinct signaling pathways are activated in response to mechanical stress applied axially and transversely to skeletal muscle fibers. *J Biol Chem* 277(48):46493–46503.
- Tatsumi R, Sheehan SM, Iwasaki H, Hattori A, Allen RE (2001) Mechanical stretch induces activation of skeletal muscle satellite cells in vitro. *Exp Cell Res* 267(1):107–114.
- Peterson JM, Pizsa FX (2009) Cytokines derived from cultured skeletal muscle cells after mechanical strain promote neutrophil chemotaxis in vitro. *J Appl Physiol* (1985) 106(1):130–137.
- Chambers MA, Moylan JS, Smith JD, Goodyear LJ, Reid MB (2009) Stretch-stimulated glucose uptake in skeletal muscle is mediated by reactive oxygen species and p38 MAP-kinase. *J Physiol* 587(Pt 13):3363–3373.
- Vandenburgh H, Kaufman S (1979) In vitro model for stretch-induced hypertrophy of skeletal muscle. *Science* 203(4377):265–268.
- Vandenburgh HH, Karlisch P (1989) Longitudinal growth of skeletal myotubes in vitro in a new horizontal mechanical cell stimulator. *In Vitro Cell Dev Biol* 25(7):607–616.
- Moon G, Christ G, Stitzel JD, Atala A, Yoo JJ (2008) Cyclic mechanical preconditioning improves engineered muscle contraction. *Tissue Eng Part A* 14(4):473–482.
- Matsumoto T, et al. (2007) Three-dimensional cell and tissue patterning in a strained fibrin gel system. *PLoS One* 2(11):e1211.
- Powell CA, Smiley BL, Mills J, Vandenburgh HH (2002) Mechanical stimulation improves tissue-engineered human skeletal muscle. *Am J Physiol Cell Physiol* 283(5):C1557–C1565.
- Crane JD, et al. (2012) Massage therapy attenuates inflammatory signaling after exercise-induced muscle damage. *Sci Transl Med* 4(119):119a13.
- Cherkin DC, Sherman KJ, Deyo RA, Shekelle PG (2003) A review of the evidence for the effectiveness, safety, and cost of acupuncture, massage therapy, and spinal manipulation for back pain. *Ann Intern Med* 138(11):898–906.
- Weerapong P, Hume PA, Kolt GS (2005) The mechanisms of massage and effects on performance, muscle recovery and injury prevention. *Sports Med* 35(3):235–256.
- Cezar CA, et al. (2014) Biphasic ferrogels for triggered drug and cell delivery. *Adv Health Mater* 3(11):1869–1876.
- Zhao X, et al. (2011) Active scaffolds for on-demand drug and cell delivery. *Proc Natl Acad Sci USA* 108(1):67–72.
- Borselli C, Cezar CA, Shvartsman D, Vandenburgh HH, Mooney DJ (2011) The role of multifunctional delivery scaffold in the ability of cultured myoblasts to promote muscle regeneration. *Biomaterials* 32(34):8905–8914.
- Hawke TJ, Garry DJ (2001) Myogenic satellite cells: Physiology to molecular biology. *J Appl Physiol* (1985) 91(2):534–551.
- Haas C, et al. (2013) Dose-dependency of massage-like compressive loading on recovery of active muscle properties following eccentric exercise: Rabbit study with clinical relevance. *Br J Sports Med* 47(2):83–88.
- Anderson JM, Rodriguez A, Chang DT (2008) Foreign body reaction to biomaterials. *Semin Immunol* 20(2):86–100.
- Franz S, Rammelt S, Scharnweber D, Simon JC (2011) Immune responses to implants - a review of the implications for the design of immunomodulatory biomaterials. *Biomaterials* 32(28):6692–6709.
- Butterfield TA, Zhao Y, Agarwal S, Haq F, Best TM (2008) Cyclic compressive loading facilitates recovery after eccentric exercise. *Med Sci Sports Exerc* 40(7):1289–1296.
- Haas C, et al. (2013) Massage timing affects postexercise muscle recovery and inflammation in a rabbit model. *Med Sci Sports Exerc* 45(6):1105–1112.
- Wang L, et al. (2014) Minimally invasive approach to the repair of injured skeletal muscle with a shape-memory scaffold. *Mol Ther* 22(8):1441–1449.
- Shvartsman D, et al. (2014) Sustained delivery of VEGF maintains innervation and promotes reperfusion in ischemic skeletal muscles via NGF/GDNF signaling. *Mol Ther* 22(7):1243–1253.
- Drust B, Atkinson G, Gregson W, French D, Binningsley D (2003) The effects of massage on intra muscular temperature in the vastus lateralis in humans. *Int J Sports Med* 24(6):395–399.
- Hovind H, Nielsen SL (1974) Effect of massage on blood flow in skeletal muscle. *Scand J Rehabil Med* 6(2):74–77.
- Hansen TI, Kristensen JH (1973) Effect of massage, shortwave diathermy and ultrasound upon 133Xe disappearance rate from muscle and subcutaneous tissue in the human calf. *Scand J Rehabil Med* 5(4):179–182.
- Koffler J, et al. (2011) Improved vascular organization enhances functional integration of engineered skeletal muscle grafts. *Proc Natl Acad Sci USA* 108(36):14789–14794.
- Shandalov Y, et al. (2014) An engineered muscle flap for reconstruction of large soft tissue defects. *Proc Natl Acad Sci USA* 111(16):6010–6015.
- Morimoto Y, Kato-Negishi M, Onoe H, Takeuchi S (2013) Three-dimensional neuron-muscle constructs with neuromuscular junctions. *Biomaterials* 34(37):9413–9419.
- Larkin LM, Van der Meulen JH, Dennis RG, Kennedy JB (2006) Functional evaluation of nerve-skeletal muscle constructs engineered in vitro. *In Vitro Cell Dev Biol Anim* 42(3–4):75–82.
- Coletti D, et al. (2007) Static magnetic fields enhance skeletal muscle differentiation in vitro by improving myoblast alignment. *Cytometry A* 71(10):846–856.
- Sakurai T, Hashimoto A, Kiyokawa T, Kikuchi K, Miyakoshi J (2012) Myotube orientation using strong static magnetic fields. *Bioelectromagnetics* 33(5):421–427.
- Rowley JA, Madlambayan G, Mooney DJ (1999) Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials* 20(1):45–53.
- Chen RR, et al. (2007) Integrated approach to designing growth factor delivery systems. *FASEB J* 21(14):3896–3903.
- Boonthekul T, Kong HJ, Mooney DJ (2005) Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution. *Biomaterials* 26(15):2455–2465.
- Reddy LH, Arias JL, Nicolas J, Couvreur P (2012) Magnetic nanoparticles: design and characterization, toxicity and biocompatibility, pharmaceutical and biomedical applications. *Chem Rev* 112(11):5818–5878.

Supporting Information

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SI Materials and Methods

Animals and Surgical Procedures. All animal work was performed in compliance with NIH and institutional guidelines. Six-week-old female wild-type C57BL/6J mice (Jackson Laboratories) were anesthetized with an i.p. injection of ketamine ($80 \text{ mg}\cdot\text{kg}^{-1}$) and xylazine ($5 \text{ mg}\cdot\text{kg}^{-1}$). For myotoxin injuries, the tibialis anterior muscles of the right legs of anesthetized mice were injected with $10 \mu\text{L}$ of $10 \mu\text{g}/\text{mL}$ Notexin Np myotoxin from Notechis Scutatus snake venom (Latoxan), using a $25\text{-}\mu\text{L}$ Hamilton syringe. Six days after notexin injection, hind limb ischemia was induced by unilateral external iliac and femoral artery and vein ligation, as previously described (12, 51). After vessel ligation, a hydrated ferrogel scaffold was placed s.c. on the tibialis anterior muscle in certain conditions, and the incision was surgically closed.

Ischemia and Perfusion Analysis. Blood perfusion measurements of the ischemic and normal limb were performed on anesthetized animals ($n = 10$), using a Laser Doppler Perfusion Imaging analyzer (PeriScan PIM II; Perimed Instruments). Entire hind limbs were scanned under basal conditions and then every other day after surgery. Perfusion was calculated as the ratio of ischemic to nonischemic limb perfusion for each animal.

Ferrogel Scaffold Stimulation. Biphasic ferrogels (7 wt% iron oxide) were fabricated with alginate covalently modified with RGD peptide (DS10, 10 RGD peptides per alginate chain), as previously described (30). Although nondegradable alginate was used to simplify the system, oxidized alginate can be used in the future to create ferrogels with controlled degradation (52). In this case, optimization of iron oxide nanoparticle size, shape, and surface charge may be necessary to ensure particle absorption by the lymphatic capillary system and clearance from the body (53). Before implantation, biphasic ferrogel scaffolds were hydrated with $100 \mu\text{L}$ PBS. Biphasic ferrogel scaffolds were then placed s.c. on the tibialis anterior muscle and stimulated for 5 min at 1 Hz every 12 h by approaching and retracting a permanent magnet with a surface field of 6,510 Gauss (K&J Magnetics, DX0Z0). The ferrogel implant and magnet were orientated so that magnetic stimulation resulted in compression of the ferrogel against the injured muscle. After retrieval at 3 d and 2 wk, scaffolds were fixed in 10% (vol/vol) neutral-buffered formalin overnight. Ferrogels were then paraffin embedded, sectioned at $7 \mu\text{m}$ thickness, and stained with H&E at the Harvard Rodent Histopathology Core. For pressure profile generation, an Instron 3342 single column apparatus (10 N load cell) configured for tensile testing was used. A custom adapter that allowed for stimulation of the ferrogel far from the load cell was attached to the top tensile grip, and the ferrogel was cyclically stimulated with a permanent magnet.

Pressure Cuff Stimulation. For mechanical stimulation using the balloon pressure cuff, a spherical low durometer urethane balloon (10–15 mm diameter 10000000FA, Vention Medical) was attached to flexible silicone tubing (1.78 mm outer diameter, 1.1 mm inner diameter; McMaster Carr) and bonded at each neck with ultraviolet cure adhesive (Loctite 3943; Henkel). The output from two solenoid valves (TE miniature switching solenoid valves, sensor technics) was joined and connected to the balloon. Valve inlets were connected to a pneumatic air pump (pneumatic pump M0019057; Parker Electronics) at the pressure and vacuum outlets. The valves were wired to two-channel relay board with two SRD-05VDC-SL-C relays that were controlled by

an Arduino Uno microcontroller developer board. The microcontroller was programmed to sequentially open the valves for 500 ms every second so that the balloon would be pressurized for 500 ms and then evacuated for 500 ms at a 1-Hz frequency. The balloon was placed inside a polycarbonate cylindrical cuff made from a 20-mL syringe body trimmed to length with the plunger removed (20-mm outer diameter, 40-mm length, adjustable wall thickness from 2 to 4 mm, using polycarbonate cylindrical liners). For pressure cuff stimulation, the mouse leg was placed in the cuff and the balloon was fixed directly above the tibialis anterior muscle outside the skin. The balloon was then inflated and deflated for 5 min at 1 Hz every 12 h. For pressure profile generation, an Instron 3342 single column apparatus (10 N load cell) configured for compression testing was used. The balloon was removed from the cylindrical cuff and cyclically inflated and deflated between two stationary compression platens separated by a distance equal to the diameter of the deflated balloon.

Histologic Assessment of Skeletal Muscle. Mice were euthanized and hind limb muscle tissues ($n = 10$) were processed for histologic analyses. Tibialis anterior muscles were fixed in 10% (vol/vol) neutral-buffered formalin overnight, paraffin embedded, and sectioned at $7 \mu\text{m}$ thickness. Sections stained with H&E were used for quantification of mean fiber diameter, inflammatory infiltrate, and fibers with centrally located nuclei. All histologic analyses performed on H&E-stained sections were performed in a blinded fashion. M1 macrophages were identified with immunostaining for mouse CCR7 (Abcam ab32527; Invitrogen A-11035). Vascular endothelial cells were identified by immunostaining for mouse CD31 (Abcam ab28364; Invitrogen A-11035). Interstitial fibrosis was assessed in picrosirius-stained sections imaged with polarized light. Quantification of CCR7 and CD31 immunostaining was performed using ImageJ.

Muscle Function Testing. Intact tibialis anterior muscles were dissected ($n = 5/\text{condition}$), mounted vertically midway between two cylindrical parallel steel wire electrodes (1.6 mm diameter, 21 mm long) attached by their tendons to microclips connected to a force transducer (FORT 25, WPII), and bathed in a physiologic saline solution in a chamber with continuously bubbled oxygen at 37°C . Muscle length was adjusted to a physiological relevant length. A wave pulse was initiated using a custom-written LabVIEW program and delivered to the stimulation electrodes via a purpose-built power amplifier (QSC USA 1310). Contractions were evoked every 5 min. Tetani was evoked at 250–300 Hz and 25–30 V, with a constant pulse width of 2 ms and a train duration of 1 s. Peak tetanic force was determined as the difference between the maximum force during contraction and the baseline level. Forces were then normalized to muscle wet weight.

Oxygen Probe. An OxyLab pO₂ instrument and implantable optical probe (Oxford Optrox) were used to measure rapid temporal changes in intramuscular dissolved oxygen and temperature during biphasic ferrogel stimulation. Briefly, the probe was inserted using a 16-g needle catheter, and the tip was carefully placed in the midbelly region of the tibialis anterior muscle. A biphasic ferrogel was then implanted s.c. above the muscle containing the probe, as before (30), and the incision was closed around the probe. Oxygen and temperature readings were recorded continuously before, during, and after biphasic ferrogel stimulation at 1-s intervals.

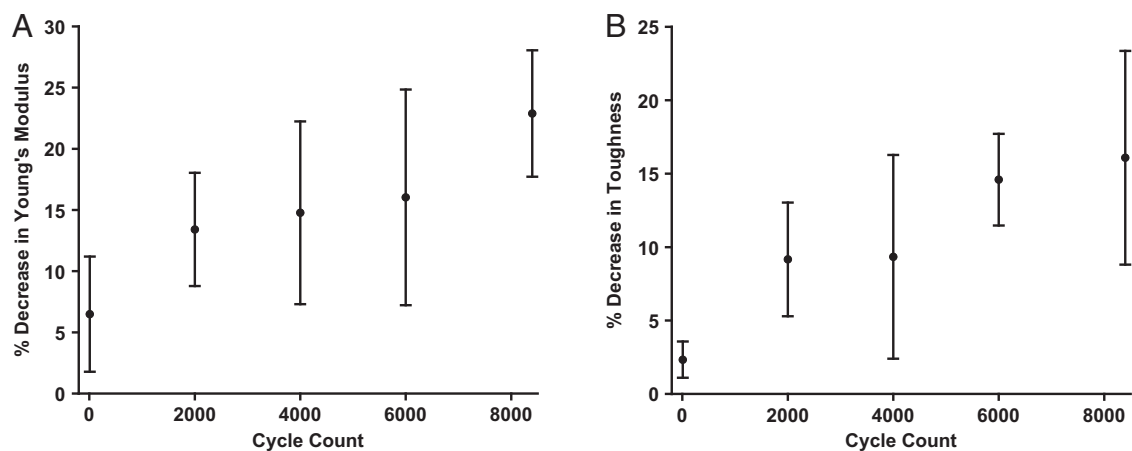


Fig. S1. Biphasic ferrogels exhibit fatigue resistance. Percentage decrease in biphasic ferrogel (A) Young's modulus and (B) toughness at 50% strain after 8,400 cyclic compressions to 50% strain, compared with the values calculated from the first cycle of compression. Values represent the mean and SD ($n = 4$).