An artificial niche preserves the quiescence of muscle stem cells and enhances their therapeutic efficacy

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A promising therapeutic strategy for diverse genetic disorders involves transplantation of autologous stem cells that have been genetically corrected ex vivo. A major challenge in such approaches is a loss of stem cell potency during culture. Here we describe an artificial niche for maintaining muscle stem cells (MuSCs) in vitro in a potent, quiescent state. Using a machine learning method, we identified a molecular signature of quiescence and used it to screen for factors that could maintain mouse MuSC quiescence, thus defining a quiescence medium (QM). We also engineered muscle fibers that mimic the native myofiber of the MuSC niche. Mouse MuSCs maintained in QM on engineered fibers showed enhanced potential for engraftment, tissue regeneration and self-renewal after transplantation in mice. An artificial niche adapted to human cells similarly extended the quiescence of human MuSCs in vitro and enhanced their potency in vivo. Our approach for maintaining quiescence may be applicable to stem cells isolated from other tissues.

The development of stem cell therapeutics has been hindered by the inability to manipulate stem cells in vitro without a loss of potency.1–2 Recent studies suggest that stem cell potency depends on the cells’ capacity to remain quiescent before their activation by regenerative stimuli such as injury.3–5 For many stem cell populations, such as skeletal MuSCs,1 hematopoietic stem cells6 and neural stem cells7, the most potent cell in terms of transplantation efficacy and the ability to repair and repopulate a tissue is the long-term–quiescent stem cell. It has been estimated that such cells can remain in the quiescent state for months in mice and years in humans.4,8–10. Stem cells reside in tissues in a specialized microenvironment or niche, characterized by a unique combination of biophysical, biochemical and cellular properties. These include mechanical properties such as stiffness, direct contact with other cell types in the niche, and molecules such as cytokines or growth factors, all of which have a critical role in regulating stem cell function in vivo.6,7,11–13. These properties have been identified as promoting quiescence in several tissue compartments.7,12,14–16.

Previous attempts to reproduce the niche in vitro have focused almost exclusively on properties that influence the dynamics of cell division, allowing studies of cell replication and cell-fate determination.11,17–21. What has not been well modeled are niche components that promote and maintain stem cell quiescence.12,14,22. Even with the best current culture conditions, as soon as quiescent cells are isolated from their in vivo niche and plated, they immediately begin to exit the quiescent state, activate (i.e., transition from the G0 stage into the cell cycle) and undergo proliferation and differentiation.1,11,23. The ability to maintain stem cells in a quiescent state in vitro would facilitate study of the biology of quiescence. In the context of cell therapies, it would preserve the potency of stem cells destined for transplantation and reduce the need to expand them ex vivo.2,23. This would be especially valuable in cases of extended culture, for example, during genetic manipulation before transplantation.

MuSCs, or satellite cells, reside in a quiescent state under the basal laminae of muscle fibers.24,25. We describe an approach to mimic the biochemical and mechanical properties of the native niche that combines a defined culture condition and a three-dimensional microscaffold assembled from extracellular matrix proteins found in the MuSC niche. We show that the resulting artificial niche enables sustained quiescence for up to a week of both mouse and human MuSCs, and enhances engraftment and self-renewal after transplantation. Our system provides a tool for studying the biology of MuSC quiescence and may aid the development of stem cell therapeutics for muscle disorders ranging from traumatic injuries to genetic degenerative diseases such as the muscular dystrophies.

RESULTS

A quiescence medium for mouse MuSCs

Consistent with previous reports2, we observed that after mouse MuSCs are isolated, they lose potency in about 2 d in vitro (data not shown). Using the Pax7-CreER and the ROSA26LacZ mouse strains to genetically label MuSCs with the luciferase reporter, we transplanted into a tibialis anterior (TA) muscle of immunocompromised recipient mice 10,000 MuSCs as ‘quiescent’ MuSCs (immediately after isolation), activated MuSCs (cultured for 3.5 d)

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or ‘myoblasts’ (cultured for 2 weeks). Noninvasive imaging for 30 d showed that quiescent MuSCs yielded the highest bioluminescence signal (Fig. 1a). Although the bioluminescent signals for MuSC populations increased over time, suggesting active cell proliferation, we cannot exclude the possibility that some of the observed differences between populations were due to differential susceptibility of the cells to apoptosis.

Based on these and previous data suggesting that quiescent cells may be more potent than actively proliferating cells1,2, we sought to define and engineer cell culture conditions that would maintain the quiescent state of freshly isolated MuSCs. To develop a suitable medium, we first identified a molecular signature of quiescent mouse MuSCs and used it to screen various media for their ability to maintain freshly isolated mouse MuSCs in the quiescent state. We selected 39 genes based on their relevance in the myogenic program and we performed a combinatorial quantitative (Q)-RT-PCR array analysis in a microfluidic chip to analyze single, freshly isolated quiescent MuSCs or single activated MuSCs that had been induced to proliferate for either 1.5 or 3.5 d in vivo by injection of cardiotoxin into muscle (Supplementary Table 1). Principal component analysis (PCA) revealed that quiescent and activated MuSCs formed distinct transcriptional clusters (Fig. 1b).

A separate analysis of the genes enriched in the two populations revealed clustering of genes highly expressed in quiescent MuSCs, defined here as ‘quiescence genes’ (e.g., Pax7, Notch2, Notch3, Hes1, HeyL, Cd34 and Foxo3) and genes highly expressed in activated MuSCs, defined here as ‘activation genes’ (e.g., Polyg, Tp53, EzH2 and Myod1) (Supplementary Fig. 1a). At the single-cell level, topological data analysis identified a molecular signature characteristic of quiescent MuSCs distinct from activated MuSCs (Supplementary Fig. 1b).

Next we used combinatorial Q-RT-PCR to screen for molecules that, when added to the medium, would promote expression of the unique quiescence transcriptional signature (Supplementary Table 1). From an initial panel of 50 compounds known or suggested in the literature to positively regulate cell quiescence, we selected the 10 molecules that showed the strongest propensity to prevent quiescent mouse MuSC proliferation (Supplementary Fig. 2). Based on the results of this screening, we chose to further test the calcitonin receptor ligand elcatonin36, the cAMP activator forskolin37; the p38 inhibitor SB203580 (refs. 28, 29), the FGF receptor inhibitor SU5402 (refs. 1, 2, 12) and TGFβ3, 5. We also found a previously unreported quiescence-promoting activity for somatostatin, the c-Met inhibitor MGCD-265 and the CDK/Aurora inhibitor JM-7706621 (Supplementary Table 2).

We then tested the effect of combinations of these compounds on MuSCs maintained for 2 d in culture (Supplementary Fig. 3). Using the combinatorial Q-RT-PCR array strategy for a set of 93 genes, we found conditions in which cultured MuSCs were very similar to freshly isolated quiescent MuSCs (Fig. 1c and Supplementary Fig. 3). We thus identified a defined, serum-free QM formulation (Supplementary Table 3) that maintained the transcriptional signature of quiescence for at least 2 d in culture (Supplementary Fig. 4a, b).

MuSCs maintained in QM had several characteristics of quiescent cells. One of the most obvious changes in activated MuSCs is a substantial increase in cell size relative to quiescent MuSCs4. We found that MuSCs cultured in QM for 2.5 d remained small, similar to the size of quiescent MuSCs (Fig. 1d). Furthermore, most of the MuSCs cultured in QM continued to express the quiescence marker CD34 and did not express the cell-cycle marker Ki67 (Fig. 1e). MuSCs cultured for 2.5 d in QM entered the cell cycle and began proliferating when switched to growth medium (GM) (Fig. 2a). However, if cultured for one or more days beyond that, they were much less responsive to GM (Fig. 2a).

Engineered muscle fibers support mouse MuSC quiescence

MuSCs isolated by fluorescence-activated cell sorting (FACS) are no longer associated with native myofibers as in the endogenous niche. Quiescent mouse MuSCs that had not been dissociated from native myofibers maintained their proliferative responsiveness for a longer time in QM compared with dissociated MuSCs (Fig. 2a). We hypothesized that the quiescent state could be prolonged by culturing MuSCs in QM on engineered muscle fibers (EMFs) that recapitulate key properties of native myofibers and the surrounding basal lamina.

Given the well-established role of substrate elasticity on the fate and
function of stem cells, we tested substrates of different elasticity using a micropost array with elasticity ranging from 2 kPa to 25 kPa, basing this range on the reported physiological ranges of elasticity of whole muscle and individual myofibers. We confirmed these values by performing atomic force microscopy (AFM) nanoindentation analysis on individual myofibers (Supplementary Fig. 4a). To determine the elasticity most conducive to mouse MuSCs remaining in the quiescent state, we maintained the cells for 2 d in culture in the presence of EdU to test for DNA synthesis and entry into the cell cycle. The propensity of the cells to remain quiescent directly correlated with substrate elasticity over the tested range (Supplementary Fig. 4b,c). Approximately 85% of the cells were quiescent at 2 kPa, whereas only ~45% remained quiescent at 25 kPa. Moreover, MuSCs on the most elastic microposts were most similar to quiescent MuSCs in terms of the percentage of cells expressing Pax7 and not expressing Myod1 (Supplementary Fig. 4d,c). We concluded that the substrate with greatest elasticity was most conducive to maintaining mouse MuSCs in a quiescent, undifferentiated state.

We also used an alternate method to generate substrates of variable stiffness. The extracellular matrix protein collagen I is biodegradable and biocompatible, and, being a lyotropic material, has uniquely tunable mechanical properties. We generated collagen-based hydrogels with elastic moduli ranging from 0.8 kPa to 2.5 kPa (Supplementary Fig. 5a). As with the micropost studies, entry into the cell cycle was delayed (Supplementary Fig. 5b). However, below ~1.5 kPa (i.e., with a collagen hydrogel formulation at 2.4 mg/ml), the activation of apoptotic programs, as judged by active caspase staining, increased (Supplementary Fig. 5c). We conclude that there might be an optimal elasticity between 1 kPa and 2 kPa, above which mouse MuSCs tend to activate and differentiate and below which they are more prone to...
undergo apoptosis. This is in accordance with a recent report showing that increased microenvironment stiffness above 2 kPa promotes myogenic cell proliferation.\(^\text{15}\)

We developed a microfabrication process on a microfluidic chip to generate EMFs with the quiescence-maintaining elastic properties identified in the micropost studies (Fig. 2b). A solution of collagen I was extruded to generate a scaffold with parallel nanofibrils, resulting from the collagen cholesteric chemical structure, and with a shape and geometry similar to those of a live myofiber (Fig. 2c and Table 1). The EMFs had an elasticity of 1.33 ± 0.18 kPa (Supplementary Fig. 6a), consistent with our previous measurements on collagen I hydrogel films. When we plated mouse MuSCs onto the EMFs (Fig. 2c and Supplementary Video 1), EDU incorporation was strongly reduced, consistent with maintenance of the quiescent state (Supplementary Fig. 6b). We also tested the mechanical stress capacity of EMFs to evaluate whether they had sufficient structural integrity for transplantation. The mechanical tensile modulus was ~27 kPa, which makes the EMFs robust to manipulation in a syringe and transplantation, similar to a myofiber (Supplementary Fig. 6c).

MuSCs are regulated by protein components of the niche, such as the extracellular matrix protein collagen VI (ref. 39). As MuSCs are closely apposed to the muscle fiber membrane in vivo, we identified muscle fiber membrane proteins that might interact with MuSCs to maintain their quiescence. We found that integrin α4β1 is expressed in the adult mouse niche on the muscle fiber membrane adjacent to quiescent MuSCs (Supplementary Fig. 7a,b). Quiescent MuSCs express VCAM\(^\text{40}\), and the primary integrin to which VCAM binds is integrin α4β1 (ref. 41). We found that integrin α4β1 was superior to other integrin heterodimers, such as α5β1, αVβ1 and α6β1, that have been implicated in MuSC biology, in terms of reducing EDU incorporation and inhibiting cell death in vitro (Supplementary Fig. 7c,d). Lamins are a key component of the basal lamina surrounding MuSCs\(^\text{47,48}\). It has been suggested that lamins are not only structural proteins of the basal lamina but are also signaling molecules that are important for the adhesion and localization of MuSCs in their niche\(^\text{48,49}\).

Based on these considerations, we coated the collagen-based EMFs with recombinant integrin α4β1 followed by recombinant laminin (Fig. 2d). When seeded onto the functionalized EMFs and cultured for 3 d, mouse MuSCs showed reduced activation as assessed by EDU incorporation, increased viability as assessed by ATP levels, and higher Pax7 and lower MyoD protein expression when compared to EMFs alone or functionalized with integrin α4β1 only (Supplementary Fig. 8a–c).

Finally, to study the specific role of elastic properties of EMFs in maintaining quiescence, we compared fully functionalized EMFs generated either as thick soft gels or as thin coatings on...
top of a rigid substrate. Consistent with previous experiments (Supplementary Fig. 5), MuSCs cultured for 36 h on thick functionali zed EMFs showed a lower degree of activation on softer substrates (Supplementary Fig. 8d). However, this difference was completely lost when we cultured MuSCs on thin functionalized EMFs, a condition where the different collagen formulations did not affect the higher stiffness of the rigid substrates to which they were attached. All of these results are consistent with the functionalized EMFs promoting a viable quiescent state by virtue of their biophysical and biochemical properties.

We next tested whether mouse MuSCs cultured on functionalized EMFs could maintain reversible quiescence in QM, as we observed for MuSCs in their native niche but not for isolated MuSCs on a two-dimensional tissue culture substrate (Fig. 2e). Indeed, functionalized EMFs facilitated the maintenance of reversible MuSC quiescence. When maintained in QM for 3.5 d, quiescent MuSCs activated in response to GM with kinetics similar to those of freshly isolated MuSCs plated in GM (Fig. 2e,f). With time, there was a gradual reduction in cell survival in QM, but even after 7 d of maintenance in QM, MuSCs could be induced to proliferate upon exposure to GM. We did not test time points beyond 7 d. Moreover, levels of ATP showed a sharp reduction when MuSCs were not associated with a fiber but not when associated with EMFs, suggesting that the EMFs promoted a sustained viability of cultured MuSCs in QM (Fig. 2g).

Figure 4 Transplant potency enhancement via the artificial niche. (a) Noninvasive in vivo bioluminescence imaging of freshly isolated mouse MuSCs (100 cells per condition) immediately transplanted into pre-injured TA muscles still associated with native myofibers (native fiber); isolated by FACS and plated onto EMFs in vitro prior to transplantation (EMF); or isolated by FACS and in suspension (no fiber) (n ≥ 4, biological replicates). (b) Noninvasive in vivo bioluminescence of MuSCs cultured for 2.5 d in QM, associated or not with a fiber as in a, prior to transplantation (50 cells per condition) and imaged weekly for one month (n = 5, biological replicates). (c) Representative bioluminescence images of a time-course analysis of one host mouse, quantified in b, that received 50 MuSCs transplanted in each TA muscle. The right leg (which is on the right side since the mouse is prone in each image) received MuSCs associated with EMFs; the left leg received MuSCs not associated with any fiber. (d) Representative immunofluorescence immunohistochemistry (IF-IHC) of luciferase expression in TA muscle cross-sections of mice imaged and quantified in b and c, isolated 40 d after transplantation. Scale bar, 100 µm. (e) Quantification of IF-IHC staining for luciferase+ fibers per cluster in TA muscles that were recipients of transplanted MuSCs. The average number of fibers per cluster per TA is shown; the number of clusters/TA was: native fibers, 4.6 ± 1.02; EMF, 2.6 ± 1.02; no fiber, 0.4 ± 0.48. Error bars, s.e.m.; *P < 0.05; **P < 0.001; ***P < 0.0001; ****P < 0.00001. P values were calculated using paired two-tailed Student’s t-test.

Figure 5 EMF maintains MuSC self-renewal capacity after in vitro manipulations. (a) IF-IHC staining of TA muscles transplanted with mouse MuSCs associated with EMFs with representative images of transplanted luciferase+ MuSCs (luciferase+ MuSCs localized between luciferase+ fibers are indicated by arrows and magnified in insets). Scale bars, 100 µm. (b) Results of noninvasive in vivo bioluminescence imaging of muscles that were recipients of transplanted luciferase+ MuSCs and reinnjured after 40 d (second injury) after the transplantation. The second injury was performed to test whether the bioluminescence signal increased as a consequence of activating and expanding luciferase+ MuSCs that were initially transplanted and that had engrafted under the basal lamina (n ≥ 4, biological replicates). (c) Quantification of the number of transplanted MuSCs expressing YFP that engrafted as stem cells. Cells were isolated and cultured in QM before transplantation in TA muscles. An injury was induced 40 d after transplantation. Ten days later, the percentage of MuSCs (VCAM+) that were donor-derived (YFP+) was assessed by FACS. (d) Noninvasive in vivo bioluminescence imaging of transduced and transplanted MuSCs. Isolated MuSCs were either cultured in EMFs or cultured in GM alone for 3.5 d. During culturing, cells were transduced with a lentivirus expressing luciferase, and 1,000 cells were then transplanted into preinjured TA muscles. Recipient mice were imaged by bioluminescence 30 d later. Error bars, s.e.m.; *P < 0.05. P values were calculated using paired two-tailed Student’s t-test.
We conclude that QM supports survival of MuSCs in the quiescent state without inducing activation and proliferation, and that this is enhanced when MuSCs are cultured on EMFs.

Preservation of the quiescence transcriptional signature

We analyzed the transcriptional profiles of single mouse MuSCs under three different in vitro conditions: maintained on EMFs and in QM, maintained on a two-dimensional substrate in QM, and maintained on a two-dimensional substrate in GM. Then we compared these profiles to the profiles of freshly isolated quiescent MuSCs. MuSCs maintained in QM and on EMFs clustered with quiescent MuSCs, whereas MuSCs cultured on two-dimensional substrates did not, regardless of whether they were maintained in QM (Fig. 2h). In a complementary approach, we generated a cell classification model using random forests, a machine-learning strategy that uses an ensemble of gene expression decision trees50. In this model, we predicted cell state as either quiescent or activated based on freshly isolated MuSC transcriptional profiles (Fig. 3a). This model performed well, with 86% accuracy during cross-validation (Fig. 3b,c). Confirming the preservation of quiescence by EMFs, 45 of 46 MuSC replicates cultured in QM on EMFs were classified as quiescent, in contrast to 8 of 33 MuSCs cultured in QM but on a two-dimensional substrate, and only 4 of 22 cultured without either EMFs or QM (Fig. 3d). Analysis of the importance of the genes used to construct this predictive model revealed that the genes most discriminating between quiescence and activation were, in this order of diminishing importance, Notch2, Heyl, Notch3, Myos1, Nfat5, Eya1, Hes1, Pax7, Hey1, Ezh2, Pten, Myf5, Cd34, Atp2a2 and Foxo3 (Fig. 3e). The single-cell distribution of expression of these genes in quiescent MuSCs was very similar to that of MuSCs cultured in QM on EMFs, as exemplified by Pax7, Notch3 and Nfat5 (Fig. 3f).

The artificial niche enhances the potency of MuSCs in vivo

We compared the engraftment potential of freshly isolated MuSCs, MuSCs in the artificial niche (the combination of EMFs and QM) and MuSCs associated with native fibers51,52. In each case, 100 cells were transplanted into TA muscles of immunocompromised mice that had been subjected to cardiotoxin-induced injury 12 h prior to create an environment that would promoteMuSC engraftment. MuSCs were all obtained from Pax7-CreER/ROSA26-LuSEAP mice that had previously been treated with tamoxifen, so the donor cells expressed luciferase, and we could assess their engraftment noninvasively. MuSCs associated with EMFs were far more potent than isolated MuSCs and nearly as potent as the same number of MuSCs associated with native fibers (Fig. 4a). These data demonstrate the importance of a niche-like environment in vivo for maintaining MuSC potency.

One characteristic of stem cell potency is the capacity of the cells to differentiate into new mature tissue16. To assess this, we cultured ~50 mouse MuSCs in the artificial niche for 2.5 d before transplantation. Bioluminescence signals in TAs transplanted with EMFs, similarly to myofibers, increased to a plateau. MuSCs alone could not engraft, likely owing to the challenging conditions of transplanting only 50 cells in a nonirradiated TA muscle (Fig. 4b,c). We confirmed by immunohistochemical analysis of the same transplanted muscles that the bioluminescence signal corresponded to regenerated muscle fibers (Fig. 4d,e).

Another important feature of stem cells is the capacity to self-renew16. Having shown that the artificial niche enhanced MuSC engraftment, we tested its effect on self-renewal, again with native-fiber-associated MuSCs as controls. First, we examined muscles that had been transplanted with MuSCs cultured in the artificial niche (as in Fig. 4) for evidence of luciferase-expressing cells in the satellite cell position. Indeed, we detected luciferase+ cells beneath the basal lamina of regenerating fibers (Fig. 5a). Second, we performed an injury, 40 d after the initial transplantation, on muscles previously injured and transplanted with luciferase-expressing MuSCs. In response to the second injury, any self-renewed, transplanted MuSCs, such as those shown in Figure 4b, would be expected to activate, proliferate and differentiate to form new muscle. Indeed, for native-fiber-associated MuSCs cultured in QM and MuSCs in the artificial niche, the bioluminescent signal increased after the second injury (Fig. 5b). Cells that had been previously transplanted without any niche not only showed very little steady-state bioluminescence before the second injury, but exhibited almost no increase after it.

To confirm that mouse MuSCs cultured in the artificial niche for 2.5 d retained self-renewal potential, we transplanted 1,000 quiescent
MuSCs expressing the reporter fluorescence protein ‘enhanced YFP’ (derived from a Pax7-CreER/Rosa26eYFP mouse) into a preinjured TA muscle. After 40 d, we performed a second injury with cardiotoxin, and 10 d later we isolated MuSCs by FACS and analyzed the number of YFP+ MuSCs. TA muscles transplanted with MuSCs associated with either native fibers or EMFs showed the presence of donor-derived MuSCs (Fig. 5e). Conversely, TA muscles transplanted with MuSCs not associated with any niche structure did not have donor-derived MuSCs. We conclude that MuSCs cultured in the artificial niche, similar to MuSCs associated with native fibers, maintain quiescence in culture and retain engraftment and self-renewal potential upon transplantation.

Finally, we tested the effect of our optimized culture conditions on the transplantation potency of MuSCs after genetic manipulation in vitro. We transduced MuSCs maintained in the artificial niche or on a two-dimensional substrate and cultured in GM (standard conditions) with a lentivirus expressing luciferase and GFP. One day later, we transplanted 1,000 MuSCs from each culture into preinjured TA muscles of immunocompromised mice. Bioluminescence measurement 30 d later showed that MuSCs maintained in the artificial niche during lenti-viral infection yielded, on average, a signal two orders of magnitude higher than MuSCs in standard conditions (Fig. 5d), suggesting that maintenance of a quiescent state by association with EMFs and treatment with QM might allow efficient genetic modification ex vivo without compromising MuSC potency.

The artificial niche supports quiescence of human MuSCs
We studied whether freshly isolated human (h)MuSCs respond similarly to mouse MuSCs in the artificial niche. First, we used FACS, as previously reported53,54, to isolate hMuSCs from surgical samples. Like mouse MuSCs, hMuSCs cultured for 2.5 d in QM modified for human cells remained small (similar to mouse EMFs but based on human proteins, and seeded Fig. 6d). Conversely, TA muscles transplanted with MuSCs not associated with any niche structure did not have donor-derived MuSCs. We concluded that MuSCs cultured in the artificial niche, similar to MuSCs associated with native fibers, maintain quiescence in culture and retain engraftment and self-renewal potential upon transplantation.

To test the transplantation potency of genetically modified hMuSCs cultured in the artificial niche, we replicated our experiments with mouse MuSCs (Fig. 5d) by transducing the luciferase gene into 1,000 hMuSCs, cultured either in our optimized conditions or in standard conditions and then transplanting them 1 d later into TA muscles of immunodeficient mice. 30 d later, hMuSCs cultured in our optimized conditions yielded, on average, a signal one order of magnitude higher than that of hMuSCs cultured in standard conditions (Fig. 6f). Taken together, these results suggest that hMuSCs can also be isolated and genetically manipulated in the artificial niche while retaining much greater potency than hMuSCs maintained under standard conditions.

DISCUSSION
Here we provide evidence that engineering a biomimetic microenvironment enables maintenance of quiescent, potent mouse MuSCs and hMuSCs. Our optimized condition consisted of a defined medium to maintain quiescence of MuSCs combined with a microscaffold in the shape of a muscle fiber. This condition replicated several biochemical and biophysical properties of the native niche: (i) geometry, (ii) elastic modulus, (iii) ECM protein composition and structural organization, and (iv) a cocktail of molecules that we found to modulate pathways involved in the regulation of MuSCs quiescence. The potential utility of our approach for therapeutic applications was shown by the enhanced engraftment and self-renewal potential of both mouse and human MuSCs cultured in the artificial niche compared to traditional conditions. In addition, MuSCs cultured in the artificial niche that we transduced with a lentiviral reporter engrafted after transplantation without loss of potency.

The loss of engraftment ability of cultured MuSCs is a major challenge in developing efficient strategies to manipulate isolated stem cells for cell therapy1,2,5,15,56. Research on MuSCs2, hematopoietic stem cells57 and neural stem cells22 has shown that very small numbers of quiescent stem cells, even single cells, can replace vast amounts of tissue; culture systems that maintain stem cell quiescence may allow these findings to be translated to clinical practice. In addition, the possibility of culturing hMuSCs for longer time periods without loss of potency in order to correct mutations associated with genetic disorders, such as muscular dystrophy, followed by transplantation of the corrected cells to replace the pathogenic tissue may enable improved stem cell therapeutics for muscle disorders55,56. Finally, transplantation of quiescent hMuSCs seeded on microscaffolds may facilitate tissue engineering to treat traumatic injuries, as in the case of volumetric muscle loss58.

METHODS
Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
M.Q. conceived and designed most of the experiments reported. T.A.R. provided guidance throughout. M.Q., J.O.B., A.D.M. and S.C.B. performed experiments and collected data. S.H. provided guidance throughout. M.Q., R.D. and J.S. designed EMF fabrication and performed EMF experiments. J.O.B. analyzed single cell gene expression data and developed the random forests model. M.Q. and R.D. designed and performed AFM experiments and analysis. M.Q., A.D.M. and S.C.B. designed, performed and analyzed single cell gene expression experiments and the screening for factors promoting quiescence of MuSCs. M.Q. and R.C. performed transplant experiments, in vivo imaging and data analysis. M.Q. and M.C.G. designed fabrication and experiments with microfluidic chips for EMF’s. M.Q. and J.S. designed the EMF and performed EMFs fabrication experiments and imaging. V.A.G. performed the experiments with the human MuSCs. J.B.S. collected.
ONLINE METHODS

Animals. C57BL/6, ROSA26^{ERT2} and B6.Cg-Foxn1nu/J mice were obtained from Jackson Laboratory. Pax7-Cre^{ERT2} mouse and ROSA26^{LacZ/cre} were provided by C. Keller, Oregon Health and Science University, Portland. Tamoxifen injections for Cre recombinase activation were performed as described previously. To control for tamoxifen injection toxicity, we injected all mice with tamoxifen. All experimental mice used were 3–6 months old. Mice were housed and maintained in the Veterinary Medical Unit at the Veterans Affairs Palo Alto Health Care Systems. Animal protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University.

Human skeletal muscle specimens. Subjects ranged in age from 38 years to 85 years. All experiments were performed using fresh muscle specimens from operative procedures. Sample processing for cell analysis began within 1 h of specimen isolation. In all studies, standard deviation reflects variability in data derived from studies using true biological replicates (i.e., unique donors). Data were not correlated with donor identity.

MusC isolation and FACS. MusCs were isolated as previously described. Briefly, muscles were collected from hind limbs, dissociated to yield a fragmented muscle suspension using a gentle MACS dissociator (Miltenyl Biotec), and digested with collagenase II (100 units per ml; Invitrogen) in Ham’s F10 medium for 90 min. After washing, a second digestion was performed for 30 min with collagenase II (100 units per ml) and dispace (2 units per ml; Invitrogen). The resulting mononuclear cell suspension was washed, filtered and stained with VCAM-biotin (clone 429; BD Bioscience), CD31-APC (clone MEC 13.3; BD Bioscience), CD45-APC (clone 30-F11; BD Bioscience) and Sca-1-Pacific-Blue (clone D7; BioLegend) antibodies at a dilution of 1:75. Streptavidin-PE-cy7 was used to amplify the VCAM signal (BD Bioscience, 1:75) and FACS-grade DAPI dilactate for nonvital cell exclusion (D3571, BD Bioscience). Cells were then filtered and stained with anti-CD31-Alexa Fluor 488 (clone W6/32; BioLegend; #318319). Unbound primary antibodies were then washed and stained with NCAM-biotin (clone 429; BD Bioscience), CD31-APC (clone TS2/16; BioLegend; #303008) and anti-NCAM-Biotin (clone HCDS6; BioLegend; #318319). Unbound primary antibodies were then washed and the cells incubated for 15 min at 4 °C in streptavidin-PE/cy7 (BioLegend) to detect NCAM-biotin. The machine was carefully optimized for purity, viability and calibration for single-cell sorting. A small fraction of sorted cells was plated and stained for Pax7 and MyoD to assess the purity of the sorted population.

Single-cell Q-RT-PCR. MusCs were FACs sorted individually into 96 well plates prepared with 9 ml RT-STA buffer (5 ml CellsDirect 2× reaction mix (Life Technologies, PN 11753-500), 0.2 µl Superscript III Platinum Taq mix, 2.7 µl nuclease-free water, 0.1 µl SUPERase In RNase inhibitor (Life Technologies) and 1 ml 10× primer mix (96 × 1 ml of 100 nM primer pairs plus 104 µl DNA suspension buffer)). Plates were spun down and stored at −80 °C to ensure complete cell lysis. Next, plates were thawed and subjected to reverse transcription (RT) (15 min at 50 °C (RT reaction)), 2 min at 95 °C (to inactivate reverse transcriptase and activate the Taq polymerase), and subjected to a pre-amplification reaction (15 s at 95 °C, 4 min at 60 °C (for 20 cycles)). Preamplified cDNAs were treated with exonuclease I (2.52 µl water, 0.36 µl buffer, 0.72 µl exonuclease I (New England BioLabs, PN M0293E)) for 30 min at 37 °C, followed by 15 min inactivation at 80 °C. Exonuclease treated cDNAs were diluted 5× in DNA suspension buffer (TEKanova, PN T0021) and stored at −80 °C. To perform the Q-RT-PCR on the Fluidigm Biomark HD, sample mixes were prepared with 2.7 µl of diluted cDNAs mixed with 3.0 l of 2× SooFast EvaGreen Supermix with low ROX (Bio-Rad, PN 172-5211) and 0.3 l of 20× DNA Binding Dye Sample loading reagent (Fluidigm, PN 100-0388), vortexed and spun down. Separately, to create the assay mix, 0.3 µl of 100 µM primer mix was combined with 2.5 µl 2× Assay Loading Reagent (Fluidigm, PN 8500073) and 2.25 µl DNA suspension buffer, vortexed and spun down. A 96.96 Dynamic Array IFC (Fluidigm) was primed on an IFC ControllerHX (Fluidigm). The primed Dynamic Array was loaded with the assay and sample mixes using an IFC ControllerHX. Finally PCR was performed on a Biomark HD, using protocol GE 96x96 PCR-Melt v2 (2,400 s at 70 °C, 30 s at 60 °C, 60 s at 95 °C hot start, 30 cycles (5 s at 95 °C, 20 s at 60 °C), followed by a melt curve). Biomark data was analyzed using Q-RT-PCR Analysis software (Fluidigm). Data were exported into Microsoft Excel for further analysis. For each amplicon, a limit of detection (LOD) was determined by running tenfold serial dilutions of embryonic cDNA in six replicates. Limit of detection was set to the lowest dilution for which all six replicates give a PCR signal. This value was set to denote the amplification of 2–10 copies of transcript per reaction chamber. Primers are listed in Supplementary Table 4.

Single-cell data analysis and molecular signature. Raw cycle threshold (Ct) values were filtered and normalized according to the manufacturer’s instructions and are represented in Supplementary Table 4. Log, (expression values) were used for all analyses. For analyses involving multiple experiments (random forests and violin plots), only genes with primers common to all experiments were used, and datasets were first batch-corrected using COMBAT. For PCA, genes were mean-centered and unit-variance-scaled. k-medians clustering with k-means++ seeding using Manhattan distance was performed for genes, using k = 2, Random forest construction was done using 8,000 trees and 25 genes with 5 random genes selected at each node. Conditional variable importance for the genes involved in random forest construction was calculated as the mean decrease in out-of-bag (OOB) accuracy of the trees after permuting that gene within sample subsets split by correlated genes with an association P < 0.2. P values for these conditional variable importance measures were based on 1,000 permutations of the response variable to mimic the distribution of variable importance when no gene has predictive value. Hierarchical clustering used Manhattan distance and complete linkage after mean-centering and UV-scaling genes. P values are approximately unbiased values calculated using multiscale bootstrap resampling with 10,000 resamples at ten sizes. Scripts generated in R computational language to analyze the data are posted at http://www.stanford.edu/~jbrett/R_Scripts/Quarta2014/.

Topological data analysis methods were used to identify the quiescent population signature in an unsupervised fashion using the Ayasdi Iris software (Ayasdi Inc.). Topological data analysis benefits from properties inherent in the classic topological methods useful for characterizing shape, as applied to point cloud data. These properties include deformation invariance in the form of relative noise insensitivity; compressed representation, as reified by the partitioning and clustering steps; and coordinate freedom that relies only on the notion of similarity between points. Although mathematical details regarding the method of construction for the topological network are described in complete form elsewhere, we summarize the approach here briefly. Two key parameters are needed for generating a topological analysis: a notion of similarity, or metric, and filter(s). For each point X, in this case a single cell, a filter function f is applied yielding some real-valued output Y. Based on the value of Y for each X, the points are binned with some overlap, the span of which is then, for each X and for all bins, a metric is used to determine the distance d(X,Y) in the original space. A partial clustering scheme is finally used to aggregate points within bins and form connections between points across multiple bins. In the topological network, the aggregated points form the nodes (and thus collections of points), and the edges appear when two aggregations in the clustering contain one or more of the same points. The method described above essentially generates Reeb graphs from the data, the result of which can be used for identification of natural segmentations of points for noisy data, and has been useful in identifying patterns in complex, high-dimensional datasets. In the present case, the data are based on population signatures across 35 genes over the single cells. For this dataset, the metric used was correlation, and the functions applied were principal and secondary components (metric-embedded). The resulting representation revealed a distinct segregation of the quiescent population. A nonparametric statistical test (Kolmogorov-Smirnov) was used to identify the most significant markers for the quiescent population relative to the entire dataset.
Single-fiber explants. Extensor digitorum longus (EDL) or flexor digitorum brevis (FDB) muscles were excised and digested in collagenase II (500 units per ml in Ham’s F10 medium) as previously described. Fibers were then washed extensively and cultured in medium containing Ham’s F10, 10% horse serum and 0.05% chick embryo extract. Every 24 h, 50% of the medium was replaced with Ham’s F10 medium with 20% fetal bovine serum (FBS). EDL fibers were cultured in suspension. Fixed fibers were stained and the number of MuSCs was quantified per fiber.

Micropost arrays. Elastomeric micropost arrays were generated with polydimethylsiloxane (PDMS) by replica molding as published elsewhere. Briefly, PDMS prepolymer was poured over a template containing an array of holes, degassed under vacuum, cured at 110 °C for 20 h, and peeled off the template. Microposts were fabricated 1 µm in diameter and 3 µm of center-to-center distance; elasticity of the microposts was changed by modulating the amount of cross-linker and their length to obtain nominal spring constants of 1.9223 nN/µm, 7.22106 nN/µm, 18.1905 nN/µm, and 32.0312 nN/µm for effective moduli of approximately 2 kPa, 6 kPa, 12 kPa and 25 kPa, respectively.

Injections. Mice were anesthetized using isoflurane through a nose cone. Muscle injury was induced by injecting 20 µl of cardiotoxin (Invitrogen) into TA muscles.

Transduction. Luciferase and GFP reporters were subcloned into a third generation HIV-1 lentiviral vector and used to transduce freshly isolated MuSCs.

Histology and immunohistochemistry. For hematoxylin and eosin staining, TA muscles were dissected and directly frozen in OCT (Tissue-Tek). For hematoxylin and eosin stain –

Atomic force microscopy. AFM was used to analyze collagen-based hydrogel properties. Gels were made from bovine tendon collagen I (Cellmatrix I-A, Nitta Gelatin). The collagen concentrations that were tested ranged from 1.8 mg/ml to 2.7 mg/ml. Varying collagen concentrations were achieved by reducing the volume of culture stock solution (3.0 mg/ml) and replacing that volume with an equivalent amount of 10x Ham’s F12 medium (Life Technologies) without sodium bicarbonate. Reconstitution buffer volumes were kept constant across all conditions (Table 1). Reconstitution buffer was composed of 2.2 g NaHCO3 (Sigma-Aldrich) in 100 ml of 0.05 N NaOH and 200 mM HEPES.

Gel films were generated according to protocol and kept on ice prior to casting onto glass surfaces for testing. Samples were given 30 min to gel at 37 °C and then rehydrated with phosphate buffer solution (PBS, pH 7.0 Life Technologies) for a minimum of 15 min prior to testing. Similarly, individual muscle fibers were adhered to glass surfaces prior to testing and submerged in PBS. Gel and muscle fiber samples were tested while remaining submerged in PBS. Short silicon contact mode probes (SHICON, Applied NanoStructures) were used to collect individual force measurements from all samples. Probes were rinsed with FBS (Life Technologies) before testing to prevent nonspecific adsorption of samples to the probe tip. Force measurements were collected using 1 µm force distances and 1 Hz scanning rates with a 0.5 V trigger point upon contact. The integral gain was set to 10 because of the submersed testing conditions. A Hertz indentation model for cone tip geometry was used to fit the data. Poisson ratios of 0.4 and 0.33 were assumed for collagen gels and primary tissue, respectively. All AFM data were collected using an Asylum Research MFP-3D-BIO.

EMF tensile testing analysis. Collagen fibers were extruded and transferred to 1.5 ml Eppendorf tubes filled with PBS. Eppendorf tubes were rinsed once with PBS prior to use to prevent nonspecific adsorption of fibers to the tube walls. Tubes were then sealed, placed on ice, and transferred to the CellScale Biomaterials Testing Facility. All tensile testing was performed on-site at the CellScale facility using the Microsquisher device. EMF samples were tested in a hydrated state. One end of the EMF fiber was fixed in place and the other was attached to the moving force transducer wire of the Microsquisher device. This enabled collection of mechanical data in a tensile rather than compressive mode. Force versus displacement curves were then converted to stress versus strain curves for calculation of the Young’s moduli.

Master mold fabrication. All photo masks were designed using SolidWorks (SolidWorks Corp.) and printed at a resolution of 20,000 dots per inch on a transparency film (CAD/Art Services). Master Mold fabrication was performed as described elsewhere. Briefly, the flow-layer master was fabricated from a combination of positive and negative photoreists using a three-step lithography process. Channel sections were fabricated from the resist material SU-8 2010. SU-8 2010 (MicroChem) was spun onto a silicon wafer (3,000 r.p.m. for 45 s), baked before exposure (1 min at 65 °C, then 3 min at 95 °C), exposed through a negative transparency mask (40 s at 7 mW/cm²), baked after exposure (1 min at 65 °C, then 3 min at 95 °C), and developed in an SU8 nanodeveloper (MicroChem). Channel sections were fabricated using the positive photoreist SJR 5740 (MicroChem). To promote photoreist adhesion, the wafer was first treated with hexamethyldisilazane (Microprime HP-Primer; ShinEtSU MicroSi, Phoenix) (1 min at 1 atmosphere). The photoreist was spun onto the patterned wafer (2,000 r.p.m. for 60 s), soft baked (1 min 45 s at 95 °C), aligned to the existing features, exposed (45 s at 7 mW/cm²), and developed (20% Microposit 2401 developer; MicroChem). The mold was then annealed (20 min at 120 °C), and hard baked (2 h at 170 °C). Low-impedance input and output channels were fabricated to allow for the rapid flushing of viscous reagents. A 60 mm layer of SU8 2075 (MicroChem) was spun onto a silicon wafer (3,000 r.p.m. for 60 s), baked before exposure (7 min at 65 °C, then 20 min at 95 °C), aligned to the primary flow structure, and exposed through a negative transparency mask (40 s at 7 mW/cm²), baked after exposure (1 min at 65 °C, then 15 min at 95 °C), and developed in an SU8 nanodeveloper (MicroChem). Control features (25 mm high) were fabricated on a separate wafer using a single lithographic step. SU-8 2025 (MicroChem) was spun onto a silicon wafer (3,000 r.p.m. for 45 s), baked before exposure (1 min at 65 °C, then 3 min at 95 °C), aligned to the primary flow structure, exposed through a negative transparency mask (40 s at 7 mW/cm²), baked after exposure (1 min at 65 °C, then 3 min at 95 °C), and developed in an SU8 nanodeveloper (MicroChem).

Microfluidic device fabrication. The microfluidic chip was fabricated as previously described. Briefly, using silicone elastomer (General Electric RTV 615) the technique of multilayer soft lithography was applied. To facilitate the release of the elastomer from the mold, all molds were treated with chlorotrimethylsilane (Aldrich). Liquid silicone elastomer (20 parts A:1 part B) was spun onto the control master (2,400 r.p.m. for 60 s) and baked in a convection oven at 80 °C for 60 min. Liquid silicone elastomer (5 parts A:1 part B) was poured on the flow master to a thickness of 7 mm, degassed, and baked at 80 °C for 75 min. The bonded elastomer was then peeled from the control mold, and access ports were punched at the flow and control inlets using a 0.055-inch punch (Technical Innovations). The device was peeled from the silicon wafer, cut to size, and sealed to a glass substrate for mechanical rigidity via plasma bonding. The final design, sized to fit a standard 22 mm × 50 mm glass coverslip, consisted of two rows of 20 culture chambers (500 µm × 300 µm × 7 mm) each, with media inlet and outlet ports and smaller (50 µm × 50 µm) flow channels connecting the culture chambers of each row. This design was chosen because it allows for the side-by-side comparison of two different culture conditions on the same chip, thus eliminating any undesired variability in handling or manipulation between experimental conditions while maintaining identical conditions within experimental groups. The photomasks, master mold and PDMS chips were fabricated at the Stanford Microfluidics Foundry based using multilayer soft lithography. After that, 18G needles were cut down to ~1.5 cm and inserted into the inlet and outlet ports where they were secured with additional PDMS. Tygon tubing (1/16 inch) was used to connect the inlet needles to a SP220I syringe pump (World Precision Instruments), which was used to control all subsequent fluid manipulations within the device.

Artificial muscle fiber fabrication. The collagen used was bovine tendon collagen I (Nitta Gelatin) or collagen solution from human fibroblasts.
(Sigma-Aldrich). All polymerization procedures were carried out using sterile techniques and sterile solutions. Polymerization was achieved by mixing monomeric collagen solution, F10 medium, and reconstitution buffer at a ratio of 8:1:1, according to manufacturer’s instructions. Lower modulus collagen was achieved by decreasing the amount of collagen solution and replacing that volume with F10 medium. All solutions and mixtures were kept on ice to slow down polymerization. Collagen fibers were then produced by extruding through a 265 fine-gauge syringe needle (Hamilton Company) with an internal needle diameter of 0.127 mm. The syringe plunger was pushed at a constant rate of 5 µl/min by a SP220I syringe pump (World Precision Instruments), volume setting to 10 µl and inner syringe diameter to 0.461 mm. All collagen fibers were extruded into warm PBS in Petri dishes or through the PDMS in microchambers. The chip chambers were precoated with 10% horse serum for 10 min and rinsed twice with PBS prior to storing the collagen fibers. The coating step was performed to prevent nonspecific adhesion of collagen fibers to the chambers. For functionalizing the collagen microfibers, mouse or human recombinant integrin α4β1 was perfused in the chip and absorbed on the surface of the collagen fiber by incubating for 1 h. The scaffold was then washed by perfusing with PBS for 30 min. A third functionalization step was performed by perfusing mouse or human laminin and incubating for 1 h. A washing step was performed by perfusing with PBS for 1 h. FACS-separated mouse or human MuSCs were then perfused in the chamber, allowed to adhere to the scaffold, cultured in the chip until fixed with 1% PFA and removed for analysis or used unfixed for transplantation. Cell density in the media perfused was titrated to obtain an average number of 10 ± 5 to 100 ± 20 (according to the experiment) MuSCs per EMF.

Transplantation. Recipient nude mice were pretrained with cardiotoxin under anesthesia 12 h before transplantation. Mice received randomly via intramuscular injection 50−1,000 MuSCs either in suspension, associated with viable muscle fibers or associated with EMFs. Live MuSCs (expressing YFP, GFP or immunostained) were counted at a fluorescence microscope before transplantation. The injection was performed with a pulled transparent glass needle precoated with FBS, which was connected with silicon tubing, preloaded with mineral oil, to a Hamilton syringe. The syringe was controlled by an automated micropump (SP220I syringe pump, World Precision Instruments). Cell suspension or individual fibers were carefully loaded in the needle under a microscope. A small opening of the skin was performed to expose the TA muscles. The needle was inserted into the proximal TA muscle and the cells or fibers were slowly injected into the muscle. The needle was left undisturbed for 5 min in the muscle. Before extracting the needle, the injection site was scaled with surgical glue (Tiessel, fibrin sealant, Baxter) to prevent leaking of cells or fibers. The skin wound was carefully sutured with one point stitch (Coated Vicryl suture, 8-0, Ethicon).

Bioluminescence imaging. Bioluminescence imaging was performed using the Xenogen IVIS-Spectrum System (Caliper Life Sciences). Mice were anesthetized using 2% isoflurane and 100% oxygen at a flow rate of 2.5 l/min by a SP220I syringe pump (World Precision Instruments). Cell suspension or individual fibers were carefully loaded in the needle under a microscope. A small opening of the skin was performed to expose the TA muscles. The needle was inserted into the proximal TA muscle and the cells or fibers were slowly injected into the muscle. The needle was left undisturbed for 5 min in the muscle. Before extracting the needle, the injection site was scaled with surgical glue (Tiessel, fibrin sealant, Baxter) to prevent leaking of cells or fibers. The skin wound was carefully sutured with one point stitch (Coated Vicryl suture, 8-0, Ethicon).

Bioluminescence imaging analysis. Analysis of each image was performed using Living Image Software, version 4.0 (Caliper Life Sciences). Briefly, a manually generated circle was placed on top of the region of interest and resized to completely surround the limb or the specified region on the recipient mouse. Imaging was performed in a blinded fashion: the investigators performing the analysis did not know the identity of the experimental conditions for the transplanted cells.

Bioluminescence analysis of ATP levels. ATP levels were measured using the CellTiter-Glo (Promega) luminescence assay as described by the manufacturer.

Scanning electron microscopy. Single myofibers or EMFs were fixed (4% PFA and 2% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.3) at 4 °C, post-fixed in 1% OsO4, and transferred to microporous (120 µm) cylindrical capsules (Electron Microscopy Sciences) for gradual dehydration in ethanol, followed by critical point drying (Tousimis). Samples were then mounted onto aluminum stubs, Au/Pd sputter-coated and imaged with a Zeiss Sigma FESEM operated at ~2–5 kV with SE detection. Hydrated samples were similarly fixed and then mounted fully hydrated onto the chamber in a Coolstage (Deben) for imaging with a Hitachi 3400N Variable Pressure (VP) SEM (Hitachi Northridge) operated at 15 kV and 50 Pa using BSE detection in VP mode.

Recombinant proteins. The recombinant proteins used in this study were, for mouse: integrin α4β1 (R&D, 7810-A6-050); integrin α5β1 (R&D, 7728-A5-050); integrin αVβ1 (R&D, 7705-AV-050); integrin α6β1 (R&D, 7810-A6-050); TGFβ1 (R&D Systems, 7666-MB-005); and laminin (Invitrogen, 23017-015). For human, recombinant proteins used were: integrin α4β1 (R&D 5668-A4-050); laminin (BioLamina, LN-211); and collagen (Sigma-Aldrich, C2249).

Immunofluorescence. Immunofluorescence analysis was performed using a Zeiss Observer Z1 fluorescence microscope equipped with a Hamamatsu Orca-ER camera or a Zeiss confocal system LSM710. Data acquisition and fiber-diameter measurements were performed using Improvision Velocity software (PerkinElmer) or Zeiss LSM ZEN software.

Antibodies and staining. Antibodies used in this study were to the following proteins, with the source of each antibody indicated: PAX7 (DSHB); Ki67 (Abcam 15580 and BD 558615); CD34 (BioLegend 343501 and BioLegend 119213); laminin (Sigma L9393, Millipore MAB1903 and One World Lab C130701); cleaved Caspase3 (Cell Signaling 9669); MyoD (Dako M3512; Santa Cruz sc-760); GFP (Invitrogen A11122 and Abcam ab31970); collagen I (Cedarlane clone 50151AP); integrin α4β1 (ABcam ab8891-100 and ABcam ab24695). EdU chemical staining was performed as indicated by the manufacturer (Invitrogen). (Supplementary Note.)

Statistical analyses. Unless otherwise noted, all statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). All error bars represent s.e.m.; **P < 0.05; ***P < 0.001; ****P < 0.0001.

Sample size. A minimal number of animals necessary to obtain statistically relevant experimental data was predicted and used. The size of groups needed was determined based on previous studies or preliminary data. The number needed per group was estimated based on expected magnitude of any measurable outcome to achieve statistical significance, and was based to achieve a power to 80% and P < 0.054. The coefficient of variation ranged from <5% to ~30%. To detect difference of 20% with a 10% standard deviation (s.d.) (using the bioluminescence assays as the limiting assay, to calculate the highest variability), four mice per group was calculated to be required (two TA muscles per mouse); with a 20% s.d. of the assay, 13 (26 muscle) mice per group; with a 30% s.d. of the assay, 20 mice (40 muscles). Thus, based on the variation used in our previous work, it was expected that 4–13 mice with each group was required.

General methods. Unless stated otherwise, sample size (n values) are reported as biological replicates of mice and/or SC isolations from separate mice performed on 28 different days. In most cases, the data presented were compiled over three years, as mice with the appropriate genotype became available. Therefore the magnitude of the effect and variability in the measurements were primary factors in determining sample size and replication of data. Although samples were not explicitly randomized or blinded, mouse identification numbers were used as sample identifiers and thus the genotypes and experimental conditions of each mouse/sample were not readily known or available to the experimenters during sample processing and data collection. The only criteria used to exclude samples involved the health of the animals, such as visible wounds from fighting. In these cases, the animals were handled in accordance with approved IACUC guidelines.
Study approval. Animals were handled and housed according to the guidelines set forth by the Veterinary Medical Unit of the VA Palo Alto Health Care System, and all procedures were approved by the IACUC prior to being performed. For human subjects, all operative specimens were obtained with appropriate written informed consent according to a protocol approved by the Stanford University Institutional Review Board.

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