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 potency1–2. Recent studies suggest that stem cell potency depends on the cells’ capacity to remain quiescent before their activation by regenerative stimuli such as injury3–5. For many stem cell populations, such as skeletal MuSCs1, 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 humans4,8–10. 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 vivo8,11–13. These properties have been identified as promoting quiescence in several tissue compartments7,12,14–16.

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 determination11,17–21. What has not been well modeled are niche components that promote and maintain stem cell quiescence12,14,22. 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 differentiation1,11,23. 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 vivo1,2,23. 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 fibers24,25. 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 ROSA26LasEAP 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), labelled 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 cells, 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., Polg, 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 elcatonin, the cAMP activator forskolin, the p38 inhibitor SB203580, the FGF receptor inhibitor SU5402, and TGF-β3,5,30. We also found a previously unreported quiescence-promoting activity for somatostatin, the c-Met inhibitor MGCD-265 and the CDK/Aurora inhibitor MJF-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 Table 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 MuSCs. 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 when in GM (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 myofilaments. We confirmed these values by performing atomic force microscopy (AFM) nanoindentation analysis on individual myofibers. To determine the elasticity most conducive to mouse MuSCs remaining in the quiescent state, we maintained the cells for 2 d 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. 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. 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 lyotrophic material, has uniquely tunable mechanical properties. In addition, collagens are components of the basal lamina, which forms a critical component of the MuSC niche in vivo. We generated collagen-based hydrogels with elastic moduli ranging from 0.8 kPa to 2.5 kPa. As with the micropost studies, entry into the cell cycle was delayed. 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. 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.

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, 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. 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.

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
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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 those 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 trees. 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 on an independent dataset (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 similar, in this order of diminishing importance, Notch2, Heyl, Notch3, Myo1s, Nfat5, Eya1, Hey1, 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 fibers. In each case, 100 cells were transplanted into TA muscles of immunocompromised mice that had previously been subjected to cardiotoxin-induced injury 12 h prior to create an environment that would promote MuSC engraftment. MuSCs were all obtained from Pax7-CreER/ROSA26LuSEAP 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 vitro for maintaining MuSC potency.

One characteristic of stem cell potency is the capacity of the cells to differentiate into new mature tissue. To assess this, we cultured ~50 mouse MuSCs in the artificial niche for 2.5 d before transplantation. Bioluminescence signals in TA muscles 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-renew. 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 regenerated 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
Table 1  Collagen matrix compositions used to generate encapsulating matrices

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Collagen stock (μl)</th>
<th>Ham's F12 (μl)</th>
<th>Reconstitution buffer (μl)</th>
<th>Collagen/media ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7</td>
<td>450</td>
<td>0</td>
<td>50</td>
<td>9:0:1</td>
</tr>
<tr>
<td>2.4</td>
<td>400</td>
<td>50</td>
<td>50</td>
<td>8:1:1</td>
</tr>
<tr>
<td>1.8</td>
<td>400</td>
<td>150</td>
<td>50</td>
<td>6:3:1</td>
</tr>
</tbody>
</table>

MuSCs expressing the reporter fluorescence protein ‘enhanced YFP’ (derived from a Pax7-CreERT2/Rosa26/YFP mouse) into a preinjured TA muscle. After 40 d, we performed a second injury with cortico- toxin, 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. 5c). 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 lentiviral 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 (Fig. 6a). We then generated EMFs similar to mouse EMFs but based on human proteins, and seeded hMuSCs onto their surface in either GM or QM (Fig. 6b,c). The EMFs facilitated the maintenance of reversible hMuSC quiescence for up to 3.5 d in QM; even at that point, the cells could exit quiescence and begin proliferating when cultured in GM (Fig. 6d,e).

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 cells37 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.

Note: Any Supplementary Information and Source Data files 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. and M.C.G. designed the EMF and performed EMF fabrication and experiments with microfluidic chips for EMFs. M.Q. and M.C.G. designed fabrication and experiments with microfluidic chips for EMFs. 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...
operativeswageduringsurgeries.M.Q.andaT.A.R.analyzeddataandwrote
the manuscript.

COMPETING FINANCIAL INTERESTS

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MuSCs were isolated as previously described. Briefly, mice were collected from hind limbs, dissociated to yield a fragmented muscle suspension using a gentle MACS dissociator (Miltenyl Biotec), and digested with collagenase II (500 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 dispase (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 Biosciences, 1:75) and FACS-grade DAPI dilactate for nonvital cell exclusion (D3571 Invitrogen). Cell isolation was performed on a BD-FACS Aria II or BD FACSAria III equipped with 488-nm, 633-nm and 405-nm lasers to obtain the MuSC population. For single-cell sorting, this cell population was sorted a second time to obtain individual cells. Human MuSCs were purified from fresh 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.

Single-cell Q-RT-PCR. MuSCs were FACs sorted individually into 96 well plates prepared with 9 μl RT-STA buffer (5 μl CellsDirect 2× reaction mix (Life Technologies, PN 11753-500), 0.2 μl Superscript III Platinum Taq mix, 2.7 μl nuclelease-free water, 0.1 μl SUPERase In RNase inhibitor (Life Technologies) and 1 μl 10× primer mix (96 × 1 μl of 100 μM 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 min 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 5x in DNA suspension buffer (TEKnova, PN T0221) 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 μl of 2× SoFast EvaGreen Supermix with low ROX (Bio-Rad, PN 172-5211) and 0.3 μl 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.
Collagen fibers were extruded and transferred. The collagen used was bovine ten.

Elastomeric micropost arrays were generated with.

For hematoxylin and eosin stain. The wafer was first treated with hexamethyldisilazane (Microprime HP-Primer; ShinEtsu MicroSi, Phoenix) (1 min at atmosphere). The photoresist 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 SUB 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. SUB 2025 (MicroChem) was spun onto a silicon wafer (3,000 r.p.m. for 45 s), baked before exposure (1 min at a separate 60 mm layer of SUB 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 3 min at 95 °C), aligned to the primary flow structure, 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 poured 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 22020 syringe pump (World Precision Instruments), which was used to control all subsequent fluid manipulations within the device.

ARTIFICIAL MUSCLE FIBER FABRICATION. The collagen was used as bovine tendon collagen I (Nitta Gelatin) or collagen solution from human fibroblasts.
Antibodies used in this study were to the follow-

Bioluminescence imaging was performed using

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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. Then 300 µl of 50 mg/ml sterile t-Luciferin (Biosynth International Inc.) dissolved in PBS was administered by intraperitoneal injection. After 23 min from the substrate injection, the mice were imaged for 30 s at the maximal light collection (f-stop, 1) at the highest resolution (small binning). Each image was saved for subsequent analysis. Imaging was performed in a blinded fashion: the investigators performing the imaging did not know the identity of the experimental conditions for the transplanted cells.

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 groups.

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