Skeletal muscle regeneration via the chemical induction and expansion of myogenic stem cells in situ or in vitro

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Muscle loss and impairment resulting from traumatic injury can be alleviated by therapies using muscle stem cells. However, collecting sufficient numbers of autologous myogenic stem cells and expanding them efficiently has been challenging. Here we show that myogenic stem cells (predominantly Pax7+ cells)—which were selectively expanded from readily obtainable dermal fibroblasts or skeletal muscle stem cells using a specific cocktail of small molecules and transplanted into muscle injuries in adult, aged or dystrophic mice—led to functional muscle regeneration in the three animal models. We also show that sustained release of the small-molecule cocktail in situ through polymer nanoparticles led to muscle repair by inducing robust activation and expansion of resident satellite cells. Chemically induced stem cell expansion in vitro and in situ may prove to be advantageous for stem cell therapies that aim to regenerate skeletal muscle and other tissues.

Skeletal muscle is the most abundant tissue in the human body and has numerous physiological functions that extend beyond locomotion to other diverse vital roles, including signal transduction. After an injury, the ability of skeletal muscles to regenerate depends on resident myogenic stem cells, such as satellite cells, which are localized beneath the basal lamina of myofibres and express the paired-box transcription factor Pax7 (ref. 2). Quiescent satellite cells are activated after muscle injury to divide, differentiate and repair the damaged tissue. However, this regeneration ability is compromised by severe acute muscle loss after traffic accidents, blast injuries, combat injuries and surgical resections, or by progressive muscle loss with aging atrophy and inherited genetic diseases such as Duchenne muscular dystrophy (DMD)4–8, resulting in disability and poor quality of life.

Muscle stem-cell-based therapies provide promising strategies for improving skeletal muscle regeneration9–11. However, the regenerative potential of muscle is limited by the paucity of autologous muscle stem cells and the need for concomitant immunosuppression of allogeneic cells. Furthermore, in vitro expansion of muscle stem cell populations is time-consuming and expensive, and the cells have diminished efficacy for engraftment10. Thus, the scarcity of cell sourcing and the lack of an effective method to expand myogenic stem cells is a major challenge for this approach for skeletal muscle regeneration. As an alternative approach, skin dermal cells may provide a convenient cell source to generate skeletal muscle cells through direct cell reprogramming by transfection with the transcription factor MyoD12, or by dermal stem cell differentiation13,14. However, the myogenic efficiency of dermal cells is relatively poor. Small molecules can modulate cell signalling and therefore manipulate cell fate through reprogramming and stem cell differentiation15. Although several small molecules have been examined for maintaining the identity of muscle stem cells16, to enhance myogenic differentiation17 and to promote muscle regeneration18,19, no cocktail has yet been developed to selectively induce and expand myogenic stem cells from dermal cell or muscle stromal cell (MusSC) populations for muscle regeneration.

In this article, we report a cocktail of chemicals that can selectively and efficiently expand myogenic cells from dermal-fibroblast-like cells and skeletal MusSCs. The myogenic efficiency is further improved by the selection of primary cells through preplating. Notably, the selectively expanded myogenic cells can be engrafted to repair preinjured tibialis anterior (TA) muscles in adult (8–12 weeks) and aged (18–20 months) mice and in the mdx mouse models of DMD. Moreover, we demonstrate that nanoparticle delivery of the chemical cocktail induces a robust in situ activation and expansion of satellite cells for adult and aged muscle regeneration.

Results

In vitro induction of myogenic cells from dermal cells by small molecules. When various combinations of chemicals20—including a cocktail (VCTFR) of valproic acid (V), CHIR99021 (C), tranylcypromine (T), forskolin (F) and RepSox (R)—were used to reprogram cultured mouse neonatal dermal fibroblasts, an unexpected finding was the appearance of myotube-like cells and contracting cell

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clusters (Fig. 1a). Immunostaining showed that these myotube-like cells were positive for skeletal muscle troponin T (TnT) but negative for cardiac myosin heavy chain, confirming the generation of myogenic cells from dermal cells (Fig. 1b). Moreover, before chemical treatment, the primary dermal cells were characterized and showed the expression of the fibroblast markers FSP1, CD90, PDGFRα and neural crest stem cell (NCSC) marker P75, and the majority (97.5%) of them were PDGFRα+ fibroblast cells (Supplementary Fig. 1).

To identify the indispensable factors in the chemical cocktail, each compound was omitted to generate different combinations of the other four, which were then used to treat dermal cells. The results showed that the number of TnT+ cells was significantly reduced when F or R was omitted (Fig. 1c). Different combinations of F and R were then screened. The combination of only F and R (FR cocktail) maximized the production of TnT+ cells, and the addition of other components from the original cocktail either reduced the number of TnT+ cells or had no effect (Fig. 1d).

A comparison was made with the demethylating agent 5-aza-2′-deoxycytidine (5-aza), which could induce transdifferentiation of certain mouse cell lines into skeletal muscle cells21. However, 5-aza treatment did not produce notable induction of TnT+ cells in this study (Fig. 1d).

Next, the dose-optimization studies determined that the combination of 20 μM for both F and R gave the highest yield (~15%) of TnT+ cells (Fig. 1e).

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**Fig. 1 | A small-molecule cocktail induces myogenic cells from dermal cells.**

a. Representative bright-field images of myotubes formed from dermal cells treated with control Fb medium (left) or medium containing the VCTFR cocktail (right) for 6 d. n = 3 samples per group, n = 5 fields of view per group. Scale bar, 100 μm.

b. TnT staining for dermal cells treated with control medium without the VCTFR cocktail (left) and with the VCTFR cocktail (right) for 6 d. n = 3 samples per group, n = 5 fields of view per group. Scale bar, 100 μm.

c, d. Quantitative analysis of induced TnT+ cells at day 10 after treatment with one chemical being removed from the VCTFR cocktail (c) and various combinations of the F and R-based cocktail (d). n = 3 samples per group.

e. Dose–effect analysis of the FR cocktail on TnT+ cell yield. n = 3 samples per group.

f. Basic culture medium containing 20 μM F and R without (none) or with the addition of the listed candidates. n = 3 samples per group.

g. TnT staining image of dermal cells in control, basic control medium (left) or induced with an optimal FR medium (right) for 10 d. n = 3 samples per group, n = 5 fields of view per group. Scale bar, 100 μm. The optimal FR medium is Fb medium containing 20 μM F and R, 50 μg ml−1 AA and 50 ng ml−1 bFGF. For c–f, data are mean ± s.d. Statistical analysis was performed using one-way ANOVA with Tukey’s post hoc test. The dashed lines indicate the comparisons to the VCTFR group, FR group, 10 μM group and none group in c–f, respectively. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
To further enhance the induction efficiency, other factors were added that have previously been used for culturing muscle stem cells, promoting skeletal muscle cell reprogramming and enhancing myogenic differentiation from pluripotent stem cells, including ascorbic acid (AA), bFGF, BMP4, IGF1, insulin and PDGF. Individually, bFGF (50 ng ml$^{-1}$), as well as AA

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Fig. 2 | Characterization of CiMCs. a, Representative bright-field images of dermal cells treated with control Fb medium (top) or FR medium (bottom) on days (D) 2, 4, 8 and 12 (from left to right). n = 3 samples per group, n = 5 fields of view per group. Scale bar, 100 μm. b, Immunofluorescence analysis of the skeletal muscle cell markers Pax7, MyoD, MyoG and Myh3 in CiMCs at days 4 and 8. n = 5 samples per group, n = 5 fields of view per group. Scale bars, 50 μm (left two columns), 100 μm (right two columns). c, The percentage of positive cells in b. n = 5 samples per group. d, RT-qPCR analysis of the indicated skeletal muscle genes of CiMCs at day 8. The fold change represents the mRNA expression normalized to the DMSO group. The organic solvent DMSO was used as a negative control, as it was used to dissolve the small molecules. n = 3 samples per group. For c and d, data are mean ± s.d. Statistical analysis was performed using two-tailed Student’s t-tests; **P < 0.01, ****P < 0.0001.
(50 μg ml⁻¹), significantly enhanced the induction of myogenic cells (Fig. 1f). When added together to FR, bFGF and AA synergistically enhanced the induction of TnT⁺ cells to about 37% of the total cell population (Fig. 1f,g). As a result, the optimized medium for inducing myogenesis from dermal cells in vitro contained 20 μM F, 20 μM R, 50 μg ml⁻¹ AA and 50 ng ml⁻¹ bFGF, hereafter referred to as FR medium.

Characterization of chemical-induced myogenic stem cells. Cell morphology changed gradually after chemical treatment (Fig. 2a).
Notably, dermal-fibroblast-like cells treated with FR medium exhibited a slender morphology at day 2, and sparse spontaneously contracting cells with short myotubes, which began to appear as early as day 4. The number of myotubes rapidly increased after that and gradually became organized into the beating, three-dimensional colonies or clusters. The contracting cell clusters on different days are shown in Supplementary Videos 1–4. By contrast, no contracting cells or myotubes were detected in the control cultures without chemical treatment.

To further characterize chemical-induced myogenic stem cells (CiMCs), the expression of markers associated with different stages of myogenesis was examined using immunofluorescence and quantitative PCR with reverse transcription (RT–qPCR). The satellite cell marker Pax7, muscle progenitor cell marker MyoD, and differentiation markers MyoG and Myh3 were highly expressed in CiMCs (Fig. 2b). The number of Pax7+, MyoD+, MyoG+ and Myh3+ cells or myotubes all increased substantially from day 4 to day 8 (Fig. 2bc). This observation suggests that the chemicals induced and expanded Pax7+ satellite cells and MyoD+ progenitor cells from dermal cells. These cells, in turn, could potentially further differentiate into mature myocytes that fuse into multinucleated myotubes. The RT–qPCR analysis confirmed that the induced cells treated with FR showed higher expression of skeletal muscle genes compared with cells treated with F or R alone (Fig. 2d).

The temporal expression of skeletal muscle genes in CiMCs was further investigated using RT–qPCR. The data revealed that myogenic genes—including Pax7, Mrf5, MyoD, Mynk, MyoG and Myh3—were all drastically upregulated at day 2 (Fig. 3a). By contrast, the expression of pluripotency genes Nanog and Oct4 of CiMCs remained undetectable throughout the 12d of the experiment (Fig. 3b) suggesting that the cells did not pass through a pluripotent state. Analysis of the cell population on days 3 and 6 using flow cytometry did not identify any SSEA1+ cells (Fig. 3c). Other vital markers for mesodermal cell types were also investigated, and markers for cardiomyocytes (Hand2), chondrocytes (Acan) and osteoblasts (Runx2) were not substantially upregulated (Fig. 3d), indicating that skeletal myogenic cells were selectively induced and expanded in FR medium.

To investigate the specificity of myogenic induction by the chemical cocktail, we analysed the global gene expression of dermal fibroblasts cultured in basic fibroblast culture medium (Fb medium) versus FR medium for 2d using DNA microarrays. The data showed that there were 385 upregulated and 378 downregulated genes (more than twofold, false-discovery-rate-adjusted \( P < 0.05 \)) for dermal cells cultured in FR medium compared with those cultured in basic medium (Supplementary Fig. 2a). Those that were upregulated by FR medium were enriched for biological processes related to development, whereas those that were downregulated were enriched for processes related to cytoskeleton organization and cell adhesion (Supplementary Fig. 2b).

CiMCs were selectively expanded from sparse dermal myogenic cells. When the dermal cells at various passages were treated with FR medium, myotubes were generated in passages 1 and 2, while almost no myotubes formed in later passages (Supplementary Fig. 3). We therefore postulated that it was stem cells or precursors in the heterogeneous dermal cell population that led to the chemically induced myogenesis.

We next sought to determine which primary cell subpopulation contributed to the chemically induced myogenesis. This was performed using a preplating technique by dividing the dermal cells into rapid-adhering cell (RAC) and slow-adhering cell (SAC) populations. This technique sorts cells by their differential adhesion to the culture dish surface. Stem cells attach to the culture dish surface weakly and slowly, whereas fibroblasts attach more firmly and rapidly. Moreover, large hair follicle cell (HFC) clusters are easily separated by low-speed centrifugation during dermal cell isolation. These three subpopulations of dermal cells (RAC, SAC and HFC) were examined after overnight seeding by staining for skeletal muscle markers (Pax7, MyoD and Myh3) and skin NCSC or skin-derived stem cell marker Sox10 (Supplementary Fig. 4a), and we found that the Pax7+, MyoD+ and Myh3+ cells were rarely observed in RAC or HFC cultures, whereas they were detected in SAC culture, indicating that dermal myogenic cells were enriched in SACs. By contrast, HFCs showed more Sox10+ cells than RACs and SACs.

Thereafter, the three isolated cell populations were treated with FR medium to determine their myogenic capabilities. About 43% of induced SACs were Myh3+, significantly higher compared with the percentage of Myh3+ cells in induced RACs (~4%) and HFCs (~1%; Fig. 4a,b), suggesting that it was the enriched myogenic cells in SACs that led to enhanced myogenesis. To determine whether NCSCs and HFCs could be additional cell sources for chemically induced myogenesis, we stained for Sox10 in chemically treated SACs and HFCs (Fig. 4c,d). The results showed that Sox10+ cells in SAC-derived cells increased slightly, but few colocalized with the Myh3+ cells. Furthermore, although more Sox10+ cells existed in the HFC-derived cell population (Supplementary Fig. 4a), Myh3+ cells were only sparsely generated with the chemical induction. These results suggested that the production of CiMCs was positively correlated with the number of enriched myogenic cells, rather than expanded from fibroblasts, dermal NCSCs or HFCs.

Further staining showed that the percentage of both Pax7+ and MyoD+ cells in FR-treated SACs was significantly increased from...
day 4 to day 8; by contrast, almost no myogenic stem cells were detected in the controls at day 8 (Fig. 4e–g and Supplementary Fig. 4b). At day 4, around 43% of Pax7+ cells and 25% of MyoD+ cells were Ki67+ proliferating cells, which slightly decreased at day 8. This confirmed the role of the chemicals in the expansion of myogenic stem cells. Subsequently, the myogenic stem cells could further dif-
differentiate and fuse into multinucleated myotubes with a striated pattern (Fig. 4h). Furthermore, chemical-induced myotubes expressed various isoforms of myosin heavy chains (MHCs), including Myh1E (adult, MF-20), Myh2 (adult, MHC-IIA), Myh3 (embryonic), Myh4 (adult, MHC-IIB), Myh7 (adult, MHC-I), and Myh8 (neonatal) (Supplementary Fig. 5). To determine whether myogenic potential could be sustained in FR medium, CiMCs were passaged every 3 d and different passages of cells were treated with FR medium. The results showed that the myogenic potential persisted in cultures for up to five passages and notably decreased thereafter, suggesting that cells could be expanded for five passages and potentially used for cell therapy (Supplementary Fig. 6). CiMCs expanded from dermal Pax7+ cells. To directly determine the contribution and fate of Pax7+ cells in response to the chemical cocktail, dermal-fibroblast-like cells were isolated from

**Fig. 5 |** The dermal Pax7+ subpopulation is the main contributor to the expanded CiMCs. a, Breeding schematic of lineage tracing and tamoxifen-inducible Pax7-creER:Rosa26-eYFP mice. Pr., promoter. b, Representative fluorescence (top) and merged fluorescence and bright-field (eYFP BF; bottom) images of Pax7 lineage-traced SACs treated with 4-OHT, FR or 4-OHT with FR (4-OHT/FR) for 12 d. n = 3 samples per group, n = 5 fields of view per group. Scale bar, 100 μm. c, Selective expansion of SACs from transgenic mice treated with FR medium on day 4 and day 8 (from left to right), n = 3 samples per group, n = 5 fields of view per group. Scale bar, 100 μm. d, Immunofluorescence staining for Pax7 (top) and Myh3 (bottom) in CiMCs from Pax7 lineage-traced of SACs treated with FR medium for 12 d. 4-OHT was added to the Fb medium during cell seeding and 24 h before the culture medium was replaced with FR medium. n = 3 samples per group, n = 5 fields of view per group. Scale bar, 100 μm. e, Immunofluorescence staining for Pax7 and Ki67 in FACS sorted eYFP− (top) and eYFP+ (bottom) cells from day-4 Pax7 lineage-traced CiMCs, which were further treated with FR medium for another 4 d after FACS sorting; from left to right, DAPI, eYFP, Pax7 and Ki67 staining, and merged image. n = 5 samples per group, n = 5 fields of view per group. Scale bar, 50 μm. f, The percentage of Pax7+ cells in e, n = 5 samples per group. g, The percentage of proliferating Ki67+ cells in Pax7+eYFP+ CiMCs at day 0 (6 h after plating) and day 4. n = 5 samples per group. For f and g, data are mean ± s.d. Statistical analysis was performed using two-tailed Student’s t-tests; **P < 0.01, ****P < 0.0001.
tamoxifen-inducible Pax7-creERT2-Rosa26-eYFP transgenic mice (Fig. 5a). The results showed that the enhanced yellow fluorescent protein (eYFP) signal was widely detected in CiMCs when dermal SAC cells were treated with 4-hydroxytamoxifen (4-OHT) and FR cocktail, but no eYFP expression was detected after treatment with 4-OHT or FR alone (Fig. 5b). These results confirmed the inducibility of eYFP reporter by 4-OHT and the expansion of Pax7+ cells by FR medium. In particular, the eYFP signal was first expressed in individual cells at day 4, and gradually appeared in myotubes/clusters thereafter, indicating that chemical-induced Pax7+ cells further differentiated and fused into myotubes (Fig. 5c).

Furthermore, to determine whether the expanded Pax7+ cells were derived from existing Pax7+ cells or other myogenic precursors, the dermal cells isolated from Pax7+ transgenic mice were seeded in FB medium containing 4-OHT for 1 d to label any existing Pax7-expressing cells with eYFP, and then cultured in FB medium for a further 2 d to remove the residual 4-OHT and prevent further labelling. The cells were then treated with FR medium for another 8 d. The results showed that the myotubes were eYFP+ (Supplementary Fig. 7a), indicating that chemical-induced myotubes were mainly derived from Pax7+ cells. Consistent with this, more eYFP-expressing myotubes formed from induced SACs than from RAGs (Supplementary Fig. 7b). Further staining demonstrated that almost all expanded myogenic cells and differentiated myocytes/myotubes were eYFP+ (Fig. 5d).

Moreover, eYFP+ and eYFP− cells were sorted using fluorescence-activated cell sorting (FACS) from transgenic CiMCs (Supplementary Fig. 8) and treated with FR medium for 4 d. Immunofluorescence analysis showed that approximately 80% of eYFP+ cells expressed Pax7 and retained a proliferative capability, as indicated by Ki67 expression at days 0 and 4, whereas no Pax7-expressing cells could be found in chemical-treated eGFP+ cells (Fig. 5e–g). Together, the results suggested that dermal-tissue-derived Pax7+ cells can be selectively and rapidly expanded by chemical induction.

CiMCs expanded from dermal cells and MuSCs from adult and aged mice. Owing to the interest and importance in determining the effect of age on the expansion of autologous stem cells for clinical application, we investigated the effects of FR medium on dermal cells and MuSCs from adult and aged mice (Supplementary Fig. 9). Similar to the findings with neonatal dermal cells, treating adult dermal cells with FR medium also generated induced myogenic cells; substantially higher numbers of myogenic cells were produced from MuSCs from adult and aged mice compared with from dermal cells (Supplementary Fig. 9). The chemical treatment induced the proliferation of Pax7+ cells from adult MuSCs, which yielded approximately 65% for Pax7+ cells at day 8 (Supplementary Figs. 9 and 10). Notably, aging reduced CiMC expansion to a certain extent, with around 36% of Pax7+ cells at day 8. Furthermore, the formation of cell clusters appeared to occur faster in chemically treated MuSCs compared with untreated MuSCs and chemically treated dermal cells.

scRNA-seq analysis of CiMCs. To further characterize CiMCs and reveal the heterogeneity of the dermal cells, we performed single-cell RNA sequencing (scRNA-seq) on four samples: neonatal dermal cells (Neo DC), neonatal dermal cells treated with FR medium for 3 d (Neo DC/FR), adult dermal cells treated with FR medium for 3 d (adult DC/FR) and endogenous adult MuSC (adult MuSC, positive control). Unsupervised clustering using Seurat revealed eight subpopulations in neonatal dermal cells with and without FR medium treatment (Supplementary Fig. 11a). After 3 d of chemical treatment, the percentage of skeletal muscle cells increased eightfold, confirming that the chemicals selectively expanded myogenic cells from heterogeneous dermal cells. Furthermore, both the adult DC/FR and adult MuSC samples were heterogeneous with several subpopulations, and the proportion of the skeletal muscle cells in both samples was much lower than in the Neo DC/FR sample. The different cell clusters were identified by marker genes obtained from differential expression testing (Supplementary Fig 11b).

To identify other markers of the Pax7+ proliferating cells, neonatal dermal cells were clustered with higher resolution to obtain more subpopulations, and differential expression testing was performed between the Pax7+ population and all of the other populations. Although three additional genes were found to be upregulated in the Pax7+ population (Spatz2, Gnat1 and Gds2), the gene expression distribution plot indicated that Pax7 was the most specific marker for the proliferating myogenic cells (Supplementary Fig. 12a). Moreover, the skeletal muscle cell cluster data from all four samples were merged computationally and pseudotime analysis was performed. The cells at the ends of the tree branches were characterized as quiescent (Pax7−Cdkn1a−), proliferating (Pax7+Ki67+) and differentiating (Pax7+Myog+) myogenic cells on the basis of marker expression (Supplementary Fig. 12b, c). After separating the pseudotime trajectory according to the original samples, the proliferating and differentiating myogenic cells at different stages were found in all four samples (Neo DC, Neo DC/FR, adult DC/FR and adult MuSC), while quiescent cells were found in only the adult MuSC sample (Supplementary Fig. 12d). When gene expression was compared between proliferating skeletal muscle cells from the Neo DC/FR and adult MuSC samples, we found 164 upregulated and 132 downregulated genes in the Neo DC/FR sample; 20 of the upregulated and downregulated representative genes are shown in Supplementary Fig. 12e. Furthermore, the Gene Ontology biological process terms enriched for the upregulated genes in the Neo DC/FR sample were broadly specified (Supplementary Fig. 12f), indicating that, although CiMCs exhibited some transcriptional differences from endogenous muscle cells, these differences did not indicate any specific biological pathways.

CiMC engraftment improved muscle regeneration. To evaluate the in vivo therapeutic utility of CiMCs for muscle regeneration, CiMCs were collected after 8 d of in vitro expansion in FR medium and injected into the TA muscles that had been preinjured by cardiotoxin (CTX) in adult, aged and mdx mice (Fig. 6a). Four weeks after implantation, force testing was performed to assess...
the functionality of the regenerated muscle (Fig. 6b,c). We found that the mean isometric tetanic forces were significantly higher for CiMC-treated TA muscles compared with the respective controls in all three models. In general, compared with adult mice, aged mice had a larger body mass and muscle mass, and the contraction force was higher, and mdx muscle had the lowest contraction.
force. Consistent with this, all muscles were excised after force testing and weighed, and CiMC-treated TA muscles had substantially higher wet muscle masses in all three models (Fig. 6d). To determine the efficiency of CiMC engraftment, DsRed-labelled CiMCs were transplanted into injured muscles. Four weeks after transplantation into all three animal models (adult, aged and mdx mice), CiMCs were integrated and formed newly regenerated myofibres with central nuclei (Fig. 6e,f). By contrast, contralateral TA muscles transplanted with DsRed-labelled dermal cells did not have DsRed+ myofibres. Notably, numerous dystrophin-positive myofibres were detected in the CiMC-grafted mdx muscles, while no dystrophin-positive fibres were found in the controls (Supplementary Fig. 13a,b). These results demonstrated that the transplanted CiMCs maintained their myogenic capability and engrafted into regenerated muscles, particularly promoting the regeneration of the muscles of aged and DMD mice.

Notably, the histological analysis of adult, aged and mdx mouse muscles revealed that the average cross-sectional area (CSA) of myofibres was increased for CiMC-treated TA muscles compared with the vehicle and DsRed+ controls. The percentage of Myh3+ cells in SACs treated with different doses of FR-nps for 10 d. n = 5 mice per group at each time point. d. Representative CMAP curves for vehicle- and FR-nps-treated muscles at days 14 and 28. Vehicle refers to the nanoparticles without drugs and served as a control. n = 5 mice per group at each time point. Scale bars, 100 μm (vertical), 1 ms (horizontal). g. CMAP amplitude of injured TA muscle treated with FR-nps or vehicle alone on days 14 and 28. n = 5 mice per group at each time point. h. Maximum tetanic force of control and FR-nps-treated TA muscles from adult and aged mice at 4 weeks. n = 5 mice per group. i. Representative isometric tetanic force curves of control and FR-nps-treated TA muscles from adult mice at 4 weeks. n = 5 mice per group. j. Muscle wet weight of control and FR-nps-treated TA muscles from adult and aged mice at 4 weeks. n = 5 mice per group. k. Representative myofibre staining of control and FR-nps-treated TA muscles from adult and aged mice at 4 weeks. n = 5 mice per group. The percentage of different myofibre types in k. n = 4 mice per group. For c, d, g, j and m, data are mean ± s.d. For the box plots in g, h, j and l, the box limits extend from the 25th to 75th percentiles; the horizontal line shows the mean value; the whiskers represent the standard deviation. Statistical analysis was performed using one-way ANOVA with Tukey’s post hoc test (d) and two-tailed Student’s t-tests (g, h, j and m); *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Type-IIA, type-IIB and type-IIX fibres (unstained by any marker, by exclusion) were present in all of the TA muscles, but type-I fibres were not. Type-IIB myofibres were the most abundant fibre type in all of the regenerated TA muscles, but there was no significant difference between CiMC-treated and control groups. However, type-IIA myofibres were slightly decreased in all of the CiMC-treated TA muscles compared with the control groups (Fig. 6h-i), confirming the better muscle recovery. Moreover, fibrosis was more severe in both aged and mdx mouse muscles for the control groups compared with that of injured adult muscles, while CiMC-treated skeletal muscles exhibited lower muscle fibrosis compared with the controls in all three models (Supplementary Fig. 13c,d). Furthermore, CiMC-treated muscles exhibited fewer macrophages compared with the controls in all three models (Supplementary Fig. 13e,f). Taken together, these results suggest that the CiMCs have an ameliorative effect on fibrosis and inflammatory responses.

A drug-releasing system promotes in situ myogenic stem cell expansion and muscle repair. To evaluate the potential effects of the chemical cocktail to local resident myogenic cells in muscle repair, a controlled drug delivery system was developed and injected into injured TA muscles (Fig. 7a). Both drugs (F and R) were loaded into biodegradable poly(d,l-lactide-co-glycolide) (PLGA)-based nanoparticles (hereafter, FR-nps). Scanning electron microscopy (SEM) analysis showed that the FR-nps were uniform round spheres with an average diameter of 427 nm and polydispersity index of 0.24 (Fig. 7b). Both chemicals were gradually released over a two-week time period, as determined using high-performance liquid chromatography–mass spectrometry (HPLC–MS; Fig. 7c). To verify whether the drug-releasing particles could selectively expand the myogenic stem cells for myogenesis, we treated SACs with different doses of FR-nps and found an increase in Myh3+ cells at day 10, with higher doses of FR-nps producing more myocytes or

**Fig. 8** Drug-loaded nanoparticles enhance muscle repair by promoting in situ satellite cell expansion. 

a, Immunofluorescence analysis of Pax7+ cells in CTX-injured muscle treated with vehicle (the nanoparticles without drugs as a control; left) and FR-nps (right) at day 3 and day 14. n = 5 samples per group at each time point. Scale bar, 20 μm. b, Quantification of Pax7+ cells at different time points after treatment. n = 5 mice per group at each time point. ‘NL’ represents the quantification of Pax7+ cells in normal TA muscle from adult mice. c, Immunostaining of Pax7 (green) and Ki67 (red) in CTX-injured muscle treated with FR-nps or vehicle at day 3. n = 5 mice per group. Scale bar, 20 μm. d, Lineage tracing of Pax7+ cells in CTX-injured muscle of Pax7-creER:Rosa26-eYFP mice. The image shows numerous eYFP+ cells around myofibres in FR-np-treated muscle at day 3. n = 3 mice per group. Scale bar, 20 μm. For b, data are mean ± s.d. Statistical analysis was performed using two-tailed Student’s t-tests. **P < 0.001, ****P < 0.0001.
myotubes, thereby recapitulating the myogenic-inducing effects of the chemicals (Fig. 7d and Supplementary Fig. 14).

To further verify the in vivo effect of drug-loaded nanoparticles on muscle regeneration, TA muscles of adult and aged mice were CTX-injured as previously described, but were subsequently injected with vehicle or FR-nps rather than cells, and then evaluated (Fig. 7e). In the beginning, the green-fluorescence-labelled nanoparticles were injected into injured muscles, which showed that the nanoparticles were locally and evenly distributed within the TA muscle 2 d after injection (Supplementary Fig. 15), but gradually degraded and became non-detectable after a month. For the muscle therapeutics, we injected 1 mg FR-nps for each TA muscle repair, on the basis of several considerations, including the amount of FR-nps required to achieve high myogenic induction in vitro, an estimated TA muscle volume, a long-term release period (three to four weeks) and the amount of nanoparticles in the muscle tissue. FR-np-treated TA muscles from adult mice had significantly higher sciatic compound muscle action potential (CMAP) amplitudes compared with muscles treated with the vehicle (that is, the nanoparticles alone as a control) at two and four weeks (Fig. 7e–g). Similar to the beneficial effects of CiMC transplantation, the mean isometric tetanic forces at week 4 were also higher for the FR-np-treated TA muscles compared with the controls in both adult and aged mice (Fig. 7h,i), as were the muscle weights (Fig. 7j) and average myofibre CSA (Fig. 7k,l), with a slightly lower proportion of type-IIA fibres compared with the controls (Fig. 7k–m). Thus, these results demonstrate that the drug-releasing nanoparticles enhanced in situ TA muscle regeneration and repair. Besides, the FR-np-treated muscles of both adult and aged mice exhibited less muscle fibrosis and fewer macrophages compared with the vehicle-treated groups (Supplementary Fig. 16), demonstrating that the FR cocktail improves muscle regeneration by modulating fibrosis and inflammation, similar to our findings with CiMC transplantation.

Next, we determined whether drug-loaded nanoparticles specifically expanded Pax7+ satellite cells in situ and accelerated muscle regeneration as FR did in vitro. Immunofluorescence staining showed substantially more peripherally localized Pax7+ cells around degenerated or regenerating myofibres in the FR-np-treated muscles compared with the control (Fig. 8a). By quantifying the number of Pax7+ cells in the region of regenerating myofibres (Fig. 8a,b), we found that, in both FR-np-treated and vehicle-treated muscle, Pax7+ satellite cells rapidly increased and peaked in number by day 3, gradually returning to basal levels by day 28. However, FR-np-treated muscle had more satellite cells compared with the controls at days 3 and 7, with an increase of greater than two-fold compared with the vehicle treatment alone. Further analysis showed that almost all Pax7+ cells were proliferating at day 3 (Fig. 8c). To directly determine whether these expanded Pax7+ cells were derived from existing Pax7+ satellite cells, the muscle injury and FR-np-delivery experiments were performed in Pax7-creER:Rosa26-eYFP mice, which enabled lineage tracing of Pax7+ cells with eYFP. FR-np-treated muscle contained more eYFP+ cells at day 3 (Fig. 8d), suggesting that FR-nps increased the proliferation and expansion of existing Pax7+ cells in the injured muscle for enhanced muscle regeneration.

Discussion

We have defined a chemical cocktail that selectively and robustly expands myogenic stem cells from dermal cells and MuSCs for the regeneration of injured adult, aged and dystrophic muscles. Furthermore, in situ delivery of these chemicals to CTX-injured muscles from adult and aged mice was achieved using a nanoparticle system, which harnessed the innate regenerative potential of the body to promote muscle regeneration. This small-molecule cocktail approach to promote muscle regeneration is preferable to genetic approaches in terms of scalability, reproducibility and clinical safety. Both cell transplantation and drug delivery approaches have the potential for translation into clinical applications.

Previously, several approaches have been explored to address the unmet needs in skeletal muscle regeneration, including biomaterials,29,31 gene editing18,19 and stem-cell-based strategies. Satellite cells are essential to achieve muscle regeneration in these approaches. However, their expansion and self-renewal potential is limited in adult muscle, and is especially decreased or exhausted in aged26,31 and DMD muscle. Furthermore, myogenic stem cells can be derived from somatic stem cells, including bone marrow mesenchymal stem cells16, umbilical cord blood mesenchymal stem cells31 and mesangioblasts30, but the differentiation efficiency remains to be improved. Pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), may provide unlimited sources for myogenic cells26–30 and, specifically, iPSCs can be used to generate myogenic cells without the ethical controversies of using ESCs. However, this approach is still limited by the lengthy and expensive reprogramming and differentiation processes, and iPSC-derived myogenic cells are immature for efficient engraftment20,28. Our findings on CiMCs and the FR cocktail help to address these challenges.

Skin dermal cells have often been chosen as a source for cell reprogramming therapies because they can be conveniently isolated using minimally invasive procedures. Here we identified the chemical cocktail FR that, in combination with bFGF and AA, can selectively induce and robustly expand myogenic stem cells from dermal cells and MuSCs in vitro. Previous research demonstrated other relevant effects of F or R on myogenic proliferation and differentiation from ESCs and iPSCs18,20,29. Furthermore, F and R as a part of a chemical cocktail or in combination with transcription factors can enhance or induce cell reprogramming, such as the conversion of human fibroblasts into neuronal25, cardiac4, skeletal muscle4 and iPSCs41,42. However, in contrast to other studies, we found that the combination of F and R robustly expanded CiMCs from dermal cells, while the efficiency is almost negligible when using F or R alone. By contrast, dermal cell populations are highly heterogeneous and exhibit anatomic and developmental variation44. Thus, different cell subpopulation(s) in dermal cells may contribute differently to chemical-induced myogenic induction and expansion. We clarified that the SAC subpopulation in skin dermal cells was highly correlated with chemical-mediated myogenic induction. We then performed lineage tracing and FACS sorting to show that CiMCs were primarily expanded from dermal Pax7+ cells by the FR medium. Furthermore, scRNA-seq analysis revealed the heterogeneity of dermal cells and the selective expansion of CiMCs. These findings provide a rational basis for using the chemically expanded stem cells and drug-delivery-based approaches for skeletal muscle regeneration.

Older individuals and patients with DMD often have progressive muscle weakness and regenerative failure due to the misregulation of satellite cells in the microenvironment45. Recent studies have demonstrated the efficacy of muscle stem cell transplant for restoring muscle functions in aged and mdx mice3,5,60. Under optimized conditions, a large number of myogenic stem cells can be obtained from dermal cells through chemical induction and expansion. These in vitro expanded CiMCs can efficiently engraft into muscles of aged and mdx mice, and significantly improve muscle functions and regeneration after transplantation. Thus, CiMC transplantation offers potential for treating patients with age-related muscle dysfunction and inherited muscle diseases, perhaps in combination with gene-editing technology. Before clinical application, further studies are needed, for example, the scalability of CiMC production and long-term evaluations of myotube survival and muscle functions.

Another highlight of this research is the development of drug-loaded nanoparticles for in situ satellite cell expansion and

NATURE BIOMEDICAL ENGINEERING | VOL 5 | AUGUST 2021 | 864–879 | www.nature.com/natbiomedeng
muscle regeneration. Notably, FR-nps can be conveniently injected into injured TA muscles, whereby the controlled release of chemicals can effectively modulate local satellite cell numbers and functions to promote the regeneration of damaged muscles, especially for aged muscle regeneration. Previous investigations have shown that pathological muscle fibrosis can retard muscle regeneration.5,45,46 We found that FR-np treatments had additional beneficial effects on muscle regeneration by reducing fibrosis, possibly due to the effects of TGF-β inhibition by R47,48. Indeed, the incorporation of macrophages into engineered tissues and the modulation of macrophage phenotype can enhance myogenesis and muscle regeneration5,49. In aged and DMD mice, immune cells may cause dysregulation of the regeneration paradigm, and tuning the macrophage phenotype improves muscle function49. In our studies, GMC- or FR-cocktail-treated muscles showed faster and better regeneration with a lower number of macrophages in adult, aged and DMD mice. Furthermore, the FR cocktail may have additional beneficial effects on immunomodulation, supported by the findings that elevated cAMP signalling and TGF-β inhibition can regulate macrophages and other innate and adaptive immune cells for muscle regeneration55,49. The immunomodulation effects of the FR cocktail and the role of immune cells in the expansion and differentiation of myogenic cells during muscle regeneration require further mechanistic investigations. Overall, the approach that we have developed harnesses and maximizes the regenerative potential of resident cells to accelerate and promote muscle regeneration, which has translational potential for clinical therapies.

Methods

Materials. PLGA polymer (50:50, inherent viscosity 0.4 dL−1 g−1) and poly(vinyl alcohol) (PVA; molecular weight (Mw) = 25,000, 88% hydrolysed) were purchased from Polysciences. Poly(ethylene glycol) methyl ether-block-poly(ɛ-capro-caprolactone) (PLGA-b-PEG, PEG average Mw = 5,000, PLGA Mw = 55,000), and dichloromethane were purchased from Sigma-Aldrich. Small molecules were purchased from Cayman Chemical.

Cell isolation and culture. Primary mouse neonatal dermal-fibroblast-like cells from C57BL/6 mice and Pax7-creER,Rosa26-eYFP mice were isolated as previously described43. In brief, the limbs and tail were removed from the euthanized newborn mice (1–3 d) before gently pulling away the skin from the body. The skin was then flattened and floated on freshly prepared trypsin (0.25% without EDTA, Thermo Fisher Scientific) overnight, and the dermis was separated from the epidermis the next day. The dermis was then cut into small pieces and digested with 0.35% collagenase II in a 37 °C water bath for 1 h. The digested mixture was filtered through a 100 µm mesh, centrifuged at 1,000 rpm for 5 min and then washed twice with Dulbecco's modified Eagle medium (DMEM). The dermal cell pellet was washed and incubated at 37 °C in a humidified, 5% CO₂ incubator overnight in low-glucose DMEM (Thermo Fisher Scientific). Then, 1 µM 4-hydroxytamoxifen (R47,48) was added to the medium during cell seeding to induce eYFP expression from Pax7 cells. Next, 1 d later, cells were washed with PBS twice and FR medium was added and changed once on day 2. On day 4, cells were dissociated with Accutase and neutralized by FBS-containing medium. Detached cells were centrifuged at 1,000 rpm for 5 min and then resuspended in the sorbitol solution (DMEM containing 25 mM HEPES, 2% FBS and 1% penicillin–streptomycin) at a cell concentration of 5 × 10⁶ cells per ml after passing cells through a 40 µm filter to remove cell clusters and debris. Single-cell suspensions were kept on ice until sorting, and dermal cells without the presence of 4-OHT and FR were used as a negative control. eYFP+ and eYFP− cells were sorted on a FACS Aria II instrument (Becton-Dickinson) after adding DAPI to exclude dead cells, and collected in sorting solution. The FACS data were collected and analysed using BD FACSDiva v8.0.2. The sorted cells were replated in FR medium and fixed at day 0 (that is, 6 h), or cultured in FR medium for 4 d, followed by immunofluorescence analysis of Pax7 and muscle marker expression.

Microarray analysis. Dermal-fibroblast-like cells were treated with basal medium (Fb medium with 50 µM AA and 50 ng ml−1 bFGF) and FR medium for 2 d. mRNA was extracted using the RNeasy Micro Kit (Qiagen) and checked for RNA quality (RNA integrity number > 7.5) using the Bioanalyzer 2100 (Agilent) before linear amplification using the Ovation Pico WTA System V2 (NuGEN). Biological replicates were treated as each biological sample was sequenced on an Illumina Genome Analyzer 2100 (Illumina) and raw data were mapped to each genome and biological samples were compared. P values were adjusted for multiple testing using the Benjamini–Hochberg method. Comparisons made in this analysis make no assumptions about the distribution of the data. In brief, adjusted P values of less than 0.05 were considered to be differentially expressed. Differentially expressed genes were submitted to DAVID for Gene Ontology enrichment analysis.

Screening small molecules for myogenic induction. Small molecules for selectively expanding skeletal muscle cells were screened using neonatal dermal-fibroblast-like cells from C57BL/6 mice that were seeded at a density of 10,000 cells per cm² in 24-well plates containing Fb medium. The next day, the original medium was replaced with a screening medium containing small-molecule cocktail Knockout DMEM (Thermo Fisher Scientific), 10% knockout serum replacement (Thermo Fisher Scientific), 10% B27 (Hyclone), 2 mM GlutaMAX (Thermo Fisher Scientific), 1% non-essential amino acids (Thermo Fisher Scientific), 1% penicillin–streptomycin (Thermo Fisher Scientific) and 20 ng ml−1 TGF-β1 inhibition by R47,48. The next day, the medium was replaced with FR medium for further screening of additional candidates that may improve myogenic efficiency, including AA (50 µM), Sigma–Aldrich), BMP4 (20 ng ml−1, Stemgent), insulin (10 µg ml−1, Stemgent), IGF-1 (50 ng ml−1, R&D Systems), PDGF (50 ng ml−1, R&D Systems) and bFGF (50 ng ml−1, Stemgent).

On the basis of the results from the screening experiments, the optimized formulation was obtained and it consisted of Fb medium with 20 µM F, 20 µM R, 50 µg ml−1 AA and 50 ng ml−1 bFGF, termed as FR medium. To determine which dermal cell subpopulations were involved in chemical-induced myogenesis, various subpopulations were tested in FR medium. For these experiments, cells were seeded at a density of 10,000 cells per cm² and cultured in Fb medium. The next day, the medium was replaced with FR medium. The medium was changed once every 2–3 d. To study the effect of passing on the myogenic expansion potential of CiMCs, CiMCs were passaged every 3 d in FR medium. Meanwhile, a portion of the cells from all passages was stored by freezing. The passed CiMCs were then treated with FR medium for 8 d, at which point immunofluorescence analysis of Pax7 and muscle markers was performed to evaluate the generation of myogenic cells. For dermal cells derived from Pax7-creER,Rosa26-eYFP mice, 1 µM 4-OHT was added in Fb medium to induce recombinase expression during cell seeding and then the medium was replaced with FR medium the next day.

Flow cytometry analysis of dermal cells. Dermal cells in suspension were stained with antibodies such as FITC-conjugated CD 90.2 (rat monocolonal antibody, Thermo Fisher Scientific), 11-0903-81, FITC (rabbit polyclonal antibody, Abcam, ab8874) and PDGFRα (rat monocolonal antibody, Thermo Fisher Scientific, 13-1401-82) (and appropriate secondary antibodies as needed), followed by flow cytometry analysis using the Guava easyCyte system with guavaSoft v.3.4.

FACS analysis of eYFP reporter cells. Neonatal dermal cells isolated from Pax7-creER,Rosa26-eYFP mice were seeded in Corning tissue–culture dishes (10 cm) at a density of 2×10⁴ cells per cm². Then, 1 µM 4-hydroxytamoxifen (R47,48) was added to the Fb medium during cell seeding to induce eYFP expression from Pax7 cells. Next, 1 d later, cells were washed with PBS twice and FR medium was added and changed once on day 2. On day 4, cells were dissociated with Accutase and neutralized by FBS-containing medium. Detached cells were centrifuged at 1,000 rpm for 5 min and then resuspended in the sorting solution (DMEM containing 25 mM HEPES, 2% FBS and 1% penicillin–streptomycin) at a cell concentration of 5 × 10⁶ cells per ml after passing cells through a 40 µm filter to remove cell clusters and debris. Single-cell suspensions were kept on ice until sorting, and dermal cells without the presence of 4-OHT and FR were used as a negative control. eYFP+ and eYFP− cells were sorted on a FACS Aria II instrument (Becton-Dickinson) after adding DAPI to exclude dead cells, and collected in sorting solution. The FACS data were collected and analysed using BD FACSDiva v8.0.2. The sorted cells were replated in Fb medium and fixed at day 0 (that is, 6 h), or cultured in FR medium for 4 d, followed by immunofluorescence analysis of Pax7 and muscle marker expression.

Microarray analysis. Dermal-fibroblast-like cells were treated with basal medium (Fb medium with 50 µM AA and 50 ng ml−1 bFGF) and FR medium for 2 d. mRNA was extracted using the RNeasy Micro Kit (Qiagen) and checked for RNA quality (RNA integrity number > 7.5) using the Bioanalyzer 2100 (Agilent) before linear amplification using the Ovation Pico WTA System V2 (NuGEN). Biological replicates were treated as each biological sample was sequenced on an Illumina Genome Analyzer 2100 (Illumina) and raw data were mapped to each genome and biological samples were compared. P values were adjusted for multiple testing using the Benjamini–Hochberg method. Comparisons made in this analysis make no assumptions about the distribution of the data. In brief, adjusted P values of less than 0.05 were considered to be differentially expressed. Differentially expressed genes were submitted to DAVID for Gene Ontology enrichment analysis.

876 NATURE BIOMEDICAL ENGINEERING | VOL 5 | AUGUST 2021 | 864–879 | www.nature.com/natbiomedeng

ARTICLES
Gene expression analysis. At the indicated time points, cells were lysed using Trizol (Thermo Fisher Scientific) and RNA was extracted according to the manufacturer’s instructions. The RNA concentration was quantified by absorption at 280 nm (Nanodrop 1000, Thermo Fisher Scientific), and an equal amount was loaded for cDNA synthesis using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). cDNA was then loaded into 96-well PCR plates with primers and the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific). B2m was used as a housekeeping gene for normalization. Thermal cycling and data acquisition were performed using the CFX96 Real-Time PCR Detection System (Bio-Rad). Data were analysed using the ΔΔt method. A list of the sequences of primers for RT-qPCR is provided in Supplementary Table 1.

Single-cell sequencing and data processing. Transcripts were mapped to the mm10 reference genome using Cell Ranger v3.1.0. Quality control was performed by selecting cells matching criteria of more than 1,000 genes detected on both libraries. The top 30 principal components were used for clustering using a shared nearest neighbour modularity optimization-based clustering algorithm with a resolution setting of 0.5. Differential gene expression testing was performed on the basis of a hurdle model, as implemented in the MAST package v1.10 (ref. [2]). Data from different samples were integrated with Seurat by projecting the expression data into a lower dimensional space through canonical correlation analysis (CCA) and then calculating and applying a transformation vector to all cells. Non-integrated data were used for differential expression testing between skeletal muscle cells from adult hindlimb and from dermal cells treated with FR. Genes with adjusted P values (Benjamini–Hochberg correction) of less than 0.01 and a log-transformed fold change of greater than 0.5 were considered to be differentially expressed.

For pseudotime analysis, skeletal muscle cells from the four samples were integrated and clustered with Seurat. A set of highly variable genes was identified by identifying the differentially expressed genes between these clusters. Dimensionality reduction using DVRtre was performed on these genes and a pseudotime trajectory was plotted using Monocle v2.12 (ref. [3]).

Cell transplantation. Twenty-four hours before cell transplantation, adult C57BL/6 mice (aged 8 weeks), aged C57BL/6 mice (aged 18 months) and mdx mice (aged 8 weeks) were anesthetized with isoflurane/oxygen inhalation, and 30 μl of PBS containing Naja nigricollis CTX Sigma-Aldrich) in PBS was injected into the TA muscle of anesthetized mice to induce injury. C1MCs (1 × 10⁷ cells) were then suspended in 30 μl of Matrigel solution and injected directly into the preinjured TA muscles. As a control, the contralateral muscles of recipient mice were similarly injured but injected with nanoparticles without drugs. Five animals per group were used for each time point. The animals were housed under temperatures of 18–23 °C, 40–60% humidity, 12 h light–12 h dark cycle, and were given food and water ad libitum.

Preparation and characterization of drug-loaded nanoparticles. Drug-loaded nanoparticles (FR-nps) were prepared using an emulsification solvent evaporation technique. In brief, PLGA/PLGA-b-PEG (50/50, w/w) was dissolved in dichloromethane to make 10% w/v solutions, then 5% (w/w) of chemicals (F-BSA, 3 mg/ml; tamoxifen (Sigma-Aldrich, T5648) diluted in corn oil (Sigma-Aldrich, C8267) was intraperitoneally injected for five consecutive days. Then, 7 d after the final injection, anaesthesia, CTX injury and FR-np injection were performed. Three animals per group were used for each time point. The animals were housed under temperatures of 18–23 °C, 40–60% humidity, 12 h light–12 h dark cycle, and were given food and water ad libitum.

In vitro myogenesis with drug-loaded nanoparticles. For selective induction of myogenic cells in dermal cells or MusCcs using FR-np, the dermal cells were seeded in 24-well plates at 10,000 cells per cm² with Fb medium. The next day, the medium was replaced with Fb medium containing 50 μM-gal3/AA and 50 ng/ml-1 bFGF. Meanwhile, FR-nps at various doses (1 mg, 2 mg and 4 mg) were added into the inserts of Transwell plates (0.4 μm pore size, Thermo Fisher Scientific) in the coculture system. Half of the Fb medium was changed every other day.

In situ regeneration with drug-loaded nanoparticles. For in situ regeneration, adult C57BL/6 mice (aged 8 weeks) and aged C57BL/6 mice (aged 18 months) were used and injured with CTX injection as described above, and 1 mg drug-loaded nanoparticles (FR-nps) suspended in 30 μl PBS was injected into an injured TA muscle. As a control, the contralateral muscles of recipient mice were similarly injured but injected with nanoparticles without drugs. Five animals per group were used for each time point. Paclitaxel (Pax7-cre-ER; Rosa26-eYFP transgenic mice were used for lineage tracing of Pax7+ satellite cells. Before performing the same procedures, 100 μl of 10 mg/ml tamoxifen (Sigma-Aldrich, T5648) was intraperitoneally injected for five consecutive days. Then, 7 d after the final injection, anaesthesia, CTX injury and FR-np injection were performed. Three animals per group were used for each time point. The animals were housed under temperatures of 18–23 °C, 40–60% humidity, 12 h light–12 h dark cycle, and were given food and water ad libitum.

Electrophysiological analysis. Before collecting muscle samples, CMAPs of each TA muscle were measured after stimulating the sciatic nerve in hindlimbs using needle electrodes as previously described. In brief, the mouse sciatic nerve was exposed to electrical stimuli (single-pulse shocks, 1 mA, 0.1 ms), and CMAPs were recorded on the TA muscle belly from 1 V. Normal CMAPs from the contralateral side of the sciatic nerve were also recorded for comparison. The Grass S88X Stimulator (Grass Medical) was used for electrical stimulation of muscle fibers, and the PolyVIEW16 data acquisition software (Astro-Med) was used for the recording.

Force measurement. The isometric tetanic force of all mice TA muscles was measured using a commercial device (Grass Tech, Astro-Med) as previously described. In brief, mice were anesthetized by isoflurane and warmed by a heating lamp during the entire procedure, the tendon was exposed and attached to a force transducer (Grass FT03 Transducer, Astro-Med), and the knee was immobilized by a stainless-steel pin. The electrical stimulation was performed using a bipolar electrode with a Grass Stimulator to the sciatic nerve. The maximum isometric tetanic force was achieved by applying single-pulse stimuli (voltage = 12 V, duration = 0.2 ms, pulse rate = 100 Hz) at an optimal muscle length, which was adjusted with 0.5 mm increments. Data were acquired and recorded using PolyVIEW16 (Grass Tech, Astro-Med). After the completion of the isometric force testing, the mouse was euthanized and the entire TA muscle was carefully dissected and weighed.

Muscle sample collection. TA muscles were collected at various time points, and fresh frozen by liquid-nitrogen-cooled isopentane (Sigma-Aldrich) for 1 min. Muscles from mice implanted with DiRed-labelled cells and Pax7-c-ER; Rosa26-eYFP mice were fixed at room temperature for 2 h in 1% paraformaldehyde, and dehydrated with 20% sucrose. Muscle cryosections were then suspended in 30 μl PBS was injected into the injured TA muscles of adult C57BL/6 mice (8 weeks). Muscle samples were collected after 2 d and 1 month, and cross and longitudinal cryosectioned before performing immunofluorescence analysis and imaging.

Histological staining. Haematoxylin and eosin (H&E) staining was performed on the basis of H&E-stained slides. Masson’s Trichrome staining was performed using standard protocols, and the total fibrotic area within a section was quantified using a threshold intensity program from ImageJ. The fibrotic index was calculated as the area of fibrosis divided by the total area of muscle.

Immunofluorescence staining. For cell immunostaining, cells were fixed in 4% parformaldehyde for 15 min and permeabilized with 0.5% Triton-X 100 in PBS for 15 min. Cells were then blocked with 5% donkey serum for 1 h and incubated overnight at 4 °C with primary antibodies (diluted in 5% donkey serum), including antibodies against Titin (mouse monoclonal antibody, DSHB, JL122, 1:10), Myh11E (mouse monoclonal antibody, DSHB, MF 20-c, 1:100), Myh2 (mouse monoclonal antibody, DSHB, SC-71-c, 1:100), Myh3 (mouse monoclonal antibody, DSHB, F1-652-s, 1:10), Myh4 (mouse monoclonal antibody, DSHB, BF-3-c, 1:100), Myh7 (mouse monoclonal antibody, DSHB, BA-D5-c, 1:100), Myh4 (mouse monoclonal antibody, DSHB, BS-61-c, 1:100), MANEX1011B(1C7) (dystrophin, mouse monoclonal antibody, DSHB, 1:100), MyoD (mouse monoclonal antibody, DSHB, 1:100), MyoG (mouse monoclonal antibody, DSHB, 1:100), Pax7 (mouse monoclonal antibody, DSHB, 1:100), Sox10 (goat polyclonal antibody, R&D Systems, AF2864, 1:200), Ki67 (rabbit monoclonal antibody, Abcam, ab16667, 1:200), FSP1 (rabbit monoclonal antibody,
Sigma-Aldrich, 07-2274, 1:200), CD90.2-FTTC (rat monoclonal antibody, Thermo Fisher Scientific, 11-9093-81, 1:100), P75 (rabbit polyclonal antibody, Abcam, ab8874, 1:100) and PDGFRα (rat monoclonal antibody, Thermo Fisher Scientific, 13-4018-1, 1:100). Next, appropriate Alexa Fluor 488-, Alexa Fluor 546- or Alexa Fluor 647-conjugated secondary antibodies (Thermo Fisher Scientific, 1:400) were used for 1 h at room temperature. Thereafter, nuclei were stained with DAPI (Sigma-Aldrich) for 10 min in the dark.

For immunohistochemical staining, the same protocol was used with minor modifications. Mid-belly transverse sections (thickness, 10μm) were fixed in 4% (v/v) paraformaldehyde for 10 min and washed with PBS for 5 min (three times), then permeabilized with 0.5% (v/v) Triton X-100 (Sigma-Aldrich) for 10 min. Slices were then blocked with 5% donkey serum in 0.1% (v/v) Triton X-100 for 1 h and incubated overnight at 4°C with primary antibodies (diluted in 5% donkey serum), including antibodies against laminin (rabbit monoclonal antibody, Sigma-Aldrich, L9393, 1:200), Pax7 (mouse monoclonal antibody, DSHB, 1:100), Ki67 (rabbit monoclonal antibody, Abcam, ab16667, 1:200) and F4/80 (rat monoclonal antibody, Abcam, ab6640, 1:200). For Pax7 staining, heat-activated antigen retrieval was performed by placing the paraformaldehyde-fixed samples in citrate buffer (pH 6.0) at 95 °C for 20 min and cooling the slides at room temperature for 20 min, followed by permeabilization and blocking before costaining with other antibodies as mentioned above. For myofibre staining, fresh frozen sections were used for staining with primary antibodies, including BA-D5 concentrate (anti-myosin heavy chain type I, mouse monoclonal antibody, DSHB, 1:100), SC-71 concentrate (myosin heavy chain type IIa, mouse monoclonal antibody, DSHB, 1:100), BF-F3 concentrate (anti-myosin heavy chain type IIb, mouse monoclonal antibody, DSHB, 1:100) and anti-laminin (rabbit monoclonal antibody, Sigma-Aldrich, L9393, 1:200), and then stained with secondary antibodies, including DyLight 405 AffiniPure goat-anti-mouse IgG2b (Jackson ImmunoResearch Laboratories, 115-475-207, 1:400), Alexa Fluor 488 AffiniPure goat-anti-mouse IgG1 (Jackson ImmunoResearch Laboratories, 115-545-205, 1:400) and Alexa Fluor 594 AffiniPure goat-anti-mouse IgM (Jackson ImmunoResearch Laboratories, 115-585-075, 1:400), respectively. All fluorescent images were taken using a Zeiss Axio Observer Z1 inverted microscope and a confocal inverted Leica TCS-SP8-SMD confocal microscope.

Statistical analysis. Values are expressed as mean ± s.d. unless otherwise indicated. The significance between two groups was analysed using two-tailed Student's t-test. For multiple comparisons, one-way analysis of variance (ANOVA) with Tukey's post hoc test was used. Statistical analysis was performed using the Origin 2018 software. P < 0.05 was considered to be significant; **P < 0.01, ***P < 0.001, ****P < 0.0001.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The main data supporting the results in this study are available within the paper and its Supplementary Information. Data for the microarray and scRNA-seq have been deposited in the Gene Expression Omnibus under accession numbers GSE158690 and GSE158691, respectively. All data generated in this study, including source data and the data used to make the figures, are available at Figshare (https://doi.org/10.6084/m9.figshare.13049690.v1).

Code availability. The custom code used is available at GitHub (https://github.com/junrensia/Jun_et_al_Nature_BME_2020/).

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Articles

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Author contributions

J.F., J. Sia, J.G.T. and S.L. designed the project and experiments and wrote the manuscript. J.F., J. Sia and P.W. performed the myogenic induction with small molecules. J.F. performed the immunofluorescence staining, histological analysis, drug delivery system preparation and animal studies. J.F. and J. Soto performed flow cytometry analysis. J. Sia and R.S. performed microarray analysis and gene expression analysis. J.F., J. Sia and J. Soto performed single-cell sequencing and data analysis. J.F. and K.E. performed HPLC analysis. J.F., L.K.L. and Y.-Y.H. performed electrophysiological analysis. All of the authors revised the manuscript and added comments.

Competing interests

The innovation related to this study has been filed for patent application by J.F., S.L. and J. Sia. (US Serial No. 30435411.2020).

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41551-021-00696-y.
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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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| Give P values as exact values whenever suitable. | ☐ |
| For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings | ☐ |
| For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes | ☐ |
| Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated | ☐ |

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection | Data analysis
--- | ---
Data collection was performed by using the PolyViWE16 Version 1.1 platform for muscle force and electrophysiological analysis, dynamic light scattering (DLS) for measuring the size and distribution of nanoparticles, the CFX96 Real-Time PCR Detection System for measuring mRNA expression, guavaSoft 3.4 for flow cytometry on Guava easyCyte, BD FACSDiva Software Version 8.0.2 for FACS sorting and NextSeq500 for single-cell sequencing. | Data analysis was performed by free or commercial software platforms, including ImageJ for measuring the cell percentage and fibrosis, Origin 2018 for bar charts or box charts and statistic analysis, the Oligo package and Limma package for microarray analysis, DAVID for gene-ontology enrichment analysis, Seurat and Monocle 2.12 for plotting single-cell sequencing, and the MAST package v1.10 for managing and analysing single-cell gene-expression data.

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Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The main data supporting the results in this study are available within the paper and its Supplementary Information. Data for the microarray and scRNA-seq have been deposited in the NCBI Gene Expression Omnibus under accession numbers GSE158690 and GSE158691, respectively. All data generated in this study, including source data and the data used to make the figures, are available from figshare at https://doi.org/10.6084/m9.figshare.13049690.v1.
Field-specific reporting
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Life sciences study design
All studies must disclose on these points even when the disclosure is negative.

Sample size
A minimum of three independent experiments were carried out for all in vitro studies. For in vivo functional tests, the sample size was five. The sample size necessary to detect a significant effect was estimated by using data from pilot studies with the following information: minimum significant effect to be detected, data variation, power (0.8) and Type-I error rate (0.05). The significance between two groups was analyzed by two-tailed Student’s t-test. For multiple comparisons, one-way analysis of variance (ANOVA) with Tukey’s post hoc test was used. Statistical analysis was performed using Origin 2018 software. A p-value < 0.05 was considered significant.

Relevant reference: Quarta M. et al. An artificial niche preserves the quiescence of muscle stem cells and enhances their therapeutic efficacy. Nat. Biotech. 34, 752–759 (2016).

Data exclusions
No data were excluded from analysis.

Replication
In all experiments, either replicate or triplicate of samples were processed in parallel. All experiments were performed independently multiple times.

Randomization
All samples and mice were randomly selected and divided into different groups.

Blinding
The investigators were blinded to group allocation during data collection and analysis, except for histological analysis and immunofluorescence staining.

Reporting for specific materials, systems and methods
We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | Antibodies            |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology         |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |
| ☑   | Clinical data         |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChIP-seq              |
| ☑   | Flow cytometry        |
| ☑   | MRI-based neuroimaging |

Antibodies

Primary antibodies:
- TnT (mouse mAb, DSHB, JLT12, 1:10), Myh1E (mouse mAb, DSHB, MF 2D-c, 1:100), Myh2 (mouse mAb, DSHB, SC-71-c, 1:100), Myh3 (mouse mAb, DSHB, F1.652-s, 1:10), Myh4 (mouse mAb, DSHB, BF-F3-c, 1:100), Myh7 (mouse mAb, DSHB, BA-D5-c, 1:100), Myh8 (rabbit pAb, Thermo Fisher Scientific, PAS-72846, 1:100), MANEX10118(1C7) (dystrophin, mouse mAb, DSHB, 1:100), MyoD (mouse mAb, DSHB, 1:100), MyoG (mouse mAb, DSHB, 1:100), Pax7 (mouse mAb, DSHB, 1:100), Sox 10 (goat pAb, R&D Systems, AF2864, 1:), Ki 67 (rabbit mAb, Abcam, ab16667, 1:200), FSP1 (rabbit pAb, Sigma, 07-2274, 1:200), CD 90.2-FITC (rat mAb, Thermo Fisher Scientific, 11-0903-81, 1:100), P75 (rabbit pAb, Abcam, ab8874, 1:100), PDGFR-α (rat mAb, Thermo Fisher Scientific, 13-1401-82, 1:100), Laminin (rabbit mAb, Sigma, L9393, 1:200), F4/80 (rat mAb, Abcam, ab6640, 1:200).

Secondary antibodies:
- Alexa Fluor 488 Donkey anti-mouse IgG (H+L) Secondary Antibody (Thermo Fisher Scientific, A-21202, 1:500).
- Alexa Fluor 546 Donkey anti-mouse IgG (H+L) Secondary Antibody (Thermo Fisher Scientific, A-10036, 1:500).
- Alexa Fluor 647 Donkey anti-mouse IgG (H+L) Secondary Antibody (Thermo Fisher Scientific, A-3157, 1:500).
- Alexa Fluor 488 Donkey anti-Rabbit IgG (H+L) Secondary Antibody (Thermo Fisher Scientific, A-21206, 1:500).
- Alexa Fluor 546 Donkey anti-Rabbit IgG (H+L) Secondary Antibody (Thermo Fisher Scientific, A-10040, 1:500).
- Alexa Fluor 647 Donkey anti-Rabbit IgG (H+L) Secondary Antibody (Thermo Fisher Scientific, A-31573, 1:500).
- Alexa Fluor 488 Donkey anti-Goat IgG (H+L) Secondary Antibody (Thermo Fisher Scientific, A-11055, 1:500).
Validation

We used standard antibodies validated by the manufacturers, and previous studies in many laboratories, including our own, and the antibodies were purchased from commercial vendors.

Primary antibodies:
TnT: On product website (https://dshb.biology.uiowa.edu/LT12).
Myh1E: On product website (https://dshb.biology.uiowa.edu/SC-71).
Myh2: On product website (https://dshb.biology.uiowa.edu/MF-20).
Myh3: On product website (https://dshb.biology.uiowa.edu/SC-71).
Myh4: On product website (https://dshb.biology.uiowa.edu/F1-652).
Myh7: On product website (https://dshb.biology.uiowa.edu/BQ-F3).
Myh8: On product website (https://www.thermofisher.com/antibody/product/MYH8-Antibody-Polyclonal/PA5-7284).
MANEX1011B(1C7): On product website (https://dshb.biology.uiowa.edu/1C7).
MyoD: On product website (http://dshb.biology.uiowa.edu/MyoD).
MyoG: On product website (http://dshb.biology.uiowa.edu/myogenin).
Pax7: On product website (http://dshb.biology.uiowa.edu/PAX7).
Sox 10: On product website (https://www.rndsystems.com/products/human-sox10-antibody_af2864).
FSP1: On product website (http://www.emdmillipore.com/US/en/product/Anti-FSP1-S100A4-Antibody,MM_NF-07-2274).
CD 90.2-FITC: On product website (https://www.thermofisher.com/antibody/product/CD90-2-Thy-1-2-Antibody-clone-30-H12-Monoclonal/11-0903-82).
P75: On product website (https://www.abcam.com/p75-ngf-receptor-antibody-ab8874.html).
PDGFR-a: On product website (https://www.thermofisher.com/antibody/product/CD140a-PDGFR-Antibody-clone-APAS-Monoclonal/13-1401-82).
Laminin: On product website (https://www.sigmaaldrich.com/catalog/product/sigma/9399-lang=en&region=US).
Ki 67: On product website (https://www.abcam.com/k67-antibody-sp6-ab16667.html).
FA/80: On product website (https://www.abcam.com/f480-antibody-cia3-1-macrophage-marker-ab6640.html).

Secondary antibodies:
Alexa Fluor 488 Donkey anti-Mouse IgG (H+L) Secondary Antibody: On product website (https://www.thermofisher.com/antibody/product/A10504).
Alexa Fluor 546 Donkey anti-Mouse IgG (H+L) Secondary Antibody: On product website (https://www.thermofisher.com/antibody/product/A10505).
Alexa Fluor 647 Donkey anti-Mouse IgG (H+L) Secondary Antibody: On product website (https://www.thermofisher.com/antibody/product/A10506).

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
Platinum-E Retroviral Packaging Cell Line from Cell Biolabs, Inc.

Authentication
Cell lines were authenticated.

Mycoplasma contamination
Cells were not tested for mycoplasma contamination.

Commonly misidentified lines
No commonly misidentified cell lines were used.

See ICLAC register
Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Neonatal CS7BL/6 and Pax7-CreER:Rosa26-EYFP mice, male and female, 1–3 days old; Adult CS7BL/6 and Pax7-CreER:Rosa26-EYFP mice, male and female, 8–10 weeks; aged CS7BL/6J mice, male and female, 18–19 months, mdx (C57BL/10ScSn-Dmdmdx/J) mice, female, 8–10 weeks. The animals were housed at temperatures of ~18–23°C, 40–60% humidity, 12-hour light/12-hour dark cycle, and were allowed food and water ad libitum.

Wild animals
The study did not involve wild animals.

Field-collected samples
The study did not involve samples collected from the field.

Ethics oversight
All animal work was conducted under protocols approved by the Animal Research Committees of UC Berkeley or UCLA.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots
Confirm that:
☑ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☑ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☑ All plots are contour plots with outliers or pseudocolor plots.
☑ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Flow cytometry analysis of fibroblasts: dermal cells were isolated from the neonatal mice, and cultured overnight and digested by accutase for flow cytometry.

FACS sorting of EYFP+ and EYFP– cells: Neonatal dermal cells isolated from Pax7-CreER:Rosa26-EYFP mice, 1 μM 4-OHT was added in the Fb medium during cell seeding. One day later, cells were induced with FR medium for 4 days, then dissociated with Accutase and neutralized by FBS-containing media before FACS sorting.

Instrument
Guava easyCyte 5 Benchtop Flow Cytometer, MilliporeSigma, 0500-5005. FACS sorting of EYFP+ and EYFP– cells: FACS Aria II instrument (Becton-Dickinson).

Software
GuavaSoft3.2; 2. BD FACSDiva Software.

Cell population abundance
The major cell population in the dermal derived cells were PDGFR-α positive cells. EYFP+ cells and EYFP– cells were collected.

Gating strategy
A single gate was created for analysis and/or acquisition. The gating process has been simplified by dragging and dropping regions. Cells without staining or labelling were used as negative control to decide the boundaries.

☑ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.