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Engineering an Injectable Muscle-Specific Microenvironment for Improved Cell Delivery Using a Nanofibrous Extracellular Matrix Hydrogel

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Abstract

Injection of skeletal muscle progenitors has the potential to be a minimally invasive treatment for a number of diseases that negatively affect vasculature and skeletal muscle, including peripheral artery disease (PAD). However, success with this approach has been limited because of poor transplant cell survival. This is primarily attributed to cell death due to extensional flow through the needle, the harsh ischemic environment of the host tissue, a deleterious immune cell response, and a lack of biophysical cues supporting exogenous cell viability. We show that engineering a muscle specific microenvironment, using a nanofibrous decellularized skeletal muscle extracellular matrix hydrogel (SkECM) and skeletal muscle fibroblasts, improves myoblast viability and maturation *in vitro*. *In vivo*, this translates to improved cell survival and engraftment and increased perfusion as a result of increased vascularization. Our results indicate that a combinatorial delivery system, which more fully recapitulates the tissue microenvironment, can improve cell delivery to skeletal muscle.

Keywords

Biomaterial; muscle; myoblast; peripheral artery disease; cell delivery; cell microenvironment; injectable

PAD affects millions of people globally.¹ This leads to decreased blood flow in the legs, which can ultimately result in non-healing ulcers and amputation. Typical therapies consist of atherectomy, stents, angioplasty, and bypass surgery; however, these are limited with only

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Supporting Information **Available**

Includes Supplemental Figures 1 through 5 and Supplemental Video 1. The material is available free of charge *via* the Internet at <http://pubs.acs.org>.

40% of PAD patients being candidates and with success rates that do not support successful procedural outcomes with one therapy alone.² The primary goal for treating PAD is to improve vascularization in the ischemic limb. Secondly, it may also be advantageous to regenerate the damaged and atrophied muscle. Skeletal myoblasts have been shown to increase angiogenesis by paracrine effects and, in tandem, can fuse with one another in damaged muscle to regenerate the tissue;^{3–4} however, they have poor cell survival and engraftment.⁵ A number of factors could be responsible: low pH, apoptotic paracrine signals, negative effects of inflammatory cells, and the lack of healthy ECM.⁵ As a result, groups inject high cell numbers; however, injecting dense concentrations of cells through a syringe and needle causes death due to extensional flow.⁶

ECM hydrogels derived from decellularized skeletal muscle have recently been developed as an injectable scaffold to promote tissue remodeling after skeletal muscle injury.^{7–8} The hydrogel contains a complex proteomic signature similar to healthy muscle ECM,^{7–8} has a nanofibrous architecture similar to the native ECM,⁹ and has shear thinning properties, which makes it ideal for injection. Cell delivery without biomaterials has resulted in poor cell survival and engraftment *in vivo*,¹⁰ but recent studies suggest that mimicking a supportive microenvironment with materials and growth factors may help combat these issues.¹¹ We hypothesized that a nanofibrous ECM hydrogel derived from decellularized skeletal muscle would create a supportive microenvironment for cell delivery to skeletal muscle. To further create a supportive niche, we also examined co-delivery of skeletal muscle fibroblasts, stromal support cells, which have been previously shown to improve myoblast viability and maturation *in vitro* through both soluble and cell contact signaling events,¹² but have yet to be tested *in vivo*. Here we show that both the complex nanofibrous ECM hydrogel scaffold and delivery with a stromal support cell improves cell viability and differentiation *in vitro*, which translated to improved cell viability, engraftment, and ischemic limb perfusion *in vivo*.

Results and Discussion

SkECM Hydrogel Improves Myoblast Viability and Differentiation *In Vitro*

To solve some of the challenges related to poor cell transplant survival, we sought to buffer cells from the forces during injection and recreate the skeletal muscle microenvironment in terms of both the ECM and supporting cells. To recreate the ECM, we took advantage of a recently developed injectable hydrogel derived from decellularized skeletal muscle ECM, which was shown to improve neovascularization and cause a shift in the inflammatory response following intramuscular injection in a rat hindlimb ischemia model.^{7,9} When the liquid SkECM is heated to 37 °C *in vitro* or injected *in vivo*, it creates a nanofibrous hydrogel,⁷ which we envisioned could be used to encapsulate cells. We first measured the physical properties at two concentrations, 6 mg/mL and 8 mg/mL (Fig. 1a–d), which we had previously found to be easily injectable, yet still form a nanofibrous gel *in vitro* (Fig. 1a,b). The 8 mg/mL solution provided a more viscous and stiffer gel than 6 mg/mL, which matched the properties of collagen gels at 2.5 mg/mL (Fig. 1c,d). To evaluate whether injection of the SkECM hydrogel and cells was feasible, myoblast viability was evaluated after resuspending cells with the SkECM hydrogel and injecting through a syringe and

needle *in vitro*. Murine eGFP myoblasts were first isolated and shown to be >95% desmin-positive (Supplemental Fig. 1). Upon suspension of the myoblasts in PBS or the SkECM hydrogel at concentrations of 6 or 8 mg/mL, the cells were approximately 99% viable at 40,000 cells/ μ L (Supplemental Fig. 2). After injection through 25, 27, and 30 G needles, the cell-material solution was evaluated *via* Trypan Blue. Viability of the cells decreased as gauge size increased in PBS; however, viability was significantly increased in the SkECM groups compared to PBS (Fig. 1e, Supplemental Fig. 3). These results are consistent with other hydrogels that were tuned to resist the forces cells experience upon injection.⁶

We sought to model cell transplantation *in vitro* by encapsulating myoblasts in the SkECM as a 3D cell-hydrogel construct, and monitoring viability in response to reactive oxygen species (ROS), which have been demonstrated as a significant contributor in transplant cell apoptosis in ischemic tissue.^{13–14} We chose to compare the SkECM hydrogel to collagen since it is the predominant component of the ECM hydrogel.⁸ We previously showed that an equivalent concentration of SkECM (6 mg/mL) compared to collagen had superior angiogenic and myogenic properties *in vivo* when delivered alone.⁷ Since the equivalent concentration of collagen produces a very dense and stiff gel, in this study, we chose to use 2.5 mg/mL collagen as a control since it matched the 6 mg/mL SkECM hydrogel's mechanical properties, which are known to influence cell behavior. Metabolic activity by an Alamar Blue assay showed that 24 hours post-encapsulation, initial viability in 3D gels of either the SkECM or collagen was not significantly different (Supplemental Fig. 4). Next, cells were either subjected to growth media or growth media containing H₂O₂ (n=6/group). After 24 hours of treatment, metabolic activity in the SkECM groups was greater than collagen gels or 2D tissue culture plastic as demonstrated by a reduction of metabolic activity by 80% in SkECM groups in comparison to 90–95% reduction of metabolic activity in collagen gels and plastic, suggesting improvements in cell survival (Fig. 1f). The complex microenvironment (multiple ECM proteins, proteoglycans, and potentially various growth factor binding sites) of the SkECM, which was previously demonstrated through quantitative mass spectrometry analysis,⁸ could be contributing to an overall increase in cell viability under these stressed conditions.

Previously, tissue culture plates coated with the liquid SkECM promoted differentiation of C2C12 myoblasts compared to a collagen coating.¹⁵ To test whether the skeletal muscle specific hydrogel could also promote myoblast differentiation when cells are encapsulated, we added differentiation media to 3D constructs and monitored differentiation with qPCR. When encapsulated for 3 days in culture, the SkECM gels significantly increases MyoD expression compared to collagen gels (Fig. 1g). Myosin heavy chain (MHC) expression increases as differentiation proceeded and was significantly higher in both 6 and 8 mg/mL SkECM compared to collagen by day 7 (Fig. 1h). Overall, the SkECM hydrogel outperformed collagen in terms of protection from ROS, and promoted increased differentiation.

SkECM Hydrogel Improves Transplanted Myoblast Survival and Engraftment *In Vivo*

Given our superior *in vitro* results with the SkECM hydrogel, we sought to test whether delivery of myoblasts in the SkECM hydrogel would help viability and engraftment *in vivo*

compared to the standard injection in saline. eGFP myoblasts were pre-labeled with a far-infrared DiR dye, and cell survival following injection of 1×10^6 myoblasts into the tibialis anterior (TA) of C57BL/6 mice was monitored for one week using an In Vivo Imaging System (IVIS). For short-term studies, up to 10 days, DiR has been shown to be useful for cell tracking and quantification.^{16–19} Cell survival, as measured by the relative total radiant efficiency, in the 8 mg/mL SkECM hydrogel was significantly increased compared to delivery in PBS (Fig. 2a). After one week, the TAs were excised and re-imaged to confirm that the cells were localized to the injected muscle (Fig. 2b). The 8 mg/mL gels showed increased myoblast cell retention within the TAs compared to PBS and the 6 mg/mL SkECM group. Immunohistochemical analysis of the excised muscle confirmed this finding (Fig. 2c). GFP colocalization with MHC showed that the cells had engrafted and differentiated into skeletal muscle. Overall, the SkECM hydrogel significantly improved cell engraftment in the TA, with the greatest increase in the 8 mg/mL concentration. Transplanted myoblasts have typically had very poor survival *in vivo* even when delivered in healthy muscle.⁵ While we focused on the treatment of ischemic skeletal muscle in our subsequent experiments in this study, these findings showing a dramatic ~20 fold increase in myoblast engraftment using an injectable hydrogel are encouraging for the delivery of skeletal muscle progenitors or stem cells in other non-ischemic conditions such as muscular dystrophies and other myopathies, where skeletal myoblast therapy has likewise suffered from poor engraftment.³

Skeletal Fibroblasts Increase Myoblast Metabolic Activity, Survival, and Differentiation *In Vitro*

In addition to the ECM, the skeletal muscle microenvironment consists of supporting cell types, such as fibroblasts. For the heart, groups have shown that cardiac fibroblasts are necessary to increase cardiomyocyte viability and promote electrical propagation, function, and engraftment.^{20–21} *In vitro* studies suggest that skeletal muscle fibroblasts could also aid in myoblast viability and increase myotube alignment.¹² We sought to investigate whether adding 10% primary mCherry fibroblasts (Supplemental Fig. 5) in the SkECM hydrogel would further improve viability and differentiation based on previous work demonstrating a similar 9:1 myoblast to fibroblast ratio to be important in *ex vivo* muscle formation.²² eGFP myoblasts were encapsulated as described above in the 8 mg/mL SkECM hydrogel given its superior cell viability. For the first experiment they were encapsulated in the bottom well of a transwell with or without skeletal fibroblasts in the upper chamber. The fibroblast group increased encapsulated myoblast metabolic activity after seven days in a transwell, indicating that paracrine activity alone of the fibroblasts positively influenced myoblast behavior in the 3D matrix (Fig. 3a). Next, myoblasts were encapsulated with or without 10% skeletal fibroblasts in 8 mg/mL gels and subjected to growth media with or without H₂O₂ to simulate ROS *in vitro*. The co-culture of myoblasts and fibroblasts had significantly higher metabolic activity under ROS conditions when normalized to their untreated control compared to encapsulated monocultures of myoblasts or fibroblasts subjected to the same conditions (Fig. 3b), suggesting co-encapsulation with fibroblasts in the SkECM hydrogel could further improve cell survival.

We next assessed whether fibroblasts could also increase differentiation of myoblasts in the SkECM *in vitro*. There was a noticeable increase in both MyoD expression on Day 3 and

MHC expression on Day 7 for the myoblast-fibroblast co-culture (Fig 3c–d). Myotubes were visually confirmed in the co-culture and appeared more elongated and multi-nucleated compared to myoblasts alone (Fig. 3e–f). By 9 days, myotubes in co-culture, but not monoculture, spontaneously contracted (Supplemental Video 1), indicating an increased maturation towards a muscle-like construct. Bi-directional soluble signaling between myoblasts and fibroblasts has been previously observed, although this was shown to reduce differentiation in 2D cultures.¹² In addition to soluble signaling, another possibility is that fibroblasts in the co-culture gel constructs contracted the hydrogel resulting in an effectively stiffer gel since the co-culture-gel constructs had a visibly smaller size. This could have increased differentiation as myoblast differentiation has been shown to increase on stiffer substrates.^{23–24} It is also possible that secreted soluble growth factors bound to the SkECM due to sulfated glycosaminoglycans present in ECM hydrogels.^{25–26} Soluble FGF-2 strongly inhibits myoblast differentiation; however if the growth factor is sequestered and thereby inhibited, myoblast differentiation is enhanced,²⁷ which could explain the differences we observed compared to previous 2D experiments with collagen.¹² This highlights the importance of not only studying cells in 3D, but also encapsulating them in a material that mimics the complex nature of the native ECM.

Combination ECM Hydrogel and Fibroblast Co-delivery Improves Myoblast Viability and Perfusion in Ischemic Muscle

Finally, we examined whether the addition of fibroblasts in the SkECM hydrogel *in vivo* could enhance myoblast viability and engraftment in a mouse hindlimb ischemia model, and whether this would increase perfusion over the typical myoblast only injection in saline. All groups received injections of 1×10^6 DiR-labeled eGFP myoblasts into the ischemic limb 7 days post-surgery and IVIS measurements were taken on day 0, 3, and 7 post-injection. Cell viability was increased in the groups containing SkECM compared to saline at day 3, and significantly increased with the addition of 1×10^5 fibroblasts on day 7 compared to saline (Fig. 4a,b). We hypothesize that this was due to the SkECM protecting cells from early apoptosis in the ischemic environment (0–3 days), followed by fibroblasts aiding in myoblast viability *via* positive cell signaling between fibroblasts and myoblasts (3–7 days onward). This trend was confirmed at 35 days post-injection by immunohistochemical analysis. The fibroblast group contained twice as many myoblasts engrafted in the TA than the myoblasts alone in either saline or SkECM (Fig. 4c). Given that myoblasts have been shown to have limited long term engraftment, sometimes even undetectable when delivered in healthy muscle,⁵ this combinatorial approach may also be useful for the delivery of other muscle stem cells such as satellite cells, which have greater inherent potential for engraftment.⁵

Perimed laser speckle contrast analysis measurements (Fig. 4d) showed an increasing trend in perfusion on day 10 and 21 post-injection for the groups containing the SkECM vs. myoblasts injected in saline, which was significant by day 35; however, there were no differences in perfusion between the myoblasts alone in the SkECM hydrogel and myoblasts and fibroblasts in the hydrogel (Fig. 4e). When analyzing the change in perfusion from day 0 (pre-injection) to day 35, both SkECM hydrogel groups significantly increased perfusion compared to saline injected groups (Fig. 4f). This was corroborated with

immunohistochemical analysis at day 35 which demonstrated increased capillary and arteriole density with both SkECM hydrogel groups (Fig. 4g–j). Previously, the SkECM hydrogel alone was shown to increase perfusion in a rat hindlimb ischemia model, which was attributed to changes in arteriogenesis rather than angiogenesis.⁸ In the current study, capillary density trended higher with increasing myoblast survival (Fig. 4h); however, arteriole density was the same for both SkECM groups (Fig. 4i,j) despite the significant increase in cell survival with the addition of fibroblasts. Other preclinical studies have suggested that arteriogenesis rather than angiogenesis may be more desirable for treating PAD patients.^{28–29} Although we cannot determine the extent to which arteriogenesis *versus* angiogenesis influenced the improved perfusion seen in the current study, it seems that the synergistic delivery of SkECM and tissue specific cells had a significant effect on capillary density at this chronic time point.

Conclusion

We show that the nanofibrous SkECM hydrogel aids in the initial delivery of cells, protecting them from the forces upon injection and a harsh ischemic environment. The material also improves blood flow to the damaged region. By further recapitulating the skeletal muscle microenvironment with skeletal muscle fibroblasts, myoblast retention and viability was increased at 7 up to 35 days post-injection, indicating a long-term effect on myoblast cell survival. This work suggests that myoblast viability and engraftment can be increased by incorporation of a complex myoblast-appropriate microenvironment, and highlights the need to recreate multiple aspects of a tissue, not just injection of a single cell type without the appropriate extracellular cues.

Materials and Methods

Skeletal muscle decellularization, neutralization, and preparation

Porcine major psoas muscle tissue was harvested from Yorkshire pigs and decellularized following a modified version of a previously reported protocol.⁷ Briefly, the skeletal muscle was cut into small pieces and decellularized in a solution of 1% (wt/vol) sodium dodecyl sulfate (SDS), phosphate buffered saline (PBS), and 0.5% penicillin/streptomycin. After 5 days in SDS solution, the tissue was then rinsed in isopropyl alcohol for an additional 24 hours to remove residual fat. The tissue was rinsed repeatedly with water and frozen at -80°C . The material was then lyophilized, milled and collected for long-term storage. The material was digested with sterile pepsin and hydrochloric acid as previously described for 2 days.⁷ The solution was neutralized on ice in sterile conditions such that the solution had a final concentration of 8 mg/mL and a pH of 7.4. The gel was aliquoted and frozen at -80°C then lyophilized for future cell experiments. The Picogreen Assay (Life Technologies, Carlsbad, CA) and 1,9- dimethylmethylene blue (DMMB, Sigma-Aldrich) assays were used to determine amount of DNA isolated (DNEasy Kit, Qiagen, Valencia, CA) from the material and sulfated GAG content respectively. Gelation was determined when the gel no longer flowed (i.e restricted from movement by gravity upon inversion) by previously documented methods.⁷ Scanning electron microscopy (SEM) was performed on gelled ECM material. Gels were fixed with 4% paraformaldehyde and dehydrated with serial ethanol

rinses. Samples were sputter coated with 7 nm of platinum (Leica SCD, Leica, Vienna) and imaged on an FE-SEM at 0.6 kV using the in-lens SE1 detector (Sigma VP, Zeiss Ltd, Cambridge, UK).

Rheological and viscosity measurements

To evaluate the storage modulus of the skeletal muscle matrix, lyophilized aliquots were resuspended to create 500 μ L of 2.5 mg/mL rat tail collagen, 6, or 8 mg/mL SkECM solutions. These solutions were allowed to gel at 37°C overnight ($n = 3$). Gels were placed on a TA Instruments ARG2 Rheometer with a 37°C preheated 20 mm parallel plate geometry and a 1.2 mm gap distance. Storage modulus was recorded at frequencies from 0.4–16 Hz, and plotted at 1 Hz. For viscosity measurements, 200 μ L of liquid SkECM was used with a gap height of 500 μ m. Data was generated for frequencies from 0.1–50 Hz.

Isolation of primary GFP myoblasts and mCherry fibroblasts

EGFP primary skeletal myoblasts and mCherry skeletal fibroblasts were isolated from transgenic mice with a C57BL/6 background, C57BL/6-Tg(UBC-GFP)30Scha/J and B6(Cg)-Tyr^c-2J Tg(UBC-mCherry)1Phbs/J respectively (Jackson Laboratory). The muscle tissue was harvested from the hind limbs of the mice, minced, and cultured on collagen-adsorbed plates. Fibroblasts were removed from the myoblast population per previously established protocols.³⁰ Subsequent rounds of preplating for approximately 15 passages separated the fibroblast population from the myoblasts. Myoblasts were stained with anti-Desmin antibody (1:100 in Staining Buffer) to confirm over 95% purity of the cell population (Supplemental Fig. 1).

Cell culture

Mouse primary eGFP myoblasts and primary mCherry fibroblasts were cultured in growth media (GM) consisting of 45% low glucose Dulbecco's Modified Eagle Medium (Life Technologies, Carlsbad, CA), 40% HAMS F-10 (Life Technologies, Carlsbad, CA), 15% fetal bovine serum (FBS, Thermo Scientific), and 0.5% penicillin/streptomycin (Life Technologies, Carlsbad, CA). bFGF at 2.5 ng/mL (Life Technologies, Carlsbad, CA) was added to myoblast cultures. For both the myoblast and the fibroblast cultures, tissue culture flasks were coated with 1 mg/mL collagen in 0.1 M acetic acid for 1 hour at 37°C and rinsed with 1 $\times$ Dulbecco's Phosphate Buffer Saline prior to seeding. The cells were culture at 37°C and 5% CO₂ and split 1:3 when 90% confluence was reached; media was changed every two days.

Cell encapsulation

The lyophilized ECM was resuspended with 500 μ L of sterile water that was supplemented with 0.49 mM magnesium chloride and 0.9 mM of calcium chloride. These suspensions were vortexed to form a homogeneous liquid and kept on ice. Total cell number and ratio was varied depending on the experiment. The liquid mixture (25 μ L) was added to a 24 well plate preheated to 40°C. The plate was incubated at 37°C and 5% CO₂ for 20 minutes. Another 25 μ L of the mixture was added directly above the previous 25 μ L and then incubated for 20 minutes again. One mL of GM was added to each well after gelation. After

one day the GM was replaced to differentiation media (DM) that consisted low glucose Dulbecco's Modified Eagle Medium containing 2% Horse Serum (Thermo Scientific) and 0.5% penicillin/streptomycin. The encapsulations were cultured at 37°C and 5% CO₂; the media was changed every two days.

Metabolic activity assays

Cells were encapsulated at 300,000 cells/25 μ L of ECM (50 μ L total, n=6) or 2.5 mg/mL collagen in 24-well plates. 1 mL of GM was added to the cells. 100 μ L of Alamar Blue reagent (Life Technologies, Carlsbad, CA) was added to the media and allowed to incubate for at least 4 hours until fluorescent readings were taken by removing 100 μ L of supernatant and added into a black 96-well plate. A BioTEK fluorescent plate reader was used to detect Alamar Blue at 560 Ex/590 Em. For assays containing reactive oxygen species, 2.5 mM H₂O₂ was premixed with the media prior to addition to the encapsulated gel. Peroxide treated groups were compared to non-treated groups to account for small variability such as cell number and cell-type differences.

Differentiation experiments

Cell-gel encapsulations were created as previously described with 300,000 cells/25 μ L, 600,000 total cells. For monoculture assays, myoblasts were either resuspended in 2.5 mg/mL rat-tail collagen, 6 mg/mL or 8 mg/mL SkECM. Myoblast cell number was fixed at 600,000 cells for co-culture groups with an additional 10% increase in total cell number (660,000) due to added mCherry skeletal fibroblasts. RNA was isolated with an RNEasy kit (Qiagen, Valencia, CA) from these gels at 0, 3, and 7 days upon adding DM. Cultures were pooled to generate enough RNA with a final 3 experimental samples per group. cDNA was generated by the SuperScript III Reverse Transcriptase kit (Applied Biosystems). Primers for MyoD (F: 5'- AGGACACGACTGCTTTCTTCA-3', R: 5'- TTAACCTTCTGCCACTCCGGA- 3'), GAPDH (F: 5'- CATCAAGAAGGTGGTGAAGC-3', R: 5'- GTTGTCATACCAGGAAATGAGC-3'), Myosin Heavy Chain GCAGAGACCGAGAAGGAG-3', R: 5'- CTTTCAAGAGGGACACCATC), and GFP (F: 5'- AAGCTGACCCTGAAGTTCATCTGC-3', R: 5'- CTTGTAGTTGCCGTCGTCCTTGAA-3'), were used to detect mRNA expression levels using SYBR Green PCR Master Mix (Applied Biosystems). Samples were run on the BioRad CFX96 Real-Time PCR Detection System. The following thermal cycle settings were used: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1min at 60°C. In monoculture assays, the gene expression was normalized to GAPDH. In co-culture assays, gene expression was normalized to GFP.

Syringe needle flow viability

Cells were resuspended in PBS, 6 mg/mL SkECM, or 8 mg/mL SkECM on ice at 40,000 cells/ μ L. 50 μ L of the solution was loaded into a 1mL syringe (Becton Dickinson). The syringe was fitted with either a 25G, 27G, or 30G needle and mounted on a syringe pump. The liquid was ejected into microcentrifuge tubes containing 1 mL PBS at 1 mL/min by a syringe pump. The cell-ECM-PBS suspension was immediately mixed 1:1 (30 μ L total) with Trypan Blue to stain dead cells (Life Technologies, Carlsbad, CA). Percent viability was

calculated by counting the number of dead cells and total cells using a hemocytometer across multiple gel formulations (n=10/group).

Cell injections and non-invasive imaging in naive murine hindlimbs

All animal procedures were performed in accordance with the guidelines established by the Committee on Animal Research at the University of California, San Diego and the American Association for Accreditation of Laboratory Animal Care. Myoblasts were stained with Xenolight DiR (Caliper, Hopkinton, MA) fluorescence dye at 320 mM for 30 min at 37°C. This concentration provided easily resolvable signal without cell toxicity. The cells were washed twice with PBS, trypsinized and spun down. 1 million myoblasts in a 25 μ L suspension of PBS, 6 mg/mL SkECM, or 8 mg/mL SkECM were kept on ice prior to animal injections. Four to six week old female albino C57BL/6 mice were used as cell delivery donors (Jackson Laboratory). The suspensions were injected *via* Hamilton syringe using a 27G needle directly into the TA of the mouse. The mice were immediately imaged under the In Vivo Imaging System Spectrum 200 (IVIS, Perkin Elmer) using the Perkin Elmer Living Image Software and thereafter each day for one week. ROIs were created and total radiant efficiency at 710 ex/760 em wavelengths was recorded each day for every mouse hindlimb. After one week of noninvasive monitoring, the mice were euthanized and the TA was excised. Once excised, the muscle was placed into the IVIS for one final end-point reading to verify the cells were localized in the TA muscle. Further histological analysis was used to quantify percent GFP-positive staining per area of muscle section.

Hindlimb ischemia surgery and laser speckle contrast imaging

Albino C57BL/6J mice were anesthetized with 5% isoflurane and lowered to 2.5% during surgery. The femoral artery and vein were ligated and removed from the right limb of the mouse. The other limb was used as an untreated control for all perfusion measurements. Immediately after surgery, perfusion measurements using the Perimed laser speckle contrast analysis system were recorded while under 2.5% isoflurane anesthesia. Images were recorded approximately 20 cm above the subject with a 5 $\times$ 5 cm area. Twenty-one images were recorded per second at a 0.17 mm resolution. Blood perfusion in the feet of the mice was recorded over time until perfusion leveled to a stable measurement (at least 20 minutes on a heated deck). Animals were required to have less than 30% blood perfusion immediately post- surgery compared to the control leg or they were not included in the study.

Myoblast injections into ischemic TAs

Seven days post-surgery, animal perfusion levels were recorded once more (pre-treatment). Cells were prepared as explained previously with Xenolight- DiR, and were either injected with 1 million myoblasts and 100,000 fibroblasts in 8mg/mL SkECM, 1 million myoblasts in 8mg/mL SkECM, or 1 million myoblasts in PBS (n=10/group, 25 μ L volumes). The animals were then imaged under the IVIS, 0, 3 and 7 days after injection. Laser speckle recordings were taken 17, 28, and 42 days post-surgery. On day 42, the limbs were excised and frozen for immunofluorescence analysis.

Immunofluorescence analysis

The excised TAs were placed in 0.5% paraformaldehyde for 2 hours at 4°C, then transferred to a 20% sucrose solution at 4°C overnight. The tissue was placed in TissueTek OCT and frozen with liquid nitrogen cooled 2-methylbutane for sectioning. Ten micron sections were taken from eight areas throughout the TA approximately 200–250 µm apart. In order to visualize eGFP, the sections were fixed again with 0.5% paraformaldehyde at room temperature for 10 minutes. The sections were blocked with 20% goat serum, and 0.3% Triton X-100 in PBS for one hour. Primary rabbit anti-GFP (1:200 Life Technologies), anti-CD31 (1:200, abcam), or anti-SMA (1:100, abcam) was diluted in blocking buffer and added to sections for one hour. The slides were rinsed 3 times in PBS for 5 minutes. Secondary antibody (Alexa Fluor 488, or 568 1:200 Life Technologies, Carlsbad, CA) was added for 30 minutes in the blocking buffer. The slides were rinsed again and Hoescht 33342 (0.1 µg/mL in DI water, Life Technologies, Carlsbad, CA) was added for 10 minutes. Slides were scanned using the Leica Ariol slide scanner and imaged using Ariol Software. The GFP positive areas using a fixed threshold and exposure time for all slides were normalized to total muscle section area and summed for each limb. Likewise, arterioles and capillaries were imaged and the number were counted and normalized to area of TA muscle.

Statistical evaluation

Significance was tested for all experiments using a 1-way ANOVA with a two-tailed distribution followed by Tukey post-hoc t-test with a 95% confidence interval. Mean fold changes and standard deviations for gene expression measurements were calculated with experimental triplicates. Syringe data was compared to a PBS control within a respective gauge size by Dunnet's post-hoc test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Disclosure

Dr. Christman is cofounder, board member, consultant, and holds equity interest in Ventrix, Inc.; and is an inventor on patent applications associated with this work and owned by UCSD.

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Figure 1. Characterization of the SkECM hydrogel and its ability to promote myoblast viability and differentiation, and engraftment

Scanning electron microscopy of (a) 6 mg/ml and (b) 8 mg/ml SkECM hydrogels showing a nanofibrous architecture. Scale bar = 1 μ m. (c) The storage modulus was measured by parallel plate rheometry for the 6 and 8 mg/mL formulations of the SkECM along with a 2.5 mg/mL collagen control (n=3/group). (d) Complex viscosity in the liquid form was also measured by parallel plate rheometry. 2.5 mg/mL collagen was used as the control and as verified here is similar to the 6 mg/mL SkECM formulation. The 8 mg/mL concentration was significantly stiffer and more viscous compared to the 2.5 mg/mL collagen and 6

mg/mL SkECM (n=3/group). (e) Myoblast viability post-injection through 27G needle in the SkECM vs. PBS. Two million myoblasts were injected in 50 μ L of 6mg/mL or 8mg/mL SkECM, or PBS through a 27G needle. Trypan blue was used to measure cell viability post-injection *in vitro* by a syringe pump (n=10/group) and demonstrated that the SkECM prevented cell death caused by the forces cells experienced upon injection through a syringe and needle. (f) Exposure to reactive oxygen species (ROS). 2.5 mM H₂O₂ was added to the media and metabolic activity was measured after 24 hours and compared to an untreated control in each respective group (n=6/group). Graph shows fractional change in metabolic activity of treated group when normalized to untreated controls, demonstrating an increase for the SkECM groups. (g–h) RT-PCR gene expression measurement of myoblasts encapsulated in either 2.5 mg/mL collagen, 6mg/mL or 8mg/mL SkECM for MyoD and myosin heavy chain (MHC) normalized to GAPDH at 3 and 7 days in differentiation media (n=3/group). The SkECM significantly increased expression of both early (MyoD) and late (MHC) markers of skeletal muscle differentiation. Values represent mean $\pm$ SD in all graphs. *p<0.05, **p<0.01, ***p<0.001 compared control.

Figure 2. SkECM hydrogel increases myoblast engraftment *in vivo*

(a) Mice hindlimbs were injected with 1 million DiR labeled GFP-myoblasts in 25 μ L of either 6 mg/mL, 8 mg/mL SkECM or PBS (n=10/group). IVIS measurements show change in fluorescence over seven days relative to the day 0 time-point. 8 mg/mL formulations consistently showed higher viability compared to 6 mg/mL and saline for days 3–7. (b) Tissue was excised to confirm that these cells remained in the tibialis anterior using IVIS. (c) GFP engraftment was measured by histology using an anti-GFP antibody stain (green) and myosin heavy chain (red). Nuclei are shown in blue. Scale bar = 200 μ m. GFP engraftment

was calculated by measuring percent of area GFP+ in each group. Both IVIS and histology measurements demonstrated that the 8 mg/mL SkECM hydrogel significantly increased myoblast engraftment and survival compared to the standard PBS injection. Values represent mean $\pm$ SD in all graphs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared control.

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Figure 3. Monoculture vs. co-culture *in vitro* comparison encapsulated in 8 mg/mL SkECM

(a) 600,000 Myoblasts were encapsulated in 50 μ L of 8 mg/mL SkECM in transwell plates. Once seeded, either 60,000 fibroblasts were placed on top with media or no cells as a control (n=6/group). Fluorescence signal by Alamar Blue was compared after one week in culture. (b) Myoblasts were encapsulated in the 8 mg/mL SkECM hydrogel with or without 10% mCherry fibroblasts. 2.5 mM H_2O_2 was added to the media and metabolic activity was measured after 12 hours and compared to an untreated control in each respective group (n=6/group). In both cases, co-culture increased metabolic activity. (c, d) RT-PCR gene expression measurement of monoculture or co-culture encapsulated in 8mg/mL SkECM for MyoD and MHC at 3 and 7 days in differentiation media. All values are normalized to day 0 time-point and GAPDH expression. (e–f) eGFP myoblasts and mCherry fibroblasts were imaged under

a fluorescent microscope (Scale bar = 100 μm). Increased differentiation was also observed by an increase in myotubes in the co-culture group (f) compared to the monoculture (e). Values represent mean $\pm$ SD in all graphs. * $p < 0.05$, *** $p < 0.001$.

Figure 4. Injection of skeletal myoblasts in a hindlimb ischemia model in either saline, SkECM, or SkECM with fibroblasts

Mice were subjected to femoral artery and vein ligation (n=10/group). After 7 days, the ischemic TAs were injected with 1 million myoblasts in either saline, 8 g/mL SkECM, or with 100,000 fibroblasts in 8 mg/mL SkECM. (a) DiR-labelled myoblasts imaged under IVIS. Representative transillumination images showing myoblast cell retention in each group by IVIS on day 0, 3, and 7 post-injection. The saline group signal was visually reduced by day 3. (b) IVIS epifluorescence measurements were recorded on day 0, 3, and 7 post-injection and all data was normalized to day 0, demonstrating a significant increase in viability in both SkECM groups at day 3, and a significant increase in the dual cell delivery group at day 7. (c) GFP+ muscle was quantified throughout the ischemic TA 35 days post injection by immunofluorescence. GFP+ signal approximately doubled in the dual cell

delivery group compared to myoblasts in saline (n=9/group). (d) Blood perfusion was measured in the ischemic limb relative to the healthy limb within each mouse (n=10/group). Representative laser speckle images of each group on day 35 are shown. Healthy limbs are on the left and ischemic/treated limbs are on the right. Areas of increased perfusion are indicated by yellow-red color. (e) Perfusion was measured for the ischemic leg relative to the healthy limb for each animal over the course of the study on day -7, 0, 10, 21, and 35 days post-injection. Gray dashed line represents administration of treatment. Significant differences were observed on day 35 for SkECM groups compared to saline. Overall change for each animal from time of treatment to day 35 is plotted in (f). (g) Arterioles (smooth muscle actin) and capillary (CD31+) staining showed increased vessels in SkECM groups *versus* saline alone (n=9/group). (h-j) Capillary and small and large arteriole densities were quantified for all treatment groups. Values represent mean $\pm$ SEM in all graphs. *p<0.05, **p<0.01 compared to saline control.