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RESEARCH ARTICLE

Tri-culture of spatially organizing human skeletal muscle cells, endothelial cells, and fibroblasts enhances contractile force and vascular perfusion of skeletal muscle tissues

Hyeonyu Kim[1] | **Tatsuya Osaki**[1] | **Roger D. Kamm**[2] | **H. Harry Asada**[1]

[1]Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

[2]Departments of Biological and Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

Correspondence
Hyeonyu Kim, Stanford Cardiovascular Institute and Department of Medicine, Stanford University, Stanford, CA 94305, USA.
Email: hyeonyu@stanford.edu

Present address
Hyeonyu Kim, Stanford Cardiovascular Institute and Department of Medicine, Stanford University, Stanford, California, USA

Tatsuya Osaki, Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

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Abstract

Constructing engineered human skeletal muscle tissues that resemble the function and microstructure of human skeletal muscles is key to utilizing them in a variety of applications such as drug development, disease modeling, regenerative medicine, and engineering biological machines. However, current in vitro skeletal muscle tissues are far inferior to native muscles in terms of contractile function and lack essential cues for muscle functions, particularly heterotypic cell–cell interactions between myoblasts, endothelial cells, and fibroblasts. Here, we develop an engineered muscle tissue with a coaxial three-layered tubular structure composed of an inner endothelial cell layer, an endomysium-like layer with fibroblasts in the middle, and an outer skeletal muscle cell layer, similar to the architecture of native skeletal muscles. Engineered skeletal muscle tissues with three spatially organized cell types produced thicker myotubes and lowered Young's modulus through extracellular matrix remodeling, resulting in 43% stronger contractile force. Furthermore, we demonstrated that fibroblasts localized in the endomysium layer induced angiogenic sprouting of endothelial cells into the muscle layer more effectively than fibroblasts homogeneously distributed in the muscle layer. This layered tri-culture system enables a structured spatial configuration of the three main cell types of skeletal muscle and promotes desired paracrine signaling, resulting in improved angiogenesis and increased contractile force. This research offers new insights to efficiently obtain new human skeletal muscle models, transplantable tissues, and actuators for biological machines.

KEYWORDS
angiogenesis, extracellular matrix, skeletal muscle tissue, tri-culture, vascularization

Abbreviations: 3D, three-dimensional; AA, aminocaproic acid; COL1, collagen I; DMEM, Dulbecco's Modified Eagle's medium; EBM, endothelial basal medium; ECM, extracellular matrix; EGM, endothelial cell growth medium; ehSMTs, engineered human skeletal muscle tissues; GFP, green fluorescent protein; hECs, human fibroblasts; hSMMs, human skeletal muscle myoblasts; HUVECs, human umbilical vein endothelial cells; IGF-1, insulin-like growth factor-1; PDMS, polydimethylsiloxane; PS, penicillin–streptomycin; RFP, red fluorescent protein; VEGF, vascular endothelial growth factor.

1 | INTRODUCTION

Engineered human skeletal muscle tissues (ehSMTs), which are three-dimensional (3D) tissue constructs made from human muscle cells and extracellular matrix (ECM), can be used for personalized drug testing platforms,[1] transplantation for regenerative medicine,[2] and muscle disease models to elucidate new pathological mechanisms.[3] The use of ehSMTs will facilitate the creation of personalized medicine and transplants for each patient while avoiding the ethical issues inherent in animal experiments and will reduce the risk of immune rejection.[4] Moreover, engineered muscle tissues can be utilized as actuators to construct biological machines. They allow such systems to generate multi-degree-of-freedom force and displacement, and can potentially provide them with the locomotive and manipulative abilities characteristic of most living organisms.[5,6] Muscle tissues on the sub-millimeter scale can meet the requirements for these micro-robots; no other engineered actuators are currently available that create such a high degree-of-freedom force and displacement in a compact body. One day they might be utilized to transport surgical micro-robots to a target organ and to perform therapeutic procedures selectively.[7–9] Using skeletal muscle myoblasts,[10–13] various cell-based actuators have been created to exploit their potential and explore applications.

For ehSMTs to be utilized in various applications as aforementioned, it is essential that the functions and structures of ehSMTs recapitulate native skeletal muscles. Previous studies have formed skeletal muscle tissue constructs using the murine cell line C2C12[10,11] and human skeletal muscle myoblasts (hSMMs).[14] However, their contractile forces are far below those of native skeletal muscles.[8,15] Myoblasts must fuse to create myotubes and then mature to become myofibers in order to be capable of generating large forces[16] and this has been difficult to achieve in vitro. Compared to native skeletal muscle formation, current in vitro systems is lacking in essential cues for maturation during cell culture. We hypothesize that enhancing the contractile force of ehSMTs requires a culture environment that more closely emulates the chemical and mechanical environment of the skeletal muscles in vivo. In particular, native skeletal muscles have various heterotypic cell–cell interactions with the surrounding cells, including endothelial cells and fibroblasts.[17] In an attempt to fill the gap between the in vitro and in vivo skeletal muscle environments, here we investigate the tri-culture effect of both endothelial cells and fibroblasts upon skeletal muscle tissue formation.

It is known that myoblasts, endothelial cells, and fibroblasts influence individual functions through complex paracrine signaling.[18–20] For example, a higher muscle differentiation rate has been observed when myoblasts are co-cultured with endothelial cells[18] or fibroblasts.[19] Also, fibroblasts induce angiogenesis and contribute to stabilizing endothelial vessels by secreting vascular endothelial growth factor (VEGF) and others.[20] However, the effects of fibroblasts and angiogenic sprouting of endothelial cells in ehSMTs are still poorly understood, especially when the three cell types are present together. Here, we aim to investigate the multi-directional effects of three-way heterotypic cell–cell interactions upon contractile forces of ehSMTs and angiogenic vascularization. To characterize the ehSMTs, we measure both contractile force and Young's modulus of the 3D skeletal muscle constructs. The role of fibroblasts in mediating the remodeling of ECM, which directly influences the contractile force of the skeletal muscle tissue,[15] is investigated. Furthermore, it is examined whether fibroblasts promote angiogenic sprouting of endothelial cells toward the 3D skeletal muscle tissues and, thereby, facilitate the growth of thicker in vitro skeletal muscles beyond the previous diffusion limit for transplantation.

The traditional method for co-culturing different types of cells involves mixing them homogeneously. However, most cells in vivo have their own organization with unique architecture, and some level of spatial compartmentalization could improve the coordination and interactions among the heterotypic cells. For example, within the body, endothelial cells form tubular blood vessels, and skeletal muscle fibers are aligned in the direction of force generation. In the muscle fascicle, fibroblasts are found within the endomysium (the surrounding ECM), in which capillaries are also embedded.[21] The location of each cell type, their spacing, the presence of direct contact, and local cell densities are major factors that affect the cell-to-cell interactions.[22,23] When mixing heterotypic cells together, these critical factors are lost. Therefore, the current work aims to create a tri-culture model where the heterotypic cells are compartmentalized in a manner that recapitulates certain aspects of native skeletal muscle. For the ehSMTs, we create a tubular vasculature with endothelial cells, aligned muscle cells in the longitudinal direction, and embedded fibroblasts in the ECM layer of collagen type I surrounding the vasculature. This particular arrangement is realized by constructing coaxial triple layers of the three cell types, in which fibroblasts exist between the inner endothelial cell layer and the outer muscle tissue layer, resulting in biochemical interactions between the vasculature and skeletal muscle through the intervening fibroblast layer. Using this organized structure, we demonstrate the effect of the distribution of fibroblasts on skeletal muscle and vasculature functions.

2 | MATERIALS AND METHODS

2.1 | Cell culture

hSMMs (Lonza) were cultured in StemLife Sk medium (Lifeline Cell Technology). Human fibroblasts (hFBs) and green fluorescent protein-expressing hFBs (GFP-hFBs; Angio-Proteomie) were cultured in FibroLife® S2 Fibroblast medium (Lifeline Cell Technology). Human umbilical vein endothelial cells (HUVECs; Lonza) or red fluorescent protein-expressing HUVECs (RFP-HUVECs; Angio-Proteomie) were cultured in endothelial cell growth medium (EGM)-Plus (Lonza). All culture media were supplemented with 1% (v/v) penicillin–streptomycin (PS; Sigma–Aldrich) and 0.2% (v/v) Normocin (InvivoGen).

2.2 | Fabrication of ehSMTs

We modified our previously published method to fabricate fascicle-inspired 3D tissues[10] to construct vascularized ehSMTs with triple layers (Figure 1A). A cavity in the 5% (w/w) gelatin (Sigma–Aldrich) sacrificial mold with 10 U/ml thrombin (Sigma-Aldrich) was created in the polydimethylsiloxane (PDMS) device by putting a large-diameter stainless-steel pin (diameter = 2.4 mm, McMaster-Carr) in the gelatin solution and removing the pin after gelation of the gelatin at 4°C. We put a mixture of hFBs or GFP-hFBs (1 million cells/ml) and 2 mg/ml collagen I into a cylindrical cavity in another PDMS mold. We inserted a thinner stainless-steel pin (diameter = 1.3 mm, McMaster-Carr) at the center of the mixture of hFBs and collagen and gelled the collagen at 37°C. Thereafter, the pin surrounded by the thin hFB-embedded collagen gel layer was removed from the PDMS mold. When the gelatin sacrificial mold and the thinner pin coated by hFB-embedded collagen gel were ready, hSMMs (15 million cells/ml) were mixed with 4.5 mg/ml of fibrinogen (Sigma-Aldrich) and 10% (v/v) growth factor-reduced Matrigel (Corning). This muscle cell and ECM mixture were injected into the cylindrical cavity in the gelatin sacrificial mold with the hFB-collagen-coated pin at the center. This PDMS device with the cell solution and the hFB-collagen-coated pin was incubated at 37°C to dissolve the sacrificial gelatin mold and gel the fibrinogen in the cell solution. The pin in the tissue was removed to create the inner channel when the cell solution was fully solidified after ~48 h. The tubular skeletal muscle tissues were cultured in the growth medium composed of StemLife Sk Medium and 1 mg/ml aminocaproic acid (AA; Sigma–Aldrich).

HUVECs or RFP-HUVECs (4 million cells/ml) were injected into the inner channel of the tubular skeletal muscle tissue when the inner channel was created. These tri-cultured tissues were incubated for 8 days with the co-culture medium, comprised of equal parts of Dulbecco's Modified Eagle's medium (DMEM; 4500 mg/L glucose; Sigma-Aldrich) and endothelial basal medium (EBM)-Plus (Lonza), supplemented with EGM-plus SingleQuots (Lonza), 1% (v/v) horse serum (Sigma-Aldrich), 1 mg/ml AA, 1% (v/v) PS, 0.2% (v/v) Normocin, 50 ng/ml insulin-like growth factor-1 (IGF-1; Sigma–Aldrich), and 1x insulin-transferrin-selenium (Life Technologies Corp).

2.3 | Measurement of the contractile force and Young's modulus

The contractile force of the ehSMTs was measured on Day 8 using a system previously published by our group.[10,24] In brief, we stretched ehSMTs using the tip of the copper cantilever (Remington Industries) with known stiffness. When the elastic force of the stretched ehSMTs and the elastic restoring force of the cantilever were balanced, we applied an electric field (2.5 V/mm; pulses of 1 ms each; for 3 s) to induce muscle contraction. We measured the displacement of the cantilever tip when it was moved to a new position by the additional contractile force. Using the measured deformation of the cantilever by the muscle contractile force and its known stiffness, we obtained the amount of contractile force in the longitudinal direction by Hooke's law. More detailed calculations of the force are provided in the previously published paper.[24] In addition, we obtained Young's modulus in the longitudinal direction of the muscle tissues when the elastic forces of the muscle tissue and the cantilever were balanced without muscle contraction. When we displaced the center of the ehSMT (length L and cross-section area A) using the cantilever (stiffness k) tip by a distance (x) and to a strain (ϵ), the deflection of the cantilever d was measured and Young's modulus of ehSMT was calculated as formula (1).

$$\frac{1}{\epsilon A}\frac{\sqrt{x^2+L/2^2}}{2x}kd. \tag{1}$$

2.4 | Histological analyses and immunofluorescence staining

ehSMTs were fixed with 4% paraformaldehyde (Santa Cruz Biotechnology) for 24 h and stored in 70% ethanol. After the tissues were embedded in paraffin using standard processing techniques (Sakura VIP 5 tissue processor), they were sectioned and analyzed by immunofluorescent staining. We used antibodies for laminin (Sigma–Aldrich) and collagen I (Sigma–Aldrich) to visualize the ECM in the tissues. The slides were heat-induced epitopes retrieved using citrate buffer (Thermo

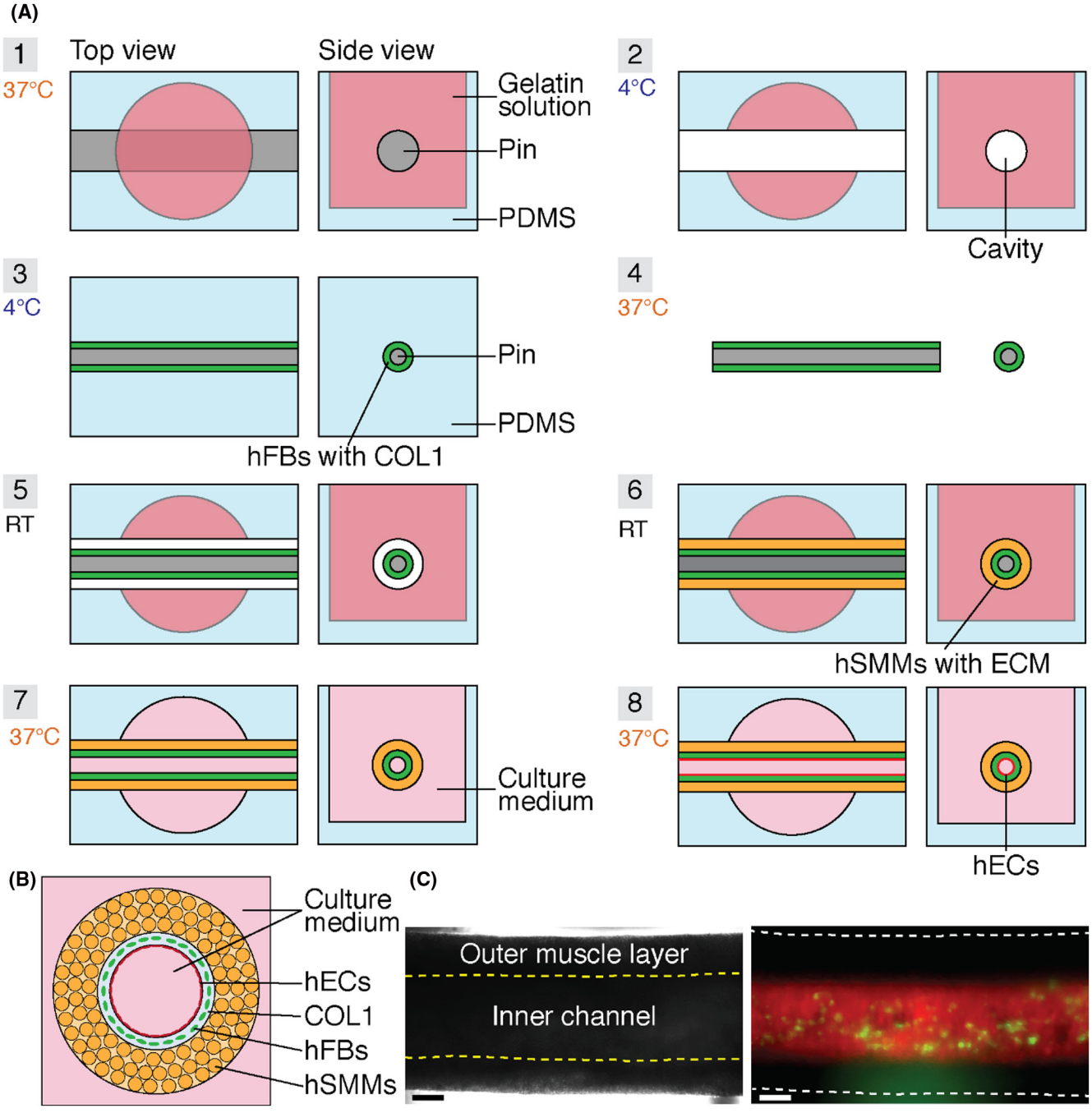

FIGURE 1 Fabrication of ehSMTs with human skeletal muscle myoblasts (hSMMs), human endothelial cells (hECs), and human fibroblasts (hFBs). (A) Protocol used to fabricate ehSMTs. The sacrificial gelatin mold with a cylindrical cavity is formed in the polydimethylsiloxane (PDMS) device, and a pin is coated by a thin layer of hFB-embedded collagen I (COL1) gel. The cavity in the sacrificial gel is filled with a mixture of hSMMs and extracellular matrix (ECM) while the coated pin is held at the center. When the solid tissue is formed in the incubator, the pin is removed to create the inner channel; it is then coated by hECs. (B) Schematic of a cross-section of ehSMTs in the coaxial triple layers: a thick outer layer composed of hSMMs, a thin center layer composed of hFB-embedded collagen I, and an inner layer composed of a monolayer of hECs. The inner channel and the outer medium space are filled with culture medium. (C) Phase-contrast image (left) of the ehSMT and fluorescent image (right) of RFP-expressing hECs and GFP-expressing hFBs in the ehSMT. The inner channel and the outer radius of the ehSMT are indicated by yellow dashed lines (left) and white dashed lines (right), respectively. The scale bar is 200 μm.

Fisher Scientific) for 20 min at 97°C and cooled for 20 min. The sections were quenched with H202 (Thermo Fisher Scientific), blocked with Rodent Block M (RBM 961, Biocare Medical) for 30 min, and incubated with the primary first antibodies (1:150) for 60 min. After washing with TRIS-buffered saline with 0.5% Tween 20

(Thermo Fisher Scientific), the sections were incubated with the Alexa Fluor 488/568 secondary antibody (1:500, Thermo Fisher Scientific) and 0.5 μg/ml DAPI (Thermo Fisher Scientific). Following another wash with DPBS, images were taken with an Olympus IX-81 inverted fluorescence microscope (Olympus Corp.). We analyzed the stained images of cross-sections of the muscle tissues by ImageJ software (NIH) to obtain the diameters of the skeletal muscle cells in the tissues. The diameter of the holes surrounded by laminin at the cross-section was considered to be the diameter of skeletal muscle cells only when it was greater than 5.5 μm, the average size of the nucleus in ehSMTs.

To compare the level of hypoxia, we incubated ehSMTs with 10 μM Image-iT™ green hypoxia reagent (Thermo Fisher Scientific) for 5 h in the incubator and imaged using an Olympus IX-81 inverted fluorescence microscope (Olympus Corp.) with the same exposure time. The intensity of the images was analyzed by ImageJ software (NIH).

2.5 | Statistical analysis

For comparing data with normal distribution and equal variances, statistical analyses were conducted using a Student's *t*-test to compare two conditions or one-way ANOVA with Tukey's post-test when there were three conditions. For data with non-normal distribution, statistical analyses were conducted using the Kruskal–Wallis test with Dunn's post hoc test. Data were considered statistically significant when the *p*-value was <.05 (*) or .01 (**).

3 | RESULTS

3.1 | Engineered human skeletal muscle tissues are fabricated to incorporate tubular vasculature and localized fibroblasts

To create ehSMTs with the spatial organization of the three cell types, we developed a new tissue fabrication method (Figure 1A) by modifying our previous method to fabricate fascicle-inspired tissues.[10] This method aligns skeletal muscle cells in the longitudinal direction of the tissues and the absence of hard contacts except at the two ends.[10] In particular, these muscle tissue constructs contain a tube-shaped vasculature composed of a monolayer of HUVECs at the center and human fibroblast-embedded collagen I layer surrounding it (Figure 1B,C). To fabricate ehSMTs with a triple-layer structure, we created a collagen layer with (Figure S1) or without fibroblasts between the outer muscle layer and the inner endothelial cell layer (Figure S2). The middle layer was developed as thin and soft as possible to prevent the fibrotic part from hindering the contraction of ehSMTs, and a 25 μm thick collagen layer was obtained using 2 mg/ml collagen I.

3.2 | Fibroblasts increase skeletal muscle contractile force and reduce stiffness

To study the effect of fibroblasts on the force generation by ehSMTs, we compared three conditions (Figure 2A): (1) a control consisting only of hSMMs and HUVECs (Control), (2) a condition having the thin collagen I layer between the hSMMs and HUVEC layers (COL1 only), and (3) a condition with the hFB-embedded collagen I layer (COL1 + FBs). Results demonstrated that the contractile force produced by ehSMTs in the longitudinal direction in the COL1 + FBs condition increased by 43% and 74% on average, respectively, compared to the Control and COL1 only conditions (Figure 2B). Conversely, inserting the acellular collagen layer reduced the contractile force. In addition, we measured Young's modulus of ehSMTs in the longitudinal direction under the three conditions (Figure 2C). These measurements showed that the acellular collagen layer significantly increased Young's modulus of muscle tissue. In contrast, Young's moduli for the Control and COL1 + FBs conditions were similar even though the COL1 + FBs case had the additional nonfunctional collagen I layer between the inner endothelial cell and outer muscle cell layers.

Since one of the main roles of fibroblasts is to synthesize and degrade ECM, Young's moduli of ehSMTs (Figure 2C) are closely related to the ECM of the tissues. Therefore, we stained collagen I and laminin of the tissues under the three conditions (Control, COL1 only, and COL1 + FBs) to investigate the effect of fibroblasts on the ECM of the ehSMTs (Figure 2D–F). By comparing the acellular and hFB-embedded collagen layers, we found that culturing with fibroblasts (COL1 + FBs condition) led to a thinner collagen layer than the COL1 only condition (Figure 2D). When we examined the ECM in the outer muscle tissue layer, we found more aggregated collagen I fibers in the case of the acellular collagen layer (COL1 only) than in the other two conditions (Figure 2E). In addition, laminin in the outer muscle layer was studied under all three conditions (Figure 2F). Results showed that there was the greatest deposition of laminin in the outer muscle tissue layer under the condition of COL1 only, while the laminin networks in the Control and COL1 + FBs conditions were similar.

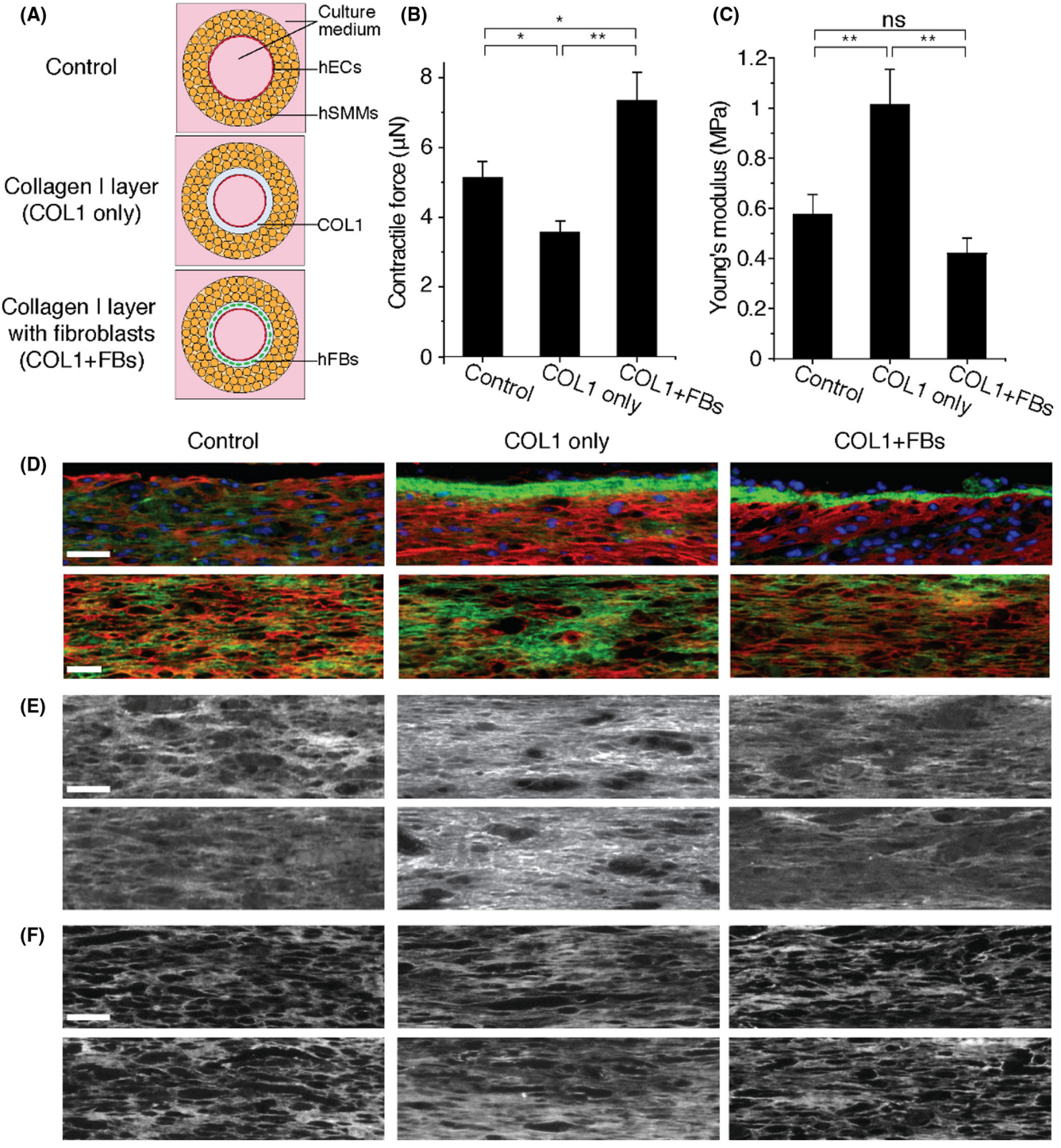

FIGURE 2 Fibroblasts increase the contractile force of ehSMTs and soften them by affecting the ECM network. (A) Schematics showing the cross-section of ehSMTs under the three study conditions. The Control condition is double-layered tissues consisting of an outer human skeletal muscle myoblasts (hSMMs) layer and an inner human endothelial cells (hECs) layer. Collagen I layer condition (COL1 only) has an acellular collagen I (COL1) gel layer in the ehSMTs in between the hSMM layer and the hEC layer. The final condition is that human fibroblasts (hFBs) are embedded in the middle collagen I layer (COL1 + FBs). (B, C) Contractile force (B) and Young's modulus (C) of ehSMTs on Day 8 under the three conditions in (A). Mean ± SE; $n = 15, 17, 9$ (B) and 13, 13, 14 (C). Kruskal–Wallis test with Dunn's post hoc test (B) and ANOVA with Tukey's post-test (C), $^{*}p < .05$, $^{**}p < .01$, ns, not significant. (D) Longitudinal cross-sections stained by laminin and collagen I in the middle collagen layer near the inner channel (top) and in the outer muscle layer (bottom). Red, green, and blue indicate laminin, collagen I, and nuclei, respectively. (E, F) Images of the longitudinal cross-section of ehSMTs stained by collagen I (E) and laminin (F) in the outer muscle layer of the three conditions in (A). The scale bars are all 50 μm.

3.3 | Culturing skeletal muscle cells with fibroblasts produces thicker myotubes

To enhance the contractile force of ehSMTs, immature myoblasts should fuse with one another to form myotubes with the striations of α-actinin (Figure S3), which then thicken as they mature. Therefore, we investigated the effect of fibroblasts on myotube diameter in ehSMTs (Figure 2A). Skeletal muscle tissues consist of functional portion-muscle cells, and a nonfunctional fibrotic portion—the ECM. In ehSMT, it is advantageous to obtain a stronger contractile force as the functional part is larger. We distinguished these two parts in ehSMTs by staining laminin, one of the main ECM proteins that encapsulate the myotube (Figure 3A). In the immunostaining images, the void circles surrounding laminin are the spaces for skeletal muscle cells, including fused myotubes and unfused myoblasts. We measured the size of skeletal muscle cells from cross-sectional images of the ehSMTs. By comparing the thickness of muscle cells under the three different conditions (Control, COL1 only, and COL1 + FBs), we found that the mean cross-sectional muscle cell diameter was significantly larger in the ehSMTs with a fibroblast-embedded collagen layer (COL1 + FBs) than the other two conditions (Figure 3B). Through the distribution of diameters (Figure 3C), we also confirmed that there were significantly larger-diameter muscle cells (greater than 14.5 μm in diameter) when ehSMTs incorporated the fibroblast-embedded middle layer (COL1 + FBs) than in the other two conditions without fibroblasts (Control and COL1 only). These results showed that ehSMTs in the COL1 + FBs condition have a lower non-functional portion, ECM, than in the other two conditions without fibroblasts.

3.4 | Fibroblasts induce angiogenesis in engineered skeletal muscle tissues

Large-scale ehSMTs are useful for a wide variety of applications as they can generate higher force as actuators and provide transplantable tissues for volumetric muscle loss patients. Vascularization of engineered tissues is beneficial for scaling up the muscle tissues by delivering nutrients and oxygen through the vessels deep within the tissue constructs where diffusion alone is insufficient. In addition, it provides human skeletal models that more closely resemble native skeletal muscles. Since fibroblasts are known to regulate both myofibers and endothelial cells in native skeletal muscle tissues,[19,20] we investigated the effects of fibroblasts on the vascularization of ehSMTs. Imaging the sectioned tissues revealed that the sprouting of endothelial cells from the inner endothelial cell layer to the outer muscle layer was more strongly induced in the presence of fibroblasts than in the control case without fibroblasts (Figure 4A,B).

To determine whether this angiogenic response was caused by hypoxia due to oxygen consumption by the fibroblasts, we compared the degree of hypoxia in ehSMTs under both conditions using reagents that fluoresce in hypoxic conditions (oxygen level < 5%). We found, however, that the presence of fibroblasts did not lead to higher levels of hypoxia than in the control case (Figure S4), and therefore suspect that the angiogenesis induced by the fibroblasts in ehSMTs was caused by paracrine signaling other than hypoxia.

3.5 | Localized fibroblasts around endothelial cells improve force and angiogenesis more effectively than when they are distributed

The uniform mixing of three cell types has been commonly used as a tri-culture method, but we compartmentalized each cell type in the ehSMTs in this study. In particular, fibroblasts were only in the collagen gel layer between the inner endothelial cell layer and the outer muscle layer. To determine the effect of the spatial distribution of fibroblasts within the ehSMTs, we homogeneously mixed hFBs with hSMMs and constructed the tubular tissues in the same way (Figure S5) to obtain ehSMTs with distributed fibroblasts (Distributed FBs condition). We compared their performance relative to the condition with no fibroblast (Control) and the localized fibroblasts in the collagen gel layer (Localized FBs condition) (Figure 5A). Our findings indicated that the contractile force of the ehSMTs was higher when fibroblasts were localized near the vasculature (Localized FBs) than when fibroblasts were uniformly distributed between muscle cells (Distributed FBs) or when there was no fibroblast (Control) (Figure 5B). Young's modulus of ehSMTs fell in the Distributed FBs condition compared to the absence of the fibroblasts (Control), but not as much as in the Localized FBs condition (Figure S6, statistically not significant). Finally, the distributed fibroblasts in ehSMTs induced angiogenesis into the outer muscle layer (Figure 5C), but it demonstrated fewer endothelial sprouts in the muscle layer than the Localized FBs condition, as shown in Figures 4B (Localized FBs) and 5C .

4 | DISCUSSION

In this study, we developed multicellular engineered skeletal muscle tissues within which human skeletal muscle

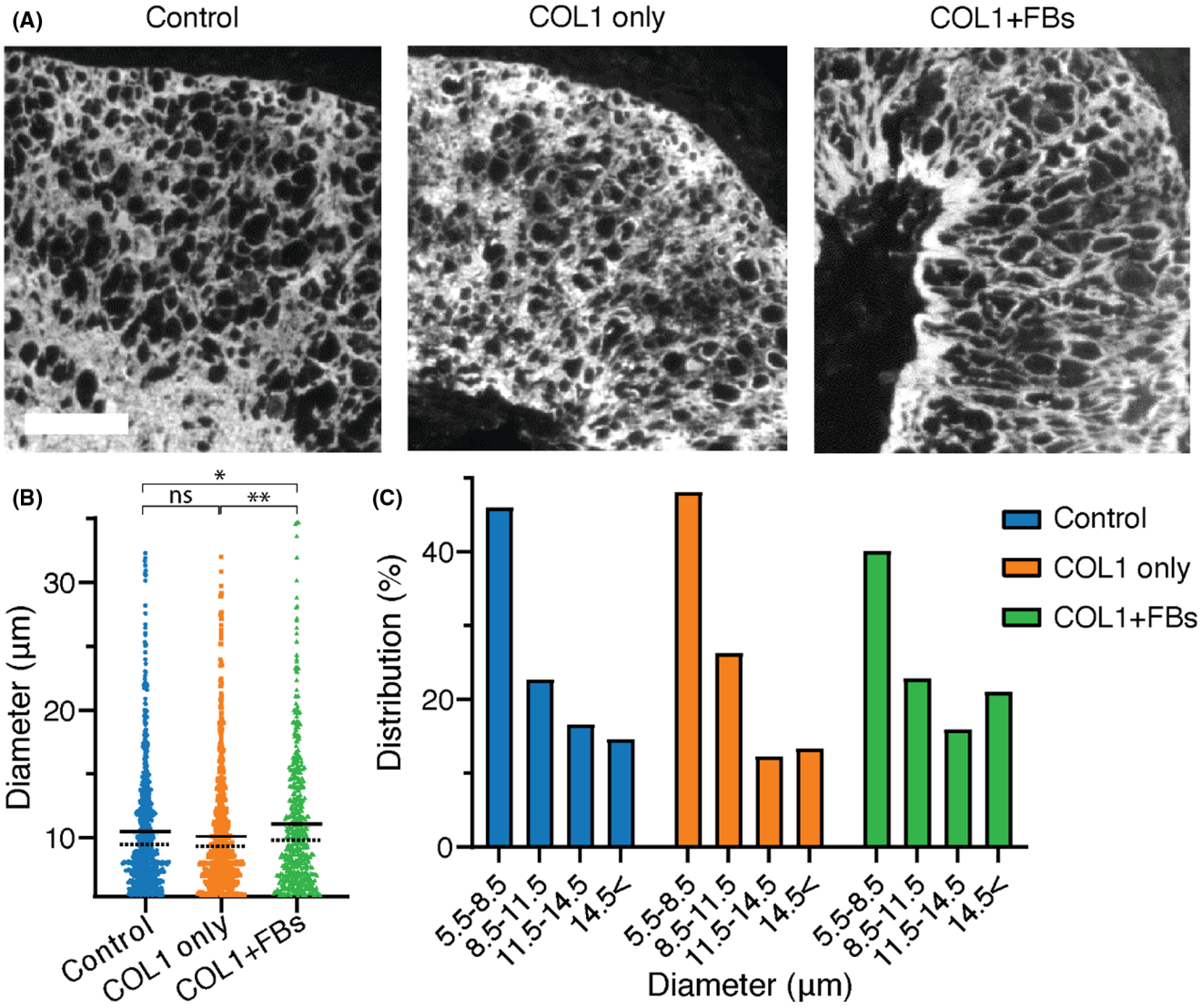

FIGURE 3 Fibroblasts make thicker myotubes in ehSMTs. (A) Representative laminin-staining images of transverse cross-sections of ehSMTs to show diameters of skeletal muscle cells under the three conditions (Control, COL1 only, and COL1 + FBs). The scale bar is 100 μm. (B, C) Mean (black lines; B), median (black dashed lines; B), and distribution (%) of the diameters (C) of skeletal muscle cells under the three conditions. $n = 897, 742, 538$. Kruskal–Wallis test with Dunn's post hoc test (B), $^{*}p < .05$, $^{**}p < .01$, ns, not significant.

myoblasts, endothelial cells, and fibroblasts are spatially organized. Using newly developed ehSMTs with distinct three coaxial layers, we found that the presence of fibroblasts in vascularized ehSMTs enhances contractile force and angiogenesis. Moreover, we confirmed that fibroblasts in ehSMTs induce softer ehSMTs through ECM remodeling and thicker muscle cells. We also found for the first time that fibroblasts localized around a vasculature enhanced the contractile performance of ehSMTs and angiogenesis into the muscle layer more than if they were evenly distributed in the tissue.

Fibroblasts are expected to affect endothelial cells and skeletal muscle cells, including myoblasts and myotubes, in ehSMTs through a variety of biochemical and mechanical mechanisms. We observed that myotube thickness, one of the important indicators of muscle maturation, was increased by adding fibroblasts to ehSMTs (Figure 3). This was likely caused by biochemical interactions between myoblasts and fibroblasts, as previous results have indicated that fibroblasts induce myoblast differentiation through IL-6.[19] Moreover, we detected more sprouted endothelial cells in the muscle tissue layer induced by fibroblasts (Figure 4), consistent with the previous observations that fibroblasts promote angiogenesis through VEGF.[20] Since endothelial cells also promote muscle differentiation through Angiopoietin-1/Neuregulin-1/ErbB2 signaling,[18] the sprouted endothelial cells penetrating between the skeletal muscle cells may help to attain higher contractile force.

In addition to communication via soluble factors, fibroblasts affect ehSMTs mechanically through their ability to

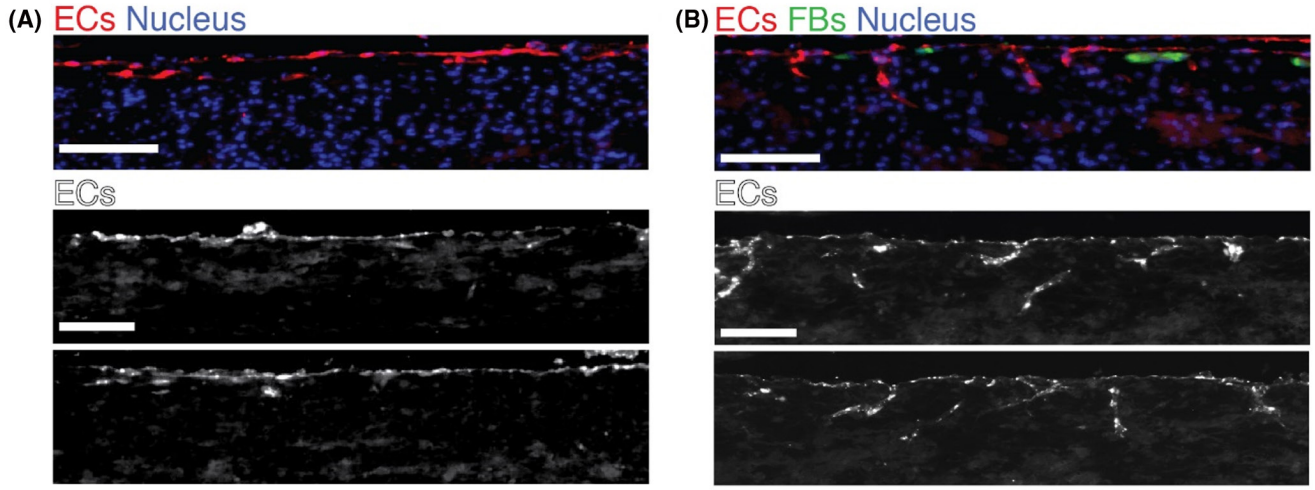

FIGURE 4 Fibroblasts (FBs) induce more angiogenesis in ehSMTs. (A, B) The longitudinal cross-sections of ehSMTs under the conditions of no FB (A) and FBs-embedded collagen I layer (B) show a monolayer of endothelial cells (ECs) in the inner channel and sprouted endothelial cells into the muscle layer. Red, green, blue, and white indicate RFP-HUVECs, GFP-hFBs, nuclei, and RFP-HUVECs, respectively. The scale bars are all 100 μm.

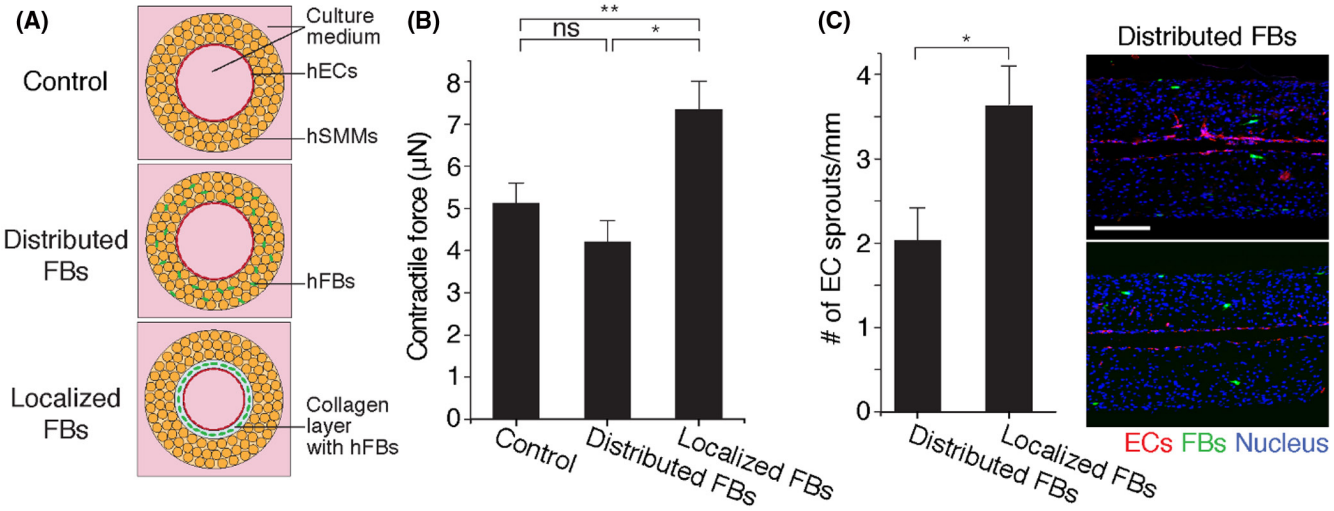

FIGURE 5 Localization of fibroblasts achieves higher contractile force and more angiogenesis than when fibroblasts are distributed among skeletal muscle cells. (A) Schematics showing the cross-section of ehSMTs under the three conditions: Control has no fibroblast, Distributed FBs has human fibroblasts (hFBs) in the human skeletal muscle myoblasts (hSMMs) layer, and Localized FBs has hFB-embedded collagen I layer in between the human endothelial cells (hECs) layer and the hSMMs layer. (B) Contractile force of ehSMTs on Day 8 under the three conditions in (A). Mean ± SE; $n = 15, 4, 9$. Kruskal–Wallis test with Dunn's post hoc test, $*p < .05$, $**p < .01$, ns, not significant. (C) Number of endothelial cell sprouts per length in the cross-section of ehSMTs under the Distributed FBs and Localized FBs conditions (left) and cross-section images showing sprouting of endothelial cells (ECs) into the muscle tissue where fibroblasts (FBs) are distributed (right). Mean ± SE; $n = 4, 4$. Student's t-test, $*p < .05$. The scale bar is 200 μm. Red, green, and blue indicate RFP-HUVEC, GFP-hFBs, and nuclei, respectively.

modulate the ECM.[25] In this study, we demonstrated how fibroblasts in the middle layer influenced the ECM network of ehSMTs (Figure 2D–F). In particular, the middle collagen layer in which the fibroblasts were embedded became thinner compared to the acellular collagen layer (Figure 2D). We hypothesize that this may be a result of fibroblast secretion of matrix metalloproteinases that degrade surrounding collagens[26] or that cell-mediated matrix compaction occurs.[27] The thicker collagen layer will increase the non-functional fibrotic part of the ehSMTs and consequently increase Young's modulus (Figure 2C), because the ECM is usually stiffer than myotubes.[28] This stiffness of ehSMTs is closely related to the contractile force of the tissue constructs since a stiff ECM is likely to hinder muscle contraction.[15] Therefore, the thinner middle collagen layer (Figure 2D) and the lower Young's modulus obtained by

adding fibroblasts (Figure 2C) may contribute mechanically to the higher contractile force (Figure 2B) together with the aforementioned paracrine signaling. Furthermore, sprouted endothelial cells in the muscle layer induced by the fibroblasts (Figure 4B) secrete and degrade the surrounding ECM, which can modify the stiffness of ehSMTs.

Young's modulus of native skeletal muscle was previously reported to be in the range of approximately 10 to 450 kPa.[29,30] Because skeletal muscle tissues are anisotropic, the mechanical properties can vary greatly depending on the orientation of the stress and the method of measurement. For example, Young's modulus of 10 kPa was obtained by indenting skeletal muscles in the transverse direction using atomic force microscopy,[29] and 450 kPa was measured using a uniaxial load frame generating tension in the longitudinal direction. When the muscle was measured in the transverse direction using this same method, the value was 20 kPa.[30] In the present study, Young's modulus in the longitudinal direction, parallel to the direction of force generation by the ehSMTs, was 420 ± 60 kPa when the fibroblast-embedded collagen I layer was present (Figure 2C), which was most similar to the stiffness of native skeletal muscles for these test conditions (Figure 2A). The average Young's moduli we obtained in this study ranged from 400 to 1000 kPa (Figure 2C), much higher than the measured Young's modulus of collagen gels,[31] fibrin gels,[32] and native skeletal muscle tissues.[29,30] This may be because the collagen fibers in our construct are largely aligned in the longitudinal direction[15] and the ECM network in ehSMTs is much denser than the acellular gels[31,32] due to cell-mediated gel compaction. Also, pretension was generated in the longitudinal direction of ehSMTs during the gel compaction,[24] and this may result in nonlinear stretch-induced stiffening of the ECM.[33] Lastly, a much thicker ECM layer between myotubes compared to the thickness of native endomysium[34] may be another reason for the higher stiffness.

When fibroblasts, known to induce angiogenesis, were more concentrated around endothelial cells, a greater number of sprouted endothelial cells were observed than when fibroblasts were distributed throughout tissues (Figure 5C). The previous study has also shown that endothelial cells recruit fibroblasts to be close to them in skeletal muscle tissue.[35] This may be attributed to more effective biochemical interactions via soluble factors between fibroblasts and endothelial cells. As mentioned earlier, fibroblasts are known to promote muscle differentiation through biochemical interactions,[19] and also, they may affect the stiffness of muscle tissues mediated by the ECM change. Although the distributed fibroblasts interacted directly with the muscle cells, the contractile force was more improved when fibroblasts were localized to the middle layer than when the fibroblasts were distributed throughout the muscle layer (Figure 5B). Based on these observations, the functional improvement of ehSMTs may be obtained more effectively by sprouted endothelial cells induced by fibroblasts than when the muscle cells directly interact with distributed fibroblasts. Further research on the influence of the distribution and density of cells on biochemical and mechanical interactions between the different cell types will be needed. In addition, the detailed mechanisms of the complex paracrine signaling between these three cell types need to be more clearly elucidated.

These ehSMTs with tubular vasculature and the fibroblast-embedded endomysium may be utilized in broader practical applications in the future, such as transplantation to patients with volumetric muscle loss, drug testing during drug discovery, or research platforms to understand the emergent behaviors resulting from the complex heterotypic cell interactions.[36] To utilize ehSMT as large-scale transplantable tissues, we will need to connect the inner channel to the microfluidic system to introduce fluid flow and generate high-density functional microvasculatures through induction of angiogenesis. In addition, ehSMTs need to be fabricated in chemically defined environments and under good manufacturing practices. To use ehSMTs as actuators of biological machines in the future, we will need control systems mediated by motor neurons to achieve desired movements and other parts of the biological machines that can perform treatment at the target site. Through these various applications of multicellular ehSMTs, this study could contribute to advances in engineering, medicine, and basic science fields in the future.

AUTHOR CONTRIBUTIONS

Hyeonyu Kim, Tatsuya Osaki, Roger D. Kamm, and H. Harry Asada designed the research, Hyeonyu Kim, and Tatsuya Osaki performed experiments and analyzed the data. Hyeonyu Kim and H. Harry Asada wrote the manuscript, and it was reviewed and edited by all authors.

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DISCLOSURES

Roger D. Kamm is co-founder of and has significant financial interests in AIM Biotech and receives research support from Amgen, AbbVie, GSK, Roche, and

Boehringer-Ingelheim. All other authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

ORCID

Hyeonyu Kim https://orcid.org/0000-0002-1303-9954
Tatsuya Osaki https://orcid.org/0000-0001-7174-0629

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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