HEALTH AND MEDICINE

Surface-directed engineering of tissue anisotropy in microphysiological models of musculoskeletal tissue

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Here, we present an approach to model and adapt the mechanical regulation of morphogenesis that uses contractile cells as sculptors of engineered tissue anisotropy in vitro. Our method uses heterobifunctional cross-linkers to create mechanical boundary constraints that guide surface-directed sculpting of cell-laden extracellular matrix hydrogel constructs. Using this approach, we engineered linearly aligned tissues with structural and mechanical anisotropy. A multiscale in silico model of the sculpting process was developed to reveal that cell contractility increases as a function of principal stress polarization in anisotropic tissues. We also show that the anisotropic biophysical microenvironment of linearly aligned tissues potentiates soluble factor-mediated tenogenic and myogenic differentiation of mesenchymal stem cells. The application of our method is demonstrated by (i) skeletal muscle arrays to screen therapeutic modulators of acute oxidative injury and (ii) a 3D microphysiological model of lung cancer cachexia to study inflammatory and oxidative muscle injury induced by tumor-derived signals.

INTRODUCTION

Mechanical forces play an essential role in morphogenesis (1–4). Graded distributions of morphogens in a developing tissue induce the contraction of actomyosin networks in a subset of cells to generate contractile forces. These locally produced forces are then transmitted to neighboring cells and integrated within the tissue to trigger global structural deformation that transforms amorphous aggregations of cells into specialized structures that enable physiological function (5, 6). This process of global shape change is regulated by physical constraints at tissue boundaries such as geometric confinement and differential adhesion strength. Mechanical boundary constraints determine the directions of principal strain in which contractile forces are focused to sculpt tissue shape and microarchitecture. This patterning of contractile forces guides diverse morphogenetic processes from early germ layer rearrangements during gastrulation to the development of the musculoskeletal system (7, 8).

Here, we describe a tissue engineering approach inspired by this fundamental biophysical principle of morphogenesis. Our method uses heterobifunctional cross-linking chemistry to spatially pattern the surface anchorage of spontaneously contracting cell-laden extracellular matrix (ECM) hydrogels. Using this technique, we demonstrate in vitro engineering and prolonged maintenance of living three-dimensional (3D) tissues that exhibit anisotropic microarchitecture and mechanical properties. Through multiscale in silico analysis of our tissue patterning approach, we reveal that cells increase their contractility as a function of polarized stresses during anisotropic tissue morphogenesis. Building upon these findings, we first demonstrate the use of our system as an in vitro platform to study the contribution of tissue anisotropy to tenogenic and myogenic differentiation of mesenchymal stem cells (MSCs). This work is followed by the development of a microphysiological system that directs spontaneous assembly of differentiated skeletal muscle tissues by human skeletal myoblasts for biochemical and morphological analysis of oxidative muscle injury. Furthermore, we demonstrate the integration of this system with microengineered drug-resistant human lung cancer tissues to construct an in vitro model of cancer cachexia. Using this multiorgan microphysiological system, our study provides the proof of principle for recapitulating the key pathophysiological features of cancer-induced muscle wasting in vitro. Last, we show the feasibility of using our muscle injury and cachexia models for preclinical drug testing by evaluating the protective effects of PARP enzyme inhibitors and dietary antioxidants.

RESULTS

Engineering principles of in vitro tissue sculpting

The orientation of aligned anisotropic tissues such as tendons and muscles is patterned by polarized tension that arises because of anchorage at tissue boundaries such as the myotendinous junctions (MTJs) (9) (Fig. 1A). These regions of differential adhesion strength act as mechanical boundary constraints that provide resistance against cellular contractile forces, resulting in the development of increased tissue tension and anisotropic tissue architecture (10). Our surface-directed tissue sculpting method emulates this configuration by using a simple 3D culture platform consisting of an open circular poly(dimethylsiloxane) (PDMS) chamber patterned with a pair of opposing nodes that provide anchoring points for engineered tissue constructs (Fig. 1B). The critical first step of tissue sculpting in this system is to selectively functionalize the surface of the anchoring nodes with a heterobifunctional cross-linker that increases the strength of adhesion between PDMS and hydrogel scaffolds commonly used for 3D culture (Fig. 1C, step 1). Next, cell-laden ECM hydrogel precursor solution is introduced into the chamber and solidified to form a tissue construct initially bound to all surfaces of

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the muscle chamber (Fig. 1C, step 2). The process of surface-directed tissue sculpting begins when the seeded cells adhere to the matrix and exert contractile forces. During culture, the cell-generated contractility induces the shrinkage of the hydrogel scaffold, which results in the detachment of the nascent tissue from the untreated, loosely adhesive surfaces (Fig. 1C, step 3). This continuous process eventually leads to the formation of a linearly organized tissue that remains tethered only to the anchoring nodes (Fig. 1C, step 4).

To demonstrate a proof of concept, we used sulfo-SANPAH (sulfosuccinimidyl 6-[(4′-azido-2′-nitrophenylamino)hexanoate] (SS) as an adhesive linker (fig. S1) to form embryonic fibroblast (3T3)–laden collagen type I hydrogel in the sculpting device (Fig. 1D). Following detachment from untreated surfaces (Fig. 1E), cell-mediated contraction deformed and compacted the tissue construct in a progressive manner, eventually transforming the initially amorphous hydrogel into a stably tethered 3D tissue strip. The sculpted construct exhibited an equilibrium geometry characterized by a relatively uniform width along the longitudinal direction (Fig. 1F). Time course analysis of compaction revealed increased cell density along the central axis of actively sculpting constructs and decreased tissue volume (fig. S2). SS-mediated adhesion between hydrogel and PDMS made it possible to maintain sculpted tissues held in isometric tension for up to 28 days with a 100% success rate (fig. S3). When the anchorage nodes were not treated with SS before cell seeding, the collagen hydrogel detached from all surfaces of the chamber within 3 days of culture and underwent isotropic contraction (Fig. 1G). Our sculpting approach also enabled construction of other simple tissue shapes by changing the number of evenly spaced anchorage nodes in the same circular chamber design (fig. S4).

**Characterization of tissue anisotropy**

The tissue constructs sculpted in our two-node system exhibited highly anisotropic cellular and ECM microarchitecture. Fibroblasts in these tissues showed an elongated bipolar morphology with an aspect ratio of approximately 7:1 and aligned within 5° of the longitudinal axis (Fig. 1, H and I). In contrast, the cells in isotropic control tissues anchored to all boundaries of the same chamber geometry were randomly oriented and exhibited heterogeneous polygonal morphologies with an average aspect ratio of 2:1 (Fig. 1, H and J). Moreover, compaction of anisotropic tissues during sculpting resulted in markedly increased density of type III collagen, which exhibited transition from an initially granular appearance to an aligned extracellular fibrillar architecture over time (fig. S6).

To compare the mechanical properties of sculpted and isotropic tissues, we performed atomic force microscopy (AFM) nanoindentation on living tissues still tethered under tension and measured the local tissue stiffness at an array of surface locations (Fig. 1M). All constructs remained adherent and isotropic after 1 day of culture, at which point the mean local Young’s modulus was roughly 700 Pa (Fig. 1N). Extending the culture period increased the surface tissue stiffness in both groups, but the extent of increase was notably different. After 9 days of culture, the sculpted tissues reached a Young’s modulus of 6.3 kPa, which was approximately 2.5-fold higher...
Next, we set out to gain a mechanistic understanding of the dynamics of anisotropic tissue sculpting in our system and their impact on tissue mechanics. To this end, we used our framework of experimental data on the evolution of macroscopic shape and microarchitecture in sculpted tissues to develop a multiscale continuum model for in silico simulation of cell contractility-driven tissue deformation in the two-node configuration (Supplementary Methods). Tissues were modeled as a continuum of representative volume elements (RVEs), each of which was constructed by connecting an active force-generating component with two passive elements to model dynamic mechanical interactions between cellular actomyosin contractility (active) and the ECM (passive) (Fig. 2A and fig. S7). The ECM elements under compression were treated as a linear elastic material, whereas those under tension were allowed to stiffen in the direction of maximum principal strain. In this system, de novo activation of the contractile force-generating component imparts stress on the passive elements, during which the ECM subjected to tension undergoes stiffening (Fig. 2B). This, in turn, increases force production by actomyosin contractility (Fig. 2B).

To execute simulations of our sculpting experiments, we defined the initial tissue geometry, boundary constraints (i.e., fixed adhesion to anchorage nodes with free detachment from all other surfaces), cell numbers, and ECM density. With the initial conditions defined, simulations were executed to allow for contractive deformation in silico, and the model was iteratively trained using experimental data on the time course of tissue compaction and shape change as a reference. As shown in Fig. 2C, the simulation results recapitulated the evolution of tissue shape observed in our experiments. Regarding tissue compaction, the simulation results estimated 72% reduction in the total volume of sculpted tissues, which was in good agreement with the experimental measurement of approximately 80% volume reduction in fibroblast-sculpted tissues upon reaching an equilibrium shape (Fig. S2). When we conducted simulations using a rectangular chamber with anchorage nodes of the same size but only 35% of the total volume of our circular chamber, the model predicted excessive narrowing and necking of sculpted tissues (Fig. 2D).

**Mechanical regulation of anisotropic tissue morphogenesis**

To analyze changes in cell contractility and tissue tension during the sculpting process, we first evaluated the magnitude and orientation of cell contractility at different stages of deformation and compaction. With the progression of tissue deformation, our simulations showed progressively increasing cell contractility throughout the tissue, but the extent of increase in the longitudinal direction ($x$-direction) was significantly greater than that in the lateral direction ($y$-direction) (Fig. 3A). Quantitatively, the average ratio of longitudinal to lateral contractility in the sculpted tissue increased from 1 to 1.8 (Fig. 3B). This was in contrast with an isotopic increase in cell contractility observed when the tissue was anchored to all boundaries of the same chamber geometry (Fig. 3A). Our simulations showed lateral packing of contractile units (Fig. 3C) akin to tissue compaction shown by our experimental data (figs. S2 and S5).

Analysis of ECM tension revealed similar patterns. Sculpting generated and increased ECM tension in the main body of tissue undergoing contractive deformation to create a highly anisotropic stress field polarized along the longitudinal direction (Fig. 3, D and E). Development of ECM tension also occurred in fully constrained tissues but in an isotropic manner (Fig. 3, D and E). According to our results, the average of stresses in all directions in the sculpted anisotropic tissues was almost twice as high as that of their isotropic counterparts (Fig. 3E). With the calculation of these stresses, our computational model allowed us to simulate and map the spatial heterogeneity of tissue stiffness of millimeter scale constructs in a manner that is not possible by measuring local stiffness at discrete locations (Fig. 3F). In our simulations of tissue sculpting, the average stiffness in the longitudinal direction ($C_x$), which coincided with the direction of principal stress, increased by more than fourfold with the maximum stiffness focused along the central axis (Fig. 3, F and G). By contrast, the stiffness in the lateral direction ($C_y$) was simulated to increase by approximately twofold, which was comparable to the extent of tissue stiffening in the fully tethered isotropic group (Fig. 3, F and G). These results were in agreement with our experimental measurements of local tissue surface layer stiffness using AFM (Fig. 1, M and N).

The results of our simulations suggest a mechanism whereby mechanical boundary constraints imposed by differential strength of tissue adhesion induce anisotropic deformation and polarized distribution of tissue-generated forces (Fig. 3H). This emergent stress polarization is sensed by cells in the deforming tissue and triggers them to increase contractile force production in the direction of principal stress (Fig. 3A). Because of the increased cell contractility, the ECM along this direction experiences higher tension and undergoes stiffening (Fig. 3, D and F), which then feeds back to
the cells to further increase their actomyosin contractility in the same direction. This contrasted with the predictions made by classical thermodynamic model-based simulation of tension-driven contractive tissue deformation. For tissue constructs that are geometrically and mechanically identical to our system, the results of this analysis showed that the magnitude of tension (and therefore stiffness) was greater for a fully constrained isotropic body in both the longitudinal and lateral directions (fig. S8).

**Sculpted tissue anisotropy modulates mesenchymal stromal cell differentiation**

Sculpting tissue architecture during morphogenesis occurs in concert with the process of cell differentiation by which stem and progenitor cells adopt tissue-specific phenotypes (11). Our theoretical predictions suggesting the critical role of stress polarization in anisotropic morphogenesis led us to ask whether the polarized mechanical environment of our two-node sculpting system influences the differentiation of stem cells in vitro. To address this question, we explored using human MSCs to form sculpted tissues as a platform to model the impact of tissue anisotropy on soluble factor-mediated contractive tissue deformation. For tissue constructs that are geometrically and microarchitecturally anisotropic seen in fibroblast-sculpted tissues (Fig. 4B). Our first objective was to confirm evidence of contractile phenotypes in MSC-sculpted tissues on the level of marker expression. At the early stage of sculpting, the contractile protein smooth muscle actin (SMA) was localized to the boundary regions near the anchorage nodes (fig. S9), but its expression became prominent throughout the tissue with the progression of sculpting. The fraction of SMA-expressing cells was greater than 60% by day 11, which was threefold higher than that measured in isotropic control tissues cultured for the same amount of time (Fig. 4B).

SMA expression has been shown to precede tenogenic differentiation of recruited progenitor cells during tendon healing in vivo (12) and skeletal myoblast differentiation both in vitro and after in vivo implantation (13). These studies led us to speculate that MSCs in our sculpted tissues might be primed for soluble factor-mediated induction of tenogenic and myogenic differentiation. To investigate this, we first sculpted MSC-containing hydrogel over 5 days using the two-node design and then treated the resultant anisotropic tissues with bone morphogenetic protein 12 (BMP-12), which is a known soluble inducer of tenogenic differentiation and tendon regeneration in vivo (Fig. 4C) (14). The differentiation of MSCs was assessed by monitoring the expression of scleraxis (SCX), a tenocyte-specific transcription factor activated by BMP-12 (15, 16). The fraction of SCX-positive cells in the sculpted tissues gradually increased in the presence of BMP-12, reaching 71% by day 18 (Fig. 4, D and F). Most of these cells displayed the typical bipolar morphology of tenocytes with spindle-shaped, elongated cell bodies (Fig. 4D, inset). In contrast, BMP treatment of isotropic control tissues for the same duration resulted in SCX activation in less than 20% of cells (Fig. 4E).

Similar experiments were conducted to investigate myogenic differentiation in MSC-sculpted tissues by using serum-free and high-insulin culture (Fig. 4G) (17). Twenty-three-day culture of the constructs under this condition led to the expression of the myogenic transcription factor myogenin by more than 80% of cells (Fig. 4, H and J). Our microfluorimetric analysis also showed a temporal increase in the level of the muscle-specific contractile protein myosin heavy chain (MyHC) (Fig. 4K). Induction of these skeletal muscle markers was accompanied by widespread coalescence of myoblast-like cells reminiscent of early cell fusion that occurs...
taneous contraction of the muscle tissues was observed (movie S1). With the proof-of-principle demonstration of engineering tissue-like structures with striated patterns of MyHC and nuclei positioned cleated myotubes aligned along the central axis (Fig. 5D). Myofiber-

MyHC-expressing cells (Fig. 5C). The nascent muscle tissues matured displaying an aligned architecture with a homogeneous population of (fig. S10). Within 3 days of culture, the sculpted constructs began to increase in response to H2 O2 treatment (Fig. 5K).

During muscle fiber differentiation (Fig. 4K). In isotropic tissues, the same media conditions induced significantly lower levels of myogenic differentiation (Fig. 4, I, J, L, and M.).

**Sculpted muscle tissues for modeling oxidative injury**

With the proof-of-principle demonstration of engineering tissue anisotropy in vitro, we next explored the use of our sculpting method as a platform technology for developing physiologically relevant preclinical models of anisotropic tissues. Building upon the demonstrated capability of our surface anchorage approach, we aimed to create a system for modeling injury and pharmacological modulation of skeletal muscle held in isometric tension. The focus of this study was to model oxidative stress-induced muscle injury, which has been implicated in a variety of clinical situations including diabetes, obesity, and muscle wasting (18–20). For increased experimental throughput and convenience, we designed muscle sculpting devices as inserts for use in standard six-well plates, each of which enabled routine formation of four 10-mm-long muscle constructs from human skeletal myoblasts embedded in collagen type I hydrogel containing 10% Matrigel (Fig. 5, A and B). The size of these constructs allows each tissue to serve as an individual replicate in standard biochemical assays such as the citrate synthase (CS) activity assay used to assess mitochondrial function in muscle tissue biopsies.

Without functionalization of anchoring surfaces with SS, tissues in this system rapidly detached and underwent isotropic contraction (fig. S10). Within 3 days of culture, the sculpted constructs began to display an aligned architecture with a homogeneous population of MyHC-expressing cells (Fig. 5C). The nascent muscle tissues matured rapidly over time, leading to the fusion of myoblasts into multinucleated myotubes aligned along the central axis (Fig. 5D). Myofiber-like structures with striated patterns of MyHC and nuclei positioned at the periphery were seen by day 11 (Fig. 5E), at which point spontaneous contraction of the muscle tissues was observed (movie S1). In isotropic control tissues, muscle contraction was only observed in sporadic individual myotubes (movie S2). We measured the level of CS in muscle tissue homogenates, which is generally reflective of functional mitochondrial content, to evaluate the bioenergetic capacity of the sculpted and isotropic constructs. Our analysis yielded an average of 120 U/g of total protein, closely approximating previously reported values (140 to 150 U/g) for gastrocnemius muscles in healthy human adults (21). CS activity in isotropic control tissues was approximately 30% lower (Fig. 5F). This significant difference in bioenergetic capacity that suggests greater mitochondrial content and more mature muscle fibers in sculpted tissues was further confirmed by microfluorimetric analysis of mitochondrial membrane potential (Fig. 5, G and H).

To simulate oxidative muscle injury in our system, we treated the engineered tissues with hydrogen peroxide. This model exhibited a linear, dose-dependent decrease in global cellular redox potential (Fig. S11) and a corresponding increase in intracellular reactive oxygen species (ROS) (Fig. 5I). Our results also indicated mitochondrial dysfunction due to the elevated production of ROS, which was evidenced by a significant reduction in CS activity with increasing concentrations of H2 O2 (Fig. 5J). Mitochondrial dysfunction due to elevated ROS drives pathological activation of poly(ADP-ribose) polymerase (PARP) family enzymes that are associated with cell death and inflammation in numerous diseases (22). In our model of skeletal muscle injury, the level of PARP-1 was indeed inversely correlated with the activity of CS as shown by its dose-dependent increase in response to H2O2 treatment (Fig. 5K).

On the basis of this finding, we explored the utility of our sculpted muscle tissues as a preclinical platform to assess PARP inhibition as a therapeutic approach for oxidative skeletal muscle injury. For this investigation, we tested the protective effects of two PARP-1 inhibitors, Olaparib and Velaparib (paribs), which were originally developed as anticancer drugs (23). Sculpted muscle tissues were pretreated...
with clinically relevant concentrations of parab for 24 hours before oxidative insult with H₂O₂ and measured CS activity 24 hours later to quantify the protective effect of the tested compounds. As shown in Fig. 5L, Olaparib pretreatment preserved CS activity levels at approximately 80% of that measured in normal sculpted tissues without H₂O₂ treatment. This observation is consistent with previous reports on Olaparib-induced improvement of mitochondrial function in skeletal and cardiac tissues (24, 25). Despite its similar mechanism of action, Velaparib produced less significant therapeutic effects (Fig. 5L). Pretreatment of the sculpted muscle tissues with the vitamin A derivative retinoic acid (RA) and the omega-3 fatty acid eicosapentaenoic acid (EPA) in combination preserved CS activity levels equivalent to uninjured control tissues (Fig. 5L), suggesting the potential benefits of natural oxidants for protection against oxidative skeletal muscle injury.

**Microphysiological model of lung cancer cachexia**

The development of our sculpted muscle tissues as a model of oxidative injury laid the foundation for the design of a microphysiologival model of cancer cachexia, a systemic wasting disorder characterized by a progressive loss of skeletal muscle tissue that affects 50 to 80% of patients with cancer (26). The pathophysiology of cancer cachexia involves multiple organ compartments, but increased ROS production in skeletal muscle driven by proinflammatory cytokines is a canonical feature, leading to oxidative injury and mitochondrial dysfunction (20, 27, 28). Despite its well-documented contribution to cancer mortality (up to 20% of cancer deaths) (29), cachexia remains a significant clinical problem for which more effective therapies and palliative treatments are urgently needed. Preclinical efforts to address this need have been hampered by the limited capacity of current animal models to mimic the pathophysiology of cancer cachexia in humans (30), calling for new approaches to preclinical modeling of human conditions.

To create a microphysiological system that enables real-time communication between skeletal muscle and cancer tissue, we engineered a microfabricated multichamber device (Fig. 6A) for compartmentalized coculture of sculpted skeletal muscle tissues with a multicellular 3D lung cancer model composed of A549 lung adenocarcinoma spheroids embedded in a 3D collagen type I hydrogel with primary human lung fibroblasts and THP-1–derived macrophages (Fig. 6B). A key clinical principle guiding our model development is the observation that cachexia is most commonly seen in patients with advanced stage disease and recurrent cancers following chemotherapy (31). To explore this phenomenon in our model, we engineered drug-resistant lung cancer tissues by treating A549 cells with cisplatin, a platinum-based chemotherapeutic agent commonly used for nonsmall cell lung adenocarcinoma (32). After 48 hours of culture, cisplatin was removed, and drug-resistant cells were expanded and used to form spheroids (Fig. S12). We did not intend to study the real-time effects of chemotherapy in the muscle tissue compartment. Rather, we aimed to simulate recurring tumors composed of drug-resistant cells that have survived previous rounds of chemotherapy and explore the broad hypothesis that these drug-resistant tumor phenotypes promote the development of cachexia.

Cisplatin-resistant A549 spheroids (cis-A549) showed elevated ROS production compared to those composed of untreated cells (Fig. 6C). When we integrated these spheroids in the 3D lung cancer compartment of our device to mimic a drug-resistant tumor, we observed a potent injurious effect in sculpted muscle tissues as illustrated by marked production of ROS throughout the construct (Fig. 6, D and E). This deleterious response occurred to a significantly reduced extent when untreated A549 cells were used to make tumor spheroids (Fig. 6, D and E), confirming the notion that drug-resistant cells are more procachetic and are therefore well suited to the development of clinically relevant in vitro models of cancer cachexia.
Baseline levels of ROS in monoculture of the muscle constructs without engineered lung cancer tissues were significantly lower than those observed in the coculture models (Fig. 6E).

Studies have shown that increased ROS promotes production of proinflammatory cytokines such as tumor necrosis factor (TNF), which are known to drive cachexia (33–35). Consistent with these findings, after 8 days of culture, the tumor spheroids derived from cisplatin-treated cells (cis-A549) began to secrete significantly higher levels of TNF than what was measured in untreated controls (Fig. 6F). The difference in TNF production between A549 and cis-A549 cells was markedly greater in 3D spheroid cultures (more than fourfold) when compared with 2D cultures of the same cells (approximately twofold difference) (Fig. 6F and fig. S13), suggesting that engineering 3D cancer constructs is a critical step in the development of in vitro cachexia models. We conducted computational analysis using the experimentally measured kinetics of TNF secretion to predict the spatiotemporal distribution of tumor-derived TNF in our device. According to the simulation, diffusive transport of TNF from the spheroid chambers induced a continuous increase in TNF concentration in the muscle compartment, reaching approximately 100 pg/ml in the media immediately surrounding the muscle tissue by day 4 (Fig. 6G). Consistent with the computational predictions, the muscle tissues in our device were seen with markedly increased nuclear translocation of p65/nuclear factor κB (NFκB) (Fig. 6H), reflecting the proinflammatory nature of the muscle injury in our model.

Oxidative injury and proinflammatory activation in our cachexia model corresponded to a significant decrease in mitochondrial bioenergetic output, as evidenced by a roughly 30% reduction of CS enzyme activity levels (Fig. 6I). An increase of more than fourfold in PARP enzyme activity levels further confirmed a mechanism of oxidative injury-driven muscle tissue mitochondrial dysfunction in our cachexia model (Fig. 6I). The collective biochemical evidence of inflammatory and oxidative injury was accompanied by abnormal changes in the tissue-level structural organization and cytoskeletal architecture of the sculpted muscle that are reflective of morphological alterations observed in cachexia-induced muscle degeneration (Fig. 6J) (36, 37). Given the pronounced increase in ROS, we explored the potential of our cachexia model for screening candidate therapeutic agents to prevent muscle wasting by pretreating sculpted muscle tissues with PARP enzyme inhibitors and the combination of RA and EPA. At the same doses used in our hydrogen peroxide model (Fig. 5L), Olaparib and Velaparib treatment had no protective effects as shown by the failure of these compounds to prevent the reduction in CS activity induced by cancer tissue coculture (Fig. 6K). Coadministration of RA and EPA, however, was effective for preserving the muscle constructs and maintaining its CS activity at levels comparable to those in the control group representing healthy muscle tissues (Fig. 6K). Further evidence of muscle tissue protection by this supplement combination was provided by immunostaining of F-actin and p65/NFκB, which revealed preservation of tissue architecture, F-actin cytoskeletal arrangement, and inhibition of p65/NFκB nuclear translocation (Fig. 6L).

**DISCUSSION**

We developed a simple method that leverages cellular contractility to sculpt the shape and microarchitecture of engineered tissues. This approach is inspired by anisotropic tissue morphogenesis,
during which differential adhesion strength at tissue boundaries patterns the spatial distribution of cellular contractile forces (1). A good example of this mechanism at work in vivo is skeletal muscle development, during which the MTJs formed with anchoring tendons act as mechanical boundary constraints that focus cellular contractile forces along the longitudinal axis of the nascent muscle (Fig. 1A) (8). To mimic the interfacial anchorage of anisotropic tissue boundaries such as the MTJ, we used heterobifunctional cross-linking chemistry to enhance the anchorage of ECM scaffolds to PDMS surfaces. SS has been used to tether ECM proteins to PDMS surfaces and engineer spatial patterns of cell adhesion (38). Here, we have demonstrated, using SS chemistry as an example, that the surface anchorage method enables tethering of millimeter scale anisotropic tissues held in isometric tension during extended culture for up to 28 days without structural failure. Other methods of surface modification that enable scaffold anchorage can be used to similar effect (39).

Our surface-directed tissue patterning approach provides an alternative to existing platforms, such as the PDMS micropillar-based system in which anisotropic tissues formed via rapid contraction in less than 24 hours are lassoed around the pillars (40). While the current iteration of our model lacks force measurement capacity of micropillar-based platforms, our sculpting method extends the time scale of tissue morphogenesis to days and weeks (Figs. 1 and 4 and fig. S3). This capability was critical to the execution of our MSC differentiation studies in sculpted tissues (Fig. 4) and will enable further investigation of how microscale events such as cell migration, ECM remodeling, and the emergence of anisotropy via gradual tissue deformations interact to pattern the spatial rearrangement of macroscopic tissues dictated by mechanical boundary constraints. An important detail of our design is the initial circular geometry, which prevents necking in the central regions of sculpted tissues tethered under tension (Fig. 2D). Necking has been reported as a mode of structural failure in engineered tissue constructs under tension (41). Our approach also permits flexibility in that the temporal dynamics of the tissue sculpting method can be easily manipulated by using cell types with different intrinsic contractility, varying the seeding density, or supplementing with exogenous factors that modulate cellular contractility.

The development of anisotropic tissue gives rise to a unique biophysical environment characterized by the polarization of tissue-generated mechanical stresses (42, 43). It is well appreciated that cells increase their contractility in response to increased stress transmitted through the ECM, such as during tissue stiffening in cancer (44). However, the degree to which polarization of increased stresses due to emergent tissue anisotropy influences cell actomyosin contractility and ECM tension across multiple scales during morphogenesis is an important question in mechanobiology. The effects of applied boundary conditions on tissue anisotropy in vitro, particularly in systems that generate uniaxial tension using various approaches, are well known (45–47). Furthermore, cells are known to respond to the tension in the cell-matrix interface generated by the resistance of the surrounding environment in a contact area-dependent fashion (48, 49). Our computational studies expand upon this foundational understanding by illustrating that contractility increases in anisotropic tissues as a function of stress polarization in a manner that is independent of contact area (Fig. 3, A, B, and H). A deeper understanding of how stress polarization due to emergent local anisotropy affects cell contractility has obvious implications not only for the regulation of tissue morphogenesis but also in the context of diseases such as cancer in which increased extracellular tension and local ECM fiber realignment converge to promote malignant phenotypes and tumor cell migration (50–52). Our tissue sculpting approach provides opportunities to create 3D culture models of these processes in which patterns of architectural and mechanical anisotropy can be rationally designed.

The emergence of tissue anisotropy during morphogenesis is coordinated with the regulation of tissue-specific stem and progenitor cell differentiation. For example, during skeletal muscle morphogenesis, myoblasts actively align before fusion and myotube assembly (53). Seminal early studies in mechanobiology and more than a decade of subsequent research on the biophysical regulation of stem cell differentiation established that tissue stiffness and cell shape determined by contact area are key regulators of fate decisions (54, 55). However, less is known regarding the influence of tissue anisotropy and stress polarization on stem cell differentiation. Our study revealed that tenogenic and myogenic lineage commitment of MSC induced by biochemical factors is markedly enhanced in sculpted tissues compared with isotropic control tissues cultured with the same medium (Fig. 4). Local stiffness measurements by AFM and our simulations suggest that sculpted tissues are approximately 2.5 times stiffer than isotropic control tissues (Figs. 1N and 3, F and G). Although the effects of this increase in tissue stiffness on MSC differentiation cannot be separated from the effect of emergent anisotropy in our sculpted tissues, it is unlikely that the magnitude of this difference can explain the increased efficiency of tenogenic and myogenic differentiation. Deeper insights gained from future investigation of these phenomena will have broad implications for the development of new technologies for directed differentiation of stem cells in tissue engineering and regenerative medicine. While MSCs are not an optimal cell source for engineering functional tendon and skeletal muscle tissues, MSC-sculpted tissues can provide a venue for researchers to investigate how the interplay between tissue mechanics, soluble factors, and emergent anisotropy regulates stem and progenitor cell differentiation during tissue morphogenesis.

We applied our tissue sculpting approach to engineer 3D-aligned skeletal muscle tissues using human skeletal myoblasts (Fig. 5). Engineered muscle tissues held in isometric tension have been used to study muscle physiology, model diseases, and test drugs (56, 57). Platforms for measuring contractility by imaging curvature of contracting muscle tissue sheets or the deflection of PDMS micropillars have also been reported (58, 59). While our sculpted muscle tissue platform does not incorporate functional measurements of contractility, it provides an adaptable method of low-cost and reproducible fabrication of human skeletal muscle tissues that provide compelling advantages over 2D myotubes and amorphous 3D cultures for routine in vitro screening of candidate therapeutics in models of acute muscle injury. Using the hydrogen peroxide exposure model of oxidative muscle injury, we demonstrated that U.S. Food and Drug Administration–approved PARP enzyme inhibitors Olaparib and Velparib targeting mitochondrial dysfunction provided marginal protection against oxidative injury (Fig. 5L). The dietary antioxidants vitamin A (RA derivative form) and the omega-3 fatty acid EPA showed a potent protective effect that preserved muscle CS activity at the level of control tissues (Fig. 5L). While not disproving the potential therapeutic efficacy of PARP inhibitors in the setting of oxidative muscle injury, our results support the idea that diets rich in natural antioxidants can promote muscle health and provide...
protection against pathology driven by elevated ROS production in skeletal muscle.

This study also inspired us to develop a microphysiological system to model oxidative muscle injury induced by tumor-derived signals. On the basis of the analysis of serum cytokine levels, inflammatory mediators produced by cells in the tumor microenvironment (e.g., TNF) have been proposed as key drivers in muscle wasting associated with cancer cachexia (27, 28). The notion that characteristics of specific tumor types (i.e., proinflammatory profile) can influence the course of cachexia independently of tumor burden challenges the historical view that cancer cachexia is tied to nutritional deficiency. However, this notion is supported by clinical examples of cachexia before the presence of detectable tumor burden or reduction of caloric intake (60). Our model may play an instrumental role in future studies of this pathophysiological basis of cachexia by enabling investigation of the discrete effects of engineered tumor tissues on skeletal muscle in compartmentalized coculture. The adaptability of our system will make it possible to examine the effect of tumor type, stage, and treatment history on cytokine elaboration and muscle injury.

To progress toward modeling the complete tumor microenvironment as a source of proinflammatory signals, it is necessary to incorporate the contribution of the tumor stromal compartment that contains tumor-associated immune cells such as macrophages that are major sources of cytokine production and serve as amplifiers of proinflammatory signals elaborated by tumor cells (27). The tumor compartments in our microphysiological model of lung cancer cachexia reflect an organotypic tumor microenvironment containing cancer cells, fibroblasts, macrophages, and stromal ECM. The measurement of increased ROS production, decreased CS activity, and increased PARP activity in our cachexia model (Fig. 6) is consistent with previous reports of PARP enzyme activation and mitochondrial dysfunction in human lung adenocarcinoma–associated cachexia (61). While analysis of changes in contractile force would be informative, we have presented biochemical and morphological evidence of muscle injury induced by engineered 3D lung adenocarcinoma tissues. It is expected that a decrease in contractile force would be observed in conjunction with oxidative muscle injury in our cachexia model. Future studies should incorporate measurement of changes in muscle tissue mass to provide an additional layer of clinically relevant assessment.

In vitro models of lung cancer–associated cachexia that use 2D cultures of myotubes and conditioned medium from lung carcinoma cells have been described previously (62–64). One study using all human cells reported that myotubes showed a lack of atrophy when exposed to cancer cell–conditioned medium (63), perhaps due to the potential issues of a low concentration of tumor-derived factors in conditioned medium and the lack of a temporal change in signal, both of which are accounted for by the low-volume coculture in our microphysiological system. Furthermore, we demonstrated that incorporating drug-resistant tumor tissues in the model contributes to increased elaboration of TNF (Fig. 6F), a known driver of cachexia (28). Our cachexia model enabled the experimental finding that a history of chemotherapy exposure and resistance in the tumor cells themselves gives rise to a proinflammatory phenotype that may be a contributing factor to accelerated development of cachexia in patients with recurring advanced malignancies.

A major question in cachexia research is the degree to which improved nutrition can decrease muscle wasting (65, 66). While caloric intake is a key factor, micronutrient density may also play a critical role by providing reserves of natural antioxidants to combat increased levels of muscle ROS in the setting of cachexia. To explore this possibility in our cachexia model, we pretreated muscle tissues with RA (vitamin A derivative) and EPA during the culture phase before the introduction of tumor tissues and showed preservation of CS activity (Fig. 6K). The potential clinical relevance of this observation warrants further investigation. While there is significant impairment of nutrient absorption, packaging, and bioavailability in the setting of advanced malignancy, these initial findings in our cachexia model suggest that if increased levels of dietary antioxidants can be delivered to muscle cells, these micronutrients may exert protective effects that function independently of overall caloric intake.

The discussion of new opportunities also allows us to identify important areas of improvement that need to be addressed in future research. First, tissue sculpting permitted by our current method is technically 2D and has only been demonstrated using planar tissues. While this approach has proven instrumental as illustrated by our muscle models, realizing the full potential of our technology may require engineering boundary constraints to create 3D patterns of multicellular contractility that faithfully mimic complex modes of structural deformation in macroscopic developing tissues. Second, modeling the process of tissue morphogenesis is not complete without considering the spatiotemporal gradients of morphogens that act in concert with mechanical cues. The lack of this important morphogenetic control may be addressed by integrating our current platform with microfluidic production and manipulation of complex biochemical gradients (67). On a related note, studies are also needed to explore the feasibility of making our engineered muscle constructs perfusable, given the critical role of vascular supply in tissue development and homeostasis (68). Future designs of muscle sculpting devices should incorporate features that allow for optical measurement of muscle tissue force production. Last, efforts should be made to achieve functional integration of our cachexia model with other organ modules such as fat and liver to study the systemic pathophysiology of cachexia in body-on-a-chip microphysiological systems.

MATERIALS AND METHODS
Experimental design
The objective of this study was to develop an easily adaptable platform technology for patterning engineered tissue anisotropy that enables the creation of tissue-engineered constructs and microphysiological systems for investigations in tissue mechanobiology, stem cell biology, and pathophysiology. The goal of our fabrication approach was to create a method that can be adapted in virtually any tissue culture laboratory, requiring only manually prepared PDMS thin slabs, biopsy punches, heterobifunctional cross-linking reagents, and ultraviolet (UV) light. We developed a computational model to simulate the tissue sculpting process with the goal of exploring how the emergence of tissue anisotropy and stress polarization influences cell contractility. The aim of our MSC study was to determine whether the biophysical environment of our sculpted tissues potentiates factor-mediated tenogenic and myogenic differentiation and to demonstrate the utility of our platform for investigation of progenitor cell differentiation in anisotropic tissue environments. We engineered sculpted muscle tissues to create a robust platform for biochemical and morphological analysis of oxidative injury and screening drug responses. Our cachexia study was designed to investigate...
the production of tumor-derived factors known to drive cachexia, test the hypothesis that drug resistant tumors are more pro cachectic, and screen the muscle protective effects of candidate therapeutics.

**Cell culture**

NIH/3T3 mouse embryonic fibroblasts [American Type Culture Collection (ATCC)] were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). Normal human lung fibroblasts (NHLF; Lonza) used for tissue sculpting experiments were maintained in complete fibroblast growth medium (FGM-2; Lonza). Human MSCs (Lonza) were expanded in complete MSC growth medium (Lonza). Human skeletal myoblasts (hSKMB; Lonza) were expanded in complete skeletal myoblast growth medium (Lonza) and differentiated in DMEM supplemented with 1% ITS+ premix (Corning) and 2% horse serum (Gibco). A549 lung adenocarcinoma cells (ATCC) were cultured in F-12K medium supplemented with 10% FBS. NHLF used for tumor-associated stromal tissues were cultured for two passages in FGM base medium supplemented with 0.1% serum and 0.5% ITS+ premix to reduce proliferation rates before tissue formation. THP-1 cells (ATCC) were maintained in suspension culture in RPMI 1640 (ATCC) supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol. THP-1 cells were treated with phorbol myristate acetate (200 ng/ml) for 6 hours before incorporation in collagen gels with NHLF to potentiate macrophage differentiation. All culture media contained 1% penicillin-streptomycin (Gibco) and were expanded in a humidified tissue culture incubator maintained at 37°C and 5% CO₂. Cells were used at passages 2 to 5.

**Tissue sculpting device fabrication**

Sculpting devices were fabricated using standard PDMS-based soft lithography. Devices for fibroblast- and MSC-sculpted tissues were 1 mm thick and contained a circular tissue chamber (8 mm in diameter) with evenly spaced semicircular anchorage nodes with a radius of 1 mm. Chambers of muscle sculpting devices were 1 mm thick, with the same sized anchorage nodes on an ellipsoid chamber tapering to a maximum width of 4 mm. Multitissue muscle sculpting inserts contained lead-in channels for gel injection in a caged configuration, although chambers can be filled by pipetting for open well culture. The human muscle tissue sculpting chamber in cachexia devices had the same size and shape as that used for sculpting skeletal muscle tissues, with the addition of six adjacent 2-mm wells designed to house tumor tissues. These wells were connected to the muscle chamber by rectangular microfluidic channels with a cross-sectional size of 300 μm × 300 μm. For device fabrication, PDMS (Sylgard, Dow Corning) base was mixed thoroughly with a curing agent at a weight ratio of 10:1. This mixture was then degassed and poured over 3D-printed device molds (Protolabs) containing the device chamber features to be fabricated. Molded PDMS was cured at 60°C for 7 hours. Alternatively, sculpting devices were fabricated manually using biopsy punches, and a 1-mm-thick PDMS slab was prepared and cured as described above. Circular tissue chambers in sculpting devices were created using an 8-mm biopsy punch. Anchorage nodes were added using a 2-mm biopsy punch.

**Surface functionalization**

SS is a heterobifunctional cross-linker that contains an amine-reactive NHS ester and a photoactivatable nitrophenylazide group. It is water soluble and reactive to amine groups and nucleophiles. Previously, SS has been used to tether extracellular matrix proteins and peptides to poly(acrylamide) and PDMS surfaces (38, 69). SS is cross-linked to PDMS via the nitrophenylazide group. During the UV treatment procedure, a highly reactive nitrene is formed from the nitrophenylazide group, and this is cross-linked to double bonds on the PDMS surface. When a collagen solution is gelled in contact with SS-treated surfaces, collagen fibers are cross-linked to the PDMS surface via the open NHS ester (fig. S1). For the PDMS surface functionalization procedure, SS was dissolved to a working concentration of 1 mg/ml in deionized water. For surface anchorage testing studies, PDMS surfaces were fully covered with SS solution. In tissue sculpting experiments, anchorage node surfaces were selectively functionalized by pipetting SS solution droplets into the nodes of dry devices placed in petri dishes. SS droplets were retained in nodes by surface tension effects while leaving the untreated surfaces of the tissue chambers dry during this procedure. To create isotropically constrained tissues, the entire chamber was filled with SS solution. After adding SS solution, devices were exposed to UV light (ELC-500 UV Cure Unit) at an optical output of 30 mW/cm² for 5 min. This solution was aspirated, and, without washing the surface, the previous step repeated for another 5 min of UV exposure. Sculpting devices were washed with phosphate-buffered saline (PBS), after which the treated surfaces were considered functionalized. Control PDMS surfaces (untreated) were subjected to the same procedure with PBS added instead of SS during the UV incubation step. Cell-laden ECM hydrogel solutions were added to sculpting devices immediately after the surface functionalization procedure.

**Device seeding and tissue sculpting**

Collagen precursor solution was prepared by mixing high concentration type I collagen with 10× DMEM, 1 N NaOH, and PBS to achieve physiological pH and osmolarity. Collagen gels were prepared at 2.5 mg/ml for fibroblast-sculpted tissues (Fig. 1) and MSC-sculpted tissues (Fig. 4). In some experiments, for characterizing tissue mechanics, gels were prepared at 5 mg/ml. For myoblast-sculpted tissues (Figs. 5 and 6), a collagen solution (5 mg/ml) with 10% Matrigel was used for hydrogel formation. Cells were gently admixed to the precursor solution at various densities depending on the study. Mouse embryonic NIH/3T3 fibroblasts were added at a density of 3 × 10⁶ cells/ml. NHLF, MSC, and hSKMB were added at a density of 2 × 10⁶ cells/ml. Before filling the sculpting chambers with cell-laden collagen solutions, individual functionalized devices were placed in a six-well plate with an underlying layer of Parafilm to prevent adhesion of the gel to the culture plate surface. The cell-laden hydrogel precursor was loaded into the PDMS culture chambers and incubated for 1 hour at 37°C for gelation. This procedure was timed to allow for rapid device seeding within 3 min of completing surface functionalization. After gelation, cultures were immersed in culture medium specific to the cell type and experiment, and the underlying Parafilm was peeled away. Tissues were maintained in six-well plates within a tissue culture incubator at 37°C and 5% CO₂.

**Multiscale continuum model**

A complete description of our multiscale continuum model and tissue sculpting simulations is available in the Supplementary Materials.

**MSC differentiation in sculpted tissues**

MSC sculpting was initiated in 10% FBS-containing FGM for 48 hours and then reduced to 2% from day 2 to day 5. To stimulate tenogenic
Cancer cachexia model
The first step in assembly of the cachexia model (Fig. 6) was seeding human muscle tissues in the same fashion as for seeding muscle tissues in six-well inserts (Fig. 5). After 6 days of muscle sculpting, stromal tissues composed of collagen type I hydrogel (5 mg/ml) embedded with 500 × 10^5 NHLF/ml and 200 × 10^5 phorbol 12-myristate 13-acetate–primed THP-1 cells were cast in the flanking microwells. After an additional 3 to 4 days of culture (9 to 10 days of muscle tissue sculpting), lung adenocarcinoma tissues were added after completion of the spheroid fabrication protocol described in the Supplementary Materials. Devices were maintained with muscle differentiation medium, which did not alter the morphological characteristics or reduce the viability of stromal and tumor tissues.

Drug testing
The initial dose range of PARP enzyme inhibitors Olaparib and Velaparib for testing was chosen on the basis of previous in vitro screening studies using myotube cultures in vitro (25). Olaparib and Velaparib were used at 100 μM in our hydrogen peroxide injury (Fig. 5L) and cachexia model (Fig. 6K). Paris were obtained as 10 mM stock solutions in dimethyl sulfoxide (Selleckchem). RA (Sigma-Aldrich) and EPA (Sigma-Aldrich) were added in combination at concentrations of 10 and 50 μM, respectively. RA and EPA concentrations were chosen on the basis of in vitro studies demonstrating anti-inflammatory and antioxidative efficacy (70, 71). In all drug testing studies, the compounds were added to muscle culture medium 24 hours before the addition of hydrogen peroxide to model oxidative injury (Fig. 5) or addition of cancer tissues to the cachexia device (Fig. 6). Compounds were replenished upon media change every 48 hours for the specified duration of these studies.

Immunofluorescence staining, microscopy, and image analysis
Tissues to be processed for staining were fixed in 4% paraformaldehyde and stored in PBS at 4°C until staining. All staining steps were performed with gentle agitation on a benchtop shaker. Constructs were permeabilized and blocked in 0.2% Triton-X and 3% bovine serum albumin (BSA) in PBS for 3 to 4 hours and incubated overnight at 4°C with primary antibodies against fibronectin (1:100; Abcam), SMA (1:200; Sigma-Aldrich), collagen III (1:50; Abcam), SCX (1:50; Abcam), myogenin (1:50; RnD Systems), MyHC (1:50; RnD systems), and p65/NFκB (1:500) was quantified by measuring the mean gray value of specified regions of interest in FIJI. The percentage of nuclear translocation for SCX, Myogenin, and p65/NFκB was quantified using FIJI according to standard methods. Cellular aspect ratios in sculpted and isotropic fibroblast tissues were calculated as the best-fit ellipse aspect ratio of phalloidin-stained cells. The percentage of SMA-positive cells and MyHC-positive cells was determined by manual counting of positive cells and automated counting of total DAPI-stained nuclei in ImageJ.

Atomic force microscopy
Colloidal probe microscopy was performed on an Asylum AFM with a MFP-3D 90-μm closed-loop XY scanner as previously described for measuring spatial variations of stiffness in lung tissue strips (72). Sculpted and isotropic tissues still anchored to device surfaces were placed on the AFM stage with an underlying pedestal of PDMS to support the tissue during nanoindentation. The tissue was bathed in warm PBS while on the AFM stage. Indentation depths were optimized by analyzing point-wise modulus values as a function of indentation depth to confirm that steady-state modulus values were obtained.

Biochemical assays
The Alamar Blue assay (Life Technologies) was used as a marker for global metabolic redox activity in muscle tissues and myotubes. CS activity levels in muscle tissue homogenates were determined using the CS Assay (Sigma-Aldrich). Cell-secreted TNF in culture medium was measured using TNF enzyme-linked immunosorbent assay (ELISA) (Abcam). Total poly(ADP-ribose) activity was analyzed in sculpted muscle tissue lysates using the HT Colorimetric PARP Apoptosis Kit (Trevigen). TNF and PARP concentrations were calculated using standard curves constructed with recombinant proteins. Enzymatic activity values calculated from standard curves were normalized to protein amount assessed by Bradford assay (Thermo Fisher Scientific).

Mitochondrial membrane potential and ROS imaging
Mitochondrial membrane potential was measured using the TMRM (tetramethylrhodamine, methyl ester) fluorescent indicator dye (Thermo Fisher Scientific) as described elsewhere (25). Real-time visualization and qualitative monitoring of ROS in living tissues were accomplished using Cell-ROX dyes (Thermo Fisher Scientific) and fluorescence stereomicroscopy (Zeiss Axio-Zoom). Fixable Cell-ROX was used for image analysis and quantification of ROS levels in sculpted muscle tissues.

Statistical analysis
After confirming that data passed normality and variance tests, statistical significance was determined using a two-tailed Student's t test.
Supplementary materials


t test for individual comparisons and is presented as the means ± SD or SEM as specified in each figure legend. The numbers of independent experiments and individual samples measured for all reported data are specified in each figure legend. To represent the significance, P values of data are specified in each figure legend. To represent the significance of comparisons, P values of P < 0.05, P < 0.01, or P < 0.001 are indicated with symbols as specified in each figure legend.

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Surface-directed engineering of tissue anisotropy in microphysiological models of musculoskeletal tissue

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