

REVIEW ARTICLE

Endothelial-mesenchymal transition in skeletal muscle: Opportunities and challenges from 3D microphysiological systems

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Abstract

Fibrosis is a pathological condition that in the muscular context is linked to primary diseases such as dystrophies, laminopathies, neuromuscular disorders, and volumetric muscle loss following traumas, accidents, and surgeries. Although some basic mechanisms regarding the role of myofibroblasts in the progression of muscle fibrosis have been discovered, our knowledge of the complex cell–cell, and cell–matrix interactions occurring in the fibrotic microenvironment is still rudimentary. Recently, vascular dysfunction has been emerging as a key hallmark of fibrosis through a process called endothelial-mesenchymal transition (EndoMT). Nevertheless, no effective therapeutic options are currently available for the treatment of muscle fibrosis. This lack is partially due to the absence of advanced in vitro models that can recapitulate the 3D architecture and functionality of a vascularized muscle microenvironment in a human context. These models could be employed for the identification of novel targets and for the screening of potential drugs blocking the progression of the disease. In this review, we explore the potential of 3D human muscle models in studying the role of endothelial cells and EndoMT in muscle fibrotic tissues and identify limitations and opportunities for optimizing the next generation of these microphysiological systems. Starting from the biology of muscle fibrosis and EndoMT, we highlight the synergistic links between different cell populations of the fibrotic microenvironment and how to recapitulate them through microphysiological systems.

KEYWORDS

biofabrication, fibrosis, in vitro 3D modeling, organ-on-a-chip, vascularization

Translational Impact Statement

No effective therapies are currently available for the treatment of muscle fibrosis, which is a key event in neuromuscular diseases. Traditionally, research on fibrosis has focused on a specific cell type (i.e. myofibroblasts), while neglecting the contribution of the vascular system. More recently, endothelial-to-mesenchymal transition has been emerging as a key feature of organ fibrosis. Analyzing the contribution of vascular damage by means of muscle microphysiological

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systems could lead to the identification of novel druggable targets blocking muscle degeneration during fibrosis.

1 | INTRODUCTION

Despite the extensive efforts to tackle fibrosis, an effective treatment is still missing.¹ Indeed, just two drugs are currently available for the treatment of fibrosis being however limited to Idiopathic Pulmonary Fibrosis.^{2,3} For the treatment of muscular fibrosis, Glucocorticoids are the only option that has shown so far efficacy in preserving muscle force and locomotion in dystrophic patients.⁴ However, Glucocorticoids are non-specific steroids that might have many side effects resulting in a contribution to muscle wasting.⁵ This lack of treatments is particularly dramatic when considering the incidence of fibrosis. Fibrosis is a pathological condition which, in the muscular context, is linked to primary diseases like muscular dystrophies, laminopathies, and neuromuscular disorders,⁶ but also volumetric muscle loss following traumas, accidents, and surgeries.⁷ It is estimated that 1 in 1000 births are cases of dystrophies⁸ and 4.5 million muscular reconstructive surgical operations are performed annually as a result of car accidents, cancer ablation, or cosmetic procedures,⁹ often resulting in the onset of fibrosis.

The lack of effective treatments for fibrosis is due to its complex nature and to the absence of proper models to mimic its onset and progression. The many cellular, biochemical, and biophysical factors involved make it challenging to understand the sequence of events leading to the disease and to find an effective therapeutic solution.¹⁰ Due to the lack of species-specificity, animal models may not be the best option to identify new drugs. Conventional 2D *in vitro* setups, while incorporating human primary cells, are limited in their ability to accurately represent native tissue architecture and functionality.¹¹ Thus, there is a need for more physiologically relevant models to efficiently identify novel therapies (such as cell-based, DNA-based, or drug-based therapies).

Advancements in bioengineering and cell biology have led to the development of 3D *in vitro* models of various tissues and organs, including muscles, which have improved our capacity to reproduce key aspects of native tissues in highly controlled experimental platforms.^{12,13} Although major progress has been made in recent years, 3D muscle models have yet to reach their full potential. By taking advantage of the high experimental control offered by these technologies, it is now possible to start deciphering complex pathological mechanisms underlying muscle fibrosis and other muscle-related diseases. An emerging hypothesis depicts the endothelium as a key player in the fibrotic process, as it can undergo a transdifferentiation known as endothelial-mesenchymal transition (EndoMT), leading to the acquisition of a fibrogenic phenotype.¹⁴ This involvement has been proven in various fibrotic tissues such as the heart, skin, kidney, and lungs. However, EndoMT in muscular fibrosis is poorly understood and under-investigated. Additionally, most of the studies are based on murine models, which might represent a limitation when

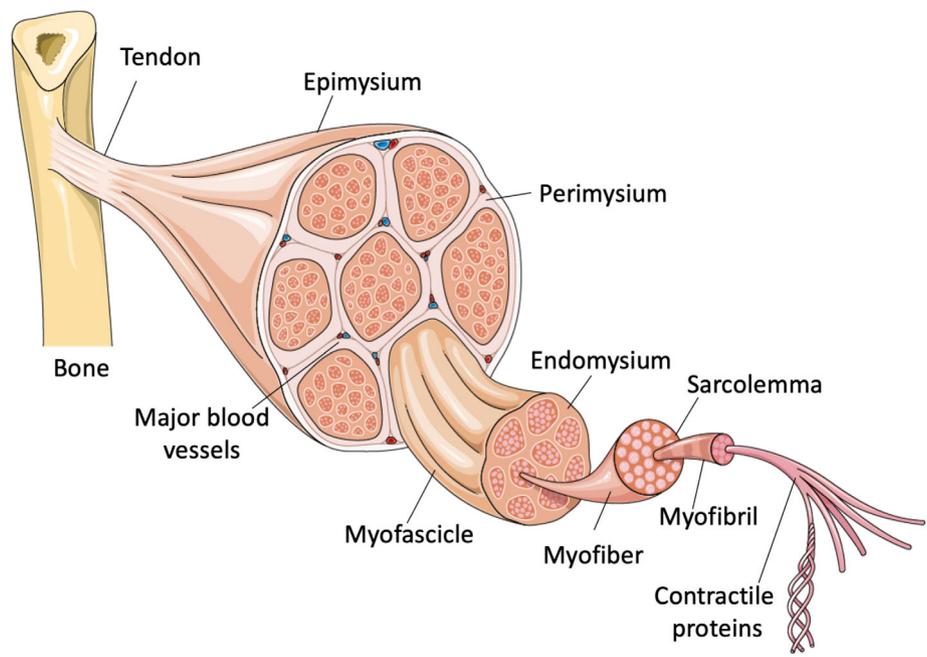
species-specificity plays a role. In this review, we explore the potential of 3D human muscle models in studying the role of endothelial cells (ECs) in muscle fibrosis and identify limitations and opportunities for their optimization. Further research on EndoMT has the potential to lead to the development of new treatments by taking advantage of microphysiological systems mimicking the architecture and functionality of the human muscle microenvironment (Figure 1).

2 | MUSCULAR FIBROSIS

Fibrosis is a pathological condition that affects various organs, including skeletal muscle, heart, liver, lungs, and kidneys.¹ It can also occur in other tissues leading to organ failure. The key hallmark of fibrosis is the excessive accumulation of extracellular matrix (ECM), which disrupts the diffusion of biochemical cues^{15,16} and causes changes in local stiffness and biomechanical parameters.^{17–19} These changes lead to cellular dysfunction and were traditionally associated with a single cell population, the myofibroblasts. The onset of fibrosis is believed to start with a tissue injury that activates an inflammatory response.²⁰ After the injury, ECM is produced as a temporary support for tissue regeneration and the number of ECM-producing myofibroblasts increases in the area. Usually, the process ends with the decline of myofibroblasts, either through apoptosis or deactivation, and restoration of normal tissue.²¹ However, if myofibroblasts persist, fibrosis develops and leads to the loss of tissue function. In the case of skeletal muscle tissue, this process results in a decline in the ability to generate contractile force.²² Myofibroblasts were initially identified as a regenerative phenotype acquired by quiescent fibroblasts.²³ Both these cell types have poorly characterized phenotypes since there is no known single marker that specifically discriminates fibroblasts or myofibroblasts from any other mesenchymal cell type. Instead, all these cell populations have in common the expression of mesenchymal markers, such as cadherin-2 (N-cadherin), cadherin-11 (OB-cadherin), fibroblast-specific protein-1 (FSP-1/S1004A), SM22 and Vimentin.²⁴ Over time, several other cell populations have been identified as myofibroblast sources thanks to cell lineage tracing studies.²⁵ It has been demonstrated that myofibroblast progenitors express Gli1 and ADAM12, like pericytes,²⁶ but also PDGFR α ,²⁷ being a marker of mesenchymal cell types like fibro-adipogenic progenitors (FAPs).^{28,29} Tie1⁺ bone marrow-derived progenitor cells were also proven to be a source of fibrotic cells in cardiac fibrosis.³⁰ Macrophages have been recently added to the list of sources of myofibroblasts³¹ together with ECs,^{32,33} as discussed in the next paragraphs of the present review.

The chain of events that leads so many different cell populations to develop myofibroblast-like properties is currently unknown. Understanding the specific local microenvironment characteristics that

FIGURE 1 Schematization of the skeletal muscle anatomy. Each myofiber contains numerous myofibrils that are made of contractile proteins. Myofibers are densely packed together forming a myofascicle. Several myofascicles constitute the muscle body. In the perimysium, which surrounds the myofascicles, and in the endomysium, which is located around the myofibers, stromal cells (e.g., vascular cells, motor neurons, fibroblasts, resident macrophages) contribute to the generation of the muscle microenvironment. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.



determine which cells will develop fibrotic properties is crucial in controlling the process and restoring tissue function before it reaches the end stage of fibrosis. For this reason, it is critical to gain insights into how certain cellular sources can act as drivers and recruit other cell populations in a fibrogenic cascade, in order to abort this process and prevent systemic disease. The endothelium is an essential cellular source to investigate in this regard. Indeed, the endothelium has not only been already associated with fibrosis in numerous tissues (e.g. liver, kidney), but it is also homogeneously distributed across the human body. Furthermore, it develops in close contact with muscle fibers, maintaining a two-way communication with them. For these reasons, vascularized muscle-specific fibrotic tissue models might be of great help to identify alternative markers of muscle fibrosis and providing more effective therapeutic options.

3 | THE IMPORTANCE OF ENDOTHELIAL CELLS IN THE SKELETAL MUSCLE

Skeletal muscle has a well-defined architecture based on bundles of parallel individual myofibers, embedded in an ECM and associated with blood capillaries and nerves. The ECM is divided into three zones: the endomysium, perimysium, and epimysium. The individual myofibers and stromal cells are dispersed in the endomysium, the inner ECM zone, forming a unit named fascicle. Each fascicle is then located in the perimysium, the middle ECM layer that supports numerous fascicles. Finally, the whole system is surrounded by the epimysium, the outer and thicker connective tissue layer.³⁴ The skeletal muscle vasculature has a hierarchical organization. Arteries penetrate the epimysium. In the perimysium, they split into a network of interconnected arterioles, which enter the endomysium. There, they branch into a meshwork of capillaries that develop in parallel to the

myofibers following the direction of the muscle.³⁵ The microcirculation of skeletal muscle is composed of two cell types: ECs and pericytes. ECs form the walls of the capillaries, which are around 200 μm in diameter. Muscle contractions exert strain on the vessel wall. ECs sense and transmit different types of external forces primarily through their cell-cell, and cell-ECM adhesions, which also generate intrinsic traction forces. ECs also respond to biochemical cues from the surrounding environment by releasing compounds, such as nitric oxide (NO) and Prostaglandins, to tune the vascular tone.²⁷ These mechanisms allow the vascular adaptation to muscular activity, ensuring the supply of oxygen and nutrients and the removal of waste by-products. It is believed that the wide set of environmental stimuli to which ECs are exposed impacts their remodeling and specialization. Different transcriptional programs are then activated defining an arterial (e.g. *FOXC* and *SOXF*)³⁶ and venous (e.g. *EPHB4*)³⁷ or lymphatic (e.g. *NR2F2*)³⁸ EC differentiation. Other transcription factors confer organ-specific attributes to ECs.^{39,40} Markers characterizing muscle ECs are the genes *E74A*, *ELK1*, *ELK4*, *GABPA*, *NRF-2*, *TSPAN7* and the secretion of the angiocrine factor *CD36*.⁴¹

Pericytes are the other cell type composing the microvasculature. They have a peri-endothelial distribution and share with ECs a basement membrane. The fine finger-like projections characterizing pericyte shape make contact with the underlying and neighboring capillary ECs. The ratio between these two populations changes according to the tissue. In the skeletal muscle, the ratio is very high (100:1 ECs to pericytes), while in the brain it is very low (1:1).⁴²

Considering their high number and ubiquitous distribution, ECs represent a relevant cell population to focus on when looking for stromal cells that contribute to the fibrotic process. Indeed, one of the key properties of ECs is their ability to undergo plasticity, which refers to their ability to change and adapt in response to different stimuli.⁴³ This plasticity is mediated by a variety of signaling pathways and

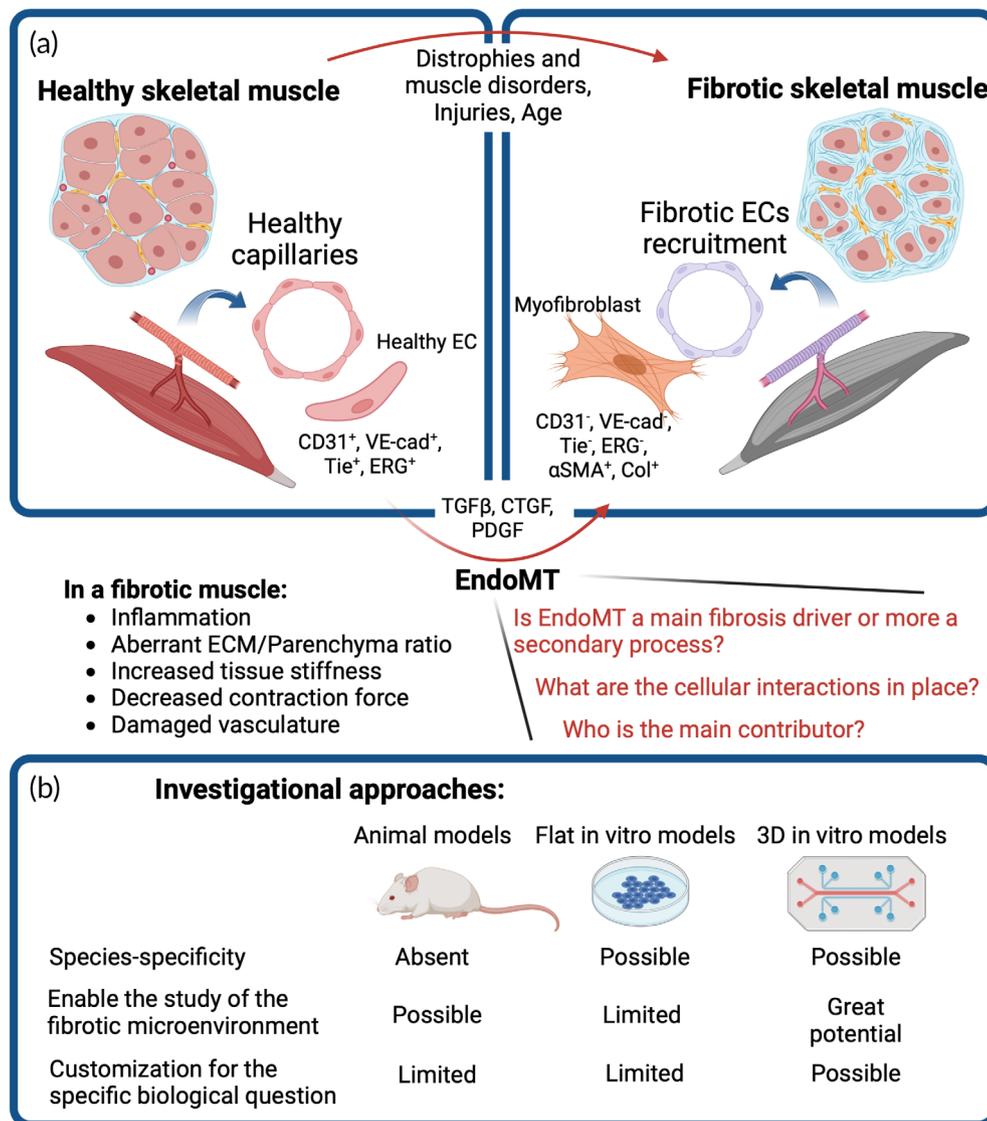


FIGURE 2 (a) Schematics showing the changes occurring in the skeletal muscle affected by fibrosis and the impact of the disease on endothelial cells (ECs). Summary of the most relevant markers and growth factors related to endothelial-mesenchymal transition (EndoMT). (b) Available approaches to study EndoMT are animal models and in vitro models. In vitro models can be divided into flat traditional cultures and 3D models. These 3D models, including microphysiological systems, showed huge advancements in recent years and are useful candidates to unveil the pathological mechanisms of cell-cell and cell-matrix communications occurring in fibrotic muscles.

transcriptional regulators that control gene expression and EC function. For example, ECs can undergo phenotypic changes in response to various types of stresses, such as inflammation,⁴⁴ hypoxia and oxidative stress,⁴⁵ and to mechanical properties (e.g. increase in stiffness observed in fibrotic tissues).⁴⁶ The most important biochemical cues associated with endothelial plasticity are TGFβ,⁴⁷ CTGF,⁴⁸ FGF,⁴⁹ and VEGFA.⁵⁰ A key example of EC plasticity is the endothelial-hematopoietic transition. During embryogenesis, hematopoietic stem cells develop from the hemogenic endothelium, and their vascular commitment is specified in a Notch-dependent manner.⁵¹ Another important example of cellular plasticity is EndoMT, the process that takes part in the embryonal formation of the tricuspid and mitral heart valves, and that gives origin to other cardiac progenitors (e.g. cardiac fibroblasts and smooth muscle cells).⁵²

Moreover, it is important to consider that EC plasticity is not limited to the embryonic phase. This concept finds confirmations in the overlapping transcriptome among different cell populations, as shown by single-cell RNA sequencing studies and fate-mapping studies.⁵³ A

recent example is the study by Cameron and colleagues which used single-cell RNA sequencing to demonstrate the presence of a mixed population of cells co-expressing pericyte and EC markers in the human skeletal muscle.⁵⁴ While pure ECs and pure pericytes have completely different transcriptional profile, the hybrid population was characterized by the expression of 257 genes in common with pure ECs and 288 genes in common with pure pericytes. These results suggested that a potential transdifferentiation between the two cell populations may be possible. EndoMT drives mature ECs out of their quiescent state^{55,56} to acquire a mesenchymal phenotype characterized by an increased migration capacity and augmented ECM secretion. Morphologically, during EndoMT ECs lose the cell-cell junctions that maintain the barrier properties of the vascular wall and undergo a cytoskeletal reorganization passing from their cobblestone-like shape to spindle-like shape without apical-basal polarity⁵⁷ (Figure 2). From the molecular perspective, EndoMT is generally identified by the reduced expression of CD31/PECAM-1, Tie1, Tie2, Von-Willebrand factor, VE-cadherin, and by the increased expression of myofibroblast

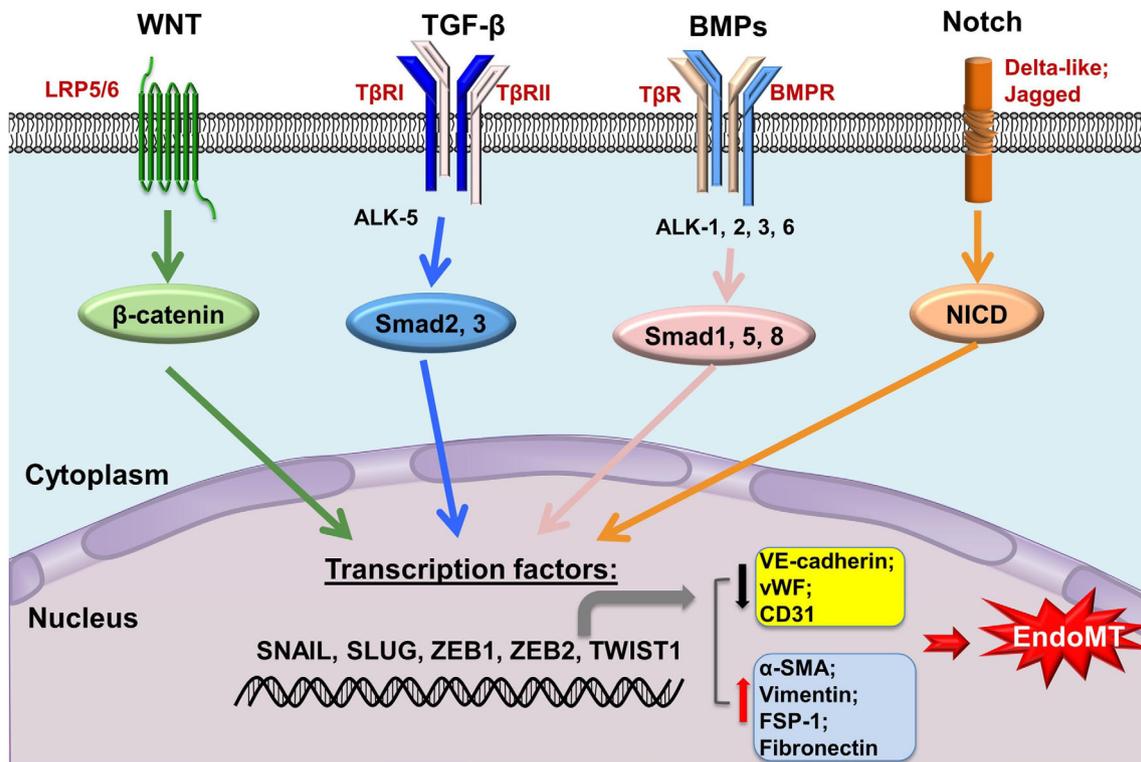


FIGURE 3 Schematic of the most important signaling pathways involved in the EndoMT initiation, namely TGF- β , BMP, Wnt/ β -catenin, and Notch. *SNAIL*, *SLUG*, *ZEB1*, *ZEB2*, and *TWIST1* are among the transcription factors that participate to the cascade. As a result, the expression of EC markers (such as VE-cadherin, vWF, and CD31) decreases and the expression of mesenchymal cell markers (like α -SMA, vimentin, FSP-1, and fibronectin) increases. Reproduced from Lu et al.⁵⁹

markers including α -SMA and FSP-1.⁵⁸ These changes are directed by four main signaling pathways, initiated by membrane receptors of TGF β , Wnt, BMP and Notch families (Figure 3).⁵⁹ The molecular cascades involve transcription factors such as *TWIST1*, β -catenin, and *SNAIL* 1, 2, 3, 5, 8, as observed in a mouse model of kidney fibrosis.⁶⁰ However, the underneath molecular process is even more convoluted, as new discoveries highlight. For example, the recent in vivo study by Mastej and colleagues showed the disruption of the combinatorial mechanism partnered by *KLF2* and *KLF4*, two transcription factors expressed by quiescent healthy ECs in the context of pulmonary fibrosis.⁶¹ The decreased expression of *KLF4* drives the upregulation of *KLF2*, which ultimately leads to an EndoMT phenotype. These changes affect the properties of ECs, such as their barrier function and the ability to undergo angiogenesis and vasculogenesis.

EndoMT was associated with inflammation, where combinations of pro-inflammatory cytokines, such as Angpt2⁶² and Nur77⁶³ are necessary to stabilize abnormal vascular remodeling.⁴⁴ Shear stress is also a fundamental factor for vascular homeostasis, and a disturbed flow or low shear stress can promote EndoMT.⁶⁴ In particular, low shear stress can induce EndoMT through a process controlled by the transcription factor *SNAIL*.⁶⁵ Finally, increased ECM stiffness is another potential EndoMT mediator. Another not fully-deciphered EndoMT promoter is represented by metabolic alterations in ECs, such as the role of fatty acid oxidation.⁶⁶

Given this wide range of promoting conditions, it becomes evident that EC plasticity is important in many physiological and pathological processes, including fibrosis,^{30,67} atherosclerosis⁶⁸ and cancer.³⁰ Indeed, EndoMT plays a critical role in maintaining blood vessel homeostasis and in promoting repair and regeneration following injury. However, its dysregulation has been linked to the development of various fibrotic diseases, including cardiac fibrosis.⁶⁹ Eventually, the disruption of the vascular integrity generates leaky capillaries, worsening the chronic inflammation present in fibrotic tissues. Given the similarities in the expression profile of ECs in both the heart and the skeletal muscle,⁴¹ there is a high likelihood that EndoMT is also involved in skeletal muscle fibrosis. Unfortunately, studies exploring this topic are limited, and there is a significant gap in our understanding of the role of EndoMT in skeletal muscle fibrosis that needs to be filled. In this scenario, 3D in vitro models could provide a major contribution. Microphysiological systems allow the generation of perfusable microvascular networks that replicate the in vivo microvasculature in terms of vessel geometry, ECM composition and dimensionality, cellular architecture and molecular profiles, also in response to biophysical stimuli (e.g. flow).^{70,71} Studies based on this technology observed increased barrier function and junctional reorganization in ECs (such as expression and localization of Occludin) in response to steady and pulsatile shear stress.⁷² Furthermore, microfluidic devices were used to correlate changes in endothelial permeability, sprouting activity, and monolayer integrity following the

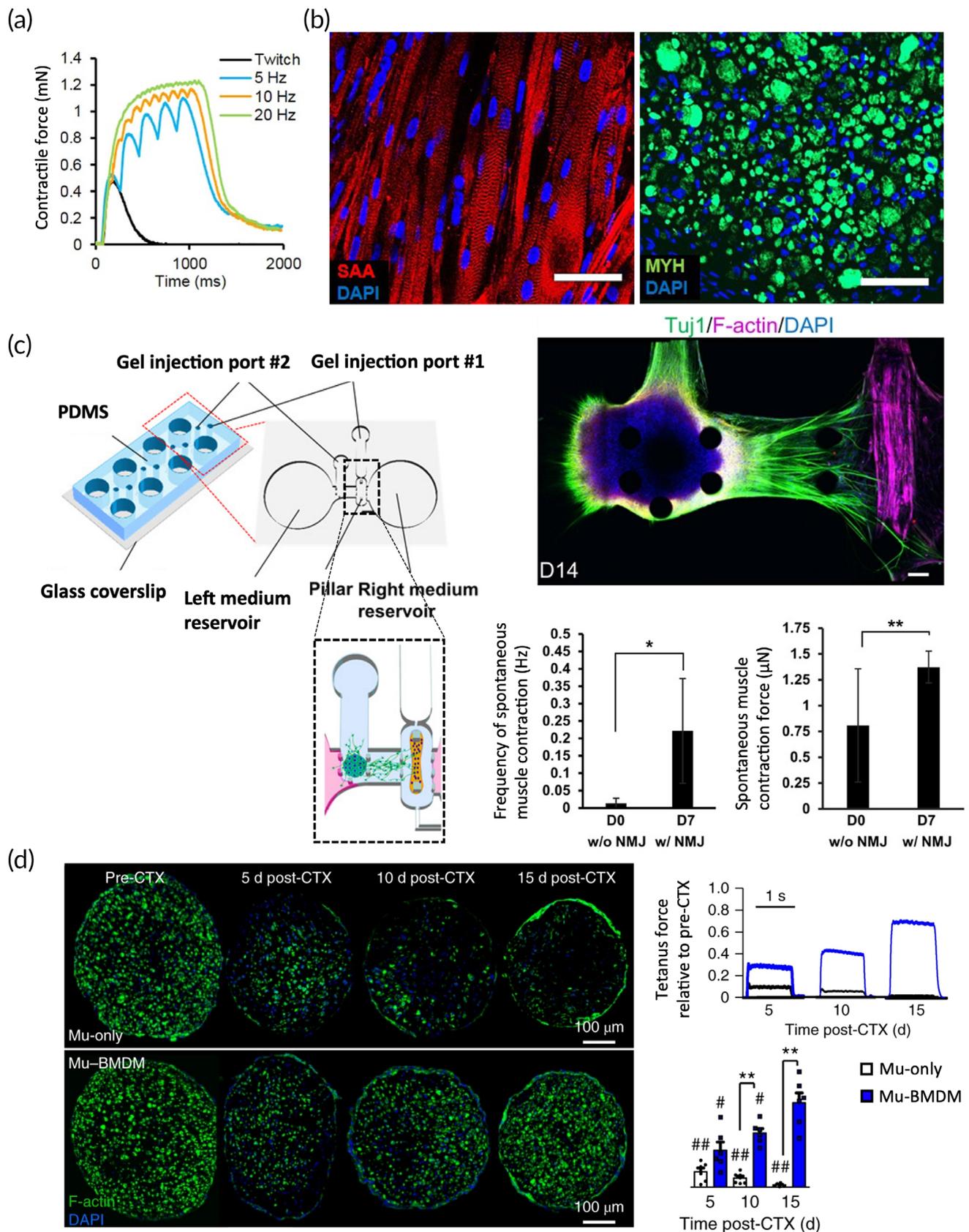


FIGURE 4 Overview of results achieved by 3D *in vitro* models of skeletal muscle. (a) Contractile force generated by an engineered 3D human muscle model upon electrical stimulation.⁸² (b) 3D bundle of human differentiated muscle cells (SAA⁺, MYH⁺) observed at different magnifications.⁸² Scale bar = 50 μm . (c) Microfluidic model of a neuromuscular junction (NMJ) showing neural-induced muscle contraction. A neuronal spheroid (Tuj1⁺) connects to a murine muscle bundle (F-actin⁺). Scale bar = 100 μm .⁸³ (d) 3D model made of a bundle of murine muscle cells (Mu-only) and muscle cells co-cultured with macrophages (Mu-BMDM). The model showed an improved regenerative capacity obtained by the co-culture after Cardiotoxin-induced damage.⁸⁴

application of fluid forces and VEGF gradients.^{73–75} Additionally, modifying the ECM stiffness it was possible to obtain changes in flow responsiveness and vessel barrier function,^{76,77} as well as in neovessel sprouting, angiogenesis-associated vessel elongation, and vascular network reorganization.^{78,79}

4 | ADVANCEMENTS OF 3D IN VITRO SKELETAL MUSCLE MODELS

In recent years, great progress has been achieved in the design of human muscle models (Figure 4a and 4b). Embedding myoblasts in a 3D hydrogel has been identified as the main approach to promote the differentiation of multinucleated myotubes packed together in a bundle.⁸⁰ Gene expression analyses demonstrated that these 3D muscle models were more similar to native muscle tissues compared to 2D cultures.⁸¹ Interestingly, several muscle-specific genes were downregulated in 2D cultures compared to 3D models, hence showing that their expression profile was closer to undifferentiated myoblasts. To achieve superior maturation, 3D muscle constructs were also coupled with electrical, optogenetic, or chemical stimulation systems to achieve an active contraction.⁸⁵ Indeed, similarly to in vivo physical exercise, electrical stimulation of 3D in vitro muscles led to a transcriptional increase of *PGC1 α* , *PDK4*, and other myokines (e.g. *NAMPT* and *ANGPTL4*). Furthermore, electrical stimulation increased the expression of contractile proteins such as myosin heavy chain, improved the synchronicity between calcium transients and contraction, and allowed to recapitulate metabolic effects like GLUT4 translocation and glucose uptake in muscle and cardiac tissues.⁸⁶ In addition, this functional activation allowed to quantify muscle force in different experimental conditions.⁸⁷

Another key feature of 3D muscle models is the presence of side populations of satellite cells, the resident pool of muscular progenitors.⁸² These cells are generally absent in traditional 2D cultures of myoblasts,^{88,89} hence demonstrating an improved capacity of mimicking specific aspects of the native composition and spatial organization of skeletal muscle. In this context, 3D muscle models overcome the limitations of 2D systems offering the chance to study how muscle cells interact with the surrounding microenvironment, namely the ECM and the other cell populations (e.g. fibroblasts, ECs, pericytes). To design physiological ECM, natural hydrogels mainly made of collagen, fibrin, Matrigel, gelatin, or their combinations have been employed. These hydrophilic matrices provide structural support to muscle cells together with molecular interactions that stimulate the maturation of myofibers.⁹⁰ This aspect is of paramount importance to study muscle pathologies where the ECM plays a role, like fibrosis.⁹¹ Hinds and colleagues tested in a 3D setup various matrix proteins, including collagen I, fibrin, and Matrigel in different concentrations, to develop skeletal muscle bundles based on neonatal rat cells.⁹² The authors compared the impact of these ECM proteins on tissue structure, generation of contractile force, and intracellular Ca²⁺ handling. As a result, a combination of fibrin (4–6 mg/mL) and Matrigel (10%–40%) was able to generate a higher force compared to lower

concentrations of the same proteins and to the collagen-based hydrogels. These results support the use of fibrin and laminin-rich hydrogels (e.g. Matrigel) for muscle modeling. Moreover, by avoiding the artificial introduction of collagen in the 3D model, it is possible to use its quantification as a fibrotic indicator. The use of artificially manufactured scaffolds offers the great advantage of knowing the biochemical composition and controlling the material stiffness. This versatility retains great value to study the role played by tissue stiffness in fibrosis progression.⁹³

Such hydrogel/cell mixtures can be seeded manually or deposited by a bioprinter. The best choice should be made to meet the requirements of the specific application. While bioprinting offers a higher degree of seeding precision, which can be exploited to ensure muscle cell alignment and promote myotube fusion, it also imposes restrictions to the device geometry and hydrogel composition. For further discussion on this matter, we suggest referring to the review by Xiang et al.⁹⁴ As an alternative solution, decellularized muscle tissues were used as 3D naturally derived-scaffolds for the generation of muscle models. These materials offer the advantage of retaining the native muscle architecture and ultrastructure.⁹⁵ However, challenges on homogeneous and controlled recellularization are posed. Additionally, the composition and biophysical properties of decellularized scaffolds cannot be controlled as finely as with the artificially constructed scaffolds.

Regarding the interaction of muscle cells with other cell populations typical of the muscle microenvironment, multiple microscale, and meso-scale devices have been designed to provide compartmentalization and allow the co-culture of tissue constructs requiring different maturation time frames. This feature enables to maintain in the same system cells with different culture conditions, facilitating the study of heterotypic communications. For instance, microfluidic models were able to integrate neuromuscular junctions (NMJ) that showed active contraction induced by a neuronal spheroid or motor neurons (Figure 4c).^{83,96} However, so far just a few models have taken advantage of this opportunity. In addition, the impact of skeletal muscle models has been limited by the absence of a stromal component. Myofibers are naturally dispersed in a supporting ECM (i.e. endomysium) where other cell types reside and provide fundamental functions. These cell populations include vascular cells, resident macrophages, and fibroblasts. These stromal cells interact with muscle fibers preserving muscle homeostasis and ensuring muscle adaptation and regeneration (Figure 4d). However, a compromised balance among these cell populations is a typical hallmark of fibrosis. For instance, during muscle injuries, the involvement of macrophages initiates an inflammatory response,⁹⁷ which then proceeds with the recruitment of fibroblasts, FAPs, and other mesenchymal progenitors. During this process, the expression level of multiple cytokines is altered, including molecules known to be involved in muscular fibrosis such as CTGF,⁹⁸ TGF β ,⁹⁹ PDGF,¹⁰⁰ and VEGF.¹⁰¹ Similar phenomena are observable in the tumor microenvironment. In particular, tumor-associated macrophages, which represent a major part of cancer immune infiltrate, can promote EndoMT and compromise the functionality of blood vessels.¹⁰² Given that myopathies are associated with inflammatory processes and macrophage infiltration, it is likely that these cells promote EndoMT and fibrosis even in these contexts.

Molecular and cellular imbalance affects the vasculature which changes its tone, molecular profile, and permeability.¹⁰³ Ignoring this complexity limits the impact that 3D *in vitro* models may have in the identification of critical pathological mechanisms of fibrosis. Therefore, we believe that the next generation of muscle models should focus on including a more complex stromal compartment to fully take advantage of the possibilities offered by these systems to study relevant biological processes.

5 | 3D IN VITRO MODELS ARE ADVANCED SOLUTIONS FOR THE STUDY OF HETEROtypic CELL-CELL INTERACTIONS

Despite still being at its infancy, *in vitro* 3D muscle modeling has already demonstrated a great potential in recreating typical cell interactions occurring in the physiological and pathological muscle micro-environment. Indeed, accumulating evidence indicates a pivotal role of stromal interactions in the pathological onset and progression of muscle fibrosis.¹⁰⁴ A relevant example of the possibility to *in vitro* study muscle-stromal interactions was provided by Juhas and colleagues, who included macrophages in a rat muscle bundle achieving an improved regenerative capacity.⁸⁴ The authors attributed this effect to the capacity of macrophages to support satellite cell proliferation and differentiation to limit myofiber apoptosis and to dampen pro-inflammatory conditions (Figure 4d). Due to their ubiquitousness and involvement in muscular diseases, ECs have also been a target of tissue engineering, which has been aiming to introduce an organized capillary network in muscle models. The presence of a mutual positive influence between human muscle cells and ECs was confirmed in an *in vitro* study by the Chazaud lab. By seeding human umbilical vein endothelial cells (HUVECs) on Cytodex beads and then embedding them with myoblasts in a fibrin-based hydrogel, the group showed that ECs were capable of pro-myogenic properties by stimulating satellite cell migration, proliferation, and terminal differentiation. In addition, the presence of satellite cells within the gel increased the number of lumenized capillaries. Moreover, capillary elongation and lumenization increased with the maturation of muscle cells.¹⁰⁵ Apelin, Oncostatin, and Periostin appeared to be three key molecular effectors correlated to myogenesis and angiogenesis, as demonstrated by antibody-blocking of these three cytokines which resulted in the inhibition of the positive effects of the co-culture. It is important to notice that the Apelin gene, which is found overexpressed by ECs in the muscle co-culture, has been recently observed to be downregulated in human pulmonary artery ECs undergoing EndoMT.¹⁰⁶ It would be interesting to clarify whether Apelin expression is affected by a dystrophic or inflamed muscle to better understand its role in EndoMT.

In another study, Gholobova and colleagues set up a 3D *in vitro* co-culture of human myoblasts and HUVECs.¹⁰⁷ Myoblasts were seeded in a fibrin hydrogel forming a 3D construct, while HUVECs were added at two different timepoints: contemporarily with the myoblast seeding or after 1 week of myoblast differentiation. The second approach resulted in a better vascular interconnection, lower

vascular degradation, and higher myotube formation. While this result demonstrates a positive interaction between differentiated myotubes and HUVECs, the level of myotube maturation obtained was lower when compared to the muscle construct cultured without ECs for the same two-week interval. This is probably caused by the necessary use of a serum-rich medium to culture ECs, while myogenesis is promoted by low-serum medium. Interestingly, Collagen IV, which is overexpressed by microvascular ECs during EndoMT, has been deposited by ECs in these models, proving that matrix protein release could be investigated in these setups. A two-step seeding approach was also used by Osaki and colleagues. The authors developed a 3D model to demonstrate the mutual cross-talk between the endothelium and muscle cells (Figure 5a).¹⁰⁸ First, C2C12/hydrogel mixtures were seeded in a cylindrical cavity created in a sacrificial gelatin template and were left differentiated for 7 days. Then, two parallel endothelialized cylindrical cavities were seeded on both sides of the muscle construct. This work showed that the angiogenesis of HUVECs increased as a result of the co-culture with C2C12 murine myoblasts. This effect seems to be related to the paracrine bi-directional signaling based on Neuregulin-1 (NRG-1) secreted by ECs and Angiopoietin-1 production by muscle cells in 5 days of co-culture. The study revealed an increase in Tie2 expression prompted by muscle co-culture. This gene is typically downregulated during EndoMT. Tie2 is a receptor tyrosine kinase that has been used for lineage-tracing studies to demonstrate the involvement of ECs in renal fibrosis.^{110,111} It has also been shown to have anti-inflammatory effects, suppressing VEGF and TNF expression, which are also targeted by the two drugs currently approved for the treatment of pulmonary fibrosis, Nintedanib and Pirfenidone.¹¹² Investigating the effects of muscle co-culture on Tie2 expression, possibly comparing healthy and dystrophic muscle constructs, or increasing Tie2 expression could be a promising approach to identifying new therapeutic strategies. Indeed, clarifying the recruitment of ECs during fibrosis is essential in finding a way to contrast pro-fibrotic stimuli. In a different setup, Kim and colleagues showed that starting the co-culture of HUVECs and human myoblasts after 2 days from the muscle seeding allowed to obtain a greater muscle contraction force, myotube fusion, and vascular network development than with the seeding of HUVECs performed at day 5.¹¹³ While these results seem to be contradictory with the previous study, it should be considered that the design is different. Indeed, two concentric cylinders were created seeding muscle cells on the outer side and HUVECs in the inner one. Taking advantage of this configuration, the authors were able to flow endothelial basal medium in the inner cylinder (the one in contact with ECs), while muscle differentiation medium was provided on the external environment, surrounding the construct. This solution allowed to provide the appropriate medium to muscle cells and ECs at the same time.

Taken together, these results demonstrate the mutual positive effects of the co-culture of muscle cells and ECs on myogenesis and angiogenesis. Additionally, it has been proven that cytokines and growth factors involved in EndoMT are impacted by 3D co-culture setups. This suggests that such tools might be the ideal solution to decipher how the fibrotic recruitment of ECs takes place in diseased muscles.

The possibility to customize the architecture of the system is another relevant characteristic of 3D *in vitro* models. This architecture can be

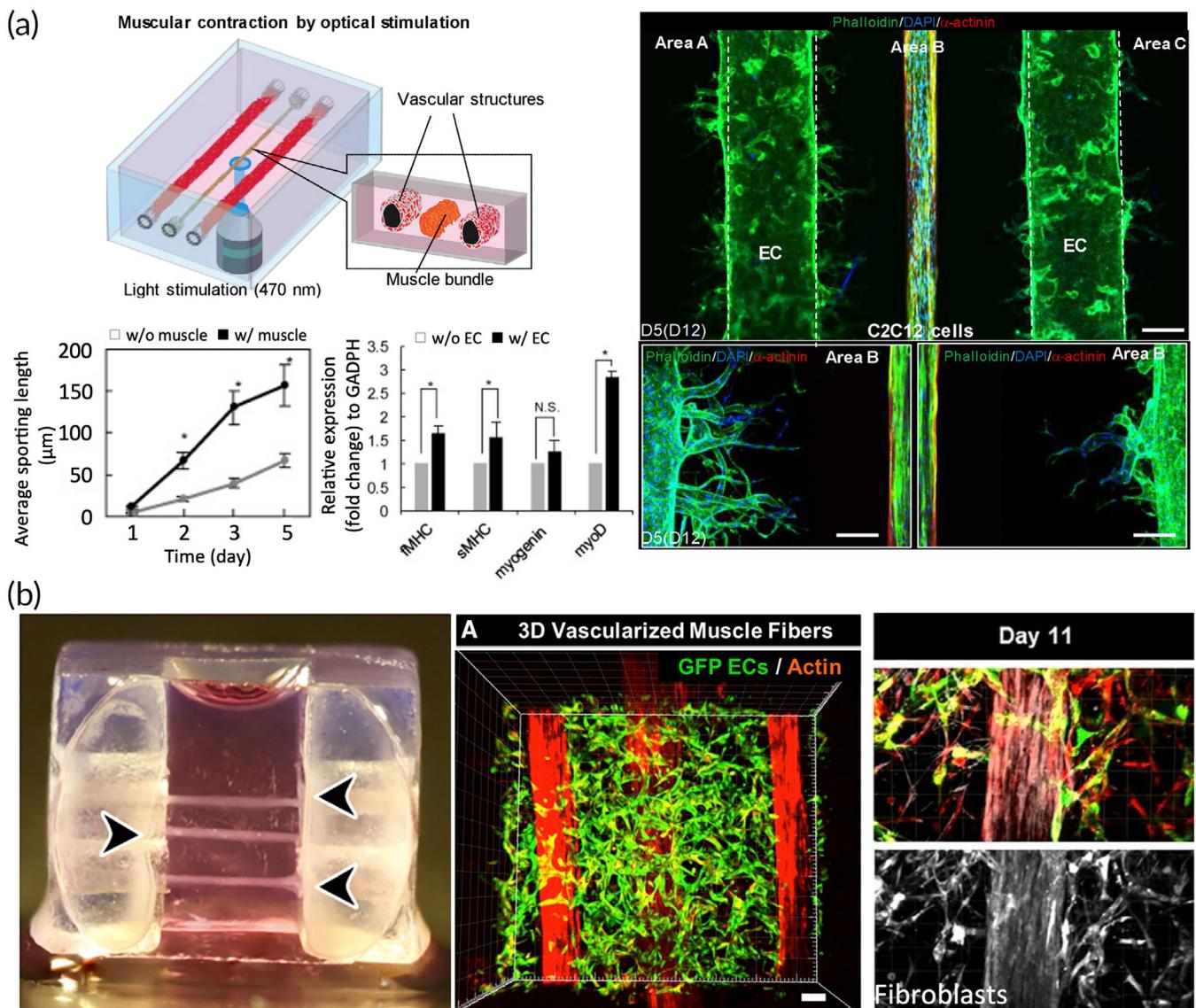


FIGURE 5 Overview of 3D *in vitro* models to analyze muscle-endothelium interactions. (a) Positive mutual cross-talk between optogenetically engineered mouse skeletal muscles and HUVECs. Scale bar = 100 μm .¹⁰⁸ (b) 3D vascularized human muscle microenvironment embedding three non-planar muscle fibers, ECs and muscle fibroblasts to study endothelial plasticity and fibrosis. Scale bar = 100 μm .¹⁰⁹

adapted to perform specific observations and to arrange cell populations in particular settings. This feature can be especially exploited to culture more than one cell population. A successful example is the concentric design used by the Asada group to develop a tri-culture *in vitro* model including human myotubes, ECs, and fibroblasts.¹¹⁴ Using a system of coaxial needles, the authors were able to culture three concentric cylinders formed by human myoblasts (outer layer), fibroblasts (middle layer), and ECs (inner layer). In agreement with previous studies, the authors confirmed an increased myotube thickness and contractile force promoted by the co-culture. Moreover, it was demonstrated that the muscle functionality improved according to the distribution of fibroblasts. Indeed, when fibroblasts were localized in the inter-layer between muscle cells and ECs, a better vascular sprouting and a higher contractile force were registered in comparison to the general distribution of fibroblasts in the same layer occupied by muscle cells. This result demonstrates the

importance of spatial cell distribution when studying cellular crosstalk, an aspect majorly exploitable thanks to the compartmentalization allowed by 3D *in vitro* models. For example, it might be relevant to try to identify a spatial pattern in the fibrotic recruitment of ECs in relation with their proximity to another cell population of the muscle microenvironment.

Overall, these models represent all the successful attempts achieved so far of implementing a stromal muscle compartment together with muscle fibers/bundles (Table 1). These models successfully showed how this approach has provided significant insights to understand cell-cell interactions within muscle tissue. Despite their success, these models have not yet been used to study the pathological evolution of the vasculature in muscular diseases, such as dystrophies. As previously discussed, ECs play a crucial role in tissue maturation and maintenance of homeostasis, making muscle models with ECs ideal for understanding their role in different pathological

TABLE 1 Summary of the most advanced 3D in vitro models of skeletal muscle integrating ECs.

Authors	Cell populations	Device	Outcomes
Juhas et al. (2018)	<ul style="list-style-type: none"> Rat myoblasts Bone marrow-derived macrophages 	3D fibrin and Matrigel-based cylindrical hydrogel with aligned myobundles and interspersed macrophages	Increased muscle regenerative capacities following cell damage
Latroche et al. (2017)	<ul style="list-style-type: none"> Human primary myoblasts Human Umbilical Vein Endothelial Cells (HUVECs) 	HUVECs seeded on Cytodex beads and cultured with myoblasts on a fibrin-based hydrogel	The co-culture improved myogenesis and angiogenesis mainly attributed to a signaling based on Apelin, Oncostatin and Periostin.
Gholobova et al. (2020)	<ul style="list-style-type: none"> Human primary myoblasts Human Umbilical Vein Endothelial Cells (HUVECs) 	Fibrin-based cylindrical hydrogel with aligned myobundles. HUVECs seeded interspersed in the bundle or around it	The muscle maturation was superior in the two-step seeding approach, where HUVECs were seeded after myotube differentiation
Osaki et al. (2018)	<ul style="list-style-type: none"> C2C12 murine myoblasts Human Umbilical Vein Endothelial Cells (HUVECs) 	Fibrin-based bundle of C2C12 created in close proximity of two HUVEC-endothelialized cylindrical cavities	Both muscle differentiation and HUVEC angiogenesis benefited from the co-culture, in a paracrine looping-signaling based on Neuregulin-1 and Angiopoietin-1
Bersini et al. (2018)	<ul style="list-style-type: none"> Human primary myoblasts Human endothelial cells Human primary muscle fibroblasts Human bone-marrow derived mesenchymal stem cells 	Microscale cylindrical muscle bundles surrounded by a fibrin-based hydrogel with ECs, fibroblasts and mesenchymal cells	Muscle-specific phenotype of ECs was identified (expression of TSPAN7, PPAR γ). Recruitment of fibroblasts regulated by paracrine signaling
Kim et al. (2022)	<ul style="list-style-type: none"> Human primary myoblasts Human Umbilical Vein Endothelial Cells (HUVECs) 	Two concentric layers with myoblasts in the outer side and HUVECs in the inner one	Longer co-culture improved muscle contraction, fusion, and vascular sprouting
Kim et al. (2022)	<ul style="list-style-type: none"> Human primary myoblasts Human Umbilical Vein Endothelial Cells (HUVECs) Human primary fibroblasts 	Three concentric layers with myoblasts in the outer side, fibroblasts in the middle layer and HUVECs in the inner one	Co-culture improved muscle functionality and differentiation. Fibroblast spatial localization impacted muscle functionality

Note: These models demonstrated their efficacy to evaluate the beneficial mutual interaction between different healthy cell types.

conditions, including inflammation or fibrosis. Moreover, an additional progress would derive from taking into account the tissue-specificity of ECs. Indeed, ECs from different tissues differ in terms of transcriptome, proteome, metabolic profile, and functionality.^{115,116} The EC tissue-specificity might be relevant when studying certain diseases and could also increase the reliability of in vitro models. Unfortunately, very few studies have dealt with EC tissue-specificity and plasticity, even though some examples have recently paved the way to implement this aspect within 3D in vitro models. The adaptation capacity of ECs was shown in a 3D triculture model of human muscle with ECs and fibroblasts (Figure 5b), whereby ECs acquired muscular phenotypic signatures when co-cultured with muscle cells.^{109,117} Since changes in EC migration are expected with the mesenchymal transition, it would be interesting to investigate them in a similar setup when a pro-fibrotic context is created. Another work focused on endothelial tissue specificity, although in a different context, was performed by Osaki and colleagues. The authors supplemented ECs with retinoic acid to obtain an endothelial barrier more similar to the blood brain barrier, useful to study drug penetration.⁹⁶ As far as we know, the usage of 3D muscle models to investigate EndoMT has not yet been exploited, while a few successful works focused on EndoMT in other tissues. Kramer and co-workers developed a microfluidic-based system embedding human microvascular endothelial cells to investigate if EndoMT exerts a role since the early stages of systemic sclerosis before the establishment of full-blown fibrosis.^{118,119} The

group observed vascular alterations upon exposure to pro-inflammatory and pro-fibrotic cytokines, such as TNF α and TGF β , mimicking pathological environments. Also, the negative effects of the two cytokines were successfully inhibited via the addition of TNF α and TGF β inhibitors showing the potential of the model as a drug-testing tool. Another recent work by Whiteford and colleagues reproduced EndoMT in a liver-on-a-chip.¹²⁰ The group co-cultured liver sinusoidal endothelial cells and hepatocytes in a microfluidic chip and observed an increased migratory behavior of ECs in a fibrotic context. Finally, Bramsen and colleagues demonstrated the EndoMT-promoting effect that glycosaminoglycans have on porcine aortic valve ECs in a 3D setup.¹²¹

Despite the importance of EndoMT in the development of fibrosis in various muscular pathologies, there is currently a lack of skeletal muscle 3D in vitro models that can replicate this process. However, given the promising results achieved in other tissues through the study of EndoMT, we believe that the time has come to develop new 3D muscle models that can help us to better understand the mechanisms behind EndoMT during fibrosis.

6 | CONCLUSIONS AND PERSPECTIVES

Over the last decade, relevant progress has been made in setting up advanced 3D models of human skeletal muscle. The convergence of

biological and engineering technologies allows to culture human muscle cells (either induced pluripotent stem cell-derived⁸⁸ or primary cells) to obtain aligned bundles of differentiated muscle fibers, showing sarcomeric organization and ability to contract upon chemical and electrical stimulation. While the culture conditions to properly expand and mature primary myoblasts have been defined, an aspect that should be considered is the dimension of the model. Successful results have been achieved with both micro¹²² and meso-scale models,¹²³ however the design should be carefully defined according to the requirements of the final analyses, such as number of cells and accessibility of the seeded chamber for cell retrieval.

The EC source is another important factor to take into consideration when developing an in vitro muscle model. HUVECs have been widely used for their high proliferation and their capacity of forming vessel-like structures. However, their relevance can be questioned. Primary ECs derived from the tissue object of the study could be a more representative cell choice than umbilical vein ECs. Indeed, ECs can be differentiated by tissue-specific gene expression profiles. In the context of skeletal muscle ECs, this was demonstrated using an in vitro 3D construct embedding primary human muscle fibers and primary human ECs. ECs acquired a muscle-specific expression of specific genes, such as *TSPAN7* and *PPARG*. Furthermore, the presence of ECs upregulated the expression of Desmin in the myofibers.¹⁰⁹

To further increase the physiological relevance, multiple cellular populations can be introduced and co-cultured in the same system. Several co-culture studies demonstrated the feasibility and potential of multicellular models with ECs,¹⁰⁹ fibroblasts,¹¹³ macrophages,⁸⁴ and neurons.¹²⁴ 3D models based on microfluidic principles are the best option to create the compartmentalization of different culture media and, at the same time, to allow cellular crosstalk and migration. The few studies that have integrated the vascular component in muscle in vitro 3D systems successfully observed the positive mutual influence between the two cell populations, improving myogenesis and angiogenesis. In this context, we think that 3D in vitro muscle models could be exploited to study EndoMT, one of the key processes contributing to fibrosis. To our knowledge, no study has addressed the role of EndoMT on human muscular fibrosis, despite it has been demonstrated to be relevant in the pathogenesis of cardiac, pulmonary, cerebral, and renal fibrosis.^{30,111,125-127} Many aspects related to this process are still not clear and, most importantly, it is not known how to stop or revert it. For example, it is still unknown how EndoMT relates with muscle tissue inflammation and how other cell populations of the fibrotic microenvironment influence it. A study on murine models showed the presence of EndoMT in skeletal muscle fibrosis and the importance of M1 macrophages for neo-angiogenesis in muscle regeneration.^{128,129} However, the authors associated this effect to resident vascular progenitors and not to differentiated ECs.¹³⁰ This consideration might explain the apparent contradiction with another study based on murine models where M1 macrophages were identified as promoters of cardiac fibrosis by inducing EndoMT via a SMAD2-dependent paracrine communication.¹³¹ Introducing human ECs and macrophages into a 3D in vitro model, along with differentiated myotubes, represents a promising approach to elucidate the relationship between endothelium and macrophages, one of the main infiltrating cells. By comparing the

effects of M1 and M2 macrophages, this model could help to clarify whether ECs are primarily affected during the early inflammatory response or the later stages of regeneration. The same approach could also be used to investigate the interactions between ECs and other cell types, such as FAPs and pericytes. Given the presence of multiple cell populations, single-cell RNA sequencing may be the most suitable analytical method, as it enables the effective identification of transitioning phenotypes, such as ECs undergoing EndoMT. Taking advantage of microfluidic principles, 3D models also offer the opportunity to analyze the perfusability of the vascular network, which can serve as a functional readout of the EC state. Moreover, these models allow to tune the flow rate and shear stress within the vascular networks. This feature would allow us to study the impact of shear stress on EndoMT, since abnormally low shear stress values were shown to promote EndoMT, although the underlying mechanisms are not fully understood. Finally, molecular compounds can be screened to assess whether they exert a therapeutic effect preventing or reverting EndoMT. Currently, Nintedanib¹³² and Pirfenidone¹³³ are the only two drugs approved for the specific treatment of idiopathic pulmonary fibrosis. Since these drugs act on a wide spectrum of growth factor receptors that are associated with EndoMT, it would be particularly interesting to investigate if they have a beneficial effect on vascularized muscle models showing signs of this mesenchymal transition.

In this review, the most relevant solutions to model in vitro 3D vascularized skeletal muscle were reported. There is no exclusive best design, these systems should be optimized according to the specific experimental requirements. Indeed, the versatility of these systems is one of the most important benefits they bring when studying a specific biological question.

In conclusion, 3D models offer several advantages over traditional 2D cultures, including the ability to mimic the complex cell-cell and cell-matrix interactions observed in vivo. These models also allow for the integration of multiple cell types and the incorporation of mechanical and biochemical cues that can influence cell behavior. We believe that this approach is particularly promising to investigate pathological mechanisms like EndoMT, which might be the result of different contributing factors. These advanced models offer superior experimental possibilities like multicellular cultures and functional readouts, improving the relevance of in vitro drug discovery. Hence, 3D muscle models could greatly accelerate the identification and development of effective therapeutics targeting EndoMT and tissue fibrosis.

AUTHOR CONTRIBUTIONS

Riccardo Francescato: Conceptualization (equal); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Matteo Moretti:** Funding acquisition (equal); supervision (equal); writing – review and editing (equal). **Simone Bersini:** Conceptualization (equal); funding acquisition (equal); supervision (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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