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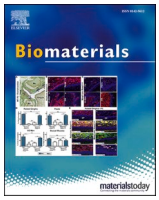

Decellularized skeletal muscle: A versatile biomaterial in tissue engineering and regenerative medicine

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ABSTRACT

A wide range of synthetic and natural biomaterials is available for skeletal muscle tissue engineering. One class of natural biomaterials consists of the extracellular matrix (ECM) from donor skeletal muscle. To obtain this ECM, the cellular compartment must be completely removed while retaining the native composition and ultrastructure of the tissue as much as possible. In this review, the progress and challenges in the field of skeletal muscle decellularization are discussed by reviewing the different decellularization methods available and by highlighting the different applications of the scaffolds. Decellularized skeletal muscle has mainly been studied in the context of regeneration with a focus on its tissue-specific morphological features as well as biochemical cues to stimulate muscle regeneration. However, in this review, the potential applications of decellularized skeletal muscle are expanded beyond the regenerative setting to demonstrate its versatility as a biomaterial. Acellular matrices are discussed as a platform to study cell-matrix interactions and drug screening. Decellularized skeletal muscle ECM can also be further processed to re-engineer its structure. An overview is presented of materials processed from decellularized skeletal muscle, ranging from injectable hydrogels, bioinks for 3D bioprinting, electrospun nanofibers to coatings for cell culture.

1. Introduction

Skeletal muscle comprises over 40% of the human body and is of high clinical importance due to its role in metabolism and movement [1]. It possesses a robust innate regenerative ability, but this regenerative response fails when a large volume of muscle is lost as a result of trauma or surgical tumor resections. The default outcome of this volumetric muscle loss (VML) is scar tissue formation compromising muscle functionality. The debilitating impact of VML on patients' lives requires the need for adequate treatment options for the repair, regeneration, or replacement of skeletal muscle tissue.

The current gold standard for the treatment of VML is autologous tissue transfer. However, this is associated with major drawbacks such as donor site morbidity, prolonged operation times and the risk of graft failure. Thus, VML represents an active field of research. Regenerative medicine attempts to provide alternative strategies for this condition and can generally be subdivided into two approaches. The first approach aims at augmenting the innate response to skeletal muscle injury, mainly through cell transplantation [2]. However, limited clinical successes have shifted the attention to the second approach, tissue engineering, in which physiologically functional tissues or organs are created in a laboratory setting. A wide range of tissue engineering strategies exist, reviewed by Ostrovidov et al. [3], and typically combines cells, scaffold materials and bioactive molecules.

A scaffold is a temporary structure that guides the applied cells and supports their 3D growth during the tissue developmental stage. A classification can be roughly made according to the composition, being either synthetic, natural or a combination of both. At present, a myriad of biomaterials for tissue engineering have been developed, using the structural, mechanical and biochemical information present in the native extracellular matrix (ECM) as a blueprint. However, up until now, no natural or synthetic material can fully replicate all features of native ECM. In this regard, decellularization as a technique to obtain native ECM is attracting more and more attention and is currently being exploited in both basic and translational research. In addition, the unique features of these natural scaffolds could also be interesting for downstream applications such as disease modeling or drug testing.

In this context, this review aims to highlight the versatility of

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decellularized skeletal muscle. After a short description of the ordered structure of skeletal muscle with its most common components, its regenerative capacity will be briefly reviewed. Then, different decellularization methods available to preserve the ECM will be discussed and the use of these matrices for regenerative purposes will be highlighted. Finally, an overview of the use of acellular matrices beyond the regenerative setting will be given to stress the broad relevance of it for different applications.

2. Skeletal muscle structure and function

Skeletal muscle is characterized by a defined structure which is inherently correlated to its function (Fig. 1) [4]. An individual muscle is surrounded by a specialized connective tissue, called the epimysium. Within a muscle, myofibers are arranged into bundles of skeletal muscle fibers, called fascicles and each fascicle is surrounded by another layer of connective tissue, named the perimysium. Each myofiber contains a highly organized cytoskeleton composed of aligned myofibrils and is surrounded by a basal lamina, called the endomysium, comprising the basement membrane. The basement membrane is a layer of ECM coating the skeletal muscle fibers [5]. These myofibers are multinucleated syncytia formed during development by fusion of mononucleated precursor cells, the myoblasts. This results in hundreds of nuclei which are located in the periphery of the muscle fiber close to the plasma membrane, whereas most of the cytoplasm is occupied by the contractile apparatus composed of myofibrils.

The physiological relevance of the connective tissue extends beyond simple structural integrity. Growth factors are stored within the connective tissue and affect cell migration, proliferation and differentiation. Moreover, these processes are further influenced by the mechanical properties of the connective tissue through integrin-mediated interactions with cells [6]. The mechanical properties depend on the protein composition of the ECM and vary substantially among different tissues. Overall, ECM constituents can be roughly divided into either fibrous-forming proteins, such as collagen or non-fibrillar proteins such as fibronectin, laminin and proteoglycans or glycosaminoglycans (GAGs) which are acidic and hydrated molecules (Table 1) [7].

In muscle, collagen is the main structural component of the ECM, representing 1–10% of the skeletal muscle dry weight [9]. It can be further subdivided into one of the 28 different members of the collagen superfamily, with the fibril-forming types I and III being the most abundant [10]. Collagen I can be found throughout all three layers of the

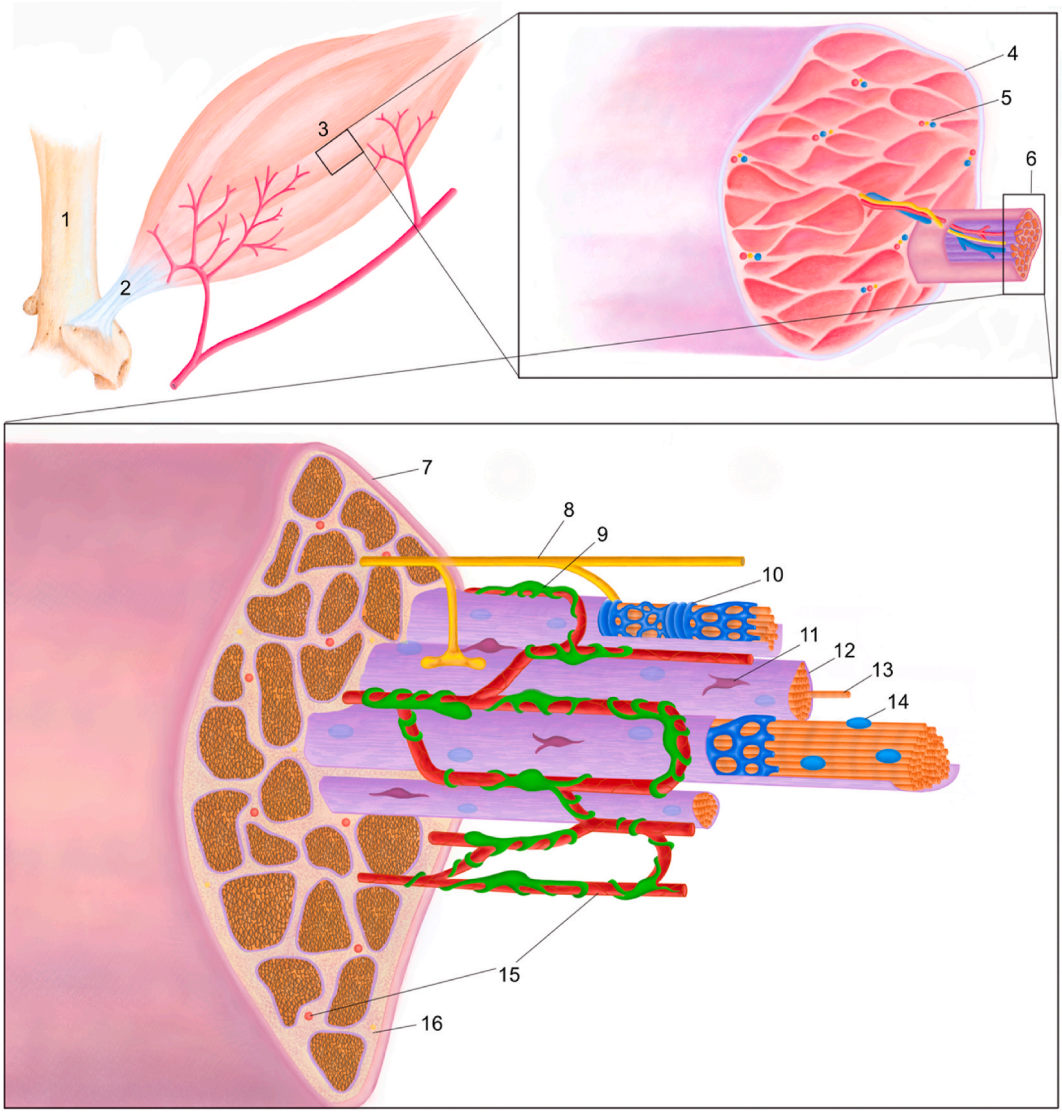


Fig. 1. Skeletal muscle structure. 1. Bone, 2. Tendon, 3. Muscle, 4. Epimysium, 5. Artery (red), vein (blue), nerve (yellow), 6. Fascicle, 7. Perimysium, 8. Nerve, 9. Pericyte, 10. Sarcoplasmic reticulum, 11. Satellite cell, 12. Sarcolemma, 13. Myofibril, 14. Muscle cell nucleus, 15. Capillary, 16. Endomysium [8]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Major ECM components of skeletal muscle.

Type	Structure	Function	Main location
Collagens			
Collagen I	Parallel fibers	Provide rigidity and tensile strength	Perimysium
Collagen III	Parallel fibers	Provide elasticity	Epi- and endomysium
Collagen IV	Network-forming fibers	Mechanical support	Basement membrane
Collagen V	Parallel fibers	Structural support for collagen I and III	Epi-, peri-and endomysium
Collagen VI	Network-forming fibers	Mechanical support; Maintenance of SC niche	Basement membrane
Glycoproteins			
Fibronectin	Globular	Binding to integrins; Lateral force transmission	Basement membrane
Laminin	Globular	Binding to integrins; Lateral force transmission	Basement membrane
Proteoglycans			
Syndecan	Heparan sulfate PG	Binding of growth factors; Maintenance of SC niche	Basement membrane
Perlecan	Heparan sulfate PG	Binding of growth factors; Maintenance of SC niche	Basement membrane
Agrin	Heparan sulfate PG	Binding of growth factors; Clustering of ACh receptors at the NMJ	Basement membrane
Decorin	Small leucine-rich PG	Regulator of collagen I fibrillogenesis	Perimysium
Biglycan	Small leucine-rich PG	Regulator of collagen I fibrillogenesis	Perimysium

ACh = acetyl choline; NMJ = neuromuscular junction; PG = proteoglycan; SC = satellite cell.

skeletal muscle connective tissue but is most predominant in the perimysium. Type I arranges itself as parallel fibers and provides the muscle with a certain degree of rigidity and tensile strength. Collagen type III is solely dispersed between the endomysium and epimysium where the flexible helical structure of the fibers gives the layers its elasticity. Other collagens known to occur in a minor quantity, are collagen type IV, V, VI, XII, XIII, XIV, XV XVIII and XXII [11].

Collagen type VI is the integral part of the basement membrane of the muscle endomysium and has been shown to influence satellite cell (SC) self-renewal together with fibronectin, another structural glycoprotein present in skeletal muscle ECM [12,13]. In addition, the proteoglycans syndecan 3, syndecan 4, perlecan and decorin have been identified as SC niche constituents as well. Many of the proteoglycans present in the skeletal muscle ECM belong to the family of small leucine-rich proteoglycans with the majority having chondroitin sulfate and dermatan sulfate GAG side chains. Proteoglycans with a heparan sulfate GAG, like collagen XVIII, perlecan and agrin, make up about 30% of the proteoglycans in skeletal muscle ECM. These heparan sulfate proteoglycans also execute a bridging function connecting the networks of collagen and laminin polymers in the muscle basal lamina [14]. Laminin represents, next to collagens and fibronectin, another structural glycoprotein present in skeletal muscle ECM with Laminin-211 being the predominant isoform. Laminin forms polymers of a heterotrimeric complex comprised of an α, β and γ chain and serves as a ligand for the dystrophin-associated glycoprotein complex and the α7β1 integrin. These connect laminin in the ECM with actin in skeletal muscle fibers and thus facilitate transmission of forces across the membrane [15]. Together, these glycoproteins stabilize skeletal muscle cells and help maintain the organized structure by providing structural support.

Skeletal muscle ECM is maintained in a dynamic way, by degradation enzymes and cells that can secrete ECM constituents. Cells responsible for the synthesis of the adult muscle ECM components are predominantly fibroblasts. Other mononuclear cells, like SCs, and even multinucleated myofibers have been shown to secrete ECM components and ECM-degrading enzymes as well. As a result, muscle cells also participate in ECM remodeling, especially during muscle injury. For example, SCs have been shown to secrete matrix metalloproteinases in culture [16], which is thought to aid them in their migration towards an injury site for initiating the repair process [17]. Furthermore, the ECM is crucial for establishing the SC niche and proper muscle regeneration depends on the cross-talk between these SCs and their microenvironment [18].

3. Skeletal muscle regeneration

Until the middle of the 19th century, it was believed that muscles did not regenerate. Nowadays, the inherent regenerative capacity of skeletal muscle is well-known [19]. It is a highly orchestrated process which can be divided into five interrelated and time-dependent phases (Fig. 2). Muscle necrosis will elicit an initial inflammatory response, which continues on a regenerative course [19]. Next, a remodeling phase will take place and finally, after a maturation phase, full restoration of the muscle can be achieved. The accompanied cellular dynamics involve both tissue-resident cells and cells infiltrating from the circulation, making the process of regeneration complex with overlapping cell-cell interactions [20].

3.1. Degenerative and inflammatory phase

Muscle injury resulting in damage of the sarcolemma triggers myofiber necrosis and increased myofiber permeability [21]. Depending on the extent of the injury, breakdown of the basement membrane will occur as well. Mononucleated cells residing in the muscle tissue are activated during the early phase of injury and provide chemotactic signals to other circulating inflammatory cells [22]. Within hours after injury, neutrophils are attracted from the vasculature and contribute to the phagocytosis of injured myofibers. In addition, these neutrophils set the stage for repair of the tissue damage by macrophages [23].

The macrophages that are recruited to the injured area can be polarized into two distinct phenotypes [24]. Initially, the pro-inflammatory M1 macrophages will remove tissue debris by producing pro-inflammatory cytokines and reactive oxygen species. Their cell numbers peak around 1–2 days after injury after which they decline and M2 macrophages become the predominant subtype. This transition coincides with the progression from inflammation to regeneration.

3.2. Regenerative phase

During the regenerative phase, a central role is played by the muscle stem cells, known as satellite cells (SCs). In the steady-state, SCs are quiescent and located between the basal lamina and sarcolemma surrounding each myofiber [25]. However, in response to the cytokines and growth factors released at the site of injury during the inflammatory phase, they are activated and re-enter the cell cycle [24]. Activated SCs express myoblast determination protein 1 (MyoD) and proliferate as myoblasts before differentiating and fusing to repair damaged muscles. A small portion of the SCs, however, will retain a quiescent state to replenish the stem cell pool.

The proliferating myoblasts can migrate bi-directionally to the regeneration site by using ECM remnants from injured skeletal muscle fibers [26]. This relies on dynamic cytoskeletal rearrangements [27,28] and gradients of chemoattractant factors released by the damaged myofibers and the recruited macrophages [29]. The myogenesis during the regenerative phase is also supported by other immune cells such as regulatory T (T_{reg}) cells and eosinophils. The latter secrete interleukin (IL)-4 which targets fibroadipogenic progenitors (FAPs). In return, the FAPs regulate T_{reg} cell dynamics by secretion of IL-33 [30]. Both the FAPs and the T_{reg} cells influence the SCs by enhancing their myogenic

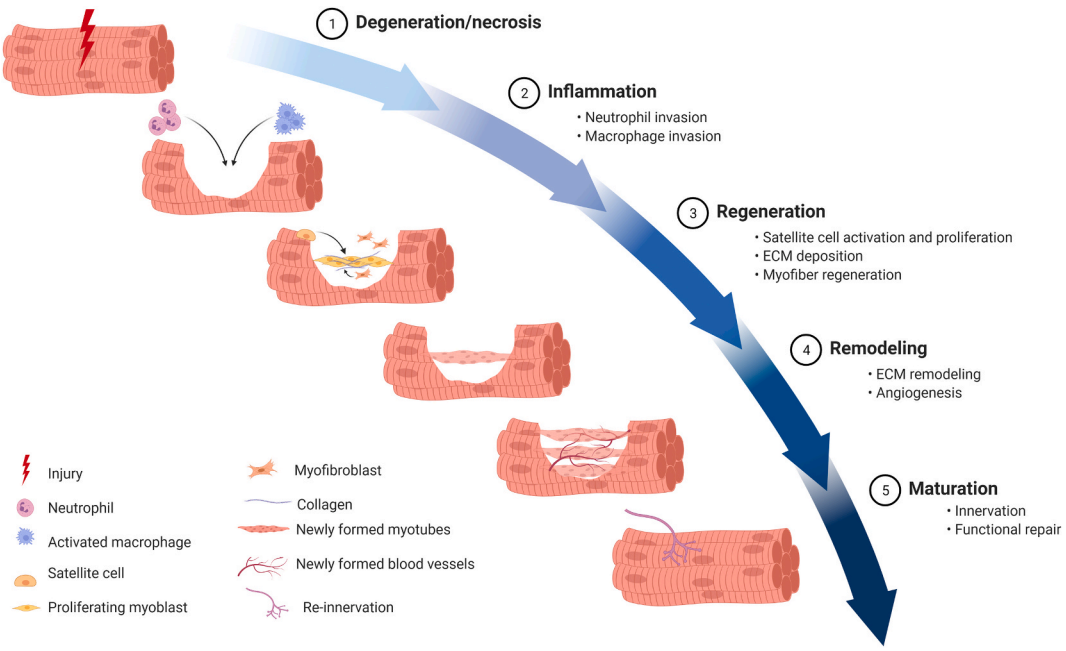


Fig. 2. Different phases of skeletal muscle regeneration.

differentiation. Myofibroblasts also play a role during tissue remodeling through their contractile properties [31,32]. Myofibroblasts are ECM-producing cells that deposit collagen during the inflammatory phase. This supports the transition to the regenerative phase by providing a scaffold for newly formed, regenerating muscle fibers [33].

3.3. *Remodeling and maturation phase*

If regeneration goes well, a transition to a remodeling phase occurs in which the temporary ECM, deposited by the infiltrated myofibroblasts, gets remodeled [34]. This coincides with capillary sprouting through the process of angiogenesis which is initiated by localized breakdown of the basement membrane and the interstitial ECM. In this way, contact between pericytes and endothelial cells (ECs) is broken and ECM-sequestered growth factors are released [35]. Finally, the proliferation and migration of capillary ECs into the surrounding tissue and formation of new vessels is facilitated. Vascular ECs gradually increase following injury, peak around 7 days post injury, and secrete several factors including angiopoietin-1 [36], insulin-like growth factor-1 [37], hepatocyte growth factor [38] and vascular endothelial growth factor (VEGF) [35]. These secreted factors further support the ongoing regenerative process by influencing both SC proliferation and differentiation.

Finally, functional recovery requires re-establishment of any lost motor innervation to the regenerated fibers as well as formation of new neuromuscular junctions. This occurs when regenerated myofibers are terminally differentiated, generally a few weeks post-injury [39]. However, the speed of reinnervation highly depends on the extent of myofiber damage. In the case of severe injury, reinnervation of a muscle is less effective and takes longer since the basal lamina, which serves as a scaffold, is disrupted. Subsequently, nerve activity further promotes the formation of a mature contractile apparatus of the regenerated muscle fibers. This is characterized by the expression of adult fast and slow myosin heavy chain isoforms and the movement of myonuclei to the periphery of the muscle fiber. At the end of muscle regeneration, regenerated muscle fibers are morphologically and functionally indistinguishable from undamaged muscles.

Since the ECM plays such an important role during several phases of muscle regeneration, the use of ECM without cells is of great interest to support regeneration in cases where the original structure has suffered too much damage. Several decellularization methods are available to obtain an acellular ECM and are discussed in the next section.

4. Decellularization methods

Efficient decellularization is usually obtained by combining different chemical, enzymatic and/or physical agents [40]. Each has a specific mode of action, as depicted in Fig. 3. Thin laminated tissues such as small intestinal submucosa (SIS) and urinary bladder matrix (UBM) can easily be decellularized by freezing and thawing followed by a brief exposure to detergents [40]. The thickness and complexity of skeletal muscle, however, warrants a more advanced decellularization process to obtain complete removal of the cellular content while preserving the hierarchical structure of the tissue. This has resulted in the development of different decellularization protocols throughout the years, as summarized in Table 2. Rodents have been the main animal model to study the impact of decellularizing agents on muscle tissue, although some studies also reported on canine or porcine tissues and more recently, results are even becoming available on human tissues. On a macroscopic level, decellularization results in a change of tissue color to white and the tissue becomes more transparent (Fig. 4). Incomplete decellularization is macroscopically visible when an area of the tissue remains pinkish.

One of the most frequently used processing methods for skeletal muscle was established at the University of Padua, combining the detergent sodium deoxycholate (SDC) and the enzyme deoxyribonuclease I (DNase I) [45–47,49,50,59,70]. The method has been demonstrated to be successful in decellularizing diaphragm and abdominal wall muscle tissue in both mice and rats. However, in comparative studies of Naik et al. it appeared that these results could not be translated to human tissues [68,69]. Upon treatment of the flexor digitorum superficialis, the zygomaticus major as well as the masseter muscle with two cycles of SDC and DNase I, DAPI staining still revealed remnants of nuclear material in the tissue sections.

Interestingly, the same studies demonstrated that two cycles of 4 h with 2% sodium dodecyl sulfate (SDS) significantly reduced the DNA content and no nuclear material could be seen with DAPI staining, while essential ECM components were preserved. These results are somehow surprising, as SDS is described as a denaturing detergent, causing disruption of the tissue ultrastructure, removal of GAGs and damage to

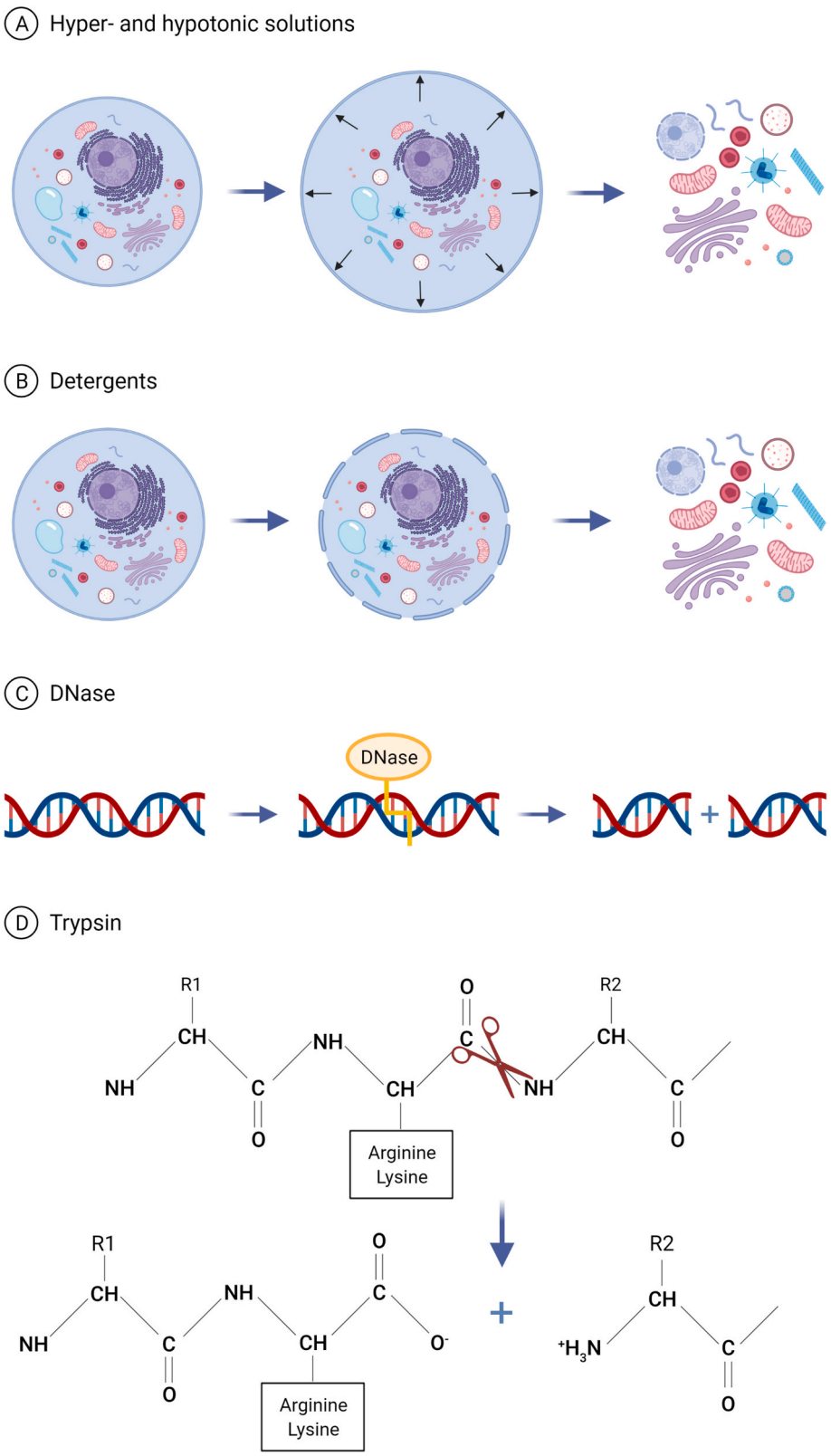


Fig. 3. Mechanism of action of commonly used decellularizing agents. A) Hypertonic and hypotonic solutions cause an osmotic shock, leading to swelling of the cells, rupture of the cell membrane and release of the cell content. B) Detergents such as SDC, SDS and Triton X-100 permeabilize the cell membrane which results in release of the cell content. C) DNase enhances the hydrolysis of deoxyribonucleotide chains. D) Trypsin cleaves peptide bonds on the C-side of arginine and lysine.

Table 2
Overview of immersion decellularization methods for skeletal muscle.

Species	Muscle origin	Decellularizing agents	Recellularization	Reference
Mouse	Extensor digitorum longus	4.2% SDC for 3 days 1% SDS for 2 days 3% Triton X-100 for 2 days 1% SDS for 2 days	C2C12 myoblasts	Borschel, 2004 [41]
Mouse	Tibialis anterior	Latrunculin B for 2 h High ionic strength salt solutions DNase I for 2 h	C2C12 myoblasts	Gillies, 2011 [42]
Mouse	Tibialis anterior	1% SDS for 2 days	N/A	Perniconi, 2011 [43]
Mouse	Latissimus dorsi	0.1% trypsin/EDTA for 24 h 1% Triton X-100 for 7 or 14 days 0.1% aprotinin DNase/RNase for 3 h	N/A	Lin, 2014 [44]
Mouse	Diaphragm	4% SDC for 4 h DNase I for 3 h	N/A	Piccoli, 2016 [45] Alvarez Fallas, 2018 [46] Trevisan, 2019 [47]
Mouse	Tibialis anterior	1% SDS for 3 days	Muscle stem cells Hematopoietic cells Endothelial cells Fibro-adipogenic progenitors Fibroblast-like cells	Quarta, 2017 [48]
Rat	Abdominal wall	4% SDC for 4 h DNase I for 3 h	Rat satellite cells	Marzaro, 2002 [49]
Rat	Abdominal wall	Three freeze-thaw cycles	N/A	Vindigni, 2004 [49]
Rat	Abdominal wall	4% SDC for 4 h DNase I for 3 h	Rat myoblasts	Conconi, 2005 [50] De Coppi, 2006 [51]
Rat	Not specified	1% SDS for 3 days	N/A	Qing, 2009 [52]
Rat	Quadriceps and hamstring	0.05% trypsin/EDTA for 1 h 1% Triton X-100 for 5 days	N/A	Stern, 2009 [53]
Rat	Lateral gastrocnemius	Chloroform for 4–5 days 2% SDS for several days	Rat BMSCs	Merritt, 2010a [54] Merritt, 2010b [55]
Rat	Latissimus dorsi	0.15% trypsin for 1 h 0.3% Triton X-100 for several days	N/A	Chen, 2013 [56]
Rat	Tibialis anterior	0.15% trypsin for 1 h 3% Triton X-100 for 3–5 days	Rat BMSCs	Corona, 2013 [57]
Rat	Quadriceps	Phospholipase A2/0.5% SDC for 18 h DNase I for 24 h	C2C12 myoblasts	Chaturvedi, 2015 [58]
Rabbit	Diaphragm	4% SDC for 4 h DNase I for 3 h	N/A	Gamba, 2002 [59]
Rabbit	Cricoarytenoid dorsalis	Freeze-thaw cycle	N/A	Fishman, 2012 [60]
Rabbit	Cricoarytenoid dorsalis	2% SDS for 4 h	N/A	Fishman, 2012 [60]
Rabbit	Cricoarytenoid dorsalis	DNase I for 3 h	N/A	Fishman, 2012 [60]
Rabbit	Cricoarytenoid dorsalis	4% SDC for 4 h DNase I for 3 h	N/A	Fishman, 2012 [60]
Rabbit	Cricoarytenoid dorsalis	Latrunculin B for 2 h High ionic strength salt solutions DNase I for 2 h	N/A	Fishman, 2012 [60]
Dog	Quadriceps and hamstring	Chloroform/methanol for 2 h 0.2% trypsin/EDTA for 2 h 2% SDC for 5 h 2% SDC for 14–16 h 1% Triton X-100 for 1 h 0.1% peracetic acid/4% ethanol for 2 h	C2C12 myoblasts Human perivascular stem cells NIH 3T3 mouse fibroblasts Human microvascular endothelial cells	Wolf, 2012 [61]
Pig	Intercostal	1% SDS for 4–5 days	N/A	DeQuach, 2010 [62]
Pig	Abdominal wall	Three freeze-thaw cycles High ionic strength salt solutions	Human ADSCs	Wang, 2013 [63]

(*continued on next page*)

Table 2 (*continued*)

Species	Muscle origin	Decellularizing agents	Recellularization	Reference
		0.25% trypsin/EDTA for 2 h 1% Triton X-100 for 5 days DNase I for 3 h		
Pig	Psoas major	1% SDS for 4–5 days	N/A	Wassenaar, 2015 [64]
Human	Abdominal wall	30 ppm Triton X-100 for 30 min 0.05% trypsin/EDTA for 1 h 2% Triton X-100/NH_4OH for 3 days	N/A	Porzionato, 2015 [65]
Human	Rectus femoris and supraspinatus	1% SDS/EDTA for 14 days DNase/RNase for 12 h	N/A	Wilson, 2016 [66]
Human	Diaphragm	4% SDC for 4 h DNase I for 4 h	N/A	Davari, 2016 [67]
Human	Masseter, zygomaticus major and flexor digitorum superficialis	2% SDS for 4 h	Human dermal fibroblasts	Naik, 2019a [68] Naik, 2019b [69]
Human	Masseter, zygomaticus major and flexor digitorum superficialis	4% SDC for 4 h DNase I for 3 h	Human dermal fibroblasts	Naik, 2019a [68] Naik, 2019b [69]
Human	Masseter, zygomaticus major and flexor digitorum superficialis	Latrunculin B for 2 h High ionic strength salt solutions DNase I for 2 h	Human dermal fibroblasts	Naik, 2019a [68] Naik, 2019b [69]

BMSC = bone marrow-derived mesenchymal stem cells; DNase I = deoxyribonuclease I; N/A = not available; SDC = sodium deoxycholate; SDS = sodium dodecyl sulfate; EDTA = Ethylenediaminetetraacetic acid.

Fig. 4. Macroscopic view of native and decellularized skeletal muscle. Scale bar represents 5 mm.

collagens [40,71]. Other studies using SDS as the only detergent for processing skeletal muscle reported mixed results in terms of ECM preservation, emphasizing the need for optimization of the detergent concentration and exposure time in addition to tissue characteristics such as species and muscle type. Fishman et al. observed that 2% SDS significantly affected the ultrastructure of the rabbit cricoarytenoid dorsalis muscle [60], while 1% SDS did not have a significant impact on the tibialis anterior muscle of mice [43] or the psoas major muscle of pigs [64]. Lin et al., on the other hand, found that a concentration of SDS as little as 0.1% in combination with 0.1% trypsin and a DNase/RNase treatment resulted in a significant loss of collagen by 27% and GAGs by 49% [44]. Moreover, a reduction of DNA content of only 86.4% was observed. When SDS was replaced by 1% Triton X-100, a more drastic decrease in DNA content of 92.2% was found and collagen was not affected by this protocol.

The first description of a combination of trypsin and Triton X-100 to process skeletal muscle was in a study of Stern et al. where it was used on small tissue slices (<500 μm) of rat quadriceps and hamstring muscle [53]. Triton X-100 mainly disrupts lipid-lipid and lipid-protein interactions rather than protein-protein interactions and is therefore classified as a milder detergent than SDS [71]. Trypsin, on the other hand, cleaves peptide bonds and can aid in the penetration of subsequent decellularizing agents, but care needs to be taken to limit exposure times to this enzyme to avoid excessive disruption of the ECM ultrastructure [40]. Concentrations of 0.05% trypsin and 1% Triton X-100 appeared sufficient to obtain acellular tissue slices in the study of Stern et al., but were found insufficient when this was repeated on intact mouse tibialis anterior muscle [42]. However, by varying the concentration of the enzyme and/or detergent, several other researchers could demonstrate the effectiveness of this decellularization protocol in rat and porcine as well as in human tissues [57,63,65].

To avoid the detrimental effects of detergents on the ECM components and tissue ultrastructure, Gillies et al. developed a method based on latrunculin B, high ionic strength salt solutions and DNase I [42]. Latrunculin B is a toxin that inhibits polymerization of actin filaments [72], whereas the salt solutions depolymerize myosin. The unique combination resulted in a drastic decrease in DNA content, which was

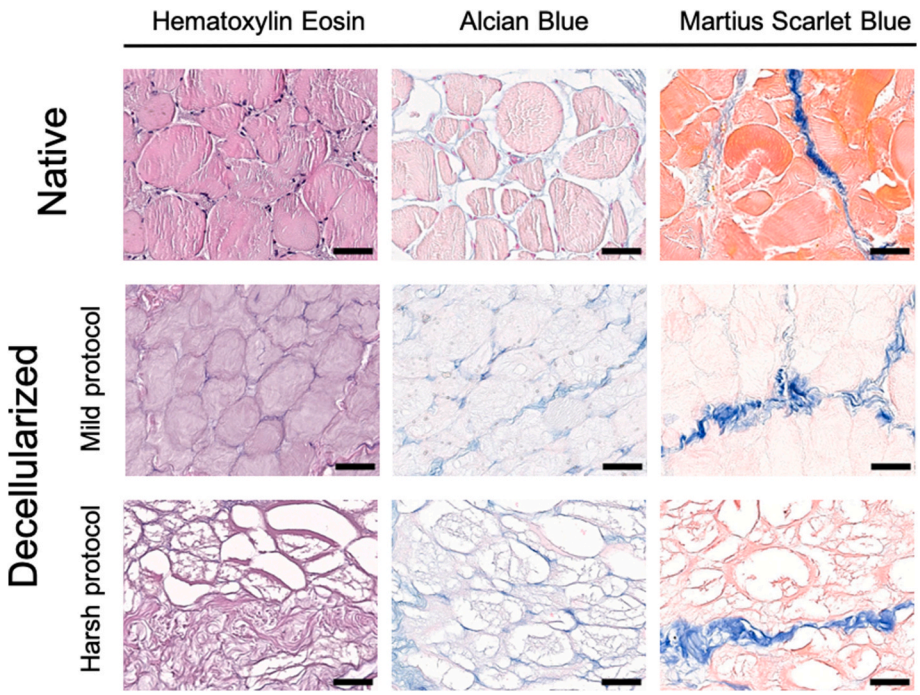


Fig. 5. Histological view of native and decellularized skeletal muscle. Microscopic comparison of native with decellularized skeletal muscle using mild versus harsh decellularization protocol. Images show cross-sections stained with hematoxylin eosin, alcian blue and martius scarlet blue to show overall structure, sulfated GAGs and collagen, respectively. Scale bars represent 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

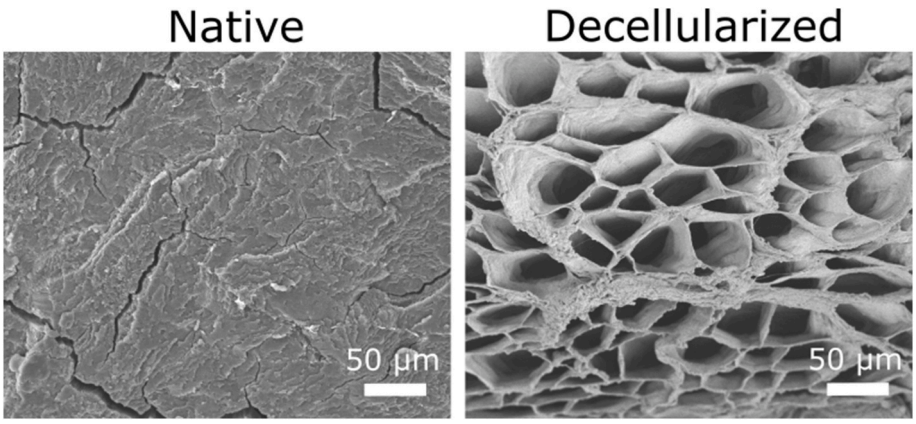


Fig. 6. Scanning electron microscopy images showing the ultrastructure of native and decellularized skeletal muscle which was processed using a protocol based on 1% SDS [74]. Decellularized skeletal muscle showing preserved honeycomb-like endomysium. Scale bars represent 50 μm, images taken at 300× magnification.

confirmed by hematoxylin-eosin, DAPI and Pax7 staining. Furthermore, collagen content was unaffected and scanning electron microscopy revealed that hollow tubular structures could be observed in the decellularized tissue. The protocol was also applied by other researchers on the lower limb of rats, where a significant decrease of collagen content was shown, together with a loss of VEGF [73]. An efficient removal of DNA, but reduced collagen content was also described for rabbit cricoarytenoid dorsalis muscle [60]. Application of this protocol on human tissue was described to be inadequate [68,69]; however, this may be attributable to the fact that frozen samples had been used, whereas latrunculin B is dependent on actin dynamics in biochemically active cells. In general, the decellularization method must be carefully chosen, where milder methods better preserve ECM elements but may be less efficient in removing cells. In contrast, more harsh methods more efficiently remove the cells, but are more likely to affect tissue structure. An example is shown in Fig. 5 with histological stainings for GAGs and collagen, illustrating structural changes. An efficient decellularization will result in a topography where spaces previously filled by cells are completely empty, which can be clearly demonstrated by scanning electron microscopy (Fig. 6).

In addition to the above-mentioned decellularization protocols, in which skeletal muscles are submerged in alternating solutions, perfusion is emerging as an attractive method to efficiently process larger tissues. By using the inherent vascular network of the organ or tissue of interest, diffusion restrictions can be overcome and decellularizing agents can be delivered deep into the organ or tissue [40]. At the same time, perfusion through the vasculature allows for efficient transportation of cellular material. While whole organ perfusion decellularization has already been described over a decade ago [75], the technique was only more recently employed to obtain acellular muscles. A limited number of studies has been carried out thus far, as summarized in Table 3.

The first study was described by Jank et al. in 2015, in which they perfused the upper limb of rats with 1% SDS for up to 50 h followed by 1% Triton X-100 for 1 h and extensive rinsing with an antibiotic solution for 124 h [76]. The protocol has then been translated to a human upper limb by increasing the exposure time of SDS to 30 days and of Triton X-100 to 15 days [77]. Although a 4.6-fold decrease in DNA content could be observed, the average value did not yet fall below the criterion

Table 3
Overview of perfusion decellularization methods for skeletal muscle.

Species	Muscle origin	Decellularizing agents	Recellularization	Reference
Rat	Upper limb	1% SDS up to 50 h 1% Triton X-100 for 1 h	C2C12 myoblasts Mouse embryonic fibroblasts HUVECs	Jank, 2015 [76]
Rat	Extensor digitorum longus	Latrunculin B for 2 h High ionic strength salt solutions DNase I for 2 h	Mouse satellite cells Mouse fibroblasts	Urciuolo, 2018 [73]
Rat	Extensor digitorum longus	4% SDC for 4 h DNase I for 3 h	Mouse satellite cells Mouse fibroblasts	Urciuolo, 2018 [73]
Rat	Extensor digitorum longus	0.25% SDS for 3 days	Mouse satellite cells Mouse fibroblasts	Urciuolo, 2018 [73]
Rat	Gracilis	Krebs Henseleit buffer for 30 min 1% Triton X-100 for 1 h 1% SDS for 1 h	N/A	Sabbagh, 2019 [80]
Rat	Gracilis	1% SDS for 3 days 1% Triton X-100 for 30 min	N/A	Sabbagh, 2019 [79]
Pig	Abdominal wall	0.02% trypsin/EGTA for 2 h 0.1% SDS for 12 h 1% Triton X-100 for 12 h 0.1% peracetic acid/4% ethanol for 2 h DNase/α-galactosidase for 30 min	N/A	Zhang, 2016 [78]
Human	Upper limb	1% SDS for 30 days 1% Triton X-100 for 15 days	N/A	Gerli, 2018 [77]

DNase I = deoxyribonuclease I; HUVEC = human umbilical vein endothelial cells; N/A = not available; SDC = sodium deoxycholate; SDS = sodium dodecyl sulfate; EGTA = Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N'-tetraacetic acid.

Table 4
Clinical results with acellular matrices in patients with VML.

Injured muscle	Acellular matrix	# patients	Outcome	Reference
Quadriceps	Porcine SIS	1	Improvement in isokinetic performance and formation of new tissue	Mase, 2010 [88]
Tibialis anterior or quadriceps	MatriStem (porcine UBM)	5	Functional improvement in 3 out of 5 patients	Sicari, 2014 [86]
Tibialis anterior, quadriceps, brachialis, biceps, rectus femoris, sartorius or hamstring	MatriStem (porcine UBM), BioDesign (porcine SIS) or XenMatrix (porcine dermis)	13	Increased force production in 11 out of 13 patients and constructive tissue remodeling in all patients	Dziki, 2016 [87]

SIS = small intestinal submucosa; UBM = urinary bladder matrix.

of Crapo et al. [40] of less than 50 ng dsDNA per mg ECM dry weight. However, the perfusion decellularization protocol holds great promise and with some further finetuning such as including a nuclease treatment or optimizing the perfusion rate, it may be feasible to obtain a clinically relevant scaffold for transplantation.

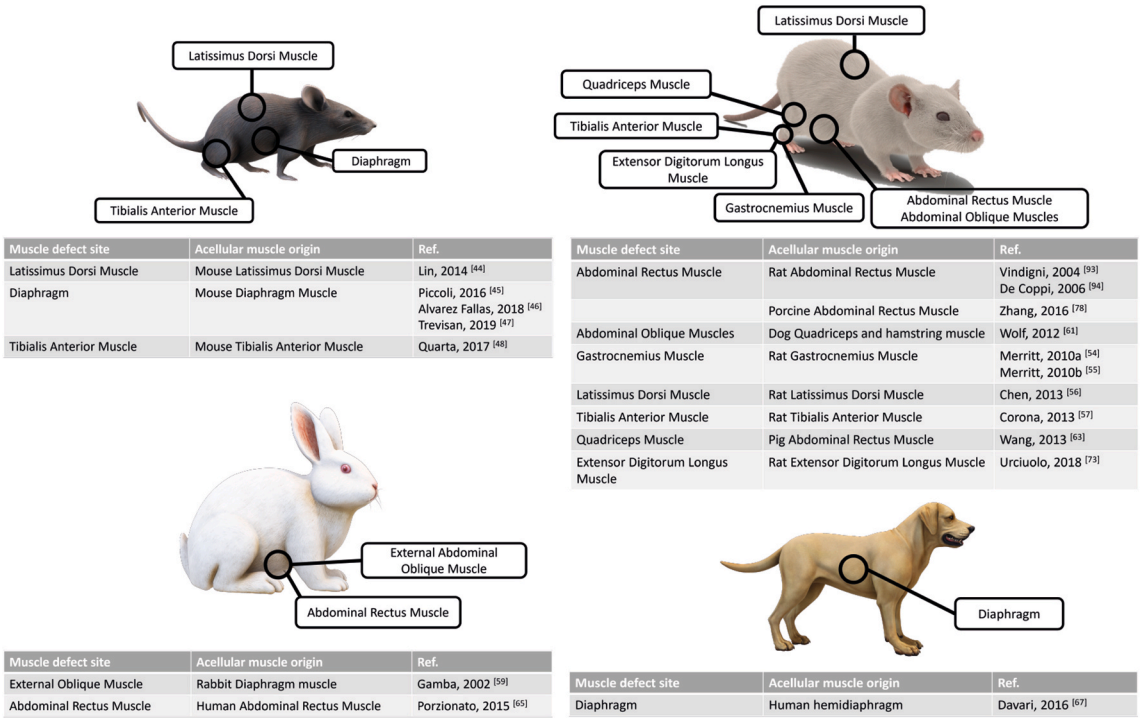
Three other groups described perfusion decellularization of skeletal muscle of pigs [78] or rats [73,79]. Zhang et al. decellularized the rectus abdominis of a pig through perfusion with a series of chemical and enzymatic treatments, including 0.02% trypsin, 0.1% SDS, 1% Triton X-100, peracetic acid, ethanol, α-galactosidase and DNase [78]. Again, a drastic decrease of DNA content of 99.5% was found upon decellularization and scanning electron microscopy revealed preservation of the ultrastructure. However, some impact on the biomechanical properties was observed since there was a significantly higher tensile stretch compared to native tissue. Moreover, total protein and GAG content were found to be decreased. Urciuolo et al. compared three protocols which were already tested for immersion decellularization, including a detergent-free method [42], a detergent-enzymatic method combining 4% SDC and DNase and a detergent-only method consisting of 0.25% SDS [73]. All three protocols resulted in a similar decrease of DNA content but differed in their impact on the myofibers. Only decellularization with 0.25% SDS was capable of completely removing the myofibers, leaving behind only the ECM. Finally, Sabbagh et al. described the shortest decellularization protocol with a duration of less than 5 h in the gracilis muscle of a rat. The combination of a Krebs-Henseleit buffer, 1% Triton X-100 and 1% SDS successfully removed the nuclear content while maintaining the structural integrity of the tissue [79].

5. Acellular matrices as scaffolds for transplantation

5.1. Non-tissue specific ECM scaffolds for regenerative repair

Initially, for soft tissue applications, commercially available ECM has been exploited originating from a variety of tissue sources and species such as human dermis (AlloDerm®, AlloPatch HD™), porcine UBM (MatriStem®, Acell Vet), porcine SIS (Restore™, FortaFlex®) and porcine mesothelium (Meso BioMatrix™) [40]. Most of these applications have been focused on rotator cuff repair [81]. For VML repair, filling the volumetric loss with a biomaterial which simultaneously sustains SC activity would be of great clinical value. Acellular scaffolds have been tested in pre-clinical VML models of abdominal repair [82,83], and musculotendinous defects in leg muscle [84,85]. Although formation of skeletal muscle by host cell infiltration as well as vascularization and innervation were observed [82–84], a complex quadriceps injury indicated that the ultimate remodeling response was solely the formation of dense collagenous tissue with small islands of nonfunctional muscle, bone and cartilage [85].

While most of the studies using these non-tissue specific ECM were conducted in animal models, a few studies have been conducted in patients as well (Table 4). In the study of Sicari et al., the effect of UBM as a biologic scaffold was first evaluated in a rodent model of VML showing *de novo* skeletal muscle fibers and associated functional improvement [86]. Similar results were reported in a parallel conducted human clinical study including five patients suffering from extremity VML. Eight layers of UBM, in total measuring about 4 mm in thickness, were implanted. Six months post-surgery, functional improvement was reported for three out of five patients. Notably, both rodent and patient



Muscle defect site	Acellular muscle origin	Ref.
Latissimus Dorsi Muscle	Mouse Latissimus Dorsi Muscle	Lin, 2014 [44]
Diaphragm	Mouse Diaphragm Muscle	Piccoli, 2016 [45] Alvarez Fallas, 2018 [46] Trevisan, 2019 [47]
Tibialis Anterior Muscle	Mouse Tibialis Anterior Muscle	Quarta, 2017 [48]

Muscle defect site	Acellular muscle origin	Ref.
Abdominal Rectus Muscle	Rat Abdominal Rectus Muscle	Vindigni, 2004 [93] De Coppi, 2006 [94]
	Porcine Abdominal Rectus Muscle	Zhang, 2016 [78]
Abdominal Oblique Muscles	Dog Quadriceps and hamstring muscle	Wolf, 2012 [61]
Gastrocnemius Muscle	Rat Gastrocnemius Muscle	Merritt, 2010a [54] Merritt, 2010b [55]
Latissimus Dorsi Muscle	Rat Latissimus Dorsi Muscle	Chen, 2013 [56]
Tibialis Anterior Muscle	Rat Tibialis Anterior Muscle	Corona, 2013 [57]
Quadriceps Muscle	Pig Abdominal Rectus Muscle	Wang, 2013 [63]
Extensor Digitorum Longus Muscle	Rat Extensor Digitorum Longus Muscle	Urciuolo, 2018 [73]

Muscle defect site	Acellular muscle origin	Ref.
External Oblique Muscle	Rabbit Diaphragm muscle	Gamba, 2002 [59]
Abdominal Rectus Muscle	Human Abdominal Rectus Muscle	Porzionato, 2015 [65]

Muscle defect site	Acellular muscle origin	Ref.
Diaphragm	Human hemidiaphragm	Davari, 2016 [67]

Fig. 7. Overview of pre-clinical VML injury models in which skeletal muscle-derived ECM scaffolds have been implanted.

biopsies showed perivascular stem cell infiltration, suggestive of an active contribution to the *de novo* formation of skeletal muscle. To extend these findings, a follow-up study including 13 patients with VML injuries at varying sites was conducted [87]. In this study, patients were treated with one of the following three porcine ECM bioscaffolds: UBM (MatriStem), SIS (BioDesign) or dermis (XenMatrix). The results of this 13-patient cohort study showed the promise of using acellular biologic scaffolds as clinically relevant functional improvement was found in 11 out of 13 patients. Also, evidence of constructive tissue remodeling was apparent as desmin-positive skeletal muscle fibers were found both at the interface and within the center of the scaffold implantation site as early as six weeks post-implantation of the scaffolds. Although these scaffolds provide a clinical benefit, neither their structure, nor their composition is similar to muscle. Therefore, muscle-derived ECM may provide additional morphological as well as biochemical cues to further stimulate muscle regeneration.

5.2. *Skeletal muscle-derived ECM scaffolds for regenerative repair*

Although ECM scaffolds such as UBM or SIS were shown to be able to fill the volumetric loss of tissue, they do not have any muscle-specific components. In addition, they lack the complex 3D organization of skeletal muscle ECM and thus attention has shifted towards tissue-matched decellularized scaffolds. The rationale for this is fourfold. First, acellular skeletal muscle would be better at instructing host and/or seeded donor cells because of the preserved 3D organization which would ultimately lead to better regeneration upon implantation [89]. Second, decellularized skeletal muscle was found to exert anti-inflammatory and immunosuppressive effects which polarizes macrophages towards an M2 phenotype [90]. Thus, the cell-mediated immune response towards acellular muscle facilitates an optimal environment for skeletal muscle regeneration, as reviewed in section 3. A third reason for using decellularized muscle is that upon degradation, biologically active degradation products are released which are known to positively affect cell migration and proliferation [91]. Importantly, these bioactive molecules are also involved in angiogenesis and hollow vessel structures were shown to be preserved upon decellularizing diaphragmatic muscle. Interestingly, these hollow vessel structures could be recognized by seeded human umbilical vein endothelial cells (HUVECs) *in vitro* and by host cells *in vivo*, resulting in functional blood vessels [46]. Lastly, intrinsic alignment of the scaffold could enable transmission of force. Consequently, several groups have started to develop decellularization protocols for skeletal muscle, of which an extensive summary was already given in section 4. As from a regenerative point of view, we will focus in the next section on the *in vivo* studies using skeletal muscle-derived ECM scaffolds for regenerative purposes, with or without recellularization prior to implantation. An overview of the different animal models used for these *in vivo* studies along with the muscle defect sites is given in Fig. 7.

5.2.1. *Acellular muscle-derived ECM scaffolds*

The first reported study on using skeletal muscle-derived ECM for skeletal muscle reconstruction focused on using diaphragm grafts for repair of a rat abdominal wall defect [59]. Unfortunately, 90 days post-surgery showed no signs of skeletal muscle reconstruction but instead remodeled into fibrous tissue. More promising results were attained using homologous, decellularized muscle ECM in a rat laceration-induced gastrocnemius muscle defect model [54]. Recovery was assessed over a period of 42 days and showed an increase in the number of blood vessels and desmin-positive areas in the ECM as recovery time increased. However, complete repopulation of the ECM center with myofibers or blood vessels was not achieved even after 42 days. Consequently, full functional recovery was not attained. Similarly, decellularized muscle matrix from rat gastrocnemius was implanted in a large defect in the gastrocnemius and compared to a type I collagen plug after 56 days [92]. With this study, improved regeneration when using the decellularized matrix compared to collagen was found, with less fibrosis, better recovery of force and more abundant muscle regeneration. However, complete functional recovery when compared to sham-operated muscles was still not achieved and a 40% reduction in force remained. Analogous strategies of using skeletal muscle ECM grafts for treating VML defects have been translated to human tissue as well. Porzionato et al. were the first to report on using a human-derived scaffold, obtained by decellularization of human abdominal rectus

muscle, in a rabbit model with an abdominal wall defect after evaluating different protocols on rat, rabbit and human muscle samples [65]. Implantation of human muscle matrix in rabbits showed good integration with host tissue without inflammatory rejection response. But recellularization of the matrix was again lacking in the core of the implant upon explanting the construct after three weeks.

Besides abdominal wall defects, other skeletal muscle defect models have been established to validate whether muscle-derived ECM can improve functional repair. For example, muscle-derived ECM has been validated in parallel to no repair and sham-operated rats, in a rat latissimus dorsi defect model to determine whether muscle function could be restored [56]. Compared to the no repair group, implanting muscle-derived ECM resulted in an improvement in muscle force. However, based on the limited neo-muscle formation, this improved muscle function was mainly attributed to bridging by the muscle-derived ECM rather than muscle regeneration. In addition, decellularized muscle-derived ECM patches have also been evaluated in the context of congenital diaphragmatic hernia as an alternative for the currently used synthetic prosthetic patches [47]. In this study, common issues related to the standard use of prosthetic materials, such as rejection or hernia recurrence, were not evident upon implantation of diaphragmatic ECM-derived patches. Furthermore, the diaphragm-derived ECM enabled re-innervation and muscle function recovery. And thus, decellularized diaphragmatic ECM-derived patches were found to be superior for supporting the repair of diaphragm ECM compared to the currently used prosthetic materials. Another study focusing on large diaphragmatic defects evaluated cryopreserved with decellularized human diaphragmatic patches in a canine model. Both grafts were found to result in a similar healing process but with fewer inflammatory cells and foreign body granulomas for the decellularized heterograft [67].

From the above-mentioned studies it is clear that the use of site-specific ECM is expanding and shows promising results for regeneration of damaged skeletal muscle. However, superiority of skeletal muscle-specific ECM scaffolds for constructive remodeling of damaged muscle tissue is still a matter of debate and was questioned based on a comparative analysis with SIS [61]. In this study, muscle ECM obtained using two different decellularization processes was compared to a non-muscle ECM derived from SIS. Despite the numerous differences in structure, composition and early host responses between muscle-derived ECM and SIS, no differences in remodeling outcome and myogenesis were found. Still, differences in muscle regeneration may become apparent when tested in a larger defect model or when evaluating functional recovery. In contrast, another study comparing perfusion-decellularized skeletal muscle ECM with porcine-derived SIS did report important differences [78]. Indeed, in the context of partial-thickness abdominal wall defects in rats, improved neovascularization, myogenesis and functional recellularization of the muscle ECM compared to SIS was found, which justifies the choice of tissue-specific ECM. Discrepancy in the findings reported in literature might be explained by the different techniques used to decellularize the tissue, as this is known to influence the composition of the obtained ECM. In addition, the extent to which the complex 3D structure is maintained will also highly influence the outcome and is also dependent on the decellularization protocol.

To address the question to what extent the selected decellularization protocol influences the *in vivo* outcome, the muscle regenerative capacity of xenogeneic acellular muscle derived from three different perfusion protocols was evaluated [73]. ECM components were found to be comparable over the tested protocols, but preservation of myofiber structure highly varied. Overall, all implanted scaffolds enabled functional recovery with apparent invasion of blood vessels, nerves and SCs. However, higher number of SCs and better restoration of myofiber type ratio were found in the acellular muscle obtained with the SDS-based perfusion protocol which presented lower myofiber preservation. And thus, a better outcome in terms of muscle regeneration and cell homing *in vivo* was attained with a harsher decellularization protocol that removes myofiber content while maintaining the ECM 3D organization. These contrasting results might be explained by the mechanical obstruction during scaffold repopulation posed by remnants of insufficiently removed myofiber cytoplasm. And thus, by comparing different protocols, new insights for optimizing the scaffolds for engineering

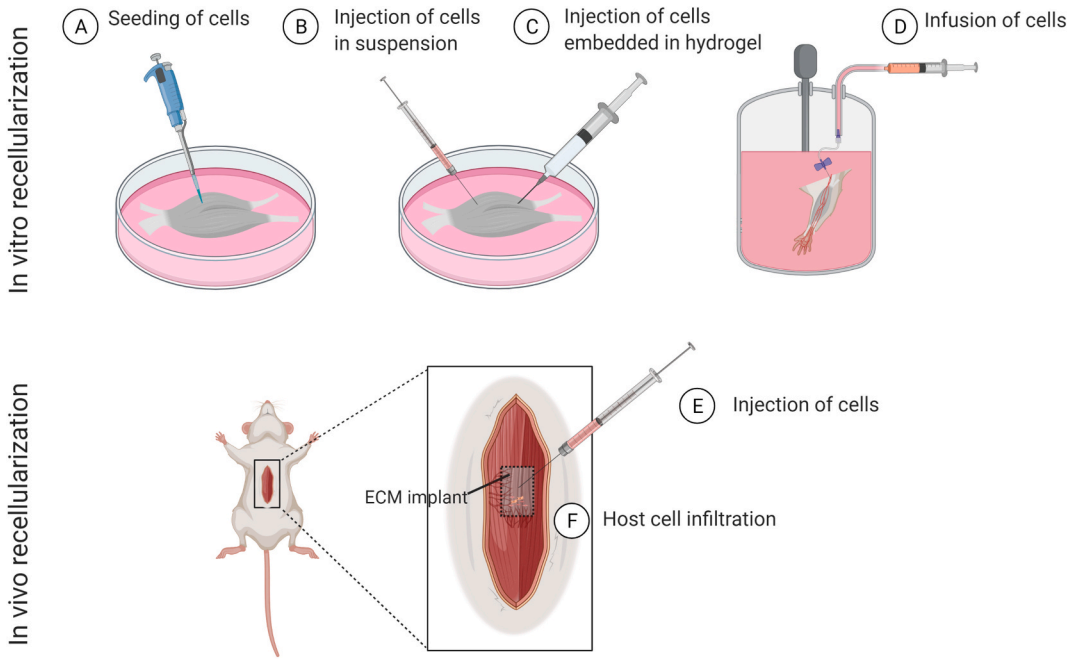


Fig. 8. Schematic representation of recellularization strategies. *In vitro* recellularization prior to implantation by means of A) direct seeding of cells on top of ECM matrix, B) micro-injection of cells suspension in ECM matrix, C) injection of cells embedded in a hydrogel into the ECM matrix or D) infusion of cells using the original vasculature. *In vivo* recellularization after implantation by means of E) direct injection of cells in ECM matrix and/or F) host cell infiltration. Recellularization can be based on using a single cell population or a combination of cell types.

skeletal muscle tissue are possible.

5.2.2. Recellularized muscle-derived ECM scaffolds

A recurring issue related to the use of acellular matrices obtained from homologous muscular tissue for functional recovery is the lack of proper recellularization, especially in the core of the matrix. Also, although not rejected, implanting these acellular matrices generally gives rise to some inflammatory response and rapid formation of fibrotic tissue. Both issues might be counteracted by adding cells to the matrices and thus recellularization strategies have extensively been studied in this field (Fig. 8) [41,42,58,68,69]. Although these *in vitro* studies already provide the field with crucial information on cell-ECM scaffold interaction, as with any implanted material, properties of the material inevitably and rapidly change *in vivo* as a result of scaffold degradation, neo-matrix deposition and host cellular remodeling events [83]. And thus, evaluating the ECM scaffolds in an *in vivo* setting is crucial for deciding on their clinical potential for treating VML defects.

One of the first *in vivo* studies reporting on the use of a muscle acellular matrix implant in combination with pre-seeding of cells aimed to evaluate the *in vivo* biocompatibility of the implant rather than its regenerative capacity within a defect model [49]. When implanting a rat acellular muscle patch in the dorsal muscle of rats, remodeling into fibrous tissue was apparent by the fourth week. On the other hand, when pre-seeded with autologous SCs, inflammatory reaction was modest and structural integrity was maintained indicating indeed an improved biocompatibility when combined with cells. The first attempt of using an acellular muscle matrix combined with seeded cells for muscle regeneration was reported in 2004 [93]. In this study, regenerated myofibers were evaluated in a rat model of full-thickness rectus abdominis muscle ablation upon implanting an autograft versus acellular rectus abdominis muscle patch seeded with autologous SCs immediately after wall reconstruction. Although the integrated patches did not herniate, the only myofibers observed to have regenerated three weeks after surgery were confined to the border regions of the implants. This experiment highlighted once more the difficulties regarding long-term survival of injected myogenic cells *in vivo*.

Optimizing the different modalities of combining cells with acellular muscle matrices has been a highly explored topic in the field. In light of such interest, seeding an acellular abdominal muscle matrix with myoblasts *in vitro,* thus prior to implantation, was explored [50]. Cells were left to attach for one day under static culture conditions and the resultant cell-matrix construct was implanted between the oblique muscles of rats up for three months. Non-seeded patches were completely replaced by fibrous tissue, while seeded patches remained structurally intact. This was in line with what had been observed in the prior biocompatibility study [49]. Seeded patches displayed abundant blood vessels and myoblasts with even electric activity arising from single motor-unit potentials. However, at the third month, thickness of the myoblast-seeded patches and their electrical activity decreased. And thus, maintaining these contractile muscle fibers for a longer time poses another challenge. As a follow-up study, the same strategy of seeding an acellular skeletal muscle matrix one day prior to implantation with rat-derived myoblasts was exploited in a full-thickness defect of the abdominal wall [94]. The outcome of surgery was followed for nine months and demonstrated progressive remodeling of the patch with skeletal muscle cells, blood vessel and nervous cell infiltration. Importantly, seeded myoblasts remained viable and present within the patch for nine months but their contribution to skeletal muscle regeneration was not assessed.

An alternative strategy to deliver cells to an acellular matrix to advance skeletal muscle regenerative capacity of the implanted construct is by injecting them directly into the implanted ECM. Since repair of a lateral gastrocnemius defect by acellular gastrocnemius muscle ECM did not result in functional recovery, the set-up was advanced by injecting bone marrow-derived mesenchymal stem cells into the ECM one week after the implantation [55]. This improved the functional outcome of the injured muscle as compared to ECM without cells. In addition, seeded ECM contained more blood vessels and regenerating skeletal myofibers than ECM without cells. Nevertheless, whether injected cells engraft or merely create an instructive environment for surrounding cells could not be determined from the data.

A more advanced approach to this strategy of delivering cells through injection was reported by Quarta et al. [48]. In this approach, decellularized tibialis anterior muscles were positioned in a bioreactor after which they were injected with a mixture of isolated cells within an ECM-based hydrogel made from milled and protease digested decellularized muscle. The bioconstructs were held in the bioreactor for a maximum of 2 h under continuous flow before being transplanted. Injected cells were a combination of five isolated populations of cells: muscle stem cells, hematopoietic cells, ECs, FAPs and fibroblast-like cells. Bioconstructs were then tested in an acute VML injury in a murine model and resulted in *de novo* myofiber formation. However, these myofibers were insufficiently innervated and thus only partial restoration of structure and *ex vivo* force production was attained. However, when combined with physical therapy, innervation was improved and *in vivo* forces were restored. Even chronic VML injuries may be amenable to treatment using this bioengineered construct, since *ex vivo* stimulation of treated muscle showed partial recovery of force production when compared to control muscles. Moreover, bioconstructs established with human muscle stem cells and muscle resident cells were able to repair and restore muscle structure and function, highlighting the translational potential of this approach.

A first *in vivo* approach using autologous native ECM obtained through perfusion decellularization was reported by Jank et al. [76]. In this study, rat and primate forearms were perfused yielding an acellular scaffold with preserved architecture and similar flexibility as compared to native joint. Next, the obtained rat matrix was repopulated with myoblasts, fibroblasts and vascular ECs through injection and infusion, respectively, followed by skin graft transplantation. After seeding, the composite tissue was further matured by applying electrical stimulation. Finally, to show applicability of the engineered composite tissue graft for surgical reconstruction after limb loss, orthotopic limb transplantation was performed onto isogenic rats. Upon anastomosis to the blood supply of the host rat, vascular channels in the graft appeared to be perfused and could withstand physiologic perfusion pressure.

A general finding regardless of the type of ECM, even when combined with a specific cell source, is the sustained or intermittent deposition of collagen in the area of transplantation. However, even in the absence of muscle fiber regeneration, functional improvements have been reported post-injury following muscle-derived ECM transplantation combined with bone marrow-derived mesenchymal stem cell injection in a tibialis anterior defect model [57]. Additionally, upon implanting the muscle-derived ECM, reduction in fiber cross-sectional area in the remainder of VML injured muscle appeared to be prevented. These results point towards a novel role of biological ECM transplantation in which deposited collagen protects the remaining muscle mass from prolonged fiber damage and putative atrophy within the remaining muscle. In this context, deposited collagen was proposed to provide structural reinforcement to the remaining muscle mass leading to a conserved response to increased mechanical stress. Targeting the remaining tissue rather than solely focusing on regenerating the damaged portion is gaining attention since insights into the complex pathophysiology after VML are expanding. Indeed, VML injuries appear to be broader than the frank loss of muscle tissue but also comprise proliferative fibrosis secondary to VML injury, the suboptimal performance of remaining muscle mass, muscle architectural perturbations of remaining muscle tissue, etc. [95]. This further supports the idea of looking at ECM-derived scaffolds not only as a way to boost muscle regeneration but also as a way to target the complex pathophysiology of the remaining tissue.

6. Acellular matrices as scaffolds to study cell-matrix interactions

Cell-ECM interactions play an essential role during all stages of skeletal muscle development and maintenance. Cells bind to the ECM through specific cell surface receptors, including integrins, syndecans and dystroglycan [96]. The key role of these interactions becomes apparent in several muscular dystrophies which are due to defects in proteins involved in cell-ECM interaction. A better understanding of these cell-ECM interactions and their biological consequences can aid in the search for novel therapeutic targets and agents. Also, the synthesis, maintenance and remodeling of the ECM involves various cell types and can thus be affected in various ways when normal tissue function is compromised in case of damage or disease.

However, the *in vivo* environment when using animal models to study these cell-ECM interactions, is often too complex and discordant going from animal studies to clinical trials. On the other hand, cell culture studies are an oversimplification of the native phenomena of cell-ECM interactions, leading to misrepresentation of cell-ECM interactions. Through tissue engineering techniques, model tissues can be designed *in vitro,* generating a powerful tool within a well-controlled environment. An interesting approach to study cell-ECM interactions is by using the isolated ECM obtained through decellularization. Indeed, many approaches, as discussed above, exist to obtain ECM and its use in combination with cells has already been described. However, its utility as a model system to study cell-ECM interactions *in vitro* is often not appreciated. As a result, studies that combine cells and skeletal muscle ECM often perform little to no analysis to evaluate how cells interact with these matrices but jump immediately into an *in vivo* model to evaluate its regenerative potential. In the following paragraphs, an overview of the few studies exploring the interactions of cells with acellular muscle matrices are given.

A simplistic way of studying cell-ECM interactions is to use solubilized powdered ECM extracts as a coating before seeding stem/progenitor cells [53]. Although the utility of such an approach has been demonstrated (see section 8), preserving skeletal muscle ECM in its native state may be better suited for studying cell-ECM interactions. For this, a mild decellularization protocol must be used, to minimize alterations of the biochemical, mechanical and structural properties of the ECM. One approach able to maintain these properties has been described and combined with C2C12 cells to evaluate the cell interactions within the matrix [42]. However, besides showing adhesion and survival of the seeded cells, further insights on how cells interact with the matrix were not studied. Similarly, Wolf et al. evaluated cell attachment, survival and morphology upon seeding muscle-derived ECM versus SIS with C2C12 myoblasts and reported the presence of elongated multinucleated myotubes upon histological examination on both scaffolds [61]. In addition, increased metabolic activity of seeded C2C12 cells over time was indicative of sustained proliferation.

A more in-depth study of cell-matrix interactions was provided by Wang et al., who evaluated integrin expression in human adipose-derived stem cells cultured on decellularized muscle and fascia samples [63]. Seeded cells adhered, proliferated, elongated and formed aligned patterns over a period of seven days of culture and expressed α_5 and β_3 integrins. These data confirmed adhesion on 3D muscle matrices. In concordance with these data, C2C12 myoblast interaction with decellularized 3D rat muscle was evaluated to study how intact muscle endomysial matrix affects cell adhesion and proliferation [58]. Cells appeared to become organized on the matrix following its native structure. Interestingly, when seeding muscle stem cells together with fibroblasts, scaffold repopulation, as well as maintenance of the $Pax7^+$ cell population, has been reported [73]. Fibroblasts are known to secrete key components of the ECM, such as collagen VI and fibronectin, which ultimately influence SC maintenance [12,97]. In addition, fibroblast presence has been shown to influence the dynamics of muscle stem cells [98]. And thus, with this study the potential of using decellularized matrices to study SC maintenance and migration *in vitro* becomes apparent.

Altogether, these studies shed a first light on the potential use of acellular muscles matrices to study cell-ECM interactions *in vitro* by re-engineering cell-derived extracellular matrices. Indeed, these matrices hold *in vivo*-like compositional heterogeneity and can thus provide a promising tool for developing a more physiological *in vitro* model. Studies using decellularized ECM to examine how ECM influences stem cell differentiation have been reported for a variety of tissues/organs, as reviewed by Hoshiba et al. [97]. However, to date, little research has been conducted using muscle-derived ECM to elucidate ECM-SC interactions and thus further research on this is warranted.

7. Acellular matrices as drug-testing platform

Animal models have been and remain the standard in preclinical testing, especially regarding toxicity testing. However, a paradigm shift from a system based on animal testing to one relying on cell-based assays is emerging. Although these cell-based assays still mainly occur in monolayer fashion, a rapid increase in the use of 3D cell culture techniques in the context of preclinical testing is occurring. The transition is encouraged by the increasing agreement that 3D systems provide a better model for physiologic conditions [99,100]. To create a 3D environment, scaffolds mimicking the ECM have been used to provide physical support on which cells can aggregate, proliferate and migrate. These scaffolds can be natural, synthetic or a combination and can be functionalized with peptides mimicking integrin attachment sites of natural ECM proteins. However, while ECM was initially thought to solely provide structural support, ECM constituents are now known to actively affect cellular behavior in a tissue-specific manner as well. It is thus not surprising that changes in the ECM components also influence the cell's response to drugs. But still, given that ECM alone has more than 300 biochemical constituents [99], the main challenge in creating 3D cultures for drug-testing platforms will be to engineer a biologically relevant tissue that recapitulates the complexity of the tissue of interest. At present, biopolymer-based 3D cultures for toxicity testing are still mainly composed of single-protein-based ECM mimics e.g. fibrin [101]. In this regard, incorporating native ECM instead of mimicking it would provide us with an intriguing model system to study not only disease dynamics but also drug response.

In drug development for intramuscular (IM) applications, a widely studied aspect concerns the drug absorption post-IM injection. To study this, decellularized skeletal muscle offers a useful system. The utility of such an approach to study drug-ECM interaction and thus to predict drug absorption after IM injection has been demonstrated using cobinamide, an antidote for cyanide poisoning [64]. In this study, the binding of the drug to decellularized porcine skeletal muscle (obtained with an immersion protocol initially developed for creating tissue-specific ECM for cell culture [62]), was reported after IM injection. Notably, the added value of using intact ECM as compared to simple ECM mimics such as type I collagen hydrogels and even more complex ones such as Matrigel or hydrogels derived from skeletal muscle was demonstrated by a lack of cobinamide binding in these ECM mimics.

Another interesting way of using ECM scaffolds for drug testing is in the specific setting of cancer. Cancer progression coincides with changes in the ECM and thus ECM has been suggested to act as a driving force in tumor progression. As a result, clinical translation of basic cancer research, in general, would benefit from having an *in vitro* model that enables the interactions of tumor cells with the ECM. Because of the evidence of the essential role of the tissue microenvironment for tumor pathogenesis, selectively targeting ECM has been set forward as a putative alternative therapeutic strategy [103]. Tissue-derived ECM from patients can be obtained through the process of decellularization. The advantage of this approach is that shape, biochemical properties but also the dysregulation of the microenvironment can be preserved. Therefore, this patient-derived ECM offers a valuable *in vitro* model for drug

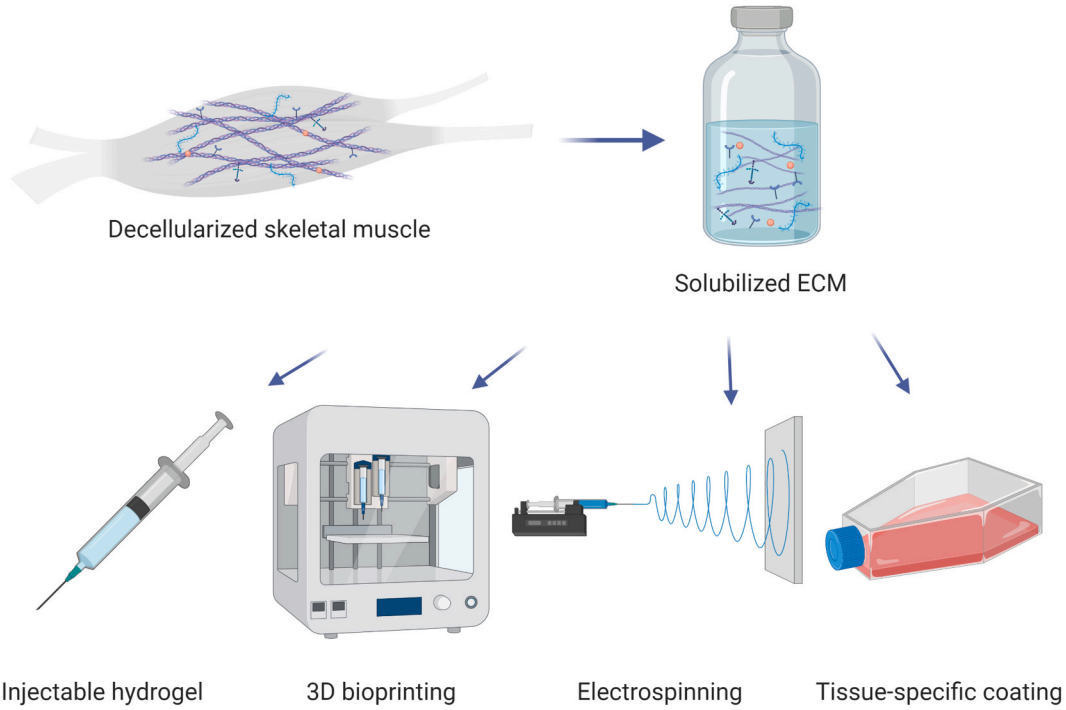


Fig. 9. Applications of solubilized skeletal muscle ECM. ECM can be used as a liquid formulation to inject or to deposit in shaped molds, 3D bioprinted to a specific structure, electrospun or used as a coating of tissue-culture plastics.

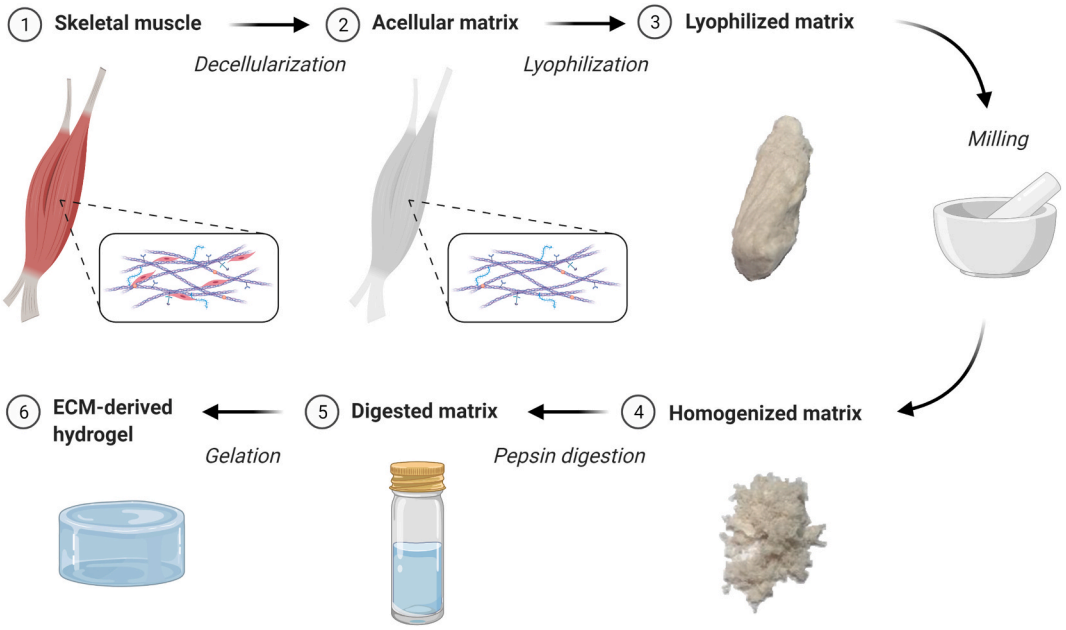


Fig. 10. Fabrication of a hydrogel from skeletal muscle ECM. First, the muscle is decellularized and lyophilized. Next, this matrix is milled and digested by pepsin. Gelation can then be induced by modifying pH and temperature.

screening and treatment outcome prediction. Such a 3D *in vitro* model focused on studying the cancer microenvironment has been developed for different tissues, including skeletal muscle. Indeed, a protocol for alveolar rhabdomyosarcoma tissue decellularization has been described and proposed as a starting point for recapitulating the disease-specific complexity [104]. Also, stiffening of the ECM as a result of cancer-associated fibroblasts has been shown to underly the development of drug resistance to BRAF inhibitors in melanoma [102]. Capturing these matrix-specific drug response modalities would aid in the development of new pharmacological therapies.

8. Skeletal muscle matrix derivatives

Intact acellular skeletal muscle is highly interesting for transplantation or *in vitro* studies requiring recapitulation of the native 3D ultrastructure, as described in detail above. However, the use of ECM in powder or liquid formulations is being investigated as well. Different routes of ECM redesign and combinations with other biomaterials can be explored in this way and open perspectives for alternative applications, as depicted in Fig. 9.

8.1. Injectable hydrogels for acute muscle defects

Decellularized tissues can be lyophilized, removing all water content while retaining the tissue-specific proteins and growth factors. Upon milling of the freeze-dried tissue, a powder can be obtained and further enzymatic digestion with pepsin allows for the formulation of a liquid hydrogel (Fig. 10) [74]. Gelation of the liquid hydrogel can then be induced by bringing the pH to physiologic level and raising the temperature to 37 °C. The importance of a proper starting material, i.e. decellularized tissue with a good balance between removal of cellular material and preservation of the ECM components, was highlighted in a study of Fu et al. comparing five decellularization protocols and the subsequent fabrication of a hydrogel [105]. The matrix resulting from treatment with SDS was unable to form a hydrogel, whereas the matrix treated with SDC followed by Triton X-100 successfully formed a 3D hydrogel scaffold. This difference was attributed to the harsh effects of SDS on the ECM, while SDC and Triton X-100 are milder detergents.

The main advantage of a hydrogel is that it appears in liquid form at room temperature, allowing it to be injected in a minimally invasive manner and making it possible to adapt to irregular defects *in vivo* before gelation occurs. DeQuach et al. used a skeletal muscle matrix hydrogel in a hindlimb ischemia model and could demonstrate a positive effect on neovascularization [106]. Moreover, significantly more muscle cells were recruited to the skeletal muscle matrix hydrogel in comparison to a collagen I hydrogel, indicating its potential of restoring muscle mass.

In addition to using a hydrogel as a stand-alone biomaterial therapy, it can also be employed to deliver therapeutic cells into scaffolds. In a study by Quarta et al., injection of muscle stem cells and muscle resident cells in suspension did not result in efficient repopulation of a decellularized scaffold due to poor cellular retention [48]. However, when using an ECM-derived hydrogel to suspend the cells, the scaffolds were repopulated and the number of *de novo* myofibers exceeded those formed in a collagen I hydrogel. The bioconstructs were further transplanted in a murine VML model and were found to enhance neovascularization, restore biomechanical force production and result in structural repair of the VML injury.

8.2. Decellularized ECM as novel bioink for 3D bioprinting

Within the field of tissue engineering, 3D bioprinting is emerging as a tool to fabricate bioengineered constructs with predefined dimensions and spatial distribution, a high degree of organization and the possibility of combining different cell-laden hydrogels, the so-called bioinks. The hydrogels should possess favorable properties in terms of biocompatibility, viscosity and long-term stability [107]. In this regard, both synthetic polymers such as polyethylene glycol and natural biomaterials such as gelatin, alginate or fibrin are currently being used to develop bioinks [108,109]. However, these bioinks lack the complexity of native ECM and are not tissue-specific.

The unique features of decellularized ECM make this biomaterial extremely attractive to serve as a novel bioink for tissue and organ bioprinting. The biomolecules retained after processing provide the required biochemical cues for cell-matrix interaction and drive migration and differentiation of the encapsulated cells [110]. Enhanced stem cell differentiation and tissue-specific functionality has already been demonstrated in constructs printed with different decellularized ECM bioinks, including skeletal muscle [111–113]. The combination of C2C12 myoblasts with a muscle-derived bioink resulted in a myogenic environment with high cell viability, alignment along the longitudinal axis and myotube formation. Moreover, better load-bearing properties and a significant increase in the formation of acetylcholine receptor clusters were observed in muscle-derived constructs in comparison to collagen constructs [111]. In a follow-up study, human skeletal muscle cells were encapsulated in a muscle-derived bioink and the 3D bioprinted constructs were subsequently used in a VML model [114]. High cell viability was again observed in the constructs and *de novo* muscle formation was seen four weeks post-surgery. Furthermore, functional improvement of 71% compared to the uninjured tissue was demonstrated. Interestingly, when the muscle-derived bioink was then combined with a vascular-derived bioink containing HUVECs into a composite structure through coaxial nozzle printing, functional recovery was increased to 85%. These findings highlight the potential of 3D bioprinting with decellularized ECM bioinks to recreate the hierarchical architecture of skeletal muscle.

8.3. Electrospinning of decellularized ECM nanofibers

Another frequently used technology in tissue engineering is electrospinning. This high-throughput system allows for fabrication of fibers in both the micro- and nanoscale. By varying the operational parameters such as rotational speed and distance to the collector, the fiber diameter, orientation and scaffold porosity can be tailored. Moreover, mechanical properties and degradation rate can be adjusted to the desired needs. Since these properties are hard to control in decellularized matrices, the fabrication of electrospun scaffolds derived from tissue-specific ECM could offer an interesting alternative. In this way, the benefits of acellular matrices containing biochemical cues can be combined with the advantages of electrospun scaffolds in terms of biophysical properties.

A recent study comparing the synthetic polymer poly-ε-caprolactone (PCL) with decellularized ECM derived from bovine skeletal muscle and a PCL-ECM blend demonstrated the feasibility of obtaining highly aligned nanofibers with these biomaterials [115]. However, upon hydration, the ECM-derived scaffolds lost their structural integrity, rendering them unsuitable for further *in vitro* or *in vivo* studies. It appeared that the addition of PCL was necessary to provide sufficient mechanical properties to the scaffolds. Furthermore, the blended scaffolds resulted in enhanced proliferation of SCs and expression of myogenic proteins compared to pure PCL scaffolds. The alignment of the scaffolds also provided topographical cues to the SCs, leading to a high degree of organization permissive for differentiation and fusion. The blended scaffolds were further evaluated in a murine VML injury model of the gastrocnemius muscle [116]. Increased myogenic differentiation and decreased fibrosis could be observed in comparison to PCL scaffolds, indicating an ongoing regenerative process. However, a functional deficit of approximately 30% was still present in all treatment groups. This might be attributed to a lack of innervation in the short follow-up period of 28 days.

The challenge remains to create ECM-derived scaffolds through electrospinning without a carrier polymer in order to more closely mimic the native microenvironment. Smoak et al. were the first to report the fabrication of such scaffolds by an adapted electrospinning process [117]. They found that homogenizing the decellularized muscle ECM is a crucial step in obtaining a uniform starting material. Subsequently, they passed the decellularized ECM through a 300 μm sieve to remove the largest particles. Without sieving, a gel-like material was formed which could not be used for electrospinning. Both random and aligned fibers were produced with the optimized method and their mechanical properties were found to be in the range of native skeletal muscle. In addition, a degradation study demonstrated that after an initial significant loss in the first 6 h, the mass remained stable for 14 days after which a gradual decrease was seen up to 8 weeks. These results are promising for long-term implantation studies, as individual ECM components commonly used to produce scaffolds tend to degrade significantly faster.

8.4. Tissue-specific coating for cell culture

Myogenic cell types such as primary isolated SCs can be expanded to obtain sufficient cell numbers for cell therapy or the development of drug screening models. However, upon removal from the native matrix, the phenotype of the cells is rapidly lost in absence of an adequate microenvironment. Therefore, protein substrates such as collagen I or gelatin are frequently used to coat tissue culture plastic in order to

improve cell attachment, survival and differentiation [118]. However, these proteins only capture a small part of the native ECM complexity. More complex compositions containing a mixture of ECM proteins such as the commercially available Matrigel are commonly used. A major drawback, however, is that this coating is derived from a mouse tumor cell line, which is still not representative of the native cell environment and precludes clinical use. Therefore, some researchers are exploring the use of decellularized ECM as an alternative.

Stern et al. demonstrated that the proliferation of both SCs isolated from rat and human skeletal muscle as well as C2C12 myoblasts was significantly enhanced when cultured on a coating derived from decellularized skeletal muscle from rats in comparison to collagen I [53]. Moreover, differentiation of the cells resulted in more mature myotubes characterized by larger diameters and more nuclei for each myotube. These findings were confirmed when using porcine skeletal muscle ECM as the source for the coating [62]. In the latter study, the differentiated C2C12 myoblasts not only exhibited a higher fusion index, the formation of the myotubes also occurred on an earlier timepoint in comparison to collagen I culture conditions. This early differentiation on skeletal muscle substrates was also observed in a study by Chaturvedi et al., along with more branched and less aligned myotubes on collagen I substrates [58]. Interestingly, no marked differences could be found in terms of cell proliferation or differentiation with respect to fibronectin substrates. Although collagen is the main component of the ECM, it seems that retention of other biologically active components is particularly important to exert a beneficial effect on the culture of myogenic cell types.

A major advantage of using decellularized ECM in cell culture, is that the tissue source to develop the coating can be matched to the cell type of interest to maximally mimic the native microenvironment. DeQuach et al. could demonstrate that the biochemical composition of the matrix remaining after decellularization is highly unique [62]. When comparing cardiac tissue to skeletal muscle tissue, a distinct biochemical profile was revealed with mass spectrometry. Differences in collagen types, glycoproteins and proteoglycans were present in the decellularized matrices. The importance of a tissue-specific ECM was further demonstrated in a study using cells isolated from skin, skeletal muscle and liver tissues [119]. Each cell type was cultured on decellularized skin, skeletal muscle and liver matrix, while collagen I served as a control. Proliferation and differentiation assays revealed that each cell type had the highest proliferation rate, maintained its phenotype and expressed specific cell markers when grown on the ECM of origin. Only cell attachment was found to be not tissue-specific.

The specificity of an ECM is not only determined by its protein composition, but also by its interaction with growth factors. Upon decellularization, tissue-specific growth factors are retained within the matrix and are subsequently released in contact with cell culture medium. In order to better control their release, Zhang et al. developed a novel substrate based on a hyaluronic acid hydrogel combined with heparin and decellularized skeletal muscle [120]. Human SCs cultured on these substrates exhibited a significantly enhanced proliferation rate and expression of myogenic proteins in comparison to gelatin and Matrigel coatings. In comparison to decellularized muscle alone, initial levels of myogenic proteins were similar, but increased on the novel substrate during the intermediate and maturation stages of myogenesis. This was attributed to a slower release of growth factors due to binding with heparin present in the novel substrate.

9. Future perspectives

With this review we aimed not only to summarize the work so far on the use of decellularized skeletal muscle for transplantation, but also to expand the view on further applications both *in vitro* and *in vivo*. Despite the many efforts that have already been made to move forward in each of these research areas, some challenges are still remaining that deserve more attention. Regardless of the final application, the first crucial step is to obtain a fully decellularized matrix. From the present literature overview, it is clear that an optimal decellularization method for skeletal muscle is still lacking. None of the described methods have led to the use of acellular skeletal muscle in a clinical setting so far, in contrast to other acellular matrices such as SIS and UBM. The high variety in decellularizing agents, concentrations, incubation times and techniques makes it difficult to draw general conclusions from the set of currently available studies. Moreover, the variety in muscle type, species of origin, defect site and animal model used make it even harder to compare studies and sometimes lead to contrasting results. There is an increasing need for more comparative studies, in particular in larger animal models, and standardization of evaluation methods. Crapo et al. [40] has provided criteria to assess the extent of decellularization from a cellular perspective. However, no criteria exist for other factors such as the effect of decellularization on ECM composition, structure and biomechanical properties. Guidelines in this direction were suggested for decellularization of peripheral nerves to enable a more standardized evaluation [121]. Several of these recommendations could be applied to other tissues as well, including skeletal muscle. In particular, we believe that a more thorough understanding of the ultrastructural and biomechanical properties of decellularized skeletal muscle would greatly advance the field.

The majority of *in vivo* studies to evaluate the regenerative potential of decellularized skeletal muscle for a large VML defect has currently been conducted in rodents (88%) [122], while only a few larger species such as dogs [84,85] and rabbits [59,65] were used. Although rodent models provide insights into the regenerative capacity of decellularized muscle for the treatment of VML injuries, their clinical relevance is limited. Taking this into consideration, results obtained in rodent models do not necessarily translate well to humans and therefore, scaling up in terms of animal model for future *in vivo* studies will be crucial to determine the true potential of ECM matrices for regeneration.

Another issue is that most implantation studies focus on neoangiogenesis and neomyogenesis, while innervation and functional recovery are less studied. Quarta et al. found that new muscle tissues generated by transplanted bioconstructs, consisting of decellularized muscle tissue scaffolds combined with muscle stem cells and other muscle resident cells, showed myofiber formation and neovascularization but insufficient innervation [48]. However, when treatment was followed by physical therapy, innervation was improved and, more importantly, *in vivo* forces were restored. These data indicate that implantation studies should pay attention to innervation as well to obtain true functional recovery. In this context, approaches such as pre-innervation may be further explored to improve implanted ECM [123].

An increasing number of studies show only partial regeneration upon implanting an acellular ECM, drawing more and more attention towards recellularization [54,65]. A major challenge remains, however, to finetune the modalities to obtain homogenous distribution of myogenic cells throughout a construct, since injection of cells might generate a considerable amount of matrix disruption. In addition, no clear evidence on which cells types and ratios to be used is available. Primary isolated skeletal muscle stem cells from the patient would seem to be the most straightforward choice of cells, but this implies taking an invasive biopsy from a healthy muscle. On the other hand, mesenchymal stem cells, in particular those isolated from adipose tissue, are more easily accessible, but raise concerns in terms of unwanted differentiation upon implantation. Other cell sources are available as well, as described in detail elsewhere [124]. However, for widescale clinical applications, the question may arise whether recellularization of acellular matrices will be cost-effective, as this complicates and delays the developmental process.

Another often neglected factor crucial for clinical applications is the effect of sterilization or disinfection on decellularized matrices. Several sterilization methods commonly used in a laboratory setting have major disadvantages, with UV sterilization having only a limited penetration depth, ethylene oxide possibly leading to cytotoxic residue and

chemicals such as peracetic acid and ethanol possibly altering the surface properties of the tissue [125]. Gamma irradiation, on the other hand, is currently a preferred method for commercial preparation of acellular tissues, but is often not available for researchers and thus cannot always be evaluated extensively in early studies [126].

When it comes to using decellularized skeletal muscle for other applications beyond transplantation, research is still in its infancy with a rather limited number of studies published. However, several primary myopathies originate due to mutations in components of the ECM [127]. Therefore, it would be of interest to have an *in vitro* model based on ECM derived from diseased tissue that is reseeded with cells from a healthy donor to study pathological alterations in cell-ECM interactions. Similar models could also serve as a platform for drug testing, contributing to the 3Rs principle.

Furthermore, derivatives of decellularized skeletal muscle offer another attractive strategy to restore muscle function after VML. In this approach, the focus is no longer on preservation of the ultrastructure of the tissue, but rather on the components. This allows for better manipulation of parameters such as composition, strength and porosity. For example, an injectable hydrogel could adapt to the irregular shape of a defect upon gelation *in vivo*, which is hard to achieve with an intact decellularized muscle. Successful results have been obtained for cardiac muscle-derived hydrogels upon injection after myocardial infarction, with a first-in-man study published [128]. Further research will have to elucidate if skeletal muscle-derived hydrogels could be used in patients as well.

Alternatively, ECM-based hydrogels could serve as bioinks for 3D bioprinting since they are easily combined with cells. By employing this technology, it might be possible to create tissue analogues which can subsequently be transplanted, overcoming the shortage of human donor tissue for transplantation. Moreover, further combination with electrospun fibers derived from decellularized muscle could lead to reinforced constructs containing topographical cues to support the regeneration process. More research in this direction will be necessary, however, since the spinnability of decellularized muscle could raise concerns. Only one research group has reported on electrospinning of ECM-based fibers without the use of a carrier polymer so far, and it will be interesting to see if these fibers are capable of restoring muscle function in a VML injury [117].

Taken together, although the field of skeletal muscle decellularization has already made a lot of progress, the majority of the research fails to progress beyond the experimental stage. The high complexity of this tissue warrants a more advanced approach such as including larger animal models to bring results closer to clinical relevance. Moreover, further exploration of decellularized skeletal muscle and its derivatives in the context of disease modeling or cell-matrix interactions might offer additional value in the search for improved therapies for muscular dystrophies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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