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Review

Vascularization of tissue-engineered skeletal muscle constructs

D. Gholobova, L. Terrie, M. Gerard, H. Declercq, L. Thorrez*

Tissue Engineering Lab, Department of Development and Regeneration, KU Leuven, E. Sabbelaan 53, 8500, Kortrijk, Belgium

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ABSTRACT

Skeletal muscle tissue can be created *in vitro* by tissue engineering approaches, based on differentiation of muscle stem cells. Several approaches exist and generally result in three dimensional constructs composed of multinucleated myofibers to which we refer as myooids. Engineering methods date back to 3 decades ago and meanwhile a wide range of cell types and scaffold types have been evaluated. Nevertheless, in most approaches, myooids remain very small to allow for diffusion-mediated nutrient supply and waste product removal, typically less than 1 mm thick. One of the shortcomings of current *in vitro* skeletal muscle organoid development is the lack of a functional vascular structure, thus limiting the size of myooids. This is a challenge which is nowadays applicable to almost all organoid systems. Several approaches to obtain a vascular structure within myooids have been proposed. The purpose of this review is to give a concise overview of these approaches.

1. Introduction

Skeletal muscle is a complex tissue with a limited capacity to regenerate upon injury. Its contractile function is exerted by aligned myofibers, formed by fusion of single-nucleated myoblasts, resulting in multinucleated fibers. Regeneration is possible by the presence of satellite cells (SC), adult muscle stem cells situated between the basement membrane of the muscle fiber and the sarcolemma (Fig. 1). Upon damage of myofibers following exercise, injury or disease, pro-inflammatory cytokines released from the skeletal muscle niche recruit inflammatory cells, such as neutrophils and macrophages which clear damaged cell debris. In turn, macrophages stimulate SCs to exit the quiescence state and to divide asymmetrically to replenish the SC pool or to provide proliferating myoblasts. Sequentially, myoblasts fuse to form multinucleated myotubes and/or fuse with damaged myofibers. At the same time, resident fibro-adipogenic progenitors deposit extracellular matrix (ECM) at the injured myofiber region [1]. Finally, the last phase in muscle tissue regeneration involves ECM remodelling and myotube maturation, which involves neuronal innervation, ingrowth of newly formed blood vessels and myofilament organization [1].

However, when a defect is too voluminous, the muscle repair process fails and scar tissue replaces the damaged area with resultant functional impairment [1]. This is the case in volumetric muscle loss (VML) caused by trauma or a surgical procedure for example. The current procedure for localized skeletal muscle repair is muscle flap transplantation. An autologous muscle flap, which contains proper vasculature, is excised at a donor site and transplanted to the damaged muscle area. Unfortunately, donor site morbidity and poor survival and integration of donor tissue at the acceptor site is generally observed [2]. The aim of skeletal muscle tissue engineering (SMTE) is to reproduce the native structure and function of muscle *in vitro* and transplant this tissue in the damaged area, avoiding removal of a muscle flap at a donor site. Different terms have been used to described the resulting constructs: bio-artificial muscle, tissue engineered skeletal muscle, muscle engineered tissue, skeletal muscle organoids, myooids etc. In this review we will use the term myooid as a general term. Besides applications in regenerative medicine, there are many other applications in which tissue-engineered skeletal muscle can be useful (reviewed in Ref. [3]). Some examples are the use as an *in vitro* model for studying myogenesis, myopathology or molecular pathways [4–6] or implementation as *in vitro* preclinical model for drug-screening and toxicity testing of compounds [7–9]. However, to create tissue-engineered constructs with a thickness exceeding the millimeter size, perfusion of the construct is essential to avoid cell death by lack of oxygen and nutrients. Current tissue-engineered muscle constructs have sizes in the range of μm to mm in diameter, which is too small to be of use in VML. This review summarizes the currently reported attempts to improve oxygen and nutrient supply through pre-vascularization strategies within tissue-engineered skeletal muscle. First, we will discuss the criteria a myooid should meet to maximally resemble *in vivo* skeletal muscle. Then, we will discuss in detail different SMTE approaches and their corresponding pre-vascularization strategy. Finally, we end with some general conclusions and perspectives.

* Corresponding author.
E-mail address: lieven.thorrez@kuleuven.be (L. Thorrez).

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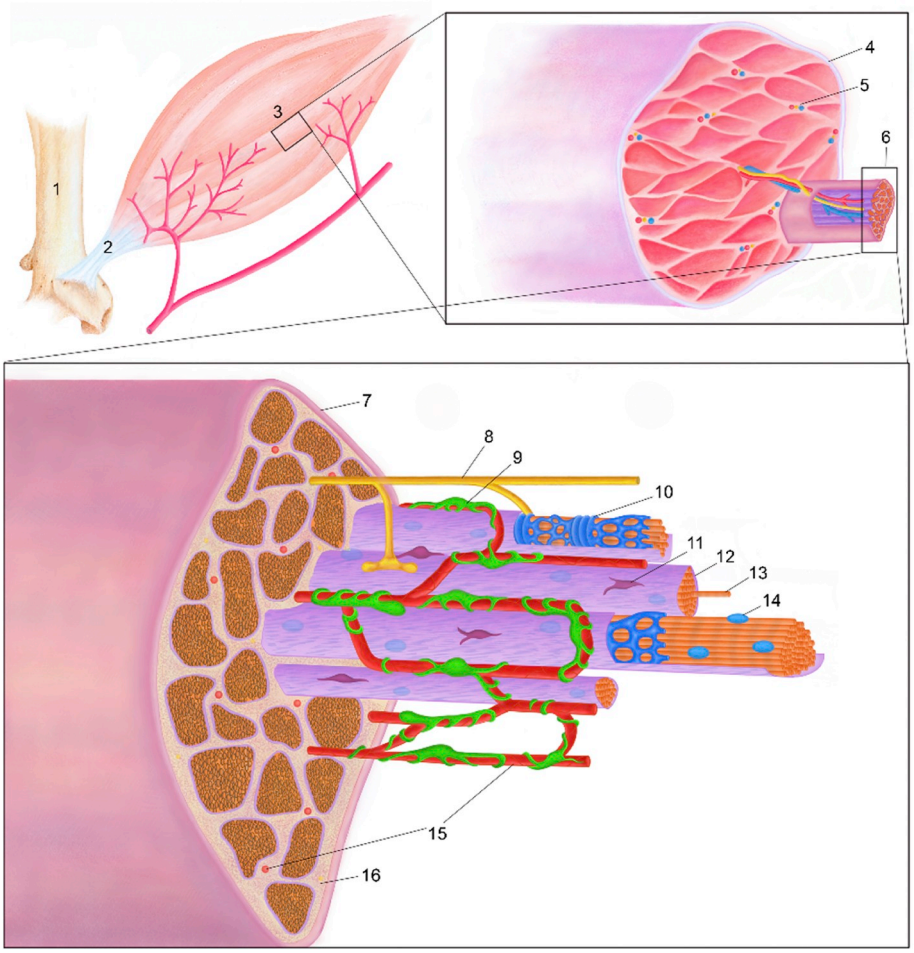

Fig. 1. Skeletal muscle structure. 1. Bone, 2. Tendon, 3. Muscle, 4. Epimysium, 5. Artery (red), vein (blue), nerve (yellow), 6. Fascicle, 7. Perimysium, 8. Nerve, 9. Pericyte 10. Sarcoplasmic reticulum, 11. Satelite cell, 12. Sarcolemma, 13. Myofibril, 14. Muscle cell nucleus 15. Capillary 16. Endomysium [46].

1.1. Skeletal muscle structure

Skeletal muscle accounts for approximately 40% of the total body weight and has an important role in body movement, thermoregulation and basal energy metabolism [10,11]. Eighty five percent of the tissue is composed of bundles of aligned multinucleated myofibers, responsible for muscle contraction and thus movement. The other fifteen percent comprise vasculature, nerves and connective tissue. Myofibers have a cylindrical shape with a diameter varying from 10 to 100 μm, and can reach a length up to 45 cm [11,12]. This elongated shape is determined by the organization of myofibrils, abundantly present in the sarcoplasm (Fig. 1). The myofibrils are composed of thick and thin myofilaments, myosin and actin respectively and are arranged repeatedly along the myofiber in sarcomeres, giving the characteristic striation pattern of skeletal muscle. Sarcomeres are the functional units of muscle fibers and are involved in muscle contraction. Besides contractile proteins, the sarcoplasm contains a mitochondrial network and the sarcoplasmic reticulum, close to the sarcolemma transverse tubular system (T-tubule) invaginations. This system is crucial for the conduction of nerve action potential to the myofiber via calcium [10] (Fig. 1).

The highly organized skeletal muscle connective tissue, the ECM, plays an important role in muscle elasticity, force transmission, cell function and regeneration [13–16]. The ECM of skeletal muscle is organized hierarchically into three fibrous layers: (i) the endomysium which surrounds a muscle fiber, is in connection with the sarcolemma, the muscle fiber membrane, (ii) the perimysium which surrounds myofiber bundles, the fascicles, and (iii) the epimysium which surrounds the entire muscle [13] (Fig. 1). The endomysium layer contains capillaries, necessary for nutrient and oxygen supply and metabolic waste removal of myofibers, and nerve branches (Fig. 1). The perimysium and epimysium are connected with the tendon in the myotendinous junction, which is important in force transmission (Fig. 1) [13]. Forces generated in the myofibers are transmitted from the contractile myofilament apparatus to the muscle ECM by integrins and the dystrophin-glycoprotein complex [1].

Collagen is the major structural protein in skeletal muscle ECM. It accounts for 1–10% of muscle dry weight and the fibrillar types I, III and IV predominate in adult skeletal muscle ECM [13], with type IV mainly in the basal membrane surrounding each muscle fiber [13]. Other ECM proteins include fibronectin, laminin, elastin and various proteoglycans [13]. ECM binds associated non-matrix molecules such as growth factors. Native skeletal muscle ECM plays an important role in muscle development during embryogenesis and regeneration. This role during embryogenesis is facilitated by directly influencing cell behavior through ECM-specific receptors on the cell surface such as integrins [16]. However, the storage and postponed release of growth factors by ECM can also indirectly influence cell behavior through growth factor signalling pathways. Several of these processes occurring during embryonic organogenesis, also take place following injury [1].

2. Challenges in mimicking native skeletal muscle

A first and obvious need in mimicking native skeletal muscle (Fig. 1) is the presence of cells that are able to differentiate into myofibers, which are consequently able to contract upon stimulation. Various tissue engineering methods to develop muscle fibers *in vitro* in a 3D

environment implement SCs, myoblasts, mesoangioblasts, pericytes, mesenchymal-derived stem cells or pluripotent stem cell-derived myogenic progenitors [17–21].

Obtaining aligned muscle fibers in myooids which are further assembled into organized muscle bundles (Fig. 1) is another key feature of engineering functional skeletal muscle tissue. Alignment allows force transduction upon electrical or mechanical stimulation. This feature can be achieved when cells are subjected to a unidirectional force during fusion into myofibers. A common approach to attain alignment is the use of attachment points which induces unidirectional tension during contraction of the hydrogel [6,9,22–25]. Alternatively, conductive micro-patterned scaffolds can be used to guide the orientation of the myofibers [26–28]. However myofibers formed in this way are relatively small and immature resulting in a lack of sufficient contractile force to replace native muscle [7].

A third challenge in SMTE is that skeletal muscle should remain under tension to avoid atrophy upon implantation. The presence of engineered tendons that can be sutured in host tissue, resulting in construct tension, can meet this need [29]. In tendon tissue engineering polymer scaffolds are often used for this matter [30]. Polymer scaffolds having the necessary mechanical and elastic properties can both provide the needed support for the tissue-engineered constructs and provide anchor sites for implantation. Scaffold use in SMTE is further discussed in section 5. Alternatively, a tendon can also be generated through tissue engineering. For details on this subject, we refer the reader to another review [30].

Scaffolds are not only important as anchor site, their structure and composition can be crucial in the survival of the tissue-engineered construct and its integration in the (damaged) host tissue. The tissue-engineered construct should provide an optimal niche for wound healing and skeletal muscle regeneration upon implantation in damaged muscle. Several strategies are being explored such as decellularized extracellular matrices derived from skeletal muscle biopsies [31–33]. This method creates an environment for wound healing by retaining the structure and composition of the native muscle ECM (Fig. 1), and is more extensively discussed in section 5.

Another important challenge in creating functional skeletal muscle, is innervation. *In vivo* innervation of myofibers starts by neurotransmitter release by motor neurons (Fig. 1). Essential in neuron-muscle interaction is the presence of a neuromuscular junction (NMJ) [6]. Stimulation of skeletal muscle is important for long-term survival, since denervation results in atrophy and functional loss [34,35]. In SMTE two different approaches have been evaluated to mimick innervation: electrical stimulation [36,37] or co-culture with neurons or nerve explants [38,39]. Both approaches improve the cytoskeletal organization in the myofibers and their response to electrical stimulation. Electrical stimulation not only has benefits for myofibers, but also on vascularization as further discussed below. We refer the reader to a review providing an in-depth overview of how skeletal muscle constructs can be innervated [40].

Finally, a major shortcoming towards clinical as well as certain *in vitro* testing applications is the limited size of the current engineered constructs. This is due to the lack of a functional vascular network, which is essential for tissue perfusion (Fig. 1). The presence of blood vessels is critical to ensure delivery of nutrients and oxygen as well as removal of metabolic waste products throughout the engineered construct. Cell survival becomes limited at a diffusion distance of about 150–200 μm from a blood vessel or culture medium [41–43]. In response to the hypoxia that arises when having a tissue engineered graft with a thickness greater than the diffusion limit, host vessels invade the implanted tissue. However, the rate of spontaneous vascular ingrowth is limited to ~5 μm/h [44], which -depending on the graft thickness-may not in due time address the metabolic need in the central area of the graft, leading to cell death. The speed of vascularization is of key importance to achieve successful transplantation. To overcome the speed limitation of host vascular ingrowth and/or maintain thicker tissues *in vitro*, an engineered pre-vascular network, would be desirable to ensure a higher survival and prolonged function throughout the construct [45]. This is discussed in detail in section 3. Vascularization approaches in SMTE are discussed throughout section 5 while alternative perfusion strategies are shortly discussed in section 6.

3. Vascularization approaches of tissue engineered constructs: angiogenesis and vasculogenesis

In vivo, vascularization of myooids can be achieved by ingrowth of existing blood vessels. This is achieved by the stimulation of angiogenesis, which is the formation of new capillaries from pre-existing vessels by sprouting of endothelial cells [47]. Angiogenesis is relatively slow, approximately 5–17 μm/h [48,49], and myooids exceeding the passive diffusion limit in any dimension will not receive sufficient nutrients and oxygen during the first days after implantation, resulting in necrosis [50]. To stimulate ingrowth of blood vessels in the myooid, growth-factor releasing scaffolds, and/or scaffolds with a specific structure or chemical composition are being used. An example of the first group is the addition of vascular endothelial growth factor (VEGF) in the scaffold [51]. This accelerates neoangiogenesis and increases microvessel density in the implanted construct [51,52]. An example of the second group is construct porosity, which also improves capillary ingrowth [53,54].

In vitro vascularization can be achieved by vasculogenesis and can result in vascular network formation in the construct. Vasculogenesis is the *de novo* assembly of endothelial progenitors into capillaries. In contrast to angiogenesis, vasculogenesis is a far less studied and understood process both in general and specifically in the field of tissue engineering [55]. In this tissue-engineering strategy, endothelial cells are added in the construct to form an *in vitro* (micro-) vascular network (Fig. 2b). The advantages of this strategy are threefold. First, the presence of this preformed endothelial network further stimulates host vessel ingrowth upon implantation [56,57]. Second, the presence of endothelial cells in the construct stimulates formation of anastomosis of *in vitro* formed endothelial networks and host vasculature upon implantation [58]. And last, this pre-vascularization improves perfusion through and thus also survival of the construct upon implantation [56,57]. The latter will also be discussed in section 6 as a separate approach to enhance vascularization. It is important to mention that regression of *in vitro* formed networks can occur upon implantation. In such cases anastomosis between *in vitro* formed networks and host vessels will not provide sufficient nutrient supply through the whole implanted constructs. Eventually, angiogenesis through the whole construct should be present to allow long-term survival [59]. *In vitro* formation of vascular networks in the construct may be attained through the mechanism of vasculogenesis as mentioned above or through angiogenesis.

Approaches for vasculogenic pre-vascularization include co-culturing of target cells and endothelial cells in 3D scaffolds or decellularized matrices, stacking of cell sheets containing endothelial cells and engineering vasculature using microfabrication techniques (e.g. 3D-printing, modular approaches, …) [22,60–65]. More details will be discussed in sections 4 and 5. Approaches for angiogenic pre-vascularization use animals as an *in vivo* bioreactor for stimulating blood vessel ingrowth in the construct (Fig. 2a) [2,61]. An example of angiogenic pre-vascularization is the use of the arterio-venous (AV) loop model [61,66]. A vein and artery are microsurgically anastomosed to an AV loop and the loop is transferred to an enclosed implantation chamber to create an isolated microenvironment *in vivo*. Upon implantation of the construct in the chamber, new blood vessels sprout from the AV loop into the construct. The AV loop technique is also implemented in clinical practice [67]. Although the AV loop in this context is not used to provide a source of vessel sprouts into the flap, it serves as a healthy donor vessel for connection with muscle flap. The AV loop model has been adapted to SMTE [2,61,66]. After

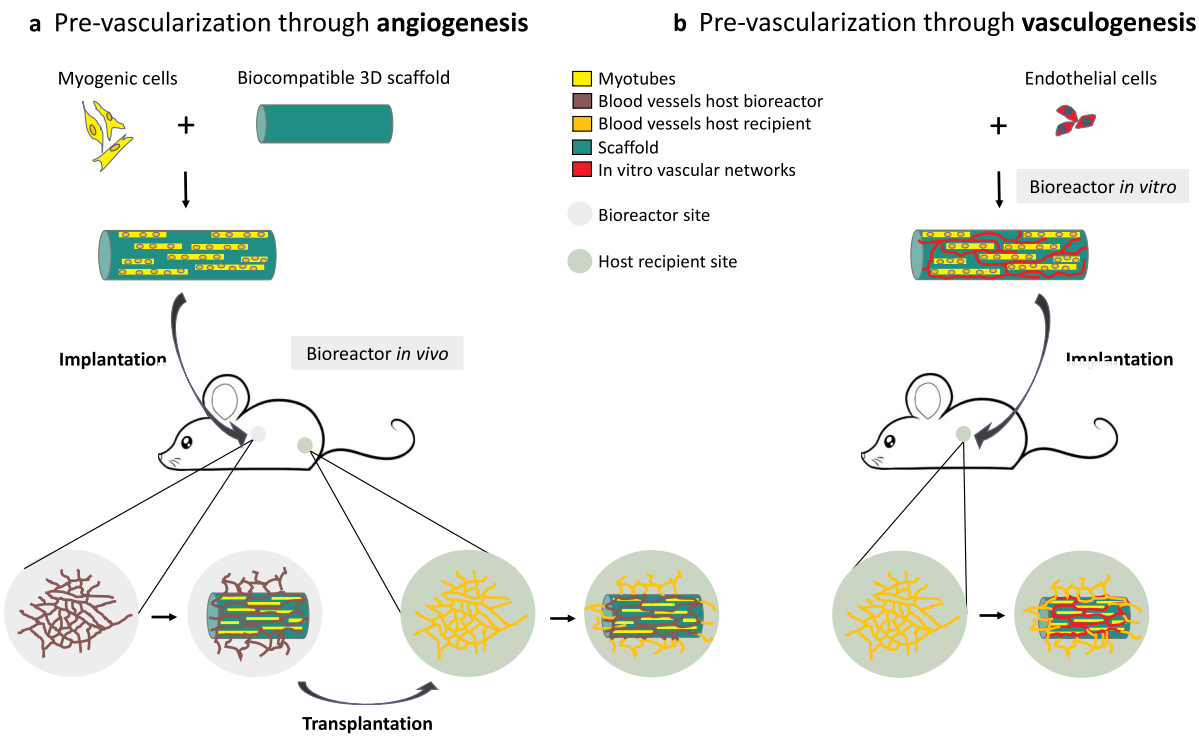

Fig. 2. Pre-vascularization strategies of tissue-engineered constructs. (a) In the pre-vascularization through angiogenesis, a construct with target cells (yellow) is implanted in a bioreactor site *in vivo* allowing for angiogenesis. Through angiogenesis and ingrowth of host vessels (dark red) the construct is provided by blood vessels. Upon transplantation of the prevascularized construct in a host recipient site, a new implantation site or animal, connection between the preformed blood vessels (dark red) and host vessels (orange) takes place. (b) In the pre-vascularization through vasculogenesis, vascular networks are formed *in vitro* by *de novo* formation of vascular networks by endothelial cells (bright red). Upon implantation in the host recipient site, preformed networks (bright red) connect with host vessels (orange), providing perfusion through the whole construct.

prevascularization of the construct by sprouting vessels from AV, it is possible to transplant the construct in the muscle defect [2] (Fig. 2a).

Other factors contribute to the success rate of the pre-vascularization strategy and anastomosis upon implantation. Presence of angiogenic factors, supporting cells such as smooth muscle cells and pericytes, hypoxia and shear stress contribute to a better vascularization outcome [56]. Below, we discuss in detail the factors that influence the SMTE process and how they can be adapted to maximize (pre-) vascularization *in vitro* and *in vivo*.

4. Cell sources for promoting myooid vascularization

Endothelial cells are epithelial cells lining blood vessels and capillaries and play a key role in the revascularization process after wound healing [68]. Various studies have demonstrated the positive effects of endothelial cell addition on vascularization, perfusion and survival of myooids *in vitro* and *in vivo* [2,57]. Different types of endothelial cells are available and are implemented in the tissue engineering field [69]. It is important to choose the proper endothelial cell source for a specific tissue engineering application. Depending on the tissue microenvironment of the isolated endothelial cells, they will have a different phenotype, gene expression pattern, structure and behavior in vascular network formation [70,71]. For example, peripheral blood-derived and cord blood-derived endothelial progenitor (EP) cells were mixed together with the mouse embryo cell line 10T1/2 in a collagen gel and implanted into cranial windows in severe combined immunodeficient (SCID) mice. While peripheral blood EP-formed capillaries regressed after 21 days upon implantation, cord blood EP-formed capillary networks remained present even 4 months after implantation. Moreover, cord blood EP capillary networks showed no leakiness and had a proper cytokine activation [72]. The most frequently used and studied endothelial cell source are human umbilical cord-derived endothelial cells (HUVECs). These cells are easy to isolate, abundant and well characterized. Furthermore, they are capable of endothelial network formation *in vitro* [2,22,57]. However to attain optimal endothelial network formation these cells can be kept in culture only a limited amount of passages (typically less than 10) to ascertain a high differential potential and stable phenotype [73] To address this problem, vascular endothelial stem cells were suggested as a cell source for vascular tissue engineering [74]. These cells have a high proliferative capacity and can produce tens of millions of endothelial daughter cells *in vitro*. In addition, they are able to differentiate into functional blood vessels upon implantation [74]. Other examples of endothelial cell sources include placental-isolated [75], tissue-specific microvasculature-derived endothelial cells, embryonic heart cells [76] and pluripotent derived endothelial cells [77].

As mentioned before, regression of *in vitro* formed endothelial networks can be observed upon implantation [59]. Several groups have successfully aimed at improving network maturation, stabilization and function of blood vessels by the addition of pericytes, mesenchymal precursor cells, fibroblasts and/or smooth muscle cells in tissue engineered constructs prior to implantation [78–80]. These cells are often used in co-culture settings with endothelial cells due to their capacity to differentiate into supportive mural cells around newly formed endothelial networks. Expression of smooth muscle actin is characteristic for this differentiation into mural cells [2,57,78,81,82]. Their presence supports *in vitro* and *in vivo* stabilization, endothelial network formation and prevents blood vessel regression. For example addition of pericytes

Table 1
SMTE models based on naturally occurring extracellular matrices as bioscaffolds. ADSC: adipose derived stem cell, ECM: extracellular matrix, MPC: muscle progenitor cell, PFP: PEGylated platelet free plasma, HUVEC: human umbilical vein derived endothelial cell, TA: tibialis anterior muscle, SIS: small intestinal submucosa, VML: volumetric muscle loss.

Material	Pre- vascularization	Implantation model	Relevant details	Ref.
Porcine SIS ECM	None	Urinary bladder resection in dogs	Acellular, neovascularization in 4 weeks	[105]
Porcine urinary bladder ECM	None	TA/quadriceps VML	Acellular, preclinical rodent model and clinical trial, functional muscle improvement	[33]
Porcine urinary bladder ECM	*In vitro*	Subcutaneous in nude mice	MPC, HUVEC and pericyte seeded; cell seeding enhanced vascularization, myofiber formation and innervation *in vivo*	[32]
porcine SIS ECM (Surgisis)	*In vitro*	Full thickness abdominal full in nude mice	HUVEC, myoblasts, human foreskin fibroblasts seeded; 14 days post-implantation host muscle integration in scaffold	[103]
Psoas major muscle ECM	*In vitro*	TA VML in rats	Addition of PFP hydrogel and ADSC to ECM improved *in vivo* vascularization	[116]

to endothelial cell culture stimulates basement membrane deposition and therefore supports further endothelial network stabilization [83]. Furthermore, addition of fibroblasts to *in vitro* endothelial cell cultures promoted lumen formation through secretion of ECM proteins. The ECM proteins not only provided structural support for cells, but also increased the hydrogel stiffness, which in turn promoted lumen formation [84]. Fibroblasts also secrete soluble factors such as basic fibroblast growth factor (bFGF), which supports vessel formation [85]. Also addition of fibroblasts to endothelial cells on a macroporous polylactic-glycolic acid (PLGA) fibrin-coated scaffold *in vitro* promoted vasculogenesis and is therefore an important cell source in pre-vascularization strategies [48]. In another example, microvascular endothelial cells were cultured on collagen discs in the presence of human foreskin fibroblasts, which resulted in vessel-like structure formations. In the absence of fibroblasts, only cobble-stone morphology of endothelial cells was observed during three weeks [86]. A beneficial effect of fibroblasts on vascular network engineering was also demonstrated in a study comparing co-cultures of myoblasts and endothelial cells with co-cultures of myoblasts, endothelial cells and mouse embryonic fibroblasts. 3D biodegradable polymer scaffolds were seeded *in vitro* with the two cell culture conditions. After transplantation, smooth muscle actin-positive fibroblasts, suggesting differentiation into smooth muscle cells, were co-localized around von Willebrand factor-positive endothelial cell networks. Two weeks post-implantation a higher number of endothelial structures and lumens was observed in the tri culture condition. Presence of large vessel structures ($> 1500\,\mu m^2$) with lumen was seen in the co-culture construct with three cell types one month after implantation, while lacking in the co-culture construct without fibroblasts, supporting the hypothesis of the supportive role of fibroblasts in vessel formation and stabilization [57]. Nevertheless, the thickness of the construct did not exceed 1 mm, so it remains unclear whether this approach could lead to thicker tissues.

5. Mimicking the extracellular matrix: different scaffolds and hydrogels and how they can stimulate (pre-)vascularization of myooids

In the following sections we will discuss the different currently used options to mimic the ECM in SMTE and how these ECM-mimics can influence (pre-)vascularization of the tissue-engineered skeletal muscle. After explaining how the ECM can influence different aspects of skeletal muscle formation, different ECM mimics will be reviewed such as cellular and acellular bioscaffolds (Table 1), hydrogels (Table 2), synthetic and biologic polymer scaffolds (Table 3) and self-assembling approaches (Table 4). A schematic overview of the different concepts to mimick ECM and engineer vascularized muscle constructs is shown in Fig. 3.

5.1. The role of the extracellular matrix in skeletal muscle

The vast majority of skeletal muscle studies focus on its contractile units, the myofibers [43]. Another important but sparsely studied component is the extracellular connective matrix (ECM). The connective tissue forms the supportive framework for the myofibers and thus plays an important role in the structural features of skeletal muscle tissue.

As discussed above, the ECM of native skeletal muscle is organized hierarchically into three fibrous layers (Fig. 1): (i) the endomysium which surrounds a muscle fiber, (ii) the perimysium which surrounds a muscle bundle and (iii) the epimysium which surrounds the entire muscle [87]. Collagen is the major structural protein in skeletal muscle ECM [88,89]. It accounts for 1–10% of muscle dry weight and the fibrillar types I and III predominate in adult skeletal muscle ECM [13]. Other ECM proteins include fibronectin, laminin, elastin and various proteoglycans [89,90]. This particular topology provides the structural framework for the blood vessel walls as well and one strategy for promoting vascularization is by mimicking this topology using an

Table 2
SMTE models based on hydrogels. HUVEC: human umbilical vein derived endothelial cell, ADSC: adipose derived stem cell, TA: tibialis anterior muscle, VML: volumetric muscle loss, SCID: severe combined immune deficiency.

Hydrogel Material	Pre- vascularization	Implantation model	Relevant details	Ref.
Collagen I	None	*In vitro*	Primary human skeletal muscle cells, mechanical stimulation for 8 days increases construct elasticity and myofiber diameter; addition of matrigel to collagen	[127]
Gelatin	None	*In vitro*	C2C12 myoblasts; Micropatterning; electrical stimulation	[133]
Gelatin	*In vitro*	*In vitro*	Primary human myoblast cell sheets and HUVEC cell sheets; thermo-responsive surface to harvest cell sheet	[132]
Fibrin	*In vitro*	*In vitro*	Co-culture primary human muscle cells and HUVECs; interspersed endothelial networks between aligned myofibers	[155]
Fibrin	*In vitro*	TA VML in SCID mice	C2C12, HUVEC and ADSC; Electrospun fibrin microthreads; electrical stimulation; 10 days post-implantation anastomosis with host vessels	[100]

Table 3
SMTE models based on polymer and naturally-derived scaffolds. AV: arterio-venous, EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, IGF: insulin growth factor, HGF: hepatocyte growth factor, HUVEC: human umbilical vein derived endothelial cell, MEF: mouse embryonic fibroblast, NHDF: Normal human dermal fibroblast, TA: tibialis anterior muscle, VEGF: vascular endothelial growth factor; VML: volumetric muscle loss.

Material	Pre- vascularization	Implantation model	Relevant details	Ref.
Collagen	*In vitro*	Subcutaneous in C57B/L6 mice	Dermal microvascular endothelial cells; Fibrin hydrogel; *in vivo* host vessel ingrowth over 8 weeks	[152]
Collagen	*In vitro*	In vitro	HUVEC and L6 myoblasts; microgrooves; endothelial network and myofiber alignment	[98]
Fibrin	None	TA VML in nude mice	C2C12 myoblasts; EDC crosslinked fibrin microthreads; HGF loaded; functional muscle improvement; host vessel ingrowth	[161]
Alginate	None	TA VML in C3H/6J mice	Primary murine skeletal muscle cells; VEGF, IGF-1 loaded; 400 μm pores; memory shaped; reduced scar formation and host vessel ingrowth	[150]
PLLA/PLGA	*In vitro*	Subcutaneous in SCID mice; intramuscular in quadriceps muscle in nude rats; anterior abdominal muscle	HUVEC, MEF, C2C12 myoblasts; matrigel loaded; endothelial co-culture promotes *in vivo* host vessel ingrowth	[57]
PLLA /PLGA	*In vitro*	1. Construct implantation around AV femoral loop 2. Flap transplantation: full thickness abdominal defect	Human skeletal muscle myoblasts, HUVEC, NHDF; Fibrin addition; enhanced cell survival; dense vascular networks	[2]

electrospinning technology as reviewed recently [91].

Besides determining topology, the ECM binds associated non-matrix molecules such as growth factors. Native skeletal muscle ECM plays an important role in muscle development during embryogenesis and regeneration. This role during embryogenesis is facilitated by directly influencing cell behavior through ECM-specific receptors on the cell surface such as integrins [16]. However, the storage and postponed release of growth factors by ECM can also indirectly influence cell behavior through growth factor signalling pathways. Several of these processes occurring during embryonic organogenesis, also take place following injury. The ECM plays a critical role in acute tissue regeneration, a highly regulated process [15]. ECM molecules such as collagens and proteoglycans orchestrate the chemotaxis of myoblasts, their proliferation and differentiation into myotubes [92]. Moreover, the degradation of the ECM by upregulated matrix metalloproteinases triggers the release of the growth factors stored in the ECM. These growth factors include the hepatocyte and fibroblast growth factors, which act on the satellite cells inducing their activation and proliferation while inhibiting their differentiation [93]. Furthermore, ECM and its associated growth factors, released upon ECM degradation, (e.g. VEGF-A and platelet-derived growth factor-BB) may also regulate each step of the angiogenic cascade which is needed to form new blood vessels after injury [94,95].

5.2. Naturally occurring extracellular matrices as bioscaffolds

The ECM-induced regeneration has sparked research for acellular ECM scaffolds in SMTE (Table 1). Bioscaffolds composed of ECM can be derived from tissues by removal of cells through chemical, enzymatic or physical methods [102]. The use of such scaffold materials has been reported to induce a strong angiogenic response upon scaffold degradation [32,33,103–105]. This neovascularization has been observed within 2 months after implantation of decellularized rat abdominal muscle seeded with myoblasts in the oblique abdominis muscles [106]. Also, for decellularized diaphragmatic muscle, the recolonization with host cells *in vivo* of left-over vascular structures has been described and was shown to result in functional vessels 15 days after orthotopic implantation [96] as shown in Fig. 4a. Furthermore, key pro-angiogenic factors involved in mouse angiogenesis were shown to be retained, albeit decreased, upon decellularization. Besides the intact angiogenic growth factors, resident in the ECM, the products released after ECM degradation may contribute to this observed angiogenesis *in vivo*. Indeed, the low-molecular-weight peptides released after ECM degradation were found to have a chemoattractant effect for endothelial cells *in vivo* [107]. So it seems that these ECM-composed bioscaffolds are an appropriate source to promote neovascularization, making an exogenous growth factor supply superfluous. Next to promoting angiogenesis, the ECM also steers other pathways leading to skeletal muscle regeneration. An essential step during skeletal muscle regeneration is the phenotypic switch of pro-inflammatory M1 macrophages to a pro-remodelling M2 phenotype [15]. M2 macrophages are an important cell source in prevention of satellite cell apoptosis [108] and are normally stimulated by immunomodulatory and anti-inflammatory factors (e.g. IL-4, IL-10) [109]. Additionally, peptides derived from the ECM have been suggested to play a crucial role in this transition as well, but the exact cues responsible for this modulation still need to be defined [109]. M2 macrophages not only have an important role in muscle regeneration, but also in vascular remodelling [108,110–112].

Once implanted, the *in vivo* degradation of scaffolds is desirable but the timing of this event is crucial. The goal is to gradually integrate the engineered tissue and promote the formation of new host tissue. Furthermore, biodegradation circumvents foreign body reaction which is a problem occurring with non-degradable biomaterials [113]. ^{14}C labelling studies were used to evaluate the degradation of an acellular SIS (small intestine submucosa) ECM scaffold during remodelling and found 40–60% scaffold removal from the site of remodelling within 4 weeks and complete removal by 60–90 days [114]. Knowledge of the degradation rate is desirable since the resulting degradation products in their turn play a supporting role in the regenerative process.

The remodelling potential of these bioscaffolds has been demonstrated in the restoration of skeletal muscle damage in animal models as well as in patients [32,33,103]. Implantation of ECM at the site of injury was associated with attraction of perivascular stem cells and neovascularization [33]. However, implantation of ECM alone did not generate a sufficient amount of *de novo formed* multinucleated myofibers to fully restore volumetric loss. A more successful approach involves seeding of myogenic cells on decellularized ECM prior to implantation (Table 1). Seeding of autologous myoblasts on homologous acellular matrix, obtained from abdominal muscle fragments, has been studied as an approach to repair abdominal wall defects [106,115]. While the acellular matrix alone resulted in fibrous tissue 30 days after implantation on the oblique abdominis muscle, myoblast-seeded patches showed a preserved structure with abundant presence of blood vessels, innervation and myoblasts. Addition of endothelial cells to the matrix further stimulates vascularization. Bladder acellular matrix scaffolds seeded with either muscle progenitor cells (MPCs) alone or MPCs with endothelial cells and pericytes in a 1:1:0.3 ratio have been compared [32]. Eight weeks after implantation subcutaneously in rats, the scaffolds with endothelial cells and MPCs displayed enhanced vascularization, striated myofiber formation and innervation as compared to MPC seeding alone. Besides bladder matrices, SIS bioscaffolds have

Table 4
SMTE models based on scaffold- and hydrogel free approach. EDL: extensor digitorum longus muscle, TA: tibialis anterior muscle, VML: volumetric muscle loss, MTJ: myotendinous junction, NMJ: neuro-muscular junction.

Material	Pre- vascularization	Implantation model	Relevant details	Ref.
Self-produced ECM	None	TA VML in Fischer 344 rat	Self-contraction into 3D; tendon anchors; MTJ; paxillin positive; *in vivo* vascularization and innervation; *in vivo* NMJ	[163]
Self-produced ECM	*In vitro*	EDL VML; TA VML in C57BL/6 mice	Self-contraction into 3D; response to electrical stimuli; functional muscle improvement *in vivo*; high pre-vascularization	[17]

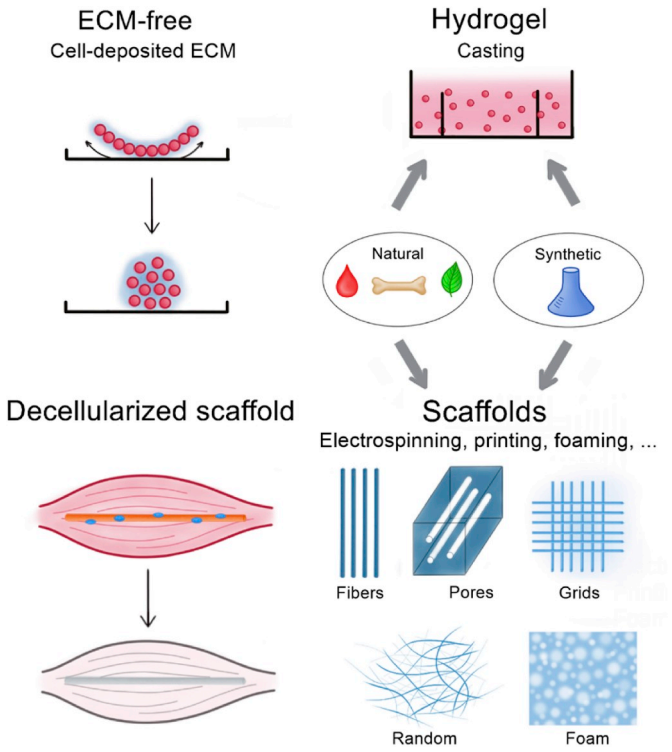

Fig. 3. Tissue engineering concepts to mimick the ECM and engineer vascularized skeletal muscle constructs. In ECM-free constructs, cell layers start to contract, which causes the layer to roll up and form a construct. In this set-up, no ECM is added, all ECM present is deposited by the cells. ECM can also be added to the tissue engineered construct based on natural - such as bone (collagen), blood (fibrin), plant-derived (alginate)- or synthetic resources. This ECM can then be cast, electrospun, printed or foamed into hydrogels or scaffolds. Cells are added during or after this process. A scaffold for tissue engineered constructs can also be made by decellularization, after which the cell-free tissue can be reseeded with cells or can be processed into another type of scaffold.

been used in the context of SMTE as well. In one approach, a triculture system of endothelial cells, myoblasts and fibroblasts was used to construct a muscle graft on SIS derived from the jejunum of pigs [103]. The resulting graft, cultured *in vitro* for a period of 3 weeks, was implanted in the site of a full thickness segment of mice abdominal wall. Two weeks post implantation, the grafts were found to be highly integrated with host muscle and were able to generate about 35% of the intact muscle force.

5.3. Hydrogels

Cell seeding on scaffolds often entails non-homogenous cell distribution throughout the construct [117]. Seeding on scaffolds can give the impression that cells are organized in 3D, while they are actually seeded on 2D surfaces in 3D scaffolds [118]. A real 3D environment is necessary for lumen formation in vascular development [119]. For homogenous seeding, hydrogels can be used to encapsulate cells (Table 2). Another advantage of hydrogels over pre-organized scaffolds is that cells are able to migrate and re-organize easily. The presence of binding and cleavable sites for cells in biological ECM-derived hydrogel materials determines migration and provides molecular cues for proper cell function and organization. Also skeletal muscle differentiation and vascularization events require cell-ECM interaction [49,120]. For example collagen type I, the most abundant ECM protein in adult animals, contains an α2β1 integrin binding site and fibrin-rich hydrogels contain α5β1 and αVβ3 integrin binding sites that promote endothelial cell morphogenesis and tube formation [121]. Next to the molecular properties, the physical properties of hydrogels also play an important role in cell behavior. Hydrogels with an elastic modulus of 12 kPa, mimicking the *in vivo* environment, enhance satellite cell proliferation and self-renewal [9]. Also for myotube formation and striation a proper stiffness is required [122]. As for vasculogenesis, an inverse relationship exists between the extent of vasculogenesis and hydrogel stiffness [122–124], favoring the use of soft hydrogels for SMTE. Several biomolecules have commonly been used to form a hydrogel for SMTE, such as collagen, gelatin, fibrin and alginate (Table 2).

First, collagen is a natural biomaterial that is an important component of skeletal muscle [88]. Collagen is obtained through extraction from animal tissues, typically bones and skin. Several collagen-based scaffolds are currently available on the market, such as Helistat (Integra LifeSciences), Instat Fibrillar (Johnson & Johnson) and Biobrane (UDL Laboratories). The major drawback of this biomaterial is its intrinsic low mechanical properties. Therefore, cross-linking strategies are used. Cross-linking may also be used to control the rate of degradation *in vitro* [125]. However, when choosing an appropriate scaffold for engineering tissue with an intrinsically aligned structure, micropatterning is required as well [98]. For this, micro grooved porous collagen scaffolds obtained through a micropatterned ice template combined with freeze drying has been explored. When coculturing vascular ECs and skeletal muscle myoblasts on the obtained micro grooved collagen scaffold, well-aligned tubule-like structures were formed and incorporated in between highly aligned muscle tissue bundles (Fig. 4c). Next to using collagen as a scaffold, it is also very suitable to be used as a hydrogel. Collagen type I hydrogels support both myogenic differentiation [126,127] and endothelial cell sprouting [55,86]. A combination of both cell types, myogenic cells and endothelial cells, in a collagen hydrogel has not been examined yet for SMTE. Collagen also supports *in vivo* blood vessel ingrowth in mice [128], albeit in combination with fibronectin, known for its important role during vascular development [129]. Implantation of pure collagen gels and sponges showed degradation and invasion of lymphocytes and macrophages over a time course of 26 weeks when implanted in rabbit muscle [130]. Collagen degradation was slower in hydrogels than in sponges and also depended on the manufacturing process. Degradation allows ingrowth of host tissue but a longer maintenance of the scaffold provides the initial support, survival and differentiation of cells upon implantation [130]. Not all collagen hydrogels are suitable for vasculogenesis: a clinically used collagen hydrogel (Zyderm) has been used for SMTE, but this bovine collagen prevented ingrowth of host blood vessels, even when high levels of VEGF were added [131].

Second, gelatin is another source of a natural biomaterial, obtained by hydrolysis of collagen. It is one of the most widely used protein-based polymers with a variety of applications in the food and

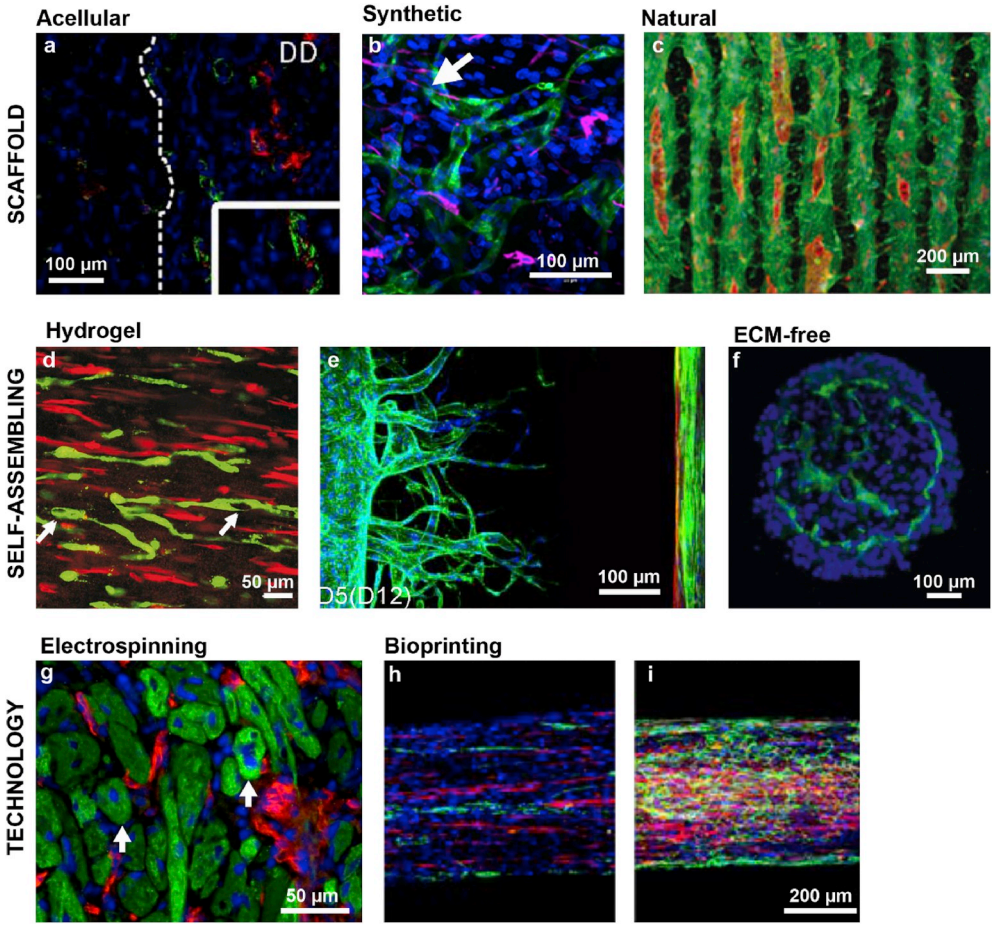

Fig. 4. Engineering vascularized skeletal muscle tissue by various strategies. (a–c) Scaffold-based strategy. A) Decellularized scaffold. Angiogenic response after implantation (15 days), alfa-SMA (green), vWF (red), DAPI (blue). Adapted from Ref. [96]. Copyright 2018, MDPI. b) Synthetic PLGA scaffold seeded with myoblasts (magenta), EC (green), DAPI (blue). Fused myoblasts are indicated by an arrow. Adapted from Ref. [97]. Copyright 2017, CellPress, Elsevier. C) Natural-derived. Preparation of microgrooved collagen porous scaffolds from micropatterned ice line template. Formation of vascularized and well-ordered muscle bundle tissue after 2 weeks of culture. F-actin (green), nuclei (blue), CD31 (red) Adapted from Ref. [98]. Copyright 2017, Mary Ann Liebert. (d–f) Self-assembling approach in the presence or absence of ECM. d,e) Hydrogel casting. D) Endothelial networks (green) between aligned myofibers (red) formed in a fibrin hydrogel under tension. Adapted from Ref. [22]. Copyright 2015, Mary Ann Liebert. E) Formation of channels in hydrogels by casting method. Muscle bundles and macrovasculature was formed by injecting respectively C2C12 cells or HUVECs within separate sacrificial channels embedded in a collagen hydrogel. Immunostaining of F-actin (green) and sarcomeric alfa-actinin (red) of muscle bundles revealed that HUVECs sprouted toward muscle tissues and formed capillary networks. Adapted from Ref. [99]. Copyright 2017, Elsevier Ltd. F) Self-assembling of monolayers of skeletal muscle and endothelial cells. Microcapillary organization of endothelial cells (green) in the skeletal muscle. Adapted from Ref. [17] Copyright 2013, Springer Nature. (g–i) Advanced technologies. G) Aligned electrospun fibrinogen/alginate scaffold. MHC (green), CD31 (red) and DAPI (blue) staining of VML defects treated with C2C12-seeded scaffolds after 2 weeks implantation. High densities of centrally nucleated myofibers (arrows) and vasculature are present. Adapted from Ref. [100]. Copyright 2018, Elsevier Ltd. h-i) 3D bioprinted prevascularized muscle construct with decellularized ECM as bioink. H) 3D bioprinted construct with ECM containing a mixed cell population. I) Endothelial and muscle cells are encapsulated in respectively vascular-derived and muscle-derived ECM bioink in the shell and core of the printing cartridge. Microvascular networks are obtained in the muscle bundle. CD31 (green), MHC (red), DAPI (blue). Adapted from Ref. [101]. Copyright 2019, Elsevier Ltd.

pharmaceutical industry. Gelatin is produced in massive quantities at low cost, is biocompatible and biodegradable. Therefore gelatin is often used as a substrate for cell growth and differentiation [132,133]. Nonetheless, gelatin suffers from the same drawback as collagen namely its mechanical weakness, resulting in the need for cross-linking or combination with other polymers and rapid degradation [134–136]. Examples of crosslinkers are chemical agents, such as glutaraldehydes, carbodiimide and genipin, and enzymes, such as transglutaminase [135,136]. Also methacrylated gelatin (GelMA) is used as hydrogel and is crosslinked by exposure to ultraviolet light [133,135]. Cross-linking with chemical agents has been shown to be related with poor cell proliferation and host tissue ingrowth. In contrast, enzymatic cross-linking allowed vessel ingrowth [136]. In general, gelatin supports myogenic differentiation and vasculogenesis *in vitro* and different pre-vascularization approaches of tissue engineered skeletal muscle constructs have been evaluated [28,132,133,136,137]. Despite *in vitro* characterization of gelatin, further research is needed to examine *in vivo* characteristics in skeletal muscle regeneration.

A third biomolecule used for hydrogels is fibrin, which has an established role in wound healing, where it serves as a provisional matrix for tissue repair. It was assumed that the use of fibrin as a scaffold material for tissue engineering approaches replicates the support during wound healing. In the same context of wound healing, fibrin is known for its pro-inflammatory features. However, in the bone regeneration field, there has been a paradigm shift from minimizing the immune response towards modulating inflammation, opening up new possibilities for the use of fibrin [138]. From a tissue engineering point of view, the high capacity of fibrin to bind growth factors such as VEGF, FGF and insulin-like growth factor, makes it an intriguing polymer for use as a scaffold for vascular tissue engineering purposes [139]. From a clinical point of view, production of autologous fibrin gel from the patient's own blood is possible to minimize immune response [140]. Our group has shown that the co-culture of muscle cells and endothelial cells embedded in fibrin hydrogels allows aligned myofiber formation and interconnected endothelial networks (Fig. 4d) [22]. Another approach using a fibrin-based 3D engineered muscle fiber bundle was recently developed to investigate the crosstalk between vasculature and a muscle fiber bundle *in vitro* [99]. In this approach, a fibrin hydrogel with C2C12 myoblasts was injected into a 600 μm thick microchannel surrounded by two 3D vascular structures in a collagen gel with similar size. This *in vitro* model was used to understand the formation of vascularized muscle tissue and to test the crosstalk between developing vasculature and engineered skeletal muscle (Fig. 4e). However, its direct application potential towards a clinical setting remains to be addressed. Fibrin hydrogels require protection against fibrinolytic enzymes, present in the medium. Also, as occurs naturally in the wound healing process, fibrin is broken down *in vivo*. An approach to counteract degradation of fibrin-based SMTE constructs is to induce

remodelling of the fibrin ECM by autologously deposited collagen by included cells [141]. Gradual degradation of fibrin can also be used as a mechanism for release of angiogenesis stimulating growth factors, such as VEGF, which improves host vessel invasion [142].

Another biomaterial is alginate, a polysaccharide which is not derived from animal tissues but from algae. It has carboxylic acid functional groups that allow covalent modification with functional peptides, such as the cell adhesion sequence arginine glycine aspartic acid (RGD) [143,144]. Furthermore it is possible to vary the cross-linking rate of alginate, resulting in alginate hydrogels with various mechanical properties [145,146]. Alginate hydrogels can be lyophilized to induce macroporosity [147,148] and are being explored as a delivery system for growth factors and cells in muscle loss animal models [144,148–150].

However, working with hydrogels alone may not be sufficient to restore large muscle deficits such as VML due to their weak mechanical properties and rapid degradation *in vivo* [151]. A combination of bioactive hydrogels and supportive scaffolds may overcome this problem. Collagen I scaffolds combined with a fibrin hydrogel containing human dermal microvascular endothelial cells supported *in vitro* vascularization. The microvascular endothelial cells formed $CD31^+$ lumenized capillaries already 3 days post-seeding. CD31 is a specific marker for endothelial cells and is involved in maintenance of endothelial cell junctional integrity, giving an indication about the network maturity [152,153]. The same scaffold was seeded with adipose-derived stem cells (ADSC) and implanted subcutaneously in mice. Cell-free scaffolds supported *in vivo* blood vessel ingrowth after 2 weeks of implantation, however ADSC addition improved vascular infiltration [152]. A cost-effective source of fibrinogen is platelet free plasma (PFP) which is obtained from human blood in a one step process. Because fibrin is rapidly degraded once implanted in the wound site *in vivo* [154], polyethylene glycol (PEG) modification can be performed to prolong the degradation time. A composite scaffold, consisting of a PEG - modified hydrogel on a decellularized muscle ECM scaffold supported robust and stable vascularization *in vivo* upon subcutaneous implantation in mice. Addition of ADSCs increased significantly the vessel density in the implanted constructs, but only a limited presence of muscle cells was observed [116].

5.4. Polymer and naturally-derived scaffolds

Scaffolds provide support and are able to deliver cells and growth factors upon implantation *in vivo*. Several scaffolds are described in the context of pre-vascularization of skeletal muscle constructs (Table 3). A distinction can be made between synthetic polymer-based and naturally-derived scaffolds.

Commonly used synthetic polymer scaffolds in SMTE are poly-(L-lactic acid) (PLLA) and polylactic-glycolic acid (PLGA). A 3D biodegradable 50% PLLA and 50% PLGA polymer scaffold with pores of 225–500 μm allows diffusion through the whole construct even in the absence of vascularization. Seeding of endothelial cells (either embryonic stem cell–derived endothelial cells or HUVECs), myoblasts and embryonal fibroblasts resulted in vessel-like structures between partially aligned, multi-nucleated myofibers after 10 days of differentiation *in vitro* [2,57]. After implantation in SCID mice, further maturation of blood vessels and myofibers was observed. The addition of human endothelial cells to the scaffolds resulted in blood vessels derived from these cells which significantly improved perfusion and implant survival *in vivo*. The presence of red blood cells in these vessels indicated anastomosis with host blood vessels. Quantification by a labeled lectin perfusion assay demonstrated that 41% of the human cell derived blood vessels were perfused [57]. However only a small portion of the implanted scaffold was occupied by interconnected vessel-like structures [82]. Further addition of fibrin, because of its pro-angiogenic properties [156], resulted in dense vascular networks and improved human cell survival [82]. Similarily, PLLA/PLGA sponges, together with fibrin, were seeded with adults ECs, human myoblast and various supporting cells [157]. This resulted in branched vessel networks that were more developed and complex than those derived from HUVECs (Fig. 4b). Furthermore, these branched networks within the engineered muscle tissue were shown to be functional, as evidenced by the anastomosis with the host vasculature within 9 days of implantation in a full-thickness defect.

These scaffolds (without fibrin) have also been implanted around a mouse vein and artery anastomosed to an AV loop [2]. Newly formed host vessels derived from the AV loop, invaded the scaffold and anastomosed with the implanted endothelial networks. One or two weeks after implantation, the muscle graft with the host vasculature ingrowth and AV loop was transplanted into an abdominal wall defect. Pre-vascularization with endothelial cells of the construct resulted in improved host integration and vascularization [2]. This study is an example of *in vivo* pre-vascularization (Fig. 2) of tissue constructs and highlight its beneficial effect on host integration and cell survival.

Compared to PLLA/PLGA polymers with randomly organized pores, scaffolds with engineered microvasculature could further improve cell function and survival in the center of the construct. A microchannel structure has been engineered in a poly(glycerol sebacate) (PGS) elastomer scaffold that allowed perfusion *in vitro* throughout the construct [158]. The fabricated microchannels could be perfused and seeded with HUVECs but they did not attach well to the PGS. At the outside of the PGS structure, adult human skeletal muscle-derived cells were seeded and permeability of PGS scaffold was demonstrated by perfusion of a myotoxic drug through the channels. As the muscle tissue was the main tissue type, this was called the parenchym site. PGS is degradable and permeable, therefore enabling vascular-parenchymal transport. However, poor myofiber differentiation and survival on the PGS was observed [158]. *In vivo* implantation experiments showed elastomer degradation and vascular host invasion 1 week post-implantation. Considering the abovementioned results, the combination of a prefabricated perfusable microvasculature with a hydrogel may be best suited to obtain large well-differentiated myooids.

As an alternative to synthetic polymers, naturally derived and ECM-based biomaterials are also evaluated in vascularization strategies for SMTE (Table 3). Examples of biomaterials are collagen, fibrin and alginate-based scaffolds. They grant-highlight > support cell attachment and are susceptible to cell-based remodelling *in vitro* and *in vivo*. The advantage of using naturally derived scaffolds is the presence of biologically active degradation products that positively affect cell migration and proliferation [159]. In contrast, degradation products from synthetic scaffolds typically do not have such positive effect. The main difference of scaffolds versus soft hydrogels, is that scaffolds provide a better initial mechanical support [31,126].

Collagen scaffolds are biocompatible and allow *in vitro* vasculogenesis when seeded with endothelial cells. To show the vasculogenic potential of collagen scaffolds *in vivo*, cell-free collagen type I scaffolds with an 80 μm pore size, combined with fibrin hydrogel were implanted and allowed host vessel ingrowth with a higher vascular volume versus fibrin alone [152]. Aligned collagen nanofibrillar scaffolds were used to imitate the ECM pattern secreted by vascular cells *in vivo* [160]. In this set-up, endothelial cells migrated in the micro-patterning direction of the nanofibrils through integrin α1 binding to collagen [160]. Moreover, collagen scaffolds allowed simultaneous differentiation of myogenic cells and endothelial cells into respectively myofibers and tubule-like structures [98].

Other biomaterials such as fibrin scaffolds, not only have pro-angiogenic properties and allow myofiber formation *in vitro*, as mentioned previously, but also support muscle regeneration *in vivo* [161]. To decrease the degradation grade by fibrinolysis of fibrin scaffolds a cross-linking strategy such as treatment with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide can be used [161]. Similar to hydrogels, growth factors such as HGF can be incorporated in the scaffold. Implantation in mice of fibrin microthreads loaded with HGF stimulated host myofiber

regeneration and enhanced vascular ingrowth [161]. Fibrin microthreads were produced by extrusion of fibrinogen and thrombin and support cell alignment [161,162]. Another way to induce myofiber alignment in the construct, is using aligned electrospun fibrin hydrogels (Fig. 4g). This hydrogel contained additionally 15% alginate. Upon seeding of mouse C2C12 on the fibrin-alginate fibers, highly aligned striated myofibers were formed able to contract spontaneously or upon electrical stimulation [100]. Implantation for 4 weeks in tibialis anterior muscle in immunodeficient mice resulted in scaffold degradation, muscle regeneration and ingrowth of host vessels. When HUVEC and adipose-derived stem cells (ADSC) were added to induce pre-vascularization, no scaffold degradation was present after implantation. Increased collagen deposition surrounding pre-vascularized scaffolds was observed preventing degradation and integration with surrounding tissue. However some evidence of anastomosis with host blood vessels was observed at 10 days post-implantation [100].

A less commonly used biomaterial in SMTE and pre-vascularization approaches is alginate [144,147,149,150]. *In vitro*, alginate scaffolds supported partial myoblast survival when modified with an RGD peptide and HGF addition [144]. Also using a macroporous alginate scaffold with 400–500 μm diameter aligned pores improved significantly the myoblast survival in comparison to nanoporous scaffolds. A macroporous alginate scaffold modified with RGD was used as vascular progenitor cell carrier in an ischemic murine hindlimb musculature model [149]. The scaffold maintained implanted endothelial cell survival, engraftment and improved angiogenesis in comparison to direct cell injection. However no analysis was performed to assess the effect of the alginate scaffold on muscle regeneration *in vivo* [149]. Macroporous alginate scaffolds have shape-memory, allowing to deliver scaffold, cells and growth factors by a minimal invasive procedure through a catheter [150]. In a tibialis anterior injury mouse model, the alginate scaffold was used to deliver myoblasts, IGF-1 and VEGF, which lowered scar formation and enhanced muscle function. VEGF addition increased angiogenesis *in vivo* at the injury site [150].

To conclude, skeletal muscle tissues have been engineered *in vitro,* using synthetic and naturally derived scaffolds, which *in vivo* partially support the survival of engineered myofiber and display some evidence of integration with host vasculature.

5.5. Scaffold- and hydrogel-free (ECM-free) approaches

ECM-free SMTE (Table 4) is based on the ability of myofiber monolayers to contract spontaneously resulting in detachment from the substrate and rolling up of the cell sheet [17,29,39,163–165]. In general, freshly isolated cells from muscle biopsies from rodents containing SCs, endothelial cells and fibroblasts are used. This heterogenous cell population is then cultured on a low attachment surface such as silicone to form cell sheets. Initial cell adherence is assured by coating the dishes with collagen type I or laminin. Once cell confluence is achieved, growth medium is changed to serum-deprived differentiation medium supporting myocyte fusion into multinucleated myotubes. Simultaneously minutien pins or tissue engineered cell anchors are pinned on the cell monolayer. No ECM is used for initial engineering, but during culture cells secrete their own ECM products. Upon spontaneous cell layer detachment, a muscle bundle is formed in between the anchors [17,29,39,163–165]. Such a muscle bundle, which mimicks the organization of mature skeletal muscle with differentiated myofibers, vascular networks and produced ECM shows spontaneous contraction *in vitro* [13]. This self-assembling approach provides a stem cell niche with close cell-cell and cell-ECM interactions, necessary for proper regeneration *in vitro*. Pax 7 + satellite cells were observed in close association with the myofibers in the muscle bundle, surrounded by laminin, resembling the situation *in vivo*. Importantly, microcapillary-like organization of endothelial cells within the skeletal muscle was observed (Fig. 4f) [17]. This level of overall maturity of the myooids is achieved by the use of freshly isolated cells, since culturing cells *in vitro* over several passages attributes to change in stem cell behavior [166,167]. In a similar approach, after 28 days *in vivo*, the muscle constructs showed an increased force generation, microvascular ingrowth and functional repair [163,165].

This approach also allows to combine different self-assembled cell constructs. Bone-tendon anchors were engineered by delamination of bone marrow-derived cell monolayers and pinned on developing muscle monolayers. This resulted in formation of myotendinous junctions (MTJ) between the tendon organoid and myooid [163]. The presence of MTJs resembling the structure of neonatal MTJs were shown *in vivo* [29]. Spinal cord explants isolated from fetal rats were also combined with muscle cell monolayers. The neural tissue developed neural extensions towards the muscle bundle with presence of acetylcholine receptors at the neuromuscular junction (NMJ). The NMJs were functional since isolated electrical stimulation of spinal cord explants resulted in muscle bundle contraction. This approach mimics *in vivo* skeletal muscle well. Experiments have only been performed with rodent or avian derived cells. It would be of great interest to evaluate the approach with human derived tissue.

6. Shaping 3D tissues by advanced technologies

6.1. Electrospinning

A highly aligned ECM provides the structural framework for the blood vessel walls. An additional strategy for promoting vascularization is mimicking this topology. One approach to achieve a highly aligned structure promoting vasculogenesis uses electrospinning technology as reviewed recently [91]. The benefit of using anisotropic topology for regulating the orientation and density of microvessel formation has been shown by several groups. For example, electrospun polycaprolactone (PCL) – cellulose fibers were found to guide morphogenesis and regulate cytoskeleton organization of HUVECs. This lead to organized capillary-like tube formation as compared to randomly oriented nanofibers [91]. Furthermore, *in vivo* vascularization by host vessel infiltration was found to be accelerated throughout the whole tissue construct resulting ultimately in rapid vascularization. However, these concepts were not yet applied to skeletal muscle tissue engineering. Besides creating fibrous topographical features in synthetic polymers, fibrous scaffolds prepared from natural polymers have been explored as well, although the application towards muscle tissue engineering is limited (Fig. 4g). One group compared the infiltration of interstitial and endothelial cells when implanting different electrospun scaffolds into the interstitial space of a rat vastus lateralis muscle [168]. Electrospun scaffolds made of collagen were rapidly infiltrated by both cell types and this resulted in functional blood vessels within 7 days. In contrast, electrospun gelatin and the synthetic electrospun polymers PGA, PLA and PGA/PLA co-polymer induced fibrosis without infiltration. Further examples on the use of electrospun scaffolds have been included throughout section 5.4.

6.2. Bioprinting

Using 3D-bioprinting, cells can be positioned into any pattern, making it a promising strategy to promote structured macrovascular network formation in engineered tissues. However, inducing precise 3D spatial cell organization to mimic the highly oriented microvascular growth is still a challenge when it comes to developing vascularized tissues. 3D bioprinting is generally performed in one of two ways: i) indirect vessel creation by means of a sacrificial hydrogel or ii) direct endothelial cell printing (e.g. droplet based bioprinting, light/laser based bioprinting, …), both of which have been extensively reviewed by others [169,170]. The concept of indirect vessel creation was demonstrated by printing a polyester based thermo-polymer as sacrificial ink to generate a vessel network in an alginate scaffold [171]. Removal of the liquid thermo-polymer by heating followed by adding gelatin to

the alginate channels created a template for highly organized microvessels. These channels were subsequently injected with HUVECs resulting in the endothelialization of the inner walls of the channels after 8 days. Similarly, Pluronic F127 was used to create a highly organized vascular network. In this approach, the Pluronic was surrounded by a castable ECM, gelatin methacrylate, and removed upon cooling. Again, when injected with HUVECs a vessel network was obtained within the structure [172]. The indirect printing method has not yet been described for a vascularized skeletal muscle construct.

While promising, the high number of intermediary steps required to obtain final cell laden vessel structures makes indirect printing techniques less desirable. Direct printing of a vascular bed out of a cell laden bio-ink can be obtained through different deposition techniques. Coaxial nozzle bioprinting is one approach to construct complex 3D networks starting from inks containing the cells of interest. Its potential for creating a 3D tissue with organized vascularization was recently shown by bioprinting endothelial cells encapsulated inside microfibers, leading to the formation of a vascular bed [173]. Subsequent seeding with neonatal rat cardiomyocytes resulted in a functional 3D muscle tissue which spontaneously contracted. Recently, this approach has been advanced by using skeletal muscle decellularized and vascular decellularized ECM bioink containing human skeletal muscle cells and HUVECs, respectively (Fig. 4 h, i). With this approach a highly structured vascularized muscle was obtained, which resulted upon *in vivo* implantation in 85% functional recovery in VML injuries [101]. Droplet-based bioprinting, another direct printing approach, achieved an improved resolution by printing features under 200 μm, which approximates the size of *in vivo* vasculature [174]. This process shows promise in creating bioprinted constructs containing multiple cell types patterned at high resolution [174]. Light/laser-based bioprinting uses lasers or UV images to print equally fine features to create specific patterns for vessel networks. The use of this technique was demonstrated to create a photopolymerized vascular structure containing HUVECs [175]. Using this approach, the endothelial cells formed lumen-like structures *in vitro* which resulted in the survival and progressive formation of the endothelial network in the prevascularized tissue *in vivo*.

7. Stimulation to improve pre-vascularization and myofiber formation in SMTE

The constructed myooids can be stimulated *in vitro* by electrical stimulation, flow perfusion or mechanical stimuli. The main goal is typically advancing myofiber maturity, however vascularization of the 3D bioengineered skeletal muscle may also be positively affected.

7.1. Mechanical stimulation

For skeletal muscles in the human body it is known that exercise induces hypertrophy, whereas a lack of adequate load results in atrophy. Mimicking mechanical stimulation of myofibers is an appealing way to stimulate myofiber maturation in SMTE. The first description of mechanical stimulation of skeletal muscle cells dates back 3 decades, with proof that mechanical forces caused myoblasts to fuse into parallel arrays of myotubes, significantly longer than myotubes that arise from static culture conditions. Furthermore, intermittent stretch caused increased protein synthesis leading to a hypertrophic response [176]. The tetanic and twitch contractile responses of tissue constructs implanted onto the latissimus dorsi muscle of mice also increased significantly after *in vitro* cyclic mechanical stimulation [177].

These findings show that muscle hypertrophy *in vivo* can be translated into *in vitro* applications as well. Current regimens exert cyclic and uniaxial forces to the engineered muscle constructs with an amplitude of stretch ranging between 6.7 and 20% of construct length with frequencies of 0.1–1 Hz for a period between 30 min and 10 days [178]. Although uniaxial stretch is mainly used in current regimes, the direction of the induced stretch needs to be considered thoroughly as it has been demonstrated that specific types of mechanical stretch activate distinct signalling pathways through mechanotransduction [179]. In addition, the effect of mechanical stress on muscle tissue seems to be broader than hypertrophy as proangiogenic factors, including vascular endothelial growth factor (VEGF), were also upregulated in response to mechanical stimulation [76].

The latter findings can be linked to the fact that angiogenesis occurs in a mechanically dynamic environment [180]. Identifying the biophysical aspects of angiogenesis regulation is therefore crucial to advance vascularization of tissue engineered products. *In vitro* studies have revealed the direct impact of uniaxial and biaxial cyclic stretch on endothelial cell activities that are involved in angiogenesis [181–183]. Bovine aortic endothelial cell migration and tube formation were found to increase in response to cyclic strain over a period of 24 h [181]. The importance of mechanical stimulation in vascular endothelial cell patterning and sprout formation have also been demonstrated *in vitro* for both 2D and 3D culture systems in which mechanical strain was found to regulate the spatial location of cell proliferation and the directionality of the sprout formation respectively [182]. In addition, 5% strain matching physiological strain resulted in increased endothelial cell survival and tubulogenesis, in contrast to pathological strain of 20% [183].

7.2. Electrical stimulation

Electrical stimulation of three-dimensional C2C12 myoblast cultures has been investigated showing enhanced sarcomere formation which improves contractile force generation [37]. Another effect of electrical stimulation is the upregulation of late muscle maturation marker MRF4, indicating accelerated skeletal muscle cell maturation [184].

In contradiction to previous studies, a negative impact of electrical stimulation upon myogenic differentiation has been shown as well. Upon electrical stimulation of chick neonatal muscle tissue, downregulation of MyoD, myogenin and acetylcholine receptor was observed [185]. Also, when applying high voltages to C2C12 engineered muscle constructs a decrease in force generation and excitability was found, while stimulating 2D cultures at high frequencies resulted in an anabolic signal resulting in increased protein synthesis [36]. Taken together, it is clear that timing and intensity of the stimulus can induce both myogenic differentiation as well as tissue damage. The thin border between both effects and the pathways behind them still need to be explored.

Also for electrical stimulation, induction of angiogenesis in skeletal muscle has been described. Already in 1976, capillary growth induction was reported in response to slow frequency stimulation of rabbit fast muscles for only 4 days [186]. A more recent report described similar results induced by electrical stimulation of rat extensor digitorum longus muscles [187]. Increased capillary proliferation were observed through NO-induced upregulation of VEGFR-2 and VEGF upon 2 and 4 days of electrical stimulation. Whether the release of NO was directly due to the electrically induced muscle activity or indirectly due to e.g. increased blood flow and shear stress after electrical stimulation, remains to be elucidated. Electrical stimulation has, to our knowledge not been applied *in vitro* to improve vascularization of myooids.

The growing interest in applying electrical stimulation to tissue engineered products has driven the search for biomaterials with high electrical conductivity (reviewed in Ref. [188]). These so-called conducting biomaterials were first based on carbon nanotubes, carbon nanowires, graphene and metallic particles. However, their intrinsic limitations such as non-biodegradability, *in vivo* toxicity and inhomogeneous distribution of the conducting particles in composite materials have driven the switch towards organic conductive polymers such as polyaniline, polypyrrole, polythiophene and their derivates. Their conductive nature allows the embedded cells and eventually the engineered tissue to be electrically stimulated. This has been

demonstrated for tissue engineered muscle using composite gelatin-polyaniline nanofibers fabricated by electrospinning [189]. When differentiating C2C12 myoblasts on those composite nanofibers were compared to myoblasts grown on gelatin nanofibers, myotube formation and maturation improved. Next, the electrical conductivity of the substrate was used to apply electrical pulses to the tissue engineered muscle. This resulted in improved functionality of the myotubes as shown by increased calcium transients and stronger contractions. While the beneficial effect of topographical cues for differentiation of myoblasts is well established, the synergetic effect of electroactive cues is only recently emerging. For example, electrospun composite scaffolds containing polyaniline in PCL fibers combined the aligned morphology of electrospun PCL and the electroactivity of polyaniline [190]. When culturing C2C12 myoblasts on such scaffolds, myotube maturation was enhanced in the aligned conductive nanofibers as compared to random scaffolds or non-conductive aligned scaffolds. The beneficial effect of combining conducting polymers with electrical stimulation makes these polymers very attractive as substrates for skeletal muscle tissue engineering.

7.3. Medium perfusion

The importance of fluid flow and associated shear stress is well established in vasculogenesis [191–193], remodelling of immature vessels [194], and maintenance of blood vessels [195]. For the remodelling in particular, differences in blood flow between vessels in a branch point are suggested to be an important regulator of blood vessel pruning [196]. Flow was also found to be a regulator of arterial-venous differentiation in chick embryo yolk sac by changing the global patterning of the arteries and veins [197]. Not surprisingly, in both physiological and pathological states, there is a close association between shear stress and the extent of vascularization [193]. This association is regulated by a combination of chemical and mechanical signalling and mediated by key endothelial cell transcription factors such as VEGF-A [198], Neuropilin-2 [199], Angiopoietin 2 [200] and several others [201]. However, the mechanism by which shear stress is sensed is not known and probably miscellaneous sensing mechanisms are involved [202]. One way to induce a fluid flow in an engineered construct *in vitro* is by using a bioreactor. Bioreactors allow the researcher to create a biomimetic cell culture environment with supply of fresh nutrients, oxygen and other chemical signals such as growth factors accompanied by the draining of metabolic waste products. A practical advantage is that they also allow the control of sterility and operating conditions such as pH and/or temperature.

To aid in understanding the association of shear stress with vascularization, bioreactors have been used in *in vitro* experiments, which have shown that *in vitro* shear stress was associated with improved endothelial cell retention of prosthetic vascular grafts [203]. Also, shear stress induced transient reorganization of cytoskeletal and adherens junction proteins [204,205]. Therefore, medium perfusion is believed to accelerate vascular tissue morphogenesis which can ultimately enhance the development of thicker tissue constructs. In addition, bioreactors also promote the viability and long-term maintenance *in vitro* of skeletal muscle precursor cells seeded and cultured within 3D porous collagen sponges [206]. Nonetheless, high shear stress as a result of perfusion may have a negative impact on the viability since striated muscles in general are known to be shear-sensitive [207]. Again, the equilibrium between a beneficial and a detrimental shear stress still needs to be thoroughly explored. To conclude, bioreactor-based 3D culture systems represent a promising approach to increase tissue functionality and applications are expected to further expand in coming years.

8. Conclusions and perspectives

In this review, we have given an overview of the different SMTE approaches and their corresponding pre-vascularization strategies. We have discussed the way pre-vascularization can be influenced by using various scaffold and cell types and by experimenting with stimulation forms and culture conditions. Vice versa we have also discussed how pre-vascularization in turn influences the survival and integration of a tissue-engineered construct in a host. Dependent on the application of the tissue engineered skeletal muscle construct(s), one SMTE strategy may be preferred over another.

For repair strategies of VML, different strategies have been described with variable success towards the (pre-)vascularization of myooids. Tissue engineered skeletal muscle can also be developed for *in vitro* disease models or pre-clinical *in vitro* models for drug screening. Also for these *in vitro* models, myooids would benefit from perfusion and stimulation for further maturation of the construct. Combining a heterogenous cell population from minced muscle, as described for the ECM-free engineering method results is an approach in which native muscle organization can be reproduced reasonably well, including a vascular network and a niche for residing satellite cells. This may be warranted for disease modelling, drug testing and developmental research. However, constructs only have a submillimeter thickness and the lack of cell expansion precludes the use of this approach for VML repair.

The use of hydrogels allows for volume creation and homogenous distribution, migration and organization of cells throughout the construct. However, working with hydrogels alone may not be sufficient to restore large muscle deficits such as VML due to their weak mechanical properties and rapid degradation *in vivo*. Therefore use of bio-active scaffolds may provide the necessary support to large myooids. The use of natural materials, both for the scaffold and the hydrogel, allows for a phased and ultimately complete degradation of the construct. Additionally, the breakdown products supply biological signals, further promoting integration of the construct into the host tissue. A combination of a hydrogel with a naturally derived scaffold, seeded with cells with myogenic potential and endothelial cells may be the most promising tissue engineering strategy for clinical purposes. Addition of other cell types and optimized experimental conditions *in vitro* such as perfusion and electrical or mechanical stimulation, may further improve construct survival and function *in vivo*. However, little is known to date about the influence of applying external stimuli on vascular networks in engineered tissues. Moreover, although stimulation strategies are known to be indispensable for enhanced muscle maturation *in vitro,* studies on this are sparse and the basic science behind it often not fully understood. For stimulation strategies, we believe the field of SMTE would benefit from the combination of conducting biomaterials and stimulation. However, the use of conducting biomaterials has not yet been studied in the context of vascularizing engineered muscle.

The role of biomaterials in tissue engineered approaches has thus far been studied in relatively simple systems, lacking the complexity of skeletal muscle tissue in terms of different cell types. At the moment, many biomaterial-based approaches have not been studied in the context of vascularization, so the question remains if these biomaterials support the development of organized (micro-)vascular networks. Also, although several approaches demonstrated success in fabricating thin, simple vascular networks, it is likely that a synergistic combination of several methods will be required to enable functional vascularization of complex tissue engineered muscle.

Recent developments in 3D bioprinting have demonstrated advantages for creation of a structured tissue, however these methods also come with disadvantages. Those need to be addressed to obtain a simple, accurate and efficient way to engineer vascularized muscle tissue. One of the major challenges is the creation of a micro-to macrovascular network mimicking the complexity of a hierarchical vascular network, including the different cell types constituting the blood vessels. The second challenge is the integration in the 3D environment composed of dense muscle tissue. Progress in this area is expected to be driven by further material or technical developments. Indeed, most

studies on bioprinting were focused on either vascularization or skeletal muscle engineering, the combined approach towards integration of vasculature in skeletal muscle is limited.

Further important obstacles preventing application of these conditions is the need for customized bioreactors and optimization of culture procedures such as cell culture media supporting the different cell types present in a more complex construct.

To conclude, we can state that considerable progress has been made in the last years towards vascularization in skeletal muscle tissue engineering, resulting in an overall increased survival of the constructs after implantation. However, it is clear that thus far a perfect solution to incorporate a vasculature in a myooid is not yet existing and many factors influencing the process are still poorly understood.

Declaration of competing interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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List of abbreviations

3D	three dimensional
ADSC	adipose derived stem cell
AV	arterio-venous
bFGF	basic fibroblast growth factor
ECM	extracellular matrix
EDL	extensor digitorum longus (muscle)
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EP	endothelial progenitor
GelMA	methacrylated gelatin
HGF	hepatocyte growth factor
HUVEC	human umbilical vein endothelial cell
IGF	insulin growth factor
IL	interleukin
MEF	mouse embryonic fibroblast
MPC	muscle progenitor cell
MRF-4	myogenic regulatory factor-4
MTJ	myotendinous junction
NHDF	normal human dermal fibroblast
NMJ	neuro-muscular junction
NO	nitric oxide
PEG	polyethylene glycol
PFP	platelet free plasma
PGS	poly(glycerol sebacate)
PLGA	poly(lactic-*co*-glycolic acid)
PLLA	poly(L-lactic acid)
RGD	arginine glycine aspartic acid
SC	satellite cell
SCID	severe combined immune deficiency
SIS	small intestinal submucosa
SMTE	skeletal muscle tissue engineering
TA	tibialis anterior (muscle)
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VML	volumetric muscle loss

Author contributions

DG, LTerrie, MG, HD and LThorrez wrote the manuscript. DG, MG and HD designed the figures and tables. All authors read and approved the manuscript.

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