HHS Public Access

Author manuscript

Biomaterials. Author manuscript; available in PMC 2022 November 01.

Published in final edited form as:

Biomaterials. 2021 November ; 278: 121173. doi:10.1016/j.biomaterials.2021.121173.

Cells, scaffolds, and bioactive factors: Engineering strategies for improving regeneration following volumetric muscle loss

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Abstract

Severe traumatic skeletal muscle injuries, such as volumetric muscle loss (VML), result in the obliteration of large amounts of skeletal muscle and lead to permanent functional impairment. Current clinical treatments are limited in their capacity to regenerate damaged muscle and restore tissue function, promoting the need for novel muscle regeneration strategies. Advances in tissue engineering, including cell therapy, scaffold design, and bioactive factor delivery, are promising solutions for VML therapy. Herein, we review tissue engineering strategies for regeneration of skeletal muscle, development of vasculature and nerve within the damaged muscle, and achievements in immunomodulation following VML. In addition, we discuss the limitations of current state of the art technologies and perspectives of tissue-engineered bioconstructs for muscle regeneration and functional recovery following VML.

Keywords

Volumetric muscle loss; tissue engineering; skeletal muscle regeneration; biomaterials; vascularization; innervation; immunomodulation

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Author Credit

This manuscript was written by IE, DW, and TAR.

Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Thomas A. Rando reports financial support was provided by Stanford University. Thomas A. Rando reports financial support was provided by National Institutes of Health. Thomas A. Rando reports financial support was provided by US Department of Veterans Affairs.

1 Introduction

Skeletal muscle injury is common following excessive exercise and traumatic incidents, such as motor vehicle accidents, orthopedic surgeries, or combat-related fractures [1–4]. Following minor injuries, skeletal muscle has a remarkable ability to regenerate, which relies on muscle stem cells (MuSCs), also referred to as “satellite cells”, and their interaction with the surrounding microenvironment [3,5]. However, volumetric muscle loss (VML) injury overwhelms the endogenous repair capacity and leads to an irrecoverable functional impairment [6–10]. Following VML, substantial loss of MuSCs diminishes the innate regenerative ability of the injured muscle. Complete removal of the extracellular matrix results in the disappearance of a scaffolding to support cell attachment and guide tissue reconstruction [11]. Moreover, VML induces chronic inflammation that causes continued tissue damage and massive fibrosis, impeding muscle regeneration [12,13]. In this context, VML leads to permanent loss of muscle and life-long disability [14].

Current clinical strategies for VML treatment are limited to free functional muscle transfer, an autologous muscle transplanted to the defect area for functional restoration [6,7]. However, autografting is often associated with donor-site morbidity, limited tissue availability, and complications such as infection and necrosis [7,14,15]. Therefore, the development of regenerative technologies, which can address current challenges and provide substitutions for autologous muscle for transplantation, is vital for VML treatment. The therapeutic success requires a large amount of muscle formation to replace the lost tissue, sufficient vascularization to supply blood flow, functional innervation to generate action potentials, and effective immunomodulation to reduce fibrosis and support tissue regeneration [16–19]. Tissue engineering approaches have tremendous potential for muscle regeneration and functional recovery (Figure 1). In this review, we will summarize current engineering strategies for VML treatment that promote myogenesis, vascularization, innervation, and immunomodulation. The advances and limitations of tissue engineering technologies, including cell therapy, scaffold design, and bioactive factor delivery will be discussed in each category.

2 Myogenesis

2.1 Cells

Cell-based therapies have great potential for treating VML injuries. Transplanted myogenic cells can form new myofibers as well as repair damaged existing fibers, thereby restoring muscle mass and function. Several studies have investigated acellular approaches for VML treatment, demonstrating improved muscle function but yielding inadequate muscle regeneration [20–22]. Systematic evaluations of muscle biomechanics and histology show that decellularized scaffolds alone are insufficient for promoting *de novo* muscle fiber formation [23–25]. Therefore, an exogenous stem cell source is likely to be necessary for effective treatment of VML injuries. Cell types that participate in normal skeletal muscle regeneration have been explored as candidates for regenerative medicine and previously reviewed in detail [26,27]. Here, we will highlight the cell sources that have shown potential, specifically for VML therapies, based on their ability to generate new muscle (Figure 2A).

MuSCs are the predominant muscle fiber forming cells in skeletal muscle tissue. Following injury, MuSCs activate, proliferate, differentiate, and fuse to repair damaged myofibers, as well as form new myofibers. As a result, MuSCs have been extensively investigated as cell sources for regenerative medicine [28,29]. Despite their scarcity in skeletal muscle, MuSCs demonstrate a dramatic proliferation potential *in vivo* [30]. Freshly isolated MuSCs transplanted into a mouse VML model yielded *de novo* muscle fiber formation and a 40% recovery of muscle mass after one month [31]. Despite their proliferative potential, it is likely that a large number of MuSCs would need to be transplanted for an improved therapeutic benefit. However, MuSCs expanded *in vitro* have a drastic reduction in engraftment and regenerative capacity when transplanted back *in vivo*, compared to freshly isolated MuSCs [32,33]. Several groups have developed *in vitro* culture conditions that enable MuSC expansion, while maintaining their therapeutic potential [34,35], but cells produced by these methods have not been transplanted into a VML model. On the other hand, various proliferating populations of myogenic progenitors and myogenic cells lines, such as L8 and C2C12 myoblasts, have been investigated for VML applications because they can be easily expanded *in vitro* [36–38]. In this review, we will refer to myogenic populations that have not been prospectively derived from MuSCs as myogenic progenitor cells (MPCs), even if poorly characterized.

In vivo, MuSCs are supported by other cells in the tissue [39,40]. A mixed cell population termed “muscle resident cells” (MRCs), made up of endothelial cells, hematopoietic cells, fibro-adipogenic progenitors, and fibroblast-like cells, has been shown to enhance the survival of MuSCs both from studies *in vitro* and in a mouse VML model *in vivo* [31]. A combination of MuSCs and MRCs doubled the recovery of muscle mass, reduced fibrosis, increased myogenesis, and significantly improved *ex vivo* muscle function compared to MuSC-only treatment. In the same study, combinatorial co-culture experiments of MuSCs with endothelial cells, MRCs, or MRCs without endothelial cells, revealed that endothelial cells were both necessary and sufficient to sustain MuSC viability, expansion, and engraftment in the VML model [31]. Minced muscle grafts, which include MuSCs and other cells residing in muscle tissue, have also been explored as cell sources for VML therapy. Minced muscle grafts transplanted into a rat VML defect yielded a 55% recovery of functional deficit measured by net torque production [41]. In a larger, porcine model of VML, autologous minced muscle grafts produced a 32% increase in strength after 12 weeks [42]. However, few areas of *de novo* muscle regeneration and significant fibrosis were observed within the defect area. Moreover, clinical limitations, including inadequate tissue availability and donor site morbidity, must be addressed as a significant volume of autologous minced muscle would be required to repair a defect at the scale of VML injuries.

Induced pluripotent stem cells (iPSCs) are attractive cell candidates for a variety of tissue engineering applications because they are easily obtained, capable of robust proliferation *in vitro*, and can be differentiated into multiple cell types [43]. iPSC-based strategies investigated for skeletal muscle regeneration include direct transplantation of iPSC-derived MPCs and *in vitro* engineered skeletal muscle constructs, where iPSCs are differentiated into myotubes *in vitro* prior to implantation *in vivo*. In a recent study, human iPSC-derived MPCs transplanted in a mouse VML model yielded an improvement in muscle contractility and donor-derived muscle fiber formation [44]. Moreover, *in vitro* iPSC-derived functional

skeletal muscle constructs have been shown to fuse with endogenous myofibers when implanted *in vivo* [45–47]. A recent study demonstrated the therapeutic potential of using multilineage engineering to create artificial skeletal muscles containing iPSC-derived myofibers, endothelial cells, pericytes, and motor neurons [48]. Although generating iPSC-derived skeletal muscle constructs, such as induced skeletal muscle (iSKM) bundles or other artificial muscles [45,48], *in vitro* and then implanting them *in vivo* is a promising approach for skeletal muscle regeneration, further investigation is needed to determine the efficacy of these therapies in a VML model. *In vivo*, iSKM bundles have been shown to progressively vascularize when implanted subcutaneously and to successfully engraft in a TA muscle, while maintaining functionality one-week post implantation. Nonetheless, clinical limitations, including potential for tumorigenicity and immunogenicity [49–51], must be addressed to fully realize an iPSC-based therapy for VML.

2.2 Scaffolds

Since VML injury also results in the removal of native ECM, endogenous and transplanted cells lack a scaffold that is necessary to regenerate organized skeletal muscle [15]. A recent study found that acellular muscle architecture is required to guide MuSCs during regeneration [52], underscoring the need for scaffolds that support myogenesis in VML therapies. Scaffolds used for enhancing skeletal muscle regeneration have been reviewed extensively [15,53–55]. Biomaterials can provide essential biochemical and biophysical cues to support cell attachment, survival, proliferation, and myogenic differentiation [53,56]. For example, MPCs encapsulated in an *in situ* casted fibrin hydrogel engrafted and formed new muscle fibers in a murine VML defect [57]. Similarly, human MPCs micropatterned on poly lactic-co-glycolic acid (PLGA) sheets, stacked to form a 3D construct, and transplanted into a murine VML defect also showed improved engraftment compared to direct cell injection [58]. Here, we will highlight some of the biomaterial properties, including composition, stiffness, porosity, and degradation, that have been shown to improve the regeneration of skeletal muscle following VML (Figure 2B).

Scaffolds for regenerative medicine can generally be characterized as natural or synthetic based on their composition [15,59]. As implied by their name, natural biomaterials are polymers derived from natural sources, such as proteins, polysaccharides, and DNA, and are often biocompatible, biodegradable, and have low cytotoxicity. Despite their biocompatibility, natural biomaterials generally have weak mechanical strength and batch-to-batch variability. Common natural biomaterials that have been used in skeletal muscle tissue engineering include collagen, fibrin, alginate, and decellularized ECM. Synthetic biomaterials, on the other hand, are more scalable and their physicochemical properties are easily tailored for their application. Unlike their natural counterparts, synthetic biomaterials must be functionalized with bioactive cues to make them biocompatible. Common synthetic biomaterials that have been used for skeletal muscle tissue engineering include polyethylene glycol (PEG), polyglycolic acid (PGA), polycaprolactone (PCL), and poly lactic-co-glycolic acid (PLGA). Although natural and synthetic biomaterials are generally associated with the respective properties and limitations mentioned above, scaffold characteristics can be altered by modifying the polymer, introducing new crosslinking chemistries, or mixing biomaterials to produce hybrid scaffolds.

Decellularized ECM (dECM) is the most widely used natural scaffold for VML applications due to the abundance of biochemical cues that are critical for tissue regeneration [60–62]. dECM scaffolds sourced from muscle, small intestine, and urinary bladder have demonstrated variable success in VML models [20,24,63,64]. Differences in ECM composition, decellularization protocols, and scaffold processing are all contributing factors to dECM variability. Other natural materials, such as keratin derived from human hair, have also demonstrated potential as scaffolds for VML therapy [65,66]. Compared to bladder dECM, acellular keratin hydrogels yielded increased muscle regeneration, decreased fibrosis, and improved functional recovery following 12 weeks post-implantation in a rat VML model [65]. Another study explored the ability of a hyaluronic acid hydrogel supplemented with laminin-111, an embryonic isoform of laminin, to support minced muscle grafts in a rat VML model [67]. Hyaluronic acid, a polysaccharide known for its anti-adhesive and anti-inflammatory properties [68], was chosen as a scaffold to effectively shield transplanted cells from the harsh immune environment. Additionally, laminin was incorporated to selectively promote the adhesion, migration, and proliferation of transplanted cells. Consistent with previous minced muscle and scaffold strategies [67,69], co-delivery of hyaluronic acid, laminin, and minced muscle yielded a functional improvement compared to an acellular hydrogel control. However, functional recovery was comparable to that achieved with minced muscle grafts without any scaffolding [67], indicating that the scaffold did not enhance the effect of minced muscle grafts alone.

Scaffold functionalization with bioactive peptides has gained significant attention for improving regeneration in both soft (e.g. skin, muscle, brain) and hard (e.g. bone, cartilage) tissue applications [70,71]. To our knowledge, peptide functionalization has not yet been explored in VML applications but has great potential for enhancing scaffold bioactivity and muscle regeneration. Unlike most natural materials, synthetic materials must be modified to promote the attachment of transplanted and endogenous cells. Synthetic biomaterials for skeletal muscle applications are generally coated, or mixed, with natural materials such as fibrin, laminin, or Matrigel, to promote cell attachment [72–74]. However, incorporating full-length ECM proteins into synthetic scaffolds can elicit an inflammatory immune response and result in undesirable changes to scaffold properties, such as surface charge and topography [75]. Functionalizing scaffolds with small peptides is an attractive alternative to improve bioactivity and overcome the aforementioned limitations associated with full-length proteins. Peptides are able to mimic the bioactivity of their full-length protein counterparts while also being easier to produce and modify under well-defined conditions. Arginine-glycine-aspartate (RGD) peptides tethered to an alginate scaffold have been shown to enhance MPC viability following transplantation into skeletal muscle [76]. In addition to improving cell attachment, peptides have also been incorporated into natural and synthetic scaffolds to promote a variety of cellular responses, including survival, proliferation, angiogenesis, and anti-inflammation [70]. RGD and IKVAV, an isoleucine-lysine-valine-alanine-valine peptide derived from laminin, have been investigated for controlling MPC behavior *in vitro* [77]. When cultured on top of hyaluronic acid hydrogels functionalized with IKVAV, MPCs demonstrated increased migration and expression of Pax7, a transcription factor involved in MuSC proliferation and differentiation, compared to cells presented with RGD.

Scaffold properties, such as stiffness, degradation, and porosity, also play critical roles in mediating tissue regeneration. Fabrication methods, such as electrospinning, pore generation, and 3D printing, as well as crosslinking techniques can be used to develop scaffolds with physical properties that closely mimic the tissue of interest. An ideal scaffold for VML therapy will likely match the stiffness of skeletal muscle with a Young's modulus of approximately 12 kPa [78]. Scaffold degradation rate is another key factor that determines the success of a regenerative medicine therapy. Ideally, the rate at which a material degrades should be proportional to the rate of new tissue formation [79]. Relative to the rate of new tissue formation, scaffolds with too slow of a degradation are prone to fibrosis and encapsulation, whereas too fast of a degradation will not provide enough physical support for cells to regenerate the injured tissue [55]. Introducing porosity into scaffolds can allow endogenous cells to participate in the regeneration process and vascularization to take place more easily [80]. Porosity becomes increasingly important for larger tissue defects, such as VML, which require rapid ingrowth of vasculature to deliver essential nutrients and remove waste. A recent study investigating the therapeutic potential of a porous scaffold in a mouse VML model found that a collagen-GAG sponge promoted a functional improvement and upregulation of factors involved in wound healing [81]. Moreover, a collagen-gelatin-laminin sponge implanted in another hindlimb VML model supported the recruitment of endogenous cells, including MuSCs, but yielded limited myofiber regeneration after 2 weeks [82].

In addition to stiffness and composition, the anisotropic, or aligned, organization of skeletal muscle can also be mimicked using scaffold fabrication techniques. Aligned scaffolds more closely resemble the architecture of skeletal muscle and have also been shown to mediate myofiber maturation *in vitro* [83]. Anisotropic materials for skeletal muscle regeneration have been investigated further and reviewed in detail [84,85]. A recent study demonstrated the importance of alignment in the regenerative response following VML in a rat model by implanting autografts in misaligned orientations [86]. Misaligned autografts, rotated at 45- or 90-degree angles, were shown to have significantly less functional recovery, fewer myofibers, and considerably more fibrosis compared to aligned autografts. Scaffolds with aligned architecture for VML applications have generally been fabricated by electrospinning and 3D printing. Aligned electrospun PCL/dECM scaffolds demonstrated increased myofiber regeneration compared to the PCL only group but did not yield significant improvements in muscle weights or functional recovery in a mouse VML model [87]. Aligned nanofibrillar collagen scaffolds implanted into a mouse VML model yielded *de novo* myofibers with 60% larger cross-sectional areas compared to dECM controls [88]. Recent advances in 3D bioprinting have drastically enhanced the fabrication of aligned skeletal muscle fibers [89,90]. Human MPCs in a 3D printed scaffold with aligned architecture showed increased muscle regeneration, improved functional recovery, and decreased fibrosis compared to non-printed groups following 8 weeks post implantation in a rat TA VML model [91]. The same bioconstruct, implanted into a pelvic floor VML model, maintained muscle volume over 8 weeks and significantly improved muscle function compared to acellular bioconstructs [92]. 3D printed bioconstructs composed of human MPCs in photo-crosslinkable dECM also demonstrated increased muscle regeneration and functional recovery in a rat TA VML model [93]. Incorporation of non-myogenic cell types

into 3D printed bioconstructs have further improved myogenesis in VML lesions [94,95]. Moreover, novel skeletal muscle 3D printing deposition strategies, including in situ and intravital bioprinting, have recently been explored and show great promise for VML therapy [96,97].

2.3 Bioactive Factors

Besides therapeutic cells and scaffolds, bioactive factors, such as growth factors, cytokines, and signaling molecules, are other key mediators of skeletal muscle regeneration [98]. The native regeneration process, including cell recruitment, proliferation, and differentiation, is orchestrated by different biochemical cues [99]. For instance, HGF has been found to be a critical factor for triggering the activation of quiescent MuSCs *in vivo* [100]. In addition, FGF-2 has been shown to be a potent inducer of MuSC proliferation [101]. Owing to their pivotal role in controlling cell behavior, bioactive factors are of great interest in regenerative medicine. Although the delivery of bioactive factors can improve myogenesis, their dosage must be tightly controlled since suboptimal dose and improper timing may result in adverse outcomes such as increased fibrosis, hypertension, and edema [102,103]. Biomaterials can enable the spatiotemporal control of bioactive factors to guide the regenerative process *in vivo*. Here, we will summarize the factors that have been used in VML therapies and their delivery strategies (Figure 2C). Moreover, additional factors, which have been shown to have therapeutic potential in the other muscle injury models besides VML, will be covered for future exploration.

2.3.1 Factors for Cell Activation and Recruitment—Owing to the remarkable capabilities of MuSCs for muscle regeneration [104], recruitment of endogenous MuSCs is an attractive approach for *in situ* muscle regeneration. Bioactive factors have been delivered to recruit host MuSCs and provide proper guidance for muscle regeneration following VML [105,106]. For example, poly(L-lactic acid) (PLLA) scaffolds releasing bioactive factors, including SDF-1α, HGF, IGF-1, and FGF-2, were investigated for their ability to improve MuSC recruitment and myogenesis following VML [105]. Only the scaffolds releasing IGF-1 or FGF-2 demonstrated a significant MuSC enrichment compared to the control scaffold without growth factors. In addition, delivery of IGF-1, FGF2, or HGF yielded more newly formed muscle fibers than control scaffold. The therapeutic effects of HGF on muscle functional recovery following VML was further investigated [106]. A sustained delivery of HGF for 48 hours to mimic its native release kinetics after injury was achieved by adsorbing HGF onto fibrin microthreads. About 91% of isometric twitch force was recovered compared with the contralateral uninjured muscle after 60 days post-injury, while the no intervention group recovered 70%. In addition, although the HGF group recovered 99% force at tetanic contraction, there was no significance between the treatment groups with and without HGF.

Besides recruiting endogenous cells, bioactive factors can be used to induce activation and promote mobilization of transplanted cells. While the therapeutic effects of such a strategy have not yet been explored in VML injury, application to other injury models demonstrate potential. For example, Wnt7a is a potent signaling protein that can promote symmetric MuSC expansion and directional migration of both MuSCs and MPCs [107–

109]. In a cryo-injured muscle model, co-delivery of Wnt7a and MuSCs in a hydrogel on top of the injury area significantly increased the migration distance of transplanted MuSCs towards the injury site to form new muscle fibers [110]. Another study that promoted the survival of transplanted MPCs and induced their migration from a scaffold placed on top of the injured tissue was investigated in a muscle laceration model [111]. HGF and FGF2, potent growth factors known to increase proliferation and prevent premature differentiation of myoblasts [112,113], were co-delivered with myoblasts [111]. In response to HGF and FGF2, myoblasts demonstrated increased migration outward from the scaffold and incorporation with host tissues, resulting in a significant reduction of defect area at 30 days post-injury. However, functional recovery was not characterized in this study [111].

2.3.2 Factors for Cell Proliferation—Achieving a large enough number of myogenic cells for regeneration is critical for VML therapy [15,56]. FGF-2 was utilized to promote the *in vivo* proliferation of L8 myoblasts [114]. In a crush injury model, L8 myoblasts, which were genetically modified to overexpress FGF-2, demonstrated a 53% reduction in apoptotic cell death and a 1.8-fold increase in proliferation compared with control myoblasts [114]. Besides promoting the proliferation of transplanted myoblasts, bioactive factors have also been used to induce endogenous cell proliferation [65,66,115]. However, direct injection of FGF-2, with or without heparin to provide a sustained release, to a crush-injured muscle showed no measurable effect on the number of proliferating cells [115]. A negligible effect of FGF-2 on cell proliferation was also found in VML treatment by delivering FGF-2 in a keratin hydrogel [65,66]. Nevertheless, co-delivery of FGF-2 and IGF-1 demonstrated a synergistic effect on improving functional recovery following VML [66]. Therefore, the delivery of inductive factors can promote myogenic cell proliferation *in vivo* and improve muscle functional recovery following VML injury.

2.3.4 Factors for Cell Differentiation and Fusion—To achieve functional muscle tissue, myogenic progenitors need to differentiate and fuse together to form mature myofibers. In the native repair process, IGF-1 plays a critical role in regulating myogenic cell differentiation and fusion [116,117]. C2C12 myoblasts cultured on a collagen scaffold with IGF-1 *in vitro* formed myotubes exhibiting a 1.5-fold increase in length and 2-fold greater nuclei per myotube compared with the myoblasts without IGF-1 [118]. The therapeutic potential of IGF-1 was further explored by transplanting the acellular IGF-1 loaded scaffold into a mouse VML model. Although the density of regenerating fibers in the IGF group increased, there was no statistical significance found compared with scaffold only group [118]. The capability of IGF-1 to induce myogenic differentiation was also investigated in a rabbit VML model [119]. IGF-1 was sustained released to the injury site by dECM scaffolds following VML. The immunohistochemical staining for myosin heavy chain (MHC) illustrated that the dECM scaffold with IGF-1 yielded a significantly increased number of newly formed myofibers and a reduction in collagen deposition compared to a scaffold only group.

3 Vascularization and Innervation

Vascularization and innervation are essential processes for muscle regeneration and functional recovery following VML injury. Vasculature, which is highly dense and

organized, provides oxygen and nutrients for skeletal muscle. Although spontaneous revascularization is triggered in response to muscle injury without any treatment, the ingrowth of blood vessels is too slow to meet the high metabolic demand of muscle regeneration in large size of defect like VML [16,120,121]. Bioconstruct vascularization is particularly challenging for VML therapy due to the large defect size and fibrosis at the injury site [122]. In addition, VML injury also results in significant axotomy of motoneurons and removal of the neuromuscular junctions (NMJs) [123]. Denervation of injured muscle results in functional impairment and muscle fiber atrophy [124]. Therefore, emerging studies have focused on developing novel approaches to promote vascularization and innervation for muscle regeneration. In this section, we will summarize current strategies to improve vascularization and innervation of skeletal muscle by cell delivery, scaffold design, and bioactive factor delivery (Figure 3).

3.1 Cells

Endothelial cells are known to play an important role in muscle regeneration [125]. Therefore, endothelial cells have been studied extensively for creating vascular networks to enhance VML therapies. *In vitro* and *in vivo* tissue engineering strategies, where endothelial cells are used to form vasculature prior to implantation or are transplanted directly, have both been investigated. Pre-vascularization of bioconstructs with human umbilical vein endothelial cells (HUVECs) was investigated in a mouse VML model [126]. Prior to implantation, bioconstructs were cultured *in vitro* to allow co-cultured HUVECs and C2C12s to form aligned vessels and myotubes, respectively. Ten days after implantation *in vivo*, pre-vascularized bioconstructs successfully anastomosed with host vasculature and were perfused with host red blood cells. However, pre-vascularized bioconstructs were surrounded by fibrosis and yielded minimal myofiber formation, unlike their non-vascularized counterparts. In another study, endothelial cells and MuSCs on a dECM scaffold were directly transplanted, without a pre-vascularization culture period, into a similar VML model [31]. Endothelial cells proliferated and contributed to vasculogenesis within the bioconstructs 21 days post implantation. Currently, both *in vitro* and *in vivo* tissue engineering approaches using endothelial cells show promise for enhancing vascularization of bioconstructs for VML therapy.

Using endothelial cells to develop functional vascular networks, whether pre-formed *in vitro* or via direct cell transplantation *in vivo*, is often a lengthy process. Microvessel fragments (MVFs) are attractive bioconstruct pre-vascularization units because they already have developed microvessel morphology, rapidly assemble into networks when transplanted *in vivo*, and can be easily isolated in large quantities from adipose tissue [127]. MVFs are inherently more complex than endothelial cells and have been shown to contain mesenchymal stem cells, which contribute to their regenerative potential [128]. Collagen hydrogels containing MVFs yielded a higher vessel density within a VML defect compared to hydrogels with adipose derived stem cells after 7 days [129]. At 14 days post injury, MVF bioconstructs contained 75% of the vessel density of uninjured controls. Moreover, perfusion of the MVF network was observed in just 7 days and was maintained at 14 days post injury. Despite evidence of anastomosis with host vasculature, bioconstruct perfusion was still low and heterogeneously distributed within the defect. More recent work

investigated the vascularization potential of MVFs with or without the addition of myoblasts in a rat VML model [130]. Moreover, more small-diameter vessels were observed in both of the pre-vascularized groups than in acellular hydrogel and autograft controls.

Similar to the aforementioned pre-vascularization strategies, using progenitor or differentiated cell populations, pre-innervation approaches for VML applications have entailed the incorporation of neurons or neural stem cells (NSCs) into bioconstructs. Addition of motor neurons to bioconstructs containing differentiated C2C12s demonstrated improved myocyte maturation *in vitro* [131]. Following implantation into a rat VML model, these pre-innervated bioconstructs maintained muscle cross-sectional area after 3 weeks, unlike the non-innervated, acellular, or non-treated control groups. In addition, pre-innervated bioconstructs enhanced the recruitment of endogenous MuSCs and the formation of NMJs near the injury area. Compared to non-innervated and acellular groups, pre-innervated bioconstructs also promoted vascularization within and outside the injury area. Another promising strategy for pre-innervating bioconstructs that has been tested in a VML model involves the transplantation of NSCs instead of differentiated neurons. A recent study demonstrated that the addition of human NSCs to human MPCs yielded improved myofiber and NMJ formation after 8 weeks in a rat VML model, compared to bioconstructs containing only MPCs [95]. The addition of NSCs to bioconstructs also yielded a decrease in fibrosis relative to MPC-only and non-treated controls. In contrast to pre-innervated bioconstructs [131], which demonstrated increased vascularization compared with their non-innervated counterparts, no significant improvements in vascularization were observed upon the addition of NSCs to MPC bioconstructs [95].

3.2 Scaffolds

The arrangement of myofibers, blood vessels, and nerves in native skeletal muscle is highly organized and essential for proper muscle function. 3D bioprinting has emerged as a promising tool for fabricating complex bioconstructs that mimic the highly organized, aligned structure of native skeletal muscle tissue [91,132]. Bioconstructs can be pre-vascularized and pre-innervated by spatially patterning a combination of multiple cell types, biomaterials, and bioactive factors. Techniques for 3D bioprinting vasculature and nerve have both been extensively reviewed for other tissue applications [121,133]. The role of spatial patterning in pre-vascularization was specifically evaluated in a rat VML model [94]. Bioconstructs composed of human MPCs and HUVECs were 3D printed in two distinct conformations; MPC-HUVEC hybrid fibers (mixed printing) and MPC fibers, each surrounded by a layer of HUVECs (coaxial printing). Coaxially printed bioconstructs yielded a significant increase in TA muscle weight compared to mixed printed bioconstructs. In addition, coaxially printed bioconstructs vascularized and anastomosed with host vasculature more than mixed printed and MPC-only groups. Muscles treated with coaxially printed bioconstructs also achieved approximately an 85% recovery of the force production of uninjured muscle. 3D printed bioconstructs, which have been pre-innervated by the incorporation of human NSCs, have also shown promise for enhancing skeletal muscle regeneration following VML [95]. Bioconstructs containing NSCs yielded a significantly higher number of NMJs at 8 weeks post implantation in a rat VML model compared to MPC-only bioconstructs. Moreover, 3D printed bioconstructs, with and without

NSCs, became highly vascularized within 4 weeks and contained more blood vessels than uninjured muscle.

In addition to their role in myofiber maturation discussed previously, aligned fibrillar scaffolds have also been investigated for their ability to enhance vascularization and innervation during skeletal muscle regeneration following VML. Compared to 3D bioprinting, where feature sizes below several microns are generally unable to be fabricated, electrospinning and shear-based extrusion methods can produce biomaterial fibers at the micro- and nano-scale [134,135]. Moreover, electrospun and shear-based extruded fibers can be aggregated in parallel to generate scaffolds with aligned architecture, more closely mimicking the architecture of native ECM [136]. Acellular, aligned nanofibrillar collagen scaffolds transplanted into a VML defect yielded improved density of perfused microvessels compared to non-aligned scaffolds [88]. C2C12s and human endothelial cells co-cultured on the same aligned scaffold also demonstrated a significant increase in perfused vessel density compared to non-aligned scaffolds in a VML model [137]. In addition, alignment was also shown to support the formation of organized vascular networks with greater anisotropy than non-alignment.

3.3 Bioactive Factors

3.3.1 Bioactive Factors for Vascularization—Angiogenic factors are well-established therapeutics for improving tissue vascularization [120,138]. Current strategies of angiogenic factor delivery in VML therapy have focused on promoting and accelerating the ingrowth of pre-existing vessels toward the injury site by recruiting endogenous endothelial or progenitor cells. For instance, IGF-1 loaded scaffolds can significantly increase the density of perfused microvessels compared to empty scaffolds following VML injury [118]. Improving muscle angiogenesis using other angiogenic factors has also been investigated in muscle ischemic injury. Sustained release of factors, including vascular endothelial growth factor (VEGF), SDF-1, FGF-2, and FGF-9, to ischemic muscle was shown to promote vascular density and improve angiogenesis at the injury site [139–144]. These factors can serve as potential candidates for VML therapy. Besides delivering angiogenic proteins, delivery of genes encoding for angiogenic factors is another interesting approach for improving vascularization during muscle regeneration. For example, mRNA encoding HGF was loaded into scaffolds along with Lipofectamine transfection agent, which enabled the intracellular delivery of the mRNA [145]. Two weeks after VML injury, the HGF encoded mRNA resulted in a 1.3-fold increase in capillary density compared with luciferase encoded mRNA [145].

In addition to inducing endogenous vessel infiltration, other applications that have not been explored in VML model, such as improving transplanted cell engraftment and stabilizing newly formed vessels, also have great therapeutic potential for VML. For example, angiogenic factors have been integrated into endothelial cell-laden bioconstructs to improve the survival of transplanted cells and stimulate *de novo* vascularization. HUVECs co-delivered with VEGF exhibited increased survival after transplantation into ischemic muscle, leading to improved tissue perfusion and muscle regeneration [146]. In addition to inducing the formation of new vessels, delivery of angiogenic factors can also promote

maturation to develop functional vascular networks [120]. The combination of factors associated with both vessel formation and maturation has been found to be a more promising approach than the delivery of single factors [147]. Nevertheless, different factors have their own time window of action during vascularization [148], suggesting that distinct release kinetics are required for each of the factors. Simultaneous delivery of VEGF and PDGF-BB did not induce stable increase in blood vessel density [148,149]. In contrast, a sequential delivery of VEGF and PDGF-BB to ischemic muscle yielded increased vessel density at 2 weeks and maturity at 4 weeks compared with single factor delivery [149]. This result suggests that delivery of bioactive factor combinations in their physiological sequences may greatly improve the regeneration process. Biomaterials, in this context, serve as a powerful technology for controlling the release kinetics of different bioactive factors to mimic the native regeneration process.

3.3.2 Bioactive Factors for Innervation—Although bioactive factor delivery is extensively explored in nerve regeneration [150], using bioactive factors to improve innervation for VML treatment is still a nascent field. A recent study investigated the therapeutic potential of IGF-1 for the innervation of VML injured muscle [118]. Scaffolds loaded with IGF-1 yielded a greater number of mature NMJs compared with empty scaffolds. Another strategy to achieve innervation for VML therapy is to induce acetylcholine receptor (AChR) clustering in bioconstructs by delivering agrin, a large proteoglycan for inducing AChR clustering [151,152]. Muscle-derived cells were seeded on scaffolds coated with agrin and cultured for 6 days *in vitro* [151]. A dramatic enhancement of AChR clustering has been found in the combination of agrin presence and cyclic stretch, providing a potential approach for VML treatment. The *in vivo* therapeutic effect for VML was further explored by physically adsorbing or chemically tethering agrin to the scaffold prior to implantation [152]. A significant increase of neuromuscular junctions and neural infiltration was found in both groups containing agrin 4 weeks after VML injury. In addition, the scaffolds that were chemically tethered with agrin achieved a further improvement of muscle regeneration compared with the physically adsorbed group due to a prolonged release profile.

4 Immunomodulation

The immune system plays a critical role in tissue regeneration. The innate and adaptive immune systems are both key mediators of the injury site microenvironment and are known to influence progenitor cell expansion, survival, and integration [153]. Immediately following injury, the innate immune response results in a cascade of inflammatory signals, infiltration of immune cells, and deposition of ECM. During normal regeneration, the inflammatory response generally resolves quickly as tissues begin to repair. However, a prolonged inflammatory response leads to pathological fibrosis, insufficient regeneration, and impairment of normal tissue function [154]. Immunomodulation is therefore critical for promoting tissue regeneration and has been reviewed extensively [153,155–157].

VML injuries result in a dysregulated immune response that causes sustained tissue inflammation, pathological fibrosis, and impaired muscle regeneration [12,17,18]. In skeletal muscle, the inflammatory response is temporally coupled with myogenesis during

regeneration [158]. Macrophages, which polarize between pro-inflammatory (M1) and pro-regenerative (M2) phenotypes during regeneration, play an important role in regulating myogenesis, inflammation, and fibrosis [159,160]. Specifically, macrophages modulate fibro-adipogenic progenitor (FAP) proliferation and differentiation into fibroblasts or adipocytes [161,162]. A prolonged inflammatory response leads to an increase in the number of FAPs and the production of extracellular matrix. Regulatory T-cells (T_{reg}) have been shown to regulate macrophage polarization towards the pro-regenerative phenotype and drive the later stages of muscle regeneration [158]. In this section, we will highlight the immunomodulatory cells, scaffolds, and bioactive factors that have been used to enhance skeletal muscle regeneration following VML.

4.1 Cells

Mesenchymal stem cells (MSCs) exhibit anti-inflammatory and immunomodulatory effects on both innate and adaptive immune cells [153]. For example, transplanted MSCs promote the transition of macrophages from the M1 to M2 phenotype, inhibit immune cell proliferation, and induce the formation of T_{reg} cells *in vivo* [163]. Therefore, MSCs have been used as an immunomodulatory cell source for enhancing regeneration in many tissue engineering applications [164]. MSCs transplanted into a rat VML defect significantly increased macrophage polarization to the M2 phenotype after 2 weeks compared to the untreated control [165]. In addition, MSC treatment yielded significantly increased muscle fiber regeneration and decreased fibrosis after 8 weeks compared to the untreated control. Transplanting MSCs with a dECM scaffold demonstrated a synergistic effect, further improving macrophage polarization and muscle regeneration [165]. In a similar VML model, MSCs derived from adipose tissue (adipose stem cells (ASCs)) in a collagen hydrogel also promoted an M2 macrophage transition and decreased inflammatory cytokines [166]. In addition, ASC treatment demonstrated improved myogenesis, increased blood flow restoration, and decreased fibrosis compared to the acellular hydrogel control. Improved myogenesis and vascularization were also observed in muscles treated with ASCs in a collagen hydrogel following a full thickness VML injury [129]. In a more severe VML mouse model, where TA and EDL muscles were removed, ASCs seeded on electrospun fibrin scaffolds yielded improved muscle regeneration and decreased fibrosis compared to acellular controls [167]. However, muscle fiber regeneration after 3 months of implantation was limited. Although few transplanted ASCs were found to fuse with host myofibers, mesenchymal stem cells are unlikely to replace a myogenic cell source.

4.2 Scaffolds

Biomaterials generally trigger an immune response *in vivo* [168,169]. Scaffold physicochemical properties play a critical role in the host immune reaction and can be altered to modulate the immune microenvironment and promote tissue regeneration. The interactions between biomaterials and the immune system have been reviewed in detail [170–173]. Immunomodulatory properties of scaffolds used for VML therapy have also been investigated. Small intestinal submucosa dECM, cardiac dECM, and muscle dECM/PCL blended scaffolds yielded an increased M2-macrophage polarization in VML models compared to untreated controls [87,165,174]. Although urinary bladder dECM implanted into a rat VML model did not recapitulate autograft macrophage polarization

dynamics over 8 weeks, M2-macrophage polarization was comparable at the final time point [175]. Mechanistic studies of the adaptive immune response in skeletal muscle regeneration following VML revealed that dECM scaffolds induce a pro-regenerative response via T helper 2 cells, which in turn guide macrophage polarization [176]. A direct comparison of natural and synthetic scaffolds implanted into a VML defect revealed divergent immune responses [177]. Specifically, M2-macrophage markers were up-regulated in dECM treated groups and down-regulated in PEG and poly(ethylene) scaffolds after 3 weeks. Synthetic scaffold groups induced a chronic neutrophil infiltrate, which was dependent on material stiffness and size. In addition to increasing neutrophil number, increases in scaffold stiffness caused decreased M2-macrophage polarization in synthetic scaffolds [177]. Similar to dECM scaffolds, natural biomaterial hydrogels, including elastin and a fibrin/laminin-111 blend, also demonstrated increased M2 macrophage polarization in VML models [178,179]. However, muscle regeneration was minimal as both approaches lacked a myogenic cell source.

4.3 Bioactive Factors

Local delivery of immunomodulatory drugs in conjunction with bioconstructs is another promising approach for enhancing tissue regeneration. FDA approved immunosuppressants have been investigated in regenerative therapies for artery, bone, and nerve tissue [180–182]. FTY70 and tacrolimus, immunosuppressants used to treat multiple sclerosis and organ rejection, respectively, have also been used to enhance regeneration following VML injury [183,184]. FTY720 loaded PLGA films implanted into a VML mouse model yielded an increased number of anti-inflammatory monocytes and M2 macrophages at the injury site, compared to PLGA films alone [183]. In addition, FTY720 treatment accelerated vascularization and yielded increased muscle regeneration. A combination therapy of minced muscle grafts and systemic delivery of tacrolimus demonstrated increased muscle regeneration and decreased fibrosis in a porcine VML model, compared to minced muscle grafts alone [184]. In addition, systemic delivery of tacrolimus moderately improved muscle function in muscles treated with minced muscle grafts. Although delivery of immunomodulatory drugs in VML therapies is still nascent, local and systemic delivery methods are both viable approaches to improve regeneration.

5 Summary and Future Perspectives

The ultimate goals of VML treatment are to fully regenerate the lost muscle and recover tissue function. To achieve these goals, a variety of tissue engineering approaches, including cell therapy, scaffold design, and bioactive factor delivery, have been developed. Although a significant improvement has been made in the regeneration of vascularized and innervated muscle with reduced scar formation, challenges still persist in this field. To investigate therapeutic efficacy, multiple animal models of VML have been developed across species and evaluated in different muscles [64]. The critical size of defects among different models ranges from 15% to 40% of muscle mass [8–10]. Besides the size of defects, there is still a lack of standard criteria in dimensions, location, and muscle type. In addition to the characterization of pre-clinical models, strategies for scale-up to yield clinical-grade bioconstructs also need to be developed. Resources of therapeutic cells

with preferred properties, such as potential for self-renewal, robust regenerative capability, and easy accessibility, limit the clinical translation of cell therapy. Future materials need to be developed with consistent composition and highly tunable properties as synthetic scaffolds and favorable biological properties as natural materials. A more comprehensive understanding of the concentrations and combinations of cellular and molecular components will facilitate the rational design of the bioconstructs.

In addition to the strategies mentioned above, exercise is an emerging approach for improving muscle regeneration. Evidence indicates that exercise, either as voluntary running or high-intensity interval training, can promote muscle formation with vascularization and innervation as well as reduced fibrosis following the transplantation of bioconstructs [31,118,185]. An immediate rehabilitation regimen post bioconstruct transplantation has been shown to promote scaffold remodeling, but may hinder regeneration and increase fibrosis [20,31,186]. Beginning exercise 1 to 2 weeks after transplantation, on the other hand, can enhance the muscle regeneration potential of the cell-seeded or growth factor-laden scaffold [118,185]. Another important goal is to restore the integration of muscle and its neighboring tendon tissues. Creation of the interface between muscle and tendon tissues requires precise imitation of the different biological and mechanical properties between distinct tissues. The development of the 3D bioprinting technologies makes it possible to have precise spatial control of bioconstruct architecture at the microscale and build up a complex myotendinous junction structure [187,188].

In summary, tissue engineering is a promising field that applies multidisciplinary strategies to develop substitutes for lost muscle tissue and restore its function. While a number of achievements have been made in skeletal muscle tissue engineering, VML therapy as a complex process is still challenging and requires systematic and sophisticated approaches. The research in biology, material science, engineering, and medicine need to converge for future clinical success.

Acknowledgements

This work was supported by grants from the NIH (AG036695, AR073248) and the Department of Veterans Affairs (I01RX001222, I01BX002324) to T.A.R. All figures created with BioRender.com.

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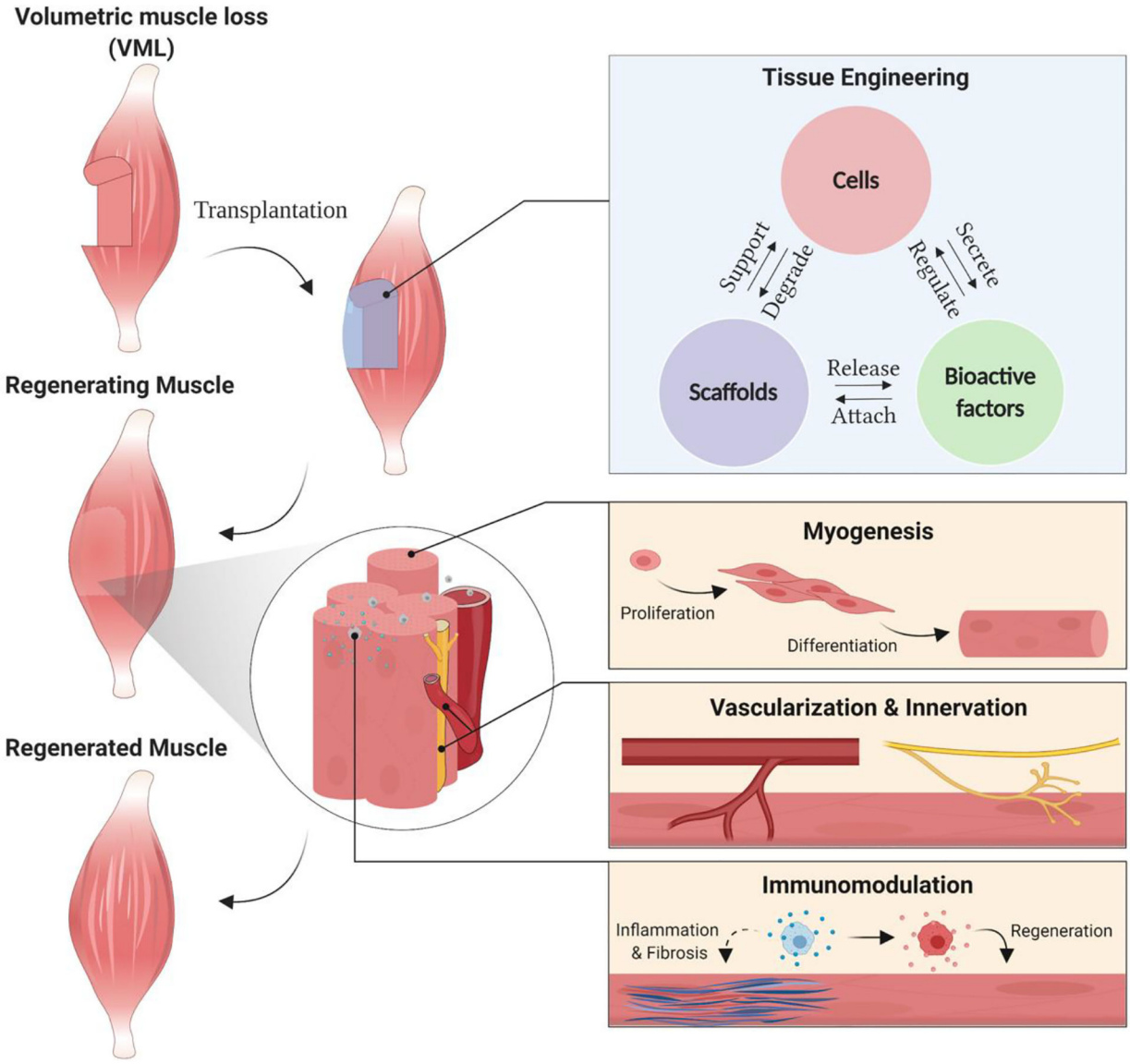

Figure 1.

Volumetric Muscle Loss (VML) results in a significant ablation of skeletal muscle. Tissue engineering strategies combining cells, scaffolds, and bioactive factors, present promising solutions for VML therapy. Muscle regeneration and functional recovery following VML requires sophisticated approaches to promote myogenesis, vascularization and innervation, and immunomodulation.

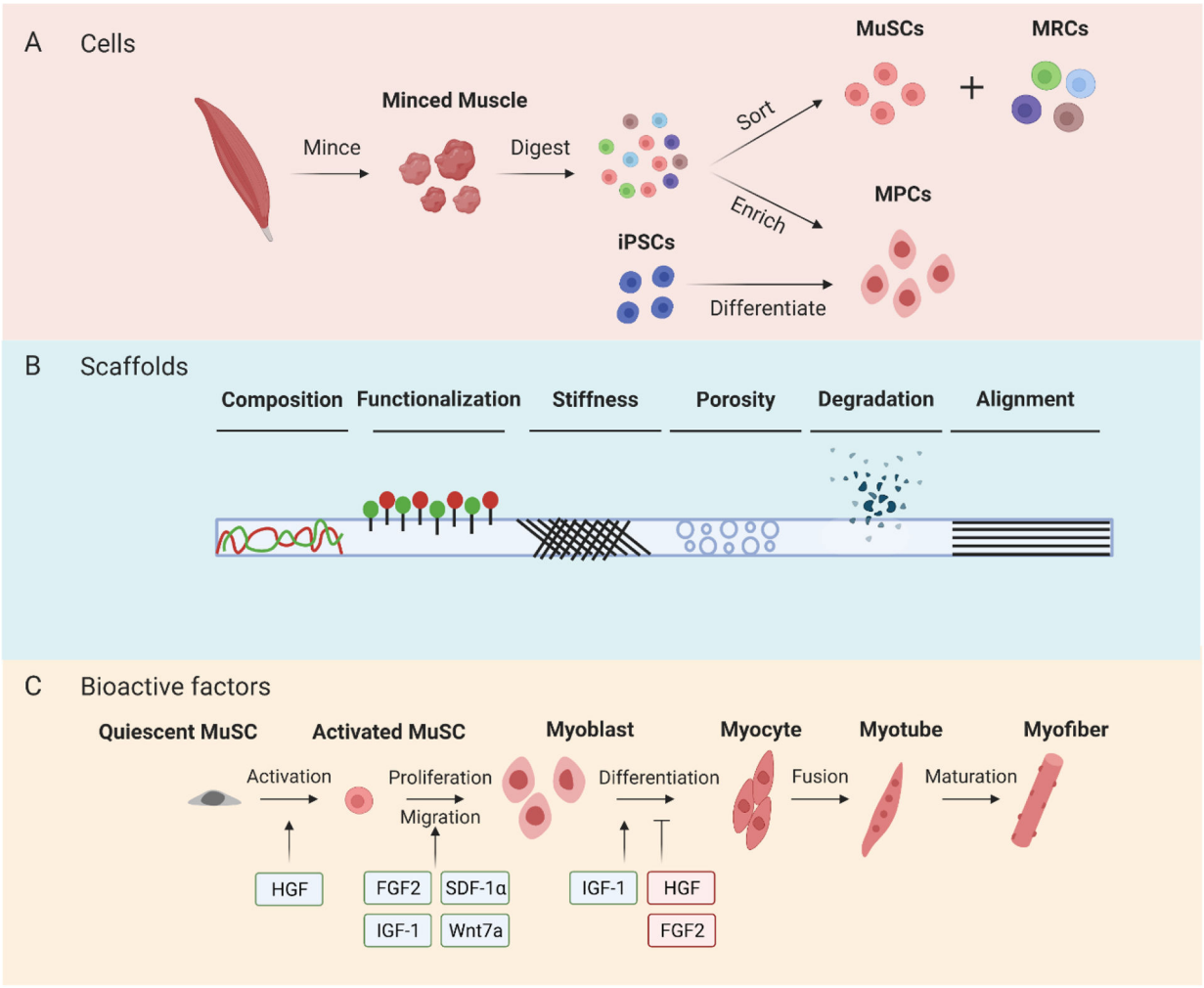

Figure 2.

Advances in tissue engineering for enhancing myogenesis following VML. (A) Myogenic cells used for VML bioconstructs are harvested from skeletal muscle or differentiated from iPSCs. Following muscle digestion, MuSCs are isolated by sorting methods whereas MPCs are purified and expanded in vitro. (B) Scaffold physiochemical properties play an important role in skeletal muscle regeneration following VML. (C) Bioactive factors can be incorporated into bioconstructs to modulate different stages of myogenesis.

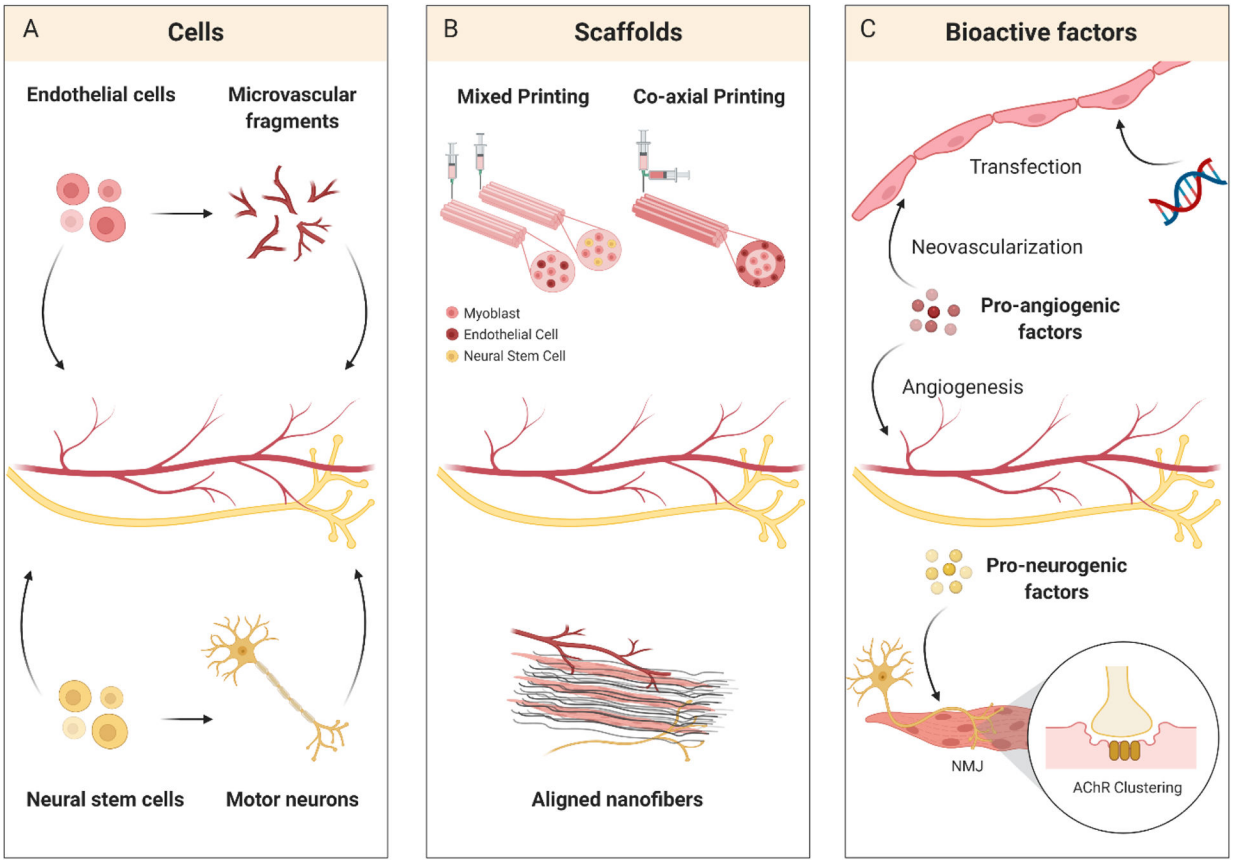

Figure 3.

Enhancing bioconstruct vascularization and innervation for VML therapy. (A) Vascular and neural cells can be incorporated into VML bioconstructs to support regenerating muscle. (B) Scaffold fabrication and patterning techniques, including 3D bioprinting and aligning nanofibers, recreate complex tissue architecture and organization. (C) Pro-angiogenic and pro-neurogenic factor delivery can recruit vasculature and nerves to improve skeletal muscle regeneration and function.