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Review

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Scaffold tissue engineering strategies for volumetric muscle loss

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Abstract

Volumetric muscle loss (VML) refers to a composite, *en bloc* loss of skeletal muscle mass resulting in functional impairment. These injuries normally heal with excessive fibrosis, minimal skeletal muscle regeneration, and poor functional recovery. Functional muscle transfer is a treatment option for some patients but is limited both by the degree of functional restoration as well as donor site morbidity. As such, new therapeutic options are necessary. *De novo* regeneration of skeletal muscle, by way of tissue engineering, is an emerging strategy to treat VML. This review evaluates available scaffolds for promoting skeletal muscle regeneration and functional recovery following VML. The use of growth factors and stem cell therapies, which may augment scaffold integration and skeletal muscle reconstitution, are also discussed. Regenerative medicine with the use of scaffolds is a promising area in skeletal muscle reconstruction and VML treatment.

Keywords: Volumetric muscle loss, tissue engineering, scaffolds, skeletal muscle regeneration

INTRODUCTION

Volumetric muscle loss (VML) following trauma or surgical resection is characterized by irreversible damage or loss of composite skeletal muscle tissue[1,2]. VML injuries can be particularly morbid when involving the lower extremities, as they significantly impair ambulation[3,4]. Critical sized loss of skeletal

muscle tissue (20% of muscle volume), as seen in substantial VML injuries, overwhelms the natural reparative and regenerative ability within skeletal muscle[3,5,6]. Instead of muscle regeneration, VML injuries normally heal with substantial fibrosis, permanent muscle damage, and poor extremity function. These sequelae significantly detract from the patient's ability to perform daily activities, ambulate, and reestablish quality of life[1,2,7-9]. An example of a patient with VML injury from a pre-tibial sarcoma is described [Figure 1].

Treatment options following extremity VML injury remain limited[2,10]. The most common treatment to restore strength across an injured muscle is free or pedicled functional muscle transfer. However, this results in incomplete functional recovery and involves donor site morbidity and weakness[11-17]. Targeted physical therapy promotes muscle regeneration and healing following VML, but only results in partial recovery of the original function[10,18]. Novel tissue engineering strategies, in place of autologous muscle transfer, are key to skeletal muscle regeneration and functional recovery following VML injuries[19-22]. This review will evaluate current tissue engineering strategies using scaffolds to promote skeletal muscle recovery in the treatment of VML.

PATHOPHYSIOLOGY OF VML

VML results in limited muscle fiber regeneration, substantial functional limitation and disability, and excessive fibrosis[1,4,23]. Muscle regeneration in VML pathology is insufficient due to the loss of essential regenerative elements such as growth factors, intact basal lamina of the extracellular matrix (ECM), and stem cells[1]. Skeletal muscle stem cells (MuSCs) are required for skeletal muscle regeneration and are activated by signals from growth factors to enter the cell cycle and proliferate in response to injury[24,25]. Broadly, skeletal muscle regeneration is initiated with pro-inflammatory M1 macrophages that phagocytose necrotic myofibers and activate quiescent MuSCs[26,27]. Anti-inflammatory M2 macrophages then replace M1 macrophages over the next week and promote tissue regeneration by supporting myoblast proliferation, growth, and differentiation[26,27]. The significant loss of MuSCs and a disrupted basal lamina in VML pathology overwhelm skeletal muscles' innate repair mechanism and result in a paucity of skeletal muscle regeneration following VML injury[4,19,22,28]. Additionally, growth factors, such as insulin growth factor 1 (IGF-1), hepatocyte growth factor (HGF), and fibroblast growth factor 2 (FGF-2), that normally activate MuSCs to enter the cell cycle and proliferate, are downregulated[28-38]. Macrophage-mediated secretion of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6), which increase myoblast proliferation and differentiation and promote muscle regeneration, is also lacking in VML pathology[24,35,39-44]. Taken together, the cells and growth factors required for myogenesis are deficient in skeletal muscle following VML, severely impairing functional reconstitution of the muscle.

In contrast, fibrogenic cytokines, such as transforming growth factor β1 (TGF-β1), are upregulated and result in a pathologic proliferation of fibroblasts and ECM components (collagen, laminin, and fibronectin), leading to extensive fibrosis[3,23,35,44]. Fibrosis further prevents normal neuron and vasculature ingrowth, resulting in denervated and ischemic muscle with little elasticity, loss of strength, and impaired contraction and relaxation[5,40]. As such, VML injuries exhibit minimal restoration of strength as myogenesis is diminished. Fibrosis further limits muscle strength and excursion, reinnervation, and revascularization within the site of VML injury.

TISSUE-ENGINEERED SCAFFOLDS FOR SKELETAL MUSCLE REGENERATION

Tissue engineering combines scaffolds, cells, and biochemical cues to aid in tissue regeneration and repair as a treatment for VML. Scaffolds are three-dimensional (3D) structural constructs that support ECM deposition, limit fibrosis, promote skeletal muscle regeneration, and augment functional muscle

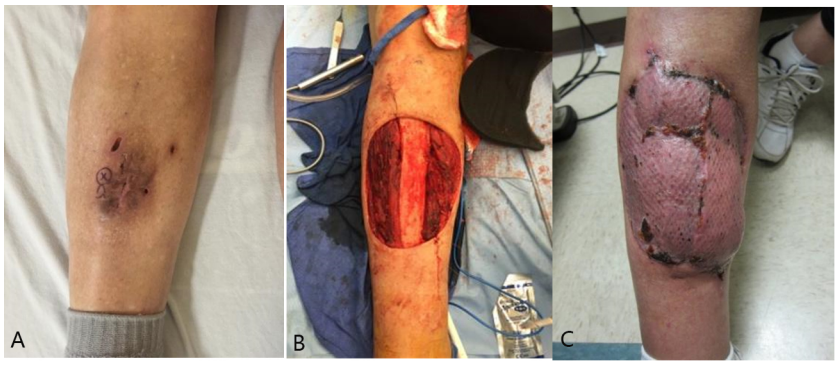

Figure 1. VML in Patient. (A) Patient presented with sarcoma in pre-tibial region; (B) Tumor extirpation results in loss of medial soleus, gastrocnemius, and tibialis anterior muscle; (C) Reconstruction utilizing non-functioning free muscle transfer. Patient exhibits permanent weakness in foot extension and flexion.

recovery[11,18,19,45,46]. Broadly, scaffolds serve as a template for tissue formation and are composed of synthetic or natural biological materials[45]. We will review tissue engineering approaches with hydrogel, acellular, nanofibrous, and electroconductive scaffolds.

The two main approaches to using scaffolds to treat VML are (1) *in vivo* skeletal muscle regeneration using scaffold development and (2) implantation of an *ex vivo* skeletal muscle construct [Figure 2][47,48]. Firstly, *in vivo* tissue engineering involves seeding of host-derived progenitor cells into the scaffold and then transplantation into the defect. Low viability, retention, and immune rejection of the seeded cells are some limitations of this technique[47,49]. For *ex vivo* tissue engineering, a functionally mature construct of contractile myofibers developed from *ex vivo* culture of scaffolds, biological factors, and progenitor cells is implanted into the muscle defect[50]. However, the contractile force produced by scaffolds and oxygen and nutrient diffusion to support cell viability is significantly lower than that of native muscle tissue[51,52]. Addressing these limitations is necessary to make scaffold therapy a reliable option for VML treatment.

Scaffold design and considerations

Scaffolds are constructed extracellular matrices that help direct muscle regeneration and optimize functional recovery [Figure 3]. The choice of material, composition of scaffolds, growth factor integration, and cellularity can all be modified in scaffold-based VML treatment[25,27]. Changes in each of these properties provide both advantages and limitations. Scaffold architecture directly modulates cell adhesion, morphology, orientation, migration, proliferation, genetic expression, and differentiation[53,54]. The porosity of scaffolds modulates nutrient exchange and oxygen transport and facilitates cell seeding, penetration, and distribution[55-58]. Balanced rates of scaffold degradation to tissue growth maintain structural stability with increasing mechanical stress until the tissue can maintain its structure without additional support[27,59]. Biologically active molecules such as growth factors and cytokines can further regulate muSC function and behavior[39,40]. Engineered scaffolds can also be utilized to deliver MuSCs and initiate direct tissue repair and regeneration in the area of injury[25,60,61].

Scaffolds as a mechanism for cell and growth factor delivery

While acellular scaffolds alone have demonstrated improvement in endogenous skeletal muscle regeneration through recruitment and proliferation of host cell populations, they result in incomplete functional recovery with sub-optimal muscle tissue regeneration and scaffold integration[60,63]. To improve muscle regeneration, scaffolds can be used to deliver growth factors to promote myogenesis following VML[25,27,64]. IGF-1 and basic fibroblast growth factor (bFGF) have previously been shown to improve

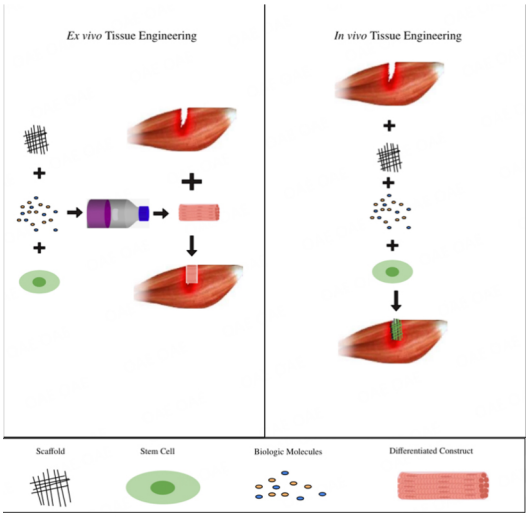

Figure 2. Tissue engineering approaches in treatment of volumetric muscle loss. In an *in vivo* approach, progenitor cells are obtained from the host to be seeded into the scaffolds, together with incorporation of biochemical cues (such as growth factors), the construct is immediately applied to the defect. In *ex vivo* tissue engineering, the same materials are first incubated together in a bioreactor so that a differentiated and functional construct is produced prior to implantation. Figure was created with Google Drawing.

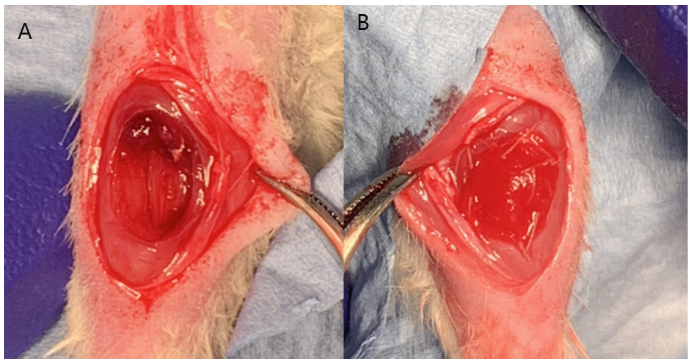

Figure 3. Scaffold implantation into VML injury in a rat. (A) VML injury to rat tibialis anterior using a 6 mm punch biopsy; (B) Acellular collagen glycosaminoglycan scaffold implantation to the defect[62].

healing following muscle injury[40,65]. In the context of VML pathology, TNF-α and IL-6, and growth factors IGF-1, HGF, vascular endothelial growth factor (VEGF), and FGF-2 have been studied for skeletal muscle regeneration following VML injury[25,66-75]. Controlled release of growth factors through scaffold materials over time is more effective than single bolus dosing and results in improved muscle regeneration[76,77]. Similarly, intramuscular injection of cells allows for local engraftment and prevents widespread distribution of cells, but local engraftment[78]. Progenitor cell populations other than MuSCs have also been studied for the reconstruction of VML, including myoblasts[79-81].

In preclinical studies, hydrogels in conjunction with IGF-1 and bFGF showed significant improvements in muscle formation and functional recovery in a murine latissimus dorsi VML model compared to hydrogels alone, hydrogels with MPCs, and keratin hydrogels with MPCs, bFGF, and IGF-1[80]. A correct ratio of cells and growth factors remains unclear. Perivascular stem cells (PSCs) and mesenchymal stem cells (MSCs) have also demonstrated improved myogenesis in the area of VML injury, and a fibrin-laminin hydrogel with MSCs improved muscle mass and myogenic marker expression[82-85]. Scaffold delivery of combinations

of growth factors and progenitor cells is a promising option for VML therapy[25,77,86].

HYDROGEL SCAFFOLDS

Hydrogels are 3D networks of hydrophilic synthetic or natural polymer chains. They are a popular choice of scaffold due to their easily manipulated physical and chemical properties that mimic the native ECM[87-89]. ECM-derived biomaterials commonly used to create hydrogel scaffolds include collagen, fibrin, keratin, polysaccharides, and alginate, but hydrogels can be synthetic or a combination of both to allow for more durability and mechanical strength[55,90-92]. An acellular hydrogel containing methacrylic acid significantly increased muscle fiber growth with a significant 1.5-fold increase in torque production, vascularization, and innervation in murine tibialis anterior (TA) VML injury model ($P < 0.01$)[93]. *In vivo* incorporation of growth factors and progenitor cells in hydrogels into the targeted area of injury can also be used to promote cell viability, myogenic differentiation, and angiogenesis. One study involving keratin hydrogels with IGF-1 and bFGF demonstrated significantly greater recovery contractile force than in keratin hydrogels with MPCs, with about 70% of native muscle force, in a murine latissimus dorsi model of VML injury[80]. Myoblasts with IGF-1, FGF, and VEGF delivered *in vivo* using keratose/alginate hydrogels and myoblasts seeded into fibrin hydrogels alone both demonstrated myogenesis, reduced scar tissue, and construct vascularization in animal models on VML[81,94-97]. Delivery of MuSCs using a polymer scaffold causes significantly higher engraftment of cells into host muscle compared to direct injection into the defect[61,98]. Muscle-derived stem cells seeded onto collagen scaffolds showed a significant 1.5-fold increase in cross-sectional area of rectus femoris muscle at 8 weeks post-injury compared to untreated VML in a murine model of VML[99]. Mesenchymal stem cells in a fibrin-laminin scaffold demonstrated an 8.2% increase in normalized muscle mass and significantly increased myofibers compared to the untreated group in a gastrocnemius-soleus murine model of VML[85]. Manipulation of biomaterials in hydrogels to influence cell behavior, improve mechanical strength, and reduce host immune response is a key area of interest. Combinations of synthetic, such as polycaprolactone, and natural materials are researched to enhance hydrogels' mechanical strength and increase myogenesis in VML models[87,91,92,100]. Adjusting crosslinking modulates hydrogel strength; chemical crosslinking reinforces mechanical strength in contrast to physical crosslinking[57,101,102]. The components of hydrogels such as collagen, gelatin, and alginate or polyethylene glycol correspond to fibrous, microporous, and nanoporous architectures, which subsequently influence cellular migration, proliferation, and nutrient exchange[57,58,103]. Biomaterials can be modified with immunomodulatory genes and the selection of biomaterial based on patient age and sexuality are two studies of interest[104,105]. Induced pluripotent stem cells (iPSCs), adult somatic cells that has been reprogrammed to become pluripotent, have gained traction due to their immunocompatibility and differentiation potential, and delivery of iPSCs using fibrin hydrogels have demonstrated improved *in situ* muscle contractility and improved engraftment of host myofibers and MuSCs in a VML murine model[106]. Hydrogels are very tunable, but the ideal biomaterial and combination of progenitor stem cells and growth factors for large-scale muscle regeneration have yet to be achieved.

DECELLULARIZED SCAFFOLDS

Decellularized scaffolds are comprised of native ECM components after the removal of all tissue cellular components[107-109] [Table 1]. As such, these scaffolds precisely mimic native tissue architecture. Skeletal muscle ECM is key to constructive remodeling in muscle regeneration as it influences cellular adhesion, signaling, and proliferation and is a major source of growth factors to recreate the complex architecture of muscle tissue[109,110]. Acellular scaffolds utilizing porcine urinary bladder ECM have demonstrated improved migration of PSCs to the injury site with *de novo* skeletal muscle cell formation and functional improvement in both a murine model for VML and three out of five patients with extremity VML injuries[108,111]. In another small-scale clinical study of 13 patients with injuries to a variety of muscles, implantation of three

Table 1. Comparison of techniques to treat VML

Technique	Advantages	Disadvantages	Takeaways
Free muscle transfer	-Some functional restoration and volume recovery -Currently used in clinics[13,23]	-Donor site morbidity -Incomplete functional recovery -Lack of donor tissue -Requires highly skilled surgical team[11-17]	-Current standard of treatment for VML -Other techniques are necessary to achieve better function and muscle volume
Acellular scaffolds	-Native ECM is retained -Augments natural recruitment of progenitor cells -Minimizes host immunogenicity -Tissue-specific ECM, has been used in small-scale clinical studies[109,111,112]	-Decellularization process must be thorough to avoid an adverse host immune response -Ability to regenerate sufficient muscle volume and restoration of function is still incomplete[27,60]	-Improvements in functionality and muscle regeneration in few clinical studies -Fast to produce and shelf-ready -May have the quickest path for approval for commercialization[112]
Cellular scaffolds	-Increased delivery of progenitor cells supports recovery and regeneration -Easily manipulated architecture and biomaterials[27,61]	Has-only been studied in *in vitro* and *in vivo* animal models, -Ability to regenerate sufficient muscle volume and restoration of function is still incomplete[27,123]	-Most promising avenue for skeletal muscle tissue engineering -Various combinations of cells, growth factors, and biomaterials can be incorporated

porcine-derived acellular scaffolds demonstrated an average improvement of 37.3% in strength, a 27.1% enhancement in functional range-of-motion tasks, and a 27.2% increase in bulk muscle at six months[112]. However, conflicting results exist regarding the use of decellularized scaffolds for the treatment of VML[11,45,63]. Porcine urinary bladder ECM in a rat TA VML injury model showed 100-fold less myosin-positive fibers compared to those in the autograft at two, eight, and sixteen weeks post-injury, indicating insufficient muscle fiber regeneration[110]. When normalized to uninjured contralateral muscles, functional recovery, defined by the maximal isometric torque of TA, in the acellular scaffold was significantly less than that of the autograft[110]. Similarly, decellularized scaffolds implanted into a rat TA VML injury model did not show *de novo* muscle regeneration, characterized by myosin-positive fibers, but instead had increased fibrotic tissue in the injury site at eight weeks post-injury compared to minced muscle scaffolds that showed substantial muscle regeneration[113]. Maximal tetanic isometric TA muscle torque was assessed *in vivo*, and similarly showed 15% more torque production with the minced muscle scaffolds compared to that of decellularized scaffolds[113].

Collectively, decellularized or acellular scaffolds may be unable to regenerate sufficient muscle tissue for VML therapy[60,110]. One study on a rat model of VML compared acellular muscle ECM and minced muscle grafts and found no appreciable muscle regeneration, increased collagen deposition/fibrosis, and reduced muSC presence in rats with acellular muscle ECM at 8 weeks[113]. Incorporation of progenitor cells into acellular scaffolds has been proposed as a solution. Progenitor cell delivery using acellular ECM, including MSCs, myoblasts, and MuSCs, has demonstrated improved skeletal muscle regeneration, functional recovery, and angiogenesis at the injury site in animal models[114-119]. The addition of murine myoblasts in a murine TA VML injury model showed a significant increase in muscle volume, mass, and myofiber density compared to scaffolds without the incorporation of myoblasts.[120] Previous rodent studies have indicated that the reduced density of MuSCs in the ECM scaffold may have contributed to the limited muscle regeneration seen after scaffold implantation[60,110,113]. Recent strategies maximize muscle growth and vessel/nerve vascularization. One study aimed to reduce scar tissue and demonstrated reduced fibrosis and improved myofiber regeneration using a decellularized muscle aponeurosis scaffold that distributed mechanical stiffness[121]. An acellular laminin-enriched fibrin scaffold demonstrated improved myofiber regeneration and an average 60% increase in torque production in a rat TA model of VML[122]. More studies are needed to determine the effectiveness of acellular and decellularized scaffolds for muscle regeneration and functional recovery.

NANOFIBROUS SCAFFOLDS

Nanofibrous scaffolds expand tissue engineering capabilities to emulate the ECM architecture at the nanometer scale and guide cell adhesion and proliferation[124]. Nanofibrous constructs, made of synthetic or natural biomaterials, have a desirable high surface area to volume ratio and high porosity and are created through electrospinning, self-assembly, or phase separation[124,125]. Nanofibers, through electrospinning, produce an anisotropic microenvironment that guides geometric myoblast alignment to favor myoblast fusion and muscle regeneration[126,127]. Electrospinning produces anisotropic, geometrically aligned nanofibers that mimic native ECM morphology and function[127,128]. Electrospun nanofiber orientation can guide MSC and fibroblast cell growth and may be preferred in tissue-engineered scaffolds[127,128]. Very recently, one study developed an injectable, anisotropic, nanofibrous hydrogel with magnetic controlled short nanofibers to guide cell alignment and organization using a remote magnetic field. These anisotropic scaffolds significantly improved the alignment of myofibers *in vivo* and functional recovery in a rat TA VML model[129].

Vascular and nerve regeneration have been explored with nanofibrous scaffolds. One study involving spatially patterned aligned myotubes from an *in vitro* co-culture of murine myoblasts and vascular endothelial cells in nanofibrillar scaffolds[130]. Implantation of the organized skeletal muscle into a mouse TA VML injury model resulted in highly organized myofibers and increased vascularization and synchronized contractility compared to endothelialized muscle tissue from non-aligned scaffolds, highlighting the potential for improvements in angiogenesis in scaffold tissue engineering[130]. Another study over core-shell composite scaffolds, with a nanofiber yarn core and hydrogel shell that are seeded with myoblasts, and demonstrated both enhanced myofiber alignment and elongation[131]. Pre-innervated scaffolds using co-cultured spinal motor neurons and myocytes in aligned nanofibrous scaffolds in a rat VML model showed greatly increased MuSCs, myocyte fusion and mature neuromuscular junction (NMJs), and muscle regeneration, indicating great potential for pre-innervated scaffolds to treat VML[21]. Cell infiltration is a key limitation in electrospun scaffolds, but adjusting biomaterial selection and variations in electrospinning and post-processing procedures are used to account for this drawback[125,132-136]. Further research is necessary to explore engineered nanofibrous scaffolds to improve spatial organization, vascularization, and innervation of regenerated muscle tissue [Table 2].

ELECTROCONDUCTIVE SCAFFOLDS

Electroconductive scaffolds incorporate conductive materials such as carbon nanotubes, graphene, and conductive nanopolymers to mimic the electrical properties of native ECM[137]. The addition of electrical properties to scaffolds enhances the regeneration of aligned myofibers, leading to contractile function recovery, which is currently missing in natural and synthetic biomaterial-based scaffolds[12,138-140]. Electrically stimulated *in vitro* skeletal muscle constructs improved contractile force, supported myoblast differentiation into myotubes, and increased the size of myobundles[141-143]. Graphene hydrogels have become increasingly popular and have been shown to improve myoblast and fibroblast proliferation and differentiation *in vitro*[144-146]. Reduced graphene oxide (RGO) with nanocomposite polymer helped myocyte differentiation and skeletal muscle regeneration, angiogenesis, and functional recovery *in vivo*[147]. Carbon nanotubes have exceptionally strong electroconductive abilities and have potential to be used for implanted cell tracking and cellular behavior sensing, but they also possess potential cytotoxicity[148-150]. Conductive nanopolymers, such as PCL, have modifiable physical properties and can be used in composite hydrogels or electrospun nanofibers to enhance myoblast differentiation and functional maturation[151-155]. Murine myoblasts cultured *in vitro* on composite gelatin-polyaniline electrospun nanofibers demonstrated improved myotube contractility[155]. More recently, an elastic, hemostatic and conductive nanocomposite cryogel composed of RGO and gelatin exhibited significant cell proliferation, myogenic differentiation, and increased repair

Table 2. Comparison of different types of scaffolds

Scaffold type	Advantages	Disadvantages
Hydrogel	-Easily manipulated physical and chemical properties that mimic the native ECM[87-89] -Composed of a variety of synthetic and natural materials (collagen, gelatin, fibrin, *etc.*) that influence cellular migration, proliferation, and nutrient exchange[57,58,103]	-Highly tunable nature of hydrogels leads to wide variability of results between studies -Limited vascularization and innervation capability compared to nanofibrous scaffolds
Nanofibrous	-Emulate the ECM architecture at the nanometer scale[124,125] -Guide cell adhesion and proliferation at the nanometer scale -High porosity and surface area: volume ratio -Improved myofiber alignment[126,127] -Capability for pre-innervated scaffolds[130]	-Electrospun scaffolds have poor cell infiltration and migration[125,132-136] -Low mechanical strength of scaffold -Less tunable than hydrogels
Electroconductive	-Enhances regeneration of aligned myofibers, leading to contractile function recovery[12,138-140] -Support contractile force and myoblast differentiation[141-143]	-Carbon-containing scaffolds possess potential cytotoxicity[148-150] -Less tunable than hydrogels

efficiency in a rat VML model[156]. An injectable electroconductive, biodegradable hydrogel with murine myoblasts showed higher myofiber density and capillary density in a rat TA VML model[157].

3D BIOPRINTING AND BIOINKS

In comparison to traditional tissue engineering strategies, 3D bioprinting using bioinks (combinations of scaffolds, cells, and growth factors) replicates the complex structure of skeletal muscle while precisely controlling the spatial positioning of cells and biomaterials [Figure 4][123,126,158]. Non-bioprinted biomaterial scaffolds fail to regain normal physiologic force generation and mature functional constructs and are limited in the ability to direct biomolecule deposition[126]. An *in vivo* nanocomposite VEGF-eluting hydrogel bioink demonstrated adherence to skeletal muscle and improved functional recovery with reduced fibrosis in a murine model of VML[159]. Other bioprinted scaffolds include a decellularized bioink that allowed for high cell viability, enhanced tissue and nerve vascularization, and functional recovery in a rat VML model and an *in vivo* colloidal foam-based porous hydrogel that showed significant functional restoration and force generation[160,161]. A methacrylated gelatin hydrogel with human adipose-derived cells, developed using an *in situ* crosslinking strategy to prevent loss of cell viability, showed improvements in hindlimb grip strength and muscular volume in a murine TA VML model[162]. One study involving a bioprinted acellular gelatin hydrogel with MPCs demonstrated TA muscle functional recovery of 82% in a rat TA VML model at eight weeks[163]. Functional neural integration of 3D bioprinted scaffolds has also recently been studied but still remains a challenge for engineered skeletal muscle[163]. A pre-innervated 3D bioprinted scaffold with human MPCs and human neural stem cells showed accelerated functional restoration by integration with host neurons and improved myofiber and NMJ formation in a rat model of VML[164]. Three-dimensional bioprinted scaffolds hold great promise, but scaffold immunocompatibility, systemic effects of implanted cells, and ability to bioprint thick skeletal muscle > 1 mm to allow for vascularization need to be investigated[126,163]. Bioprinting patient-derived stem cells and the development of various combinations of bioinked materials and cells require further direction and study[126].

BARRIERS TO CLINICAL TRANSLATION

Current research on tissue-engineered skeletal muscle constructs for VML is mostly limited to small animal models [Table 3]. A few human clinical studies on decellularized ECM scaffolds derived from animal tissues have shown limited success in the restoration of muscle function[8,112]. Implantation of an acellular porcine-derived scaffold in a 13-patient cohort study showed an average improvement of 37.3% in strength, 27.1% improvement in functional range-of-motion tasks, and 27.2% increase in bulk muscle at six months[112]. Muscles injured included the anterior tibial compartment, brachialis, biceps, deltoid, quadriceps, rectus femoris, sartorius, and hamstring, with an average tissue deficit of 66.2% and ranged from 25%-90%

Table 3. Comparison of animal models of VML

Animal model	Advantages	Disadvantages
Mouse	-Cost-effective and readily available[108] -Easily reproducible injury -Ability to obtain a large cohort	-Smaller scale defect than seen clinically[171] -Limited translational capacity to humans
Rat	-Larger than mouse model -Easily reproducible injury -Physiologically, morphologically, and genetically more similar to humans compared to mice	-Smaller scale defect than seen clinically[171] -Limited translational capacity to humans
Sheep /Pigs /Canine	-Larger size defect for more clinically relevant applications	-Limited by price, resources needed to care for them -Few large animal models

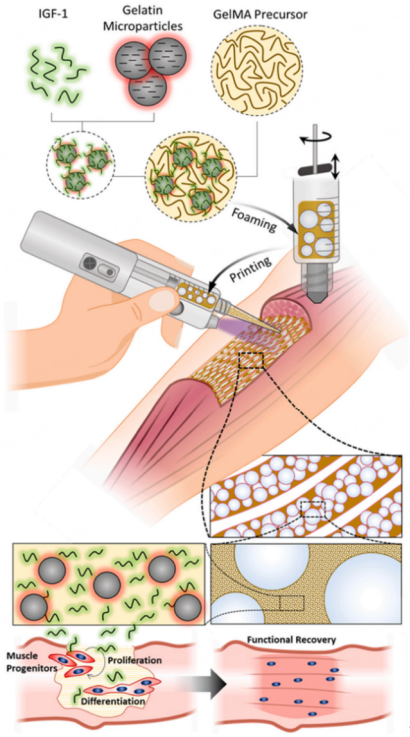

Figure 4. Diagram of a handheld 3D printer used to print scaffolds directly into the muscle defect[165].

compared to the contralateral muscle at enrollment into the study. By 24-28 weeks, strength testing ranged from -17.88% to 136.1% and improvements in force production in 8 of the 13 patients and overall significant improvement of 37.3% ± 12.4% and range-of-motion in at least one task improved by 27.1% ± 10.5% for all the patients[112]. By eight months, bulk muscle, identified by dense tissue on imaging, showed an average increase of 27.2%. Prior to ECM implantation, all the patients had personalized physical therapy regimens and standard-of-care treatments as well. However, the range of improvements varied widely among patients, and it is difficult to compare between patients with different injured muscles. The improvements in strength, range of motion, and bulk muscle, although positive, are still insufficient to fully restore muscle functionality to its pre-injury state.

Here, it is important to mention the incorporation of physical therapy to augment functional recovery following VML. Some studies in rodent models have demonstrated synergistic improvements in muscle strength by adding an exercise regimen[10,165,166]. One study involving a 3D bioprinted scaffold composed of gelatin methacryloyl with colloidal foam-like porosity incorporated progressive aerobic exercise using an 8-week treadmill running regimen and found a significant 25% improvement in tetanic gastrocnemius strength compared to the same treatment group without exercise in a murine gastrocnemius VML model[165]. Scaffold implantation, in combination with exercise training, synergistically improved functional recovery[165]. Similarly, one study involving a rat TA VML injury model alone, without the use of scaffolds, found a 17% improvement in maximal isometric torque after providing free-reign access to running wheels[166]. Another study evaluated early rehabilitation therapy of passive range of motion in a murine posterior compartment VML model and found 3-fold reduced muscle stiffness compared to VML alone[10].

In addition to the role of physical therapy, the use of electrical stimulation on muscle regeneration can further augment functional recovery. Intermittent electrical stimulation can potentially enhance the strength of the remaining muscle post-VML injury. The previous study involving early rehabilitation incorporated a regimen of passive range of motion with electrical stimulation and demonstrated 32% greater isometric plantarflexion torque compared to VML alone and 21% greater compared to range of motion therapy alone[10]. Clinically, early mobilization and therapy lead to improved function and recovery[166]. Further preclinical studies that incorporate exercise and physical therapy with scaffold implantation can hopefully translate to improved functional recovery in clinical patients. Challenges still remain for VML therapy to gain greater functional improvements and large volume muscle tissue.

The ideal scaffold with the optimum microarchitecture (porosity, elasticity, biodegradability, anisotropic), progenitor cell population, and combination of growth factors to effectively guide myogenesis *in vivo* is yet to be designed[131,167]. Vascularization, innervation, and immunocompatibility are essential for scaffold success, and no tissue engineering technology has been fully successful[60,64,167]. Force generation by engineered muscle tissue is reduced on strength testing compared to that of natural muscle[126]. Regeneration of large quantities of aligned myofibers for clinically sufficient functional restoration following scaffold implantation has yet to be achieved[126]. A better understanding of intricate spatiotemporal events in skeletal muscle regeneration and subsequent application to tissue-engineered scaffolds are needed[12,60]. Successful engineered scaffolds for tissue regeneration necessitate the formation of large volumes of autologous myoblasts, growth-factor delivery to support integration and survival of implanted cells *in vivo*, vessel and nerve vascularization, and immunomodulation to prevent excessive scar[22,120,139,168-170]. If scaffolds are cellular, rejection following scaffold implantation must also be considered. Scalability and accurate representation of tissue engineered constructs in VML animal models to human patients present another major challenge in clinical applications[170]. Muscle defects in mice and rats, the most used VML models, are much smaller than those seen clinically, and increasing scaffold size for clinical use will need effective strategies to promote angiogenesis, myogenesis, and neural integration within the construct. Variability in animal models, anatomic location and creation of muscle defects, and muscle function and recovery assessment tools can all lead to variable preclinical results, further limiting the translation from these studies to clinical settings[171]. Additionally, the pathway to industrialization and commercialization of tissue-engineered scaffolds requires improvements in efficient, quick, and cost-effective methods of manufacturing with thorough clinical trial testing that shows acceptable patient safety and clinical effectiveness from regulators and clinicians[158,172].

CONCLUSIONS AND FUTURE DIRECTIONS

While there is a current paucity of options for VML treatment, tissue engineering techniques offer opportunities to promote myogenesis and fibrosis following VML injury. The current standard of care using

autologous functional muscle transfer is limited by the degree of functional recovery and donor site morbidity. Development of effective treatments to address large deficits in skeletal muscle mass is hopeful with tissue engineering. Bioengineered scaffolds can mimic native ECM and incorporate biophysical and biochemical cues to guide host cellular responses and functions, resulting in improved functional recovery. Translation into human patients has been achieved in thirteen patients so far with an acellular scaffold and physical therapy[111,112]. Clinical translation of scaffold treatment in patients with VML injuries could resemble the following paradigm: wound debridement, assessment of strength and range-of-motion, tissue-engineered scaffold implantation, and lastly, functional muscle flap coverage. With bioengineered scaffold implantation, patients with extremity VML injuries can achieve improved muscle functionality and, subsequently, a better quality of life. A combination of extensive physical therapy, scaffold implantation, and functional muscle transfer has the potential as a viable treatment option for VML. Although many challenges remain, further research in this area may allow for scaffolds to emerge as clinically useful treatment modalities for VML injury.

DECLARATIONS

Authors' contributions

Made substantial contributions to writing the manuscript: Zhu C, Sklyar K, Karvar M
Provided revisions, guidance, and direction: Endo Y, Sinha I

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Conflicts of interest

Indranil Sinha reports a relationship with InPrint Bio LLC that includes equity or stocks. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. All other authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

All animal procedures were approved by the Institutional Animal Use and Care Committee of Harvard Medical School (Protocol number: 2016N000375).

Consent for publication

Not applicable.

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