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Vascularized and Innervated Skeletal Muscle Tissue Engineering

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Abstract

Volumetric muscle loss (VML) is a devastating loss of muscle tissue that overwhelms the native regenerative properties of skeletal muscle and results in lifelong functional deficits. There are currently no treatments for VML that fully recover the lost muscle tissue and function. Tissue engineering presents a promising solution for VML treatment and significant research has been performed using tissue engineered muscle constructs in preclinical models of VML with a broad range of defect locations and sizes, tissue engineered construct characteristics, and outcome measures. Due to the complex vascular and neural anatomy within skeletal muscle, regeneration of functional vasculature and nerves is vital for muscle recovery following VML injuries. This review aims to summarize the current state of the field of skeletal muscle tissue engineering using 3D constructs for VML treatment with a focus on studies that have promoted vascular and neural regeneration within the muscle tissue post-VML.

Graphical Abstract

Tissue engineered treatments for volumetric muscle loss (VML) span a range of construct compositions, preclinical models, and functional outcomes. Promoting vascular and neural regeneration following VML is vital to ultimate muscle regeneration and function. This review summarizes tissue engineering solutions to VML with a focus on incorporating vascular and neural regenerative components.

Keywords

skeletal muscle regeneration; tissue engineering; volumetric muscle loss; vascularization; neuromuscular junction

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1. Introduction

1.1 Incidence and Major Causes of Volumetric Muscle Loss

Skeletal muscle comprises around 40% of total human body mass and is the most abundant tissue in the human body^[1]. It has a high regenerative capacity following traumatic injury except in cases of volumetric muscle loss. Volumetric muscle loss (VML) is a defect where the muscle's natural repair mechanisms are overwhelmed leading to chronic functional deficits in the affected muscle^[2–5]. Causes of VML include combat injury, trauma (e.g. car accidents), tumor ablation, bone fracture fixation, and degenerative disease^[3,6]. The incidence of VML in the general US population and the associated economic burden are difficult to estimate since there are no surgical or billing codes specifically dedicated to VML^[7]. Estimates obtained from referencing surgical therapies (e.g. free functional muscle transfer), physical and occupational therapies (e.g. gait training and strength exercises), and advanced wound care are little more than educated guesses and may be highly inaccurate. However, some analyses have been performed for military personnel with extremity battlefield wounds. Among medically-retired military service-members, VML is a major cause of service-disqualifying disability, contributing to over 90% of muscle conditions leading to long-term disability and resulting in a projected lifetime disability cost of \$341,300 per individual^[3]. Of US servicemembers with extremity battlefield wounds from 2001–2005, 53% involved penetrating soft-tissue wounds and 26% were bone fractures, the majority of which (82%) were open fractures with severe soft tissue damage^[8]. These results were similar to those of previous wars^[8,9]. Among the military patient population, 67% of total cases of VML were in the lower limb (Figure 1a) and 58% of patients also had a bone fracture, 14% had a nerve injury, and 5% had a vascular injury in the limb with the VML^[3].

1.2 Biology of VML

VML is characterized by a persistent functional deficit and unlike other causes of muscle injury such as freeze injury, crush, toxin, ischemia reperfusion, and eccentric contractions which fully recover their function after 1–2 months, untreated VML injuries do not regain their full functional capacity^[7]. The native regenerative potential of skeletal muscle through its resident satellite stem cell population has been extensively reviewed elsewhere^[10,11]. In brief, muscle follows a predictable timeline in its response to injury, progressing through phases of degeneration and inflammation, regeneration, and fibrosis^[4,11]. The degeneration and inflammation phase begins within minutes of muscle injury and extends 1–2 weeks post-injury. In this phase, injury-induced disruption of the sarcolemma and basal lamina allow for the influx of extracellular calcium which causes autodigestion and necrosis of myofibers followed by immune cell invasion. Approximately 1 week post-injury, muscle regeneration begins and peaks at around 2 weeks post-injury. In small injuries like contusions or sprains, the regeneration phase is characterized by satellite cell differentiation to myoblasts and fusion with other myoblasts or the remaining mature myofibers. In tandem, low-level fibrosis deposited by fibroblasts bridges the gap between still-functional myofibers in order to transmit force along the muscle as well as guide the regenerating myoblasts along the injury site. The fibrosis and scarring phase begins around 2 weeks post-injury and increases for up to 4 weeks post-injury and is characterized by increased fibroblast-mediated collagen deposition. There have been numerous prior reviews describing the disruptions to

this regenerative pathway caused by VML injuries (Figure 1b)^[4,5,7,12–15]. After the large-scale loss of tissue typified by VML, fibroblasts deposit a large volume of scar tissue faster than the rate of myoblast fusion and regeneration, ultimately preventing muscle regeneration within the defect site^[4]. This results in a significant functional deficit to the damaged muscle that persists over time.

Changes to gene expression within a muscle post-VML also have a significant impact on regenerative potential. Aguilar et. al. utilized RNA-sequencing to investigate molecular changes within untreated VML-injured muscle and those treated with a minced muscle graft. In untreated controls they found that inflammation-related genes and genes associated with ECM deposition and fibrotic remodeling remained upregulated up to 28 days post-injury^[16]. Interestingly, despite finding significant improvements in both muscle strength and histological regeneration of the defect within the treated muscles, they found no difference in gene expression compared to untreated controls. Persistent upregulation of genes associated with fibrosis (*collagen 3*, *MMP2*, *TGFβ1*, *PDGFRα*, *PDPN*) and negative regulation of myogenesis (*Id2*, *MSC*, *Snai1*, *MSTN*, *BMP1*) was present in both the treated and untreated groups at similar levels. This indicates that histologic and functional regeneration of VML-injured muscle is not sufficient to alter the pro-fibrotic gene expression profile of the muscle at 28 days post-injury. Further research is required on the potential for continued remodeling of the muscle tissue and associated changes in gene expression at later time points.

1.3 Understanding Functional Deficits in VML: A Multifaceted Injury

The exact causes of the significant functional deficits observed after VML remain somewhat obscure. Aside from the frank loss of muscle tissue and associated contractile proteins typified by VML, there are a variety of detrimental impacts to the remaining muscle body that lead to sub-optimal strength output by the muscle. In an analysis of 15 preclinical studies, Corona, Wenke, and Ward identified a trend describing the relationship between the strength deficit of VML-injured muscles and the extent of initial injury^[17]. Using regression analysis of VML-induced strength deficits compared to initial percent VML, they found that a VML-injured muscle's force output was significantly lower than what would be expected from the loss of muscle mass alone (Figure 1c). Specifically, the observed strength deficits were 60% higher per percent VML than would be predicted by muscle tissue loss alone. This observation was maintained across various preclinical animal models of VML and at a range of time points up to 4 months post-injury. It was hypothesized that the increased functional deficits were due to persistent detrimental impacts to remaining muscle including denervation, disorganization of myofiber architecture, and impaired force transmission to the tendon.

Further elucidating this trend, Corona et. al. assessed strength deficits and the extent of motoneuron axotomy in a rat tibialis anterior (TA) VML model with an initial 20% TA mass loss^[17]. In addition to confirming that the 20% mass loss yielded higher strength deficits ranging from 45–90%, by labeling motoneurons innervating the entire TA prior to and immediately following the VML injury they found that there was no motoneuron death within the spinal cord due to VML but 57–79% of motoneurons had undergone significant

axotomy and lost interaction with the TA muscle. Notably, this loss of motoneuron interaction with the muscle was present as early as 3 days post-injury and remained constant up to 21 days post-injury. Cross-sections of the injured TA revealed an increase in the number of myofibers in the healing muscle over time, which combined with the constant level of motoneuron axotomy suggests the occurrence of motor unit reorganization and expansion with each motoneuron innervating multiple myofibers. Further research is required on the impact of VML-induced motoneuron axotomy and motor unit expansion and the capability to engineer functional muscle-nerve interactions post-VML. Tissue engineered muscle constructs that incorporate methods to encourage neural infiltration and the formation of functional neuromuscular junctions post-VML are a promising avenue to restore interaction between the muscle and nerve post-VML.

1.4 Preclinical Models of VML

Tissue engineered muscle grafts have been implanted in a broad range of preclinical models of VML spanning many muscle types within mice, rats, rabbits, dogs, and pigs (Figure 2) [18]. Variability of preclinical models occurs both within a species, where for example VML defects in mice have been evaluated in at least six different muscles, as well as across species as the defect size scales larger to approach that of VML defects in humans. These VML models have been extremely useful for understanding the biology of injury as well as for testing therapeutic strategies. Experiments in mice and rats provide for the highest throughput and facilitate the study of cell survival, histoarchitecture of the regenerated muscle, vascular and neural ingrowth, and functional regeneration. Scaling-up biomaterial-based interventions from defects where the average mass of lost tissue is < 20 mg in mice or < 160 mg in rats to clinically relevant scenarios, which may easily be 3 or 4 orders of magnitude larger, is non-trivial. Biomaterial-based treatments of VML in larger preclinical (e.g. dog, pig) and even clinical models have generally resulted in functional improvements although histologic assessments have shown remarkably modest muscle regeneration.

The actual size of the VML defect may not be the only factor driving inconsistencies in reporting therapeutic benefits. The lack of standardization in muscle type and location may also be a factor. For example, biomaterial strategies are agnostic to muscle types, which range from fusiform to bipennate. When comparing studies with differing results it is important to keep in mind the differences in anatomy presented by varying muscles within a single animal as well as anatomical differences between species. Differences between species become particularly relevant when studying regeneration of the neuromuscular junction (NMJ). Striking differences in NMJ morphology and size exist between vertebrate species with an increase in postsynaptic folding and focusing of the NMJ at a single site on the myofiber as one progresses from fish to mammals^[19]. Human nerve terminals and NMJs are among the smallest among vertebrates and contain significantly higher levels of postsynaptic membrane folding which in humans increases the synaptic area by 8 times.

Although a broad range of preclinical models of varied VML defect sizes have been investigated along with the associated damage to vasculature and innervation, there is still no universal consensus on the definition of a critical sized defect in skeletal muscle, which might be utilized as a benchmark control and means of standardization in study design. A

recent study by Anderson et. al. was the first to define a critical sized skeletal muscle defect within the mouse quadriceps and characterized changes to fibrosis, macrophage infiltration, myofiber formation, vasculature, and innervation across a range of defect sizes^[20].

When muscle is resected longitudinally (as opposed to use of a biopsy punch) or defects are partial-thickness it is difficult to maintain consistency in the defect size. Approaches to maintain reproducibility have included tattooing a region of fixed dimensions prior to removal^[21], measuring the weight of muscle removed incrementally during surgery^[22,23], measuring the functional deficit as muscle is removed incrementally during surgery^[24], and recording the total removed muscle weight per animal^[25]. To reproducibly assess differences in muscle damage and regeneration at different VML defect sizes, Anderson et. al. utilized biopsy punches with diameters ranging from 2 to 4 millimeters (corresponding to approximately 4–32% of the quadriceps wet weight) to create full-thickness defects in the mid-belly of the quadriceps with the leg in an extended position. They assessed tissue damage up to 28 days post-injury^[20]. Fibrosis, persistence of CD68+ macrophages, and presence of myofibers with relatively small cross-sectional area increased over time with increasing defect size. By 28 days post-injury, the 2 mm size defects exhibited lower amounts of amounts of fibrosis and had similar levels of centrally-nucleated myofibers to uninjured controls demonstrating its status as a sub-critical sized defect with healing potential.

Defects of 3 mm diameters were further investigated for neural and vascular regeneration. Transgenic Thy1-YFP mice, whose motor neurons express yellow fluorescent protein (YFP), were used to visualize innervation within the defect site up to 28 days post-injury. Although motor neurons were visible in uninjured muscle tissue, significant autofluorescence in VML defects prevented visualization of neurons within the injured muscle. Postsynaptic acetylcholine receptor clusters demonstrated marked differences in morphology between control and injured muscle and were classified into three categories: normal pretzel, abnormal fragmented, and newly-formed. No clusters with a normal pretzel morphology were visible within the defect site at either 14 or 28 days post-injury. Cluster morphology within the defect remodeled over time with significantly more clusters demonstrating a fragmented morphology at 14 days post-injury, decreasing over time to contain more of the newly formed clusters 28 days post-injury. Of the clusters present at 28 days, 44.3% had an abnormal fragmented morphology and 55.7% appeared to be newly formed clusters. Despite remodeling in cluster morphology over time, no clusters were associated with a regenerated motor neuron. Total vascular volume within the defect site was quantified 28 days post-injury using Microfil and micro CT and demonstrated that injured muscle had a larger vascular volume as well as perfused vessels with a larger diameter compared to uninjured controls.

Although significant in its definition and characterization of a skeletal muscle critical sized defect, the previous study did not measure force deficits due to injury, which is a defining feature of VML and standard for the field. They also utilized adult female mice between 3 and 9 months of age and assessed VML injury to the quadriceps muscle alone. When translating the 3 mm critical size defect model to future studies, potential differences in anatomy and regeneration between various different muscle types as well as differences due

to species, age, and sex must be considered. Larger animals generally have different muscle fiber type compositions than smaller animals, with a higher prevalence of slow twitch myofibers^[26]. Young rodents have a higher density of satellite cells^[27] with differences in remodeling post-VML^[23] and may therefore require a larger critical sized defect than older animals. Differences in muscle anatomy and regeneration due to sex must also be considered as it has been shown that over 3,000 genes are differentially regulated between male and female muscle^[28]. Fiber type composition, myofiber cross-sectional area, fatigue recovery, and endurance testing differ greatly between men and women in various muscles and sex-based differences in skeletal muscle composition are present across species^[26]. Male myofibers generally have a lower type I composition, a larger cross-sectional area, and are more fatigable than those in females, and women typically exhibit longer endurance and faster recovery from muscle exhaustion than men which may have implications for sex-based differences in regeneration following injury. Future studies on the applicability of the 3 mm critical sized defect to alternative preclinical models and potential differences due to muscle type, age, sex, and species would be of great benefit to the field.

Significant advancements have been made in the use of pre-vascularized constructs within VML-injured muscle, including successes in characterization of *in vitro* vascular development, anastomosis with host vasculature post-implantation, and the development of several useful methodologies for *in vivo* vascular quantification. The majority of those studies have utilized a mouse abdominal defect model^[29–35], however, and it is unclear if either the constructs implanted or the success of vascular regeneration would translate to muscles with a different anatomy or larger animal models with a higher vascular demand post-injury. As described below, different muscles within the body have varying degrees of heterogeneity in capillary density throughout the tissue and constructs that successfully anastomose with host vasculature within one defect model may not perform as well elsewhere in the body or in the process of scaling up to treat a human VML defect. Further research on the versatility of pre-vascularized constructs under development as well as their feasibility for use in large animal models is required.. These differences between species in muscle anatomy, vascularization, and innervation must be considered when comparing preclinical models to each other and when developing a tissue engineered muscle graft for VML treatment in humans.

1.5 Current Clinical Treatment of VML

Optimal VML treatment depends on the location and size of a specific VML defect. The functional deficits associated with a small VML defect may be ameliorated by hypertrophy of surrounding muscles via physical therapy while muscles with larger VML defects do not respond to physical therapy^[16]. Current treatment options for large-scale VML are limited and include transfer of an autologous free muscle flap^[2,36], muscle transposition^[2,3], or amputation and power bracing^[36], all of which have major limitations. Donor site morbidity, lack of donor tissue, and the need for a highly skilled surgical team complicate VML treatment and decrease positive patient outcomes^[6,36]. In battlefield injuries, free or rotational muscle flaps are often utilized as soft tissue coverage in the treatment of severe type III open tibia fractures but do not recover the strength deficit of the injured muscle surrounding the fracture^[3]. Tissue engineering strategies aim to fill the muscle tissue volume

and enable dense regeneration of vascularized and innervated muscle as well as to recover the full function of VML-injured muscle. An understanding of skeletal muscle vascular biology, neural biology and the impediments to regeneration caused by VML is vital to successful tissue engineered treatments.

2. Tissue Engineering Strategies to Regenerate Skeletal Muscle

A range of approaches for the development of tissue engineered skeletal muscle have been reported with previous excellent reviews summarizing the current state of the field^[18,37–44]. Tissue engineered skeletal muscle approaches fall into four main categories: acellular scaffolds, cells sheet-derived engineered muscle grafts, minced muscle grafts, and tissue engineered muscle grafts (Figure 3)^[45]. Each of these may be combined with growth factors or other pro-regenerative molecules or peptides to improve the regenerative response. There are benefits and drawbacks to both the acellular or cell-based approaches to skeletal muscle tissue engineering, with acellular scaffolds providing a lower regulatory hurdle and faster translation to the clinic^[45] while cell-based approaches often result in significantly more histological muscle regeneration post-VML compared to acellular counterparts^[25,46]. Minced muscle grafts have excellent engraftment potential but require *in vitro* expansion or the use of large volumes of host muscle tissue.

Cell sources for skeletal muscle tissue engineering have been reviewed elsewhere^[45,47,48] and include satellite cells or other muscle-derived precursor cells, perivascular stem cells, bone marrow-derived mesenchymal stem cells, umbilical cord mesenchymal stem cells, adipose-derived stem cells, induced pluripotent stem cells, and embryonic stem cells. Major considerations when selecting a myogenic cell source include expansion capacity, immunogenicity, translatability, differentiation potential, and engraftment potential *in vivo*. Muscle precursor cells are frequently combined with a material scaffold to provide mechanical support and assist in cell retention within the defect, or acellular scaffolds are implanted that are designed to promote endogenous muscle repair.

The following sections will provide a brief overview of each of the four approaches.

2.1. Cell sheet-derived engineered muscle grafts.

The delivery of monodispersed cells traditionally involves the injection of a muscle cell suspension with or without a hydrogel carrier^[45,49]. However, this strategy, which does not provide significant structural support, is not particularly suitable for the treatment of VML defects. Instead, implantable cell sheet-derived 3D constructs have been developed by culturing muscle-derived cells in monolayer until confluence and subsequent delamination and formation of a cylindrical 3D construct^[50,51]. One study cultured primary mouse muscle cells in monolayer for approximately 8 days and implanted the delaminated monolayer as a 3D construct in a mouse VML model of the extensor digitorum longus muscle^[51]. Muscle and vasculature were present within the explanted graft after 15 days *in vivo*. In a second study, rat primary muscle precursor cells were cultured as monolayers that were then delaminated and rolled into cylindrical constructs with bone marrow cell-derived bone anchors at each end^[50]. The constructs were then implanted in a rat 30% VML model of the tibialis anterior and assessed after 28 days, at which they exhibited the formation of small

myofibers and the presence of associated vasculature and nerves. The above cell sheet-derived engineered graft approaches benefit from a high cell density within the constructs and promising results within small VML defects *in vivo*, but may be limited in their ability to scale-up to clinically relevant sizes and can be difficult to surgically manipulate due to the lack of a scaffold.

2.2 Acellular Scaffolds

There has been much discussion on the ideal biomaterial for skeletal muscle tissue engineering scaffolds, which has been the focus of several previous reviews^[18,39,44,52]. The two main biomaterial categories are natural versus synthetic materials with hybrid materials as a third smaller category. Natural materials used for skeletal muscle tissue engineering include decellularized extracellular matrix (dECM) from various organ and donor sources as well as ECM components and other naturally-derived materials such as fibrin, hyaluronic acid, laminin, collagen, chitosan, silk fibroin, alginate, agarose, keratin, and gelatin. Synthetic materials used for skeletal muscle tissue engineering include polypropylene, poly-(lactic acid), poly-(glycolic acid), poly-lactic-co-glycolic acid, poly(ϵ -caprolactone), polyurethane, and polyethylene glycol. Natural materials are bioactive, may contain pro-myogenic growth factors, and are biocompatible but have limitations in batch-to-batch variability, immunogenicity, and a lack of precise design control. Synthetic materials offer improvements in the tunability of mechanical cues and nanoscale topography as well as consistency between batches but may be limited in their biocompatibility, cell adhesion, and need for functionalization due to limited bioactivity. Hybrid materials are combinations of natural and synthetic biomaterials and are promising options for combining the bioactivity of natural materials with the precision of synthetic materials, but they may also retain the limitations of both categories. Biomaterials in all categories can be utilized with a broad range of fabrication methods that each provides different mechanical properties, degradation timeline, topographical cues to cultured cells, and *in vivo* engraftment potential. Generally, naturally derived biomaterials have resulted in improved muscle, vascular, and neural regeneration with significant emphasis placed on the method of fabrication and presence of alignment cues, native growth factors, myogenic stiffness, biocompatibility, and degradation timeline.

There are currently conflicting reports on the ability of acellular scaffolds to consistently promote muscle regeneration and functional recovery post-VML. Acellular scaffolds are more clinically translatable with a lower regulatory burden and acellular decellularized ECM (dECM) scaffolds have the potential to modulate the immune response and macrophage polarization to promote muscle regeneration^[53]. Despite these benefits, acellular scaffolds used to treat VML defects often lack histological evidence of muscle regeneration. There has been a broad range of preclinical studies comparing the efficacy of acellular or cell-seeded dECM^[21,46,54–59] with mixed results. There have also been two recent clinical trials evaluating the regenerative potential of acellular dECM within human VML defects^[58,60–62]. In an effort to address significant variability in reported results, the efficacy of using acellular dECM to treat VML injuries was systematically evaluated in a large 2015 study with 120 rats (8 rats/group)^[46]. Acellular dECM was implanted into two different defect models, a musculotendinous junction (MTJ) model and a VML model, and compared

to untreated or autograft-treated controls at a range of time points up to four months post-treatment. In the MTJ model, the implanted ECM scaffold completely resorbed without muscle tissue remodeling. In the VML model, the implanted ECM scaffold improved muscle function by 17% but remodeled into fibrous tissue lacking significant *de novo* myofiber formation and muscle regeneration (Figure 4c). Physical rehabilitation via voluntary cage wheel running did not improve the regenerative response of the decellularized ECM treatment. The ability of the implanted scaffold to improve contractile properties of the muscle without correlated muscle fiber regeneration within the defect area may be explained by scaffold mediated functional fibrosis and has been demonstrated repeatedly^[22,25,46,63–65]. The implanted scaffold likely functions as a mechanical bridge between intact muscle fibers, transmitting the contractile force across the defect and subsequently resulting in a higher measured functional outcome. This is in contrast to force production by regenerating myofibers within the defect, which is the ultimate goal of a tissue engineered treatment for VML.

The first use of a tissue engineered construct for VML treatment in humans was 2010 case report where a single patient with a blast-induced VML injury to the thigh was treated with acellular decellularized porcine ECM. The patient demonstrated improved isokinetic performance four months post-treatment and evidence of muscle formation in CT imaging nine months post-treatment with no histology shown^[58]. Following initial testing in mouse quadriceps VML defects with moderate regeneration following 6 months of dECM treatment^[54], a recent study assessed 13 human patients with VML injuries at a range of anatomical locations with an average muscle tissue deficit of 66.2% and evaluated the efficacy of an acellular scaffold composed of porcine dECM to promote muscle regeneration^[54,60]. Measurements of force production, improvements in functional task performance, and electromyographical assessments up to six months post-operatively demonstrated that patients showed an average of 37.3% improvement in muscle strength, 27.1% improvement in range of motion, and some electrophysiological improvements compared to pre-operative values^[60]. Regeneration and remodeling of the tissue within the defect site was demonstrated through histology of biopsies and CT or MRI imaging (Fig. 4a). Disconnected islands of muscle tissue were present within the defect site in addition to vasculature, mobilization of perivascular stem cells, and some neurofilament (Figure 4b). Although the study incorporates robust functional testing and CT data there was limited histological analysis shown. Since muscle tissue engineering data in humans is scarce, the field would benefit from a more detailed quantification of the histological data including muscle, vascular, and nerve density across the biopsy sample as well as a detailed representation of variability among patients.

2.3 Minced Muscle Grafts (MMGs)

A third category investigated pre-clinically for VML treatment is the use of minced muscle grafts. In this approach, autologous or allogeneic muscle is removed via biopsy, minced into small pieces and often expanded *in vitro*, and placed in the VML defect^[16,22,66–68]. Minced muscle grafts can be easily generated from the patient's own muscle and result in excellent muscle regeneration but require *in vitro* expansion or the use of large volumes of host muscle tissue. Due to the need to limit the volume of biopsied muscle removed from the

patient, the minced muscle must be expanded for this approach to be feasible for the treatment of large defects. To maximize the regenerative potential of small muscle biopsies, the potential to decrease the percent composition of minced muscle by mixing it with pro-regenerative hydrogels and growth factors is an area of active investigation. Expansion capacity of autologous minced muscle within a collagen hydrogel at a range of muscle compositions was investigated and compared to grafts composed of 100% autologous minced muscle (Figure 5a,b)^[66]. It was found that reducing the percent of muscle by 50% retained its ability to promote functional improvement post-VML but the regenerating defects treated with 50% minced muscle contained less *de novo* myofiber regeneration than the 100% grafts and regenerating myofibers had smaller cross-sectional areas. The potential for improving outcomes of VML treatment with 50% minced muscle grafts via incorporation of laminin-111, a muscle extracellular matrix protein, within a hyaluronic acid hydrogel has also been investigated (Figure 5c)^[67]. The 50% minced graft with laminin-111 and hyaluronic acid resulted in improved function compared to untreated controls but was not significantly different from treatment with 50% minced grafts lacking laminin-111 or lacking hyaluronic acid. *De novo* muscle regeneration remained less than that observed in injuries treated with 100% minced grafts, indicating a need for further research in the expansion and treatment potential of minced muscle grafts composed of less than 100% muscle. In addition, it was recently shown that VML-injured muscles treated with a 100% autologous minced muscle graft have no difference in gene expression compared to untreated controls, with upregulation of pro-fibrotic and inflammation-related genes 28 days post-injury (Figure 5d)^[16]. Although a promising option, more research is required on efficient *in vitro* expansion methodologies of minced muscle grafts and overall scale-up potential prior to clinical translation.

2.4 Tissue Engineered Muscle Grafts (TEMGs)

Tissue engineered muscle grafts (TEMGs) leverage the benefits of acellular and cells-only approaches through the combination of a translatable pro-myogenic cell source with a biomaterial scaffold. Despite their significant regenerative potential, TEMGs may be limited, however, by long *in vitro* pre-culture times, issues with cell distribution within the scaffold, and limited expandable myogenic cell sources. There has been extensive research on the use of various TEMGs to treat VML defects in a range of preclinical models. Similar to the cells-only approach, there has been a broad range of cell sources incorporated into TEMGs including satellite cells or other muscle-derived precursor cells^[21,33,56,69–73], mesenchymal stem cells^[74,75], adipose-derived stem cells^[76–78], and human pluripotent stem cells^[79–81] (Figure 6). Scaffold materials used in the development of TEMGs include dECM^[21,55,56,74,82], collagen^[83,84], hyaluronic acid^[67,85], fibrin^[24,25,49,70,77], poly(lactic acid)/poly(lactic-co-glycolic acid)^[32,86], silk fibroin^[87], keratin^[71,72], gelatin^[88,89], and methacrylated gelatin^[90]. Regenerative potential of TEMGs following implantation within VML defects has varied widely and comparison between studies is complicated by the broad range of cells and scaffold materials as well as differences in *in vitro* pre-culture time, construct size, and the preclinical VML defect model used. Although TEMGs are limited by the regulatory hurdle and biological variability inherent to cell-based constructs, the combination of pro-myogenic cells with a biomaterial scaffold present has demonstrated increased histological regeneration of VML-damaged muscle compared to acellular

constructs in addition to improving muscle contractile function. Additionally, the incorporation of a biomaterial scaffold enhances construct durability and ease of surgical manipulation compared to cells-only constructs. Overall, TEMGs are a promising option for the treatment of VML.

2.5 Functional Outcomes

VML is characterized by persistent loss of function over time and functional testing following treatment of VML injuries is a standard assessment. Different studies have tested functional output at a range of time points post-VML from 2 weeks^[67] to 6 months post-injury^[65]. Functional measurements primarily involve electrical stimulation of a nerve near the graft and subsequent measurement of the muscle's twitch force, maximum isometric force, maximum specific force, and/or the force frequency relationship. Measurement of the force frequency relationship enables quantification of the muscle's ED₅₀, the frequency of stimulation that elicits its half-maximal contraction amplitude. Often, contralateral uninjured muscles are used as a comparison and forces may be presented as a percent of the contralateral. Ex vivo force measurement is also used to measure muscle contractility independent of innervation, whereby explanted muscle is tethered on both ends and directly stimulated^[21,65]. In some cases, there were no differences between treatment groups following neural stimulation but ex vivo force measurement did show differences^[21]. An alternative measure of muscle function that is less often utilized in tissue engineering approaches post-VML is the use of electromyography (EMG) and compound motor action potential (CMAP)^[59,61,86,91]. In EMG, surface or needle electrodes are placed above or within the muscle and measure motor neuron electrical signals and electrical activity within the muscle in response to nerve activity. Due to the importance of neural regeneration post-VML to ultimate graft integration and function, EMG is a useful tool for muscle tissue engineers and should be more widely utilized to measure functional recovery post-VML.

The vast majority of studies quantifying functional outcomes post-VML in preclinical models have analyzed muscle function at the muscle's optimal length (L_0) and measured standard functional outcomes such as maximum isometric contraction, specific force, twitch force, and force-frequency curves. As mentioned previously, there has been recent discussion on the discrepancy between improved functional outcomes post-implantation despite low and variable *de novo* myofiber regeneration, in particular with respect to implantation of acellular dECM scaffolds^[22,25,46,63–65] calling into question the utility of these measurements to determine the extent of regeneration. It has been postulated that functional improvements due to acellular dECM implantation may be due to scaffold mediated functional fibrosis^[46,64]. A significant recent study by Passipieri et. al. tested this hypothesis by utilizing a novel computational model to investigate the biomechanical mechanisms underlying improved functional outcomes despite limited histological regeneration following implantation of acellular and cell-seeded dECM scaffolds in a rat latissimus dorsi (LD) model^[65]. They found that increased contractile force measurements of the treated LD (acellular and cell-seeded) compared to non-repaired controls 2 months post-injury were primarily due to increased volume of tissue in the defect region and subsequent improved passive force generating ability. Despite improvements in *de novo* myofiber regeneration near the implant/host interface by cell-seeded grafts and an ~87%

recovery of maximum isometric force, 96% of the total measured force recovery was due to passive force generation and differences in measured contractility between acellular and cell-seeded grafts were likely due to graft thickness alone. The model also highlights the contribution of lateral force transmission to contractility of non-repaired VML-injured muscle, which should be a consideration by future studies on VML. In a substantial result, simulated forces predicted by the model demonstrate a significant decrease in the contribution of passive force generation to total measured contractile force when measurements are taken at 80% of the muscle's optimal length. When measured at a suboptimal length, implanted grafts would require active force generation by myofibers within the defect in order to show marked improvements in overall muscle contractility. They also demonstrated decreases in the contribution of passive force generation with increased defect size. The predicted results must be validated experimentally but if true could improve measurement of graft function post-VML through the use of suboptimal muscle length and more accurately reflect histological results.

3. Engineering Vascularized Skeletal Muscle

3.1 Anatomy of Muscle Vasculature

Due to the high metabolic demands of skeletal muscle, tissue engineering techniques for skeletal muscle regeneration must consider the design constraints imposed by the limits of oxygen diffusion. Previous studies with pancreatic islet cells and lung-derived fibroblasts have shown that the limiting oxygen diffusion distance in order to prevent necrotic cores in cultured tissues is 150–200 μm ^[92–94]. Native muscle myofibers have diameters ranging from approximately 30–100 μm ^[5,95], which, when combined with the diffusion distance of oxygen, demonstrate the need for perfusable vasculature within the large-scale regenerating muscle environment post-VML. The dense and organized structure of vasculature within native skeletal muscle is optimized for efficient nutrient transfer throughout the entire tissue and must be considered in tissue engineered constructs. Blood vessels within skeletal muscle are highly organized to provide optimal nutrient and gas diffusion to myofibers with arterioles and venules branching into terminal arterioles and terminal venules that are perpendicular to the skeletal muscle myofibers (Figure 7a)^[96]. These terminal arterioles and venules are interspersed and alternate with one terminal venule present between two terminal arterioles, and a terminal arteriole is present approximately every 1 millimeter down the length of each myofiber. The terminal arterioles and venules then branch into tortuous capillaries that run parallel to the myofibers and form organized networks called microvascular units^[97]. To further the efficiency of gas transfer from blood vessels to muscle cells, capillaries are embedded within grooves in the myofiber sarcolemma and mitochondria within the myofiber interior congregate around these grooves, minimizing the required distance of gas and fatty acid diffusion^[96].

Microvascular units (MVUs) are the smallest unit of control for vascular perfusion in skeletal muscle tissue and consist of a terminal arteriole and the group of 20–30 capillaries that it supplies^[98] (Figure 7b). Each MVU covers approximately 1 millimeter of the myofiber length after which a new MVU supplied by a different terminal arteriole begins. The width of a MVU is approximately 500 μm and it extends around 100 μm deep into the

muscle, and there are approximately 20 MVUs per milligram of skeletal muscle tissue^[98]. Capillaries within an MVU are 4–10 μm in diameter and have a tortuous architecture along the myofibers which functions to increase the capillary to myofiber surface area and maximize oxygen diffusion to the muscle^[97,99]. Skeletal muscle is a highly plastic tissue with the capacity to adapt to increased oxygen and nutrient demands^[99]. In resting muscle, the skeletal muscle microvasculature and MVUs are intermittently and unequally perfused. The skeletal muscle vasculature dilates in response to exercise with changes in oxygen demand sensed by muscle microcirculation and transmitted upstream to larger vessels inducing vasodilation and vasomotion, rhythmic oscillations in blood vessel diameter that impact blood flow. In addition, muscle vasculature and blood flow is modified in response to exercise by local factors released by the muscle as well as neurovascular changes to increase vessel perfusion^[96,100]. When regenerating a large volume of skeletal muscle tissue after injuries such as VML, the microvascular architecture and capacity of the muscle vasculature to adapt to changing environmental and exercise demands should be considered.

A variety of mechanisms to quantify the extent of vascularization are utilized in the analysis of skeletal muscle biology. These may also be applied to the quantification of vasculature in engineered muscle constructs and regenerated muscle post-VML. For an excellent review of quantification methods of skeletal muscle vascularity see Olfert and Baum et al^[100]. Capillary density (CD) is perhaps most frequently used and is defined as the number of capillaries per millimeter squared when counted in cross-section. Increases in skeletal muscle CD have been linked to increased muscle functional performance, mitochondrial volume density, and maximal aerobic capacity and decreased skeletal muscle CD is associated with poor prognoses for a variety of diseases including peripheral arterial disease, diabetes, cachexia, and chronic obstructive pulmonary disease^[100]. Despite the utility of CD to measure muscle vascularity, it does not account for changes in the capillary to myofiber surface area caused by differences in capillary vessel tortuosity or myofiber diameter. Capillary length density, the length of capillaries per unit volume of muscle fibers, incorporates vessel tortuosity but requires more technically challenging longitudinal sectioning and quantification methods. The capillary to fiber ratio (C/F ratio) is counted in cross section similar to CD but more accurately represents differences in capillary surface area and overall vascular density due to changes in myofiber area. Use of CD alone does not capture the impact of changes to myofiber cross-sectional area which can adapt in response to exercise and tissue remodeling after injury^[100]. A final consideration when assessing muscle vascularity is the heterogeneity of vascular density throughout the depth of the native muscle tissue. Different muscles within the body have varying degrees of heterogeneity in both the muscle fiber type as well as capillary density throughout the tissue (Figure 7c)^[100]. Tissue engineered constructs for skeletal muscle regeneration post-VML should consider the native capillary density and C/F ratio pattern for the specific muscle being treated, and analysis of regenerating muscle should consider differences in capillary density across a section of the native tissue. Engineered constructs with tunable vascular density or incorporation of a vascular density gradient depending on the intended site of implantation would be a potentially beneficial addition to the field.

3.2 Strategies to Enhance Vascularization of TEMGs

Approaches to engineer vascularized skeletal muscle with the use of implantable 3D constructs fall into two main categories: muscle constructs relying on host vessel infiltration and constructs that are pre-vascularized *in vitro* prior to implantation with or without an incorporated myoblast cell source (Figure 8). Scaffold material may encourage host vascular infiltration, and constructs designed to promote vascularization post-VML have included fibrin, decellularized ECM, collagen, and combinations of poly-(lactic acid) and poly-lactic-co-glycolic acid. Fibrin in particular has a demonstrated pro-angiogenic potential^[101] and has been utilized in combination with other biomaterials to promote vascularization *in vivo*. Defect size is also a critical determining factor in the feasibility and practicality of host vessel infiltration. While host vessel infiltration may be sufficient for the survival of implanted grafts within smaller defects in mice and rats, larger animal models of VML and human patients would likely require an implanted vascular source for graft survival over time^[102]. Significant work has been done with both approaches to engineer vascularized muscle yielding promising results with the potential for clinical translation.

3.2.1 Host Vessel Infiltration—Infiltration of the host blood vessels into an implanted construct is impacted by a variety of factors including ischemia within the defect site, paracrine signaling by regenerating muscle cells, pro-angiogenic biomaterial scaffolds, and mobilization of immune cells. Approaches to encourage host vessel infiltration often utilize microsurgical techniques to graft portions of major host vessels onto implanted muscle constructs or the implantation of muscle constructs adjacent to a major vessel. Early studies utilizing host vessel infiltration were mainly concerned with supporting the survival of implanted muscle tissue and focused on graft survival over time while later studies prioritized analysis of the infiltrating vasculature itself and developed advanced quantitative techniques to do so. A variety of skeletal muscle tissue engineering studies that rely on host vessel infiltration post-VML have been summarized in Table 1. There are also numerous studies investigating host vessel infiltration following muscle injuries other than VML (ischemia, myotoxin, etc) that are outside the scope of this review.

The feasibility of encouraging host vasculature to infiltrate an implanted muscle construct was initially assessed with a microsurgical arteriovenous (AV) loop implantation model. In one study, rat or human muscle was implanted within the groin in a semi-sealed chamber connected to host AV vessels. Gross examination of the tissue demonstrated survival of vascularized muscle tissue 6 weeks post-implantation^[103]. A second study further elucidated the potential of the AV loop and examined the potential of host vessels to vascularize an implanted acellular fibrin scaffold. After vascularizing the scaffold *in vivo* for 2 weeks, they then injected fluorescent primary rat myoblasts into the scaffold site to test its ability to support muscle growth *in vivo* over time^[104]. Myoblasts implanted into vascularized scaffolds were visible up to 8 weeks post-implantation while control myoblasts implanted into the contralateral groin lacking an AV loop were not detectable. Yet another study utilized a different technique to promote vascularization of an implanted muscle construct by creating a fibrin scaffold seeded with primary rat myoblasts and implanting it within a cylindrical silicone chamber surrounding the host femoral artery and vein^[102]. Three weeks post-implantation, host vessels had infiltrated the muscle construct and the explanted

construct contained desmin positive muscle cells and was contractile when electrically stimulated.

Other studies have attempted to encourage host vessel infiltration by transecting a major blood vessel and attaching the transected end to the implanted construct. To vascularize and innervate a construct composed of primary rat myoblasts, Williams et. al. implanted the construct along the biceps femoris muscle of rats and attached transected ends of the sural nerve and blood vessel to the implant^[105]. After just one week post-implantation they saw host vessels within the graft. In a unique animal model enabling intravital imaging of blood vessel infiltration, Juhas et. al. implanted engineered muscle bundles composed of primary rat satellite cells in fibrin into a mouse dorsal skinfold window model after two weeks of *in vitro* pre-cultivation (Figure 9a,b)^[73]. The dorsal skinfold model enabled direct visualization of constructs and blood vessels through a glass coverslip in the live animal and a broad range of quantification techniques. Host blood vessels rapidly infiltrated the implanted constructs with pre-differentiated constructs that contained more mature muscle cells inducing a higher blood vessel density over time than undifferentiated constructs that contained an immature satellite cell-like phenotype. Additionally, the infiltrating blood vessels were perfused by host blood flow after just one week *in vivo*, which is visible via video. To further quantify the blood vessel maturity, they also quantified the cross-sectional lumen density of the blood vessels and found that constructs containing more mature muscle cells had an overall higher lumen density. The average lumen diameter increased over time to reach 7 μm , similar to native hindlimb muscle, and the average rate of vessel ingrowth in pre-differentiated constructs was 18.9 ± 2.1 vessels $\cdot\text{mm}^{-2}\cdot\text{day}^{-1}$. Although their muscle constructs were not implanted in a VML model, the dorsal skinfold window model is an important advancement for the field due to its ability to enable live intravital imaging and detailed analysis of host vessel infiltration into implanted muscle constructs.

Another interesting approach to encourage host vessel infiltration is the use of gene therapy to encourage vessel growth. Zhou et. al. utilized primary rat myoblasts transfected to overexpress human vascular endothelial growth factor 165 (VEGF-165) or human stromal cell-derived factor 1 (SDF-1) on a calf skin-derived collagen sponge to encourage vessel growth after implantation in a back muscle defect^[106] (Figure 9c). They implanted four groups of cells pre-cultured on the collagen sponge: non-transfected myoblasts, VEGF-165 transfected myoblasts, SDF-1 transfected myoblasts, and a 1:1 ratio of VEGF-165 and SDF-1 transfected myoblasts. After up to 8 weeks post-implantation they found that the while the VEGF-165 and SDF-1 transfection groups had more vessels than non-transfected controls they contained varied degrees of vascular infiltration to the graft. The combined co-culture of VEGF-165 and SDF-1 myoblasts, however, had enhanced vascular infiltration than either growth factor group alone as well as larger vascular lumens. Upon analysis of microvascular density they found that capillary density in all three transfection groups decreased slightly over time as the tissue remodeled, with each group displaying different temporal changes in capillary density over the eight week regenerative period.

Host vessel infiltration serves as a useful tool to demonstrate viability and regenerative potential of tissue engineered muscle constructs during initial testing within small VML defects. Reliance on host vasculature for graft survival when scaling to large, clinically-

relevant defect sizes is not feasible and alternative methods of promoting construct vascularization are vital to clinical translation. Despite this eventual need, studies that utilize host vessel infiltration in small defect sizes are relatively high throughput and provide useful and important information on early-stage muscle regenerative potential of an engineered construct. Additionally, critical vascular quantification metrics have been developed through the use of host vessel infiltration that can inform future studies within larger defects that contain an implanted vascular source.

3.2.2 Pre-Vascularized Constructs—Pre-vascularized constructs for skeletal muscle tissue engineering include both constructs containing vessels alone and co-cultures of vessels and muscle cells. Major skeletal muscle tissue engineering studies that rely on pre-vascularized scaffolds for VML treatment have been summarized in Table 2. Early successes in vascularized skeletal muscle tissue engineering included the development of a 3D tri-culture system containing cell sources for the three major components of vascularized muscle: skeletal muscle cells, human endothelial cells, and pericytes to support the developing vascular network^[29]. Mouse embryonic fibroblasts were used as a pericyte cell source and it was found that inclusion of fibroblasts in the 3D construct significantly improved vascular maturity over time and that fibroblasts differentiated to express smooth muscle actin and were located around endothelial cells as a support for vessels. Constructs implanted in a mouse abdominal wall VML defect model contained perfusable human vessels after 2 weeks as well as desmin and myogenin positive muscle, with tri-culture constructs resulting in greater vascular perfusion in both lectin perfusion and luciferase injection assays (Figure 10d).

Further work on the tri-culture system utilizing C2C12 myoblasts, GFP-positive human umbilical vein endothelial cells (HUVECs), and human foreskin fibroblasts (HFFs) investigated the impact of *in vitro* culture time on vascular maturity and integration following implantation in an abdominal wall defect model^[31]. Tri-culture scaffolds were compared to empty scaffolds or those containing just C2C12s and all implanted constructs were evaluated after 2 weeks *in vivo*. Grafts implanted after different pre-culture times displayed differences in vessel alignment and maturity 2 weeks post-implantation with increased incubation time *in vitro* causing more mature morphology post-implantation. The three-week pre-culture group exhibited the most mature morphology with parallel, aligned myofibers and vessels, similar in morphology to native abdominal muscle. Functionality of the implanted vessels was assessed through tail-vein injection of fluorescent dextran and tri-culture constructs resulted in improved blood flow through implanted grafts compared to acellular or myoblast-only constructs. Blood flow, blood vessel density, C/F ratio, and smooth muscle actin density increased with *in vitro* pre-culture time among the tri-culture groups. Interestingly, the signal of implanted GFP-HUVECs decreased in the three-week pre-culture group despite the increase in overall blood flow, indicating remodeling of the more mature implanted vasculature and replacement by the host.

To further investigate vascular network formation within 3D constructs, Lesman et. al. directly compared vascular morphogenesis of HUVEC:HFF co-cultures versus HUVEC:HFF:C2C12 tri-cultures in 3D constructs of varied scaffold composition (50% PLLA:PLGA, fibrin and thrombin at different concentrations, composite of PLLA:PLGA +

fibrin)^[30]. Quantification of vascular network maturity via live cell imaging of fluorescent vessels over 7 days of *in vitro* culture was performed through a maturity scoring system that classified the extent of network connectivity into four categories (Figure 10a,b). In an interesting result, they found that the incorporation of myoblasts slightly impeded *in vitro* vascular network maturity over time compared to the co-culture lacking myoblasts. They evaluated integration of the co-culture constructs with host vasculature *in vivo* by implanting co-cultures on a range of scaffold compositions in an abdominal wall defect for 10 days and determined that while both fibrin and composite scaffolds with cells resulted in some anastomosis with host vessels, the composite scaffolds containing both PLLA:PLGA and fibrin resulted in the highest amount of perfused host vessels within the graft regardless of whether or not cells were included. Although significant perfusion of infiltrating host vessels was visible along with some mosaic vessels composed of mouse and human cells, few of the implanted RFP+ human vessels were perfused with dextran at 10 days (Figure 10e).

To avoid the lengthy *in vitro* cultivation times of pre-vascularized constructs and develop a more clinically translatable VML treatment, Pilia et. al. assessed the vascular regenerative potential of freshly isolated microvascular fragments (MVFs) versus adipose derived stem cells (ASCs) in a collagen scaffold implanted in a rat VML model of the TA muscle for 1 or 2 weeks^[78]. MVFs and ASCs were isolated from rat epididymal fat pads and while MVFs were freshly isolated prior to implantation, ASCs were cultured and utilized at passage 2. VML defects treated with MVFs contained vessels earlier with a higher vessel density than those treated with ASCs or acellular controls but all treatment groups had a vessel density that remained lower than control native skeletal muscle. Vascular perfusion measured via DiI systemic injection was present as early as 7 days in the MVF group and after 14 days in the ASC group, although total vascular perfusion was low and unevenly distributed throughout the defect. MVFs isolated from GFP+ rats were then used to determine whether perfusable vessels within the defect were derived from implanted MVFs or host vessel ingrowth and the majority of vessels within the defect area were GFP-positive. Although overall vascular perfusion was low in this study, it is noteworthy that MVFs freshly isolated on the day of surgery enabled significant vascularization of implanted constructs and is a step toward a more clinically translatable treatment.

The vascular potential of MVFs in 3D muscle constructs was further investigated by a recent study that utilized co-culture of freshly isolated primary rat MVFs and *in vitro* expanded primary rat myoblasts in a collagen gel for regeneration of a large VML defect to the rat biceps femoris muscle^[107]. Constructs containing MVFs with or without myoblasts were pre-cultured for 4 days *in vitro* before implantation and compared to muscle autografts or the empty defect. Interestingly, the *in vitro* constructs containing both MVFs and myoblasts had increased vascular network formation with a higher number of branches and total vessel length compared to MVF-only constructs (Figure 10c). This is in contrast to previous research comparing 3D tri-culture to co-culture systems where the inclusion of myoblasts slightly impeded vascular network formation^[30]. After 2 weeks post-implantation, micro-CT angiography demonstrated that all treatment groups had equal vascular volumes, primarily composed of small vessels. Defects treated with constructs containing MVFs had a higher proportion of small vessels compared to empty or autograft groups. Both MVF treatment groups contained high levels of fibrosis, adipose infiltration, low levels of muscle

regeneration, and poor maintenance of tissue volume within the defects demonstrating a need for further research on the capability of muscle constructs pre-vascularized with MVFs to improve regeneration post-VML.

Utilizing a unique host vessel infiltration technique, Shandalov et. al. investigated the potential to promote increased perfusion and anastomosis of their pre-vascularized constructs by following a two step implantation protocol^[32]. The construct was cultured *in vitro* for 10 days then first implanted around the mouse femoral artery and vein for 1–2 weeks, after which it was transferred as a vascularized axial flap to an abdominal wall VML defect site. The study used a 50% PLLA:PLGA scaffold and tested differences between acellular constructs and those containing C2C12s alone, co-culture of HUVECs and human dermal fibroblasts, or a tri-culture of C2C12s, HUVECs, and fibroblasts. In the cohort of constructs that had been implanted around femoral vessels for 1 week, tri-culture constructs had the highest capillary density and functional vessel density (FVD) while among those that had been implanted for 2 weeks, both tri-culture and C2C12 monoculture constructs had high CDs and FVDs. Interestingly, for both implantation timelines the HUVEC-fibroblast co-culture and acellular constructs resulted in comparatively low CD and FVD. The perfusion rate and perfused vascular volume in implanted grafts were quantified through the use of ultrasound imaging following injection of a microbubble contrast agent. One week of implantation increased both the perfusion rate and perfused vascular volume of tri-culture constructs compared to other groups while 2 weeks of implantation caused both tri-culture and C2C12 monoculture constructs to have highly perfused vascular volumes with no difference in perfusion rate among all three groups. Overall, earlier vascular network maturity was seen in implanted tri-culture constructs with monoculture muscle constructs following close behind given an extra week of implantation around femoral vessels. This indicates that implanted myoblasts promote vasculogenesis by femoral vessels compared to co-culture constructs containing vessels alone. Despite differences between groups after incubation around femoral vessels, fewer differences were seen following transfer to an abdominal wall VML defect. Tri-culture constructs that had incubated around femoral vessels for 1 week had the highest CD and vessel size but no difference was seen between groups for constructs that had incubated around femoral vessels for 2 weeks. Overall, these results indicate that tri-culture constructs have more rapid integration with host vessels when transplanted as a vascularized flap pre-incubated *in vivo*. Constructs containing myoblasts or pre-cultured vessels alone have the potential for equal vascular integration post VML when the initial incubation time around host femoral vessels is increased.

The impact of vessel alignment on engraftment within VML-injured muscle as well as methods to tune that alignment were investigated by Rosenfeld et. al^[34]. 3D co-cultures of HUVECs and neonatal human dermal fibroblasts on gelatin-based scaffolds were cultured for 4 days then subjected to cyclic strain (10% strain, 1 Hz) for an additional 4 days of culture, resulting in vessels aligned diagonal to the direction of strain. Static strain was applied to other constructs prior to cell seeding and maintained for the entire 8-day culture period, resulting in vessels aligned parallel to the direction of strain. After implantation for 2 weeks in an abdominal wall VML defect model, statically-strained constructs preserved their pre-implantation vessel alignment and vessels were on average 2.5 times longer than vessels in implanted unstrained control constructs. Furthermore, by comparing statically-strained

constructs implanted with vessels either aligned with or perpendicular to host musculature, they demonstrated that vessel alignment with host muscle cells provides improvements in integration and mechanical durability upon implantation. Native skeletal muscle is a highly organized tissue with myofiber and vascular alignment and this study provides strong evidence for the importance of promoting blood vessel alignment in engineered muscle constructs implanted in VML defects.

Our group recently demonstrated that electrospun fibrin hydrogel scaffolds induce alignment of a vascular network formed by 1:1 HUVEC:ASC co-culture after 11 days *in vitro*^[108]. When cultured with C2C12 myoblasts, the electrospun scaffolds promoted dense muscle and vascular regeneration in a mouse VML defect of the TA muscle. To enable scale-up to larger defect sizes, we tested the ability of pre-vascularized scaffolds lacking myoblasts to anastomose with host vessels after a 10-day implantation within the mouse TA VML defect. Implanted human vessels anastomosed with host vessels and mosaic vessels were visible that were partially of human and partially of mouse origin (Figure 10f). In addition, implanted human vessels that were perfused with mouse red blood cells were visible within the defect. Despite integration with host vasculature, the implanted pre-vascularized scaffolds were ringed with a collagen boundary that prevented full integration with the host musculature. Upon further investigation we determined that significant collagen was deposited by the ASCs during *in vitro* culture prior to implantation. Previous work utilizing adipose-derived MVFs in a large VML defect reported similar high levels of collagen in MVF treatment groups^[107], indicating a need for further research into the regenerative potential of adipose-derived MVFs and ASCs within the highly fibrotic VML environment.

In a step forward for clinical translation of pre-vascularized constructs, tri-culture constructs containing primary human adult venous endothelial cells (ECs), smooth muscle cells, and myoblasts have been developed and tested in an abdominal wall VML defect model^[33,35]. In a direct comparison to HUVECs as an endothelial cell source, it was found that the constructs containing adult human ECs with adult supporting cells had a higher vessel network complexity in both co-culture and tri-culture than those with HUVECs^[35]. Upon implantation in an abdominal wall defect for 9 days, human vessels at the implanted graft perimeter were mainly replaced by host vessels that had vascularized more than 80% of the implants. The host vessels had infiltrated an average of 2 mm into the grafts with no difference based on EC source. Genetic modification of primary human adult venous endothelial cells and primary human adult venous smooth muscle cells transduced to express angiopoietin 1 (ANGPT1) and vascular endothelial growth factor (VEGF), respectively, resulted in increased vessel elongation and maturation in 3D constructs *in vitro* compared to constructs containing non-transduced cells^[33] (Figure 10g–j). An abdominal imaging window was developed to facilitate intravital imaging of vessel growth within the defect over time and is a beneficial tool for further research in the vascularization of implanted constructs post-VML. Constructs with genetically modified cells also resulted in faster host vessel infiltration and increased total vessel length within the defect up to 7 days post-implantation. Interestingly, the implanted endothelial cells decreased within the graft area over time and this occurred more rapidly with genetically modified cells than controls.

There has been significant recent success in the development and characterization of pre-vascularized muscle constructs both *in vitro* and *in vivo*. As stated above, due to the high metabolic demands of skeletal muscle tissue, any large-scale VML defect will likely need an implanted vascular source to sustain the regenerating muscle tissue. Previous work described above has characterized differences in vascularization and muscle regenerative potential related to cell sources, *in vitro* pre-cultivation time, scaffold composition, the application of mechanical strain, and pre-incubation around major host vessels *in vivo*. A broad range of quantification metrics have been developed including the use of trackable fluorescent endothelial cells, vascular perfusion, intravital imaging techniques, analysis of blood flow rate, and micro-CT angiography, among others. Despite the broad successes in the field thus far, the majority of studies evaluating pre-vascularized constructs *in vivo* post-VML have been limited to a rodent abdominal wall defect model and it is as yet unclear if similar results would be generated in other muscle types that may have significant variations in anatomy. In addition, there is a strong need to evaluate the above pre-vascularization technologies in larger animal models of VML that have an increased vascular demand.

4. Engineering Innervated Skeletal Muscle

4.1 Biology of the Neuromuscular Junction

The neuromuscular junction (NMJ) is the site of contact between motor neurons and the myofibers within skeletal muscle that they innervate. Motor neuron axons travel from the central nervous system to skeletal muscles primarily unbranched and upon reaching the target muscle they branch extensively to contact individual myofibers at the NMJ^[19]. The primary purpose of the NMJ is to convert motor neuron action potentials into muscle contraction. In response to motor neuron action potentials and subsequent release of acetylcholine, myofibers undergo membrane depolarization, the opening of voltage-gated calcium channels, and an increase in intracellular calcium concentration to trigger activation of the contractile apparatus^[109]. Postsynaptic acetylcholine receptors (AChR) are organized into dense clusters optimized to intercept the acetylcholine signal from the nerve terminal. The highly specialized NMJ structure ensures the efficiency of signal transfer across the synapse between a motor neuron and skeletal myofiber and effective muscle contraction.

4.1.1 Pre- and Postnatal Development of the Neuromuscular Junction—

During prenatal development of skeletal muscle tissue, primitive clusters of acetylcholine receptors form in the center regions of developing myofibers prior to innervation and independent of neuronal signals in a process termed pre-patterning^[110,111]. The density of acetylcholine receptors within pre-patterned myofibers during embryonic development is approximately 1000 receptors/ μm^2 . Pre-patterned cluster formation and stabilization is partially induced by the myofiber membrane-associated cytoplasmic protein rapsyn^[110]. Rapsyn forms aggregates with acetylcholine receptors in the developing myofiber membrane, facilitating cluster formation, and mice lacking rapsyn do not form AChR clusters within their muscles. The muscle specific receptor tyrosine kinase, MuSK, is also vital to proper AChR clustering and NMJ formation and mice lacking MuSK also have no clusters of AChR. As motor neuron axons grow into the developing muscle, AChR clusters at synaptic sites contacted by a nerve are stabilized and enlarged while AChR clusters not

contacted by the infiltrating nerve axons disperse over time (Figure 11). At this stage in development the AChR clusters are each poly-innervated by two or more axons. Agrin, a large heparan sulfate proteoglycan, is secreted by the nerve terminal and is vital for AChR cluster stabilization and prevention of AChR dispersal^[110]. In fact, mice lacking agrin die in utero or at birth due to NMJ dysfunction^[112]. The AChR density at synaptic sites increases dramatically as the muscle develops to approximately 10,000 receptors/ μm^2 and in adult muscle can reach 20,000 receptors/ μm^2 at the NMJ^[113]. Non-synaptic AChR, by contrast, are present at a low density of <10 receptors/ μm^2 .

During postnatal development, neurons are pruned to single contact at each synaptic site and as the muscle matures, around half of the motor neurons innervating the developing muscle degenerate^[110]. The AChR clusters mature and undergo a morphological change from an immature oval-shaped plaque morphology to a more mature pretzel shape^[113]. This transition is caused by folding of the postsynaptic membrane and localization of dense AChR clusters at the fold crests^[110]. Additionally, as the muscle matures, locations along the nerve terminal outside of the synaptic cleft are covered by a synaptic non-myelinating Schwann cell. Interestingly, ECM proteins within the neuromuscular synapse differ from the general basal lamina surrounding myofibers outside of the synapse. The two major basal lamina proteins, collagen IV and laminin, have different isoforms within the synaptic region. Differences between synaptic and extrasynaptic ECM proteins has been reviewed elsewhere^[113] and synaptic ECM proteins have been shown to play a vital role in NMJ development.

4.1.2 Mature Neuromuscular Junctions in Adult Muscle—The adult vertebrate NMJ exhibits a complex pretzel morphology due to folds within the postsynaptic membrane that correlate with the spatial location of nerve terminal boutons to enable a high efficiency of signal propagation^[19]. The folds penetrate approximately 1 μm into the cytoplasm of the muscle cell and significantly increase the area of the postsynaptic membrane. Each terminal bouton is 1–5 μm wide and upon activation releases numerous 50 nm diameter membrane-bound synaptic vesicles containing acetylcholine to be received by the highly dense postsynaptic AChR clusters. The synaptic cleft, the space between the presynaptic nerve terminal and postsynaptic myofiber membrane, is approximately 50 nm wide and filled with large molecules that ensure NMJ ultrastructural organization and efficient signal propagation from nerve to muscle^[111]. In adults, each myofiber is innervated at its NMJ by a single axon from a motor neuron^[111]. Motor neurons branch within the muscle tissue to innervate multiple myofibers, termed its motor unit, and myofibers within a motor unit are generally distributed throughout the muscle to ensure uniform contraction.

Native neuromuscular regeneration and repair of NMJs can occur following peripheral nerve lesions depending on the severity of injury^[111]. Nerve axons undergo Wallerian degeneration whereby the axon cell membrane degrades, axon cytoskeleton and organelles disintegrate, and terminal Schwann cells invade the NMJ synaptic cleft with the debris cleared by recruited and resident macrophages. Interestingly, it has been shown that despite severe disruptions to pre-synaptic nerve axons following denervation, the number of acetylcholine receptors remains unchanged up to 20 days post-injury then drops by 50 and 70% at 30 and 60 days post-injury^[114]. Despite this, after denervation the AChR become

dispersed from their synaptic clusters and are present throughout the myofiber surface^[115]. Regeneration begins at the axon proximal end and in small injuries, the regenerating axon grows following its previously occupied location to preferentially re-innervate original synaptic locations or axons may sprout from neighboring NMJs^[116]. Following severe injury where the basal lamina is disrupted, regenerating nerves may innervate new locations along the myofiber with the potential for poly-innervation. As the regenerated NMJ matures, reorganization occurs to ensure mono-innervation and AChR cluster arrangement. Concurrent muscle regeneration is vital for proper maintenance of regenerated NMJs and re-innervated NMJs on basal lamina ghosts lacking myofibers decline over time. In addition, satellite cell function has been connected to NMJ regeneration post-injury with reductions in postsynaptic morphology, loss of postsynaptic myonuclei, and deficits in NMJ reinnervation linked to satellite cell depletion^[111].

4.2 Strategies to Enhance Neural Ingrowth and Formation of NMJs in TEMGs

The formation of neuromuscular junctions in tissue engineered muscle constructs both *in vitro* and post-implantation is important for ultimate function of the engineered muscle. Significant research has been performed on NMJ biology, development, and regeneration using *in vitro* NMJ models and is described briefly below. *In vivo* formation of NMJs has included the incubation of 3D skeletal muscle constructs along major nerves, neurotization, and the use of rehabilitative exercise. The following sections will provide an overview of *in vitro* engineered NMJs and focus on studies promoting NMJ formation *in vivo* within VML-injured muscles. Methods and biomaterials utilized to encourage neuromuscular regeneration post-VML are summarized in Table 3.

4.2.1 Engineered NMJs In Vitro—The development of *in vitro* NMJs has been the focus of previous reviews and has provided a vital tool for the analysis of NMJ development and function^[117]. Co-culture of neurons and myoblasts both in monolayer or 3D constructs has enabled *in vitro* NMJ formation where electrically induced nerve stimuli result in measurable post-synaptic potentials and contractile activity in myofibers^[118–120]. To demonstrate that this interaction functions via conventional NMJ interactions, nerve-induced myofiber activity can be prevented by application of the AChR blocker D-tubocurarine^[121]. Dixon et. al. recently described the development of the first fully human 3D nerve and muscle co-culture system using human primary skeletal myoblasts and human induced neural stem cells within a collagen/Matrigel scaffold^[122]. In a recent study by Vila et. al., the first human patient-specific tissue engineered *in vitro* NMJ system was developed using primary human skeletal muscle cells and muscle-derived hiPSCs differentiated into motoneurons within a collagen/Matrigel scaffold^[123]. To monitor NMJ function, they utilized photosensitive motoneurons to form light-sensitive NMJs and developed an optical stimulation platform to quantify NMJ activity and muscle contraction. The significant advancements in the development of human NMJs within 3D *in vitro* constructs in recent years have provided useful tools for patient disease monitoring and drug screening and could provide significant benefit to studies assessing neuromuscular regeneration following VML.

In vitro AChR clusters are a vital postsynaptic component of the NMJ and have been induced in both monolayer and 3D culture of myoblasts through the use of various

pharmacologic agents (including agrin and laminin) as well as via electrical stimulation^[124]. Agrin is present in the developing and adult NMJ responsible for stabilizing AChR clusters and has been extensively researched for its ability to induce AChR clustering and promote NMJ formation in cultured myotubes^[125–133]. Laminin is an extracellular matrix protein present in skeletal muscle with various isoforms present at different stages of development as well as within the neuromuscular synapse versus extrasynaptically^[113]. Significant variability exists in protocols of agrin application with concentrations ranging from 10 ng/ml to 10 µg/ml and inconsistencies in time point and duration of application. Despite variability in application, it remains clear that agrin application provides a significant stimulus to cultured myotubes to cause clustering of AChRs. Interestingly, this differs from the native biological function of agrin during embryonic development, which as described above does not induce AChR clustering itself but rather stabilizes the pre-formed clusters in the developing muscle. Additionally, the morphology of agrin-induced clusters appears limited to an immature plaque shape as opposed to developing into a mature cluster pretzel morphology as demonstrated by an interesting study by Bruneau et. al^[132]. In that study, cluster formation induced by both agrin and laminin was evaluated with AChR fluorescence tracked over time. Significant differences in cluster morphology were present when induced with the two agents and it was demonstrated that agrin application following an initial laminin application stimulated acetylcholine receptors to transfer from a pretzel-shaped laminin-induced cluster to the more immature agrin-induced cluster. This effect was seen after just one hour of agrin application and resulted in the complete disappearance of the larger laminin-induced clusters after just 24 hours. Other studies have found that the combined application of agrin and laminin induced significantly more AChR clustering than agrin or laminin alone^[126,133]. The potential of agrin pre-treated constructs to promote innervation post-implantation was assessed using a fibrin gel containing C2C12 myoblasts and 1 µg/ml agrin^[125]. Following subcutaneous implantation near the peroneal nerve for up to 8 weeks, implanted constructs that had been pre-treated with agrin exhibited significantly more nerve infiltration *in vivo*. While this study is a promising first step, further investigation is required on the potential for agrin or other pharmacologic agents to encourage neuromuscular regeneration within the severely damaged VML environment.

4.2.2 Neurotization Post-VML—Neurotization is the process of surgically re-innervating muscle fibers by insertion of a donor nerve within the muscle tissue^[115]. Clinical use of neurotization to repair peripheral nerve injuries is limited as it is considered an inefficient method to repair innervation at the motor endplate^[115]. Despite this, neurotization continues to be a useful experimental tool to study innervation of implanted engineered tissues including skeletal muscle. Few studies have directly investigated neurotization as a method to innervate an implanted 3D construct in VML-injured muscle. Most studies assessing the benefits of neurotization for muscle tissue engineering have utilized non-VML models such as denervation of host muscle via nerve transection^[134], subcutaneous implantation^[135], and other non-muscle defect models^[136]. Due to differences in neural infiltration and NMJ recovery between small muscle injuries versus after VML with its associated gross loss of the basal lamina, further investigation is necessary on the innervation potential of neurotization within the extensively damaged VML environment.

The potential regenerative benefits of neurotization were characterized post-VML and compared to non-neurotized controls in a notable recent study. Kaufman et. al.^[86] implanted a pre-vascularized 3D muscle construct within a mouse abdominal wall VML defect model and nerve infiltration was encouraged via neurotization of the femoral nerve within the femoral bundle, which was transferred to the implanted construct. In control constructs, the femoral vessels were transferred to the implant without the femoral nerve. After 6 weeks spontaneous electrical activity, a marker of poor muscle innervation, was measured via EMG within the implant region. Neurotized grafts had no spontaneous activity while 5 out of 7 control non-neurotized grafts presented with spontaneous activity. Compound motor action potential (CMAP) was measured following stimulation of the transferred portion of the femoral nerve and compared to uninjured muscle as well as non-neurotized controls. CMAP amplitude for neurotized samples reached 0.6 mV whereas control samples had no measurable CMAP. Both neurotized grafts and controls contained β 3-tubulin-positive neurofilament with significantly more area coverage in neurotized grafts. Longitudinal sections enabled visualization of NMJ size and morphology and although smaller than within uninjured muscle, NMJs with overlapping neurofilament or synaptophysin and acetylcholine receptor clusters were visible within the implanted constructs in both groups (Figure 12). The promotion of increased innervation within implanted 3D muscle constructs post-VML is significant and NMJs were visualized longitudinally, allowing for proper assessment of NMJ size and morphology. Despite this, no quantification of NMJ size, morphology, or extent of neural and acetylcholine receptor cluster overlap was reported and would be of benefit to the field.

4.2.3 Rehabilitative Exercise Post-VML—Physical rehabilitation is often considered to be beneficial for muscle healing and accelerates the repair of small, acute muscle injuries via immune modulation, promotion of vascularization, release of pro-myogenic growth factors, and reduction of fibrosis^[137–139]. Following VML, physical rehabilitation has been used clinically in an attempt to promote increased muscle strength in the remaining muscle and synergist muscles in order to offset the functional deficit caused by the injury but results have been limited and physical therapy has not resulted in clinically meaningful improvements in range of motion, limb function, or muscle strength post-VML^[15,16]. The emerging field of regenerative and rehabilitative medicine aims to combine the rehabilitative effects of physical exercise with regenerative outcomes and *de novo* tissue formation. Application of this approach to VML injured muscle has been recently reviewed^[15,140].

To investigate the potential of physical rehabilitation to enhance regeneration of the lost muscle tissue, Aurora et. al. utilized a rat VML defect of the TA muscle and assessed the regenerative impact of 1 or 7 weeks of voluntary caged wheel exercise on the injured muscle beginning 1 week post-injury^[137]. After 7 weeks of exercise, maximal torque output of TA muscles in exercised rats was 17% higher than in sedentary rats. Despite improved function, muscles in exercised rats contained significantly more fibrosis within the defect region compared to those of sedentary rats and had upregulated pro-fibrotic genes. Exercise also resulted in double the amount of centrally-nucleated myofibers within the remaining muscle compared to sedentary controls, an indication of chronic injury and remodeling. Clinical use of physical rehabilitation following ECM treatment of VML-injured muscle has also

resulted in improved functional outcomes^[60,62] and may be a useful tool for encouraging neural infiltration post-VML.

Quarta et. al. recently directly assessed the impact of exercise on neural regeneration following a VML defect in mice of the TA muscle through the use of either voluntary caged wheel running or higher intensity treadmill running^[21]. Using caged wheel exercise, it was determined that mice regained their pre-injury daily running habits 1 week post-injury. To further validate this, forced treadmill exercise within the first week post-injury resulted in delayed muscle regeneration and increased fibrosis while exercise delayed by one week post-injury resulted in accelerated myogenesis and decreased fibrosis. Following treatment with 3D constructs composed of primary mouse satellite cells and muscle resident cells on a decellularized ECM scaffold and one week of recovery post-injury, mice were exercised on treadmills for one hour per day over a period of 3 weeks. Exercised mice demonstrated a 20% improvement in gait analysis and significant improvements in neural-stimulated and *ex vivo* force output compared to sedentary controls. In addition, exercised mice had increased levels of AChR clusters and NMJs within the defect compared to muscles in sedentary mice (Figure 13a–c). Interestingly, differences between groups was more pronounced in the distal portion of the muscle, perhaps due to variability in NMJ location and motor endplate patterning of the native muscle tissue. While significant in its detailed analysis of functional output and NMJ formation following exercise, this study would benefit from further analysis of the morphology and maturity of regenerating NMJs through the use of longitudinal sectioning and image analysis of the entire NMJ structure.

The impact of voluntary caged wheel exercise on muscle regeneration post-VML was further investigated by Nakayama et. al., who implanted either topographically aligned or randomly-oriented collagen scaffolds within a mouse VML defect of the TA muscle and began voluntary exercise 1 week post-injury for a period of 2 weeks^[84]. The presence of AChR clusters and NMJs was quantified in muscle within 500 μm or 1000 μm of the implanted scaffold (Figure 13d,e). Aligned scaffolds with exercise resulted in increased levels of AChR clusters and NMJs in muscle within a 500 μm distance from the scaffold. Randomly-oriented scaffolds with exercise demonstrated increases in AChR clusters and NMJ formation when the analysis was expanded to include muscle within a 1000 μm distance from the scaffold. Although it is significant that exercise increased neural regeneration within muscle at varying distances surrounding the defect, it would be more informative to quantify neural regeneration within the defect and the implanted scaffold itself. In addition, analysis of nerve-evoked muscle function would further demonstrate any improvements to neural regeneration and muscle function due to exercise. Future studies on the regenerative potential of rehabilitative exercise should include a broad array of quantitative measures including both muscle function and histological regeneration as well as neurally-stimulated force output and/or EMG with CMAP. To robustly assess neural regeneration post-VML and provide further understanding of this complex injury environment, studies should also quantify nerve infiltration to the defect site, the presence of NMJs (with overlapping AChR cluster and nerve stains), and 3D NMJ morphology and size.

5. Conclusion and Future Directions

In this review we have summarized the current state of the field of skeletal muscle tissue engineering for VML treatment with a focus on engineering vascularized and innervated muscle. Significant variability exists in the composition of engineered muscle constructs, preclinical VML defect models, and measured outcomes following implantation. In order to effectively compare results between studies, the field would benefit from increased standardization of defect models and outcome measures. Native skeletal muscle has highly organized vascular and neural networks which must be considered when engineering skeletal muscle constructs and analyzing *in vivo* data.

Host vessel infiltration or pre-vascularization of constructs prior to implantation are reliable methods to promote vascularization of implanted constructs. Significant progress has been made in the field of vascularized skeletal muscle tissue engineering but continued research on pre-vascularized construct translatability to varied muscle types and within large animal VML defect models is required. Promoting neural regeneration post-VML is a relatively early area of research and primary methods to encourage innervation of regenerating muscle post-VML are neurotization and rehabilitative exercise. Although both methods have resulted in moderate successes, there is a need for further research on innervation post-VML and for the incorporation of improved quantification techniques to measure nerve ingrowth and NMJ morphology and maturity within the defect site. Intravital imaging has recently enabled real-time monitoring of vascular infiltration post-VML and would be an exciting and beneficial method for assessing nerve ingrowth as well. Additionally, growth factor release from implanted constructs within VML defects has been investigated for its pro-angiogenic potential. Growth factor release is an obvious choice to promote neural regeneration as well. Based on current publications, there is no evidence that it has been investigated in the VML environment to date. Future studies should focus on the development of novel technologies to encourage robust vascular and neural regeneration following VML as well as useful quantification metrics. Future studies should also incorporate robust quantification of immature, developing vasculature and nerves and enable a correlation between the regeneration state of the tissue and any improvements in functional outcome in order to delineate minimum requirements for functional regeneration post-VML (Figure 14). Further investigation into the presence of potential synergistic effects of regenerating vasculature and nerves in the post-VML environment may also improve the design of engineered treatments.

Future studies developing treatments for VML should also consider differences in regenerative potential between acute and chronic VML defects. Most prior studies have evaluated engineered skeletal muscle constructs within acute VML defects, where the VML injury was created immediately before construct implantation. In the clinical reality, however, chronic VML is much more common whereby a VML injury is sustained and treatment is only available months or even years later. Few studies have directly compared the regenerative potential of an engineered muscle construct in acute versus chronic VML^[21] and significant differences in regeneration are likely due to variation in the muscle regeneration timeline. In addition to incorporating consideration of variability due to the age,

sex, and anatomical location of preclinical models, future studies should also consider treatments that would translate well to a chronic VML environment.

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Biography

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References

- [1]. Janssen I, Heymsfield SB, Wang Z, Ross R, J Appl Physiol 2000, 89, 81. [PubMed: 10904038]
- [2]. Garg K, Ward CL, Hurtgen BJ, Wilken JM, Stinner DJ, Wenke JC, Owens JG, Corona BT, J. Orthop. Res 2015, 40.
- [3]. Corona BT, Rivera JC, Owens JG, Wenke JC, Rathbone CR, J. Rehabil. Res. Dev 2015, 52, 785. [PubMed: 26745661]
- [4]. Turner NJ, Badylak SF, Cell Tissue Res 2012, 347, 759. [PubMed: 21667167]
- [5]. Grasman JM, Zayas MJ, Page RL, Pins GD, Acta Biomater 2015, 25, 2. [PubMed: 26219862]
- [6]. Sicari BM, Agrawal V, Siu BF, Medberry CJ, Dearth CL, Turner NJ, Badylak SF, Tissue Eng. Part A 2012, 18, 1941. [PubMed: 22906411]
- [7]. Corona BT, Wenke JC, Ward CL, Cells Tissues Organs 2016, 202, 180. [PubMed: 27825160]
- [8]. Owens BD, Kragh JF, Macaitis J, Svoboda SJ, Wenke JC, J. Orthop. Trauma 2007, 21, 254. [PubMed: 17414553]

- [9]. Owens BD, Kragh JF, Wenke JC, Macaitis J, Wade CE, Holcomb JB, J. Trauma - Inj. Infect. Crit. Care 2008, 64, 295.
- [10]. Charge SBP, Rudnicki MA, *Physiol. Rev* 2004, 84, 209. [PubMed: 14715915]
- [11]. Huard J, Li Y, Fu FH, J. Bone Joint Surg. Am 2002, 84-A, 822.
- [12]. Passipieri JA, Christ GJ, *Cells Tissues Organs* 2016, 202, 202. [PubMed: 27825153]
- [13]. Greising SM, Rivera JC, Goldman SM, Watts A, Aguilar CA, Corona BT, *Sci. Rep* 2017, 7, 1. [PubMed: 28127051]
- [14]. Bursac N, Juhas M, Rando TA, *Annu. Rev. Biomed. Eng* 2015, 17, 217. [PubMed: 26643021]
- [15]. Greising SM, Dearth CL, Corona BT, *Cells Tissues Organs* 2016, 202, 237. [PubMed: 27825146]
- [16]. Aguilar CA, Greising SM, Watts A, Goldman SM, Peragallo C, Zook C, Larouche J, Corona BT, *Cell Death Discov* 2018, 4, DOI 10.1038/s41420-018-0027-8.
- [17]. Corona BT, Flanagan KE, Brininger CM, Goldman SM, Call JA, Greising SM, *Muscle and Nerve* 2018, 57, 799. [PubMed: 29144551]
- [18]. Sarrafian TL, Bodine SC, Murphy B, Grayson JK, Stover SM, *Vet. Surg* 2018, 47, 524. [PubMed: 29603757]
- [19]. Slater CR, *Int. J. Mol. Sci* 2017, 18, DOI 10.3390/ijms18102183.
- [20]. Anderson SE, Han WM, Srinivasa V, Mohiuddin M, Ruehle MA, Moon JY, Shin E, San Emeterio CL, Ogle ME, Botchwey EA, et al., *Tissue Eng. Part C Methods* 2019, 25, 59. [PubMed: 30648479]
- [21]. Quarta M, Cromie M, Chacon R, Blonigan J, Garcia V, Akimenko I, Hamer M, Paine P, Stok M, Shrager JB, et al., *Nat. Commun* 2017, 8, 15613. [PubMed: 28631758]
- [22]. Kasukonis B, Kim J, Brown L, Jones J, Ahmadi S, Washington T, Wolchok J, *Tissue Eng. Part A* 2016, 22, 1151. [PubMed: 27570911]
- [23]. Kim JT, Kasukonis BM, Brown LA, Washington TA, Wolchok JC, *Exp. Gerontol* 2016, 83, 37. [PubMed: 27435497]
- [24]. Grasman JM, Do DM, Page RL, Pins GD, *Biomaterials* 2015, 72, 49. [PubMed: 26344363]
- [25]. Gilbert-Honick J, Iyer SR, Somers SM, Lovering RM, Wagner K, Mao H, Grayson W, *Biomaterials* 2018, 164, 70. [PubMed: 29499437]
- [26]. Haizlip KM, Harrison BC, Leinwand LA, *Physiology* 2015, 30, 30. [PubMed: 25559153]
- [27]. Yin H, Price F, Rudnicki M. a, *Physiol. Rev* 2013, 93, 23. [PubMed: 23303905]
- [28]. Welle S, Tawil R, Thornton CA, *PLoS One* 2008, 3, DOI 10.1371/journal.pone.0001385.
- [29]. Levenberg S, Rouwkema J, Macdonald M, Garfein ES, Kohane DS, Darland DC, Marini R, van Blitterswijk CA, Mulligan RC, D'Amore PA, et al., *Nat. Biotechnol* 2005, 23, 879. [PubMed: 15965465]
- [30]. Lesman A, Koffler J, Atlas R, Blinder YJ, Kam Z, Levenberg S, *Biomaterials* 2011, 32, 7856. [PubMed: 21816465]
- [31]. Koffler J, Kaufman-Francis K, Yulia S, Dana E, Daria AP, Landesberg A, Levenberg S, *Proc. Natl. Acad. Sci. U. S. A* 2011, 108, 14789. [PubMed: 21878567]
- [32]. Shandalov Y, Egozi D, Koffler J, Dado-Rosenfeld D, Ben-Shimol D, Freiman A, Shor E, Kabala A, Levenberg S, *Proc. Natl. Acad. Sci* 2014, 111, 6010. [PubMed: 24711414]
- [33]. Perry L, Landau S, Flugelman MY, Levenberg S, *Commun. Biol* 2018, 1, DOI 10.1038/s42003-018-0161-0.
- [34]. Rosenfeld D, Landau S, Shandalov Y, Raindel N, Freiman A, Shor E, Blinder Y, *Proc. Natl. Acad. Sci* 2016, 113, 3215. [PubMed: 26951667]
- [35]. Perry L, Flugelman MY, Levenberg S, *Mol. Ther* 2017, 25, 935. [PubMed: 28279644]
- [36]. Grogan BF, Hsu JR, *J. Am. Acad. Orthop. Surg* 2011, 19, 35.
- [37]. Mertens JP, Sugg KB, Lee JD, Larkin LM, *Regen. Med* 2014, 9, 89. [PubMed: 24351009]
- [38]. Qazi TH, Mooney DJ, Pumberger M, Geißler S, Duda GN, *Biomaterials* 2015, 53, 502. [PubMed: 25890747]
- [39]. Wolf MT, Dearth CL, Sonnenberg SB, Loba EG, Badylak SF, *Adv. Drug Deliv. Rev* 2015, 84, 208. [PubMed: 25174309]
- [40]. Cittadella Vigodarzere G, Mantero S, *Front Physiol* 2014, 5, 362. [PubMed: 25295011]

- [41]. Ngan CGY, Quigley A, Kapsa RMI, Choong PFM, J. Tissue Eng. Regen. Med 2018, 12, e1. [PubMed: 28066991]
- [42]. Juhas M, Bursac N, Curr. Opin. Biotechnol 2013, 24, 880. [PubMed: 23711735]
- [43]. Juhas M, Ye J, Bursac N, Methods 2015, 99, 81. [PubMed: 26455485]
- [44]. Fuoco C, Petrilli LL, Cannata S, Gargioli C, J. Orthop. Surg. Res 2016, 11, 86. [PubMed: 27460672]
- [45]. Sicari BM, Dearth CL, Badylak SF, Anat. Rec 2014, 297, 51.
- [46]. Aurora A, Roe JL, Corona BT, Walters TJ, Biomaterials 2015, 67, 393. [PubMed: 26256250]
- [47]. Pantellic MN, Larkin LM, Tissue Eng. Part B Rev 2018, 24, ten. TEB.2017.0451.
- [48]. McCullagh KJA, Perlingeiro RCR, Adv. Drug Deliv. Rev 2015, 84, 198. [PubMed: 25049085]
- [49]. Kim JH, Ko IK, Atala A, Yoo JJ, Sci. Rep 2016, 6, 38754. [PubMed: 27924941]
- [50]. VanDusen KW, Syverud BC, Williams ML, Lee JD, Larkin LM, Tissue Eng. Part A 2014, 20, 2920. [PubMed: 24813922]
- [51]. Carosio S, Barberi L, Rizzuto E, Nicoletti C, Del Prete Z, Musarò A, Sci. Rep 2013, 3, 1420. [PubMed: 23478253]
- [52]. Nakayama KH, Shayan M, Huang NF, Adv. Healthc. Mater 2019, 8, 1.
- [53]. Sadtler K, Estrellas K, Allen BW, Wolf MT, Fan H, Tam AJ, Patel CH, Lubner BS, Wang H, Wagner KR, et al., Science (80-.) 2016, 352, 366.
- [54]. Sicari BM, Rubin JP, Dearth CL, Wolf MT, Ambrosio F, Boninger M, Turner NJ, Weber DJ, Simpson TW, Wyse A, et al., Sci. Transl. Med 2014, 6, 1.
- [55]. Conconi MT, De Coppi P, Bellini S, Zara G, Sabatti M, Marzaro M, Franco Zanon G, Gamba PG, Parnigotto PP, Nussdorfer GG, Biomaterials 2005, 26, 2567. [PubMed: 15585259]
- [56]. Corona BT, Ward CL, Baker HB, Walters TJ, Christ GJ, Tissue Eng. Part A 2014, 20, 705. [PubMed: 24066899]
- [57]. Corona BT, Wu X, Ward CL, McDaniel JS, Rathbone CR, Walters TJ, Biomaterials 2013, 34, 3324. [PubMed: 23384793]
- [58]. Mase VJ, Hsu JR, Wolf SE, Wenke JC, Baer DG, Owens J, Badylak SF, Walters TJ, Orthopedics 2010, 33, DOI 10.3928/01477447-20100526-24.
- [59]. Turner NJ, Yates AJ, Weber DJ, Qureshi IR, Stolz DB, Gilbert TW, Badylak SF, Tissue Eng. Part A 2010, 16, 3309. [PubMed: 20528669]
- [60]. Dziki J, Badylak S, Yabroudi M, Sicari B, Ambrosio F, Stearns K, Turner N, Wyse A, Boninger ML, Brown EHP, et al., Nat. Regen. Med 2016, 1, 1.
- [61]. Sicari BM, Rubin JP, Dearth CL, Wolf MT, Ambrosio F, Boninger M, Turner NJ, Weber DJ, Simpson TW, Wyse A, et al., Sci. Transl. Med 2014, 6, 234ra58.
- [62]. Gentile NE, Stearns KM, Brown EHP, Rubin JP, Boninger ML, Dearth CL, Ambrosio F, Badylak SF, Am. J. Phys. Med. Rehabil 2014, 93, 1. [PubMed: 24355993]
- [63]. Chen XK, Walters TJ, J. Plast. Reconstr. Aesthetic Surg 2013, 66, 1750.
- [64]. Garg K, Ward CL, Rathbone CR, Corona BT, Cell Tissue Res 2014, 358, 857. [PubMed: 25300647]
- [65]. Passipieri JA, Hu X, Mintz E, Dienes J, Baker HB, Wallace CH, Blemker SS, Christ GJ, Tissue Eng. Part A 2019, 00, ten. tea.2018.0280.
- [66]. Ward CL, Ji L, Corona BT, Biores. Open Access 2015, 4, 198. [PubMed: 26309796]
- [67]. Goldman SM, Henderson BEP, Walters TJ, Corona BT, PLoS One 2018, 1.
- [68]. Li MTA, Willett NJ, Uhrig BA, Guldberg RE, Warren GL, J. Biomech 2014, 47, 2013. [PubMed: 24280565]
- [69]. Madden L, Juhas M, Kraus WE, Truskey GA, Bursac N, Elife 2015, 4, e04885. [PubMed: 25575180]
- [70]. Page RL, Malcuit C, Vilner L, Vojtic I, Shaw S, Hedblom E, Hu J, Pins GD, Rolle MW, Dominko T, Tissue Eng Part A 2011, 17, 2629. [PubMed: 21699414]
- [71]. Passipieri JA, Baker HB, Siriwardane M, Ellenburg M, Vadhavkar M, Saul JM, Tomblyn S, Burnett L, Christ GJ, Tissue Eng. Part A 2017, 23, 556. [PubMed: 28169594]

- [72]. Baker HB, Passipieri JA, Siriwardane M, Ellenburg M, Vadhavkar M, Bergman CR, Saul JM, Tomblyn S, Burnett L, Christ GJ, Tissue Eng. Part A 2017, 00, DOI 10.1089/ten.TEA.2016.0457.
- [73]. Juhas M, Engelmayer GC, Fontanella AN, Palmer GM, Bursac N, Proc. Natl. Acad. Sci 2014, 111, 5508. [PubMed: 24706792]
- [74]. Qiu X, Liu S, Zhang H, Zhu B, Su Y, Zheng C, Tian R, Wang M, Kuang H, Zhao X, et al., Stem Cell Res. Ther 2018, 9, DOI 10.1186/s13287-018-0821-5.
- [75]. Merritt EK, Cannon MV, Hammers DW, Le LN, Gokhale R, Sarathy A, Song TJ, Tierney MT, Suggs LJ, Walters TJ, et al., Tissue Eng Part A 2010, 16, 2871. [PubMed: 20412030]
- [76]. Kesireddy V, Int. J. Nanomedicine 2016, 11, 1461. [PubMed: 27114706]
- [77]. Gilbert-Honick J, Ginn B, Zhang Y, Salehi S, Wagner KR, Mao H-Q, Grayson WL, Cell Transplant 2018, 27, 1644.
- [78]. Pilia M, McDaniel JS, Guda T, Chen XK, Rhoads RP, Allen RE, Corona BT, Rathbone CR, Eur. Cells Mater 2014, 28, 11.
- [79]. Maffioletti SM, Sarcar S, Henderson ABH, Mannhardt I, Pinton L, Moyle LA, Steele-Stallard H, Cappellari O, Wells KE, Ferrari G, et al., Cell Rep 2018, 23, 899. [PubMed: 29669293]
- [80]. Choi IY, Lim HT, Estrellas K, Mula J, Cohen TV, Zhang Y, Donnelly CJ, Richard JP, Kim YJ, Kim H, et al., Cell Rep 2016, 15, 2301. [PubMed: 27239027]
- [81]. Rao L, Qian Y, Khodabukus A, Ribar T, Bursac N, Nat. Commun 2018, 9, 1. [PubMed: 29317637]
- [82]. Corona BT, Machingal MA, Criswell T, Vadhavkar M, Dannahower AC, Bergman C, Zhao W, Christ GJ, Tissue Eng Part A 2012, 18, 1213. [PubMed: 22439962]
- [83]. Ma J, Holden K, Zhu J, Pan H, Li Y, J. Biomed. Biotechnol 2011, 2011, 1.
- [84]. Nakayama KH, Alcazar C, Yang G, Quarta M, Paine P, Doan L, Davies A, Rando TA, Huang NF, npj Regen. Med 2018, 3, 16. [PubMed: 30245849]
- [85]. Rossi CA, Flaibani M, Blaauw B, Pozzobon M, Figallo E, Reggiani C, Vitiello L, Elvassore N, De Coppi P, FASEB J 2011, 25, 2296. [PubMed: 21450908]
- [86]. Kaufman T, Kaplan B, Perry L, Shandalov Y, Landau S, Srugo I, Ad-El D, Levenberg S, Am. J. Transplant 2019, 19, 37. [PubMed: 29856531]
- [87]. Chaturvedi V, Naskar D, Kinnear BF, Grenik E, Dye DE, Grounds MD, Kundu SC, Coombe DR, J. Tissue Eng. Regen. Med 2016, DOI 10.1002/term.
- [88]. Hagiwara K, Chen G, Kawazoe N, Tabata Y, Komuro H, J. Tissue Eng. Regen. Med 2016, 10, 325. [PubMed: 23554408]
- [89]. Ju YM, Atala A, Yoo JJ, Lee SJ, Acta Biomater 2014, 10, 4332. [PubMed: 24954910]
- [90]. Hosseini V, Ahadian S, Ostrovidov S, Camci-Unal G, Chen S, Kaji H, Ramalingam M, Khademhosseini A, Tissue Eng. Part A 2012, 18, 2453. [PubMed: 22963391]
- [91]. Han N, Yabroudi MA, Stearns-Reider K, Helkowski W, Sicari BM, Rubin JP, Badylak SF, Boninger ML, Ambrosio F, Phys. Ther 2015, 96, 540. [PubMed: 26564252]
- [92]. Colton CK, Cell Transplant 1995, 4, 415. [PubMed: 7582573]
- [93]. Laschke MW, Harder Y, Amon M, Martin I, Farhadi J, Ring A, Torio-Padron N, Schramm R, Rücker M, Junker D, et al., Tissue Eng 2006, 12, 2093. [PubMed: 16968151]
- [94]. Folkman J, Hochberg M, J. Exp. Med 1973, 138, 745. [PubMed: 4744009]
- [95]. Webster MT, Manor U, Lippincott-Schwartz J, Fan CM, Cell Stem Cell 2015, 1.
- [96]. Kusters YHAM, Barrett EJ, Am J Physiol Endocrinol Metab 2015, 310, E379. [PubMed: 26714849]
- [97]. Granger DN, Senchenkova E, Inflammation and the Microcirculation: Colloquium Series on Integrated Systems Physiology: From Molecule to Function, Morgan & Claypool Life Sciences, 2010.
- [98]. Emerson GG, Segal SS, J Appl Physiol 1997, 82, 42. [PubMed: 9029196]
- [99]. Hepple RT, Med. Sci. Sport. Exerc 2000, 31, 117.
- [100]. Olfert IM, Baum O, Hellsten Y, Egginton S, Am. J. Physiol. Heart Circ. Physiol 2016, 310, H326. [PubMed: 26608338]
- [101]. Van Hinsbergh VWM, Collen A, Ann. N. Y. Acad. Sci 2001, 936, 426. [PubMed: 11460496]

- [102]. Borschel GH, Dow DE, Dennis RG, Brown DL, Plast. Reconstr. Surg 2006, 117, 2235. [PubMed: 16772923]
- [103]. Messina A, Bortolotto SK, Cassell OCS, Kelly J, Abberton KM, Morrison W. a, FASEB J 2005, 19, 1570. [PubMed: 16014398]
- [104]. Bach AD, Arkudas A, Tjiawi J, Polykandriotis E, Kneser U, Horch RE, Beier JP, J. Cell. Mol. Med 2006, 10, 716. [PubMed: 16989731]
- [105]. Williams ML, Kostrominova TY, Arruda EM, Larkin LM, J. Tissue Eng. Regen. Med 2013, 7, 434. [PubMed: 22328229]
- [106]. Zhou W, He D-Q, Liu J-Y, Feng Y, Zhang X-Y, Hua C-G, Tang X-F, J. Tissue Eng. Regen. Med 2015, 9, 1404. [PubMed: 23365046]
- [107]. Li MT, Ruehle M, Stevens H, Servies N, Willett N, Karthikeyakannan S, Warren GL, Guldberg R, Krishnan LN, Tissue Eng. Part A 2017, 23, 989. [PubMed: 28372522]
- [108]. Gilbert-Honick J, Somers SM, Grayson WL, in Biomater. Stem Cells From Basics to Appl (Eds: Garipcan B, Denizli A), Palme Yayinevi, Istanbul, Turkey, 2018, pp. 143–153.
- [109]. Kuo IY, Ehrlich BE, Cold Spring Harb. Perspect. Biol 2015, 7, 1.
- [110]. Witzemann V, Cell Tissue Res 2006, 326, 263. [PubMed: 16819627]
- [111]. Liu W, Chakkalakal JV, The Composition, Development, and Regeneration of Neuromuscular Junctions, Elsevier Inc., 2017.
- [112]. Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, Sanes JR, Cell 1996, 85, 525. [PubMed: 8653788]
- [113]. Singhal N, Martin PT, Dev. Neurobiol 2011, 71, 982. [PubMed: 21766463]
- [114]. Frank E, Gautvik K, Sommerschild H, Cold Spring Harb. Symp. Quant. Biol 1976, 40, 275. [PubMed: 1065530]
- [115]. Konofaos P, Wallace RD, J. Reconstr. Microsurg 2015, 31, 481. [PubMed: 26220432]
- [116]. Schiaffino S, Partridge T, Skeletal Muscle Repair and Regeneration, 2008.
- [117]. Smith AST, Passey SL, Martin NRW, Player DJ, Mudera V, Greensmith L, Lewis MP, Cells Tissues Organs 2016, 202, 143. [PubMed: 27825148]
- [118]. Umbach JA, Adams KL, Gundersen CB, Novitch BG, PLoS One 2012, 7, 3.
- [119]. Chipman PH, Zhang Y, Rafuse VF, PLoS One 2014, 9, DOI 10.1371/journal.pone.0091643.
- [120]. Morimoto Y, Kato-Negishi M, Onoe H, Takeuchi S, Biomaterials 2013, 34, 9413. [PubMed: 24041425]
- [121]. Guo X, Gonzalez M, Stancescu M, Vandenberg HH, Hickman JJ, Biomaterials 2011, 32, 9602. [PubMed: 21944471]
- [122]. Dixon TA, Cohen E, Cairns DM, Rodriguez M, Mathews J, Jose RR, Kaplan DL, Tissue Eng. Part C Methods 2018, 24, 346. [PubMed: 29739270]
- [123]. Vila OF, Uzel SGM, Ma SP, Williams D, Pak J, Kamm RD, Vunjak-Novakovic G, Theranostics 2019, 9, 1232. [PubMed: 30867827]
- [124]. Lozano R, Gilmore KJ, Thompson BC, Stewart EM, Waters AM, Romero-Ortega M, Wallace GG, Acta Biomater 2016, 45, 328. [PubMed: 27554016]
- [125]. Ko IK, Lee BK, Lee SJ, Andersson KE, Atala A, Yoo JJ, Biomaterials 2013, 34, 3246. [PubMed: 23391495]
- [126]. Zhang BGX, Quigley AF, Bourke JL, Nowell CJ, Myers DE, Choong PFM, Kapsa RMI, Dev. Neurobiol 2016, 76, 551. [PubMed: 26251299]
- [127]. Scott JB, Ward CL, Corona BT, Deschenes MR, Harrison BS, Saul JM, Christ GJ, Front. Pharmacol 2017, 7, 1.
- [128]. Ferns MJ, Campanelli JT, Hoch W, Scheller RH, Hall Z, Neuron 1993, 11, 491. [PubMed: 8398142]
- [129]. Bian W, Bursac N, FASEB J 2012, 26, 955. [PubMed: 22075647]
- [130]. Tourovskaia A, Li N, Folch A, Biophys. J 2008, 95, 3009. [PubMed: 18502803]
- [131]. Lee LK, Kunkel DD, Stollberg J, BMC Neurosci 2002, 3, 1. [PubMed: 11825337]
- [132]. Bruneau EG, Macpherson PC, Goldman D, Hume RI, Akaaboune M, Dev. Biol 2005, 288, 248. [PubMed: 16256100]

- [133]. Burkin DJ, Kim JE, Gu M, Kaufman SJ, J. Cell Sci 2000, 113, 2877. [PubMed: 10910772]
- [134]. Kang S-B, Olson JL, Atala A, Yoo JJ, Tissue Eng. Part A 2012, 18, 1912. [PubMed: 22559300]
- [135]. Criswell TL, Corona BT, Wang Z, Zhou Y, Niu G, Xu Y, Christ GJ, Soker S, Biomaterials 2013, 34, 140. [PubMed: 23059002]
- [136]. Dhawan V, Lytle IF, Dow DE, Huang Y-C, Brown DL, Tissue Eng 2007, 13, 2813. [PubMed: 17822360]
- [137]. Aurora A, Garg K, Corona BT, Walters TJ, BMC Sport. Sci. Med. Rehabil 2014, 6, 1.
- [138]. Ambrosio F, Ferrari RJ, Distefano G, Plassmeyer JM, Carvell GE, Deasy BM, Boninger ML, Fitzgerald GK, Huard J, Tissue Eng. Part A 2009, 16, 839.
- [139]. Brutsaert TD, Gavin TP, Fu Z, Breen EC, Tang K, Mathieu-Costello O, Wagner PD, BMC Physiol 2002, 2, 1. [PubMed: 11818034]
- [140]. Rando TA, Ambrosio F, Cell Stem Cell 2018, 22, 306. [PubMed: 29499150]
- [141]. Zhang J, Hu ZQ, Turner NJ, Teng SF, Cheng WY, Zhou HY, Zhang L, Hu HW, Wang Q, Badylak SF, Biomaterials 2016, 89, 114. [PubMed: 26963901]
- [142]. Jank BJ, Xiong L, Moser PT, Guyette JP, Ren X, Cetrulo CL, Leonard DA, Fernandez L, Fagan SP, Ott HC, Biomaterials 2015, 61, 246. [PubMed: 26004237]
- [143]. Choi Y-J, Jun Y-J, Kim DY, Yi H-G, Chae S-H, Kang J, Lee J, Gao G, Kong J-S, Jang J, et al., Biomaterials 2019, 206, 160. [PubMed: 30939408]
- [144]. Aurora A, Wrice N, Walters TJ, Christy RJ, Natesan S, Acta Biomater 2018, 65, 150. [PubMed: 29128541]
- [145]. Corona BT, Garg K, Ward CL, McDaniel JS, Walters TJ, Rathbone CR, Am. J. Physiol. Physiol 2013, 305, C761.

Figure 1. Incidence and biology of VML.

a) Distribution of VML location in the body among medically retired military servicemembers. (Reproduced with permission from [3]). **b)** Differences between native skeletal muscle regeneration (left) and deposition of scar tissue following VML (right). (Reproduced with permission from [5]). **c)** Percent strength deficit post-VML in various pre-clinical models. Models include rat, mouse, and pig defects of the quadricep, TA, LD, EDL, and gastrocnemius muscles. Actual values are significantly higher than theoretical predictions based on mass loss alone. (Reproduced with permission from [7]).

Figure 2. Preclinical models of VML.

Various models of VML have been developed in mice, rats, rabbits, dogs, and pigs at a range of anatomical locations across species and with differences in muscle anatomy and pennation.

Figure 3: Tissue engineered skeletal muscle techniques are either acellular or cell-based. Four general approaches exist for engineered skeletal muscle: acellular scaffolds, cell sheet-derived engineered muscle grafts, minced muscle grafts, and cell-seeded scaffolds (here termed Tissue Engineered Muscle Grafts; TEMGs).

Figure 4. Acellular decellularized ECM treatment in VML defects may promote regeneration with some variability.

a) Computerized axial tomography images of human quadriceps VML defect pre-implantation (*top*) and 8 months following treatment with acellular decellularized ECM demonstrating the presence of new tissue within the defect region.^[60] **b)** Histological analysis over time of muscle biopsies from human VML patients treated with acellular decellularized ECM. (Reproduced with permission from [60]). **c)** Minimal regeneration over time in a rat TA VML model following treatment with acellular decellularized ECM (urinary bladder matrix). (Reproduced with permission from [46]). MF20: pan-isoform myosin; MHC: myosin heavy chain.

Figure 5. Minced muscle grafts of varied composition within VML defects enable histological regeneration but have a persistent pro-inflammatory gene expression profile.

a,b) Collagen hydrogel containing 0–100% MMG within a rat VML defect for 8 weeks and quantification of myofiber number and diameter. (Reproduced with permission from [66]).

c) Histological analysis of a rat VML defect treated with a hyaluronic acid (HA) hydrogel supplemented with laminin-111 (LMN-111) with or without 50% minced muscle graft at 8 weeks post-implantation. (Reproduced with permission from [67]). **d)** Heatmap of

differentially expressed genes comparing rat TA VML defects with no repair to those treated with 100% MMG up to 28 days post-injury demonstrating persistent similarities in transcriptional response regardless of treatment. (Reproduced with permission from [16]).

MF20: pan-isoform myosin; Con: Control; HA: hyaluronic acid; LMN-111: laminin-111.

Figure 6. Tissue engineered muscle grafts developed for VML treatment have incorporated a broad range of cell sources.

a) Primary human muscle progenitor cells within fibrin and Matrigel. (Reproduced with permission from [69]). **b,c)** Human induced pluripotent stem cell-derived myoblasts within fibrin and Matrigel. (Reproduced with permission from [81]). **d)** C2C12 mouse myoblasts within electrospun fibrin. (Reproduced with permission from [25]). **e)** Primary rat satellite cells and muscle progenitors within fibrin and Matrigel. (Reproduced with permission from [73]). MF20: pan-isoform myosin; Lam: laminin; BTX: α -bungarotoxin; SAA: sarcomeric α -actinin; MHC: myosin heavy chain.

Figure 7. Vascular Anatomy within Skeletal Muscle.

a) Highly organized vasculature with dense capillary networks run parallel to myofibers forming microvascular units optimized for nutrient transfer. *Inset:* Capillaries are embedded within myofiber sarcolemma, where mitochondria congregate at the contact site between capillary and myofiber. (Reproduced with permission from [96]). **b)** Terminal venule (Ve) and terminal arteriole (Ar) branching into capillaries (Ca) within a microvascular unit in skeletal muscle. Capillaries follow a tortuous path and parallel the myofibers. (Reproduced with permission from [97]). **c)** Changes in capillary density (black dots) are visible across the gastrocnemius muscle. (Reproduced with permission from [100]).

Figure 8: Engineering Vascularized Skeletal Muscle.

Strategies to vascularize tissue engineered constructs include host vessel infiltration (left) and implantation of pre-vascularized constructs (middle and right).

Figure 9: Host vessel infiltration to various implanted engineered muscle constructs.

a,b) Dorsal skinfold window containing constructs composed of primary rat satellite cells in fibrin. Vessels are visible in real time infiltrating constructs. (Reproduced with permission from [73]). **c)** Angiogenic gene-modified primary rat myoblasts in collagen implanted within VML defect. i) control; ii) VEGF+ myoblasts; iii) SDF+ myoblasts; iv) VEGF+SDF+ myoblasts. (Reproduced with permission from [106]). **d)** Mouse myoblasts on electrospun fibrin scaffold implanted in VML defect. (Reproduced with permission from [25]). **e)** Acellular aligned collagen scaffolds implanted in VML defect followed by exercise. MHC: myosin heavy chain. (Reproduced with permission from [84]).

Figure 10: Pre-vascularized constructs for VML treatment composed of varying cell populations and demonstrating a variety of *in vitro* and *in vivo* quantification methods.

a) Classification of *in vitro* network maturity from single endothelial cells to a fully-developed vascular network. **b)** Differences in *in vitro* vascular morphology between endothelial cell + fibroblast co-culture compared to myoblast+ endothelial cell + fibroblast tri-culture. Dense desmin+ myotubes are visible in tri-culture. (Reproduced with permission from [30]). **c)** Comparison of vascular network morphology in *in vitro* constructs containing GS1-Lectin + freshly-isolated MVFs (i) in combination with GFP+ primary rat myoblasts or (ii) MVFs alone. (Reproduced with permission from [107]). **d)** Comparison of perfusable vessels within abdominal wall VML defects via lectin (i) and luciferin (ii) perfusion. VML treatments contained either myoblast monoculture, myoblast + endothelial cell co-culture, or myoblast + endothelial cell + fibroblast tri-culture. (Reproduced with permission from [29]). **e)** Co-culture of RFP+ HUVECs and HFFs implanted in abdominal wall VML defect. Perfusion with FITC-dextran demonstrates anastomosis of host and implanted vessels. (Reproduced with permission from [30]). **f)** Immunohistochemistry demonstrating anastomosis of a human vessel (Hu-CD31) from pre-vascularized electrospun scaffolds (ASCs + HUVECs) with a host vessel (Ms-CD31) within a TA VML defect. (Reproduced with permission from [25]). **g)** Abdominal imaging window (AIW) provides novel method for intravital confocal imaging of vascular infiltration into an abdominal wall VML defect.

h) Visualization of host vessels (blue; Ms CD31), perfused vessels (red; dextran), and implanted human endothelial cells (green; Hu ECs) below the abdominal imaging window. Implanted tri-culture constructs contained human endothelial cells, smooth muscle cells, and myoblasts and were compared to those angiopoietin 1– and VEGF-expressing tri-cultures. Host vessel perfusion with TRITC-dextran (i) enables quantification of functional host vasculature (j) when compared to total Ms CD31+ host vessels. (Figures **g-j** reproduced with permission from [33]). HUVEC: human umbilical vein endothelial cell; GFP: green fluorescent protein; α SMA: α -smooth muscle actin; RFP: red fluorescent protein; ANGPT1: angiopoietin 1; VEGF: vascular endothelial growth factor.

Figure 11: Pre- and Post-Natal Development of the Neuromuscular Junction.

During embryonic development the immature muscle is pre-patterned with immature plaque-shaped AChR clusters prior to nerve contact. Following polyinnervation, nerve-secreted agrin stabilizes contacted AChR clusters while extrasynaptic clusters disperse. During postnatal development, neurons are pruned to single contact with myofibers and agrin helps to refine AChR clusters morphology. Adult skeletal muscle is highly organized with one neuron innervating each myofiber at the NMJ, which exhibits a mature, pretzel morphology with direct overlap between the presynaptic nerve terminal bouton and the postsynaptic AChR clusters.

Figure 12. Improved functional outcomes and NMJs within VML-injured muscle following neurotization.

a) Abdominal wall defects treated with a neurotized pre-vascularized construct. **b,c)** Compound muscle action potential (CMAP) of native muscle, neurotized graft, and non-neurotized control. CMAP amplitude of neurotized grafts was significantly higher than non-neurotized control. **d)** Longitudinal view of NMJ morphology present within the defect site. TUB: tubulin; ACHR: acetylcholine receptors; SYN: synaptophysin. (Figure reproduced with permission from [86]).

Figure 13. Improved functional outcomes and NMJs within VML-injured muscle following rehabilitative exercise.

a-c) Primary mouse satellite cells and muscle resident cells in TA VML defects had improved force output and NMJ formation following treadmill exercise. (Figure reproduced with permission from [21]). **d,e)** Acellular aligned collagen scaffolds implanted in TA VML defects had increased NMJ formation in muscle surrounding the defect area following voluntary caged wheel exercise. (Figure reproduced with permission from [84]). SynPh: synaptophysin; NFL: neurofilament; αBTX: α-bungarotoxin.

Figure 14. Integration of implanted pre-vascularized and neurotized engineered skeletal muscle construct into host musculature.

VML treatment with an *in vitro* pre-vascularized muscle construct results in anastomosis with host vasculature and dense perfusable vessels. Neurotization enables host neural infiltration to the implanted construct with regenerating neuromuscular junctions. Vasculature and nerves within the construct region show a mid-stage regenerating phenotype post-VML, with lower vascular density and organization, poly-innervation, and immature plaque-shaped neuromuscular junctions.

Table 1.

Tissue engineering studies utilizing host vessel infiltration to vascularize implanted muscle constructs post-VML.

Cells	Scaffold	Animal Model	Vascular Analysis	Major Results	Ref
Primary rat myoblasts	Fibrin	Construct implanted around rat femoral vessels	Gross examination of blood flow, histology	Vascular infiltration and muscle survival 3 weeks post-implant, construct contractile	[102]
Rat or hu muscle tissue	----	Muscle in semi-sealed chamber connected to host AV loop (rat)	Gross examination	Tissue survived 6 weeks post-implant and appeared vascularized	[103]
Primary rat myoblasts	Fibrin	Myoblasts injected into scaffold site pre-vascularized <i>in vivo</i> by host AV loop for 2 wks	India Ink injection, histology	Host vessel infiltration enabled myoblast survival for 8 wks	[104]
Primary rat myoblasts	----	Construct sutured to tendons of biceps femoris with transected sural nerve and blood vessel attached	Histology	Host vessels within graft after 1 wk	[105]
Primary rat satellite cells	Fibrin	Construct implanted in dorsal skinfold window (mouse)	Intravital imaging, CD, cross-sectional lumen density, immunostaining	Infiltrating blood vessels were perfused by RBCs after 1 wk; pre-differentiated muscle induced higher blood vessel density & cross-sectional lumen density	[73]
Primary rat myoblasts (transfected to express VEGF/SDF)	Collagen sponge	Construct implanted in back muscle defect (rat)	Gross examination, histology, CD	Co-culture of VEGF and SDF transfected myoblasts induced higher capillary density and larger lumens than each growth factor alone	[106]
C2C12s	Fibrin +/- tethered HGF	VML defect in TA muscle (mouse)	Gross examination, histology, immunostaining, CD	Higher CD in treatment groups over time compared to controls	[24]
----	Porcine decellularized ECM	Abdominal wall defect (rat)	Histology, vessel number	Vessels containing RBCs present in implanted graft at 8 weeks	[141]
----	Collagen (aligned or randomly-oriented)	VML defect in TA muscle (mouse)	Histology, immunostaining, isolectin perfusion, perfused vascular density, CD	Scaffold implantation + exercise significantly increased revascularization	[84]

Table 2.

Tissue engineering studies that utilize pre-vascularized constructs to vascularize implanted muscle constructs post-VML.

Cells	Scaffold	Animal Model	<i>In Vivo</i> Vascular Analysis	Major Results	Ref
C2C12s, HUVECs, embryonic fibroblasts	1:1 PLLA:PLGA sponge	Ab. wall defect (mouse)	Histology, lectin perfusion, luciferase injection, CD	Fibroblasts stabilize vessels; pre-vascularization improves survival <i>in vivo</i>	[29]
C2C12s, GFP-HUVECs, HFFs	Porcine dECM	Ab. wall defect (mouse)	Mean vessel diameter, intravital imaging, dextran perfusion, CD, C/F ratio, smooth muscle actin	Constructs pre-cultured for 3 weeks had faster anastomosis and maturation upon implantation, followed by replacement by host vessels	[31]
C2C12s, GFP/RFP-HUVECs, HFFs (co-culture vs. tri-culture)	1:1 PLLA:PLGA +/- fibrin	Ab. wall defect (mouse)	Vessel length, vessel diameter, % area CD31, immunostaining, dextran perfusion, vascular network length/area, histology	<i>In vitro</i> vessel maturity higher with co-culture compared to tri-culture; composite PLLA:PLGA + fibrin with or without cells resulted in most perfused host vessels within graft <i>in vivo</i>	[30]
Primary ms muscle progenitors, endothelial cells, + fibroblasts	----	TA and EDL muscle defects (mouse)	Histology	Muscle cells and vasculature present within defect after 2 wks	[51]
Primary rat GFP+ MVFs or primary rat ASCs	Collagen	TA muscle defect (rat)	Histology, CD, DiI perfusion	MVF treatment groups had faster vascular growth and higher vessel density; Vessels within defect were perfusable but overall perfusion level was low	[78]
C2C12s, HUVECs, human dermal fibroblasts	1:1 PLLA:PLGA sponge	Initial implantation around femoral vessels then transfer to abdominal wall defect (mouse)	Gross examination, histology, CD, dextran perfusion, immunostaining functional vessel density, ultrasound following contrast agent injection, vessel circumference	Tri-culture constructs have more rapid and complete integration with host vessels	[32]
C2C12s, HUVECs, mouse embryonic fibroblasts	Rat dECM	Rat forearm transplant	Gross examination, histology, immunostaining, intraoperative blood pressure (radial artery)	Perfusable vessels formed <i>in vitro</i> within graft were perfused by host blood upon implantation; measurable blood pressure; red blood cells present in graft vessels	[142]
GFP+ HUVECs, neonatal human dermal fibroblasts	Gelatin-based sponge or fibrin	Ab wall defect (mouse)	Histology, immunostaining, pro-angiogenic factor secretion, CD, dextran perfusion, vessel length	Static stretch induced aligned, vertical vessels <i>in vitro</i> ; Static constructs maintained vessel alignment post-implantation, were perfusable, had inc. vessel length; Vessel alignment with host muscles improved mechanical outcomes	[34]
Primary rat myoblasts (GFP+) +/- Primary rat MVFs	Collagen	VML in biceps femoris (rat)	Histology, micro-CT angiography, vascular volume, vessel diameter, Microfil perfusion	MVFs + myoblasts had better vessel network <i>in vitro</i> than MVF alone; No difference in vascular volume between groups <i>in vivo</i> ; Significant collagen within MVF treated defects	[107]
Primary mouse satellite cells and muscle resident cells	Mouse dECM	VML defect in TA muscle (mouse)	Histology, immunostaining, # blood vessels/field	Constructs containing muscle resident cells (including ECs) resulted in significantly more blood vessels in the defect	[21]
HUVECs, ASCs	Fibrin	VML in TA muscle (mouse)	Histology, immunostaining, % area CD31	Anastomosis and perfusion of implanted human vessels with host vessels; ASCs on pre-vascularized scaffold are source of significant collagen in defect	[25]

Cells	Scaffold	Animal Model	<i>In Vivo</i> Vascular Analysis	Major Results	Ref
Primary hu. venous endothelial cells, hu. dermal fibroblasts, hu. myoblasts	1:1 PLLA:PLGA + fibrin	Ab wall defect (mouse)	Histology, dextran perfusion, systemic ms-CD31 stain, fluorescent hu. ECs, total vessel length, vascularized area, host vessel invasion radius, laser speckle blood flow imaging	Adult primary ECs form more mature vascular network than HUVECs; 3D tri-culture with adult hu. ECs successfully anastomosed with host vessels post-VML	[35]
Primary hu. venous endothelial cells ^{ANGPT1+} and smooth muscle cells ^{VEGF+} , Primary hu. myoblasts	1:1 PLLA:PLGA + fibrin	Ab wall defect (mouse)	Gross examination, histology, Intravital imaging, total vessel length, dextran perfusion, systemic ms-CD31 stain, fluorescent hu. ECs	Constructs with transduced ECs and SMCs resulted in faster host vessel infiltration, increased total vessel length, and faster loss of implanted human ECs	[33]
Hu. myoblasts, HUVECs	Porcine dECM	TA muscle defect (rat)	Gross examination, histology, Immunostaining, vessel number, hu. CD31+ cells	Pre-vascularized constructs fabricated via coaxial 3D printing significantly increased vessel number and CD31+ cells post-VML compared to mixed printing	[143]
Primary hu. ASCs	PEGylated platelet free plasma hydrogel & porcine dECM	TA muscle defect (rat)	Histology, immunostaining, # blood vessels, # pericytes	Implanted constructs with ASCs had higher CD31+ cells; ASCs from implanted constructs homed to perivascular space within defect	[144]

Table 3.

Studies that assess neurotization or rehabilitative exercise and subsequent neural regeneration post-VML.

Cells; Scaffold	Animal Model	Innervation Stimulus	<i>In Vivo</i> Innervation Analysis	Major Results	Ref
Primary rat muscle progenitors and bMSCs	VML defect in TA muscle (rat)	Neurotization (peroneal nerve)	Immunostaining, neural electrical stimulation	Treated group had increased contractile force; nerve branching from host to graft visible with NMJs	[50]
C2C12s, HUVECs, human dermal fibroblasts; 1:1 PLLA:PLGA + fibrin	Ab wall defect (mouse)	Neurotization (femoral nerve)	EMG, CMAP, immunostaining, β 3-tubulin+ area	Neurotized grafts lacked spontaneous EMG activity, had measurable CMAP, and higher β 3-tubulin+ area; NMJs present in both groups	[86]
Acellular porcine decellularized ECM	VML defect in TA muscle (rat)	Voluntary caged wheel exercise	Neural electrical stimulation, histology	No improvement in histology or function due to exercise	[46]
Minced muscle graft	VML defect in TA muscle (rat)	Voluntary caged wheel exercise	Neural electrical stimulation, myofiber glycogen depletion post-neural stim., histology	Exercise resulted in lower fibrosis within defect, increased torque output, and increased macrophage presence	[145]
Primary ms. satellite cells and muscle resident cells; Murine decellularized ECM	VML defect in TA muscle (mouse)	Voluntary caged wheel exercise or treadmill exercise	Gait analysis, neural electrical stimulation, histology (cross-section), AChR clusters/myofibers, % NMJs	Exercise improved force output and NMJ formation within defect compared to sedentary	[21]
Acellular aligned collagen	VML defect in TA muscle (mouse)	Voluntary caged wheel exercise	Histology (cross-section), # of AChR clusters, # of NMJs	Exercise increased # of AChR clusters and NMJs near the defect region	[84]

Table 4.

Table of Acronyms

AChR	Acetylcholine receptor
ANGPT1	Angiopoietin 1
ASC	Adipose-derived stem cell
AV	Arteriovenous
BMP1	Bone morphogenic protein 1
C/F	Capillary to fiber ratio
CD	Capillary density
CMAP	Compound motor action potential
EC	Endothelial cell
ECM	Extracellular matrix
EMG	Electromyography
FVD	Functional vessel density
GFP	Green fluorescent protein
HFF	Human fetal fibroblast
HUVEC	Human umbilical vein endothelial cell
Id2	Inhibitor of differentiation 2
LD	Latissimus dorsi muscle
MMP2	Matrix metalloproteinase 2
MSC	Musculin
MSTN	Myostatin
MTJ	Musculotendinous junction
MVF	Microvascular fragment
MVU	Microvascular unit
NMJ	Neuromuscular junction
PDGFR α	Platelet-derived growth factor receptor α
PDPN	Podoplanin
PLGA	Poly(lactic-co-glycolic) acid
PLLA	Poly(L-lactic acid)
RFP	Red fluorescent protein
SDF	Stromal cell-derived growth factor
Snai1	Snail family transcriptional repressor 1
TA	Tibialis anterior muscle
TGF β 1	Transforming growth factor β 1
VEGF	Vascular endothelial growth factor
VML	Volumetric muscle loss
YFP	Yellow fluorescent protein