Biomimetic Scaffolds for Regeneration of Volumetric Muscle Loss in Skeletal Muscle Injuries

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ABSTRACT

Skeletal muscle injuries typically result from traumatic incidents such as combat injuries where soft-tissue extremity injuries are present in one of four cases. Further, about 4.5 million reconstructive surgical procedures are performed annually as a result of car accidents, cancer ablation, or cosmetic procedures. These combat- and trauma-induced skeletal muscle injuries are characterized by volumetric muscle loss (VML), which significantly reduces the functionality of the injured muscle. While skeletal muscle has an innate repair mechanism, it is unable to compensate for VML injuries because large amounts of tissue including connective tissue and basement membrane are removed or destroyed. This results in a significant need to develop off-the-shelf biomimetic scaffolds to direct skeletal muscle regeneration. Here, the structure and organization of native skeletal muscle tissue is described in order to reveal clear design parameters that are necessary for scaffolds to mimic in order to successfully regenerate muscular tissue. We review the literature with respect to the materials and methodologies used to develop scaffolds for skeletal muscle tissue regeneration as well as the limitations of these materials. We further discuss the variety of cell sources and different injury models to provide some context for the multiple approaches used to evaluate these scaffold materials. Recent findings are highlighted to address the state of the field and directions are outlined for future strategies, both in scaffold design and in the use of different injury models to evaluate these materials, for regenerating functional skeletal muscle.

1.0 CLINICAL NEED FOR SCAFFOLDS TO REGENERATE SKELETAL MUSCLE

Large skeletal muscle defects profoundly impact the quality of life of patients by significantly reducing the functionality of the injured muscles [1]. Skeletal muscle injuries typically result from traumatic incidents such as those presented from excessive exercise, contusions, lacerations, surgical resection or reconstruction, or combat injuries [2-4]. Approximately 35-55% of all sports injuries involve skeletal muscle damage to the myofiber and/or connective tissue [5]. Further, there are about 4.5 million reconstructive surgical procedures performed annually as a result of traumatic injury, car accidents, cancer ablation, or cosmetic procedures [6, 7]. On the battlefield, extremity injuries are present in 54% of cases, with 53% of these injuries involving soft tissue damage [8, 9]. Many of these injuries involve the loss of at least 20% of a given muscle's mass, which is termed volumetric muscle loss (VML) [1, 10]. These injuries result in billions of dollars in health care expenses, as well as the development of different surgical techniques and medical devices to repair them [11-14].

While skeletal muscle has an innate repair mechanism that can repair small wounds, the magnitude of critically sized injuries or VML defects limits these mechanisms from repairing large wounds [2]. Further, there are no treatments available that attempt to harness or direct this endogenous regenerative pathway. There are several established protocols for the treatment of small-scale skeletal muscle injuries such as RICE (resting the wound with a combination of ice, compression, and elevation) [13-15]. However, larger-scale injuries such as those presented by VML often require specialized surgical solutions [1, 16]. The current standard of care for large-scale VML injuries is an autologous tissue transfer from an uninjured site, where surgeons remove the damaged muscle tissue and graft healthy muscle from a donor site unaffected by the traumatic incident [7, 17]. Regardless of the level of success of these procedures, there is always

a loss of muscle strength and function, usually due to the formation of scar tissue [10, 16, 18]. While autologous tissue transfers are the best available treatment option, as many as 10% of these surgeries result in complete graft failure due to complications such as infection and necrosis [17, 19]. In the case of extensive traumatic injuries such as those presented by military personnel, there may be limited donor site availability [18, 20]. Often these extensive injuries result in amputation, requiring the use of prosthetic devices to regain partial functionality [10]. As such, there is a significant need to develop off-the-shelf biomimetic materials that can direct skeletal muscle tissue formation within these large defect sites to facilitate functional tissue regeneration [1, 18].

2.0 SKELETAL MUSCLE ANATOMY

Skeletal muscle is one of the most abundant tissues in the human body and accounts for 40% to 45% of the total body mass [21, 22]. It coordinates locomotion through attachment points to the skeleton and its macroscopic structure consists of organized networks of myofibers, blood vessels, nerves, and connective tissue. The working contractile unit of skeletal muscle is the sarcomere which consists of interposing filaments of actin and myosin [23]. Multiple myoblasts fuse together to form myofibrils which are structures with repeating sarcomeres along the length of the myofibril. A bundle of myofibrils form the basic structural element of muscle and is called the myofiber (Figure 1). Myofibers range between 20-100 μm in size and are the conglomeration of multiple myofibrils [21, 23]. Ultimately, the nuclei of these myoblasts translocate to the periphery of the myofibers residing just beneath the sarcolemma, or cell membrane of myofibers. Similarly, myofibers within a muscle organize into larger structures referred to as fascicles [21] and in general, the anatomical structure of skeletal muscle can be

thought of as a series of self-contracting cables that organize into a complex hierarchical structure. Functionally, myofibers are classified as slow or fast twitch fibers, which in part is due to differences in the metabolic activity of these fibers. Broadly, slow twitch fibers utilize oxidative metabolic pathways and fast twitch fibers utilize glycolytic metabolic pathways, and also differ physically by varying myosin heavy chain proteins within the contractile apparatus of the fibers (for a review, please see [24]).

Each myofiber is surrounded by a basement membrane referred to as the basal lamina (Figure 1). This dense structure is composed of a variety of basement membrane proteins such as type IV collagen, laminin-2, and fibronectin and is essential for guiding the endogenous regenerative pathway of skeletal muscle [25, 26]. In addition to these basement membrane proteins, the extracellular matrix is also composed of sulfated glycosaminoglycans and proteoglycans, such as heparan sulfate, as well as type I collagen, which assist in maintaining the structural integrity of skeletal muscle [27, 28]. Skeletal muscle progenitor cells, termed satellite cells (SCs), reside in the interstitial space between the sarcolemma of myofibers and the basal lamina. These cells are identified by expressing paired box gene 7 (Pax7) and are typically quiescent in mature, healthy tissue [2, 29-31]. Satellite cells account for 2-7% of all myofiber nuclei, but they are the primary cell type responsible for the regeneration of skeletal muscle tissue [32].

3.0 NATIVE REGENERATIVE PATHWAY OF SKELETAL MUSCLE

Skeletal muscle regeneration is a complex process that involves an inflammatory response, growth factor signaling, SC mediated repair of the injury, and fibroblast infiltration, ultimately leading to either functional regeneration or scar tissue formation. These processes

have been loosely classified into three phases: (1) the destruction/inflammatory phase, (2) the repair phase, and (3) the remodeling phase (Figure 2) [10, 13, 21, 33, 34].

3.1 Destruction/Inflammatory Phase

Traumatic injury to skeletal muscle leads to the rupture of the sarcolemma which exposes the intracellular contents of the myofiber to large amounts of extracellular calcium. This triggers the activation of calcium-dependent proteases and initiates myofiber necrosis [21, 34, 35]. Necrosis occurs along the length of the damaged myofiber until it reaches a contraction band, a dense band of cytoskeletal material that is designed to halt the necrosis of injured myofibers [13, 36, 37]. Almost immediately after injury, neutrophils migrate to the injury site and assist in the degradation of damaged myofibers. Macrophages quickly migrate to the injury site and become the predominant cell type present in as little as two days post-injury [38]. One of the most critical cytokines secreted from the inflammatory cells is nitric oxide, whose functions include: (1) the facilitation of further degradation of myofibers present in the wound environment [33, 38], (2) the release of growth factors such as hepatocyte growth factor (HGF) from the surrounding matrix [33, 39-42], (3) the induction of vasodilation of nearby blood vessels to recruit additional inflammatory cells [13, 43, 44], and (4) the inhibition of macrophage polarization into an anti-inflammatory, fibrosis promoting M2a macrophage [45]. While macrophages migrate to the wound site within two days of injury, they also modulate the cytokines they release over time. Initially, pro-inflammatory M1 phenotype macrophages assist in the phagocytosis of necrotic myofibers and also support satellite cell survival and proliferation [33, 38]. Over the next 3-7 days, M1 macrophages are replaced by M2 macrophages, which are believed to enhance the regenerative response of tissues by promoting myoblast proliferation,

growth, and differentiation [10, 33, 38]. Persistence of M1 phenotype macrophages will continue to secrete cytokines such as interferon gamma (IFN- γ), which, in addition to stimulating macrophage phagocytosis, also inhibits the recruitment of M2 macrophages, or the differentiation of naïve macrophages to the M2 phenotype [46]. One of the key factors that M2 macrophages secrete is interleukin-10 (IL-10), which increases myoblast proliferation and overall skeletal muscle regeneration [47, 48].

3.2 Repair Phase

The repair phase of skeletal muscle regeneration is comprised of two sub-phases: the recruitment and proliferation of SCs (Figure 2B) and the differentiation of SCs into mature muscle tissue (Figure 2C). The recruitment/proliferation phase is initiated upon mechanical injury of muscle fibers when HGF is rapidly released from the extracellular matrix (ECM) of the muscle fiber itself, stimulating the activation and proliferation of SCs as well as the chemotaxis of SCs to migrate to the injury site [40, 41, 49, 50]. HGF has been found to be the sole growth factor capable of bringing SCs back into the cell cycle [51-53]. Other factors, such as fibroblast growth factor 2 (FGF2) and fibroblast growth factor 6 (FGF6) are also released from the ECM, which also stimulate SC proliferation to gather enough progenitor cells to repair the injury [13, 50, 54-58]. Because of their location between the sarcolemma and the basal lamina, SCs typically migrate longitudinally from the same myofiber to the injury site rather than laterally from other intact fibers [2, 29, 59]. However, once injuries result in the rupture of the basal lamina, lateral migration of SCs to the injury site does occur [60, 61]. SCs are extremely efficient at repairing muscle and are capable of generating thousands of myoblasts from a small number of satellite cells [62, 63]. Once SCs reenter the cell cycle (i.e. activate), they begin to

express myogenic factor 5 (Myf5) and will express myogenic differentiation factor-1 (MyoD) when they are committed to myogenic differentiation [2, 64-67]. Additionally, once SCs are committed to differentiation they are referred to as myoblasts, and begin to lose Pax7 expression [22, 68].

The second sub-phase of regeneration, the differentiation phase, occurs when myoblasts begin to express myogenin, fuse with other myoblasts, then mature into myofibers (Figure 2C) [65, 68]. This process is inhibited by the growth factors associated with the proliferation phase of muscle regeneration, mainly HGF, FGF2, and FGF6, highlighting the importance of the temporal expression of growth factors in skeletal muscle regeneration [2, 50, 69, 70]. Specifically, there is evidence that prolonged systemic supplementation of HGF will inhibit healing, showing a need for the growth factor to be present for no longer than 48-72 hours [50, 71, 72]. Instead, the process of myoblast differentiation is stimulated by the insulin-like growth factor (IGF) family [73, 74]. This family (consisting of members IGF1 and IGF2) is unique in that each growth factor will stimulate both the proliferation and subsequent differentiation of myoblasts [75-78]. Both *in vitro* and *in vivo* studies have suggested that IGF1 is more potent in skeletal muscle regeneration than IGF2, either due to growth factor activity or the increased availability of its receptor, insulin-like growth factor receptor-I (IGFR1) [79, 80]. Neutralizing the function of IGF1 was found to inhibit skeletal muscle regeneration, highlighting the importance of this factor in the regenerative response [81]. Injections of exogenous IGF1 into skeletal muscle injuries in aged animals restores the proliferative capacity of SCs, making it a potent factor in skeletal muscle regeneration, and a necessary factor for enhancing the endogenous regenerative response of skeletal muscle [82-84]. Further, the expression of IGF1 in the macrophage population appears to drive macrophages towards an M2 phenotype, in addition

to stimulating myoblast fusion [85]. IGF-mediated cell-fusion events occur after the initiation of myogenin expression which also terminates MyoD/Myf5 expression [2, 30, 68, 86]. Nascent myofibers are characterized by centrally aligned nuclei and as these cells mature and generate more contractile proteins the nuclei gradually translocate to the periphery of the myofiber [2, 13, 30, 66].

3.3 Remodeling Phase

The final stage of muscle regeneration is the remodeling phase and is a balance between the culmination of the differentiation sub-phase of muscle regeneration and the infiltration of fibroblasts into the injury site. Regenerating myoblasts will fuse with the existing musculature at the interface of the injury or to the ECM (Figure 2D) [10]. The infiltration of fibroblasts can begin as early as the inflammatory phase and supports regeneration by repairing and replacing connective tissue that was damaged by the injury [13, 87]. While the connective tissue synthesized by fibroblasts is important in providing stability for the regenerating tissue and replacing damaged or lost ECM, collagen deposition often remodels into scar tissue and limits the organization of the regenerated tissue (Figure 2 E-G) [10, 13, 87, 88]. The balance between these two antagonistic responses dictates the functional outcome from the regenerative pathway. In general, small injuries remodel without the presence of scar tissue, but as the injury becomes larger the fibroblast infiltration increases such that there begins to be a significant amount of collagen deposition, ultimately impeding the functional regeneration of skeletal muscle tissue.

In injuries resulting from VML, there are no regenerative cues present due to the complete destruction or removal of the basal lamina and surrounding connective tissue (Figure 2E). This results in a loss of traditional signaling that indicates the need for SCs to migrate into

the injury site. Therefore, there does not appear to be a large influx of myoblasts into these injuries [13, 89]. Because of the lack of SC infiltration into the injury site, the infiltrating fibroblasts dominate this stage of regeneration, resulting in large amounts of collagen deposition [90, 91]. Ultimately, the deposition of collagen I into the wound site results in the formation of scar tissue (Figure 2G), limiting the ability of the muscle to uniformly contract, and resulting in a loss of function, as observed from the patient [10, 88, 92]. In addition to the loss of mechanical function observed in patients with VML injuries, these injuries also result in significant losses of muscle volume, which can generate physical deformities that may impact the psychological health of the patient.

3.4 Cell Types Involved in Skeletal Muscle Regeneration

Concurrent with the repair phase of skeletal muscle regeneration, blood vessels, nerves, and fibroblasts infiltrate the injury site [2, 10, 34]. Skeletal muscle is a highly vascularized tissue and as such, its revascularization is vital for successful regeneration [93, 94]. The close proximity of SCs and endothelial cells in skeletal muscle facilitates paracrine signaling between these cell types to support both angiogenesis and myogenesis [95, 96]. Muscle is not only highly vascularized, but also heavily innervated to facilitate the cooperative contraction of myofibers to conduct uniaxial force [34]. Factors such as IGF1 have been shown to induce nerve sprouting and HGF and FGF2 have been shown to be potent angiogenic factors, suggesting that the growth factor cascade in skeletal muscle regeneration affects a variety of cell types in addition to myoblasts [97-102]. In addition to mediating the deposition of collagen, scar tissue formation and functionally impaired regeneration [12, 13, 36, 103], fibroblasts infiltrate during the early in the inflammatory stages of skeletal muscle regeneration along with SC recruitment [13, 87]. The

interactions between these two cell types are critical for proper regeneration. It is believed that fibroblasts mediate regeneration through repairing and replacing connective tissue that was damaged by the injury. Finally, as reviewed in 3.1, macrophage signaling plays a critical role in skeletal muscle regeneration. Inflammatory M1 macrophages secrete cytokines such as IFN- γ and tumor necrosis factor α (TNF- α), which support the phagocytosis of damaged myofibers, in addition to stimulating the *in vivo* survival and proliferation of myoblasts. However, these same factors inhibit myoblast differentiation, so it is essential that the spectrum of macrophage polarization shifts to an M2 dominate phenotype. These M2 macrophages secrete several cytokines, including IL-10, which is an extremely potent factor that stimulates the regeneration of skeletal muscle tissue [47].

4.0 CURRENT TISSUE ENGINEERING STRATEGIES AND LIMITATIONS

There are two main approaches to develop constructs for skeletal muscle regeneration: i) the *in vitro* development of engineered skeletal muscle constructs to augment repair, or ii) the development of scaffolds to regenerate damaged skeletal muscle tissue *in vivo* [104-107]. Materials designed to guide regeneration of skeletal muscle can be further classified as either acellular or cellular; with the latter incorporating myoblasts or SCs into their matrix. The utilization of the scaffolding material as a delivery vehicle for progenitor cells allows for an exogenous regenerative response to complement endogenous regeneration by augmenting the number of cells available to regenerate large scale injuries. Conversely, acellular strategies invoke the endogenous regenerative response by delivering sustained amounts of growth factors to stimulate the recruitment and proliferation local progenitor cells into the injury site.

To direct skeletal muscle growth, biomimetic scaffolds must facilitate cell alignment, promote skeletal muscle formation, and stimulate vascularization and innervation. Cell

alignment is one of the most critical factors in skeletal muscle regeneration due to the unique geometric architecture of muscle fibers [21, 108-111]. Fibrous elements within scaffolding materials have been shown to direct cell alignment along the length of the scaffold's surface [112-115]. Strategies to promote skeletal muscle formation and angiogenesis include the incorporation of growth factors and peptide sequences within the scaffolding material [93, 116, 117]. Sustained delivery of growth factors improves skeletal muscle regeneration compared to single bolus dosing, suggesting that the controlled release of growth factors from scaffold materials is an efficient delivery method for these molecules [73, 83, 93]. Finally, scaffolds need to balance degradation with structural and mechanical support of the injury site such that enough structural stability is present within the scaffold material to support mechanical loading until the regenerated tissue is stable enough to withstand loading independently [4, 91, 118].

4.1 *In Vitro* Skeletal Muscle Development

Engineered skeletal muscle constructs have been developed to study skeletal muscle formation *in vitro* to develop high content models for drug screening applications and ultimately to replace or augment damaged tissue *in vivo* [21, 107, 108]. These tissues are typically prepared by seeding myoblasts onto a protein surface, commonly fibrin, which is remodeled into muscle tissue [80, 119-124]. Skeletal muscle constructs engineered *in vitro* can spontaneously contract and have been electrically stimulated to induce tetanic contractions, demonstrating that these constructs can accurately mimic skeletal muscle function [122]. Vandenburg and colleagues developed a platform technology that generates skeletal muscle constructs in the bottom of 96 well plates between two polydimethylsiloxane (PDMS) posts as a high content model for drug screening applications. Each individual well is capable of being electrically stimulated and

assessed for tetanic force production [125-127]. One of the limitations of these skeletal muscle constructs is the inability of nutrients to diffuse to the innermost layers of the tissue. While this is not a problem for small skeletal muscle constructs used in drug screening applications, constructs approaching 0.4 mm in thickness result in the formation of a necrotic core making it unsuitable for implantation [122, 128]. Recently, a study using a heterogeneous population of cells isolated from the muscles in mice hind limbs demonstrated that vascular networks could be formed *in vitro* to support the survival of these constructs for at least 40 days in culture [129]. Another recent study investigated myoblasts seeded into fibrin hydrogels to produce bundles of biomimetic engineered muscle components up to 1 cm in length. These constructs generated functional skeletal muscle tissues after 2-4 weeks in culture [124]. Remarkably, these bundles also repaired themselves after cardiotoxin insult *in vitro*, demonstrating that the SC niche was present within these constructs. Vascularization of these constructs was achieved *in vivo* by implanting the scaffolds perpendicular to the intact *panniculus carnosus* muscle. However, it is currently unclear whether these bundles can engraft into existing musculature and contribute to their overall force production.

Larger skeletal muscle constructs ranging between 1 and 4 cm in length have been implanted to observe engraftment, vascularization, and functional recovery of VML injuries. Implanting *in vitro* skeletal muscle constructs near existing blood vessels greatly enhances their vascularization [130]. Successful *in vivo* engraftment significantly increases the force output of these constructs, demonstrating that these constructs are capable of integrating with the surrounding tissue to remain viable and enhance their force production [131]. Finally, *in vitro* skeletal muscle constructs approximately 4 cm in length, termed “skeletal muscle units” (SMUs), were implanted into a rat VML model where 33% of the *tibialis anterior* (TA) muscle was

removed. While these SMU constructs supported some vascular and nervous tissue integration as well as a significant increase in TA force production after 28 days, the forces generated in these muscles remained significantly below baseline force values [132].

The major limitation of these methods is the lack of vascularization of *in vitro* skeletal muscle constructs. Dennis and Kosnik showed that myoblasts need to be within 150 μm of a source of oxygen to proliferate or differentiate, limiting the size of viable constructs without a functional vascular network [128]. Further, these constructs are generated by seeding myoblasts and fibroblasts on protein surfaces and then they are differentiated into functional tissue. This requires 3-4 weeks of culture time for the formation of a robust ECM to support construct contractility [107]. The ability to generate contractile constructs from a patient's own cells is a promising option [107, 127], and likely the only cell source for such regenerative strategies; however, nearly a month would pass between obtaining the biopsy and implantation. Therefore, these constructs might be better suited for reconstruction of chronic conditions rather than for the repair of acute VML injuries.

4.2 Biomaterials for Skeletal Muscle Regeneration

Scaffolds for skeletal muscle regeneration have been made from a variety of materials, ranging from synthetic polymers such as poly(glycolic acid) (PGA)[133] and poly- ϵ -caprolactone (PCL) [109]; to decellularized ECM materials [134]; to natural polymers such as alginate [93], collagen [113], and fibrin [91]. In addition to the use of a variety of materials as acellular scaffolds, strategies for skeletal muscle regeneration have utilized a variety of cell sources [105, 108]. The incorporation of cells into scaffolds has significantly increased the survival of implanted cells in the injury site, which was previously below 5% [135, 136]. While

the source of these cells can vary with different scaffolds, in general SCs or myoblasts derived from SCs in culture have been investigated to regenerate skeletal muscle injuries [105, 137]. The cell sources used, *in vivo* functional outcomes, and the limitations of each material are summarized in Table 1. The sections below discuss recent advances in the design of scaffolds for the regeneration of VML injuries and are organized based on the composition of their scaffolding materials.

4.2.1 Synthetic Polymers

A variety of synthetic materials have been used as scaffolds for skeletal muscle regeneration such as PGA [133, 138], poly(lactic acid) (PLA) [139], their copolymers (PLLA/PLGA) [140], and PCL [109, 141, 142]. Synthetic materials offer several advantages to natural polymers such as their ability to have precisely tuned mechanical and structural properties that can be tailored to each tissue engineering application [115, 143, 144]. Synthetic polymers can be readily fabricated into a variety of geometries such as individual fibers or electrospun meshes with aligned or random nanofiber orientation [145-147]. Myoblasts seeded onto electrospun meshes with aligned nanofiber orientation generate highly aligned cultures that, when cultured in differentiation medium, readily fuse into aligned myotubes [109, 148]. Incorporating electrically conductive materials within synthetic scaffolding materials facilitated more efficient electrical stimulation of myoblast cultures, further improving myoblast differentiation compared to aligned fibers without stimulation [149-151]. Synthetic scaffolds can easily be fabricated with multiple growth factors incorporated into their bulk structure to facilitate the controlled release of these factors into a potential injury site [152].

The mechanical stability of synthetic polymer scaffolds that have been used for skeletal muscle applications [153-155] have been used previously with successful outcomes as a structural support material with which to repair abdominal hernias [156, 157]. In these applications, several studies noted the beginning of a vascular network forming in as little as 6 weeks after implantation of PGA meshes, suggesting that these scaffold materials have a modest ability to stimulate angiogenesis [138, 158]. However, a common criticism of synthetic materials is that their surfaces do not always readily support cell attachment [142]. Due to their inherent suboptimal bioactivity, many strategies exist to functionalize the surfaces of synthetic polymer scaffolds such as the addition of collagen or decellularized ECM to modulate tissue responses [109, 153]. Another limitation of synthetic materials is that they typically stimulate a foreign body response as characterized by an increase in the number of foreign body giant cells [159]. This foreign body response induces a prolonged inflammatory response, as well as a sustained M1 macrophage polarization; ultimately resulting in attenuated regeneration [160, 161]. The limited bioactivity of scaffolds made from synthetic polymers limits their utility for treating VML injuries because of the need for these scaffolds to guide functional tissue regeneration, rather than to trigger a sustained inflammatory response.

An alternate approach to the suboptimal bioactivity of synthetic scaffolds has been to use them as a sacrificial layer to support ECM deposition from myoblasts. Using this approach, a polyurethane (PU) foam is generated from a hardened sugar template, and the ECM is synthesized from rat myoblasts [162]. While the PU foam was coated with fibronectin to facilitate myoblast attachment, type I collagen was detected via immunostaining; and laminin and type IV collagen were detected via tandem mass spectroscopy after 4 weeks of culture. The mechanical strength of this engineered ECM is currently reported to be approximately one third

of decellularized skeletal muscle derived ECM (M-ECM), however changes to the growth medium or the inclusion of fibroblasts may facilitate an increase in ECM production to increase the mechanical properties as well as the morphological similarities between the engineered ECM and native M-ECM.

4.2.2 Decellularized Scaffolds

Decellularized ECM has been harvested from a variety of tissues such as the small intestine, urinary bladder, dermis, and pericardium and has been used as a biomaterial because it contains many matrix proteins and growth factors thought to be critical for tissue regeneration in their native configuration [163-165]. While ECM-derived scaffolds are thought to contain all of the proteins and growth factors necessary to direct tissue regeneration, care needs to be taken with the decellularization process, since the detergents used can significantly affect the protein and growth factor content within the scaffold [166-169]. Further, incomplete decellularization can result in the persistence of DNA fragments in the material which have the potential to induce an inflammatory response [170]. Interestingly, there does not appear to be site-specific advantages between the decellularized ECM source site and the site of implantation in skeletal muscle applications. The regenerative response in a rodent partial thickness abdominal wall defect due to M-ECM was equivalent to the regenerative response using a small intestine submucosa (SIS) scaffold [28]. One advantage of using ECM derived scaffolds is that they are angiogenic and serve to promote vascularization of skeletal muscle injury sites [16, 171]. However, there are conflicting reports regarding the efficacy of a variety of acellular ECM scaffolds, including M-ECM, dermal ECM, and SIS to enhance the functional recovery of a large muscle defect. Investigations looking at remodeling events 1-2 months post-injury reported modest myoblast infiltration into the injury site, although it appears to be somewhat disorganized

[172-175]. There is no consensus whether these additional myofibers contribute to the functional recovery of the muscle 2 months post-injury. Some studies report no improvements in force generation [176], while others report a significant improvement in functional recovery [177]. The addition of bone-marrow derived mesenchymal stem cells (MSCs) to ECM scaffolds significantly improved functional recovery and blood vessel formation compared to acellular ECM scaffolds (approximately 85% and 75% of the force produced in the contralateral leg, respectively) [178]. However, MSCs have not been shown to engraft into skeletal muscle tissue so the exact mechanism of MSC-mediated increases in recovery are not clear [178]. Decellularized bladder matrices seeded with muscle derived cells that were differentiated prior to implantation also significantly increased the functional recovery of VML injuries 2 months post-injury two fold by engrafting into the uninjured tissue [89, 179]. Long term remodeling, between 3-6 months post-injury is required for acellular urinary bladder matrix (UBM) and SIS scaffold mediated functional recovery [180, 181]. Interestingly, Corona *et al.* observed an increase in the force production of their untreated injury control groups over time (18.6 ± 0.2 N/kg body weight at 2 months compared to 19.8 ± 0.6 N/kg body weight at 6 months) [90], suggesting that the scar tissue deposited in the injury site may be reorganizing over time to facilitate some amount of contractility [90, 181]. In human clinical trials, there have been several accounts of successful partial restoration of mechanical function in four of six patients using SIS and UBM ECM scaffolds to treat VML defects [18, 134]. While ECM scaffolds have been shown to significantly enhance skeletal muscle repair in VML defects, there remains a significant reduction in mechanical function compared to uninjured muscle. As ECM based scaffolds are derived from complex tissue environments, the exact composition of these materials is not well

characterized. While these materials have the ability to direct a regenerative response after longer time points, their mechanism of action remains an active area of research.

An alternative strategy to the use of decellularized matrix could be to utilize minced skeletal muscle tissue that has not been decellularized. In this approach, morselized muscle tissue was implanted within a VML defect, resulting in an approximate 55% restoration of the functional deficit of unrepaired muscle controls [182]. These materials may be advantageous for use in traumatic injury where the damaged tissue is still present, but they would not be a viable therapy for injuries where the tissue was completely destroyed or lost. Extended recovery times of 4 months did not appreciably increase the contractility of injured muscles relative to 2 months post injury, suggesting a shorter window of remodeling for cellular constructs than acellular scaffolds. Improvements to this promising therapy may require that the materials actively direct the reorganization of muscle fibers to facilitate their alignment with the surrounding healthy tissue.

4.2.3 Natural Polymers

Naturally occurring polymers such as alginate [93, 98, 183], collagen [113, 184, 185], and fibrin [20, 91, 140] have been used extensively in skeletal muscle engineering. Unlike synthetic polymers, natural polymers possess intrinsic bioactive signaling cues and can form complexes with carbohydrates to form molecules such as heparan sulfate proteoglycan which bind growth factors to enhance cell migration, proliferation, or differentiation [186-189]. In addition, growth factors can adsorb directly to some natural polymers. Alginate is derived from seaweed and was originally used as an encapsulation agent for a variety of cell types [190] and due to its amenability to chemical alterations to promote cell adhesion [191], as well as its

tunable mechanical and structural properties [192-194], it has become increasingly used as a scaffold for skeletal muscle regeneration. Alginate gels with stiffnesses between 13 and 45 kPa were found to maximize myoblast proliferation and differentiation [195]. Additionally, alginate facilitates the controlled release of a variety of growth factors important to skeletal muscle regeneration, such as HGF [136, 183], FGF2 [136, 183], IGF1 [93, 196], and vascular endothelial growth factor (VEGF) [93, 196-198]. Alginate gels loaded with HGF and FGF2 significantly increased myoblast viability within the construct, as well as the migration of myoblasts out of these scaffolds, demonstrating that alginate can deliver myoblasts to skeletal muscle [136]. When translated to a mouse TA laceration model, myoblast-seeded gels loaded with HGF and FGF2 significantly reduced the wound size 30 days after injury with an increased number of regenerating myofibers. It was found that the combination of growth factors and the myoblasts produced this effect, but individually each growth factor only supported a modest regenerative response [183]. More recently [199], alginate gels incorporating VEGF and IGF1 have been implanted into ischemia-induced skeletal muscle injuries to address skeletal muscle regeneration by improving the early vascularization and myoblast survival within the injury sites [93, 197]. Functionally, these studies demonstrated that the delivery of VEGF and IGF1 increased the force production 4-5 fold compared to blank alginate gels 7 weeks after injury, approximating the force production of contralateral leg controls [93]. While this model of injury is not as severe as VML injuries, bulk alginate scaffolds loaded with multiple growth factors can significantly improve the regeneration of skeletal muscle injuries by improving the overall vascularization and myoblast differentiation *in situ*. However, alginate gels have only been implanted on the surface of injury sites [199], and their ability to direct skeletal muscle regeneration without the presence of the existing skeletal muscle ECM is not known.

Collagen, particularly type I, belongs to a family of ECM proteins found in connective tissues such as dermis, tendons, and blood vessels [200]. Scaffolds made from type I collagen have been used in a wide variety of tissue engineering applications including skin, cartilage, bone, tendon, skeletal muscle, and nerve [188, 201]. Collagen gels have been used in skeletal muscle regeneration as a cell delivery vehicle since intramuscular injections of myoblasts result in the rapid death of the delivered cells [135]. Myoblasts implanted within a collagen gel have been shown to migrate out of the scaffold and fuse with the existing myofibers present at the periphery of the injury site [202, 203]. Disorganized myofibers form as a result of the amorphous polymerization process of the collagen lattices, resulting in less optimal healing of muscle injuries [204]. Acellular collagen sponges also supported disorganized repair of large muscle defects. However, a large amount of adipose tissue was observed 12 to 24 weeks post-injury, which further limited the amount of organized functional recovery [205]. To improve the alignment of regenerating myofibers, collagen gels can be fabricated with aligned pore structures through controlled freeze-drying processes. These modified scaffolds facilitated aligned myotubes along the direction of the pores, which when integrated into a large muscle defect, were capable of producing force upon electrical stimulation [113]. However, because of the lack of a robust regenerative response observed with implanted collagen scaffolds, collagen materials were customized with molecules such as heparin to achieve controlled release of growth factors into the injury site [206, 207]. In fact, the functional recovery of muscles damaged by acute ischemia was significantly increased 1 month after injury by adding a VEGF-loaded collagen scaffold, demonstrating that scaffolds can contribute to regeneration by supplying necessary growth factors to the wound site (53% vs. 75% of the force produced before VML defects) [208]. Other growth factors such as HGF, IGF1, and FGF2 have been added to denatured collagen gels

to increase SC recruitment into the injury site [209]; however, no functional measurements were performed on these regenerating muscles and data was obtained only at early time points.

Fibrin has been used extensively as a scaffold material in tissue engineering because of its intrinsic bioactivity and role as a provisional matrix during the initial stages of wound healing [189, 210]. Fibrin is a branched microfibrillar polymer formed when activated thrombin cleaves two small peptides from fibrinogen allowing fibrinogen to self-assemble into a complex fibrillar network. Fibrin gels have been used as the provisional seeding matrix for many *in vitro* skeletal muscle constructs as described above [211-213]. Despite completely degrading within 3 weeks post-injury, fibrin gels promote myoblast survival and differentiation into myofibers that can integrate with uninjured tissue [20, 214, 215]. Implanting a myoblast populated fibrin gel near an existing blood vessel greatly enhances the vascular perfusion of these constructs. These primary myoblasts formed vascularized functional mature skeletal muscle tissue three weeks after implantation [130]. In a different approach to vascularization of skeletal muscle constructs, endothelial cells were seeded into fibrin-PLLA/PLGA constructs in addition to fibroblasts and myoblasts. This tri-culture supported a complex vascular network, suggesting the need for multiple cell types to be present in cell constructs for skeletal muscle regeneration [140]. The addition of PLLA/PLGA to these tri-culture scaffolds was done in part to augment the stability of fibrin constructs, as uncrosslinked fibrin is rapidly remodeled. Another strategy to decrease the rate of proteolytic degradation of fibrin has been to add polyethylene glycol (PEG) groups to the bulk structure of fibrin. In addition to modulating the structural properties of the material, PEGylation can be used as a strategic loading mechanism for a variety of growth factors such as IGF1, to facilitate controlled release of therapeutic molecules to enhance skeletal muscle regeneration [216].

To increase the guided organization of skeletal muscle regeneration, our laboratory pioneered the use of fibrin microthreads, which are morphologically similar to the hierarchal cable-like structure of skeletal muscle. Fibrin microthreads facilitate cell alignment along the longitudinal axis of the material [217] and can be loaded with growth factors such as FGF2 [218] to create discrete structures that can be combined with microthreads loaded with other factors to generate customized composite scaffolds to enhance tissue regeneration. The combination of a bioinspired scaffold material and a highly myogenic human cell line allowed for more complete endogenous muscle regeneration in a mouse model of VML injury (55% vs. 90% of the force produced in the contralateral leg) [91]. While we observed a reduction in the deposition of scar tissue 3-4 months post-injury, histological analyses of tissue harvested at early time points suggested that the microthreads were largely degraded within 2 weeks of implantation and many of the regenerated myofibers in the wound site at later time points exhibited some degree of misalignment with respect to the native muscle tissue [91]. We developed a method to attenuate the degradation rate of fibrin microthreads using carbodiimide crosslinking chemistry to enhance the *in vitro* persistence of these scaffolds [219]. Interestingly, discrete structural and mechanical properties were developed by modifying the pH environment of the carbodiimide crosslinking reaction, suggesting that the efficiency of carbodiimide crosslinking can be regulated both through controlling the pH environment as well as the total crosslinking time [219].

4.3 Limitations

There have been many significant successes in developing materials to guide skeletal muscle regeneration. One of the strengths of synthetic materials is the ease in which they are fabricated, as well as the flexibility to construct scaffolds in a variety of controlled geometries to control myoblast growth and differentiation [109], despite needing functionalization with natural materials to improve myoblast attachment or regenerative outcomes [153]. Care must also be taken during the fabrication process to avoid making materials that induce a foreign body response [159, 220]. Decellularized ECM materials have been shown to significantly improve functional outcomes in VML defects. However, they do not appear to achieve complete alignment between healthy and regenerating tissue, likely due to the volume of defect space. While natural scaffolds are typically more bioactive than unmodified synthetic materials, bulk scaffolds that form through random protein polymerization do not generate uniform cell alignment (Figure 3B). Applying static axial stretching techniques to natural polymers may facilitate the alignment of protein fibrils within the bulk scaffold, which will direct uniaxial alignment of cells. However to date, these materials have not yet been applied to VML injuries [219, 221]. Fibrin microthreads have been shown to direct aligned muscle regeneration (Figure 3C), but these microthreads degrade rapidly *in vivo* [91]. While crosslinked fibrin microthreads should attenuate this degradation rate, they have not yet been implanted to assess their *in vivo* degradation kinetics.

Further, many of the models of skeletal muscle injury utilize ischemia [93, 216], toxin [196], or crush induced [222] injuries. These methods of muscle injury leave the basal lamina intact which allows SC recruitment to the injury site due to chemotactic factors being released by the basal lamina [1, 54, 91]. The combination of the early recruitment of SCs to the injury site, as well as the persistence of the regenerative template of the basal lamina, allows these injuries

to completely regenerate within 3-4 weeks [2, 13, 21, 25]. In contrast, VML injuries result in the complete destruction or removal of the basal lamina, eliminating the template for SCs to regenerate organized functional skeletal muscle tissue [1, 91, 172, 223]. Table 2 briefly summarizes the methods and limitations of each of these animal models.

Finally, implanted cell-seeded constructs have utilized a variety of cell types, sourced from several different species. Myoblasts, or expanded SCs, are a promising cell type for cell-based therapies as they can functionally integrate into the existing musculature in the host. Further, they seem to be the most likely cell source for autologous therapies, which would ultimately be required for many of these injuries. Generally, studies have either involved human myoblasts in immune compromised animals [91], or allogeneic myoblast sources from litter mates to limit rejection responses [179]. However, expanding human myoblasts to sufficient numbers for therapeutic benefit *in vitro* is nontrivial [59], so other cell types such as MSCs have been investigated [178]. While the paracrine effects of MSCs have been well characterized in other systems [224], the ability of these cells to differentiate *in situ* into skeletal muscle tissue has not been established, which is critical for the repair of large skeletal muscle defects. Establishing cell culture methods and techniques to facilitate the long-term *in vitro* expansion of myoblasts would increase the clinical feasibility of these cells. However, the ability of acellular scaffolds to repair and regenerate skeletal muscle tissue should not be overlooked when considering clinical therapy options. The positive results seen to date clinically suggest that acellular scaffolds can also improve force production outcomes [134]. Ultimately, tissue engineering solutions will likely include a combination of biomimetic scaffold materials to guide tissue growth along with a cell source to enhance the repair of large scale defects such as those presented in VML injuries.

5.0 CONCLUSIONS AND FUTURE DIRECTIONS

Tissue engineering scaffolds for VML injuries have advanced from passive materials implanted that provide structural support to inductive scaffolds that direct endogenous tissue regeneration. In addition to developing SMUs designed to replace lost skeletal muscle tissue *in vitro*, skeletal muscle constructs are a powerful model system to investigate biological questions regarding skeletal muscle formation and regeneration *ex vivo*. Novel strategies have been developed to incorporate essential growth factors and to direct skeletal muscle regeneration and these materials have expanded our knowledge on how VML injuries repair *in situ*. However, future scaffolds must support more efficient regeneration by enhancing SC recruitment (i.e. migration) to the defect site to recruit more progenitor cells since the matrix cues present in most conventional muscle regeneration studies are destroyed or missing in VML injuries (Figure 3C). Additional myoblasts may be required to supplement the endogenous regenerative process, to ensure complete functional regeneration. This would require novel cell culture techniques to preserve myoblast plasticity *in vitro*, and to enable these cells to differentiate and fuse with the existing musculature *in vivo*. Regardless of the source of the cells (endogenous or exogenous), future scaffolds must also direct the alignment of myoblasts within the defect site to facilitate seamless integration between healthy and regenerating tissue (Figure 3). Additionally, care must be taken to select a clinically relevant animal model to appropriately draw conclusions on effects of a given treatment on VML injuries. Finally, controlled structural and mechanical properties are necessary to ensure that scaffolds persist long enough to support organized functional skeletal muscle regeneration.

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DISCLOSURES

GDP discloses that he is a co-founder and has an equity interest in Vitathreads L.L.C., a company that has licensed intellectual property associated with fibrin microthreads.

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TABLE AND FIGURE LEGENDS

Table 1. A summary of the scaffold materials and cellular components used in skeletal muscle regeneration.

Table 2. A summary of the different animal models used to study skeletal muscle regeneration.

Figure 1. Schematic illustration showing the hierarchical arrangement of skeletal muscle tissue. Skeletal muscle tissue is a highly organized series of cable like structures, with the smallest functional unit referred to as a myofibril. Bundles of myofibrils form myofibers, which in turn are grouped together to form fascicles, which are bundled to form the muscle belly.

Figure 2. Schematic representation of native skeletal muscle regeneration. (A) SCs can be identified by their position between the sarcolemma and basal lamina of mature skeletal muscle. (B) Upon injury, SCs are recruited to the wound site and begin to proliferate. (C) These proliferating SCs will eventually fuse together to form immature myofibers, (D) which will fuse with the existing healthy myofibers, and will then mature by expressing more contractile proteins to regenerate functional tissue. In VML injuries, (E) the basal lamina is destroyed or removed, facilitating the migration of fibroblasts into the wound site. (F) In the absence of proper signaling cues, fibroblasts begin to deposit collagen, (G) ultimately leading to massive scar tissue formation.

Figure 3. Schematic representation of scaffold mediated repair of volumetric muscle loss (VML). (A) VML injuries span large portions of the muscle belly, and (B) can be repaired using bulk scaffolds to fill this void space. These scaffolds support tissue repair, but functional regeneration is limited by disorganized myofiber and scar tissue formation. (C) Scaffolds with precisely engineered topographic cues to direct aligned tissue growth, such as microthreads, will guide organized myofiber formation for more robust functional tissue regeneration.

Table 1. Tissue engineered scaffolds used in skeletal muscle regeneration

<u>Scaffold Material</u>	<u>Cellular Component</u>	<u>Functional Outcomes</u>	<u>Limitations</u>	<u>References</u>
Poly(glycolic acid) (PGA)	Rat myoblasts	Formation of multinucleated myotubes and some capillary	Insufficient vascularization for tissue formation	[133, 138, 158]
Poly-ε-caprolactone (PCL)	Human and mouse myoblasts	Protein coatings facilitated myoblast proliferation and fusion	Scaffold alone does not support myofiber growth	[109, 142]
Decellularized scaffolds	Acellular	Support cell infiltration, nascent myofiber formation, and functional recovery	Long incubation times required to observe functional recovery	[18, 28, 90, 134, 172-175, 177, 180]
Decellularized scaffolds	Rat myoblasts and human mesenchymal stem cells	Shorter time points to functional outcomes in acellular scaffolds	Not all cell types integrate into muscle defect. Cell sourcing may become a limiting factor	[16, 89, 171, 176, 178, 179, 181]
Minced skeletal muscle	Native tissue	Support functional recovery of injury	Does not support robust alignment of nascent myofibers	[182]
Alginate	Rat and mice myoblasts	Reduced wound size 1 month after injury, supporting enhanced angiogenesis	Scaffold did not replace damaged tissue	[183, 196, 199]
Alginate	Acellular	Support functional recovery and vascularization of nascent myofibers	Utilized ischemia-induced skeletal muscle injury. Scaffold did not replace damaged tissue	[93, 197]
Collagen	Rat and mice myoblasts	Scaffolds support cell growth and integration with host tissue	Disorganized muscle regeneration with little data collected on functional recovery	[113, 202-204]
Collagen	Acellular	Scaffolds support myoblast infiltration	Little information obtained from long term (> 1 month) implantation studies	[205, 206, 208, 209]
Fibrin	Rat and mice myoblasts	Scaffolds promote angiogenesis and myoblast survival, differentiation, and integration with host tissue	Scaffolds rapidly degrade and support disorganized muscle regeneration with limited data on functional recovery	[20, 130, 140, 214, 215]
Fibrin	Human myoblasts	Longitudinal topography of scaffolds facilitate myoblast integration with host tissue to stimulated aligned regeneration	Rapid scaffold degradation may attenuate muscle regeneration	[91]

Table 2. Animal models used in skeletal muscle regeneration

<u>Injury Model</u>	<u>Mechanism of Injury</u>	<u>Limitations</u>	<u>References</u>
Laceration	Laceration of the belly of the skeletal muscle and opposing stumps are sutured together	Small size of injury may encourage spontaneous regeneration	[183]
Ischemia	Ligation of artery that vascularizes the skeletal muscle	Muscle matrix remains intact after injury and may serve as a template for endogenous regeneration	[93, 197, 216]
Cardiotoxin	Support cell infiltration, nascent myofiber formation, and functional recovery	Long incubation times required to observe functional recovery	[196]
Crush induced	Blunt trauma to crush skeletal muscle without inducing any injury due to laceration	Muscle matrix remains intact and in place after injury and may serve as a template for endogenous regeneration	[222]
Volumetric muscle loss	Large amount of tissue is completely removed from skeletal muscle	Variability exists in the size, as well as location, of the defect, making direct comparisons between studies difficult	[89, 91, 172, 177, 178, 180, 223]

Treatment of volumetric muscle loss requires scaffolds to actively direct functional regeneration

Figure 1

Figure 2

Figure3

Treatment of volumetric muscle loss requires scaffolds to actively direct functional regeneration