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Mini Review: Biomaterials in Repair and Regeneration of Nerve in a Volumetric Muscle Loss

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Abstract

Volumetric muscle loss (VML) following a severe trauma or injury is beyond the intrinsic regenerative capacity of muscle tissues, and hence interventional therapy is required. Extensive muscle loss concomitant with damage to neuromuscular components overwhelms the muscles' remarkable regenerative capacity. The loss of nervous and vascular tissue leads to further damage and atrophy, so a combined treatment for neuromuscular junction (NMJ) along with the volumetric muscle regeneration is important. There have been immense advances in the field of tissue engineering for skeletal muscle tissue and peripheral nerve regeneration, but very few address the interdependence of the tissues and the need for combined therapies to repair and regenerate fully functional muscle tissue. This review addresses the problem and presents an overview of the biomaterials that have been studied for tissue engineering neuromuscular tissues associated with skeletal muscles.

Keywords

Neuromuscular junction; Nerve regeneration; Muscle regeneration; Tissue engineering

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I. Introduction

Explosion or blast trauma, bullet wounds, road traffic accidents, contusion injury (as in sports), or even debridement or removal of dead muscle tissue in compartment syndrome (CS) can cause penetrating soft tissue injuries leading to VML injuries, which leads to functional loss and cosmetic deformities[1–4]. VML can be defined as traumatic loss or surgical removal of the skeletal muscle with functional impairment and is often associated with neuromusculoskeletal damage [5–7]. Various neural components (Figure 1) such as peripheral and intramuscular nerves, NMJ, innervation to muscle fibers, and motoneurons-muscle fiber signaling can be damaged or lost from the muscle tissue due to VML[8,9]. Without prompt surgical intervention, profound consequences beyond the frank loss of muscle can occur. This includes extensive fibrosis, loss of muscle contractility, range of movement, strength, necrosis, amputation, and a marked loss of quality of life of the patient[5,10–12]. Moreover, traumatic bone fractures and osteosynthesis are the main contributors for soft tissue damage, including approximately 70% of peripheral nerve lesions[13]. Furthermore, vascular lesions due to musculoskeletal intervention operations affect up to 8% of cases and lead to 17.5% of traumatic nerve injuries. Long-distance nerve lesions and axon damage fail to regrow and reinnervate target muscles. Nerve defects longer than 8mm in size could not regenerate, motor functional recovery is virtually nonexistent, and necessitate a nerve transplantation[13]. If the regenerating axons do not reach the targeted muscle within a specific time period, neuromuscular and motor functions will certainly fail[14].

Compounding this problem, nearly 6 million fractures occur in the US annually with more than 3% consisting of open fractures, increasing the possibility of CS and VML[15]. Global MSK disorders (including VML and fractures) afflict >1.7 billion people worldwide, including >130 million Americans and up to $873.8B in medical care costs[16,17]. This will likely rise by 400% by 2050 as life-expectancy increases[18]. Thus, there is a great need to treat MSK, CS, and VML disorders to improve patient’s quality of life, reduce societal burden, and escalating healthcare costs.

Thus, repair and regeneration of tissues lost from VML injuries require activation, proliferation, and differentiation of a resident pool of stem cells known as satellite cells[19–21]. These cells are activated for myofiber regeneration following inflammation or hematoma formation following muscle injury. There is either maturation of regenerated muscle fibers with muscle function recovery or fibrous tissue formation and impaired muscle function in cases of severe trauma (VML)[12,20,22]. Fibrous tissue should be surgically debrided for regeneration of functional tissue, debridement further leads to loss of the muscle increasing the compartment volume (As seen in Figure 2 by Corona et al.) [12]. In such cases, vascularization and innervation are vital for functional muscle regeneration. Therefore, there is not only an urgent need for therapeutic modalities that promote satellite cells for muscle tissue regeneration, but also promote neuronal and endothelial cells for inducing peripheral nerve repair for functional tissue regeneration.

II. Pathophysiology associated with VML

The pathophysiology of sudden loss of muscle in VML injuries is highly ordered[22]. Primary order effects of VML are particularly challenging because skeletal muscle is incapable of extensive fiber regeneration after VML injuries[3,7]. Satellite cells and the basal lamina are the main components for effective muscle healing, that must remain intact[23–25], otherwise, VML leads to ablation of these critical components and the remaining musculature is unable to heal the damaged tissue. Improving the strength of the VML-injured muscle depends on: (1) number of remaining muscle fibers, (2) activation of survived fibers, and (3) promotion of new muscle fiber formation and density[22]. Furthermore, inflammation must be optimally resolved or regeneration deteriorates into fibrosis[26]. VML injuries result in exacerbation of the inflammatory phase of fracture healing[27], indicated by elevation in a cluster of markers (CD3+) lymphocytes and CD68+ macrophages in the fracture callus at 3 and 14 days post-injury in rats, respectively[28]. A common treatment for inflammatory response includes surgical debridement. Surgical debridement not only leads to increase in compartment volume (Figure 2 E), but also results in upregulation of inflammatory and fibrotic transcriptional pathways for at least one month after surgery in-vivo[12]. The current clinical care strategy for VML involves the use of free muscle transfer (i.e., muscle flaps) for bone coverage in animal models followed by extensive physical rehabilitation[22,29]. Yet, these procedures are not intended to restore muscle function clinically[30] and experience donor site morbidity and secondary surgery that adds to surgical complexity and cost[30].

While the general homeostasis and regenerative capacity of skeletal muscle depends on the presence of neural and vascular influence, the innervation of neuromuscular junctions depends on the sympathetic neurons which are of crucial importance for the integrity and function of nerve–muscle contact. A recent study presented the distribution and functions of sympathetic neurons in mouse skeletal muscle as shown in Figure 3. It is also controlled by various regulatory factors along with extracellular matrix protein synthesis and degradation[31–33]. VML can affect or damage the related peripheral nerves, intramuscular nerves and innervation at NMJs[8,29].The functional loss caused by VML can deteriorate over time due to the limited regenerative capacity of these large defects affecting muscle fiber and its neural components. This leads to the loss of the contractile proteins and the failure of excitation-contraction coupling, resulting in neuromuscular strength deficits[8].

The peripheral nerve injury causes cascade of physiologic events including Wallerian degeneration at the distal ends[34] and retrograde degeneration as well as initiation of the regenerative process at the proximal ends of the peripheral nerve[35]. The peripheral nerve can regenerate by itself to some extent, but in cases of severe injury (as expected in VML) there is a retraction of the axonal terminals and initiation of a significant inflammatory response which leads to either limiting or complicating (scar formation) the regenerative capacity of the nerves[35]. Chronic axotomy of the motoneurons, and denervation of the Schwann cells and muscle fibers can further affect the functional capacity of the muscle[8]. The denervation also activates protein degradation pathways that lead to muscle atrophy by exceeding the protein synthesis[9,31,36,37]. Due to the aggravated muscle atrophy

and complicated intrinsic regenerative capacity, surgical intervention or tissue engineering strategies become necessary for full recovery in these cases.

III. Molecular Pathways Associated with Neuromuscular Degeneration in VML

VML injury promotes degeneration and loss of function progressively after initial site injury due to acute tissue loss. Several molecular pathways are involved in the progression of muscle degeneration post VML injury. The degeneration of muscle tissue appears to have a similar pathophysiology as Duchene's Muscular Dystrophy. The reperfusion of vasculature initiates the downward cycle of muscle tissue degeneration owed to the accumulation of reactive oxygen species (ROS). Upon injury, initial ischemia develops within 4–8 hours of ischemic shock inducing irreversible neuromuscular damage. Return of oxygen upon reperfusion of muscle produces a burst of oxygen free radicals, nitric oxide species, and cellular and inflammatory infiltrate which causes swelling[38,39]. This can induce oxidative damage to the varied cell types making up the muscles being re-vascularized. Oxidative damage and regulation of oxidative free radicals play a central role in regenerating the injured muscle.

Neuromuscular degeneration following severe injury revolves around the perpetual downward cycle of inflammation, oxidative stress, fibrosis, and resultant interruption of the neuromuscular regeneration machinery. Several key markers such as transforming growth factor signaling (TGF), WNT (Wingless-related integration site) signaling pathway, nuclear factor erythroid 2-related factor 2 (NRF2), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), are interconnected in their role to regulate inflammation, fibrosis, and oxidative stress[40]. This comes down to an increase in WNT/TGF signaling that impedes myogenesis and neurogenesis. Wnt/β-catenin cascade activates TGFβ signaling which is a crucial step for proper muscle repair. TGF signaling pathway plays a molecular role in neuromuscular tissue degeneration and its expression level is a representation of various levels and stages of inflammation (Figure 4). TGFβ along with the Mitogen-activated protein kinase (MAPK) family can phosphorylate SMADs leading to activation of collagen transcription shown in Figure 4. Hence, the TGFβ and MAPK pathway lead to the synthesis of extracellular matrix and lead to fibrosis[41]. WNT signaling is one of the central signal pathways that is involved in the perpetuation of fibrosis[41].

Oxygen free radicals and inflammatory signaling (Figure 5) trigger the expression of NRF2 signaling to regulate the impact of oxidative stress[42]. This centers on improving mitochondrial function under these conditions. NRF2 has also been implicated in mitochondrial health when it triggers the PGC1-α pathway[42,43]. The keap1-NRF2 pathway (Figure 5) is critical for promoting antioxidant expression to mediate pro-oxidant metabolites while being a key anti-inflammatory signaling molecule after traumatic tissue injury. In the NMJ of skeletal muscle of wild type and aged rats, NRF2 deficiency[44] was associated with reduced mitochondrial oxygen consumption, increased mitochondrial ROS production, increased protein nitrosylation, cellular redox dysregulation, and reduced acetylcholine receptor expression. On the other hand, PGC1-α is a key receptor of several

myokines in myoblasts that play several roles in bone and neuronal tissue health and offer protection from oxidative damage. Furthermore, it regulates NRF2 transcription in a regulatory feedback loop and plays a central role on the expression of myokines needed to stimulate muscle and neuronal cell formation of NMJ. In addition, PGC1-α rescue overcame oxidative damage during VML injury and rescued the pathophysiology of the induction of persistent loss of muscle tissue[45].

IV. Current Strategies for Neuromuscular Repair and Regeneration Related to VML

There are several studies that focus on regeneration of VML, but very few investigated the NMJ and the peripheral nerve regeneration regarding muscle tissue. This section will focus on the current grafting and tissue engineering strategies for NMJ in VML and vascular tissue loss models. Autologous tissue grafts and physical therapy are the current gold standards for VML injury repair. A healthy muscle graft (i.e, Latissimus dorsi muscle or Gracilis muscle) is transplanted in clinical studies from the donor site with or without neurorrhaphy to the site of the injury to restore the lost muscle tissue[31,46]. Limited donor tissue, donor site morbidity, graft failure due to infection or necrosis, and need for multiple surgeries are the main limitations to the autologous tissue grafts[31,47,48].

For nerve injuries related to VML, skeletal muscle graft could work as a nerve conduit and enhance nerve tissue regeneration in short nerve gap injuries (sheep femoral nerve 5mm and rat sciatic nerve 2mm), and not for long nerve gap defects, as studied in-vivo[49–51]. Muscle transplantation with neurorrhaphy can have complications such as tubular collapse of the nerve, poor regeneration, and scar tissue proliferation. Several pre-clinical animal studies have used artery, vein, and tendon autografts for peripheral nerve regeneration which presented similar results to the muscle autografts[49,51–54]. Yet, nerve loss in VML is still a critical challenge clinically in functional muscle regeneration.

Physical therapy helps improve the function and strengthen the remaining muscle by releasing growth factors, modulating immune response, promoting vascularization, and reducing scar formation[31,53,55]. Manual as well as mechanical stimulation has been shown to induce peripheral nerve regeneration, improving the muscle fiber reinnervation along with better functionality in various studies[56–60]. Although, these studies should be very carefully planned and executed in a clinical setting to benefit the tissue rather than causing additional motor or sensory damage[56]. Also, exercise, physical therapy, and mechanical or manual stimulation may not be a viable option for certain severe injury cases and would need additional surgical manipulation for better tissue repair and regeneration.

V. Need for Tissue Engineering

Tissue engineering involves the utilization of scaffolds, cells, and bioactive molecules known as the tissue engineering triad for repair and regeneration of injured tissues. Several in-vitro studies showed that the myoblast survival, migration rate, and myogenic regeneration were greatly enhanced by the scaffold designing designing along with growth factor delivery[1,21,61]. Similar is the case for nerve regeneration, especially

in VML where the defect is large, in-vivo[49,62,63]. It has been proven that scaffolds mimicking the extracellular matrix environment and microarchitecture of the tissue lead to favorable results. The combination of appropriate stem cells along with growth factors in a microenvironment mimicking the extracellular matrix of a muscle will result in improved regeneration of skeletal muscle tissue. Thus, tissue engineering is needed for muscle tissue along with the affected neuromuscular tissue in a VML injury for a fully functional tissue regeneration. But the combination of these three different aspects together has yet to be completely explored. Clinical studies show neural innervation of the muscles and reestablishment of the NMJ are important aspects in regeneration of the muscular tissue. This not only avoids atrophy in the newly regenerated muscle tissue at later stages[9,31,64], but can also influence muscle fiber growth, alignment and type[65]. The extent of reinnervation of the NMJs have not been studied in a VML injury model after treatment, but Wu et al indicated a weaker force output in such regenerated tissues[37]. This can be attributed to the loss of neural function as well as increased fibrous connective tissue growth and failure of complete regeneration of the muscle tissue[37,66].

Recent studies in-vitro use co-culture of neurons and muscle cells to study the mechanism and interrelation of these tissue types for completely functional muscle tissue regeneration in a large sized defect[31,67–69]. We will focus on the tissue engineering triad regarding cells, and bioactive molecules along with biomaterials that have been studied for muscle tissue regeneration and are also common with or support neuronal growth.

VI. Cell and Molecular Approaches For Muscle Tissue Regeneration Supporting Neuronal Growth

The incorporation of stem cells have been an important aspect of tissue engineering and has been widely studied for muscle and nerve tissue regeneration. For example, treatment of Tibialis Anterior (TA) using cardiac stromal cells have ameliorated muscle fibrosis and promoted endogenous regeneration of dystrophin-deficient muscle in mdx mice via exosome stimulation of satellite stem cells, a model of Duchenne Muscular Dystrophy (DMD)[70,71] which is currently under clinical trials[72,73]. Other studies illustrate the essential role that satellite cells play in myogenesis[74]. Satellite cells have been very popular for skeletal muscle tissue regeneration, also for neuromuscular tissue, satellite cells and Schwann cells are believed to support neuronal growth. Transplantation of satellite cells have shown to promote myoblast survival and proliferation in-vivo, but clinical studies have not been that successful, as mentioned earlier, VML produces tissue loss greater than the regenerative potential of the native tissue, preventing functional restoration to the limb[31]. Mesenchymal stem cells (MSCs) are multipotent stromal cells with the ability to divide into different tissue types. It is believed to be a source of trophic mediators[75–77] and can modulate various tissue functions including musculoskeletal tissue[78,79] and peripheral nerves[75,80,81]. MSCs promote angiogenesis which enhances the healing by increased vascularization to the regeneration site. MSCs have a stimulatory effect on Schwann cell population by increasing the neurotrophic expression which in turn increases myelinization and axonal regeneration[82–84]. Furthermore, MSCs can reduce scar tissue formation, while adipose derived stem cells have been used for nerve tissue regeneration[85–87]. However,

mesenchymal stem cells have increased rejection due to culture time for high cell numbers, exposure to serum prions and peptides, low viability, and increased senescence[88].

Along with cellular approaches, the controlled release of growth factors along with the biomaterials has been explored and used in various tissue regenerative therapy for promoting faster growth. Vascular Endothelial Growth Factor (VEGF) has been proved to promote angiogenesis[61,89,90], while VEGF and Insulin like growth factor (IGF-1) have shown to enhance vascular tissue formation in ischemic muscle tissue enhancing muscle tissue regeneration[21,91,92]. VEGF has also been shown to promote axonal regeneration by upregulating expression of nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF)[89,93]. Basic fibroblast growth factor (bFGF) has shown an improvement in functional recovery and innervation of muscular tissue with minimum fibrotic tissue[94]. Several studies have shown that FGF increase in myelin axon, increased functional motor return, nerve amplitude and muscle action potential, and improved axonal regeneration in in-vivo models for up to 10mm of nerve repair[49,95–97]. Neurotrophic factors such as NGF and BDNF secreted from Schwann cells are naturally released in the nerve regeneration cascade[95,98]. NGF has shown to promote axonal regeneration by increasing the number of Schwann cells at the injured axon end, for improved nerve repair[49,99]. It has also been seen that improving the expression of neuromodulators like GDNF promotes angiogenic factors which improves muscular regeneration along with the NMJ restores[100].

Exogenous growth factors that target these conditions have severe side effects (ectopic bone formation, prolonged inflammation, and pain)[101] and poor efficacy (high dose to low cell density) clinically [102,103]. Finally, drug delivery (e.g., nitrates) or gene therapy vehicles (e.g., viral vectors) impede endothelial cell (EC) function owed to cytotoxicity, immune toxicity, nitrate tolerance, and altered gene expression[104–107]. Therefore, cellular approaches require added microenvironmental and structural support to improve the rate and quality of healed muscle. Various in-vitro and in-vivo studies show targeted markers including myokines[108]; such as the aminobutyric acid family (GABA, BAIBA)[109], interleukin family, neurturin[110], and Brain-Derived Neurotrophic Factor (BDNF), lipidomics of signaling lipid mediators[111]; including prostaglandins (PGs) and leukotrienes (LTs) families, and myogenic genes (especially MyoD, Myogenin, and MyH2) that play a critical role in muscle regeneration[112,113]. Neurturin is involved in motor nerve recruitment and NMJ remodeling, GABA is involved in metabolic regulation via brown adipose tissue markers, and BAIBA is a muscle-derived osteocyte survival factor also implicated in the reduction of insulin resistance and skeletal muscle inflammation. These myokines are thought to be released following activation of the PGC-1α receptor due to stressors, nutrient scarcity, degeneration due to age, injury, and disease and act via retrograde signaling[109,114,115].

VII. Decellularized ECM Approaches

Decellularized extracellular matrix (DECM) approaches (with or without bioprinting) have also been attempted in-vivo[26,31,116]. In a pig model of VML, DECM could not abate inflammatory signaling while fibrosis could not be averted[12]. Rodent VML studies have demonstrated virtually no Pax7 positive cells within the defect two weeks after

implantation of various biomaterials, pointing to material-induced fibrous encapsulation and/or inadequate chemo-traction as primary deficiencies in host satellite migration[26]. This was further demonstrated by autologous muscle flap paste mixed with DECM and fixated using fascia, leading to scarring in some aging animals[117]. Moreover, pre-fabricated scaffolds or decellularized transplant muscle modifications during surgery to fit the defect dimensions and use of silk sutures between biomaterial/ transplant and surrounding muscle[118] can add surgical time or micro-structural mismatch with surrounding tissues in mouse animal models. Further disadvantages include mechanical properties and chemical composition (e.g., degradation rate, stiffness/rigidity) that are generally harder to control in ECM treatments[119]. Thus, control over the properties of the micro- structure and bioactivity of therapies are needed to improve the healing process.

VIII. Biomaterials Used in VML and Peripheral Nerve Regeneration

These properties can be more precisely controlled in natural and synthetic biomaterials. However, disadvantages include challenges in cellular attachment, the potential degradation into byproducts that impede regeneration, and the potential formation of fibrous capsules due to an inflammatory response have occurred[119,120].

Collagen is the most abundant extracellular matrix protein found in many tissues and can promote cellular proliferation and tissue healing[27,49]. Purified collagen is FDA approved and is used for many applications. Collagen has shown to promote muscle cell survival and differentiation. Studies have shown myoblast differentiation to myofibers and integration to the tissues[31,121]. Collagen has also shown good properties for peripheral nerve regeneration and is used as a nerve conduit (commercially available as NeuroGen1) [49]. Kroehne et al showed that collagen scaffolds enhanced myotube differentiation and when implanted in an excised muscle in a mice model, the myotubes aligned and integrated to the host tissue following better healing along with electrical stimulation to the muscle[122]. Collagen scaffolds still has drawbacks as they are easily degradable and lack mechanical stiffness. Other natural biopolymers such as fibrin and chitosan have also shown capabilities to promote myoblastic activity[123,124]. Fibrin microthread scaffolds were shown to support the growth of myofibrils in healing of a VML in a mouse model as compared to a nanoporous fibrin gel[124]. Fibrin nerve conduits also show to increase the axonal regeneration due to its porous structure that allows the neurotropic factors to penetrate through it and also its degradability avoids nerve compression[86]. Myotube differentiation along with acting as a nerve conduit suggests that these materials can induce neuromuscular regeneration in a VML. Engineered gelatin hydrogels promote cell growth, form Glycosaminoglycan (GAG)-like structure, and are biodegradable[125–133], For instance, placing an osteoactivin doped gelatin (without cross-linker) hydrogel scaffold was used in rat VML, however, no effect was observed on neuromuscular regeneration[134]. This was likely owed to the need for modification to structurally support and promote rapid new muscle and vasculature.

As compared to natural biopolymer, synthetic biopolymers such as poly-L-lactic acid (PLLA), poly (lactic-co-glycolic acid) (PLGA), and poly-caprolactone (PCL)[135–140] can be modified to improve the mechanical properties, degradation rate and controlled release of

growth factors to induce neuromuscular and eventual myogenic regeneration. PLGA is FDA approved and has widely been used as suturing material for scaffolds. Studies have found out that PLGA improves cell filtration and promotes angiogenesis and hence a material of interest for VML injury cases[137,141–143]. Luis et al showed that PLGA proved to enhance peripheral nerve regeneration in-vitro but had limited capabilities when used in a large gap model (10mm) in a rat sciatic nerve[144]. PCL is an FDA approved biomaterial and has been explored for musculoskeletal tissue regeneration. PCL has been preferred for use in slow drug delivery applications and where more structural strength is needed because of its slow degradability rate[137,145–147]. To modify and control the degradability rate along with the mechanical properties, copolymers PCL along with PGA and PLA are widely used[148]. Electrospun PCL along with natural biopolymers like chitosan has been explored to improve the myoblast cell proliferation, differentiation, and alignment of cells to form myotubes in-vitro[137,149,150]. However, these synthetic polymers have poor bioactivity and cell affinity. These scaffolds can cause immune or foreign body response due to the polymer or result of its degradation products[31,151]. PCL and PLGA scaffolds are even fabricated using 3D printing or additive manufacturing techniques for different geometries which help modify the mechanical properties, porosity of the scaffold, degradation rates, and elasticity which can affect the cellular response to the surface in terms of bioactivity, biocompatibility, local cellular responses, immune or foreign body responses[137,152,153].

Carbon nanotubes & polypyrrole (PPy) have been widely explored for neural tissue regeneration due to their electroconductivity. These materials boosted axon regeneration and neuromuscular recovery for end to end nerve repair. The combination of nanotubes along with mesenchymal stem cells have been seen to enhance the expression of neuronal markers, and growth factors are important for neuronal growth[154,155]. Incorporation or doping of such electrically conducted material has shown to improve the properties of various biomaterials. For example, PPy along with poly-d,llactic acid (PDLLA) has shown to enhance nerve regeneration in a rat sciatic nerve in-vivo[156].

Further, biopolymer surface modification with single peptides lack multi-functionality to mimic ECM[157], have low half-lives and lack thermal stability[158], while mini-proteins limit angiogenesis via low VEGF activity, thereby limiting their use for tissue healing[159]. While therapies should attenuate fibrosis and inflammation for improving the regeneration of muscle[20,160], most of the above approaches have not adequately addressed this issue as an intrinsic property of the biomaterial or via adequate drug delivery.

IX. Conclusion and Future Perspectives

There are few studies which have combined different techniques to overcome the complex anatomy issue for the VML repair and regeneration. The study performed by Novakova et al mentions the use of tissue engineered skeletal muscle units to promote regeneration and better integration of tissues with the underlying muscle tissue in a VML using a sheep model[161]. In combination with the skeletal muscle units, they also use an engineered neural conduit for better bridging and innervation of the skeletal muscle units to the adjacent tissues. Sheep bone marrow stromal cells were used to create the engineered 3D grafts and conduits in-vitro and implanted in the sheep. The resultant led to proper linear alignment of

newly regenerated muscle tissue fibers with no necrotic core[161]. It is not well understood if the newly generated muscle tissues can withstand similar forces as normal.

With the complexity of the tissue, it is well understood now that a single technique or a material may not mimic the intrinsic cascade of events for the regeneration of a tissue as seen in Figure 6. The tissue engineering triad involving scaffolds, cells, and bioactive molecules has changed with understanding of various aspects. The effect and role of the composition of biomaterials and its biomechanics play a very important role and can enhance the regenerative capacity for a certain type of cell to grow along with the growth factors. Along with that the tissue engineering architecture also plays an important role and with the development of 3D printers, complex geometries controlling various factors inside it is now possible to attempt to mimic these structure-function relationships.

Thus, there is a vital need for a new approach for improving the outcome of VML defect healing by novel scaffold chemistry, architecture, and method of intra-defect treatment that will yield improved autologous healing rates and quality of regenerated muscle tissue along with related endothelial and neuronal growth. The therapies we propose here are clearly needed to promote functional muscle tissue regeneration, vascular and nerve regeneration, reduce inflammation, and shorten recovery time from VML[22]. Our group and others have consistently demonstrated muscle strength to be the primary determinant of functional capacity in humans. Thus, providing structural support and promoting autologous healing will lead to muscle regeneration, functional restoration, and greatly improve the outcomes of patients afflicted with VML.

Investigation into tissue engineered constructs for neuromuscular repair has increased in recent years due to the discovery of the interdependence between muscle and neuronal tissues. Various studies tried different techniques for co-culture of skeletal muscle and neuronal cells to achieve this. Cvetkovic et al. upgraded the 2 dimensional co-culture by building a modular cellular system made by multi-layered tissue rings which contained differentiated skeletal muscle myotubes mixed with ECM for the first layer and motor neuron aggregates mixed with ECM proteins in another layer making a 3D construct (Figure 7)[27]. This co-culture was amenable for both cell types. The use of 3D printing provided flexibility as well as better integration to the co-culture design which helped in functional NMJ behaviour which can lead to physical, mechanical as well as functional muscle tissue repair and regeneration[27]. One method currently under investigation for repair of VML injuries involves using preinnervated tissue-engineered muscle constructs[162]. These constructs were created by plating mouse myoblasts on a PCL aligned nanofiber sheet to form a myocyte layer, then plating dissociated motor neurons, harvested from the spinal cord of Sprague Dawley rat pups, on top of the myocyte layer to form a co-culture. Co-cultures were differentiated for 14 days and then implanted into a 20% VML defect in the Tibialis Anterior of athymic rats for one and three weeks. The co-cultured scaffolds resulted in a significant increase in muscle cross sectional area after 3 weeks compared to non-innervated aligned myocyte scaffolds. While this method is useful for determining the relationship between muscle and peripheral motor neurons, it lacks clinical relevance as it involves harvesting motor neurons from the host and the pre-cultured scaffolds cannot be quickly modified in response to unforeseen changes in spatial distribution during surgical

implantation. Additional Studies have been conducted over constructs composed of murine myoblasts seeded on an acellularized porcine bladder matrix and kept in a bioreactor for one week[163]. The constructs were then implanted into latissimus dorsi defects involving a 50% critical loss of the latissimus dorsi muscle in nude mice. This method had promising results for treating VML related injuries including increased myofiber formation, angiogenesis, and neurovascular bundle density two months after implantation. Despite these positive results, this method also involves the use of a bioreactor and the resultant scaffolds are not easily modifiable to match spatial requirements.

There has been a lot of ongoing research in tissue engineering for muscle regeneration, for not only the use of cells but also the advancement in biomaterials and growth factors needed to enhance the cellular proliferation and differentiation in a VML model. With these advancements, one needs to also address the question of regeneration of the NMJ along with the muscle tissue to better mimic the natural tissue function and maintain the tissue morphology by avoiding atrophy. Incorporation of hybrid or combination of biomaterials and growth factors that direct the growth and support the regeneration of these two different tissue types which are closely interrelated is very important. The ongoing research in 3D bioprinting of tissues, using a variety of biomaterials, stem cells, in situ bioprinting, and bioactive molecules, can be designed to replicate the natural cascade of growth of various tissue types.

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Highlights

- Volumetric muscle loss (VML) often leads to damage to neuromuscular components
- Regenerative capacity of skeletal muscle is under neurogenic & vascular influence
- Nerve loss still remains a critical challenge in functional muscle regeneration
- Need for studying the extent of reinnervation of neuromuscular components in large defects
- Tissue engineering use biomaterials to mimic structure-function relationship in VML

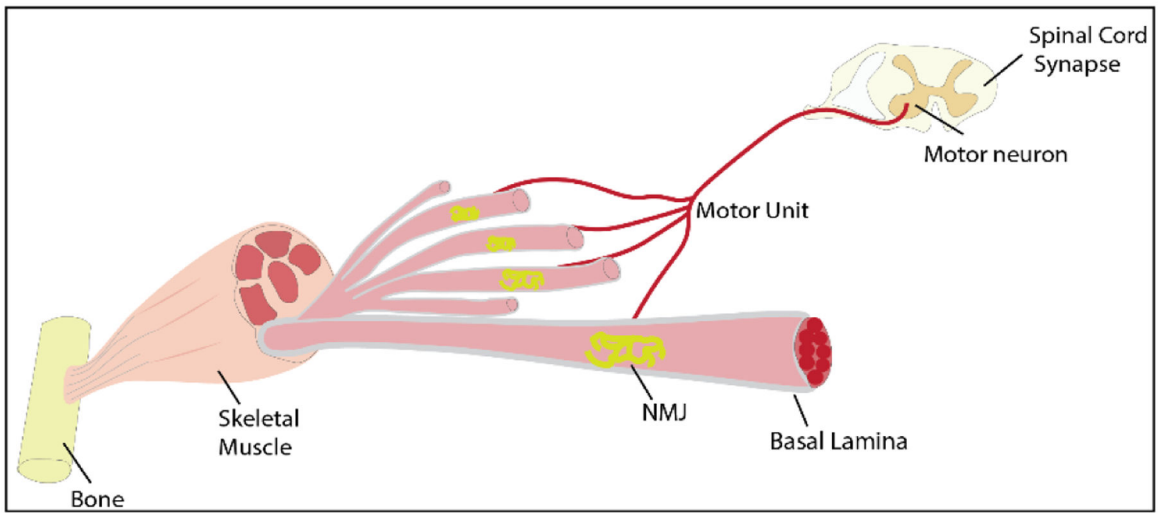

Figure 1:
Illustration of the Neuromuscular Junction in Skeletal Muscle

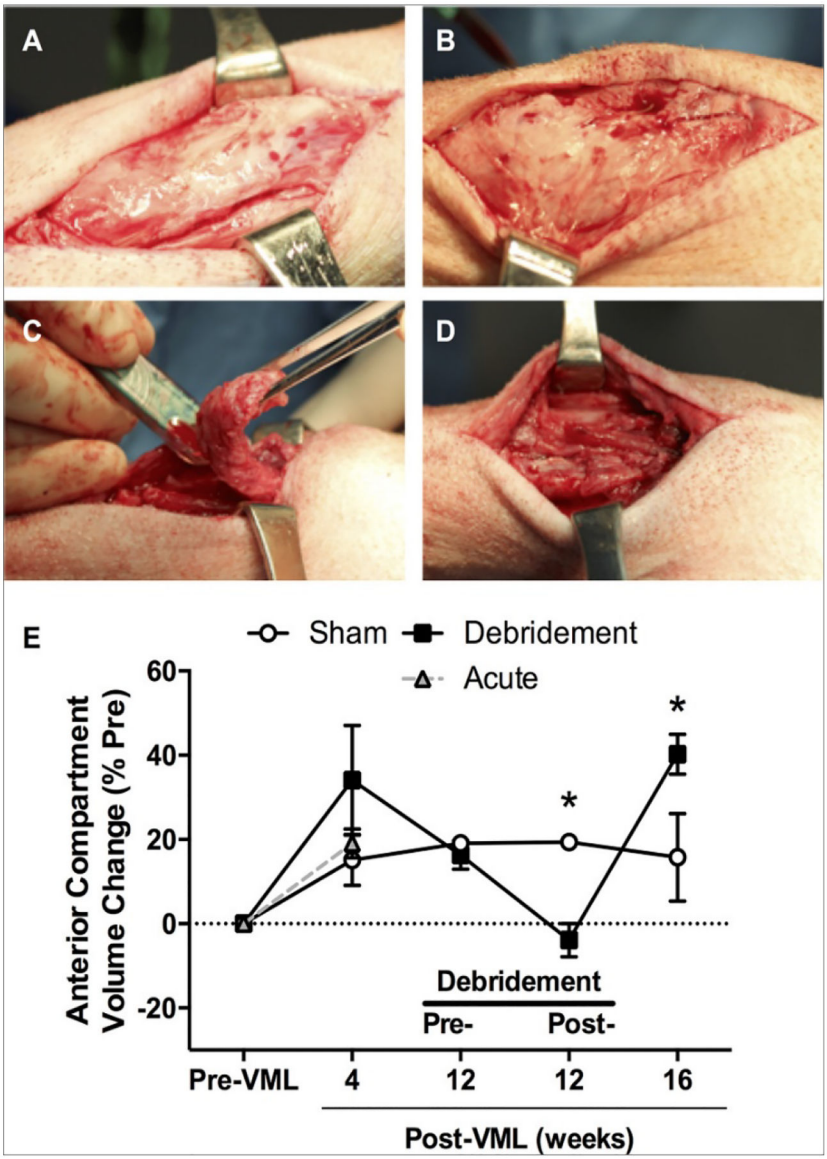

Figure 2:

Debridement induces increase in compartment volume. Peroneus tertius muscles underwent volumetric muscle loss (VML) injury. (**a** & **b**) Twelve weeks after VML injury, fibrous tissue enveloped the anterior compartment (**c** & **d**) at which time the overlaying fibrous tissue was surgically debrided. (**e**) Lower limb anterior compartment volume was measured using computed tomography imaging at the times specified. Values are means ± SE. Image taken from Corona, B.T., J.C. Rivera, and S.M. Greising, Inflammatory and Physiological Consequences of Debridement of Fibrous Tissue after Volumetric Muscle Loss Injury. Clinical and translational science, 2018. 11(2): p. 208–217.

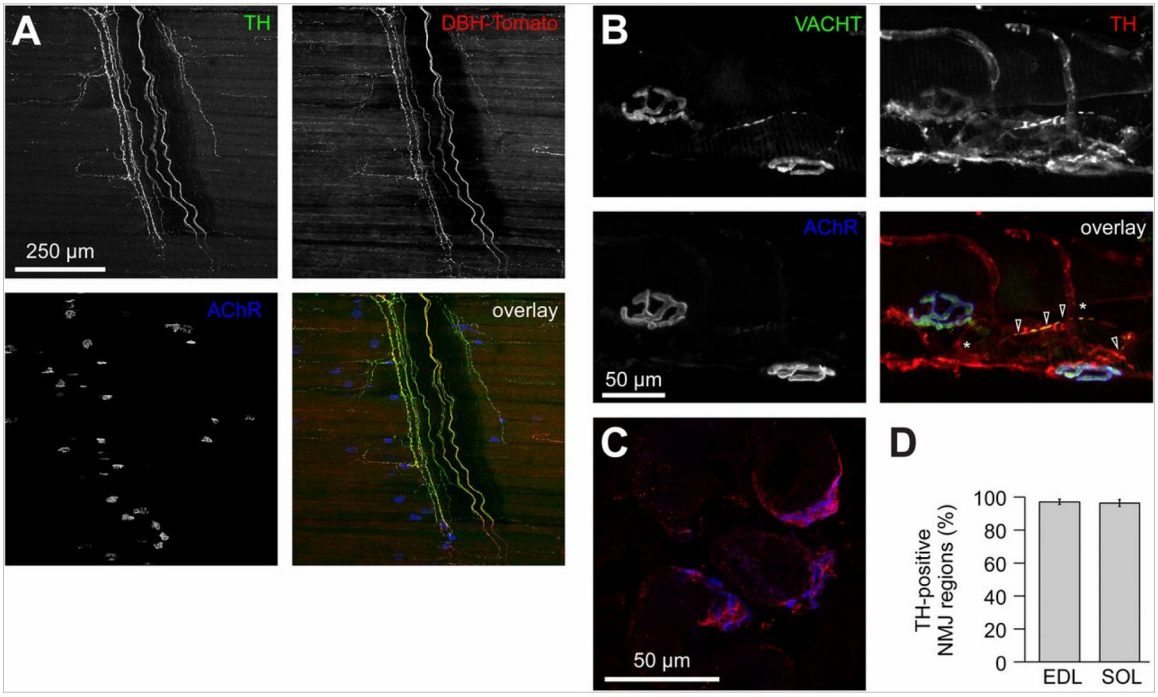

Figure 3:

Distribution of sympathetic neurons in skeletal muscle. (*A*) Diaphragm muscle of a DBH-Tomato mouse expressing Tomato protein in sympathetic neurons was co-stained with anti-TH antibody and BGT-AF647 (AChR). Signals from TH, Tomato, and BGT are depicted in the overlay in green, red, and blue, respectively. Three-dimensional maximum projection of a confocal z stack of a representative region is shown. All channels were brightness/contrast-enhanced. (*B*) Longitudinal sections of wild-type EDL muscles were labeled against VACHT, TH, and BGTAF647. Signals from these markers are depicted in the overlay in green, red, and blue, respectively. Three-dimensional maximum projection of a confocal z stack of a representative region is shown. All channels were brightness/contrast-enhanced. (*C* and *D*) EDL and soleus muscles were sectioned transversally, stained with BGT-AF555 (blue in overlay) and anti-TH antibody (red in overlay), and then imaged with confocal microscopy. (*C*) Representative confocal brightness/contrast-enhanced optical section from EDL. (*D*) Quantification of TH-positive NMJ regions from EDL and soleus (SOL) muscles. Mean ± SEM ($n = 4$ muscles each). Negative controls lacking primary antibodies showed 0.7 ± 0.7% (mean ± SEM, $n = 4$ muscles) in EDL and 0.0% (mean ± SEM, $n = 4$ muscles) in soleus of TH-positive NMJ regions. Image taken from Khan, M.M., et al., Sympathetic innervation controls homeostasis of neuromuscular junctions in health and disease. Proceedings of the National Academy of Sciences, 2016.

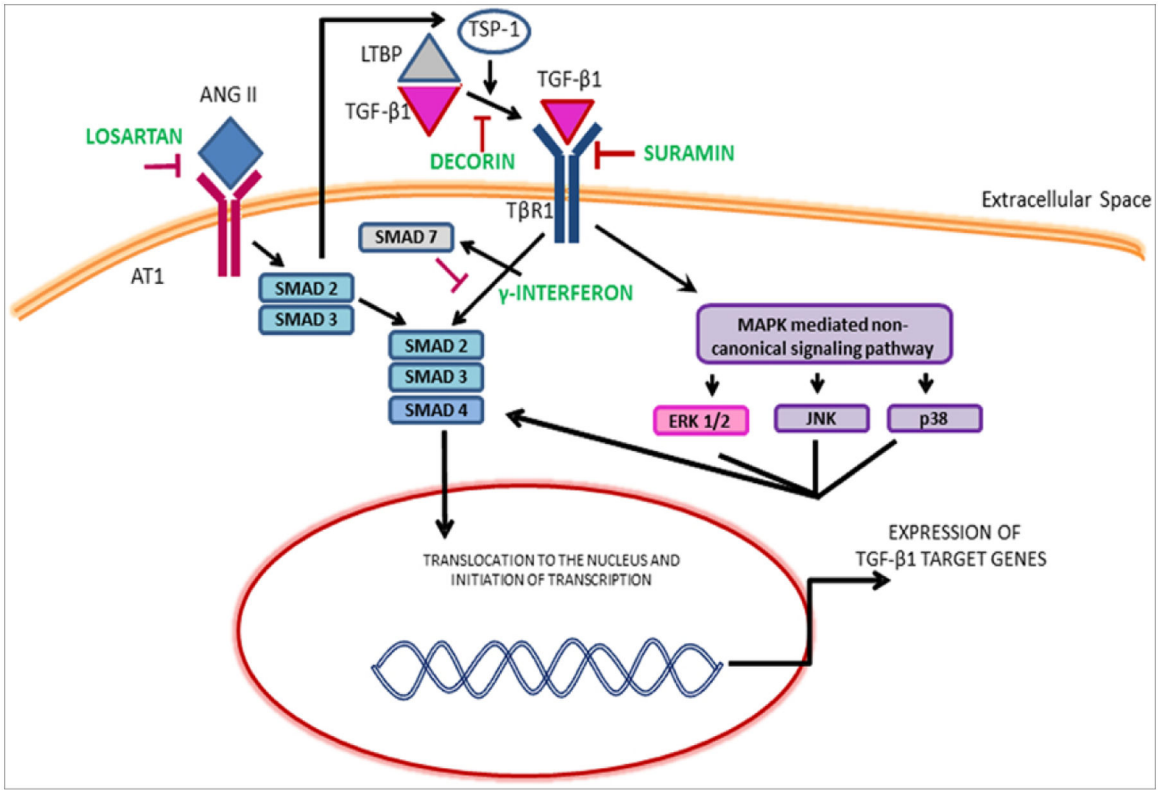

Figure 4.

(Garg, 2015): Illustration of the TGF-β1 signaling pathways and the mechanism of therapeutics. ERK, Extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; LTBP, Latent transforming growth factor binding proteins; MAPKs, Mitogen-activated protein kinase; TSP-1, Thrombospondin-1. Image taken from K. Garg, B.T. Corona, T.J. Walters, Therapeutic strategies for preventing skeletal muscle fibrosis after injury, Front. Pharmacol. 6 (2015) 87. https://doi.org/10.3389/fphar.2015.00087.

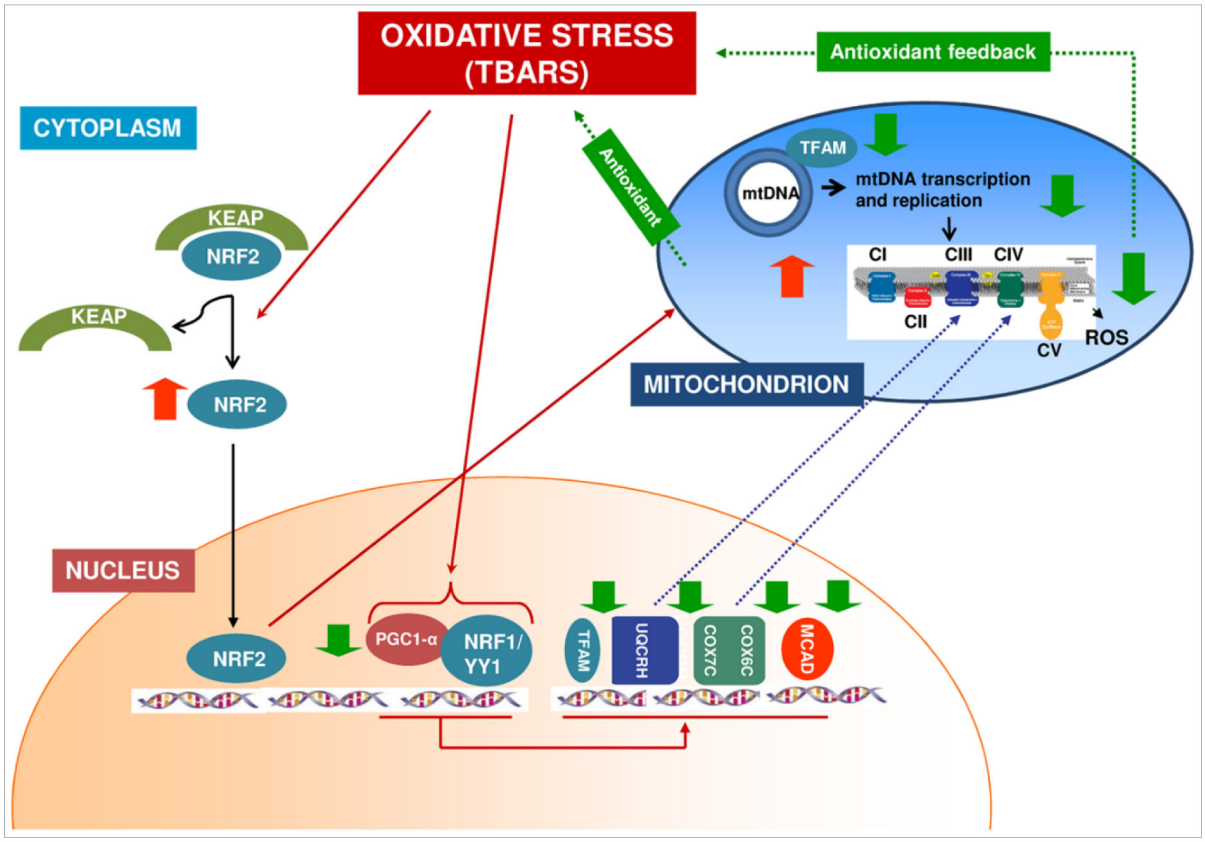

Figure 5:

Supposed mechanism of PGC1-α/NRF1-NRF2 pathway anti-oxidative stress cellular defense in chronic kidney disease patients in peritoneal dialysis treatment. Oxidative stress alters the interaction of Kelch-like ECH-associated protein 1 (Keap1) and Nuclear factor erythroid-derived 2-like 2 (Nrf2), thereby liberating Nrf2 activity from repression by Keap1. NRF2 migrates into the nucleus where it activates the transcription of Superoxide dismutase 2, mitochondrial (SOD2). At the same time, oxidative stress causes the down-regulation of Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1-α) and Nuclear respiratory factor-1 (NRF-1) with the consequent down-regulation of PGC-1α downstream target genes (TFAM, COX6C, COX7C, UQCRH and MCAD). The reduced TFAM expression causes a decrease in mitochondrial transcription and replication. The downregulation of all these factors suggests the decrease in mitochondrial OXPHOS activity to reduce ROS accumulation and creating an antioxidant feedback. Image taken from Zaza, G., et al., Downregulation of Nuclear-Encoded Genes of Oxidative Metabolism in Dialyzed Chronic Kidney Disease Patients. PLOS ONE, 2013. 8(10): p. e77847.

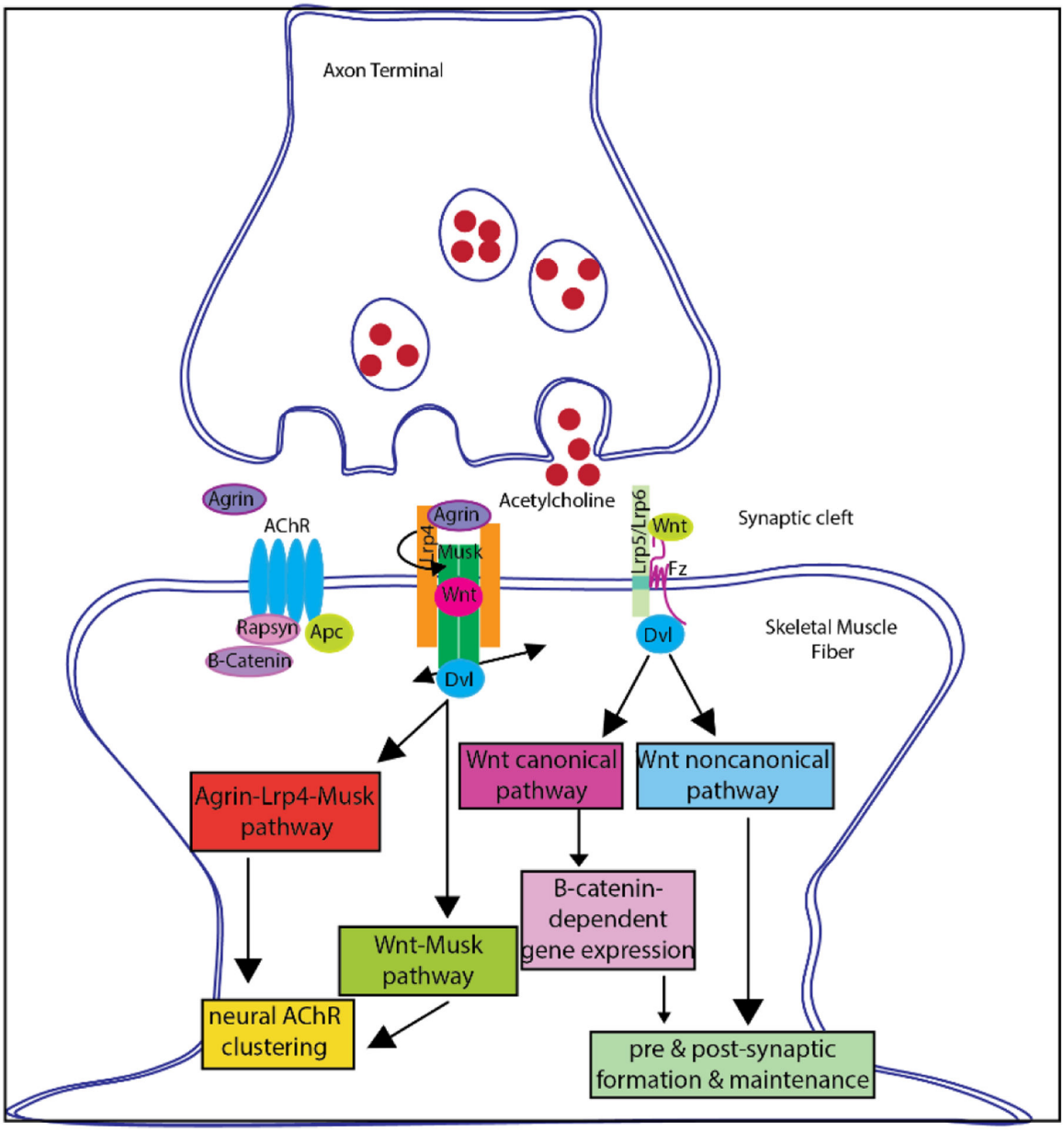

Figure 6:
Illustration of cell signaling cascades involved in Neuromuscular junction regeneration & remodeling

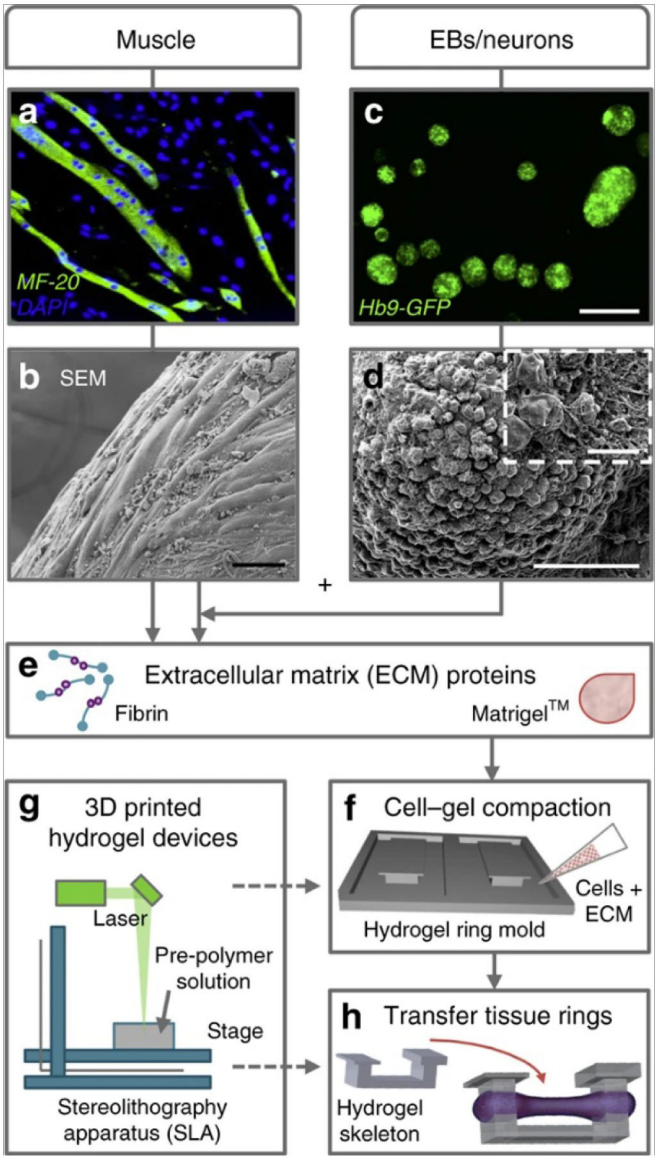

Figure 7:

Skeletal muscle cells and motor neurons were combined into a fabricated 3D co-culture system. C2C12 myoblasts were differentiated into multinucleated myotubes (**a**) and combined with extracellular matrix (ECM) proteins to create an engineered muscle ring tissue (**b**). In parallel, mouse embryonic stem cells (HBG3 mESCs) were differentiated into motor neurons (MNs) through the formation of embryoid bodies (EBs) (**c** and **d**) and then combined with the engineered muscle tissue and ECM proteins (**e**) on 3D-printed hydrogel devices (**f** and **g**). Once the multi-layered rings sequentially compacted and fused together, they were then placed on a stationary hydrogel skeleton (**h**). Scale bars, 50 μm (**b** and **d**), 500 μm (**c**), and 10 Î¼m (**d**, inset)[1]. Image taken from Cvetkovic, C., et al., A 3D-printed platform for modular neuromuscular motor units. Microsystems & Nanoengineering, 2017. 3(1): p. 17015.