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Exploring the role of biological factors in skeletal muscle tissue engineering

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Abstract
A number of conditions both congenital and acquired, such as volumetric muscle loss (VML) and muscular dystrophy can be treated through skeletal muscle tissue engineering. To optimize regeneration in skeletal muscle tissue constructs, the appropriate biological factors with properties such as increasing differentiation and proliferation, aiding in satellite cell activation, improving cell survival and inducing angiogenesis should be used in conjunction with taking into consideration and manipulating the effects that certain systems such as the immune system and the gut microbiome have on skeletal muscle regeneration. These biological factors include IGF-1, VEGF, HGF, myostatin inhibitors, PDGF-B, FGF-2 (also known as bFGF), insulin, and androgens. In skeletal muscle tissue engineering, these biological factors stimulate the utilized cell sources (myoblasts and satellite cells) and injured skeletal muscle tissue to promote regeneration. The purpose of this review is to discuss the specific effects of these biological factors on the utilized cells and their usage in skeletal muscle tissue engineering. The effects that the aforementioned systems have on skeletal muscle regeneration and potential methods to manipulate these systems for improved regeneration will also be discussed. The paper will also highlight the challenges associated with the use of these biological factors. The use of bacteriophages to modulate the levels of gut bacteria that influence muscle regeneration, as well as incorporating polarized M2 macrophages into *in vitro* tissue constructs to blunt host immunosurveillance mechanisms are proposed, which do not yet appear in the scientific literature.

Keywords
Tissue engineering, Biological factors, Skeletal muscle, Myoblasts, Satellite cells, Volumetric muscle loss, Muscular dystrophy, Proliferation, Differentiation

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1. Introduction

Skeletal muscles make up ~ 45% of the mass of an adult human body and are essential for body movement (1). Muscular dystrophy is a type of muscle disease (myopathy) that leads to progressive loss of muscle mass and impairs muscle strength, and its global prevalence is estimated to be 3.6 per 100000 people (2). Despite the molecular origins of Duchenne muscular dystrophy (DMD), which is the most common type of muscular dystrophy, being known for years there is currently exists no curative treatment. The main treatment shown to be effective in slowing down the progression of muscular dystrophy and increasing the lifespan of patients is the use of corticosteroids (5). Another muscle disease is volumetric muscle loss (VML), defined as the loss of skeletal muscle tissue, which results in functional impairment in the body. Common reasons for VML are physical trauma and surgeries (e.g., aggressive tumor ablation) (1,3,4). In addition to VML and muscular dystrophy, prolonged denervation of muscle is another clinical situation that leads to a significant loss of muscle tissue. Currently, the most utilized conventional method of treating the loss of muscle tissue is the transplantation of muscle from elsewhere in the patient's body to replace the muscle lost (4). Even though it is a widespread surgical procedure, transplantation of muscle from elsewhere in the body is related to complications such as loss of function and volume of muscle in the donor site of the body (1). Therefore, there is an urgent need to develop effective therapies to treat these diseases. A potential method to treat muscular dystrophy is through skeletal muscle tissue engineering (4).

Skeletal muscle tissue engineering can provide clinical substitutes for transplantation of muscle in lieu of transplanting muscle taken from from elsewhere in the body (1,4). Skeletal muscle tissue engineering includes the combined application of engineering, cell transplantation and materials science to create substitutes for skeletal muscle transplantation. In this way, the implantable matrix or scaffold can restore and maintain the function of the tissue it was created to mimic (6). In this field, it is important for new scaffolds and cells to mimic the properties of natural skeletal muscle tissue. In addition, biological factors are essential to create successful bioengineered skeletal muscle tissues. Thus, the use of biological factors helps to control cell behavior that is important to produce a functional tissue as part of a successful tissue engineering strategy. Biological factors are signaling molecules that play crucial roles in stem cell proliferation and differentiation into skeletal muscle (7). In particular, biological growth factors (e.g., IGF-1, VEGF) are commonly utilized in skeletal muscle tissue engineering (7,8). The importance of VEGF in tissue engineering is due to its capability for inducing angiogenesis while IGF-1 is important because it promotes proliferation in a multitude of cell types (7,8). In addition to these biological factors certain systems in the body such as the gut microbiome also play a role in skeletal muscle regeneration (9). This review paper will discuss the role of skeletal muscle tissue engineering for treatment of skeletal muscle damage or loss. It will also explore the recent advancements in the usage of biological factors in skeletal muscle tissue engineering, highlight the systems that affect skeletal muscle regeneration and discuss how they can be manipulated to improve regeneration following transplantation of skeletal muscle tissue constructs, scaffolds or matrices.

2. Skeletal muscle tissue

2.1. Overview of skeletal muscle physiology

There are over 600 skeletal muscles in the human body which are connected to the bones via tendons. These muscles enable locomotion in the body through contraction and relaxation (1). This locomotion is regulated by muscle fibers that are located in each muscle and are formed from undifferentiated myoblasts (muscle stem cells) fusing with each other into long, cylindrical structures called myotubes. Figure 1 shows the structure of skeletal muscle tissue. The most important organelles constituting the muscle fibers are myofibrils which consist of actin and myosin filaments in repeating units called sarcomeres. When the muscle contracts, actin filaments are pulled towards myosin shortening the sarcomere and generating contractile force (1). Additionally, each individual muscle fiber is coated by a layer consisting of several proteins such as collagen, laminin, and fibronectin called the basal lamina (10). Laminins and collagen in the basal lamina provide structure and strength to muscle fibers while simultaneously participating in cellular signaling (11). Another important role of the basal lamina is in accommodating satellite cells that are wedged between the basal lamina and the muscle fiber (11,12). Satellite cells are the inactive forms of myoblasts and they play an important role in muscle regeneration (12). Physiologically, the muscle fibers inside the native skeletal muscle tissue are aligned with each other to generate force. The engineered skeletal muscle tissue also needs to share these properties and focus on the alignment of muscle fibers (1,10).

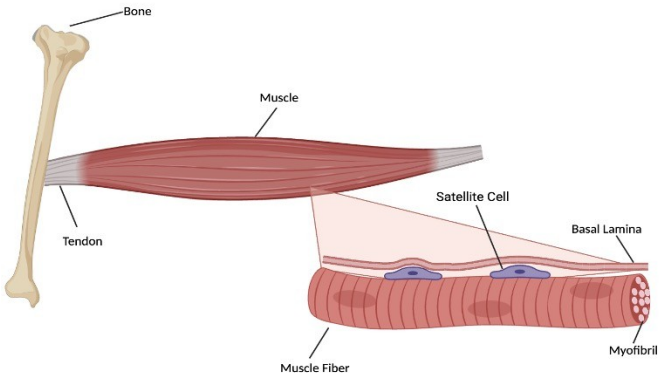

Figure 1: Structure of skeletal muscle tissue. This figure was created using *BioRender.com*.

The architecture of the cells and organelles constituting skeletal muscle endow it with mechanical and biological properties that enable the generation of a force necessary to produce a movement. These mechanical properties allow skeletal muscle tissue to withstand dynamic loading (10). Additionally, vascularization is an important biological property that enables skeletal muscle to perform ATP generating metabolism; thus, skeletal muscle tissue is highly vascularized to meet its high energy requirements (13). Capillary networks in skeletal muscle also have a high density of 600 capillaries per mm of muscle tissue and run parallel to muscle fibers which enable optimized nutrient and oxygen delivery to the muscle fibers (13). Overall, mimicking these properties is important to design implantable

and functional skeletal muscle tissue constructs, scaffolds or matrices.

2.2. Current treatments in skeletal muscle injuries/damage/loss

The most significant diseases/conditions that can be treated through skeletal muscle tissue engineering are VML and muscular dystrophy. As previously mentioned, there is no curative treatment for muscular dystrophy and all current treatments aim to prolong the lifespans of patients and maintain their quality of life. The main therapeutic drugs for DMD are the corticosteroids, namely prednisone or deflazacort. Administration of prednisone at a daily dose of 0.75 mg/kg of body weight or deflazacort at a daily dose of 0.9 mg/kg of body weight has been shown to be effective in slowing down the progression of DMD (5,14). These corticosteroids are often combined with frequent pulmonary, cardiac and orthopedic monitoring, assisted ventilation devices, physical therapy and specialized diets. The combination of these approaches alleviates some of the side effects of corticosteroid usage while also increasing the lifespan and quality of life of patients (14). On the other hand, the standard treatment for VML involves replacing the damaged or lost muscle with well vascularized and innervated healthy muscle tissue from elsewhere in the body. The procedure of choice for this is a muscle flap where a muscle is transferred with its neurovascular supply remaining intact. For this, either a free functional muscle transfer (FFMT) or traditional muscle flap is used with FFMT being utilized more as it allows for transfer of muscle from anywhere in the body compared to traditional muscle flaps which have a limited transferrable range. FFMT differs from traditional muscle flaps since in this procedure, the veins, arteries and nerves are cut and later sewn back together at the transplant site. This procedure has limitations such as operative times of 4-6 hours (compared to 1-2 hours for traditional muscle flaps), donor site morbidity and extended rehabilitation times. However, the main limitation of autologous muscle transplantation is that it can rarely restore premorbid muscle function (15). Due to these limitations, there is an urgent need to develop alternative therapies to restore skeletal tissue architecture and function.

3. Skeletal muscle tissue engineering

Several criteria need to be fulfilled to create a successful scaffold for skeletal muscle tissue engineering purposes. The artificial extracellular matrix (ECM) created must enable the infiltration of cells into the scaffold and additionally allow nutrient and oxygen transfer into the tissue. To achieve this the scaffold created must be porous with pores that are sized between 20 and 100 μm (10). Additionally, due to the dynamic loading that skeletal muscle tissue must withstand, engineered skeletal muscle should have similar mechanical properties such as stiffness. Stiffness values of 30-50 kPa, in addition to giving scaffolds the ability to bear dynamic loads, also aid the proliferation and differentiation of muscle cells (16). When considering the material to be utilized in skeletal muscle tissue engineering, piezoelectric materials that can convert mechanical energy to electrical energy should also be considered. These materials generally have adequate mechanical properties to support skeletal muscle function in addition to generating electrical signals due to the deformation that occurs in the material after movement such as muscle contraction and relaxation (17,18). These electrical signals can support the differentiation of muscular progenitor cells as shown by the increase in gene expression for factors that control

myogenic differentiation in myogenic cells grown in poly-3-hydroxybutyrate/poly-β-alanine (P3HB/PBA) scaffolds with similar effects being observed in other piezoelectric materials such as polyvinylidene fluoride (PVDF) (17,19). In addition to the material utilized, tissue engineered skeletal muscle should be highly vascularized in a manner that is similar to native muscle tissue so that the energy and oxygen needs of the muscle cells can be met. Additionally, tissue engineered constructs should be capable of innervation after *in vivo* transplantation, which is essential to prevent muscular atrophy (20). To create tissue engineered skeletal muscle, a biopsy is taken from the patient, stem cells are isolated and replicated *in vitro*. These stem cells are then seeded onto a scaffold which ideally fulfills these essential criteria for properties such as porosity and stiffness while also incorporating biological factors (Figure 2). The seeded scaffold should then be preconditioned for the native environment of muscle tissue through methods such as hypoxic preconditioning that can increase vascularization, or mechanical preconditioning that can accelerate the organization and maturation of tissue (21,22).

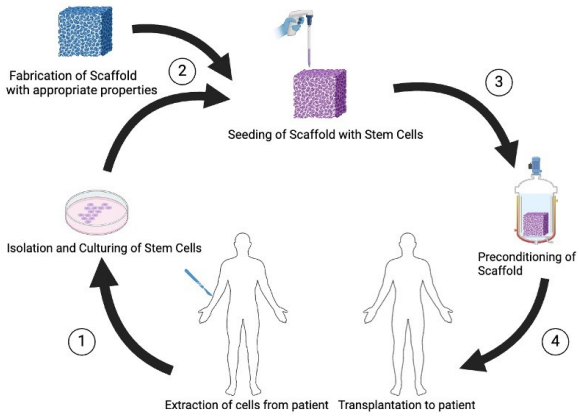

Figure 2. The conventional process of creating tissue engineered skeletal muscle. This figure was created with *BioRender.com*.

An alternative to conventional scaffolds utilizing tissue engineering is self-assembling cell sheet engineering. In this method cells secrete their own extracellular matrix (ECM) and assemble into a 3D tissue instead of being seeded onto a hydrogel or scaffold . The most prominent technique in cell sheet engineering of muscle tissue involves the usage of a thermoresponsive polymer such as poly(N-isopropylacrylamide) to generate self-organized muscle sheets which can then be detached from the substrate by decreasing the temperature and then be assembled into multi-layered tissue sheets (23) . Other techniques to harvest intact cell sheets, such as manipulating surface charge or biochemistry have been developed. A trigger-free technique that relies on cells' capability to weaken their adhesion forces to a culture surface through cell-cell and cell-ECM interactions has been developed to enable cell-sheet harvesting through a scraping action (24). These self-organized models offer the advantage of already being encased in cell-secreted ECM, allowing circumvention of commercially available ECM proteins, which tend to produce inconsistent results. However, this method

has limitations, such as a longer formation time and the inability to automate production (23).

4. Common stem cell types used in skeletal muscle tissue engineering

Multiple stem cells types can used in skeletal muscle tissue engineering. Among the stem cell lineages that have myogenic potential are embryonic stem cells (ESCs), induced Pluripotent Stem Cells (iPSCs), and mesoangioblasts (12). Besides the cells that are known to have high myogenic potential, cell lineages such as mesenchymal stem cells (MSCs) promote angiogenesis, provide anti-apoptotic effects and regulate myoblast cell cycle withdrawal. These effects are tightly controlled through the secretion of paracrine factors such as VEGF, MMPs, IGF-1, HGF and FGF-2 (25). In addition, the usage of cells such as adipose-derived stromal vascular fraction (SVF) cells in conjunction with myogenic cells such as myoblasts have been shown to create tissue engineered skeletal muscle with superior vascularization and innervation potential (20). Similarly, the integration of neural stem cells into bioprinted skeletal muscle tissue constructs at a ratio of 300:1 (muscle precursor cells:neural stem cells) was shown to promote neuronal differentiation and neuromuscular junction formation (26). However, the most important stem cells for skeletal muscle tissue engineering are satellite cells and myoblasts. Myoblasts are usually formed through the activation of satellite cells. Satellite cells are normally quiescent; however, in response to damage they are activated and turn into myoblasts, and then start proliferating through a combination of asymmetrical and symmetrical divisions (12). Due to the widespread usage and efficiency of myoblasts and satellite cells in skeletal muscle tissue engineering, the focus of this paper will be on these cell types.

5. Role of biological factors in skeletal muscle tissue engineering

Biological factors play an important role in controlling cell behavior. They control and influence cell activities such as migration, proliferation and differentiation. Through the proper application of these biological factors, the proliferation and differentiation of stem cells around the defect area can be enhanced, which contributes to the improvement of tissue regeneration (7,8). Some biological factors such as VEGF also have angiogenic effects and can be used to promote angiogenesis in tissue engineering constructs (7). Table 1 indicates the list of biological factors and their effects on the cells.

Table 1. List of biological factors that have potential to be used in skeletal muscle tissue engineering and their effects.

Biological factor	Effects
VEGF	Promotes angiogenesis, protects myoblasts from apoptosis and promotes proliferation and differentiation.
IGF-1	Protects myoblasts from apoptosis and promotes proliferation and differentiation of myoblasts
HGF	Activates quiescent satellite cells, promotes proliferation and inhibits differentiation at low doses, inhibits proliferation and promotes differentiation at high doses.
Myostatin İnhibitors	Activates satellite cells and promotes proliferation and differentiation of myoblasts.
PDGF-B	Promotes proliferation of myoblasts and shown to promote angiogenesis together with FGF-2
FGF-2	Promotes proliferation of myoblasts and reduces apoptosis. Promote angiogenesis together with PDGF-B
Androgens	Promotes proliferation and differentiation of myoblasts
Insulin	Promotes formation of skeletal muscle tissue

5.1 Vascular Endothelial Growth Factor (VEGF)

VEGF is widely known to be an important factor for the stimulation of angiogenesis and has been demonstrated to induce the formation of large vascular networks *in vivo* which could not be formed in its absence (27). In addition to its angiogenic potential VEGF also plays an important role in protecting differentiating myoblasts from apoptosis. While myoblasts are forming muscle tissue *in vitro* some myoblasts undergo apoptosis while others continue the process of differentiation into myotubes (28). VEGF was able to protect C2C12 myoblasts from apoptosis during myogenesis. Inhibition of VEGF signaling through receptor antagonists increased myoblast death rate by 3.5 times. VEGF was also demonstrated to be able to increase myoblast migration by 5 times (28). In addition to these effects a subtype of VEGF called VEGF-B significantly promoted the proliferation of C2C12 myoblasts by increasing the expression of cyclin D1 and PCNA (29). VEGF-B also stimulated the differentiation of myoblasts into skeletal muscle *in vitro* by increasing the expression of the genes such as MyoG and MyoHC that are characteristic of skeletal muscle cell differentiation through the PI3K/Akt pathway (29). The PI3K/Akt pathway, when activated, prevents apoptosis, aids in cell cycle progression and promotes proliferation. However, activating this pathway may promote carcinogenesis (30). However, overall, VEGF promotes angiogenesis, proliferation and differentiation while also protecting myoblasts from apoptosis.

5.2 Insulin and Insulin Like Growth Factor-1 (IGF-1)

Similarly to VEGF, IGF-1 also has the ability to protect cells from apoptosis. IGF-1 secreted from adipose stem cells (ASCs) protected C2C12 myoblasts from myostatin induced apoptosis, with IGF-1 being responsible for most (82.5%) of the antiapoptotic effects on myoblasts cultured *in vitro* (31). When applied to C2C12 myoblasts using a nanoclay prolonged release mechanism IGF-1 increased the thickness and number of myotubes and also increased the number of viable cells in a dose dependent manner, thereby demonstrating that it led to increased proliferation of the myoblasts (32). Separately, IGF-1 was also shown to be able to increase myoD expression in C2C12 myoblasts treated with myostatin suggesting that it aided in myoblast differentiation (31). Similarly, an increase in the differentiation of non-myostatin treated myoblasts was also observed in another study (32). The product of IGF-1, Insulin, also promoted regeneration of skeletal muscle. In a study using rats with scalding injuries insulin was shown to increase the number of nuclei expressing myoG in the short term in both non-injured and injured rats' skeletal muscle and was also shown to increase the number of nuclei expressing myoD in the long-term suggesting that, similarly to IGF-1, insulin supports myogenesis (33). Overall, IGF-1 can protect myoblasts from apoptosis while also promoting differentiation and proliferation and insulin can promote the formation of skeletal muscle tissue.

5.3 Hepatocyte Growth Factor (HGF)

Hepatocyte growth factor (HGF) is a growth factor that is believed to play a role in satellite cell activation. In native muscle tissue, it is present in the ECM in an inactive form and is cleaved and activated when injury occurs (34). Exogenous administration of HGF at a dose of 50 ng to injured rats increased the number of myoblasts by ~ threefold showing that it does indeed play a

role in the activation of satellite cells, however, multiple injections of HGF over multiple days also inhibited the differentiation of these myoblasts (35). In a different study, a lower dose of HGF applied to C2C12 myoblasts and human myoblasts at 2 ng/mL increased the number of cells, showing that it promoted the proliferation of myoblasts while leading to a decrease in the number of myoblasts that were committing to differentiation and fusing with each other in both cell types. Conversely, in the same study, a concentration of 10 ng/mL of HGF decreased cell number suggesting that it inhibited proliferation however it increased the expression percentage of the myoHC and myoD genes which led to increased fusion in both cell types, showing that it promoted differentiation. It was suggested that the dose-dependent nature of HGF's effects might be due to c-Met receptor expression levels (34). Additionally, it was shown HGF, when used together with IGF-1 on C2C12 myoblasts and rat MSCs being co-cultured in 3D fibrin-collagen gel matrices, was able to increase the expression of myHC2 in a manner that was statistically significant after 2 days suggesting that it promoted differentiation of the cells in skeletal muscle. Other myogenic markers such as ACTN2 were also analyzed; however, no statistically significant conclusions were reached due to high standard deviations (36). Overall, HGF exercises dose dependent effects on differentiation and proliferation while also playing a role in the activation of quiescent satellite cells.

5.4 Myostatin inhibitors

Myostatin is a growth factor that belongs to the TGF-β (Transforming Growth Factor-β) family of growth factors. The application of myostatin to human myoblasts *in vitro* activated the Smad3 pathway resulting in the decrease of myoD expression, which, in turn, led to an inhibition of myoblast differentiation (37). In addition to activating Smad3, myostatin also increased the expression of a cyclin dependent kinase-2 (CDK2) inhibitor called p21 while simultaneously decreasing the expression of cdk2. These effects resulted in cell cycle arrest at the G1 checkpoint and thus inhibited the proliferation of myoblasts (37). Due to these detrimental effects of myostatin, factors that inhibit its activity such as Cripto (a regulator of early embryogenesis) and the anti-myostatin antibody, RK35, present promise in promoting the proliferation and differentiation of myoblasts. When mdx mice (which do not express dystrophin) were injected with a hyaluronic acid-porcine skeletal muscle ECM hydrogel combined with RK35, a localized increase in muscle weight, muscle cross-sectional area and emyHC (embryonic myosin heavy chain a key marker for skeletal muscle regeneration) expression occurred (38). Additionally, the injection of the hydrogel with RK35 served to increase the amount of Foxp3+ T cells which triggered an immune response that promoted myogenesis (38). Taken together, these responses suggest that this method of RK35 application promotes muscle regeneration. In a separate study, the application of exogenous Cripto to isolated myofibers was found to reduce the number of Pax7+/MyoD- quiescent satellite cells after 48 hours suggesting that Cripto played a role in satellite cell activation (39). After 72 hours Cripto also increased the amount of Pax7-/MyoD+ cells that were committed to differentiation showing that in addition to activating satellite cells, Cripto also promoted the differentiation of those activated cells (39). In a different study, the administration of Cripto protein encapsulated in polyethylene glycol-fibrinogen hydrogel

spheres to mice whose tibialis anterior muscle was injured using cardiotoxin, resulted in the greatest increase in area percentage of emyHC expressing cells and was able to increase the size of myofibers in addition to increasing the number of myofibers possessing central nuclei; thereby suggesting that Cripto also promoted the regeneration of muscle *in vivo* (40). Overall, myostatin inhibitors activate satellite cells while also playing a role in promoting differentiation and proliferation.

5.5 Platelet Derived Growth Factor-B (PDGF-B)

PDGF-B (platelet derived growth factor-B) was recently shown to be secreted from myotubes and myoblasts (41). When primary myoblasts were exposed to exogenous PDGF-BB for 6 days, the number of myoblasts was greater compared to the control group, suggesting that PDGF-B increased the proliferation of myoblasts (41). In the same study, it was also demonstrated that PDGF-B increased the diameter of myotubes, expression of myHC in myotubes and the force generated by myotubes (41). Overall, PDGF-B can promote the proliferation of myoblasts.

5.6 Fibroblast Growth Factor-2 (FGF-2)

FGF-2 (fibroblast growth factor-2 also known as basic fibroblast growth factor) is another growth factor that can be utilized in skeletal muscle tissue engineering. When myoblasts from the line L8 which were genetically modified so that they overexpressed FGF-2 were encapsulated in alginate spheres and administered to rats injured through crushing injury, an increase in muscle proliferation and a reduction in apoptosis of muscle cells was observed (42). Additionally, in a different study, it was found that the delivery of FGF-2 naked DNA together with PDGF-B naked DNA induced the formation of new blood vessels and increased the relative area of regenerating muscle in rabbits with ischemic injury to their hindlimbs (43). Overall, FGF-2 can promote proliferation of myoblasts and protect myoblasts from apoptosis while also promoting angiogenesis when used in conjunction with PDGF-B.

5.7 Androgens

Androgenic steroids are male sex hormones that are known to have anabolic effects on skeletal muscle. The application of DHT (dihydrotestosterone) to C2C12 myoblasts being cultured *in vitro* increased the percentage of cells in the S-phase and also increased creatine kinase activity (44). In a different study utilizing L6 cells (another line of murine myoblasts), testosterone application increased the number of cells in the S-phase and the expression of myoG and myHC (45), suggesting that DHT led to an increase in both cell proliferation and differentiation. Androgens promote differentiation through the PI3K/Akt pathway (a pathway that VEGF and IGF-1 also act on through different receptors) since inhibition of the pathway prevents the positive effects of testosterone on differentiation (46). Overall, androgens can promote the proliferation and differentiation of myoblasts.

6. Effects of gut microbiome

It has recently been discovered that the gut microbiome has effects on skeletal muscle. Certain metabolites such as D-Malate produced by the gut microbiome affect skeletal muscle growth. Dietary supplementation of D-Malate, which is produced by certain bacteria such as Arthobacter, Brevibacterium, Corynebacterium, Pseudomonas, Bacillus, and Acinetobacter, decreased the muscle mass of mice through its inhibitory effects on

angiogenesis in muscle (9). In another study it was found that in mice of increasing frailty index scores and age, which is correlated with lower muscle mass and density, the Rikenellaceae group of bacteria which contains the genus Alistipes were overrepresented (47). In a separate murine study it was found that an age associated increase in Sutterella bacteria, was correlated with the loss of gastrocnemius muscle mass. A decrease in Barnesiella bacteria, which is a genus that has positive effects on muscle mass was also age-associated (48). However, the elimination of the aforementioned bacteria that have negative effects on muscle growth for improved muscle regeneration after transplantation cannot be achieved through administration of antibiotics as antibiotics would result in an overall decrease in all gut bacteria including those with positive effects on muscle mass such as Barnesiella. This reduction in all types of gut bacteria would have an overall negative effect on muscle regeneration. Muscular atrophy was observed in 6-8 week old germ-free mice (49). Specifically engineered bacteriophages can be used instead of antibiotics to target and reduce certain bacterial genera that negatively impact muscle mass, thereby improving muscle regeneration after tissue engineered muscle transplantation into patients. This approach avoids affecting the population of bacteria that positively influence muscle regeneration.

7. Effects of the immune system

The immune system also plays a critical role in the regeneration of skeletal muscle. After skeletal muscle suffers damage, M1 pro-inflammatory macrophages are recruited to the damage site by cytokine secretion. These M1 macrophages are associated with the removal of debris from the site as well a further expression of cytokines that promote the proliferation of satellite cells. This early proliferative phase is regulated by interferon-gamma (IFN-γ) which leads to macrophages expressing the M1 pro-inflammatory phenotype and further leads to increased proliferation of satellite cells while also inhibiting their differentiation. ~ 3-4 days after muscle injury anti-inflammatory cytokines such as IL-10 are released which prompt the transition from the M1 macrophage phenotype to an M2 macrophage phenotype. In this stage, proliferation is suppressed while differentiation is promoted as myotube formation, ECM production and angiogenesis contribute to the restoration of skeletal muscle (50). Delivery of exogenous M1 macrophages or the cytokine macrophage-colony stimulating factor (M-CSF) to aged mice was able to increase the rate of recovery of muscle function following atrophy due to hindlimb unloading (51). Moreover, in another study utilizing mice, it was found that mice lacking PPAR-γ, a transcription factor which is required for the production of GDF3 in M2 macrophages, exhibited diminished regeneration after cardiotoxin injury—a deficit that could be counteracted through exogenous GDF3 administration. This study also found that when myoblasts were cultured in the presence of recombinant GDF3, their proliferation was slightly reduced, while their fusion into myotubes was increased, indicating that GDF3 is a potent inducer of myotube formation. This once again highlights the integral role of macrophages for muscle regeneration (52). Considering the importance of the immune response and both types of macrophage in muscle regeneration an important question to be considered is if skeletal muscle tissue constructs should incorporate certain components of the immune system such as M2 macrophages so that immunosurveillance mechanisms

following transplantation are blunted. The incorporation of M2 macrophages that have been polarized *in vitro* or the incorporation of certain signaling molecules such as IL-10 which can stimulate M1 to M2 macrophage transition into skeletal muscle tissue constructs can be beneficial to muscle regeneration following transplantation. The blunting of immunosurveillance mechanisms will prevent an overly-aggressive inflammatory response from occurring after transplantation. This effect is very important because exceedingly high levels of inflammatory signaling can diminish the regenerative capacity of muscle (53). Even though, as mentioned previously, M2 macrophages have a slight inhibitory effect on the proliferation of muscle precursor cells, this effect likely would not be significant compared to their benefits, as the transplanted skeletal muscle tissue constructs would already have a substantial number of cells seeded into them and incorporate some of the aforementioned biological factors that promote proliferation.

8. Perspectives

Despite the significant potential of these biological factors, there are some challenges and difficulties associated with the application of some of these factors, as shown in Figure 3.

Figure 3: Summary of challenges and future directions. This figure was created using BioRender.com.

The first challenge stems from the fact that peptide-based growth factors and hormones such as IGF1 have short elimination half-lives only lasting for a few hours after being released into the body (54). The short half-lives of these biological factors make maintenance of therapeutic levels over extended periods of time harder (55). To solve this problem the biological factors may be administered to the site through multiple injections over a time interval (55). However, this would be expensive and logistically implausible (7). Another solution would be the implementation of a slow-release mechanism into scaffolds such as the nanoclay IGF-1 release mechanism mentioned previously which enabled the sustained release of IGF-1 over 15 days (32). Besides nanoclays, other methods, such as the use of silica nanocomposites, can also enable a continuous, gradual release of certain compounds. This was evidenced in a study where porous silica nanocomposites were used to continuously release selenium—an element which can contribute to improved

muscle regeneration but is toxic at high doses (56).

Another limitation is that the release of some of these factors may lead to unintended off target effects. It was shown that long-term insulin administration promotes muscle regeneration, but also leads to higher insulin resistance in rats (33). Similarly, high serum concentrations of IGF-1 are also related to increased insulin resistance (41). Androgens have been shown to cause an undesirable increase in masculine characteristics and serious, potentially life-threatening adverse effects, such as hepatic rupture, particularly in studies involving female athletes (58). A potential solution to this problem would be to ensure that these biological factors remain localized to the tissue construct of scaffold.

Yet another challenge is associated with the usage of these biological factors that may increase cancer risk. In a case control study, it was found that there was an association between higher levels of IGF-1 in the blood with higher lung cancer risk in humans (59). Additionally, due to Smad3 (which is activated by myostatin) signaling having a potential role as a tumor suppressant, inhibition of myostatin might also lead to an increased cancer risk (37,60). This can especially be a problem if the cells being utilized in the scaffold are already tumorigenic such as iPSCs (12). VEGF plays an important role in the early stages of tumor development, progression, and metastasis (61). Along with IGF-1 and androgens, VEGF is linked to the PI3K/Akt pathway, which is associated with drug resistance in certain cancers (30). Altogether, this could potentially increase the risk of a dangerous resurgence of cancer in patients with a recent history of cancer, such as those suffering from VML due to aggressive tumor ablation, or lead to cancer in patients without a prior diagnosis. The increased risk of tumorigenicity is an adverse effect of the biological factors because of their abilities to promote cell proliferation, survival, and angiogenesis. This risk of carcinogenesis can be mitigated by localization of these biological factors to the implanted scaffold. Additionally, careful risk evaluation, proper dosing of biological factors, and frequent diagnostic checks on patients receiving these factors should be incorporated into post implantation protocols.

There is currently a lack of clinical trials in humans for the usage of these biological factors. For future research, clinical trials will provide more insightful data to better understand the specific mechanisms of action of these biological factors. Overall, considering the present challenges it is important that further research be conducted in developing better prolonged release mechanisms for biological factors and increasing the localization of these factors and preventing them from entering the general circulation. Moreover, future research should investigate the effects of manipulating gut microbiome composition using engineered bacteriophages on skeletal muscle regeneration, as well as the impact of incorporating immune system components into skeletal muscle tissue constructs to blunt immunosurveillance mechanisms.

9. Conclusion

Volumetric loss of muscle tissue after physical trauma and muscular dystrophy can be treated using engineered skeletal tissue implants. Incorporating relevant biological factors into tissue constructs or scaffolds can increase the differentiation of stem cells to muscle tissue, aid in the activation of quiescent satellite cells, increase the number

of proliferating cells, increase stem cell survivability (protecting the stem cells from apoptosis) and promote angiogenesis. The biological factors that fulfill most of the aforementioned criteria are IGF-1, VEGF and Myostatin Inhibitors. These are among the most promising for use in skeletal muscle tissue engineering. Despite the positive effects of all biological factors mentioned in this review, challenges still remain in their usage such as some of the factors having unintended side effects, increased probability of carcinogenesis or having very short elimination half-lives. To overcome these challenges further research should be conducted into increasing the localization of biological factors to muscle tissue and preventing them from entering circulation and developing prolonged release systems. Some other important directions for future research that have not yet appeared in the literature include investigating the effects on muscle of using bacteriophages specifically engineered to eliminate gut bacterial genera that inhibit muscle regeneration, and examining the effect of explicitly incorporating certain immune system components, such as M2 macrophages, into *in-vitro* assembled skeletal muscle tissue constructs to blunt host immunosurveillance mechanisms upon implantation.

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