External physical and biochemical stimulation to enhance skeletal muscle bioengineering

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External physical and biochemical stimulation to enhance skeletal muscle bioengineering

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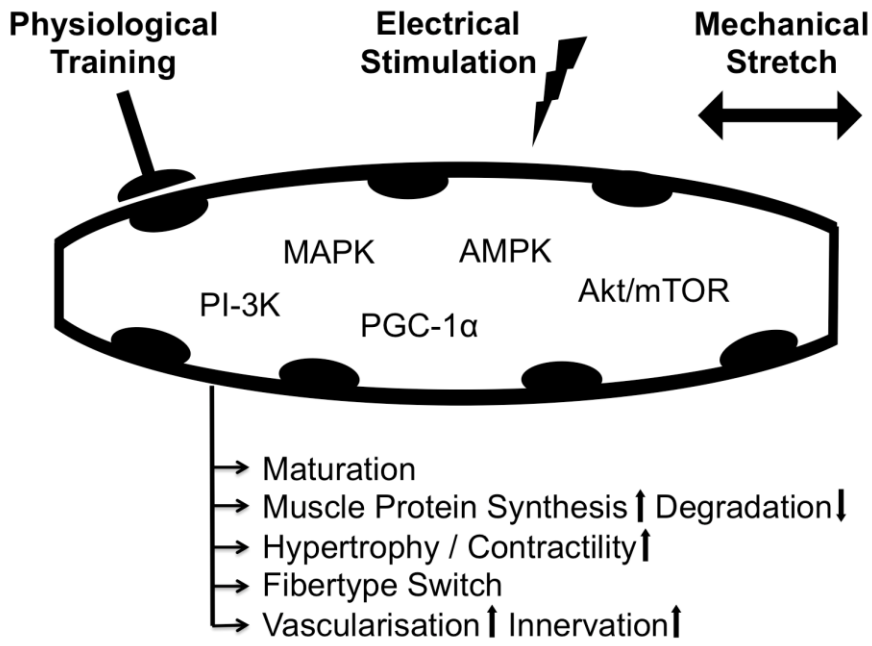
1. Abstract

Purpose of review: Cell based muscle tissue engineering carries the potential to revert the functional loss of muscle tissue caused by disease and trauma. Although muscle tissue can be bioengineered using various precursor cells, major limitations still remain.

Recent findings: In the last decades several cellular pathways playing a crucial role in muscle tissue regeneration have been described. These pathways can be influenced by external stimuli and they not only orchestrate the regenerative process after physiologic wear and muscle trauma, but they also play an important part in aging and maintaining the stem cell niche, which is required to maintain long-term muscle function.

Summary: In this review article we will highlight possible new avenues using external physical and biochemical stimulation in order to optimize muscle bioengineering.

2. Graphical Abstract



3. Introduction

The loss of contractile muscle tissue remains to be a major medical problem, since systemic muscle disease and localized muscle damage cause significant loss in quality of life of affected patients. Urinary incontinence caused by damage to the sphincter muscle is one of the major clinical challenges in urology of the 21th century [1]. The most common form of incontinence is stress urinary incontinence due to muscle and nerve damage resulting from vaginal delivery, aging or tumor therapy [2]. With a growing elderly population and an increased number of men treated for prostate cancer, new therapeutic approaches for continence recovery are needed.

Current treatment options include pelvic floor muscle training, pharmacological treatments, or surgical intervention [3]. The long-term efficacy of these options is often not satisfying and they are associated with a number of possible side effects [4].

The replacement of the damaged sphincter muscle with engineered muscle tissue for functional recovery is proposed as a new treatment option with little or no side effects. Cells currently investigated for use in regeneration of the sphincter muscle are committed muscle satellite cells [5-8] and adult stem cells, including skeletal muscle derived stem cells (MDSCs)[9], bone marrow mesenchymal [10], and adipose-derived stem cells (ADSCs)[11, 12]. Promising results were achieved with satellite cells or muscle precursor cells, which have been widely investigated for muscle regeneration for a variety of genetic and acquired muscle disorders [8, 13-15].

The term “satellite cell” linked with muscle tissue was first used in 1961 by Alexander Mauro, who observed a group of mononucleated cells at the periphery of adult skeletal muscle myofibres by electron microscopy [16]. Already in this first report Mauro suggested an involvement of these cells in muscle growth and regeneration. In the following years scientific evidence confirmed the mitotic activity [17] and its rapid activation following muscle injury [18]. After tracking the satellite cells during the next steps of muscle regeneration a map of myogenesis could be drawn; satellite cells showed transformation to myoblasts [18] which in turn would form myotubes [19]. They not only showed the ability to give rise to myoblasts, they were also capable of self-renewal through asymmetric and symmetric division [20]. Satellite cells are postnatal stem cells, exclusively dedicated to the formation of myotubes. Based on this observation, satellite cells are also referred to as muscle precursor cells (MPCs) more recently.
The process of muscle regeneration through MPCs has been subject to extensive research over the last years, with the aim to not only understand the physiology, but to also develop strategies to influence proliferation and differentiation in experimental and clinical settings. We currently understand that this process is primarily driven by growth factors, which are altered by tissue injury or exercise [21, 22]. Predisposing factors for the outcome of the subsequent regeneration are intactness of innervation, extent of vasculature, hormones and nutrition [23]. This dynamic interplay between MPCs and their environment, often referred to as niche, plays a crucial role in their utilization in regenerative medicine.

Regenerative medicine aims to learn from natural processes and to apply these established strategies to comply with all of the demands of MPCs during regeneration and growth, in order to ultimately engineer a tissue resembling functional muscle. However, a complex interplay of soluble factors, metabolic optimization and biophysical stimulation will be needed to optimize muscle fiber regeneration. Depending on the nature of the approach, these factors can be applied in different chronologies; cells can be treated *in vitro* before or during implantation. Regeneration can also be enhanced *in vivo* after implantation. Unfortunately, the full potential of these technologies has not been realized today.

The goal of this review is to highlight possible pathways to improved muscle tissue engineering for clinical application by evaluating the physiologic and pathologic cellular changes in muscle wasting and regeneration. It can reasonably be assumed, that the ideal protocol will consist of a combination of different strategies.

4. Metabolic optimization

4.1. Muscle fiber types

Plasticity of skeletal muscle is facilitated by adaptations of the metabolic and contractile fiber type [24]. In rodents, slow-twitch, high endurance type I and IIa fibers are clearly distinct from fast-twitch, high peak force type IIx and IIb muscle fibers [25]. In humans, the fiber types are restricted to type I, IIa and IIx, and a number of hybrid fiber types [25]. Defined neuronal activation patterns dominantly regulate the metabolic and myofibrillar properties of muscle fibers [26]. For example, continuous motor neuron firing, resulting in low-amplitude intramyocellular calcium levels, promotes a slow-twitch fiber type [26]. Inversely, a sporadic motor neuron activation linked to high amplitude of sarcoplasmic calcium spikes favors the expression of a fast-twitch fiber-specific gene program [26]. In both cases, clearly distinct paradigms of physical activity underlie the differential motor neuron activation. Thus, endurance and resistance training are associated with a switch towards a higher proportion of slow- and fast-twitch muscle fibers, respectively. Importantly, the phenotype of the muscle fibers by far exceeds the obvious difference in the contraction kinetics. On one hand, slow-twitch muscle fibers mostly use oxidative metabolism of glucose, lipids and lactate to synthesize ATP for long, sustained contractions [25]. Moreover, a pronounced tissue vascularization, elevated myoglobin levels and improved import mechanisms for these three energy substrates all contribute to the high endurance phenotype of these fibers [25]. Finally, a cell-autonomous remodeling of intramyocellular calcium handling and the neuromuscular junction ensure a persistent switch in the fiber type [25]. Most strikingly however, slow-twitch muscle fibers exhibit a massive proliferation of both intramyofibrillar as well as subsarcolemmal mitochondria concomitant with a corresponding boost in mitochondrial function. The increase in heme-containing proteins, e.g. many of the respiratory chain proteins, and the pronounced tissue vascularization confer the typical red color to the oxidative muscle beds with a high number of slow-twitch muscle fibers [25]. In contrast, muscles with a high proportion of fast-twitch muscle fibers appear more whitish. These fibers primarily rely on anaerobic glycolysis to generate the ATP required for fast-twitch contractions with a high peak force [25]. Accordingly, this type of contraction cannot be sustained for a prolonged time and hence, fast-twitch fibers exhibit a higher fatigability compared to slow-twitch muscle fibers. Moreover, due to the predominant dependence on anaerobic metabolism of glucose, fast-twitch muscle fibers are low on mitochondria in terms of number and activity. Importantly however, fast-twitch muscle fibers have a high potential to undergo hypertrophy: the increase in fiber, and therefore also of the muscle cross-sectional area, allows the synthesis and deployment of additional contractile elements and as a consequence, an increase in peak force [25]. Hypertrophy in these fibers is mainly driven by a shift in the balance between protein synthesis and degradation, favoring the former process [27].

4.2. Molecular regulation of muscle plasticity

Surprisingly, the underlying molecular mechanism that differentiates between the fast- and slow-fiber type programs is unknown. In particular, the machinery that interprets the different calcium transients in fast- vs. slow-twitch muscle fibers remains largely elusive. Nevertheless, several important key players for the metabolic and myofibrillar adaptations in either direction have been identified. In slow muscle fibers, the calcium/calmodulin-dependent protein kinase (CaMK) and the protein phosphatase calcineurin A (CnA) are intimately involved in the calcium-dependent signaling cascade resulting in the oxidative, high endurance program [26]. Various

sensors of the altered metabolic demands in these muscle fibers furthermore promote the same phenotype [28]. For example, a shift in the ratio between intracellular AMP and ATP leads to an activation of the AMP-dependent protein kinase (AMPK). Similarly, the relative levels of NAD+ and NADH determine the activity of the protein deacetylase sirtuin 1 (SIRT1) in a catabolic context. Inversely, high substrate levels lead to the activation of the mammalian target of rapamycin (mTOR)- and protein kinase B (PKB/Akt)- controlled signaling pathway, that promotes protein biosynthesis and other anabolic pathways [27]. In contrast, myostatin-signaling through activin receptors, in particular type IIB (ActRIIB), limits growth of muscle mass [27]. The neuroendocrine milieu during and after exercise also result in muscle adaptations, in particular by catecholamine-signaling via β2-adrenergic receptors, glucocorticoid and thyroid hormone signaling through the nuclear glucocorticoid receptor and the thyroid hormone receptor [28], respectively, or the adipokines adiponectin and leptin [29]. The mechanical stress in a contracting muscle fiber is translated by various signaling effectors, for example the p38 mitogen-activated protein kinase (p38 MAPK) or increased levels of reactive oxygen species (ROS) [28]. Adaptive angiogenesis and endothelial remodeling are induced by hypoxic signals as well as nitric oxide (NO). These and most likely other signaling pathways determine the muscle fiber phenotype and hence function. It however is unclear how these pathways are coordinated and integrated. Intriguingly, all of the signaling pathways that are engaged in endurance training at some point impact on the gene expression or post-translational modification of the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) [28, 30]. This protein is a transcriptional coactivator and therefore has the intrinsic ability to engage a multitude of different transcription factors. Accordingly, once activated, PGC-1α controls many, if not all of the transcriptional programs that are required for a high endurance phenotype in skeletal muscle. In addition, PGC-1α activity is modulated by competition with co-repressor proteins such as NCoR1 [31]. Furthermore, PGC-1α also exerts negative effects on gene regulation, for example by indirectly reducing the activity of the nuclear factor κB (NFκB) in the control of pro-inflammatory gene expression [32, 33]. As a consequence of activation, PGC-1α promotes mitochondrial biogenesis and oxidative metabolism in all tissues with a high energetic demand. More specific for muscle, PGC-1α also regulates calcium handling, glucose uptake, lactate oxidation, glycogen and intramyocellular lipid replenishment, neuromuscular plasticity, vascularization and other biological programs. This is culminating in greatly improved fatigue resistance in muscle fibers with elevated PGC-1α due to a metabolic and myofibrillar switch towards oxidative type IIa and to a lesser extent, type I muscle fibers [34]. Inversely, muscle tissue devoid of a functional PGC-1α allele exhibits preferential fast fiber-specific properties in terms of myofibrillar gene expression and mitochondrial function [35, 36]. In addition, muscle-specific knockout mice for PGC-1α suffer from activity-dependent muscle fiber damage, local and systemic inflammation, dysregulated glucose homeostasis and other signs of pathological inactivity [35, 36]. The relative levels and activity of PGC-1α thus constitute a regulatory nexus for muscle fiber plasticity.

Intriguingly, since elevation of PGC-1α is sufficient to induce an endurance-trained phenotype in skeletal muscle and thereby improves muscle functionality, increased levels of PGC-1α improve a number of different muscle pathologies. For example, muscle-specific transgenic expression of PGC-1α reduced fiber damage and ameliorated muscle performance in animal models for Duchenne muscular dystrophy [37], denervation-induced fiber atrophy [38], sarcopenia [39], a mitochondrial myopathy [40] and, in a Zebrafish model, muscle wasting elicited by high doses of statin drugs [41]. While the exact mechanism for the therapeutic effect of muscle PGC-1α in

these pathologies with completely different etiologies is unclear, it is conceivable that the PGC-1α-dependent trained phenotype could broadly alleviate major symptoms of all of these diseases, namely impaired fiber integrity and contractility. In fact, PGC-1α not only rectifies the energy crisis that is observed in many muscle pathologies, but also stabilizes intramyocellular calcium handling, improves vascularization, elevates detoxification of reactive oxygen species, reduces tissue inflammation, inhibits protein degradation and affects neuromuscular junction plasticity. Strikingly, muscle PGC-1α affects both post- as well as pre-synaptic properties by activating a hitherto unknown retrograde signal from the muscle fiber to the motor neuron. As a result, increased motor neuron branching, acetylcholine-containing synaptic vesicles and bigger mitochondria are achieved in the motor neuron endplate [42]. In addition to the resulting functional improvement, muscle PGC-1α also promotes a morphological stabilization by elevating the length and number of synaptic folds per neuromuscular junction [42]. Thus, even though many mechanistic aspects underlying the therapeutic effect of muscle PGC-1α remain enigmatic, this coactivator constitutes an attractive target to improve a wide variety of muscle pathologies.

4.3. Therapeutic modulation of muscle fiber types and metabolism

General loss of muscle mass is the main problem in many muscle diseases, in particular those that are characterized by a rapid, lethal muscle wasting process as observed in cancer cachexia, amyotrophic lateral sclerosis or Duchenne muscular dystrophy [43]. Nevertheless, in many pathologies, muscle fibers of different types are selectively affected and therefore, a more targeted therapy might be more efficient in these cases [44, 45]. For example, a reduction in fast-twitch, glycolytic muscle fibers is predominantly involved in sarcopenia, the muscle wasting in the aging process. Accordingly, elderly patients are progressively losing the ability to quickly react to imbalances and thus are more prone to falls and as a consequence, to bone fractures. Similarly, slow-type muscle fibers are also more refractory towards steroid myopathy, respiratory muscle loss in chronic obstructive pulmonary disorder and heart failure, cancer and AIDS cachexia, type 1 diabetes or sepsis. Inversely, loss of neuronal input or mechanical loading most often results in a preferential loss of slow-type muscle fibers, e.g. in limb immobilization (casting), spinal cord injury and paraplegia, bed rest or microgravity [44, 45].

In a number of pathologies, increased loading in the form of physical activity is an efficient intervention for prevention and therapy, for example subsequent to limb immobilization, bed rest or sarcopenia. However, in other muscle diseases, patients are not able to exercise or even exhibit exercise intolerance, thus showing a detrimental effect of contractions on fiber integrity. Besides anabolic steroids and growth factors, which both harbor the potential for many serious side effects, therapeutic options for the treatment of muscle diseases are currently scarce to non-existent. However, several pharmacological and biological modalities are currently in development. Inhibition of myostatin signaling, which activates fast-twitch muscle fibers [46], underwent several iterations with limited clinical success. Recently however, blockade of the activin receptor type IIB emerged as a more promising strategy to reduce muscle wasting [47]: several entities are currently in clinical trials for different indications [48]. Similarly, hypertrophy of fast-twitch muscle fibers could potentially likewise be promoted through an activation of the Akt/mTOR pathway. Experiments in transgenic animals with constitutively activated mTOR however revealed a detrimental outcome on muscle health [49] and therefore, such therapies would have to be carefully designed. Finally, a systematic comparison of the transcript

expression signatures of human muscle atrophy in spinal cord injury and fasting revealed ursolic acid as a compound that, in subsequent experiments, reduced denervation-induced muscle atrophy in mice [50]. However, the mechanism underlying this effect currently is unclear; likewise, proof of efficacy in humans is still lacking.

Several pharmacological entities have been proposed as endurance exercise mimetics: for example, the polyphenol resveratrol increases oxidative metabolism and endurance capacity when fed to mice [51] or rats [52]. Resveratrol was postulated to exert these effects through activation of SIRT1 [53]; more recent reports however have shown a resveratrol-dependent activation of AMPK [54] and/or cAMP-degrading phosphodiesterases [55]. Regardless of the exact mechanism, resveratrol signaling through these three proteins ultimately converges on PGC-1α [51, 53-55]. Similar to resveratrol, AICAR, an activator of AMPK, also promotes a high endurance phenotype in mice [56]. Similar to SIRT1 that deacetylates PGC-1α [57], AMPK phosphorylates and thereby activates this coactivator [58]. In contrast, a synthetic ligand for the peroxisome proliferator-activated receptor β/δ (PPARβ/δ) by itself was not sufficient to increase endurance capacity [56]. However, PPARβ/δ activation amplified the effect of endurance training [56]. In transgenic mice with a muscle-specific overexpression PPARβ/δ fused to the VP16 activator domain, a boost in oxidative metabolism and endurance capacity was observed [59] that is highly reminiscent of the effect of PGC-1α in the same context [34]. It therefore is conceivable that PPARβ/δ might be an important binding partner for the coactivator PGC-1α in the regulation of an oxidative muscle phenotype.

Despite impressive experimental evidence, the application and efficacy of these endurance mimetics in human patients is questionable. For example, in most human trials conducted so far, resveratrol had no or only a minor effect on several metabolic parameters [60]. Importantly, resveratrol-dependent extension of life span was restricted to specific mouse lines indicating that genetic factors could contribute to the efficacy of resveratrol [61]. AICAR-mediated activation of AMPK might be limited by the poor bioavailability and short half-life of the compound and the increase in serum lactate and uric acid. Moreover, AMPK in general stimulates catabolic programs, inhibits protein synthesis and activates autophagy – these effects might obviously be detrimental in a long-term therapy [62, 63]. Furthermore, it is still unclear whether PPARβ/δ exerts pro-cancerogenic effects in certain tissues and contexts [62, 63]. Finally, the concept and possibility of developing a true exercise mimetic that elicits all of the beneficial effects of bona fide physical activity is highly controversial: based on known drugs, it is hard to imagine that a pharmacological agent can exert a broad, multi-organ effect, regulate 1000 or more genes, exhibit a low to non-existent LD50 and thus very large therapeutic window without any major side effect at the same low costs as exercise [63]. Therefore, genetic engineering of muscle tissue and/or muscle precursor cells used for stem cell therapy might provide a better option for many muscle pathologies. For example, muscle-specific overexpression of PGC-1α [34], CaMK [64] or CnA [65] all promote a complete fiber-type switch towards slow-twitch fibers, accompanied by an increase in endurance capacity. Importantly, the effects of overexpression of these genes transcend skeletal muscle, e.g. the transcriptional induction of vascular endothelial growth factor (VEGF) expression to promote endothelial remodeling and vascularization by muscle PGC-1α [66]. Likewise, muscle PGC-1α-dependent expression of the myokine Fndc5/irisin results in browning of white adipose tissue [67] and neurogenesis in the hippocampus [68]. Thus, genetic engineering of muscle by activators of fast- or slow-twitch fiber function as a therapeutic

modality for muscle diseases should be aggressively explored under the consideration of therapeutic windows, specificity and selectivity [69].

5. Biophysical stimulation of engineered muscle tissue

Engineering of functional skeletal muscle requires a broad understanding of muscle physiology, and of repair and functional adaptation mechanisms in particular. The dynamic relationship between progressive muscle work and hypertrophy or, conversely, immobility and atrophy is well studied [70]. Physiological stresses acting on striated muscle can be mimicked by mechanical stimulation (stretch) to adjust passive muscle loading and by electrical stimulation (pacing) to trigger active muscle contraction. By applying these physical forces *in vitro* or by intensifying them *in vivo* a specific gene and protein expressions pattern can be activated, leading to an accelerated growth and development of the engineered muscle.

5.1. Mechanical stimulation

One possibility to mimic *in vivo* exercise in skeletal myofiber cultures is the application of mechanical stimulation by stretch. Goldberg et al. proposed the development of tension (either passive or active) as the critical event in initiating compensatory growth of skeletal muscle [71]. Their observations revealed, that passive stretching of the soleus or diaphragm delays protein degradation and increases muscle mass, while chronical muscle unloading results in a decreased muscle mass [72]. Vandenburgh demonstrated *in vitro* that intermittent stretch results in increased protein synthesis as a basis for a hypertrophic response [73] and leads to an increase in myotube diameter and creatine kinase activity [74]. Beyond that, constant tension applied to embryonic skeletal muscles leads to differentiation of fibers in a tissue culture environment [73]. Eventually, satellite cell activation, which is a crucial step in muscle repair and regeneration, has been observed *in vitro* two hours post-stretch [75]. These findings were early evidence for the fact that the same biochemical processes accountable for muscle hypertrophy *in vivo* were also applicable *in vitro*. During further experiments these principles were used in a computer-controlled bioreactor system to precondition engineered muscles built from MPCs seeded on collagen-based scaffolds. After seven days of *in vitro* training the samples showed longitudinally organized cells with first myofiber formation and superior myosin expression. Pre-conditioned samples were then implanted to the latissimus dorsi muscle, where they demonstrated significantly higher tetanic contractions [85].

5.1.1. Hypertrophy

Under normal physiological conditions, forces are applied to muscle fibers in a longitudinal direction. Due to the longitudinal growth of the bones, uniaxial strain and stretch is also the dominant force affecting maturing myoblasts and muscle fibers during embryogenesis. However, the construction of computer controlled and programmable motors allows not only variation of the loading-axis, but also of the stretching protocols, thereby allowing to study the effects of different regimes on muscle physiology and hypertrophy response *in vitro* [76-78]. Diverse modalities of mechanical stimulation have been studied for muscle tissue engineering, including static stretch, ramp stretch (i.e. discrete start, continuous increase in stretch amplitude), and cyclic stretch (i.e. intermittent stretching with a given shape, amplitude, frequency, and duration of pauses) [79]. Most *in vitro* muscle stretching systems mimic physiological stimulation of

muscle fibers *in vivo*; therefore they exert cyclic and uniaxial forces to the engineered muscle. The amplitude of stretch varies between 6.7-20%, with frequencies of 0.1-1 Hz [80]. The duration of stimulation in the present studies ranges between 30 min and 10 days [80]. The studies applying a regimen in this range could show an increase in myofiber length (2-4 fold)[81], diameter (12-30%)[82, 83], protein expression (myosin heavy chain, 8 fold) [84] and contractility (75 fold) [85] when compared to the static control. Machingal at el. treated volumetric muscle loss injuries in mice with muscle precursor cell implantation. The animals receiving mechanically preconditioned cells achieved a 44% higher muscle force two months post injury when compared to animals receiving mechanically unstimulated muscle precursor cells [86]. Noteworthy are two studies utilizing modified regimens (biaxial stretch [87], 2 and 4% ramp stretch [88]) showing negative effects of stretch on striated muscle *in vitro*. However, considering the vast amount of *in vitro* and *in vivo* data proving the strong link between muscle growth and exercise, one has to conclude that the question is not whether to stimulate, but rather which is the optimal regimen with regard to the desired tissue in regenerative medicine.

5.1.2. Phenotypic differentiation

As discussed earlier, a critical distinction can be made between fast twitch myofibres, which mainly express Type II myosin heavy chain (MHC) isoforms and slow-twitch fibres, which express Type I MHC isoforms. When engineering muscle tissue, the composition of the tissue is crucial in order to match the physical needs for successful clinical application in regenerative medicine. Remarkably, applications of different mechanical stimulation-regimens have been shown to affect the phenotypic differentiation between the two muscle fiber types. Analogous to human studies, where a shift from fast MHC isoforms to slower isoforms was observed in response to (endurance) exercise [89], *in vitro* application of appropriate mechanical stimulation like stretch has been shown to lead to an up-regulation of Type I and down-regulation of Type II fibres [90, 91]. This shift was more distinct with increased stretch. Engineering of skeletal muscle with a high amount of Type I fibre for urinary sphincter replacement remains a major goal in urological tissue engineering and makes this observation essential for future strategies.

5.1.3. Mechanotransduction and metabolic changes

Mechanical stimulation of skeletal muscle influences growth, morphology and differentiation through intracellular pathways. The molecular mechanism of this action remains unclear. However, several pathways have been proposed: mechanical forces can a) be transmitted to the cells via the extracellular matrix (ECM), b) be generated within the cytoskeleton of individual cells and c) be mediated by stretch-activated calcium channels [92]. Integrins show the ability to communicate mechanical signals from the environment into the interior of the cell [93]. Activation of an intracellular signaling cascade is the response to mechanical stimulation and includes PI-3K, mTOR, mitogen activated protein kinases (MAPK), calcium, glycogen synthase kinase and AMP activated kinase [94]. There is an overlap of these pathways with metabolic signaling (see chapter 4).

An additional consideration is the activation of satellite cells, which can also be stimulated through mechanical stimulation. Two growth factors appear to be of great importance in this process. One of them is a splice variant of the insulin like growth factor 1 (IGF-1); increased levels of IGF-1 mRNA have been measured following stretch [95] or damage [96]. The other important factor is the hepatocyte growth factor (HGF); increased levels of HGF could be observed in cell

culture after stretch [75] and in serum after resistance training [97]. Moreover, stretch activation could be abolished by adding anti-HGF antibody to stretched cultures [75].

5.2. Electrical Stimulation and Electromagnetic Fields

The characterization of developing, wounded, pathologic and normal tissue can be done according to its electric properties including voltage, resistance, current, capacitance and conductance [98, 99]. Measurements on normal tissues can give information on the orientation of the cells, on fat and moisture content, tissue damage, tissue freshness—or time since death [100]. Besides that, electric currents play a major role in the healing process in wounds and amputations. An injury disrupting an epithelial layer immediately generates endogenous electric fields (EFs), which play a dominant guidance role in directing cell migration in epithelial wound healing [101]. Around skin and corneal wounds, electric current flow is orientated towards the wound in order to attract cells into this area [101]. Therefore electric fields are not only useful for characterization of engineered tissue in regenerative medicine, but utilization of electric fields holds also promise as strategy for improvement and enhancement of constructed tissue.

In skeletal muscle, the important role of innervation is well understood and it is known to play a major role in directing the process of terminal differentiation of skeletal muscle cells [102]. Denervation of mature skeletal muscle results in gradual loss of tissue mass (atrophy), impairment of functional properties and satellite cell death [103] [104]. One strategy to mimic physiological action of motoneurons *in vitro* is the use of electrical stimulation. The main objectives, analogue to mechanical stimulation, are increased proliferation and survival rates, desired differentiation and improved functionality. The advantages over other alternatives mentioned so far are a relatively simple equipment design and minimal invasiveness. In respect to safety requirements for regulatory standards the toxicity of chemicals and the immunogenicity are negligible. The physiological range for the applied current is between nA and mA and for the electrical potential difference between mV and V. Devices can be designed to apply direct current (DC) or alternating current (AC) fields [99]. DC devices apply a continuous electric field to cell chambers via direct contact of agar bridges, while AC systems deliver a bidirectional electric field and are either capacitively coupled (CC, parallel electrode plates generating an electric field in-between without direct contact to the cell culture) or directly coupled via electrodes placed inside the cell chamber [99]. The heart of each AC set-up is a stimulator that permits control of pulse width, pulse amplitude (voltage), pulse frequency, and duration of each pulse. An alternative principle of electric field delivery for AC, which is mainly utilized in bone and cartilage tissue engineering, is inductive coupling [105]. By delivering pulsed current to two coiled wires, a pulsed electromagnetic field can be applied to the culture dish which is placed in-between [105]. With few modifications AC and CC devices can also be installed for *in vivo* treatment [106, 107].

5.2.1. Electric Field application and Cell/Tissue response

Differing tissue response has been observed depending on the type of stimulation (AC or DC) and on the regimen applied. Basically, DC field stimulation influences cell orientation, migration and morphology [99]. Typically, cells have a net negative charge and by applying a direct current they are moved by electrophoresis. These physical properties can be used in tissue engineering to guide cells to predefined positions, or to orient cells in particular directions (muscle fibres)[100]. However, proliferation, differentiation and function can be stimulated both by DC and AC fields. Of particular note is the special susceptibility of skeletal muscle to electrical stimulation: unlike all other tissue types, an external electrical current with the accurate frequency (0.5-5Hz), pulse

amplitude and duration can evoke an action potential in skeletal muscle cells releasing calcium ions from the sarcoplasmatic reticulum and a subsequent contraction of myotubes [108]. A skeletal muscle stimulating bioreactor with a corresponding stimulation protocols has been described by Donnelly et al. [109].

By placing myoblasts in a cardiac milieu with cardiac-like electrical current pulses Pedrotty et al. observed a significant increase in proliferation rates, without any effect on differentiation, proposing the activation of the L-type calcium channel-dependent pathway [110]. Likewise Serena et al. could demonstrate activation of satellite cells through electric stimulation and subsequent NO release [111]. Regarding differentiation, Langelaat et al. not only confirmed an accelerated sarcomere assembly and advanced maturation by electrical stimulation [112, 113], they also found striking differences depending on the cell sources: primary muscle progenitor cells (MPCs) were much more susceptible to the electrical stimulus and expressed higher levels of mature myosin heavy chain (MHC) isoforms than C2C12 cell lines. The effect of electrical stimulation was optimal when started on the second day of differentiation after myotube formation for MPCs [112] and on the 4th day for C2C12 myoblasts [114], respectively. Due to higher levels of MHC and higher numbers of sarcomeres after electrical stimulation Ito et al. could observe a 4.5 fold increase in force compared to untreated myoblasts [114]. Analogue *in vivo* results were presented by Distefano et al. by implanting muscle-derived stem cells into a mouse model of Duchenne Muscular Dystrophy: animals treated with neuromuscular electric stimulation for 4 weeks demonstrated a 2-fold increase in number of dystrophin-positive myofibres and had a shortened recovery time as compared to untreated control animals with transplanted muscles. Enhanced strength and increased vascularity were found independent of muscle stem cell implantation in electrically stimulated animals [115].

In addition to a higher protein expression, there is evidence for a fast-to-slow change in phenotype expression with high frequency burst [116] or chronic low frequency stimulation [117] [118]. A slow-to-fast change can be achieved by removing the stimulation. Some data suggest that electric stimulation also influences the extracellular environment; changes in collagen composition were observed depending on the electric voltage and frequency applied [119]. This variation might be caused by changes in expression of matrix metallopeptidases. However, the consequence of this observation is not as clear as it is for the significant increase of vascular endothelial growth factor (VEGF) mRNA levels in surrounding tissue after electrical stimulation [120]. Although most studies observe primarily positive effects on skeletal muscle, electrical stimulation can also alter cellular metabolism negatively, impair cell viability and even induce cell death depending on frequency- and voltage set-up [121].

6. Conclusion

The ultimate goal in tissue engineering of skeletal muscle is to engineer contractile tissue of the desired fiber type, thereby augmenting the damaged muscle in function. Metabolic optimization and mechanical or electrical stimulation offer many new avenues to optimize the engineering of functional skeletal muscle tissue. Through targeted interventions into key pathways, muscle cells can be altered to influence the maturation process, the production of muscle specific proteins and muscle fiber type choice. Indirectly, these actions also influence the environment by supporting innervation and vascularization in proximity of the engineered construct.

However, naturally occurring muscle regeneration is a complex, orchestrated process respecting chronological order and dose dependency. The current research is still fare away from mimicking

this natural biological function. Therefore, more research on individual factors is warranted to later choose a limited number of the most valuable pathways for future application.

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