Aus dem Zentrum für Muskuloskelettale Chirurgie und dem Julius-Wolff Institut der
Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Functional Evaluation of Skeletal Muscle Regeneration Following Severe Crush Trauma and the Therapeutic Application of Specialized Tissue Engineering in the Rat

zur Erlangung des akademischen Grades Doctor medicinae (Dr. med.)

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von

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Foreword

Partial results of those presented in this thesis have previously been published.

I, Janina Kueper, participated in the following publication:

Pumberger M, Qazi TH, Ehrentraut MC, Textor M, **Kueper J**, Stoltenburg-Didinger G, Winkler T, von Roth P, Reinke S, Borselli C, Perka C, Mooney D, Duda GN, Geißler S. Synthetic niche to modulate regenerative potential of MSCs and enhance skeletal muscle regeneration. Biomaterials. 2016 May 10.

No texts, illustrations or tables have been taken from the publication and used in this thesis. The methodology presented in this piece of work reflects that of the publication in greater detail to comply with the format of a doctoral thesis.

The details of my contribution to the publication can be found in the subsequent ‚Declaration of Publications' on page 75.

Of note, the Figure 3 from this dissertation was reused from a work titled 'Dose–Response Relationship of Mesenchymal Stem Cell Transplantation and Functional Regeneration After Severe Skeletal Muscle Injury in Rats' by Tobias Winkler, Philipp von Roth, Georg Matziolis, et al. published on March 1, 2009, by the journal 'Tissue Engineering Part A'. A copyright license (license number: 4251920949127) was acquired by me on December 18, 2017, for use in this dissertation, from the Licensed Content Publisher 'Mary Ann Liebert, Inc.'.

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Abstrakt

Einleitung: Schwere Skelettmuskeltraumata sind ein häufiges klinisches Problem was zu langfristigen Schmerzen und eingeschränkter Mobilität führen kann. Trotz des Pools an muskelspezifischen Stammzellen, die als Satellitenzellen bezeichnet werden, die zur Proliferation, zum Wachstum und zur Differenzierung stimuliert werden, um Muskelfasern nach einer Verletzung zu reparieren, ist die Rückkehr zur Vorverletzungsfunktion oft unmöglich. Wir stellten die Hypothese auf, dass die Transplantation von mesenchymalen Stromalzellen in einem flexiblen Konstrukt, ergänzt durch stimulierende Wachstumsfaktoren, den regenerativen Prozess durch parakrine Modulation der posttraumatischen Mikrozellularumgebung unterstützen kann.

Methodik: Es wurde Ratten ein Quetschtrauma des Skelettmuskels zugefügt. Posttraumatisch erhielten die Ratten entweder intramuskuläre Injektionen der Wachstumsfaktoren Insulin-Wachstumsfaktor-1 und des vaskulär-endothelialem Wachstumsfaktors und / oder autologe mesenchymale Stromalzellen, die zuvor in einer Knochenmarkaspiration gewonnen worden waren, oder ein speziell konstruiertes poröses Alginat, angereichert mit den eben genannten Wachstumsfaktoren und / oder mit mesenchymalen Stromalzellen. Die fast twitch sowie die tetanische Kontraktionskraft der Tiere wurden an den Tagen 7, 28 und 56 nach dem Trauma mittels einer elektromechanischen Stimulationsvorrichtung gemessen.

Ergebnisse: Alle Versuchsgruppen zeigten am Tag 7 nach der Verletzung eine signifikante Abnahme der Kontraktionskraft, mit geringen Unterschieden zwischen den einzelnen Gruppen. Im Gegensatz dazu unterschieden sich die Kontraktionskräfte zwischen der Kontrollgruppe, der leere Alginate transplantiert wurden, und der mit Wachstumsfaktoren und/oder Stromalzellen bereichterten Alginaten transplantierten Gruppen am Tag 28 nach der Verletzung signifikant. Die höchste Kraft wurde in der Versuchsgruppe gefunden, in der Alginate mit Wachtumsfaktoren und mesenchymalen Stammzellen transplantiert wurden, gefunden. Sie hob sich signifikant von den anderen ab (p (Alginat) $<0{,}001$; p (Alginat + GF) $= 0{,}003$). Zwischen den Versuchsgruppen, die 28 Tage nach dem Trauma ausgewertet wurden, und den Gruppen, die 56 Tage nach dem Trauma ausgewertet wurden, konnte keine signifikante Zunahme der Muskelkraft beobachtet werden.

Schlussfolgerung: Wir konnten bestätigen, dass die Transplantation eines mit Wachstumsfaktoren angereicherten und mit autologen mesenchymalen Stammzellen besetzten porösen Alginats zu signifikant verbesserten funktionellen Ergebnissen führt. Spezialisiertes Tissue Engineering, das

auf der Transplantation von Zellen und wachstumsfördernden Faktoren beruht die mittels eines flexiblen Biomaterials transplantiert werden können, dürfte eventuell eine Lösung für nosokomiale Muskelschäden sein, die während eines chirurgischen Eingriffs entstehen können.

Abstract

Introduction: Skeletal muscle trauma is a common condition which may result in long term pain and disability. Despite the pool of muscle-specific stem cells termed satellite cells, which are stimulated to proliferate, grow and differentiate to repair muscle fibers upon injury, return to pre-injury function is often impossible. We hypothesized that the transplantation of Mesenchymal Stromal Cells (MSCs) in a synthetic niche supplemented by stimulatory growth factors may support the regenerative process through paracrine modulation of the post-traumatic microcellular environment

Methods: A crushed-muscle injury model was implemented in rats. Upon completion, rats received either intramuscular Injections of the growth factors (GF) Insulin Growth Factor-1 and Vascular Endothelial Growth Factor and/or autologous MSCs which had previously been harvested in a bone marrow aspiration, or a specially engineered porous Alginate enriched with the before mentioned growth factors and/or seeded with MSCs. Animals were sacrificed at 7-, 28- and 56 days following trauma and their fast twitch- and tetanic contraction forces were measured via an electromechanical stimulatory device.

Results: All experimental groups showed significant decreases in contraction strength at day 7 following injury, with little difference amongst groups. On the contrary, fast twitch and tetanic contraction forces differed significantly between the Alginate-alone control group and the groups transplanted with with Alginates seeded with MSCs and Alginates enriched with GFs and seeded with MSCs at day 28. The highest relative force was found in the latter group, which differed significantly from the others (p (Alginate) <0.001; p (Alginate + GFs) $= 0.003$). No significant increases in muscle force could be observed in between the groups evaluated at 28 days following trauma and the groups evaluated at 56 days following trauma.

Conclusion: We could confirm that the transplantation of a porous Alginate enriched with growth factors and seeded with autologous Mesenchymal Stromal Cells resulted in significantly improved functional outcomes. Tissue engineering, which relies on the transplantation of cells and growth factors conducive of regeneration seeded on scaffolds which support their survival and release into the microcellular environment, may be a solution in particular to nosocomial damage created by incisions necessary during a surgical procedure.

1. Introduction

1.1 Skeletal Muscle Development, Physiology, and Regeneration

Skeletal muscle, the tissue responsible for movement and posture, comprises 40-50 % of a human beings body mass with its approximate 640 muscles within the human body (1, 2). It is a complex composite structure. Within it, various components including blood vessels, nerves, myofibers and connective tissue come together to allow for ambulation, movement, facial expressions and other voluntary muscular contractions that define the physical human condition.

An overview of the architecture and contraction of skeletal muscle is displayed in Figure 1.

Skeletal muscle forms when so-called myoblasts, muscle precursor cells, fuse. This results in long, multinuclear myotubes. Fused myoblasts form the myofiber, which constitutes the basic structural element (3). The myofibers themselves contain sarcomeres, the basic functional units responsible for muscular contraction (4).The developmental stage of any given myofiber may be estimated by examining it's individual myotubes: centralized nuclei point towards youth, whilst peripheral nuclei indicate maturity.

Myofibers may be broadly divided into two types based on their type of myosin heavy chain, the principal motor protein of skeletal muscle (5):

- Type I myofibers, also called oxidative slow twitch fibers, are thin, red, highly perfused and responsible for low intensity exercise, extended duration activities such as endurance running or cycling. They rely on oxidative phosphorylation for their supply of energy and are highly durable.
- Type II myofibers, also called glycolytic fast twitch fibers, are responsible for high intensity exercise, slow duration activities such as sprints and powerlifting. They are further divided into Type II A fibers which contain some myoglobin and garner their energy from oxidative phosphorylation, as Type I fibers, but additionally contain a high number of mitochondria and glycogen. This allows for a sustained delivery of energy in case the oxidative energy supply becomes depleted. Type IIB fibers on the other hand contain high amounts of glycogen and phosphocreatine, readily available metabolites for the conversion into energy. Since these metabolites are limited in terms of their time-intensive replenishment, Type IIb muscles fatigue faster than Type I or Type 2a fibers. The

lack of a dense capillary network and myoglobin in Type 2b fibers result in an off-white color of the muscle itself.

The contractile properties and subsequent transfer of force depend on the distribution between the two types of fibers within any given muscle (6, 7). These isoforms define a muscle's functional profile, but make for no difference in myofiber development.

Irrespective of fiber type, skeletal muscle requires a well formed vascular network that allows for a consistent supply of nutrients and oxygen whilst allowing a removal of metabolic waste products such as lactic acid. Skeletal muscle may require as much as 80 % of cardiac output during strenuous exercise, compared to approximately 20 % at rest (8). Primary arteries give rise to obliquely angled feeder arteries which branch out into an arteriolar network. This network enters the perimysium and subsequently transforms into a capillary network upon contact with the endomysium. The capillary network is interconnected and runs in parallel as well as in connection with the venous and the lympathic networks. Damage to any of the vessels responsible for the supply of nutrients and oxygen, waste disposal, and removal of deoxygenated blood may severely affect the viability of individual myofibers or the muscle as a whole depending on the scale of the damage. Repair and regrowth of these vessels is therefore of paramount importance in skeletal muscle regeneration (7).

To allow for movement, individual mofibers require stimulation from a motor neuron. A single motor neuron may innervate several fibers in order to coordinate contraction (9). Depending on the frequency of the stimulations called action potentials, one may differentiate between so-called fast twitch contraction, which describes a singular contraction, and tetanic contraction, which describes a sustained contraction. The frequency of action potentials required to achieve a tetanic contraction varies depending on the myofiber type predominantly present in the muscle (10).

Sheaths of tissue compartmentalize skeletal muscle: the endomysium encloses individual myofibers, whilst the perimysium joins several myofibers together into a singular compartment. The epimysium, the most fibrous and coarse of layers, holds the entire muscle, and allows for synergistic contraction (3). Tendons, as extension of the extracellular fibrous casing of the muscle, allow for a transfer of contractile forces on to the skeletal system, resulting in movement.

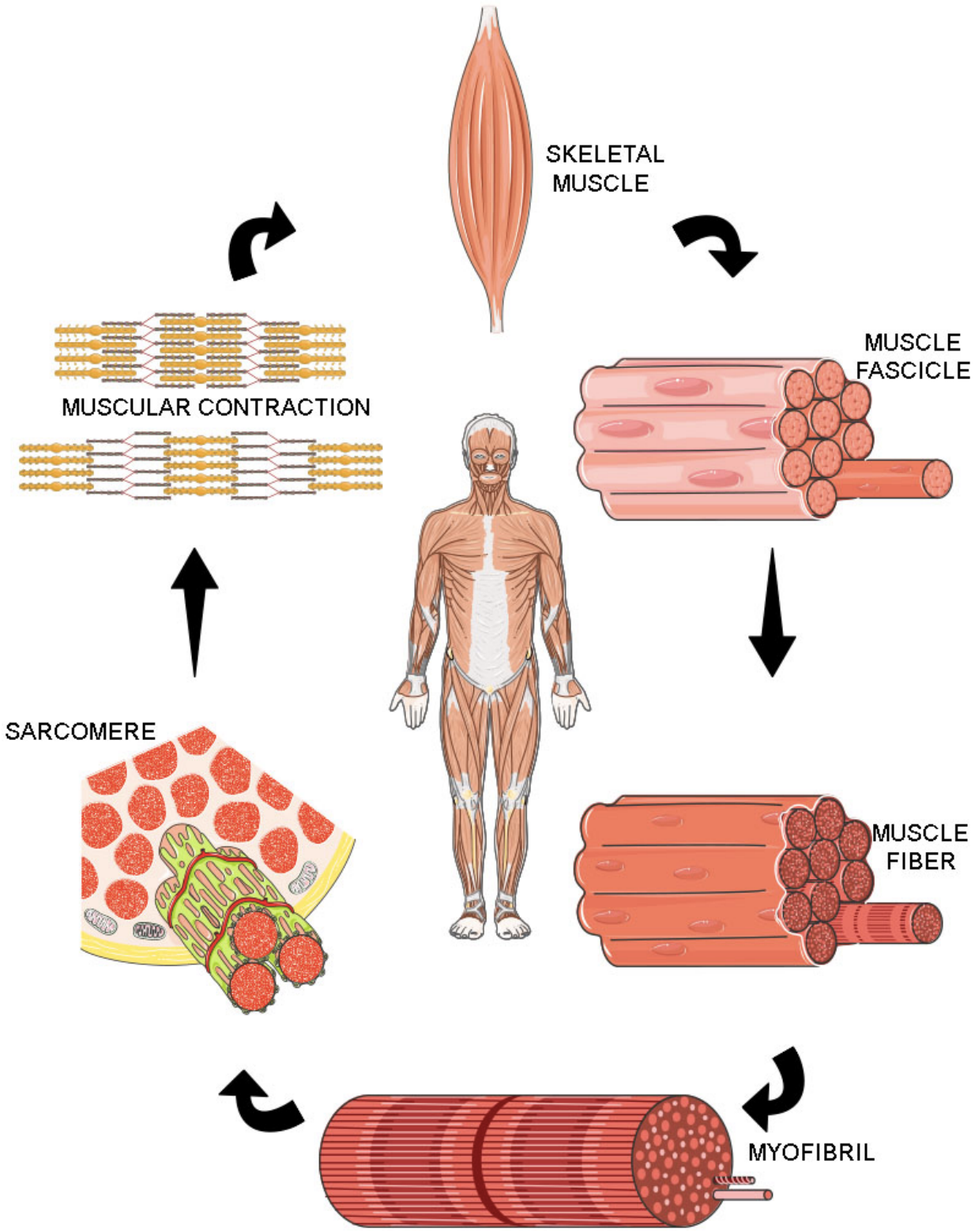

Figure 1. Skeletal muscle architecture and contraction. The human body's skeletal muscle can be subdivided (in orders of magnitude) into fascicles, fibers, myofibrils, and sarcomeres. Synergistic muscular contraction allows for the maintenance of posture and movement.

1.2 Skeletal Muscle Injury: An Overview

Skeletal muscle injuries greatly affect the quality of life of those suffering from them and may, in some instances, result in additional medical complications or death. The burden of musculoskeletal disease overall was estimated to have increased by almost 50 % in the years from 1990 to 2010 (11). Though rare genetic muscular dystrophies may become increasingly rare with the increased availability and implementation of early diagnoses and preimplantation diagnostics, the aging population more than makes up for this decrease (12). The sharp expansion in many countries' octogenarian population has resulted in a startling increase of musculoskeletal disease, resulting in it occupying the 4th rank in the list of conditions that dominate the global disease burden (11, 13).

Injuries of skeletal muscle may occur during recreational activities or in the workplace, be inflicted upon a patient as a necessary step in a surgical procedure or be the result of continuous minor trauma coupled with structural issues such as in muscular dystrophy. Functional recovery following injury depends on the affected individuals' capacity for muscular regeneration. The individual's capacity in turn depends on a number of different factors, including their health status, diet, sleep quality, sex, and age (14-20). Decreases or loss of function have a significant impact on patients' lives, restraining their mobility and independence. Improving the outcomes of muscular injury are therefore of great clinical- and economic significance.

Skeletal muscle injuries can occur in a direct or an indirect form. Whilst damage such as tears and contusions as well cuts and lacerations constitute direct trauma, indirect trauma of the skeletal muscle is defined as complete or incomplete muscle strains where a disproportionate amount of tensile force applied to the muscle cause its myotendinous junctions to rupture (3, 21). Moreover, skeletal muscle injuries may affect different subcomponents of the muscle. Muscle trauma may disrupt a structural unit's organization and integrity, and damage surrounding extracellular matrix, blood vessels, and nerves. The structures affected by an injury are major indicators of the capacity for regeneration. Neuromuscular diseases for example constitute special cases. Duchenne muscular dystrophy for example results from the faulty production of the protein Dystrophin which stabilizes muscle membranes by anchoring the actin cytoskeleton in place, thereby maintaining structural integrity (22). Amyotrophic lateral sclerosis on the other hand is a disease characterized my progressive muscle wasting-a result of spinal motor neuron death caused, amongst other things, by defective autophagy mechanisms (23). In both instances, the prognosis is dire. Damage that exceeds a certain threshold is permanent, and inevitably leads to death.

Following any kind of trauma, skeletal muscle initiates a healing process. Skeletal muscle has an excellent intrinsic potential for regeneration due to a pool of dormant precursors of myoblasts termed satellite cells (24). These cells are situated in between the basal lamina and the sarcolemma (25). Satellite cells will abandon their quiescent state if prompted to do so by indicators of muscle strain, be it physiologic or pathologic. Once activated, they migrate to the site of strain. By means of extensive proliferation, differentiation, and fusion into new myotubes, satellite cells have the ability to repair the muscle and restore its function (26, 27). Once their mission is complete, the remaining satellite cells proliferate in order to replenish their population before returning to their quiescent state (24). These processes integrate in to the overall regenerative process which is initiated upon injury.

The regenerative process of skeletal muscle generally encompasses three stages: disintegration, repair, and remodeling.

1. During stage 1, the regenerative process is initiated by the rupture of affected membranes and an increase in cytosolic calcium, which triggers the necrosis of damaged muscle fibers. The necrosis of said fibers leads to an activation of localized mononuclear cells. This activation results in a release of chemokines such as a number of interleukins, insulin like growth factor, prostaglandins, and tumor necrosis factor alpha, which attract additional immune cells (28, 29). The cytokines in combination with the tissue acidification caused by cellular necrosis stimulate the pain response and result in capillary vasodilation (30). Within 48 hours of injury, Neutrophils begin invading the muscle. Shortly thereafter, macrophages follow suit (31).
2. During stage 2, the inflammatory response develops in full in response to the necrosis, which results in the phagocytosis of the cellular debris by macrophages. These macrophages secrete a variety of cytokines such as IL-1,IL-6, and TNF-alpha which in addition to reinforcing the inflammatory process stimulate the aforementioned satellite cells (32). As described above, they are usually quiescent cells which are activated to enter the cell cycle, differentiate into myoblasts, proliferate and fuse with pre-existing myofibers (33, 34). In parallel to the reconstruction of the muscle itself, neovascularization begins to lay the way for a return to normal circulation. The deposition of collagenous tissue by fibroblasts results in a bridging of the muscular injury, if not yet a complete return to full contractility.
3. During stage 3, regeneration of the damaged nervous endings and the maturation of the

myofiber bring the regenerative process to an end. This stage takes the longest of all. Following axon disruption and Wallerian degeneration, Schwann cells proliferate in parallel with axonal regrowth guided by neurotrophic factors and closely regulatory expression of gene networks involved in regeneration (35). Regeneration of the motor nerves and motor end plate present a definitive limit on more severe muscle injuries which involve neurovascular structures as well as genetic neuromuscular conditions.

The regenerative potential of skeletal muscle in general is limited by the extent of the trauma and the available pool of satellite cells. The formation of fibrotic scar tissue and fatty degeneration within the muscular unit as well as the heterotopic ossification of the surrounding connective tissues as side effects of the regenerative process may lead to decreased tensile strength and muscular dysfunction. These adverse outcomes may be enhanced by old age, which appears to results in few to no active satellite cells, and immobilization, which has a similar effect (36, 37).

1.3 Conservative and Pharmaceutic Therapeutic Approaches to Skeletal Muscle Injury

Few advances have been made at the clinical level concerning the treatment of skeletal muscle trauma in the past decades. Traditional therapeutic approaches for skeletal muscle trauma most commonly practiced in healthcare today are based upon conservative treatments according to the RICE protocol (Rest, Ice, Compression, Elevation) and/or prescription of nonsteroidal anti-inflammatory drugs (NSAID) or COX-2 inhibitors (3, 38). Frequently, interventions are supplemented with physiotherapy (39). The goal in general is to minimize muscle necrosis, decrease hemorrhage and edema, reduce pain and allow for a maximal intrinsic regenerative response from the muscle itself.

Some of the aspects of the RICE protocol are more controversial than others. Select studies examining the RICE protocol for muscular injuries in murine models found that short-term use of cryotherapy and rest in particular could attenuate overall tissue damage and capillary density, decrease intra-compartmental pressure, decrease the rate of ischemia reperfusion injury, increase vascularization and result in a shorter inflammatory period as well as a better structured regenerated muscle fiber (40-44). Possible reasons for these improved structural results may be due to reductions in the amount of oxidative stress the regenerating muscle is exposed to during the inflammatory response (45). These results however have been highly contested. Rest in particular is often used as way of modeling muscle wasting resulting from disuse, with the muscle showing signs of regenerative efforts such as satellite cell activation after as few as 7 days of

immobilization (46). Icing results in vasoconstriction, and thereby physically delays the migration of immune cells to the site of injury. This may result in a retarded regenerative process, resulting in decreased fiber size and increase in fibrous scar tissue (47). Compression and elevation aim to similarly decrease the blood flow and may be assumed to therefore share some of the shortcomings of icing. The application of heat, in contrast to ice, was found to be helpful for the regenerative process of skeletal muscle. The stages of regeneration appeared to be accelerated, with the vasodilation of blood vessels likely facilitating the migration of immune cells (48). Low level laser therapy was similarly found to be conducive of regeneration following muscular strain caused by endurance training in a rat model (49).

The use of Non-Steroidal-Anti-Inflammatory-Drugs (NSAIDs) or COX-2 inhibitors, whilst popular, has recently come under fire and should be avoided beyond the immediate post-traumatic period. Several studies demonstrated adverse outcomes of both groups of medications, impairing tissue remodeling through the down-regulation of several anti-apoptotic proteins involved in the regenerative process (50-52). Ibuprofen in particular has been shown to decrease myofiber thickness when administered in a chronic fashion, though no adverse effects on the tendon could be identified (53).

Physiotherapy, in contrast to pharmacologic or RICE interventions, still has broad support from clinicians. Whilst mobilization of an injured muscle should proceed carefully, exercise may prevent decreases in the number of myofibers compounded by disuse following trauma. Early mobilization has generally been favored since it allows for an earlier intervention into the earlier stages of skeletal muscle regeneration(40, 54). Specifically, it may reduce the adverse effects of the inflammatory response following an injury and thereby improve vascularization (55, 56). Long term physiotherapy may be effective in the remodeling stage, allowing for a reordering of scar tissue to allow for a maximization of force generation and transduction . Additional, physiotherapy strengthens the uninjured muscle, allowing for partial compensation of the functional deficits. This has been demonstrated in a particularly convincing manner by studies that examined the influence of physiotherapy on the outcome of volumetric muscle loss injuries. Exercise in the form of voluntary wheel running in a rat study was found to increase muscle weight following the administration of a volumetric muscle loss injury (57). Nonetheless, physiotherapy is not an option for every kind of muscle injury. Patients with severe injuries or comorbidities may be unable to comply with an exercise regime, and patients with muscular dystrophy may worsen their condition by incorrectly administered physiotherapy. In these cases, massage as the softest form of

physiotherapy may be a last resort (58).

None of the traditional therapeutic approaches have been proven ideally suited to optimize muscle regeneration following trauma in humans, though the diversity in injuries, the varied demographic make-up of the patient population and a lack of compliance may be instrumental in the lack of a scientific consensus.

1.4 Surgical Therapeutic Approaches to Skeletal Muscle Injury

Rarely, operative treatment of muscular damage exhibiting specific characteristics has been found beneficial. Among the injuries that are indicated for surgery are complete tears or strains of the muscle belly, or problematic locations of the damaged muscle with few or no agonist muscles, (3, 59). During these interventions, muscle's may be transposed, transplanted or debrided in the hope of restoring some function.

Autologous muscle transfer is the most frequently performed intervention in volumetric muscle loss injuries or injuries which damage a major motor nerve. The intrinsic regenerative potential here is minimal. For this procedure, healthy muscle is grafted from a donor site adjacent to the injury site and 'rerouted' in order to service the function the injured muscle had. The most frequently performed autologous muscle transfers are those of the gracilis and the latissimus dorsi muscles (60-62). These procedures are most commonly used to address issue with elbow flexion and to allow for smile restoration following a facial nerve palsy. The outcome, in instances of nerve palsy, relates directly to how well the neurovascular anastomosis heals. Another example of autologous muscle transfer is the so-called Whiteside surgery. A severe trauma of the abductor muscles of the hip can lead to a substantial loss of function resulting in postural instability, restricted ambulation, and a limp. In this clinical instance, the Whiteside technique calls for the transfer of anterior portions of the gluteus maximus to the greater trochanter, and a transfer of the posterior portion to the anterior capsule of the hip (63). This allows for a return of functional hip abduction, and a reduced or absent limp.

If the area surrounding the traumatized skeletal muscle is too heavily involved in the injury, the final option of surgical intervention is a free functional muscle transfer. Here, a vascularized muscular flap is transposed to allow for defect coverage, or, more importantly in the context of this thesis, for functional reconstruction. The muscle's chosen as donors depend on the extent of

the tissue defect, as well as the function of the muscle to be replaced. The rectus femoris and the gracilis muscles are most commonly transplanted in posttraumatic lower extremity defects (64). Additional muscles which may be used are the extensor brevis, gastrocnemicus, rectus, rectus femoris, and serratus muscles. Partial latissimus and rectus flaps round out the options for defect reconstruction (65).

Though these kinds of flaps have the ability to restore function at least in part, they have several disadvantages. First, many patients develop extensive pain and may suffer additional complications stemming from the donor site (64). Second, the vascular anastomosis between the free flap and the connecting local vessel may fail. This phenomenon is particularly well documented in free flaps used for head and neck reconstruction. Even though muscle function (and therefore contractility) are not in the foreground, failure of anastomosis in combination with additional complications such as infection may result in graft loss in 4-10 % of all cases (60, 66, 67). Third, the length of the operative time and the complexity of free muscle flaps automatically limit the patient pool eligible for such a procedure. Patients who may most benefit from such reconstructions may have to be stabilized and rehabilitated extensively before returning to a health status where they would be capable of withstanding the stressor associated with such a treatment.

1.5 Miscellaneous Other Novel Therapeutic Approaches to Skeletal Muscle Injury

A plethora of novel experimental treatment protocols have developed quite recently. With a spectrum of new approaches, some have proven better than others in both animal models and (if proven safe) human patients.

Many approaches appear to have been driven by the desire to develop an affordable, easily available, commercially advantageous product aimed in particular at athletes. Despite the initial enthusiasm many of these ventures receive, few options have been found truly beneficial to healing. Neither trials of hyperbaric oxygen therapy nor of therapeutic pulsatile ultrasound treatments or platelet-rich plasma injections could confirm a consistent positive effect on muscle regeneration, though all three received considerable attention from commercial entities (68-71).

With a recent increase in popularity of Chinese traditional medicine, select groups have sought out the efficiency of acupuncture in addressing musculoskeletal disorders. Researchers were particularly interested in its utilization for patients who were unable to perform physiotherapy due

to severely limited mobility or overall health. Surprisingly, acupuncture was found to have a similar effect, increasing satellite cell proliferation and IGF-1 levels and thereby enhancing skeletal muscle regeneration when paired with electrical impulses (72, 73). The applicability of this method is naturally limited to less severe skeletal muscle injuries.

Electrical impulses and electrical stimulation without the component of acupuncture have been another object of study. Though the target group of patients most eligible for this kind of intervention is similar to that of acupuncture, many studies have focused primarily on patients suffering from muscular dystrophies. Original work on elderly healthy subjects showed an increase in the maturation of precursor cells into myofibers (74). Unfortunately, those kinds of results could not be recapitulated in patients suffering from neuromuscular disorders. In three separate studies examining the effect of neuromuscular stimulation on overall strength of different muscle groups in patients affected by muscular dystrophies, only one study found a favorable effect (75-77). One possible explanation is that neuromuscular stimulation may be less effective in individual's who's pool of satellite cells is already depleted from chronic degenerative disease. Patient selection and further studies analyzing the biological mechanisms of neuromuscular stimulation are paramount for further development for this field of therapeutics.

Neuromuscular conditions, with their lethal outcome, have been another focus of research and development in recent years. With the advent of the gene editing tool CRISPR-Cas9, many patients and scientists have become hopeful that efficient, and above all safe gene therapies may finally be on the horizon (78). Though significant ethical- and technological hurdles remain, a significant change in the gold standard of treatment appears to be on the horizon for those affected by genetically caused skeletal muscle degeneration (79).

1.6 Prior Studies of Skeletal Muscle Regeneration in our Laboratory

Due to our group's clinical affiliations, an early interest developed in developing an intervention for skeletal muscle injuries that may be translated from bench- to bedside. Early studies focused on bolus injections of MSCs compared to saline injections in a rat model (80). Due to the favorable functional outcome of the rats treated with MSC injections, the following studies aimed to elucidate the mechanisms behind the improvement of muscular strength. Following labeling of MSCs with so-called very small iron oxide nanoparticles, MSCs could be localized at the site of injection over several weeks, though the overall retention at the site of injury was unclear (81). A

subsequent study could demonstrate a sustained dose-response relationship between the number of MSCs injected, and the amount of functional improvement observed during muscle force measurements (82). To further standardize our injury model and allow for consistency across experiments, the group analyzed the time course of regeneration following the application of our crush model (83). This study and its subsequent follow-ups provided the baseline values of untreated rat skeletal muscle regeneration for the analysis performed in this thesis, and the related publication for the sake of an overall reduction in the number of animals used. The efficiency of the crush model, the consistency of the functional outcome as captured by the group's customized measurement device, and the Mesenchymal Stromal Cells (MSCs) which proved so beneficial in skeletal muscle regeneration were adapted for the study at hand based on the published experience and utility of the methods developed over a span of several years.

1.7 Hypothesis

MSCs circulating in the blood stream have been found to support regeneration following a depletion of the satellite cell pool as it occurs for example in muscular dystrophy, though the effect on the recovery of function is unknown (84, 85). Our laboratory has performed extensive studies evaluating the effect of MSCs and various growth factors (GF) on skeletal muscle regeneration in the past (80, 82, 86, 87). The retention of MSCs and GFs at the site of injury however was unclear. Thus, we hypothesized that the beneficial effects of MSCs and GFs on skeletal muscle regeneration could be improved by allowing for their controlled spatiotemporal colocation and release. This study aimed to investigate which mode of MSC and GF delivery would significantly support muscle regeneration and aid in the overall healing process, resulting in an improved functional outcome.

We therefore compared the intramuscular injections of MSCs and/or GFs with transplantation of MSCs seeded on porous alginate scaffolds optionally capable of a concomitant, controlled release of stimulatory IGF-1 and $VEGF_{165}$. Blank Alginates and Alginates containing only the growth factors or the MSCs were used as controls.

2. Materials and Methods

4-month old female Sprague Dawley rats (Charles River Laboratories, Sulzbach, Germany) were utilized for this study. The rats were allowed ad libitum access to food and water and were housed under constant conditions at the Institute for experimental medicine under professional animal care and supervision. All surgical procedures were performed at the earliest one week after arrival to allow for some familiarization with their new environment. The rats weighed 200-250 grams at the time of the first surgery. Every experimental group was originally allocated 10 animals,. Every experimental group is represented by a minimum of 7 animals in the final statistical analysis. All experimental procedures were performed in accordance with the policies and principles established by the German national animal Welfare Code, the Animal Welfare Act and the NIH Guide for Care and Use of Laboratory Animals. The study was approved by the German Federal ministry of science and research (Reg.-Numbers G 251/06, 0234/03).

2.1 Overview of the Experimental Design

Following a time of familiarization to their environment, all rats included in this study received bilateral tibia biopsies as shown in part A) of Figure 2. Subsequently, the bone marrow aspirate was cultured in vitro to allow for the proliferation and quality control of the MSCs utilized in some of our experimental groups. In parallel, the alginates required for some of our groups were produced in the laboratory. The bolus injections and specialized alginates were manufactured. These steps occurred in sync and are displayed in part B) of Figure 2. After a period of recovery for the rats, all animals received a crush injury of the left soleus muscle as shown in part C) of Figure 2. The rats were then randomly allocated to one of two interventions: bolus injections or alginate placement. This is shown in part D) of Figure 2. Finally, immediately following the crush injury, all rats received some form of treatment. The researchers were not blinded to group allocation due to the obvious visual differences between the treatment groups of the experiment. Overall, there were six treatment groups as shown in part E of Figure 2:

1. Bolus injection of IGF-1 and $VEGF_{165}$
2. Bolus injections of IGF-1, $VEGF_{165}$, and MSCs
3. Placement of a blank Alginate
4. Placement of an Alginate seeded with MSCs
5. Placement of an Alginate seeded with IGF-1 and $VEGF_{165}$
6. Placement of an Alginate seeded with IGF-1, $VEGF_{165}$, and MSCs

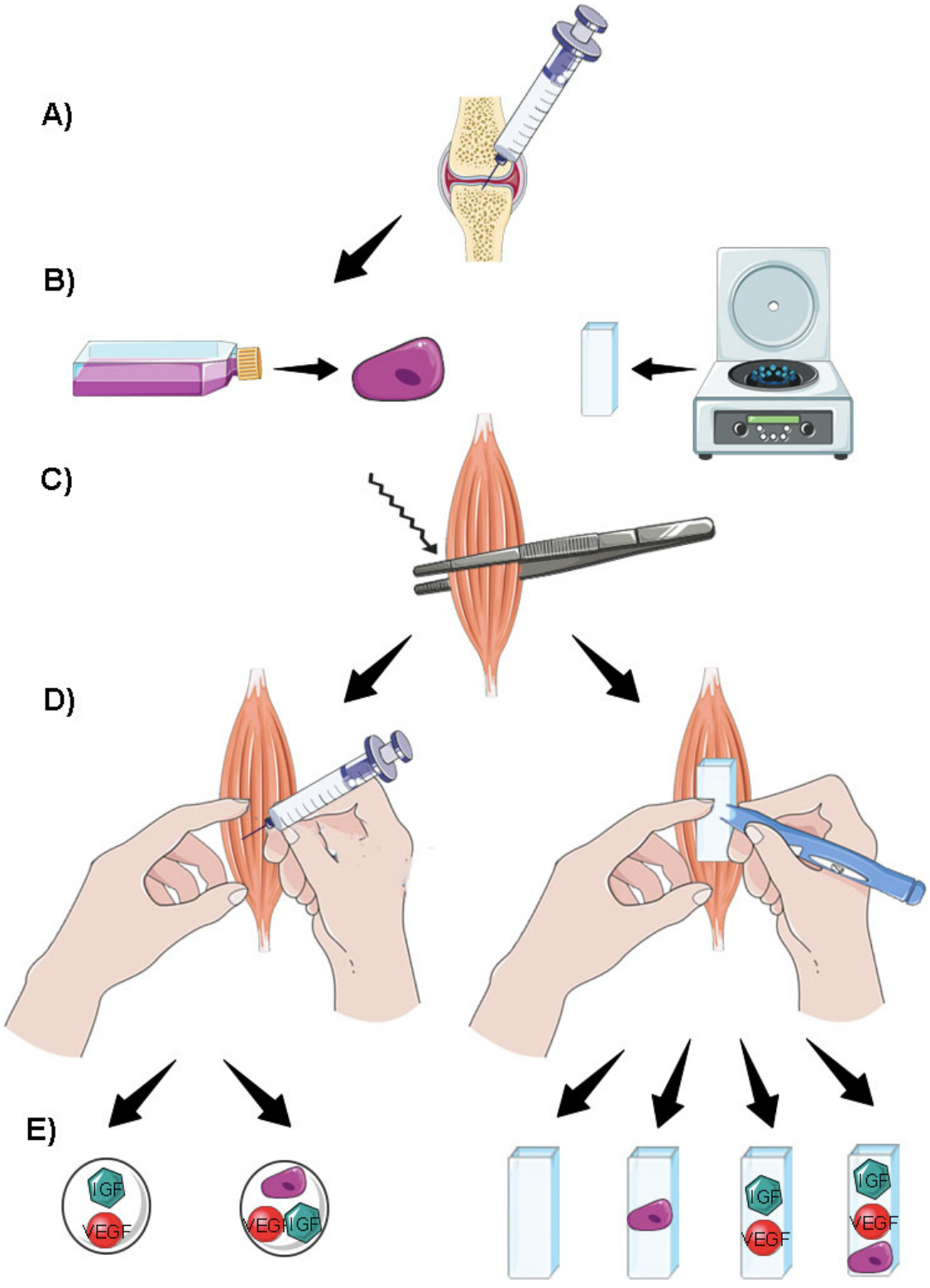

Figure 2. Overview of methods. A. Tibia biopsy with bone marrow aspiration. B. Concomitant culturing of MSCs and production of alginate scaffold. C. Crush injury to the left and right soleus muscles. D. Division into injection groups and scaffold groups. E. The injection groups contain either just GFs or GFs and MSCs. The scaffold groups contain either nothing, just MSCs, just GFs, or a combination of GFs and MSCs.

2.2 In Vivo Procedures

2.2.1 Mesenchymal Stromal Cell Procurement

The rats were initially anesthetized with 5 % isoflurane in 70 % N2 Gas and 30 % O2 Gas. Both hind limbs were shaved and disinfected thoroughly.

The animals were subsequently positioned on their backs and remained under general anesthesia with a constant flow of 3.25 % isoflurane. Intra- and postoperative pain was managed by administering 0.3 ml of a solution of the local anesthetic Rimadyl® mixed with isotonic NaCl at a ratio of 1:3 per bolus injection to the neck fold of the rat subcutaneously. The surgical tibia biopsy was then performed on the right and the left hind limb with the animal in a supine position. A 15 mm long incision was made medially of the patellar ligament parallel to the tibia as shown in Figure 3.

Figure 3. Incision. Placement medial to the patellar ligament.

The medial trochanter of the tibia was exposed and a round defect spanning approximately 2 mm was created by the perpendicular perforation of the bone with a no. 20 scalpel. A 20-gauge needle was then carefully inserted centrally down the shaft were bone marrow was aspirated as shown in Figure 4.

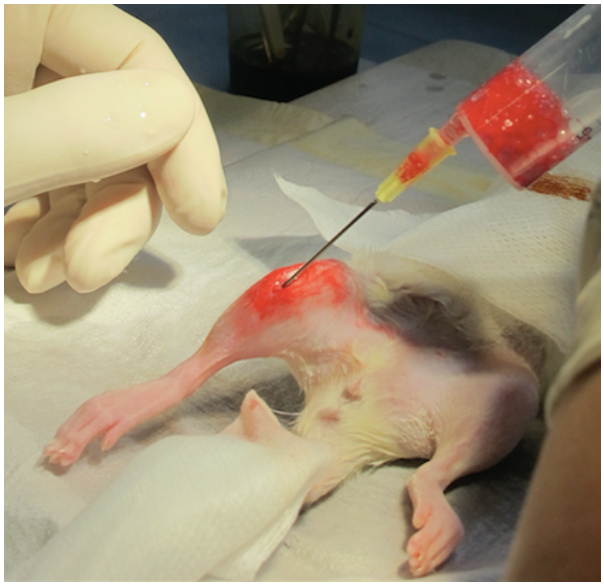

Figure 4. Bone marrow aspiration. Insertion of a 20 G needle followed by aspiration.

The aspirate was immediately immersed in 20 ml of cell culture medium and transported to the laboratory to be transferred to the incubator. The skin incision was closed with 3-0 prolene sutures and the rat was placed back in its cage were it remained under careful observation.

Additional administration of a local anesthetic was considered if the rat exhibited signs of pain.

2.2.2 Induction of Muscle Trauma

The rats were initially anesthetized with 5 % isoflurane in 70 % N2 Gas and 30 % O2 Gas. Their left hind limbs were shaved and disinfected.

The animals were placed in a prone position and received the identical anesthetic- and analgesic care as described for the tibia biopsies. A 20-30 mm long skin incision was made longitudinally spanning the length of the lower medial hind limb. The skin muscle was incised from the Achilles tendon to the proximal attachment of the soleus muscle. The neurovascular bundle crossing the mid-length of the soleus muscle was visualized to be purposely excluded during the induction of the crush trauma as to avoid denervation. Curved artery forceps whose jaws were covered by polyethylene tubes to omit lesions of the fascia were used to crush the soleus muscle lengthwise beginning just proximal of the insertion at the posterior calcaneus and advancing towards the origin with the exception of the trapezoid area adjacent to the neurovascular bundle. Standardized pressure was exerted upon the muscle in 3 steps distally and 2 steps proximally of the bundle by closing the forceps to the third degree and keeping it in place without additional tension or pull on the muscle for 20 seconds at each placement. The amount of pressure applied if the forceps is

closed to its third stage was measured to be 112 N (SD 5.1) at preliminary examinations with a material testing device (Zwick 1455 – Zwick GmbH, Ulm, Germany).

Once the application of the standardized crush trauma was concluded, the fascia was longitudinally incised to release pressure within the muscle. Depending on the group assignment of the animal, the procedure was concluded with multiple lavages and by either the transplantation of an alginate or a bolus injection. Both procedures are described in greater detail below. The operation was concluded with the closure of the wound and the rat was placed back in its cage under careful observation.

As described in the outline of the procedure of the tibia biopsies, additional administration of a local anesthetic was considered if the rat exhibited signs of pain.

2.2.3 Application of Alginates

The transplantations of the alginates were always performed immediately following the implementation of the trauma model.

The alginates remained sterile within their wells and were removed with a lever immediately after the incision of the soleus fascia. They were placed along the medial portion of the soleus, parallel to the anatomical alignment of the muscle fibers, extending across the neurovascular bundle. The gastrocnemicus muscle was then carefully placed back above the soleus, thereby returning to its physiological position and creating a protective niche for the alginate. To close the wound, the muscle and the skin were approximated by 3-0 prolene sutures.

2.2.4 Application of Bolus Injections

The bolus injections were always performed immediately following the implementation of the trauma model.

The syringes with the previously prepared solutions were kept on dry ice and removed from their sterile container 5 minutes prior to their injection to allow the suspension to thaw. Following the application of the crush trauma, a cannula was inserted into the soleus muscle distal from the origin below the tibial plateau and the solution was then slowly injected throughout the length of the muscle over the course of ten seconds whilst being maneuvered parallel to the muscle fibers. To close the wound, the muscle and the skin were approximated by 3-0 prolene sutures.

2.2.5. Muscle Force Measurements

The method utilized for the muscle force measurements has been previously described (87).

The rats were initially anesthetized with 5 % isoflurane in 70 % N2 Gas and 30 % O2 Gas. They then received a weight adapted intra-peritoneal injection of Ketamin and Xylazin (2.5:2 ml/gram bodyweight). Their hind limbs and lower backs were shaved and disinfected, and the animals were placed in a prone position for surgery. The biomechanical evaluation was performed alternately between two animals, always beginning with the uninjured control muscle in the right hind limb, then proceeding to the previously crushed muscle of the left hind limb. The surgical procedure to prepare the muscle for measurement was identical for both sides.

First, a 5-centimeter-long incision along the medial aspect of the curvature of the hind limb was made through the skin and the skin muscle. The soleus muscle was dissected and and its tendon separated from the Achilles tendon. Conclusively the sciatic nerve was dissected and exposed. A silk suture was secured within the soleus muscles' tendon.

Following this, the rat was transferred to the testing apparatus. The knee joint was pierced by a cannula which was set within the frame of the contraption, the ankle joint was enclosed within a heavy wire gateway and the toes were constrained with tape to ensure the complete fixation of the lower extremity as shown in Figure 5. The tendon of the soleus muscle was connected to the transducer of the muscle force using the previously thusly placed silk suture firmly fastened to both sides.

Finally, the sciatic nerve was placed in the clasp of the stimulator (Experimetria, Budapest, Hungary) and coated with 1 ml of isotonic NaCl solution to allow for improved conductivity.

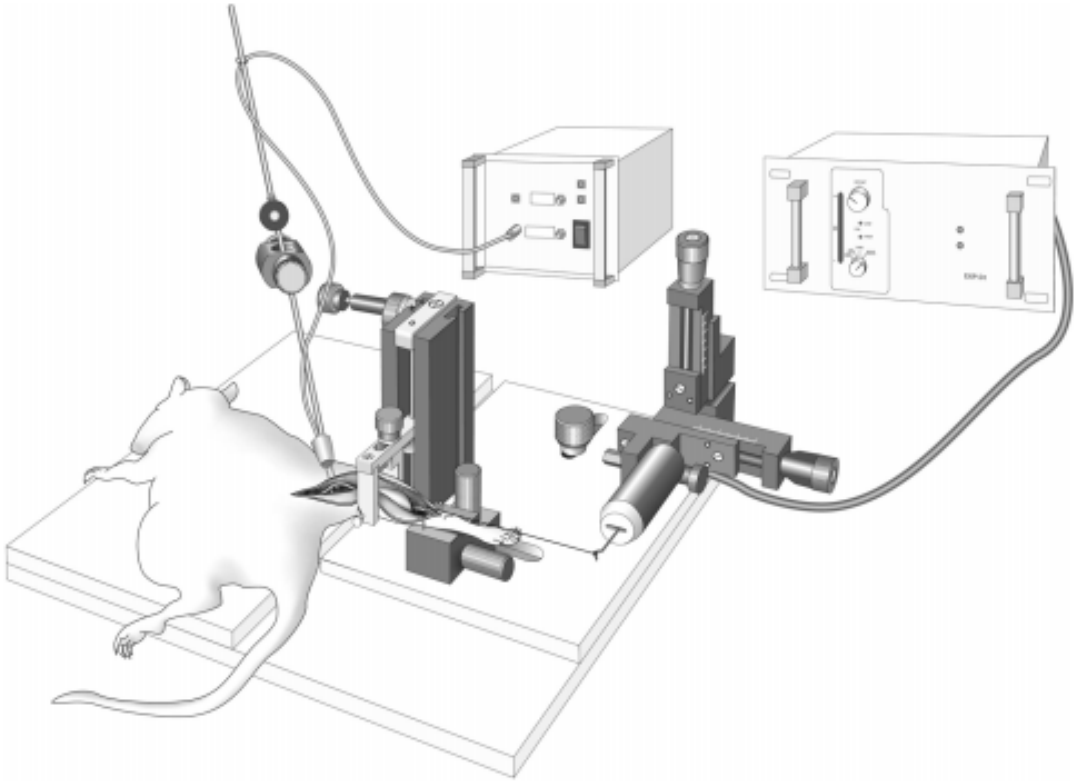

Figure 5. Muscle force measurement. The anesthetized rat is placed on a rigid board following the dissection of the soleus muscle and the connection of its tendon with a force transducer via silk sutures. The electrical stimulator can be viewed on the left. The force transducer connected to a software which records all contractions can be seen on the right.

The software for measurement was subsequently launched on the computer.

After ensuring a pre-existing tension of the muscle of 0.1 N was being applied through the force transducer, the bipolar stimulation of the sciatic nerve was initiated. The computer program controlled stimulator consequently began its electrical impulses following a previously programmed protocol in two different modes: 5 initial pulses at 9 mA/75 Hz lasting 0.01 seconds to record the force of the fast twitch properties, and 5 ensuing pulses at 9 mA/75 Hz lasting 3 seconds to record the tetanic force properties of the soleus muscle. Each pulse during both modes was separated by 5 second pause intervals without stimulation. The force of the contraction of the soleus muscle ensuing the electrical stimulation of the sciatic nerve by the stimulator caused a pull on the force transducer which was consequently transcribed and graphically displayed in real time on the computer interface Upon the completion of the series of stimulations, the data obtained was saved on an external hard drive for later evaluation.

An example of a right muscle force measurement can be viewed in Figure 6A. The right muscle was the uninjured muscle, and therefore represents the control. The trend lines above the measurements indicate an increase in force with increasing numbers of fast twitch contractions, and a decrease in force with increasing numbers of tetanic contractions. These observations relate to the excitation and respective subsequent fatigue of the muscle.

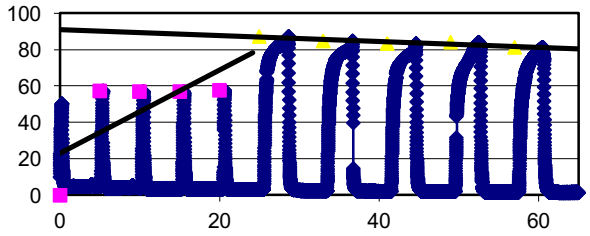

Figure 6A. Example of a right muscle (control group) force measurement. Five measurements of fast twitch contractions can be viewed on the left. Five measurements of tetanic contractions can be viewed on the right. Trend lines are included above both sets of measurements.

An example of a left muscle force measurement can be viewed in Figure 6B. The left muscle was the injured and subsequently treated muscle, and therefore represents one of six treatment groups. Similar increases and decreases in fast twitch and tetanic contraction respectively can be observed as in the uninjured right control. The overall strength however is markedly diminished.

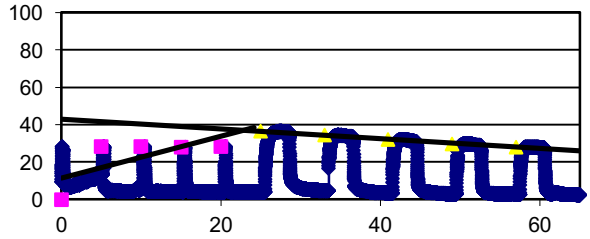

Figure 6B. Example of a left muscle (treatment group) force measurement. Five measurements of fast twitch contractions can be viewed on the left. Five measurements of tetanic contractions can be viewed on the right. Trend lines are included above both sets of measurements.

2.3 In vitro Procedures

2.3.1 Mesenchymal Stromal Cell Culturing

The cells derived from the bone marrow aspiration were transferred into 20 ml of Dulbecco's Modified Eagle Medium (DMEM: 10 % fetal calf serum, 1 % Penicillin-Streptomycin), seeded in 75 cm2 cell culture flasks and left to multiply at 37 degrees Celsius and 5 % CO2. The DMEM was

exchanged at regular intervals of 3-4 days to optimize cell expansion. Upon reaching 60-70 % confluence in their flasks the cells were trypsinized (0.25 % trypsin-'Sigma, Germany') and split. Cells were confirmed to be MSCs if they displayed surface markers CD29, CD44, CD105, CD73, CD166, CD90, and RT1A, and no CD45, CD34 and RT1B during flow cytometry.

2.3.2 Alginate Fabrication

The production of the implantable alginates was performed in several steps.

First, ultrapure alginates (Novamatrix, Oslo, Norway) in the form of MVG alginate as the high molecular weight component (HMW: 250 kDa) and LVG alginate as the low molecular weight component (LMW: 50 kDA) were diluted to 1 %. Subsequently, 1 % of the sugar residues were oxidized with sodium periodate (Sigma-Aldrich, St Louis, MO) at room temperature in the dark following the addition of a sterile magnetic stirrer as previously described (88). Ethylene glycol (Fischer, Pittsburgh, USA) was used to stop the reaction following a 17-hour period of oxidation. Next, the alginate solution was dialyzed for 72 hours (MWCO 3500 Da, Spectra/Por®), frozen at -20 C, and lyophilized. As previously described, G4RGDSP peptides (Commonwealth Biotechnology, Richmond, USA) were utilized to modify the alginate solution by a factor of 10 (10 peptide molecules per alginate chain)(89). Following these substitutions, the alginate solution was once more filtered, lyophilized, and stored at -20 C.

Second, pending the production of implantable Alginates, alginate stock solution was prepared by adding 16.5 ml of a mixture of 1 % penicillin/streptomycin in DMEM (5 ml penicillin/streptomycin in 500 ml DMEM) to respectively 0.33 g of HMW- and LMW alginate in a glass bottle to obtain a 4 % polymer solution. A sterile magnetic stirrer was added. The solution was lightly shaken and then placed on a magnetic stirrer plate at 220 rpm on a 4 °C work bench and stirred over a duration of 26 hours to allow for the complete dissolution of the alginate.

Third, the alginate solution was transformed into either blank alginate scaffolds (1), growth factor enriched alginate scaffolds (2) or alginate scaffolds enriched with (3) growth factors and seeded with MSCs (3).

(1) For a 1 ml sample of blank alginate, 500 µl of alginate stock solution was rapidly added to a syringe with a previously prepared solution of 460 µl of DMEM + 1 % P/S solution and 40 µl of CaSO4. The contents were mixed thoroughly. The blend was subsequently poured on to a glass slide, with another sterile glass slide being placed 2 mm above with slight pressure for 30 minutes

to allow for the gelation of the alginate. Gels intended for the production of Alginates seeded with MSCs proceeded to be cut into 8x3x2 mm bricks and frozen at -80 C for 24 hours. Following this, the Alginates were lyophilized to induce porosity to allow better cellular engraftment into the gel.

(2) For a 1 ml sample of growth factor enriched alginate, the growth factors were retrieved from their storage space of – 80 °C on crushed ice and left to thaw for 30 minutes. Recombinant human IGF-1 and recombinant human VEGF165 (R&D systems, USA) were the GF chosen to enrich the Alginates. Two syringes were prepared: one containing a mixture of 500 µl of alginate stock and 60 µl each of IGF- and VEGF-stock (the stock contained 60 µg GF/ml of alginate solution), the second syringe holding 460 µl of DMEM + 1 % P/S solution and 40 µl of CaSO4. The two mixtures from the syringes were combined into one in a rapid motion. The contents were mixed thoroughly. The blend was subsequently poured on to a glass slide, with another sterile glass slide being placed 2 mm higher with slight pressure for 30 minutes to allow for the gelation of the alginate.

(3) For a 1 ml sample of Alginates seeded with MSCs, the process as seen at point (1) and/or (2) was repeated. Subsequently, 1 million autologous MSC were removed from their flasks. They were centrifuged and combined with 20 ml of DMEM after the removal of the supernatant. Following this, the mixture was pipetted onto the frozen porous scaffold and incubated at 37 degrees Celsius and 5 % CO2 for 20 minutes. Additional 500 ml of DMEM were then added. The scaffolds seeded with MSC were subsequently allowed a supplementary 4 hours in the incubator to allow for cellular engraftment and infiltration of the porous Alginate.

An Alginate being prepared for implantation was seeded with 50 µl of a $20x10^6$ MSCs/mL cell suspension.

Following production scaffolds were immediately placed into a thermodynamically stable container, transported to the animal operating theatre and transplanted upon the open muscle crush injury of the rat.

2.3.3 Injection Bolus Production

Two different kind of bolus injection were prepared: injections totaling 6 µl containing only VEGF and IGF-1 (1), and injections totaling approximately 10 µl containing MSC as well as recombinant VEGF and IGF-1 (2).

(1) For the production of a singular bolus injection containing solely growth factors, 3 µl each of VEGF and IGF-1 (R&D Systems, Abdingdon, UK) were combined in a 5 ml syringe.

(2) For the production of a singular bolus injection containing both growth factors and MSC's, the process as seen at point (1) was repeated. Subsequently, 1 million autologous MSCs were removed from their flasks. They were centrifuged and combined with the mixture of VEGF and IGF-1 following the removal of the supernatant.

The probes were placed on ice following their production and immediately transferred into a thermodynamically stable container, transported to the animal operating theatre where they were injected along the length of the crushed soleus muscle.

2.4 Statistical Analysis

Statistical analysis was performed using SPSS 22.0 (IBM, Armonk, New York). No power analysis was performed to determine the sample size, since we relied on previous experience with group sizes to minimize the number of animals required for statistically significant results in accordance with the 3-R-principles (i.e. Replacement, Reduction, Refinement).

To test for normality, the Shapiro-Wilk test was utilized. To assess the homogeneity of variances, the Levene test was employed. Unpaired Student's t-tests or one-way analysis of variance were applied to determine statistical significance for two- or more than two groups respectively. Bonferroni's or Dunnett T3 p-value adjustment multiple comparison procedures were utilized for equal- and unequal variances respectively. Muscle force measurements were analyzed as percentages recording the comparative force of the traumatized and subsequently treated left muscle normalized to the right control muscle.

Results are presented as percentages representing the reduction of muscular force of the left muscle compared to the healthy right control (uninjured muscle contraction strength) ± the standard error of the mean. The measurements at the latter time points (28-days ± 56-days post-injury) were compared to the initial muscle force measurements within the identical treatment groups at 7-days post-injury.

$p < 0.05$ was decided to be the mark of statistical significance.

3. Results

133 4-month old female Sprague Dawley rats were included in this analysis (at least n=7 per group). A number of animals were excluded from the study due to complications stemming from anesthesia which resulted in death (n=14), disease (specifically a case of alopecia of unknown cause; n=1), or faulty measurement due to bugs with the software system or mechanical issues with the force transducer (n=2). The overall number of animals remaining in each treatment group is shown in Figure 7. As may be observed, the majority of animals had to be excluded due to death related to complications from anesthesia. Coupled with this one may observe a greater reduction in numbers in groups performed first in the timeline of experimentation, namely 7-day groups of the two treatment groups focused on injections. This likely correlates with the author's experience level. Despite reductions in numbers, the results were considered significant enough by the animal protective authorities to not warrant any replacement animals for the treatment groups from which animals were lost.

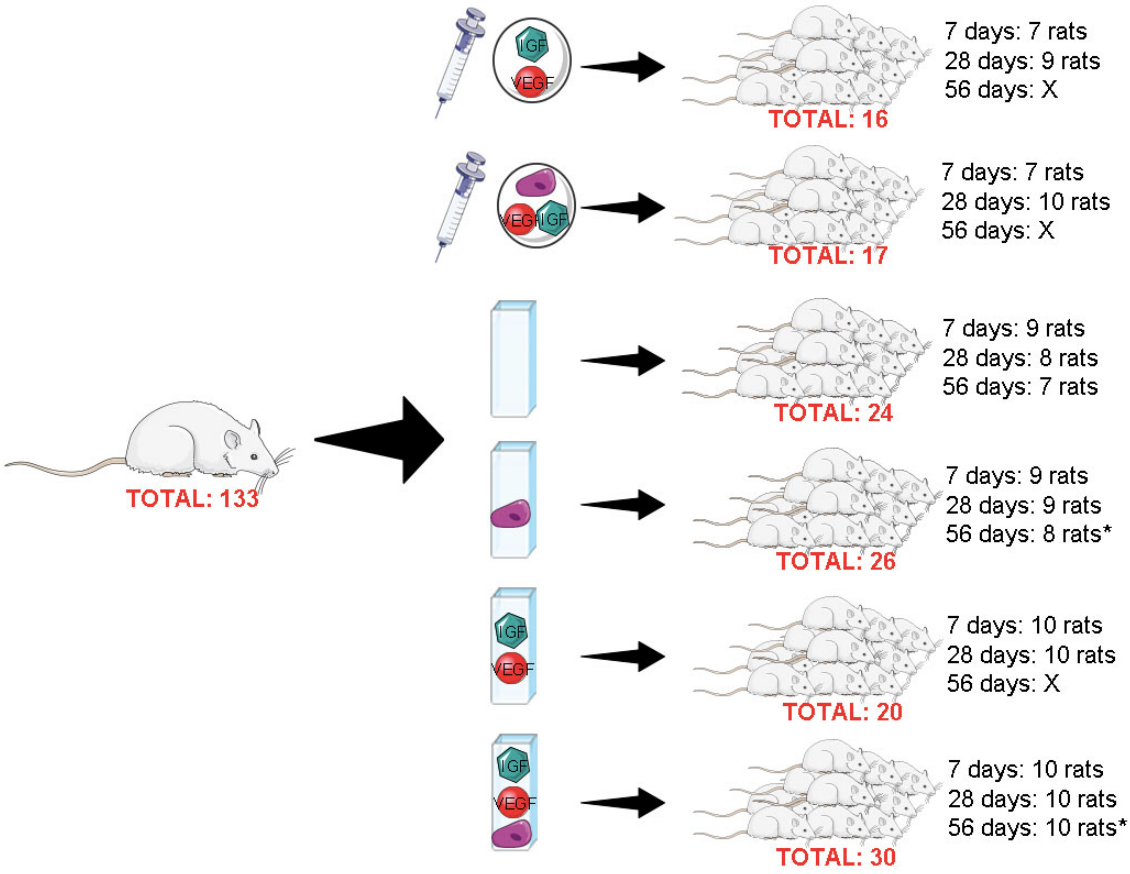

Figure 7. Division of rats into treatment groups. The animals were randomly allocated to one of 6 treatment groups: Injection of GFs, Injection of both GFs and MSCs; or insertion of blank Alginate, Alginate with MSCs, Alginate with GFs, Alginate with both GFs and MSCs.

Due to legal specification of the responsible authorities, no animals remained entirely untreated. As previously mentioned, our group had previously investigated skeletal muscle regeneration in an untreated rat model (83). This data was regarded as a sufficient baseline, and to allow for a reduction in the overall number of animals used for this study, redundancy in publication was avoided.

Furthermore, the authorities requested we perform 56-day measurements exclusively on treatment groups that appeared to have promising results at an earlier evaluated time point. Therefore, only two time points were evaluated following the inducement of muscular trauma in the Alginate + GFs group as well as the Injection of GFs- and Injection of GFs + MSCs groups:

- 7 days, and
- 28 days.

Three separate time points were evaluated for the groups consisting of Alginates alone, Alginate + MSCs, and Alginate + GFs + MSCs:

- 7 days,
- 28 days, and
- 56 days.

The time interval between the retrieval of MSCs and the application of them within bolus injections and alginates varied between 2-8 weeks.

For the sake of clarity, this section begins with a visual overview of the compounded results grouped by the time point when the animals were analyzed. This can be viewed below in Figures 8 and 9. It then proceeds with the individual treatment which have been grouped together depending on the content administered within the injection or the alginate: GFs, MSCs, or both. The individual results are displayed both in figure and table form. To conclude, an overview of the individual developments of muscle strength within the individual treatment groups across the time points at which they were analyzed is displayed and discussed.

Detailed information about the figures such as group sizes, statistical analysis, and the error bars is included in the figure legens.

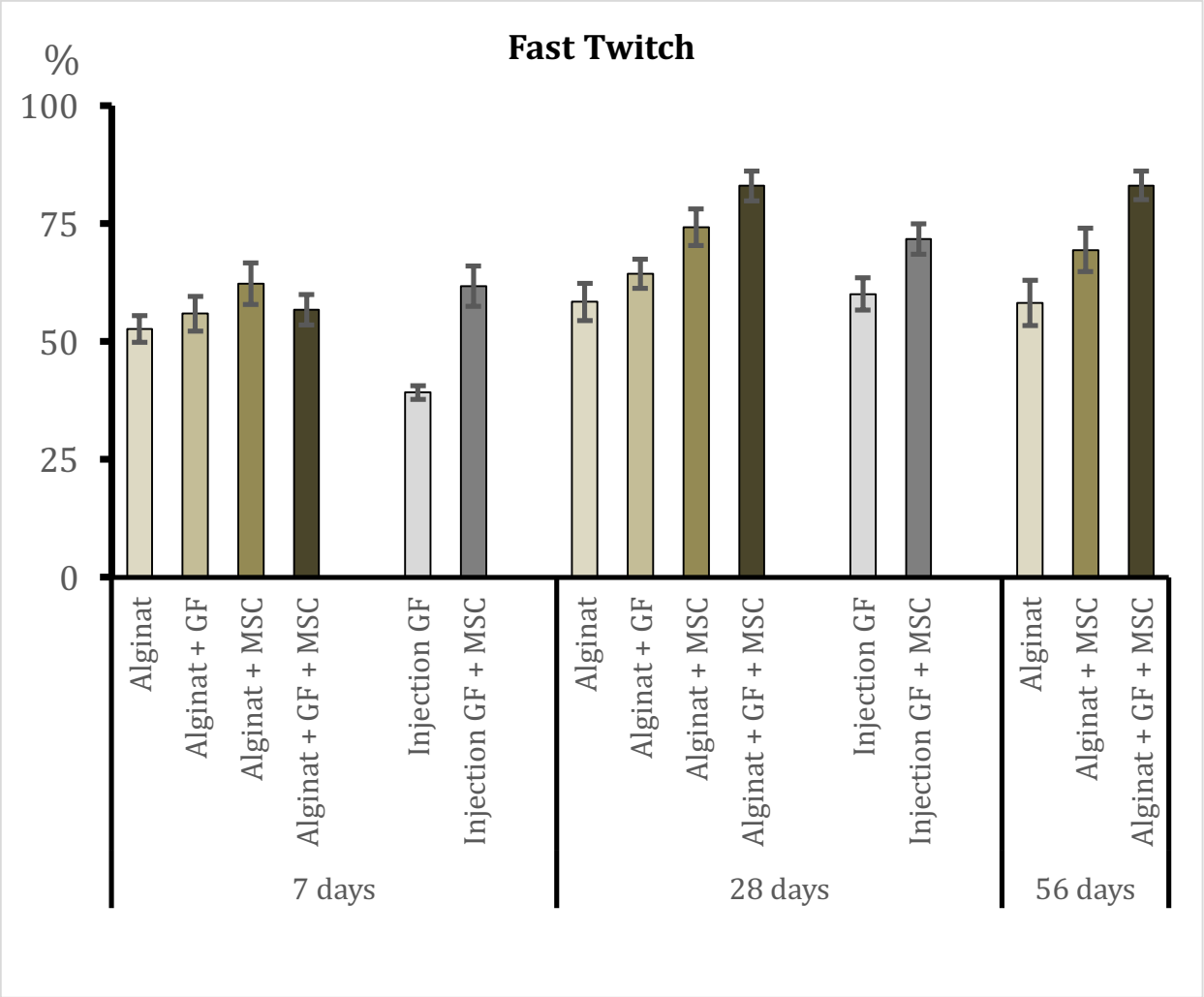

Figure 7. Functional fast twitch recovery of injured soleus muscle. Functional regeneration was assessed by measuring fast twitch forces of the left soleus muscle at days 7, 28 and in some cases 56 post injury. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

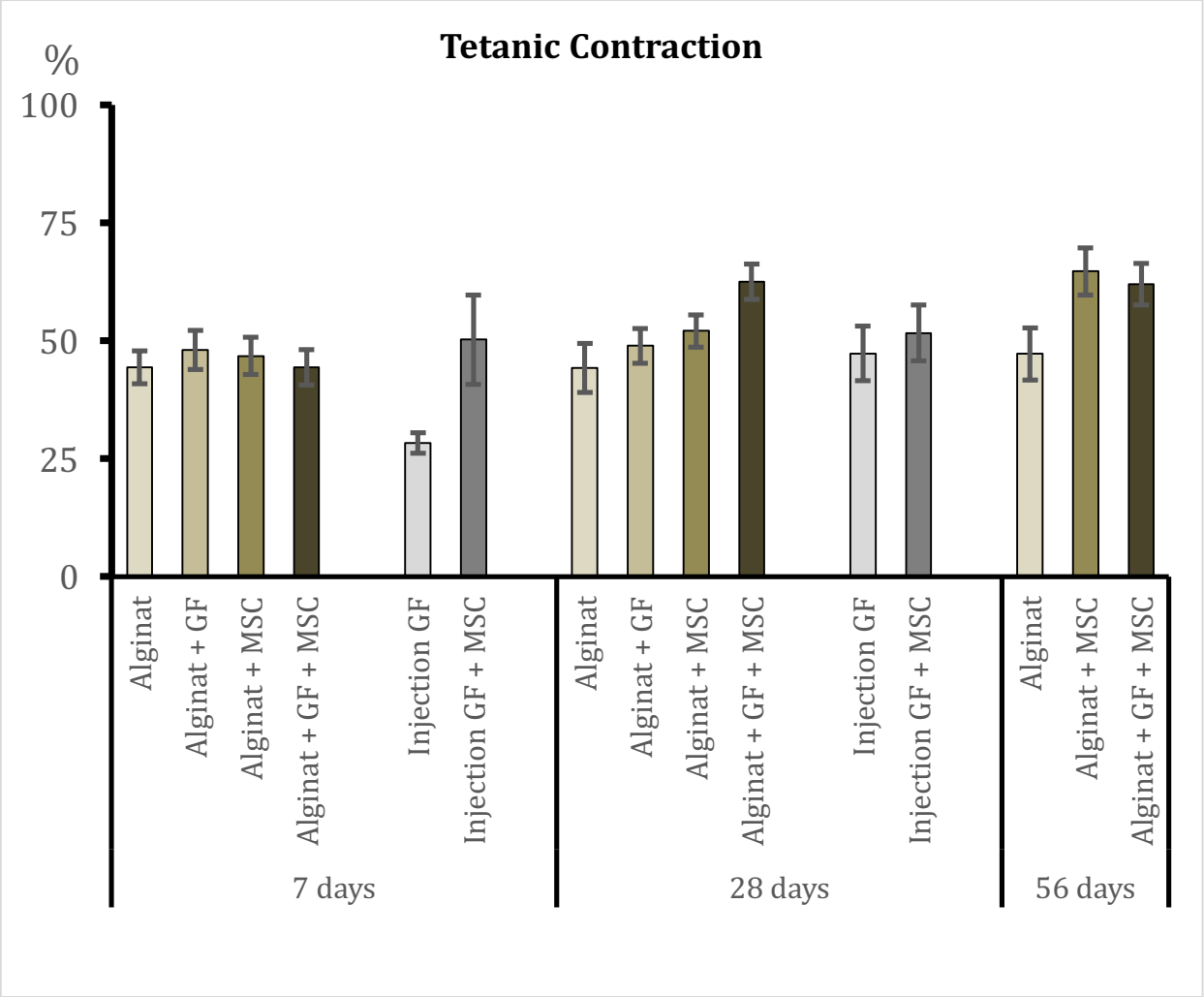

Figure 8. Functional tetanic contraction recovery of injured soleus muscle. Functional regeneration was assessed by measuring fast twitch forces of the left soleus muscle at days 7, 28 and in some cases 56 post injury. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

3.1 Effect of Alginate Implantation on Skeletal Muscle Regeneration

The results of the muscle force measurements are displayed in Table 1.

Following the application of the alginate succeeding the induced muscular trauma on day 0, fast twitch contractile force showed an increase by an average 5 % in between posttraumatic days 7 and 28. The tetanic force showed no difference.

Fast twitch contractile force subsequently showed no increase in between posttraumatic days 28 and 56. Tetanic force showed an increase by an average 3 % in between posttraumatic days 28 and 56.

Neither the mild overall increase in fast twitch nor the mild overall increase in tetanic force at days 28 or 56 were found to be statistically significant when compared to the initial measurements at day 7. This means that muscle force generally did not recover beyond the baseline status following injury at day 7.

Table 1. Results of the alginate treatment group. A total of 24 rats were part of this treatment group (7-day: 9; 28-day: 8; 56-day: 7). There was no statistically significant difference in muscle force measurement at days 28 or 56 for the fast twitch or tetanic contractions when compared to the measurement at day 7.

Alginate (control)						
	Fast twitch			Tetanic force		
	7 days	28 days	56 days	7 days	28 days	56 days
Mean (in %)	**53**	**58**	**58**	**44**	**44**	**47**
SEM	**3**	**4**	**5**	**3**	**5**	**5**
Statistical Significance (compared to day 7)		**0.863**	**0.973**		**1.000**	**1.000**

To exclude negative effects of the blank alginate scaffold on the healing process, the muscle force was compared with historical data available stemming from prior studies within this group (83). No adverse effect could be observed between groups with or without the alginate, confirming it's general biocompatibility.

3.2 Effect of Growth Factor Delivery on Skeletal Muscle Regeneration

The results of the muscle force measurements of the Injection of GF group are displayed in Table 2.

First, an injection of GFs was attempted to allow for a baseline when comparing the differing methods of release and placement of IGF-1 and $VEGF_{165}$.

Following the application of the injection succeeding the induced muscular trauma on day 0, fast twitch contractile force showed an increase by an average 21 % in between posttraumatic days 7 and 28. The tetanic force showed an increase by an average 19 % within the same time frame..

Fast twitch contractile force subsequently showed no increase in between posttraumatic days 28 and 56. Tetanic force showed an increase by an average 3 % in between posttraumatic days 28 and 56.

Both the increase in fast twitch as well as the overall increase in tetanic force at day 28 were found to be statistically significant when compared to the initial measurements at day 7. This means that the overall muscle force substantially recovered beyond the baseline status following injury by day 28. Of note, both the values of the treatment group's fast twitch and the tetanic contraction force were substantially below that of the blank alginate at day 7.

Table 2. Results of the injection of GF treatment group. A total of 16 rats were part of this treatment group (7-day: 7; 28-day: 9; 56-day: 0). There was a statistically significant difference in muscle force measurement at day 28 for the fast twitch or tetanic contractions when compared to the measurement at day 7.

Injection of GFs				
	Fast twitch		Tetanic force	
	7 days	28 days	7 days	28 days
Mean (in %)	**39**	**60**	**28**	**47**
SEM	**1**	**3**	**2**	**6**
Statistical Significance (compared to day 7)		**<0.001**		**0.016**

GFs have a short half-life in vivo and rapidly lose their bioactivity. To address the shortcoming of bolus injections, where one has to potentially use multiple, high dose GF injections for clinical translation, an alginate scaffold was used to locally provide a sustained in vivo release of IGF-1 and $VEGF_{165}$.

Following the application of the alginate seeded with GFs succeeding the induced muscular trauma on day 0, fast twitch contractile force showed an increase by an average 4 % in between posttraumatic days 7 and 28. The tetanic force decreased by an average 3 %.

Neither the mild overall increase in fast twitch nor the mild overall increase in tetanic force at day 28 were found to be statistically significant when compared to the initial measurements at day 7. This means that muscle force generally did not recover beyond the baseline status following injury at day 7 by day 28.

The results of the muscle force measurements of the Injection of GF group are displayed in Table 3.

Table 3. Results of the Alginate + GF treatment group. A total of 20 rats were part of this treatment group (7-day: 10; 28-day: 10; 56-day: 0). There was no statistically significant difference in muscle force measurement at day 28 for the fast twitch or tetanic contractions when compared to the measurement at day 7.

Alginate + GFs				
	Fast twitch		Tetanic contraction	
	7 days	28 days	7 days	28 days
Mean (in %)	**56**	**59**	**48**	**45**
SEM	**4**	**7**	**4**	**6**
Statistical Significance (compared to day 7)		**0.094**		**0.875**

No significant difference was observed between the different treatment groups and the blank alginate treatment groups at days 7 and 28 ($p>0.338$).

The results of the fast twitch and tetanic contraction force measurements of the injured and treated left soleus muscle normalized to the uninjured right muscle at day 7 post-injury are displayed in Figures 9 and 10.

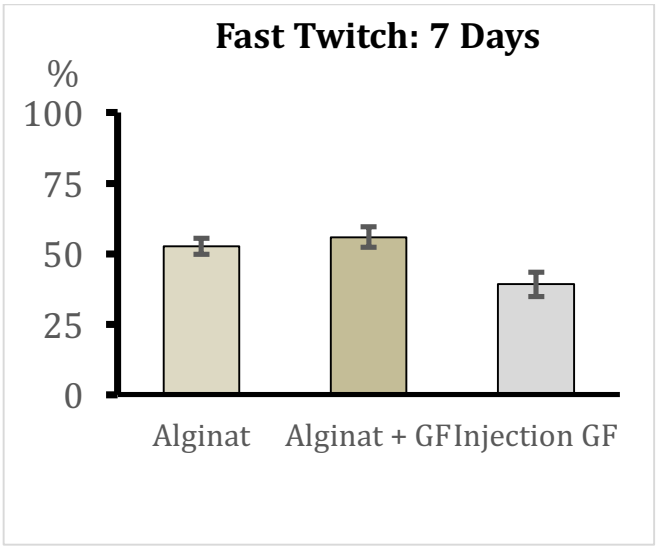

Figure 9. Functional fast twitch recovery of injured soleus muscle at day 7. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

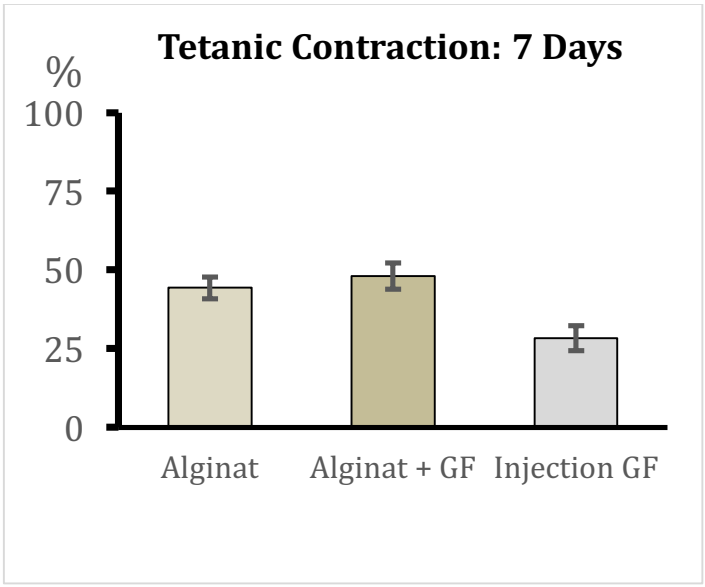

Figure 10. Functional tetanic contraction force recovery of injured soleus muscle at day 7. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

The results of the fast twitch and tetanic contraction force measurements of the injured and treated left soleus muscle normalized to the uninjured right muscle at day 28 post-injury are displayed in Figures 11 and 12.

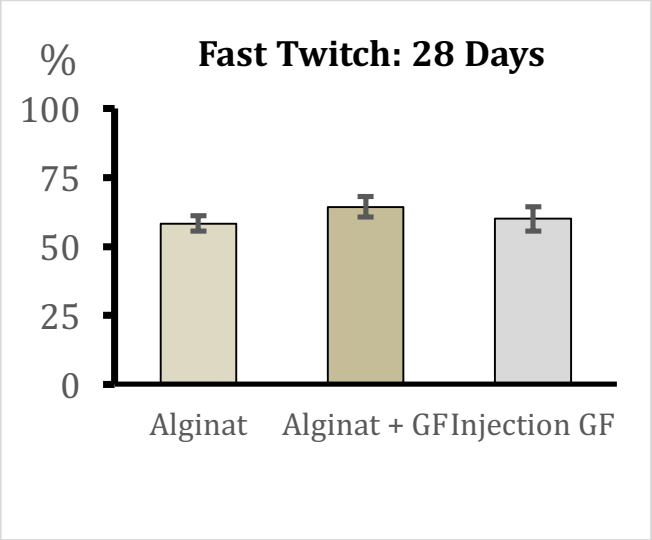

Figure 11. Functional fast twitch recovery of injured soleus muscle at day 28. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

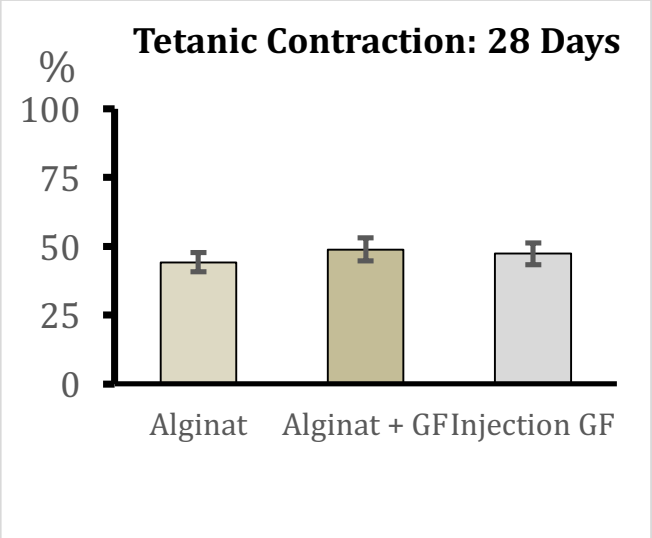

Figure 12. Functional tetanic contraction force recovery of injured soleus muscle at day 28. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

3.3 Effect of Mesenchymal Stromal Cell Delivery on Skeletal Muscle Regeneration

The results of the muscle force measurements of the application of an Alginate containing just MSCs are displayed in Table 4.

Following the application of the Alginate seeded with MSCs succeeding the induced muscular trauma on day 0, fast twitch contractile force showed an increase by an average 12 % in between posttraumatic days 7 and 28. The tetanic force showed no change within the same time frame.

Fast twitch contractile force subsequently showed a decrease by an average 5 % in between posttraumatic days 28 and 56. Tetanic force showed an increase by an average 18 % in between posttraumatic days 28 and 56.

Neither the overall increase in fast twitch as well as the stagnant tetanic force at day 28 were found to be statistically significant when compared to the initial measurements at day 7. This means that the overall muscle force did not substantially recovered beyond the baseline status following injury by day 28. Whilst this remained the same for fast twitch force at day 56, the increase of tetanic force by day 56 days following injury by contrast was found to be significant.

Table 4. Results of the Alginate + MSC treatment group. A total of 26 rats were part of this treatment group (7-day: 9; 28-day: 9; 56-day: 8). There was a statistically significant difference in muscle force measurement at day 56 for the tetanic contractions when compared to the measurement at day 7.

Alginate + MSC						
	Fast twitch			Tetanic contraction		
	7 days	28 days	56 days	7 days	28 days	56 days
Mean (in %)	**62**	**74**	**69**	**47**	**47**	**65**
SEM	**4**	**9**	**9**	**4**	**6**	**5**
Statistical Significance (compared to day 7)		**0.521**	**1.000**		**1.000**	**0.028**

A significant difference in fast twitch recovery was observed between the Alginate + MSC group and the blank alginate treatment group at day 28 ($p= 0.024$). A significant difference in tetanic force recovery was observed between the Alginate + MSC group and the blank alginate treatment group at day 56 ($p= 0.028$). The results of the 56 day group are discussed in depth in Section 3.5.

The results of the fast twitch and tetanic contraction force measurements of the injured and treated left soleus muscle normalized to the uninjured right muscle at day 7 post-injury are displayed in Figures 13 and 14.

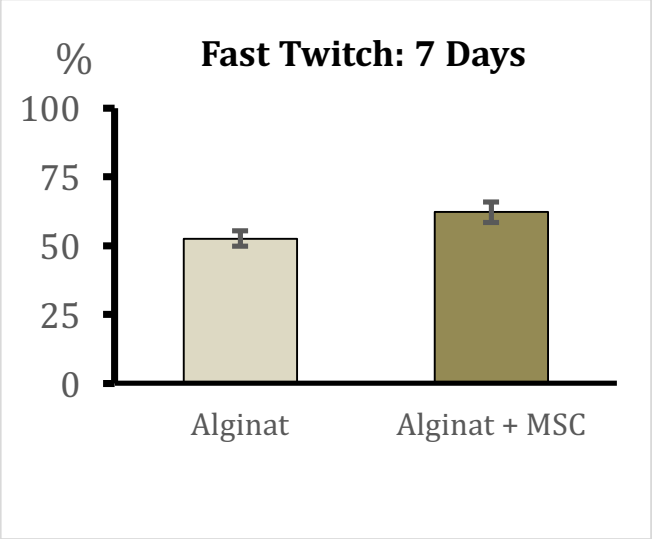

Figure 13. Functional fast twitch recovery of injured soleus muscle at day 7. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

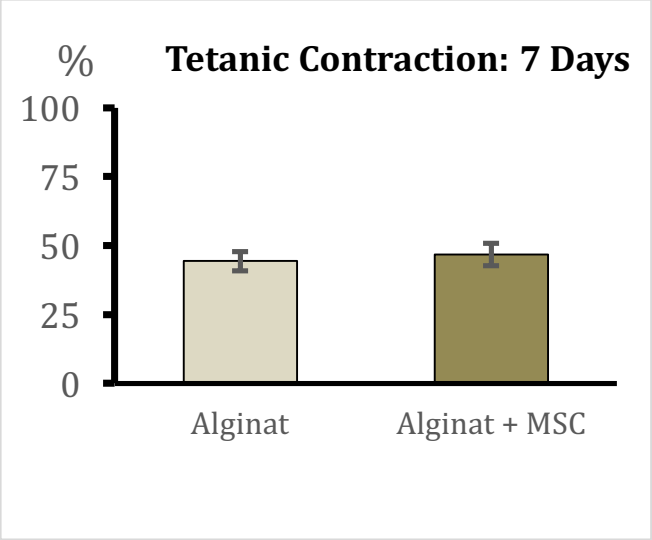

Figure 14. Functional tetanic contraction force recovery of injured soleus muscle at day 7. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

The results of the fast twitch and tetanic contraction force measurements of the injured and treated left soleus muscle normalized to the uninjured right muscle at day 28 post-injury are displayed in Figures 15 and 16.

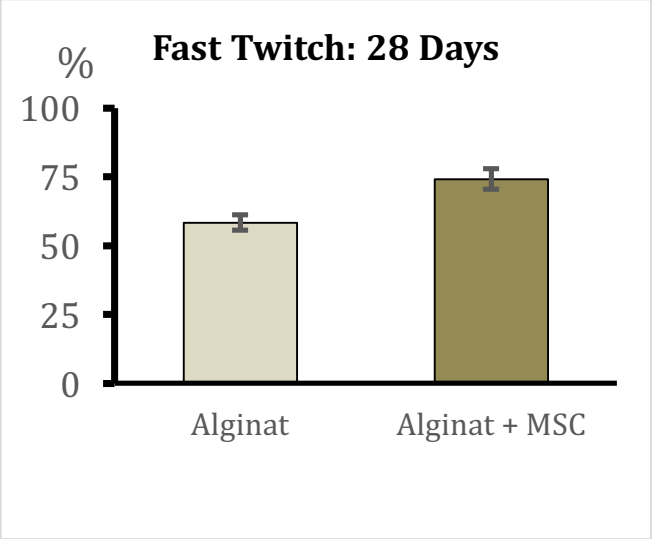

Figure 15. Functional fast twitch recovery of injured soleus muscle at day 28. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

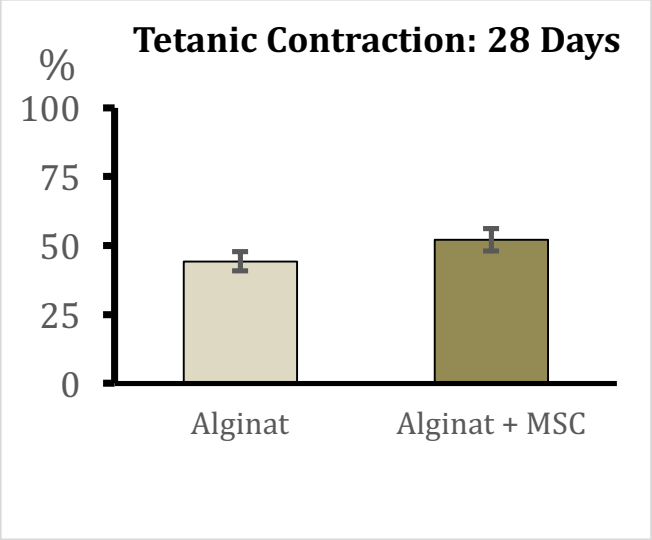

Figure 16. Functional tetanic contraction force recovery of injured soleus muscle at day 28. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

3.4 Effect of Combined Growth Factor and Mesenchymal Stromal Cell Delivery on Skeletal Muscle Regeneration

The results of the muscle force measurements of the application of the combined delivery of GFs and MSCs as an injection are displayed in Table 5.

Following the application of the injection succeeding the induced muscular trauma on day 0, fast twitch contractile force showed an increase by an average 10 % in between posttraumatic days 7 and 28. The tetanic force showed an increase by an average 2 % within the same time frame..

Neither the increase in fast twitch as well as the increase in tetanic force at day 28 were found to be statistically significant when compared to the initial measurements at day 7. This means that the overall muscle force did not substantially recover beyond the baseline status following injury by day 28.

Table 5. Results of the injection of GF + MSC treatment group. A total of 17 rats were part of this treatment group (7-day: 7; 28-day: 10; 56-day: 0). There was no statistically significant difference in muscle force measurement at day 28 for the fast twitch or tetanic contractions when compared to the measurement at day 7.

Injection of GF + MSC				
	Fast twitch		Tetanic force	
	7 days	28 days	7 days	28 days
Mean	**62**	**72**	**50**	**52**
SEM	**4**	**3**	**10**	**6**
Statistical Significance (compared to day 7)		**0.074**		**0.896**

No significant difference was observed between the different treatment groups and the blank alginate treatment groups at days 7 and 28 ($p>0.160$).

The results of the muscle force measurements of the application of the combined delivery of GFs and MSCs within an Alginate are displayed in Table 6.

Following the application of the Alginate succeeding the induced muscular trauma on day 0, fast twitch contractile force showed an increase by an average 26 % in between posttraumatic days 7 and 28. The tetanic force showed an increase by an average 18 % within the same time frame.

Both the increase in fast twitch as well as the increase in tetanic force at day 28 were found to be statistically significant when compared to the initial measurements at day 7. This means that the overall muscle force substantially recovered beyond the baseline status following injury by day 28.

Both fast twitch and tetanic contraction force subsequently remained the same posttraumatic days 28 and 56. The difference between the overall force at day 56 remained statistically significant when compared to the initial measurements at day 7.

Table 6: Results of the Alginate + GF + MSC treatment group. A total of 30 rats were part of this treatment group (7-day: 10; 28-day: 10; 56-day: 10). There was a statistically significant difference in muscle force measurement at days 28 and 56 for both the fast twitch tetanic contractions when compared to the measurement at day 7.

Alginate + GF + MSC						
	Fast twitch			Tetanic contraction		
	7 days	28 days	56 days	7 days	28 days	56 days
Mean (in %)	**57**	**83**	**83**	**44**	**62**	**62**
SEM	**3**	**3**	**3**	**4**	**4**	**4**
Statistical Significance (compared to day 7)		**0.001**	**0.001**		**0.010**	**0.034**

A significant difference in fast twitch recovery was observed between the Alginate + GF + MSC group and the blank alginate treatment group at day 28 (p= 0.001). Similarly, a significant difference in tetanic force recovery was observed between the Alginate + GF + MSC group and the blank alginate treatment group at day 28 (p= 0.018). The results of the 56-day group are discussed in depth in Section 3.5.

The results of the fast twitch and tetanic contraction force measurements of the injured and treated left soleus muscle normalized to the uninjured right muscle at day 7 post-injury are displayed in Figures 17 and 18.

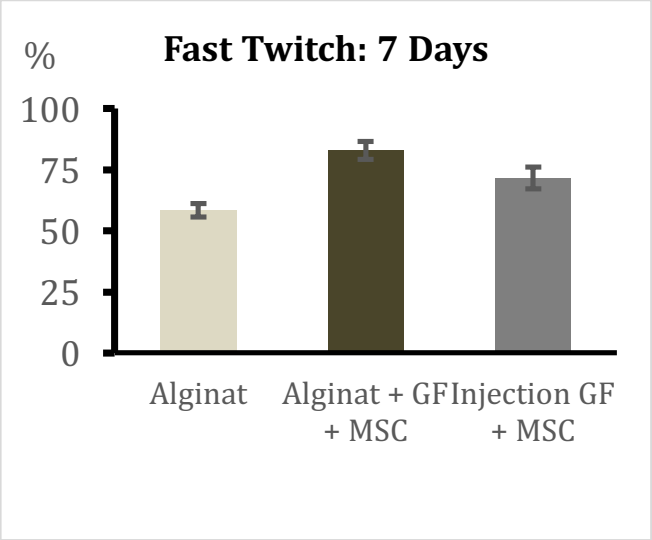

Figure 17. Functional fast twitch recovery of injured soleus muscle at day 7. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

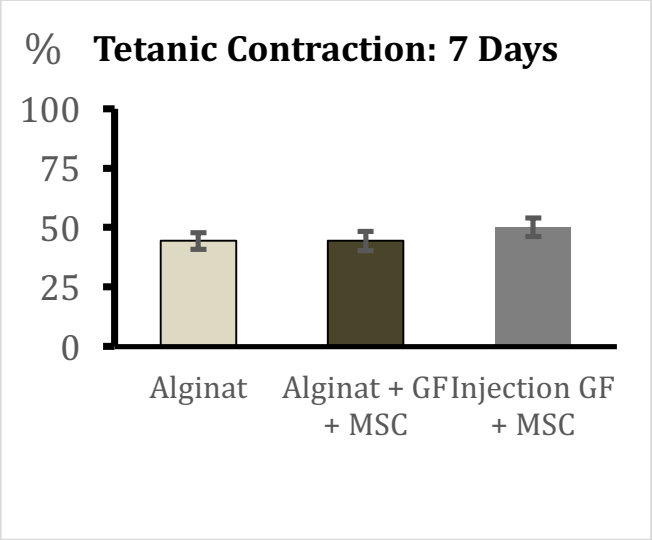

Figure 18. Functional tetanic contraction force recovery of injured soleus muscle at day 7. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

The results of the fast twitch and tetanic contraction force measurements of the injured and treated left soleus muscle normalized to the uninjured right muscle at day 28 post-injury are displayed in Figures 19 and 20.

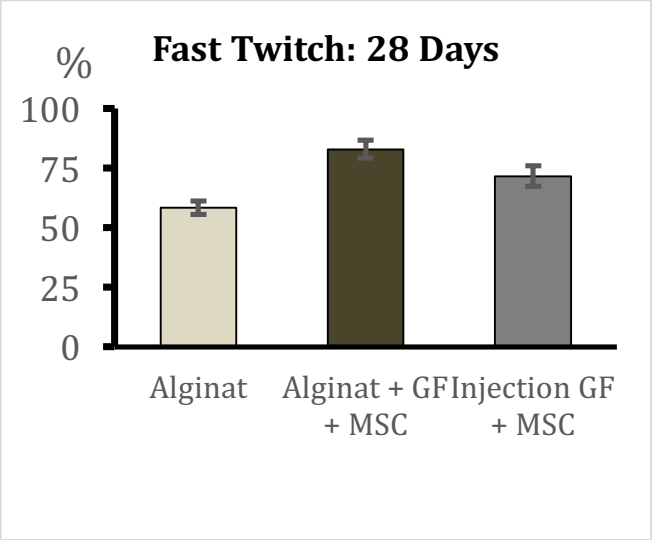

Figure 19. Functional fast twitch recovery of injured soleus muscle at day 28. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

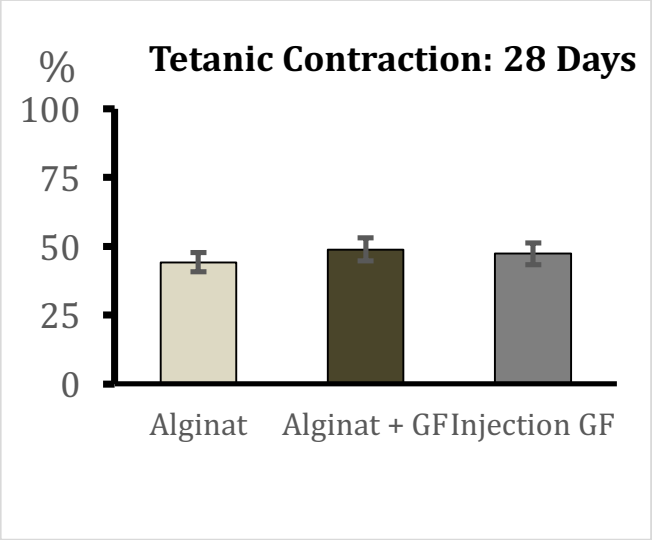

Figure 20. Functional tetanic contraction force recovery of injured soleus muscle at day 28. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

3.5 Long-term Effect of Combined Growth Factor and Mesenchymal Stromal Cell Delivery on Skeletal Muscle Regeneration

The legal specification of the responsible authorities only permitted us to monitor the long term effects two groups: Alginate + MSC and Alginate + GF + MSC.

A significant difference in fast twitch recovery was observed between the Alginate + GF + MSC group and the blank alginate treatment group at day 56 ($p= 0.028$). Similarly, a significant difference in tetanic force recovery was observed between the Alginate + GF + MSC group and the blank alginate treatment group at day 56 ($p= 0.047$).

For the Alginate + MSC treatment group, no significant difference in fast twitch recovery was observed between the Alginate + MSC group and the blank alginate treatment group at day 56 ($p= 1$). However, a significant difference in tetanic force recovery was observed between the Alginate + MSC group and the blank alginate treatment group at day 56 ($p= 0.028$).

The results of the fast twitch and tetanic contraction force measurements of the injured and treated left soleus muscle normalized to the uninjured right muscle at day 56 post-injury are displayed in Figures 21 and 22.

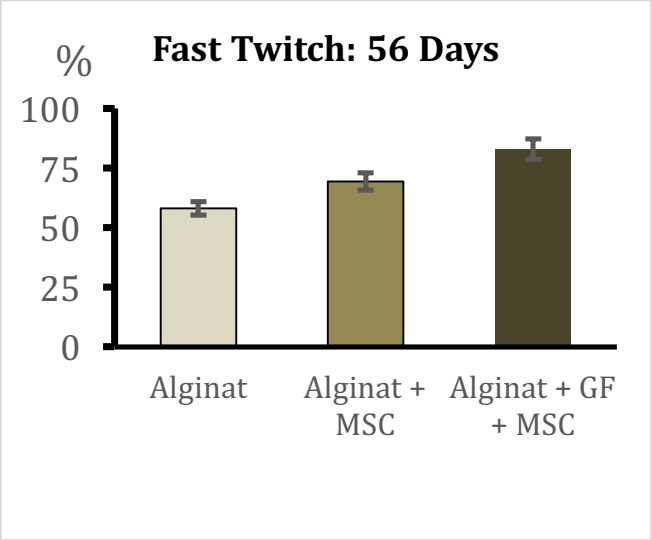

Figure 21. Functional fast twitch recovery of injured soleus muscle at day 56. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

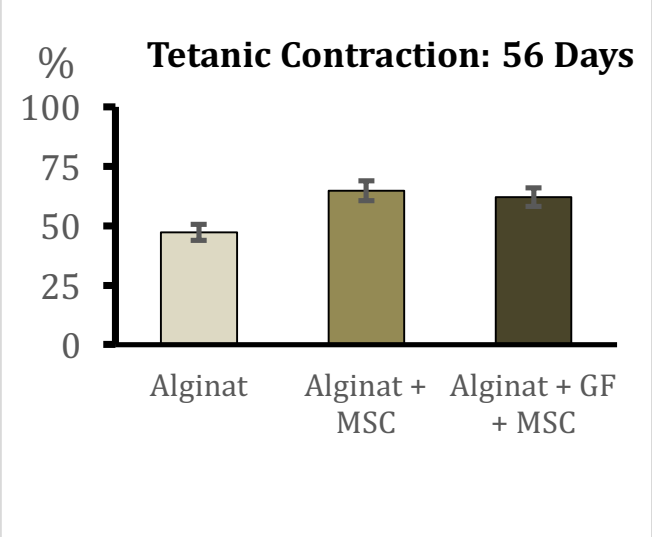

Figure 22. Functional tetanic contraction force recovery of injured soleus muscle at day 56. The measurement was normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard error of the mean.

3.6 Progression of Skeletal Muscle Regeneration over Time

The results of the fast twitch and tetanic contraction force measurements of the injured and treated left soleus muscle normalized to the uninjured right muscle at days 7, 28 and 56 post-injury are displayed in Figures 23 and 24. They are presented in the context of historic measurements of Sodium Chloride and MSC injections, which were evaluated at day 28 only.

The injection of Sodium Chloride solution and the injection of MSC represent historical data previously collected by the group.

Injection of Sodium Chloride alone resulted in a fast twitch force of 59 % (standard deviation: 12 %), and a tetanic contraction force of 39 % (standard deviation: 10 %). Injection of MSCs alone resulted in a fast twitch force of 72 % (standard deviation: 13 %), and a tetanic contraction force of 53 % (standard deviation: 8 %). The results of both treatment groups were found not to be statistically significant when compared to the implantation of a blank alginate ($p=0.8$).

The results are pooled by treatment group to allow for an overview of individual muscle force recovery within the group over time.

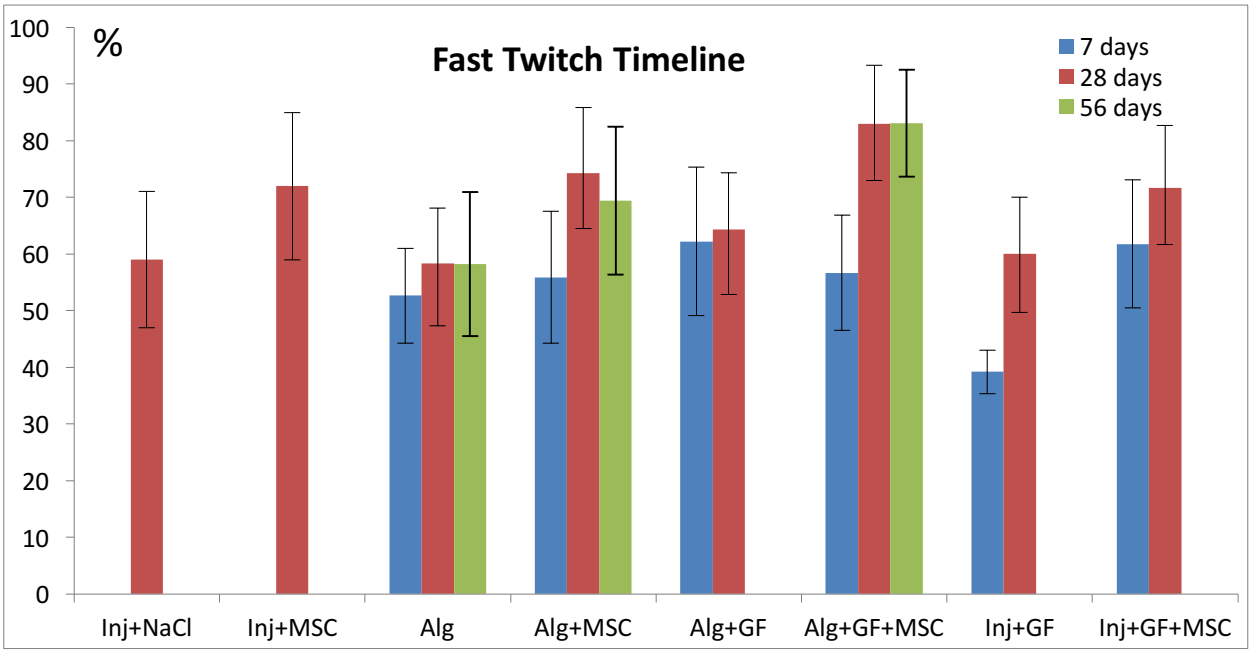

Figure 23. Timeline of fast twitch recovery. The measurements were taken at days 7, 28, and 56. They were normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard deviation.

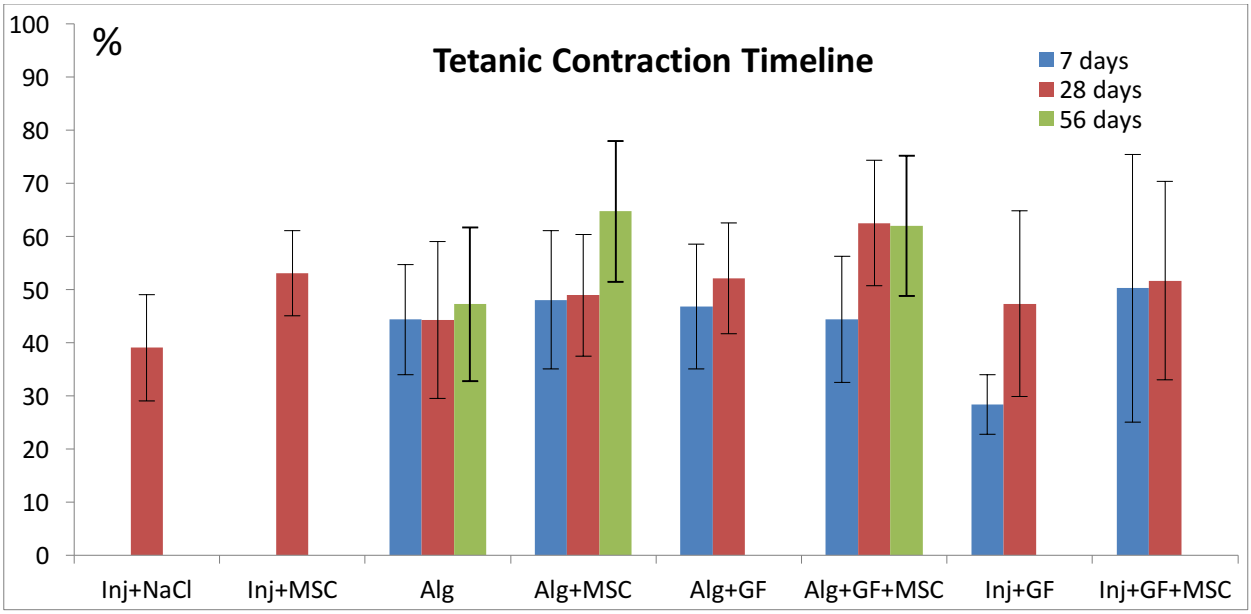

Figure 24. Timeline of tetanic force recovery. The measurements were taken at days 7, 28, and 56. They were normalized to the uninjured (intact) contralateral right muscle. All measurements are in percent (%). Bars represent mean values and error bars represent standard deviation.

4. Discussion

We could confirm our hypothesis that the transplantation of Alginates enriched with GFs and seeded with MSCs leads to improved functional outcomes following trauma.

Overall, both the fast twitch- and the tetanic force were significantly diminished in all groups at day 7. Both the lowest fast twitch force as well as the lowest tetanic force at this time point were found following the injection of GFs on day 7 post-trauma, which brings up questions over how GFs may interfere in the regenerative cascades in a manner that actually harms the outcome.

At day 28, the fast twitch contraction force differed significantly between the control Alginate group and the groups transplanted with Alginates seeded with MSCs and Alginates enriched with GFs and MSCs. The fast twitch force was found to be highest in the group transplanted with Alginates enriched with GFs and MSCs, which was significantly higher than the groups transplanted with Alginates alone. Groups transplanted with Alginates seeded with MSCs alone also showed significantly higher fast twitch contractile force compared to the Alginate controls at that time point. Similarly, the highest tetanic contraction force at 28 days following trauma was found in the group transplanted with Alginates enriched with GFs and MSCs. This was found to be significantly higher than the tetanic contraction force of the control group,

Overall, the group treated with Alginates enriched with GFs and seeded with MSCs showed the strongest increases of both fast twitch- and tetanic contraction forces from day 7 to day 28. The control Alginate group, likely mirroring the natural conditions of muscle regeneration without therapeutic intervention, showed a low increase in fast twitch force and no increase in tetanic muscle force at day 28 compared to the performance at day 7 following trauma.

4.1 Tissue Engineering Therapeutic Approaches to Skeletal Muscle Injury

As broad solutions to a variety of muscular injuries, growth factor- and cell delivery via bolus injections and tissue engineering strategies have captured scientist's interests.

Bolus therapies rely on the injection of a variety of growth factors- and/or cells with mesenchymal- or muscular characteristics in a systematic or localized manner. Unfortunately, the complexity of the post-traumatic cellular environment appears to result in unsatisfactory outcomes when individual proportions of growth factors are selectively boosted to unnaturally high levels at unphysiological time intervals (90, 91). This is something we could confirm with our own results,

with bolus injections of of IGF-1 and $VEGF_{165}$ resulting in reduced muscle force at day 7 following crush injury when compared to a blank Alginate. Whilst this decrease could be recovered by day 28, the GFs clearly had an adverse effect on the immediate recovery of our rat's soleus muscle. This supports the scientific consensus that aimless injection of variable doses of GFs may do more harm than good during skeletal muscle regeneration.

In contrast to simple bolus injections, tissue engineering of skeletal muscle attempts to recreate the three-dimensional tissue by seeding muscle cells or precursors and extracellular matrix components upon a scaffold which is subsequently implanted into the damaged area (92, 93). This may be combined with a spatiotemporally defined release of GFs.

Within tissue engineering, one needs to differentiate between two classical approaches: the *in vitro* strategy and the *in vivo* strategy.

In vitro strategies have focused primarily on the preproduction of functional tissue which may then be transplanted into a patient as whole. If developed to the maximum of its possibilities, this technique would allow for patient specific transplants which do not induce an immunogenic response and are perfectly shaped for the muscular defect in question, making it an attractive option for larger volumetric defects resulting from tumour resections or blast trauma (94). In vitro muscle is grown by culturing cells on an appropriate biomaterial until the resulting unit resembles skeletal muscle in at least some of its major properties (contractility, shape etc.). Whilst ideally the most patient-adaptable strategy, it bears several disadvantages.

First, fabricating large pieces of any vascularized tissue has presented a major challenge to engineers. Whilst recent developments in the geospatial architecture of biomaterials via 3D-printing technology have offered some respite by pre-printing some basic channels which may be seeded by endothelial cells, they do not yet represent the status quo (95). The size of a nonvascularized construct is always intrinsically limited since cell survival may be threatened by a lack of nutritional and respiratory support which environmental diffusion is incapable of compensating for (96, 97).

Second, the correct alignment of cells that allow for the formation of myofibers are a biologic problem which has yet to be resolved. Cells may not necessarily arrange in the polar manner required. One possible solution to this may be multi-layered scaffolds specifically designed to

allow for a growth and differentiation of precursor cells in a manner that preserves the structural integrity of the muscle (98). But even then, deposition of fibrous collagen and scar formation may disrupt the arrangement both of the remaining as well as the regenerating myofibers. Modulation of scar formation is therefore an important aspect of regeneration, be it through manipulation of the local niche through the use of extracellular matrices or the application of small molecules to mitigate fibrosis via the simulation of myogenic differentiation (99, 100).

Third, the innervation of any unit of muscle created in a petri dish has thus been unsatisfactory. Nerve cells are notoriously difficult to successfully cultivate in vitro, and a combination of muscular and neuronal culturing techniques has thus far not been attempted.

Fourth, for the reasons given above, the myofiber density, arrangement and alignment necessary to allow for even minimal functionality is out of the question. Without said density the engineered construct would be incapable of absorbing and transferring the forces physiologically imbued on a muscle as a functional unit.

Several successes have been recorded in the creation of artificial human skeletal muscle and the transplantation of larger pieces of engineered muscle (101-103). Nonetheless, the implementation of such extensive, cost-, labor- and time intensive measures would be mostly restricted to cases of volumetric muscle loss following severe trauma or extensive surgeries such as tumor operations and have little application in the minor but much more common forms of muscular injury.

In vivo strategies, in contrast, have focused primarily on the in situ implantation of cells and/or growth factors. This was the strategy our group focused on. The hypothesis here is generally that the cells and factors transplanted stimulate or enhance the local healing process by inducing biochemical cascades that favour regeneration over fibrosis. Ideally, the cells may even engraft unto the skeletal muscle and physically contribute to the formation of new tissue, as demonstrated by some of our group's previous studies (86). Unfortunately, this strategy also has its disadvantages.

First, the cells and growth factors are not locally bound and may travel the body rather than remain at the location where they were transplanted. This leads to several problems. There is a risk that the cells and growth factors may never contribute to the injury itself. On top of this, the cells and

growth factors may have systemic effects on other types of tissue or the organism as a whole who's effects may not be accurately predicted in animal models.

Second, cells which are directly transplanted into the immediate vicinity of an injury risk attacks from immune cells which are a part of the physiologic response to trauma (3). These attacks may severely diminish cell number and viability.

To address both of these challenges, these cells and growth factors may be primarily transplanted via a biomaterial scaffold that houses them. Scaffolds form a protective housing around the cells whilst allowing for a consistent, time sensitive release of any chemicals and growth factors inserted into them. Ideally, the combined mechanical and biochemical properties of these hybrid biomaterials result in a recruitment of local cells conducive to regeneration.

4.2 Selection of Scaffold

In tissue engineering, scaffolds play a vital role in supporting the cells and GFs which combined represent the triad of the field. Without cell survival, the utility of tissue engineering is minimal. Scaffolds must therefore be not only biocompatible and biodegradable to avoid an immune response or an adverse inflammatory (foreign body) reaction by the host, but also be able to adapt to a tissue's biomechanical- and physiological properties. As discussed above, one must differentiate between in vitro tissue engineering, where an artificial tissue is created in the laboratory over a significant period of time, and in situ tissue engineering, where cells and growth factors are combined on a scaffold. Whilst in vitro skeletal muscle engineering aims to cover significant volumetric defects and ideally replace an area of muscle loss in its entirety, the goal of in situ skeletal muscle engineering attempts to support the intrinsic regenerative process through paracrine signaling (104).

Since our model of muscle injury doesn't aim to result in volumetric muscle loss, we chose to create our scaffold keeping in mind that this form of in situ skeletal muscle engineering may be more applicable to a greater number of muscular injuries and more easily brought from bench- to bedside.

Biomaterials such as collagen, poly-l-lactic acid, decellularized extracellular matrix, gelatin, hyaluronic acid and polycaprolactone have a number of positive attributes such as electrical conductivity and/or structural features conducive of cellular alignment and a pro-myogenic

environment. Alginates however appeared ideally suited to our study given their high porosity conducive of cell survival- and proliferation and their mechanical properties which result in a protective, nurturing environment supportive of uninterrupted paracrine signaling by the seeded cells (105).

4.3 Selection of Growth Factors

In this study, we decided to enrich the scaffold of our choice with the GFs IGF-1 and $VEGF_{165}$. The regeneration of skeletal muscle involves a time sensitive release of a variety of inflammatory, pro- and anti-apoptotic cytokines, and angiogenic- as well as neurogenic growth factors. GFs such as Epidermal Growth Factor, Platelet Derived Growth Factor, VEGF, and IGF-1 all bear important roles during the regenerative process and operate by regulating the extracellular environment, allowing for recruitment and stimulation of myogenic-, angiogenic- and synaptogenic progenitors, and playing a role in the immunomodulation immediately following the injury (106).

Though it is tempting to assume that increases in any of these growth factors could result in an automatically improved outcome, select studies-as well as our own results- point to the contrary. The potential side effects of unwittingly affecting the dynamic of these growth factors in the wrong manner is not to be underestimated given the delicate balance of the regenerative process. Overexpression of Transforming Growth Factor-beta, usually responsible for the regeneration and restructuring of the extracellular matrix, and Fibroblast Growth Factor, usually a promitogenic force increasing the proliferation of satellite cells and mesenchymal cells, have been shown to result in an inhibition of myogenic differentiation, significantly slowing the regenerative process (107).

The GFs in this study were utilized primarily with the objective of supporting the transplanted MSCs, and thereby the MSC-driven paracrine modulation of the regenerative process. VEGF and IGF-1 utilized as treatment in murine ischemic models of muscle injury have repeatedly been shown to decrease apoptosis and increase cell recruitment, thereby supporting myogenic-, angiogenic- and neurogenic regeneration (108-110).

As anticipated, our groups transplanted with Alginates enriched with GFs and seeded with MSCs outperformed all others. To optimize our effort of boosting the regenerative process, we chose to engineer a porous alginate hydrogel capable of providing a safe microenvironment for the MSCs seeded on it and allowing for a sustained release of the GFs it was enriched with.

4.4 Selection of Cells

A wide variety of cells have been used successfully for cell therapy of muscle injuries. We decided to utilize MSCs for our experiments for two reasons.

First, our laboratory has experience utilizing MSCs in attempts to bolster the regenerative process of skeletal muscle (80, 86).

Second, MSCs are easily obtained (most commonly from the bone marrow, umbilical tissue, dental pulp, skeletal muscle- or adipose tissue), as well as robust and simple to expand in the laboratory.

MSCs have been identified to be multipotent cells capable of differentiating into adipose-, cartilaginous-, myogenic- and bone tissues several years ago (111, 112). Multiple studies, including some of our own, have attempted to influence muscle regeneration by utilizing autologous MSCs and have shown promising results by successfully supporting the healing process following muscle injury (82, 86, 87). MSCs have been found to primarily take on a paracrine role by releasing cytokines that modulate the inflammatory response, reduce the apoptosis of muscle cells and induce blood vessel growth (29, 113). One major downside of MSC's may be the decline of their regenerative properties with age. This is particularly problematic in view of the fact that most of the musculoskeletal disease burden stems from the elderly population. Patient derived induced pluripotent stem cells may, at least in part, be an answer to this dilemma (114, 115).

Embryonic stem cells and patient derived induced pluripotent stem cells have recently been experimentally induced to differentiate into myogenic progenitors which have shown success in boosting the regenerative process and replenishing the satellite cell pool (116, 117). Rather than supporting muscle regeneration through paracrine signaling, these cells appear to differentiate and engraft within the damaged tissue, physically contributing to the reconstitution of the injured area. Embryonic stem cells however suffer the issue of ethical procurement, whilst induced pluripotent stem cell require significantly greater time commitments and costs.

4.5 Rat Models of Skeletal Muscle Injury

Due to the complexity of the biology behind skeletal muscle injury and regeneration, a number of animal models have been established over the years. The most common animals used by far are

murine models, specifically rats and mice. A variety of different models of skeletal muscle trauma have been established. They can broadly be grouped into physiological-, mechanical-, chemical-/toxic- and thermal- and occlusive- stressor. Every model differs in the extent of the application of the amount of force, the amount of toxin and so forth, making them nearly impossible to compare and correlate amongst each other.

In rats, the most commonly traumatized muscles are the soleus-, the gastrocnemicus- and the extensor digitorum longus muscles. Other habitually traumatized muscles included the tibialis anterior-, the triceps surae-, the plantaris and the rectus muscle.

Less commonly used methods of trauma induction in rats, though in differing variations, are physiological trauma by exercise, ischemia reperfusion injuries and thermal trauma by burning or freezing of the muscle (118-123).

Mechanical trauma is more frequently performed and generally induced manually via a crush injury which is generally presented as a scenario were the surgeon manually closes a forceps or clamp over the muscle of the animal and keeps it closed for an assigned amount of time. This is the methodology adapted for our technique. Though the the force with which the trauma is induced can be approximated for instrumented clamps which are locked firmly into place, such efforts can not be undertaken for studies which utilize forceps for trauma induction. The application of force here is solely reliant on the surgeon, therefore being highly unpredicable in terms of the amount of trauma and damage done upon the muscular tissue (47, 87, 124, 125). Mechanical trauma induced by a machine may be administered through a variety of mechanisms. These machines are usually designed and built at the institutions where the research is being performed, making the results inherently difficult to reproduce. This is a shortcoming our measurement apparatus suffer from as well. Additional techniques such as the dropping o weights and hammers have been constructed, with calculations being made to measure the standardized amount of force applied to the muscle (126-128).

Finally, the chemical/toxic approach was designed for a standardized amount of fluids, for example Bupivacain Hydrochloride or the snake venom Notexin, to be injected directly into the muscular tissue, thereby causing a dysfunction of Ca2+ channels resulting in the necrosis of cells (129-131). The problem with this method is its disjuncture from the physiological processes which form the natural response to a skeletal muscle injury as it most commonly occurs in human beings.

Though select groups have attempted to design a standardized method of trauma induction, most

have relied heavily on processes improvised solely for the premise of the experiment at hand. Hence, the development of standardized procedures for the induction of muscle trauma in animal models remains an expansive area for future research. Overall, animals bred and utilized for testing in the medical field are of incalculable value and should not be turned to unless certain measures of standardization which translate to a level of possible comparability of results in between studies which perform animal experiments such as skeletal muscle trauma upon similar premises is affirmed. Greater standardization amongst these procedures would lead to improved settings to transfer collective experimental research work and elevate it to the clinical level, for the profit of the patient.

In this vein, the crush injury model used in this study should be retained. It has been utilized for many years, and should be reused in further analyses to allow for a cohesive, reproducible, and most importantly comparable body of literature.

4.6 Measures of Outcome

Most studies examining muscle regeneration tend to quantify progress (or lack thereof) in a histological manner. Amongst other things they measure the areas consisting of fibrotic tissue, assess the number of novel muscle fibers and blood vessels, and examine the stages of neuronal regeneration. Whilst these parameters allow for an interesting overview of the regenerative process, they do not represent the scope of functional recuperation.

Human functional outcomes following muscle injury are more commonly used as a final end point in studies, though they frequently display great variations depending on the type of muscle injury, the treatments assessed, the patients' compliance, as well as the patients' basic characteristics such as age, sex, and co-existing conditions. Given the importance of an eventual clinical translation of the kind of tissue engineering examined in our study given the current unsatisfactory option for treatment of muscular injury, prioritizing the functional outcome in studies analyzing protocols of cell- and/or biomaterial based therapies for skeletal muscle regeneration appears of great relevance.

Unfortunately, muscle force measurements require a fairly complex set-up and a long operative time. This occasionally results in the premature death of animals prior to the time of their intended sacrifice. In addition, the validity of histological results following maximal contraction induced by electrical stimulation as in our version of muscle force measurements may be questionable

given the injuries that kind of forceful contraction may result in.

4.7 Timeline of Regeneration

Knowledge of the natural timeline of muscle healing in humans is of great value as a manner of guidance of therapeutic interventions. Murine models of skeletal muscle injury have allowed some introspection into the phases of regeneration. As discussed, the regenerative process is generally divided into three stages: disintegration, repair, and remodeling. In murine models, the stage of disintegration lasts 1-3 days, followed by a repair phase of 3-4 weeks. The remodeling phase lasts the longest, spanning 3-6 months. Histological markers and cellular analyses indicate that the repair phase and the remodeling phase appear to overlap (3, 132).

The remodeling phase depends largely on the reintegration of a muscular unit into habitual movement i.e. contraction. This relies on renewed stimulation by intact nerves, a stable supply of nutrients by a well-distributed vascular network, and a subsequent restructuring of immature myofibers and a decrease in fibrotic scar tissue resulting in reformed functional structural units (3, 83, 133).

The relatively slow process of neuronal regeneration may be a limiting factor considering its importance in allowing for a well-coordinated, even contraction of a muscle. Uneven or entirely missing neural stimulation appears to result in continuous regenerative efforts following the initial degeneration of individual muscle fibers, exhausting the satellite cell pool and resulting in poor histological outcomes (134-136).

Given our study's time points of evaluation, one may assume that the animals were in the early repair phase at 7 days following trauma, the late repair phase at 28 days following trauma, and well into the remodeling phase at 56 days following trauma. We found that both fast twitch and tetanic contraction force appeared to increase the most between our treatment groups' 7- and 28 day intervals. Given the expected relatively fast processes of cellular recruitment and vascularization primarily of the repair phase and the following relatively slow processes of scar remodeling and neuronal regeneration, this was an expected result. Both the fast twitch and the tetanic contraction forces of our treatment groups increased insignificantly or decreased in between the 28- and the 56 day intervals following trauma. This may indicate a negative impact of certain aspects of the remodeling phase such as the development of fibrosis on the overall functional outcome at that point in time.

Long term analyses of muscular strength following the completion of the remodeling phase may be of interest but were not within the scope of this investigation.

4.8 Outlook

Compared to our prior work which examined the outcome following the injection of an identical dose of MSCs transplanted on our scaffolds (10^6 cells), our groups transplanted with Alginates seeded with MSCs were found to perform almost identically at the 28-day evaluation point as groups injected with MSC alone did in that particular study at the 21-day evaluation point. Our groups transplanted with Alginates enriched with the GFs IGF-1 and $VEGF_{165}$ however well outperformed the group from our prior work injected with MSC alone at the identical time points, with an average 9 % increase of fast twitch force and an average 10 % increase of tetanic contraction force between the two groups from the two separate studies (80).

This highlights the importance of the interplay between the biomaterials, cells and GFs utilized for specialized tissue engineering. Future studies should identify the details of the microcellular environment created by such artificial niches and more closely identify the effects of the individual components to allow for modifications which render this kind of an experimental intervention even more effective.

Given the overall burden of musculoskeletal injuries in modern health care, innovative strategies that individually address the particularities of the heterogeneity that constitutes muscle injuries should be continued to be developed. Translation from the bench- to the bedside are of paramount importance for the development of novel therapies to address skeletal muscle injury. Whilst strategies such as the in situ tissue engineering in this study may be particularly useful to mitigate nosocomial muscle injuries caused by necessary surgical interventions, in vivo engineering of 3D synthetic muscle tissue may be the solution for larger scale volumetric muscle loss and commercially available injectables may form a new basis for the treatment of minor muscular injuries such as strains. Treatments should aim to be as case-specific as possible, and cause as little interruption as possible to the patient.

4.9 Conclusion

All in all, our study found that the functional outcomes of the group of animals treated with Alginates enriched with the GFs $VEGF_{165}$ and IGF-1 seeded with MSCs were superior all others. The activation and reinforcement of the endogenous regenerative response appeared to be

unparalleled. The ancillary stimulation of the MSCs by the GFs appears to increase their success in supporting the intrinsic regenerative process following injury to the skeletal muscle. Whilst 'restitutio ad integrum' was unable to be achieved in our study, future studies may able to achieve this goal through optimizations of the cells, GFs, and the scafoold used in this experiment. The successes of the kind of in situ tissue engineering represented in this study should lead to further experimentation examining the effects of this kind of treatment protocol on a variety of different muscular injuries in terms of cause, severity and structural change. Treatment protocols which are cost- and labor effective and easily implemented at little expense to the patient would improve the clinical translation of all novel approaches to skeletal muscle regeneration.

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Affidavit

“I, Janina Kueper, certify under penalty of perjury by my own signature that this thesis on the topic ‚Functional evaluation of skeletal muscle regeneration following severe crush trauma and the therapeutic application of specialized tissue engineering in the rat’ is my own work. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources. All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) where indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM are my own work for which I claim responsibility. My contributions to any publications related to this dissertation correspond to those that are specified in the subsequent joint declaration with my supervisor. All publications resulting from this thesis and which I am author of correspond to the URM and are my own work for which I will answer. The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me. I fully understand the rights and responsibilities stated therein.”

_____ _____

Date Signature of the Doctoral Candidate

Declaration of Publications

Janina Kueper had the following share in the following publication:

Publication 1: Pumberger M, Qazi TH, Ehrentraut MC, Textor M, **Kueper J**, Stoltenburg-Didinger G, Winkler T, von Roth P, Reinke S, Borselli C, Perka C, Mooney D, Duda GN, Geißler S. Synthetic niche to modulate regenerative potential of MSCs and enhance skeletal muscle regeneration. Biomaterials. 2016 May 10.

Contribution Details: I performed all muscle force measurements included in Publication 1 and assisted during the surgeries for the bone marrow biopsies and transplants of alginates or injections. For the muscle force measurements, I assisted with the dissection of the muscle and the placements of a suture in the tendon. I then tested the experimental set-up. Following this, I took full responsibility of the animal, placed it in the experimental apparatus, attached the animal to the force transducer, and completed the muscle force measurement. I then saved all results, and organized and integrated them into a master document with all necessary notes following the conclusion of the measurements. The animals' care (marking for identification, postoperative care etc.) as well as the animals' anesthesia were my additional main tasks. The statistical analysis of a subset of my data resulted in Figures 4 A and B in the above-mentioned publication and their associated paragraph, numbered 3.4 and titled 'Transplanted synthetic niche enhances contraction forces of injured muscles'. I was of assistance during the preparation of the manuscript.

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Signature, Date and Stamp of the Supervisor

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Signature of the Doctoral Candidate

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publications

1. Du JY, Aichmair A, **Kueper J**, Lam C, Nguyen JT, Cammisa FP, Lebl DR.. Incidental durotomy during spinal surgery: a multivariate analysis for risk factors. Spine (Phila Pa 1976). 2014 Oct 15;39(22):E1339-45. doi: 10.1097/BRS.0000000000000559.

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