Improving the regeneration of injured muscle

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Chapter 1

General introduction

1.1 Introduction

Skeletal muscle engineering is a challenging field that can significantly contribute to clinical applications for a wide variety of muscle injuries such as strains, trauma, muscular dystrophies, and congenital malformations (figure 1).

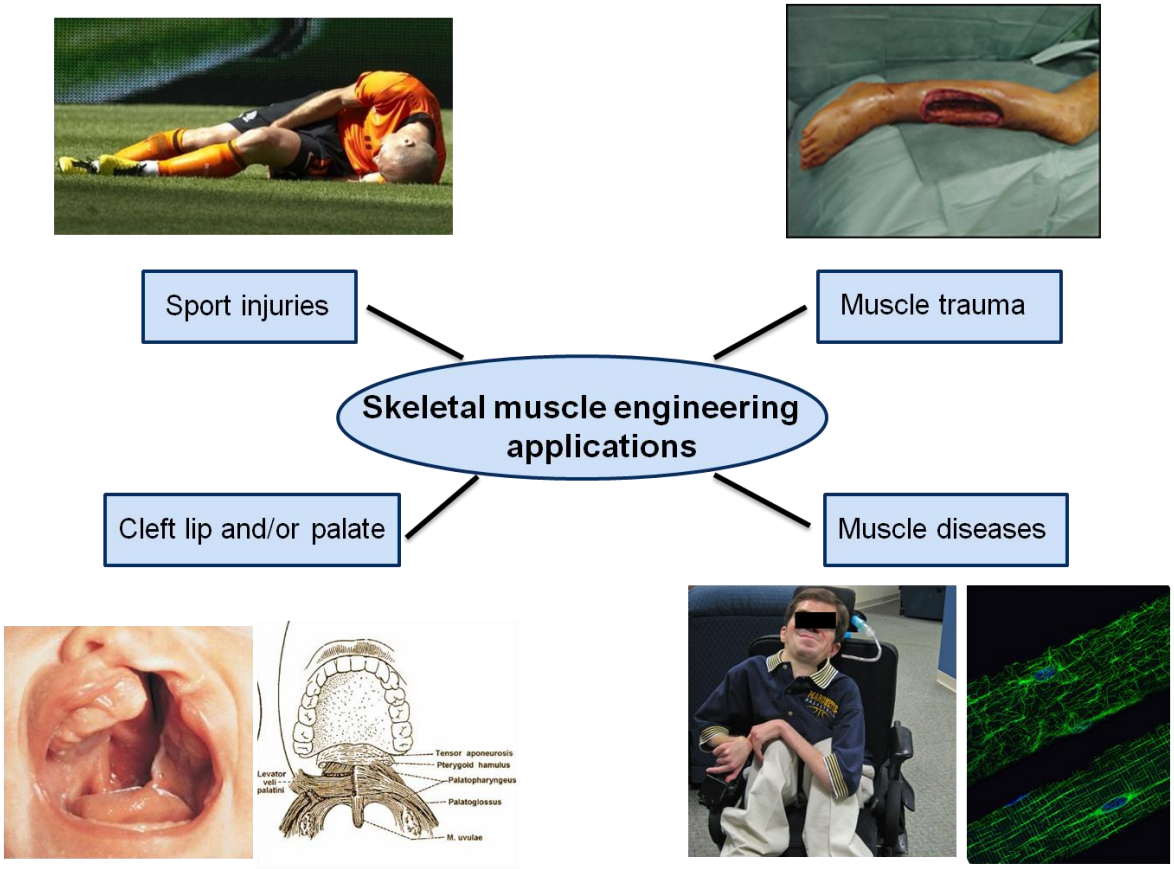

Figure 1. Possible therapeutic areas for skeletal muscle engineering. *The treatment of strain injuries in sports, muscle defects after trauma, genetic muscle diseases such as Duchenne muscular dystrophy, and congenital malformations such as cleft lip and/or palate can benefit from skeletal muscle engineering.*

Muscle strain injuries occur in sports with high intensity sprinting such as football, rugby and soccer, and have an incidence of about 30%. One of the most common affected muscle group is the hamstring and treatment is still not optimal as shown by the recurrence rate of 30%.[1,2] Loss of muscle tissue commonly occurs in patients with large wounds such as military personnel, victims of car accidents and gunshots, and in surgical patients. The standard treatment for these patients is the

transplantation of autologous muscle tissue. However, this leads to new defects that may lead to muscle fibrosis. Eventually these patients still end up with a permanent physical handicap.[3,4] Muscular dystrophies are inherited myogenic disorders and show progressive muscle wasting and weakness. Duchenne muscular dystrophy (DMD) is the most common form affecting about 1 out of 3,500 male newborns. It is characterized by the absence of the protein dystrophin, which provides structural strength to the muscle tissue. Currently, no effective treatments are available for any of the muscular dystrophies. Gene and stem cell therapy might offer new solutions, but still major problems exist regarding safety and the delivery into all affected muscles.[5-7] Malformations of muscle tissue such as in cleft lip and/or palate (CLP), are characterized by disorganized muscle fibers and impaired function. About 45% of the CLP patients show clefts in the soft palate.[8] These patients have difficulties with feeding and speech, and surgery is required to close the defect. However, in many patients speech and feeding problems persist after surgical closure, which is often due to the formation of fibrotic tissue in the levator veli palatini (LVP), the major muscle of the soft palate.[8-11]

In all of these muscle disorders, a main problem is the formation of fibrotic tissue after restoration.[11] This prevents the regeneration of oriented muscle fibers, and therefore impairs full functional recovery. Fibrosis is also one of the main causes of recurrent strain injuries. Present therapies are often insufficient to treat muscle injuries because of the development of fibrosis. In the field of tissue engineering and regenerative medicine, new approaches are being developed with the ultimate aim to restore muscle function by improving muscle regeneration and reducing fibrosis.

1.2 Skeletal muscle regeneration

In order to design and optimize treatment strategies for muscle disorders, muscle development and regeneration have been studied extensively (reviewed in chapter 2). Briefly, muscle regeneration occurs in three phases: inflammation, regeneration, and remodeling, which may lead to

fibrosis.[11,12] During inflammation, macrophages phagocytose necrotic debris, and produce factors that, together with factors released from the extra cellular matrix (ECM), start the regeneration phase.[11-14]

Skeletal muscles regenerate by the activation of a small population of stem cells, which are associated with the myofibers.[15,16] These satellite cells are able to migrate to the site of injury where they proliferate extensively and subsequently differentiate to form new muscle fibers. This process is regulated by many growth factors such as insulin-like growth factor (IGF)-I, fibroblast growth factor (FGF)-II, and hepatocyte growth factor (HGF).[11-13] A crucial event during regeneration is the self-renewal of satellite cells to replenish their numbers for future regeneration cycles, which is a key characteristic of all stem cells.[17] Factors in the satellite cell niche are crucial for self-renewal and regeneration (reviewed in chapter 3). The direct contact of the satellite cell with the myofiber and the basal lamina appears to be essential for maintaining their stem cell status.[18] Loss of contact with the niche leads to proliferation and differentiation of the satellite cell.

Fibrotic tissue is often formed during the final remodeling phase, which contributes to incomplete functional recovery and recurrent muscle injuries.[11,12,19] A key regulator of fibrosis in many tissues is transforming growth factor (TGF)-β.[20] Thus, prevention of fibrosis might be achieved by decreasing TGF-β activity in the tissue.

1.3 Strategies to improve skeletal muscle regeneration

In order to prevent the formation of fibrotic tissue, and to optimize muscle regeneration, several strategies have been developed (figure 2, reviewed in chapters 2 and 3). Firstly, growth factors that stimulate muscle regeneration can be injected into the muscle defect.[21-24] Secondly, satellite cells alone, myofibers including satellite cells, or other cell types with myogenic capacity can be injected into the injured muscles.[25-33] All these approaches partly improve regeneration in muscle injury models such as strains, contusions, and lacerations. However, for the regeneration of large muscle defects such as after trauma or clefts of the

soft palate, the injection of growth factors and/or satellite cells is not sufficient. This type of defects, require three-dimensional scaffolds that serve as a template for migrating satellite cells, and guide regenerating myofibers across the defect. Several types of scaffolds have been used either or not loaded with growth factors and/or cells.[34-37]

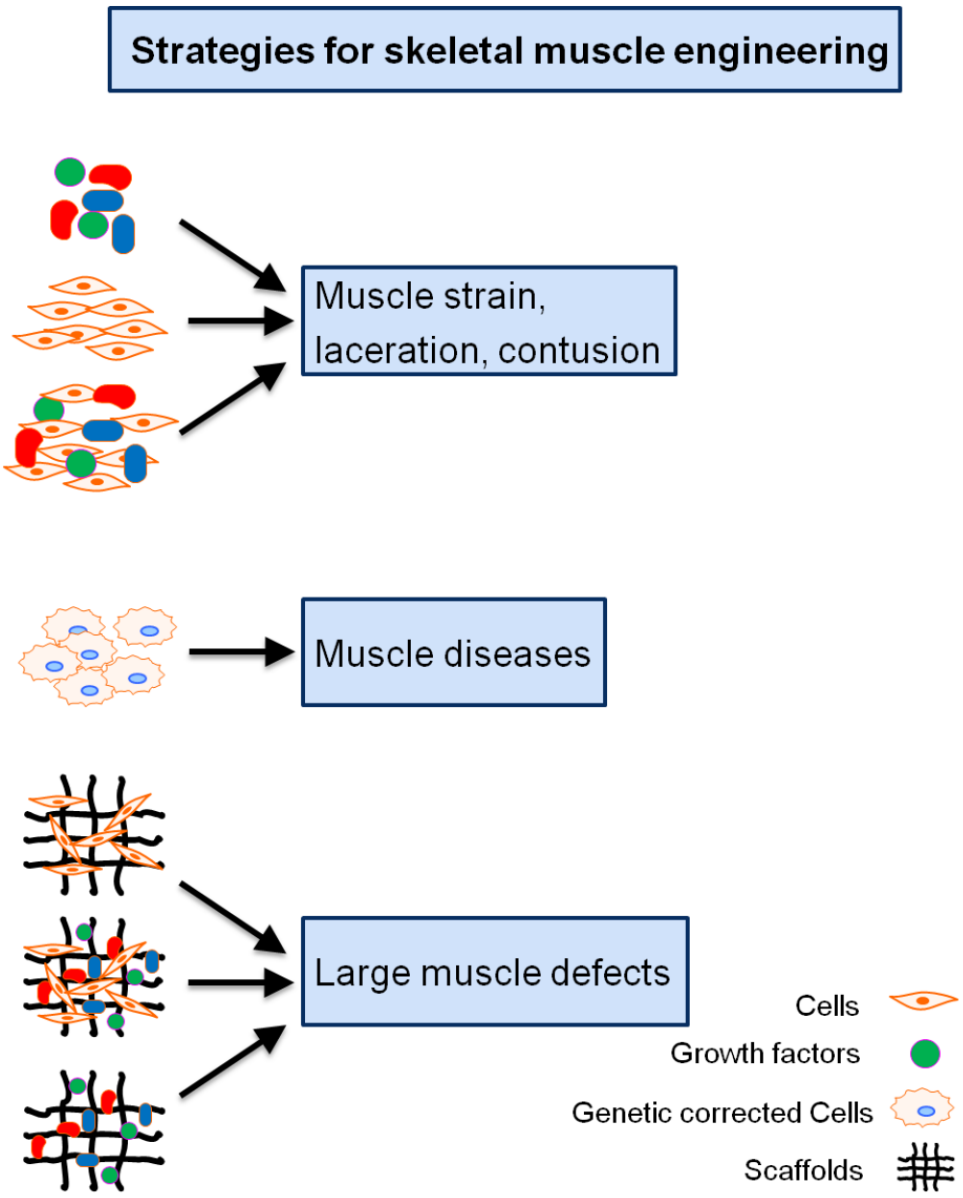

Figure 2. ***Strategies for skeletal muscle engineering.*** *For strains, contusions and lacerations, growth factors and/or cells can be injected directly into the defect. For genetic muscle diseases, genetically transformed (stem) cells need to be used. For large muscle defects, scaffolds loaded with cells and/or growth factors are needed to provide structural cues for tissue regeneration.*

However, muscle fibers are generally not able to grow into the scaffold, which is eventually replaced by fibrotic tissue. Loading the scaffolds with cells generally improves the outcome but major problems still exist in satellite cell isolation and culture, and in their survival and migration after transplantation.[28,29,38,39] To overcome these problems, scaffolds need to be developed that attract resident satellite cells towards the defect, and support their proliferation and differentiation into functional muscle fibers.

1.4 Aim of the study

Models for full-thickness muscle defects that generate fibrotic lesions are lacking up to now. Such models are required to develop approaches to regenerate muscle defects after surgical trauma or clefts of the soft palate. As the implantation of scaffolds loaded with satellite cells still faces major problems in the isolation and culture of these cells, we chose a different approach. The first aim was to develop a fibrosis model by making full-thickness muscle defects and then to implant scaffolds loaded with factors that attract resident satellite cells and reduce fibrosis.

In chapter 4, we developed a new model for recurrent strain injuries in which fibrotic tissue is mimicked by the implantation of cross-linked collagen scaffolds. This model can be used to evaluate new treatment modalities for existing fibrosis. In chapter 5, we developed a new muscle injury model in which large fibrotic lesions form spontaneously. This model can be used to test scaffolds loaded with growth factors that improve muscle regeneration and prevent fibrosis. In order to improve muscle regeneration, scaffolds with stromal derived factor-1α (SDF-1α) were implanted. SDF-1α regulates satellite cell migration.[40,41] The results of this study are also presented in chapter 5. In order to prevent fibrosis, the activity of TGF-β, the main fibrosis-inducing factor, needs to be reduced. Decorin, a leucine-rich proteoglycan, is able to bind TGF-β and thereby reduces its activity.[21,42,43] We tested scaffolds with decorin and SDF-1α in our fibrosis model to simultaneously promote satellite cell migration and prevent fibrosis (chapter 6). Cultured satellite cells, or

muscle progenitor cells (MPCs), can also be used for therapy. *In vivo*, niche factors from the myofiber and the basal lamina regulate satellite cell behavior.[44] Therefore, we investigated the effect of ECM molecules from the basal lamina on the myogenic potential of MPCs in 2D and 3D cultures *in vitro* (chapter 7). In chapter 8, we made a start to translate the results of these studies towards a therapy for the repair of the muscles in the soft palate. As a first step, we compared the myogenic capacity of MPCs isolated from a limb muscle and a craniofacial muscle. Finally, the most important results of this thesis and suggestions for future research on muscle regeneration are discussed in chapter 9.

In summary, the research aims of this thesis are:

- To develop new fibrosis models to study the effect of implanted scaffolds on muscle regeneration (chapters 4 & 5).
- To test scaffolds loaded with growth factors for their ability to improve muscle regeneration and to inhibit fibrosis (chapters 5 & 6).
- To develop a 3D culture system for satellite cells to analyze the effects of ECM components on their myogenic capacity (chapter 7).
- To compare the myogenic capacity of satellite cells derived from a limb and a craniofacial muscle (chapter 8).

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Chapter 2

Skeletal muscle development and regeneration

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Ruurd Torensma
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Stem Cells and Development 16:857–68 (2007)

Abstract

In the late stages of muscle development, a unique cell population emerges that is a key player in postnatal muscle growth and muscle regeneration. The location of these cells next to the muscle fibers triggers their designation as satellite cells. During the healing of injured muscle tissue, satellite cells are capable of forming completely new muscle fibers or restoring damaged muscle fibers. A major problem in muscle healing is the formation of dysfunctional scar tissue, which leads to incomplete functional recovery. Therefore, the identification of factors that improve the process of muscle healing and reduce the formation of scar tissue is of great interest. Because satellite cells possess the capability of self-renewal, a unique feature of stem cells, they play a central role in the search for therapies to improve muscle healing. Growth factor-based and (satellite) cell-based therapies are being investigated to treat minor muscle injuries and intrinsic muscle defects. Major muscle injury that involves the loss of muscle tissue requires the use of scaffolds with or without (satellite) cells. Scaffolds are also being developed to generate muscle tissue *in vitro*. These approaches aim to restore the structure and function of the injured muscle without dysfunctional scarring.

2.1 Introduction

Skeletale muscle represents nearly half of the total body mass and thus is the most abundant tissue of the human body. The skeletal muscles induce smooth and coordinated body movements through their attachment to the skeleton. To ensure proper function, the skeletal muscles are highly vascularized and extensively innervated. A skeletal muscle is composed of many bundles of myofibers, which are the functional units. A single myofiber is derived from the fusion of numerous myoblasts and therefore contains many nuclei. Each myofiber contains many myofibrils, which are composed of repeating sarcomeres. A sarcomere is an arrangement of the contractile proteins myosin and actin, which form the thick and thin filaments, respectively (figure 1). These proteins are key elements for the contractile properties of skeletal muscle. For skeletal muscle to contract, the myofibers depolarize as a consequence of nerve activation. This results in the release of intracellular calcium from the sarcoplasmatic reticulum. Calcium causes binding of myosin to actin, and subsequently contraction of the myofibers and the entire skeletal muscle. Most human skeletal muscles contain a mixture of three different types of myofibers. Type 1 myofibers are slow twitch and fatigue resistant, type 2A myofibers are fast twitch and moderately fatigue resistant, and type 2B myofibers are fast twitch and not fatigue resistant. The proportions of these myofibers within skeletal muscles is dynamic and can change throughout life.[1–4]

Skeletal muscles are able to self-regenerate after injury. Crucial cells in this process are the satellite cells, which are located between the sarcolemma and the basal lamina of the myofiber.[5,6] After injury these cells are activated; they proliferate and eventually fuse to the damaged myofibers or fuse together to form new myofibers.[1,7–9] Injury and diseases such as Duchenne muscular dystrophy (DMD) lead to impaired muscle function. The formation of a dysfunctional scar tissue during regeneration may account for this problem. Thus, the identification of factors that influence the regeneration process of injured muscle is of great interest. The aim of this review is to give an overview of muscle development and regeneration, as well as how this knowledge is now

being used to develop treatment modalities for major muscle injuries or muscle disease.

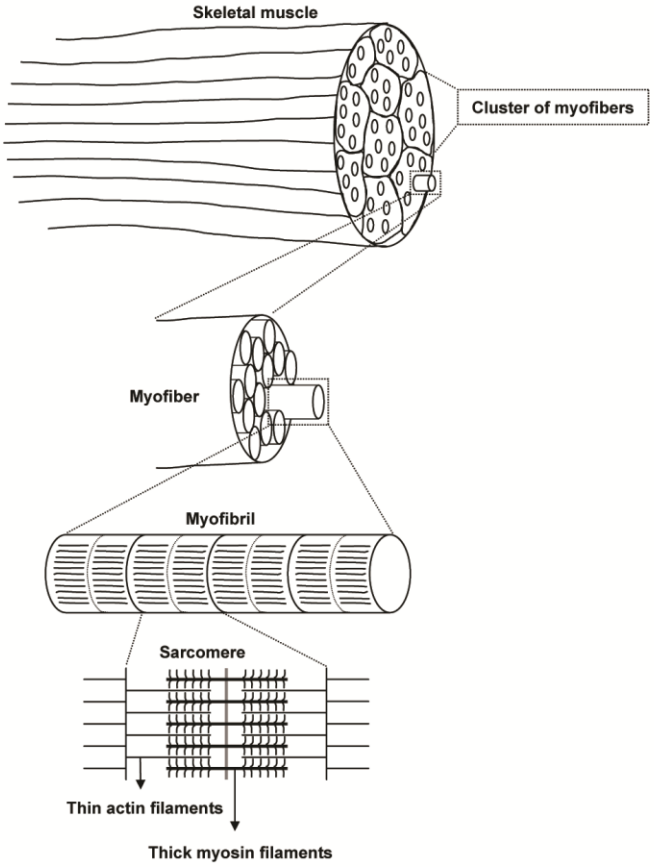

Figure 1. The structure of skeletal muscle. *Skeletal muscle is made up of clusters of myofibers. A single myofiber is composed of many myofibrils, which contain repeating sarcomeres. Each sarcomere contains the proteins actin and myosin, which represent the thin and thick filaments, respectively. These proteins are responsible for muscle contraction.*

2.2 Embryonic myogenesis

2.2.1 Somite development

In the early stages of embryonic development, the major function of gastrulation is to create a mesodermal layer between the ectoderm and the endoderm. The mesoderm forms the blood, blood vessels, bones, cartilage, connective tissue, and the muscles of the body trunk. On either side of the neural tube, this mesoderm is divided into the axial mesoderm (notochord), intermediate mesoderm, paraxial mesoderm, and the lateral plate mesoderm.[10] With the exception of the craniofacial muscles, nearly all embryonic skeletal muscles are derived from the paraxial mesoderm. First the paraxial mesoderm separates into cell clusters, called the somites, starting at the head region and sequentially added caudally. Cells

of the ventral part of the somites undergo an epithelial-to-mesenchymal transition, thereby forming the sclerotome, which eventually forms the vertebrae and ribs. In the chick, this process is characterized by the down-regulation of Pax3 and Pax7, two members of the family of paired/homeodomain transcription factors (figure 2). Members of this family play an essential role in embryonic organogenesis.[11] Cells of the dorsal part of the somites maintain Pax3 and Pax7 expression, and form the dermomyotome. This dermomyotome is responsible for the musculature and the dermis and is divided into an epaxial and hypaxial part, which forms the deep back muscles and the intercostal, abdominal, and limb muscles.[10]

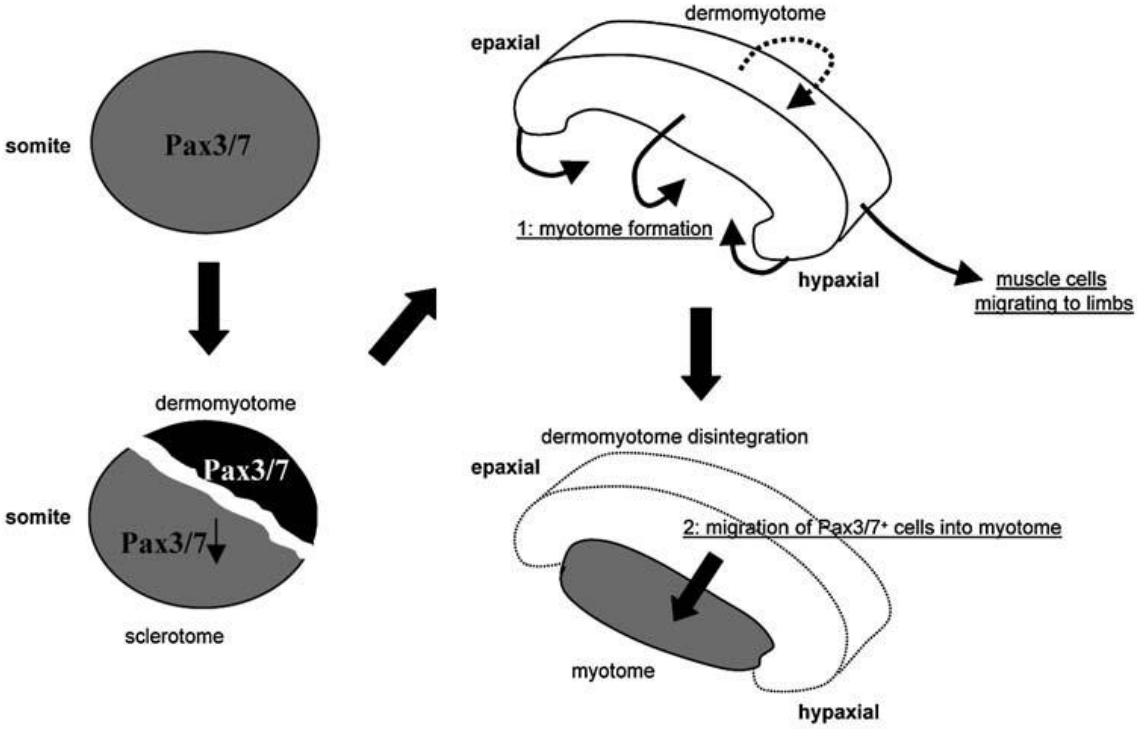

Figure 2. The formation of the somites and the myotomes. *The paraxial mesoderm, which gives rise to most of the skeletal muscle, segments into the somites. Eventually the somites differentiate into a sclerotome and a dermomyotome. In the chick, Pax3 and Pax7 expression is down-regulated in the sclerotome, but in the dermomyotome both are maintained. After the formation of the sclerotome and the dermomyotome, muscle progenitor cells delaminate from the four edges of the dermomyotome. These cells down-regulate their Pax3 expression and up-regulate the expression of myogenic regulatory factors, such as Mrf5, Myf4, MyoD, and myogenin. This results in the differentiation and fusion of the muscle progenitor cells, which leads to the formation of the myotome. In time, the dermomyotome disintegrates and muscle progenitor cells, expressing Pax3 and Pax7, migrate into the myotome. These cells contribute to the massive muscle development in the embryo and give rise for most of the satellite cells.*

2.2.2 Myotome development

A crucial step in the formation of skeletal muscle is the appearance of the myotome (figure 2). First, muscle progenitors cells delaminate from the four edges of the dermomyotome.[12] In addition, muscle progenitor cells migrate into the limb buds. It has been described that c-Met, a tyrosine kinase receptor that binds hepatocyte growth factor (HGF),[13] and Pax3 are major contributors to this delamination and migration, because mouse embryos lacking functional c-Met and Pax3 do not form skeletal muscle in the limbs. At the edges of the dermomyotome, Pax3 is also important to the survival of these cell.[14–17] These delaminating progenitor cells down-regulate Pax3 and become myoblasts through the action of the myogenic regulatory factors (MRFs), a family of basic helix–loop–helix transcription factors that regulate myogenesis. These myoblasts increase their expression of Myf5, Mrf4, and MyoD,[18–20] and differentiate into myocytes through the action of myogenin, Mrf4, and MyoD.[21] The myocytes eventually fuse and mature into multinucleated muscle fibers forming a continuous muscle layer, the myotome. It is known that these processes are influenced by signals from adjacent structures. Sonic hedgehog (Shh) and Wnt proteins, both representing a family of secreted signaling molecules, are involved in muscle development. These proteins are released from the neural tube, notochord, and surface ectoderm, and provide stimulatory signals during myogenesis. Bone morphogenic proteins (BMPs), another family of secreted signaling proteins involved in developmental processes, are released from the neural tube and the lateral plate mesoderm and inhibit myogenesis.[1,22–25]

2.2.3 Embryonic muscle and satellite cells

Given that the dermomyotome progressively disintegrates[10] and the myotome is already post-mitotic, these structures cannot account for the massive muscle development in the embryo. Several groups describe that cells expressing Pax3 and Pax7, but not the myogenic markers, migrate from the central dermomyotome directly into the myotome. During muscle development, these cells contribute to muscle growth and are maintained within the muscle mass. Before skeletal muscle forms in the limb buds, these precursor cells probably proliferate extensively to create

the skeletal muscle tissue.[26–29] As mentioned before, c-Met and Pax3 are involved in the migration of cells from the somite into the limb buds, and only upon arrival in the limb do these cells start to express MyoD and Myf5.[30] Furthermore, the Pax3- and Pax7-positive cells derived from the central dermomyotome also give rise to most if not all satellite cells, which emerge during the later phases of embryonic development.[26–28] Although the paraxial mesoderm in the embryonic body is completely segmented into somites, in the head it is incompletely segmented into seven mesenchymal structures, called somitomeres. Most head muscles such as the matistactory, jaw opening, and eye muscles, are derived from the paraxial head mesoderm, but the tongue muscles are derived from the somites.[10,31] In addition, differences have been described in the regulation of myogenesis in the head and these are reviewed elsewhere.[1,31,32]

During embryonic development, two distinct types of skeletal muscle fibers appear. The first muscle fibers that emerge are called primary or embryonic fibers; the secondary or fetal fibers arise later. The primary and secondary fibers have distinct morphological and biochemical properties and can be classified into slow-twitch and fast-twitch fibers.[33–36] Moreover, it seems that this commitment is independent from the surroundings and occurs in the somite.[37] Toward the end of embryogenesis, the satellite cells appear. They are the major players in postnatal muscle growth and regeneration.

2.3 Muscle regeneration

In general, adult skin wound healing occurs in three overlapping phases: inflammation, tissue formation and tissue remodeling.[38] Wound healing proceeds similarly in other tissues such as muscle.[2] A drawback of wound healing often is the formation of scar tissue (fibrosis), which can give esthetic as well as functional problems.

2.3.1 Fetal tissue regeneration

In contrast with adult skin wound healing, fetal wound healing of the skin can occur without the formation of scar tissue. Since this observation, the

search for factors that contribute to scarless healing was of great interest because these factors have a promising role in preventing scar formation in adult wound healing.[39–41] In the fetus, rapid wound closure is induced without scab formation and inflammation and with specific cytokine levels. Additionally, matrix deposition is rapid and similar to the uninjured fetal skin, whereas the extracellular matrix is rich in hyaluronic acid. Several studies indicate that the fetal environment is not essential for scarless tissue repair and that intrinsic factors of the tissue itself are vital.[42,43] However, not all fetal wounds heal without scar tissue, and healing takes place only in fetuses up to a certain gestational age.[44–46] Also wound size is important, as the extent of scarring increases with increasing wound size.[47,48] In contrast to the healing of fetal skin, wounds in gastric tissue, intestine, and nerve tissue always heal with scar formation.[49–51] Moreover in diaphragmatic wounds, muscle regeneration is absent, and scar tissue forms.[52] With the exception of this study, there is little further information on fetal muscle healing.

2.3.2 Adult tissue regeneration

Similar to the healing of adult skin, the healing of adult muscle injury caused by trauma occurs in three overlapping phases. As described, these phases include inflammation, tissue formation, and tissue remodeling resulting in the formation of scar tissue (fibrosis). After muscle injury, disruption of the myofiber plasma membrane initiates an influx of extracellular calcium, leading to calcium-dependent proteolysis.[53–55] This results in necrosis and degeneration of damaged myofibers, which is restricted to the damaged site through the formation of a contraction band that seals off the defect.[56] At the site of injury, blood vessels are also damaged, allowing the invasion of inflammatory cells. Factors are released in the injured muscle tissue that attract and activate inflammatory cells, which secrete chemotactic factors to attract more inflammatory cells. Neutrophils are the first inflammatory cells at the site of injury, and later macrophages arrive to phagocytose muscle debris.[1] Interestingly, satellite cells and macrophages interact to amplify chemotaxis and thereby enhance inflammation. Macrophages may also support satellite cell survival by cell–cell contact and the release of

soluble factors.[57] Additionally, macrophage infiltration leads to increased satellite cell proliferation and differentiation. After macrophage depletion, muscle regeneration is completely absent.[58] In conclusion, macrophages are not only important for the resolution of necrosis but are also involved in the induction of muscle regeneration.[59]

2.3.3 Satellite cells

At the site of injury, many growth factors are expressed and several of these are able to activate satellite cells. Examples are members of the fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β) families, insulin-like growth factor-I and -II (IGF-I, IGF-II), hepatocyte growth factor (HGF), and interleukin-6 (IL-6). The functions of these factors are reviewed extensively elsewhere.[1,2,22,60] Normally, satellite cells are quiescent, located between the basal lamina and the sarcolemma of myofibers, and express Pax7.[61,62] Pax7 in combination with M-cadherin (figure 3), a calcium-dependent cell adhesion molecule, c-Met, or other markers should be used to identify satellite cells.[22] However, up to now, there is no unique marker for quiescent or activated satellite cells. Upon injury, activated satellite cells either migrate to adjacent myofibers if the basal lamina is destroyed or migrate under the basal lamina to the site of injury.[22] The activation of satellite cells is similar to embryonic myogenesis, which is controlled by Pax3, Pax7 and Myf5 (figure 4).[63] First the activated satellite cells up-regulate either MyoD or Myf5, but eventually these factors are co-expressed. During this stage, the satellite cells become proliferative, and are also known as myoblasts. Down-regulation of Pax3[63] and Pax7,[61,64] and up-regulation of myogenin and Mrf4 lead to terminal differentiation of these myoblasts. Pax3 and Pax7 activate myogenin via up-regulation of MyoD, but Myf5 is able to activate myogenin directly. Ultimately, these differentiated myoblasts either fuse to each other, creating new myofibers, or fuse to existing damaged myofibers for repair.[1,7–9] These myofibers are still small, and the nuclei are located near the center of the myofiber. Maturation of these myofibers is characterized by an increase in size, and the movement of the nuclei to the periphery.[22] Next to growth factors, Notch, Shh, and

Wnt, which are important in muscle embryogenesis, may also be involved in satellite cell activation and postnatal muscle regeneration.[60]

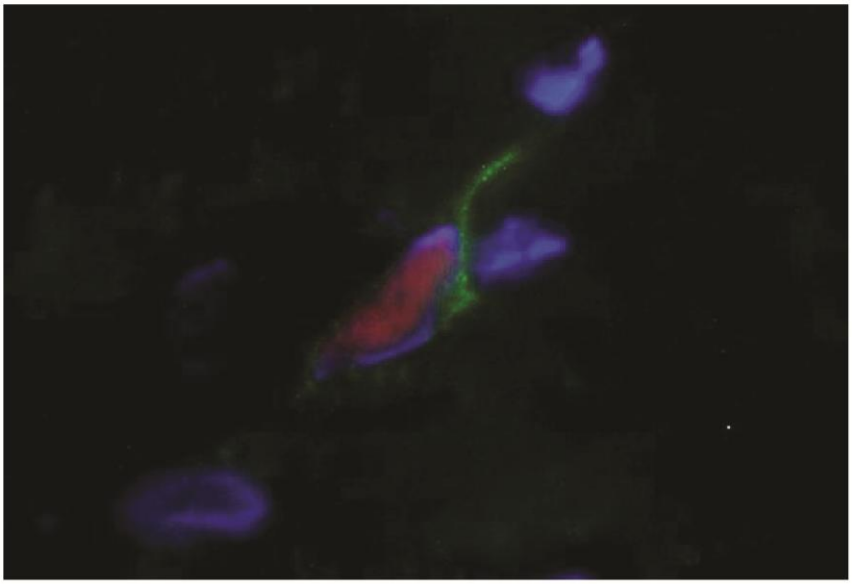

Figure 3. Identification of a satellite cell in rat muscle by immunostaining with Pax 7 (red), M-cadherin (green) and DAPI (blue). (S. Grefte, unpublished results).

Another essential factor in the function of satellite cells is the muscle environment. In aging muscle, regeneration is less efficient and the number of satellite cells declines. Because exposure of aged mice to serum of young mice restores muscle regeneration effectively,[65] the aged stem cells must still have retained their regenerative capacity.[65–67] These local factors are unknown, and further research is needed to identify them. Besides satellite cells, other cell types seem to be involved in muscle regeneration. Some studies suggest a minor role for non-muscle stem cells and for muscle-derived progenitor cells other than satellite cells.[1,68] However, the exact functions of satellite cells and other cell types in regenerating muscle defects remain unclear. Moreover, evidence exists that satellite cells constitute a heterogeneous population.[69] Also, satellite cell markers such as M-cadherin and Myf5 seem to be heterogeneously expressed,[70] but Pax7 is expressed in almost all quiescent satellite cells.[61,62]

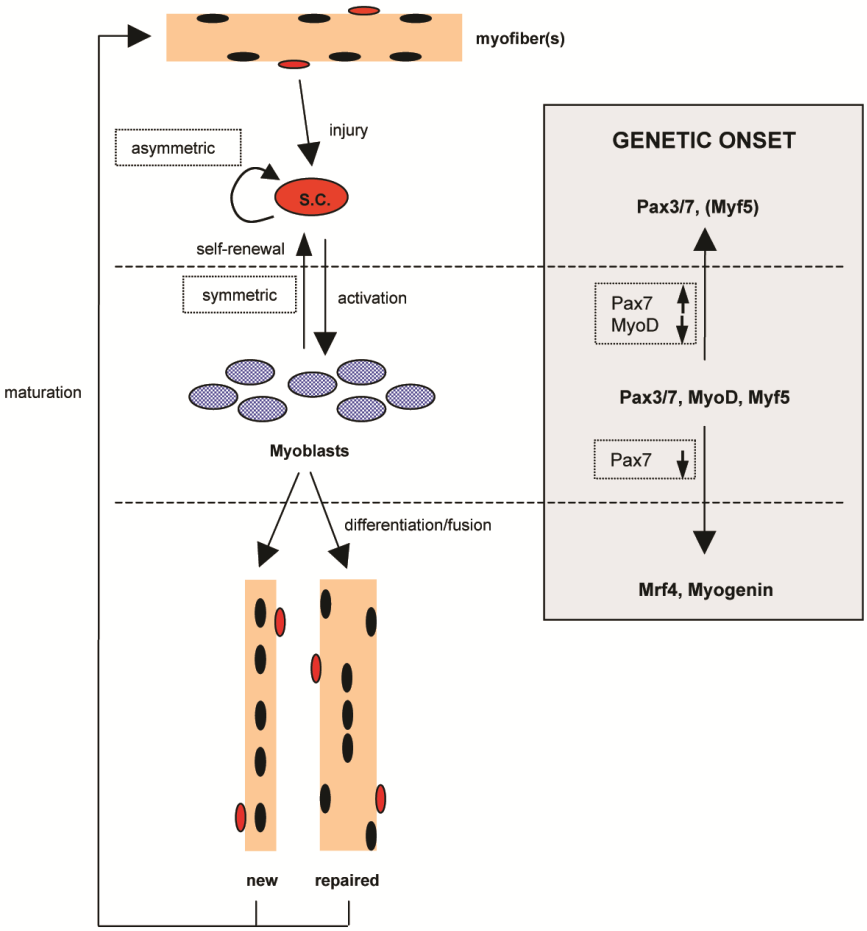

Figure 4. The role of satellite cells during muscle regeneration. *After injury, quiescent satellite cells expressing Pax7 migrate to the site of injury. Environmental signals such as growth factors activate the satellite cells to become myoblasts. These myoblasts are proliferative and express Pax7, MyoD, and Myf5. Down-regulation of MyoD and maintenance of Pax7 expression might be involved in the self-renewal of satellite cells.*[61,64] *Differentiation of the myoblasts is marked by the down-regulation of Pax7 and up-regulation of Mrf4 and Myogenin. Finally, fusion of the differentiated myoblasts occurs to create new myofibers or to repair damaged myofibers. Maturation of these new or repaired myofibers is characterized by an increase in size and the movement of the nuclei to the periphery.*

2.3.4 Self-renewal of satellite cells

A crucial characteristic of satellite cells is the capacity of self-renewal, which is a unique feature of all stem cells. Without self-renewal the number of satellite cells would decline after repetitive muscle injury and also during normal tissue turnover. Direct evidence for the self-renewal of satellite cells was provided using genetically labeled myofibers, but the exact mechanism has not yet been determined.[71] Two mechanisms have been proposed that result in satellite cell maintenance.[68,72]

Asymmetric division results in the formation of two daughter cells, of which one remains quiescent while the other undergoes myogenic activation and differentiation. Alternatively, symmetric division results in the activation and proliferation of all daughter cells. While the bulk of these proliferated cells down-regulate Pax7 and differentiate into new myofibers, a few cells retain Pax7 expression, return to their quiescent state, and repopulate the satellite cell pool.[61,64] In Pax7 knockout mice, satellite cells are still present directly after birth, but their number declines during postnatal development. It was also shown recently that in Pax7 knockout mice apoptosis of satellite cells occurs.[63] In contrast, Pax3 is essential for the survival of cells in the hypaxial dermomyotome as described before.

2.3.5 Fibrosis

At the site of injury, fibrosis may also occur leading to the formation of scar tissue.[73] Similar to skin wound healing, a provisional matrix provides an initial extracellular matrix for cell invasion. In time, (myo)fibroblasts begin to produce extracellular matrix components like fibronectin, followed by type III collagen, and ending with excessive production of type I collagen. Finally, after tissue remodeling and apoptosis of the myofibroblasts, a nearly acellular scar tissue is formed.[3] It is unknown whether regenerated myofibers will eventually fuse and fully regenerate the muscle tissue, but most muscle injuries heal without dysfunctional scar tissue. In contrast, excessive fibroblast proliferation may occur in large muscle injuries, resulting in a scar that limits full muscle regeneration.[74,75] Using a clonal population of muscle-derived stem cells it was shown that myogenic precursor cells are able to differentiate into myofibroblasts after muscle injury.[76] TGF-β1 is involved in scarring during wound healing in the skin.[38] In muscle, TGF-β1 is highly expressed at the site of injury, and it is able to induce myofibroblast differentiation of muscle-derived stem cells *in vitro*.[76] This suggests that some of the (myo)fibroblasts responsible for scar formation might be derived from myogenic cells such as satellite cells.

In summary, the function of satellite cells during muscle regeneration is regulated by many growth factors and cytokines. Their

capacity of self-renewal provides a source of satellite cells for muscle regeneration throughout life. Although most muscle injuries heal without dysfunctional scar tissue, this may occur in large muscle defects

2.4 Improving muscle regeneration

2.4.1 Growth factor-based therapy

The effects of growth factors on the activation, proliferation, and differentiation of satellite cells have been reviewed elsewhere.[1,2,22,60] Growth factors with stimulatory effects might be used *in vivo* to enhance the regeneration of muscle tissue. Indeed, it appears that the administration of growth factors after muscle injury may improve the healing process. In mouse models for muscle strain,[77] contusion,[78] and laceration[79,80] injuries, direct injection of IGF-I and FGF-2, and to a lesser extent nerve growth factor (NGF), improved healing of the muscle. This was indicated by an increase in the number and the diameter of regenerated myofibers. Additionally, the strength of the myofibers improved. The administration of decorin, an inhibitor of TGF-β, also induced muscle healing, and in combination with IGF-I it seems to be the best strategy to improve muscle healing.[80] However, based solely upon the strength of the myofibers, the administration of decorin alone showed the best improvement. These conflicting results stress the use of histological as well as functional parameters to evaluate muscle regeneration. Because TGF-β is considered to be involved in fibrosis, the inhibition of this growth factor might reduce scar formation during muscle regeneration. In fact, the administration of decorin indeed inhibits fibrosis.[80] In contrast, it was also shown that administration of FGF-2 did not improve muscle regeneration.[81] Therefore, it is important to exactly identify which growth factors enhance muscle regeneration *in vivo* and also at which concentration, location, and time point they should be administered.

Thus, growth factors seem to improve muscle healing after minor injury. However, for intrinsic muscle defects such as DMD or large muscle defects, growth factor-based therapy might not be the appropriate

strategy. To treat DMD, the use of cell-based therapy is a better solution. For major muscle injuries, scaffold-based therapy to fill up the large defect is the best method. These scaffolds can also be loaded with growth factors and/or myogenic cells.

2.4.2 Cell-based therapy

To regenerate muscle through the delivery of exogenous cells such as myofibers or satellite cells, it is crucial that these cells not only regenerate the damaged muscle but also replenish the satellite cell pool. This allows a long-term normal tissue maintenance and regenerative capacity. Although there is evidence that the dystrophic muscle environment is hostile for muscle regeneration,[82] the mdx mouse model has been used extensively to study muscle regeneration after cell transplantation. The mdx mouse lacks dystrophin, a structural protein that is mutated in DMD patients. Repetitive injections of notexin after the transplantation of clones of myoblasts into irradiated mdx nu/nu mouse muscle resulted in new myofibers of donor origin.[83] The formation of new muscle after repetitive muscle damage indicates that a new satellite cell population was established.[83] Labeling studies showed that myoblasts repaired the injured muscle fibers after transplantation and formed new satellite cells.[84,85] Furthermore, labeled satellite cells were detected in host muscle fibers, and isolated satellite cells from these fibers were also able to become active and to proliferate.[86] Grafting of myofibers that contain satellite cells resulted in the regeneration of damaged muscle and the expansion of the satellite cell pool.[71] Moreover, grafting the satellite cells, which were isolated from these myofibers, generated clusters of new myofibers. This indicates that the isolation of satellite cells from the myofiber does not impair the myogenic potential of these cells.[71] Additionally, a pure population of myogenic cells expressing Pax3, Pax7, and CD34 contributed to muscle regeneration and the formation of new satellite cells.[87] However, cultured satellite cells gradually loose their myogenic potential, and the transplantation of these cells leads to less efficient muscle regeneration.[87,88] Freshly isolated satellite cells may induce muscle regeneration more efficiently, but the small number of isolated satellite cells might be a problem. Therefore methods for

culturing satellite cells must be developed in which they maintain their myogenic properties.

Unfortunately, it has also been shown that the majority of the myoblasts die after transplantation in mdx mice.[89–91] Inflammation may be involved because infection of the myoblasts with a retroviral vector containing the IL-1 receptor antagonist partly prevented the observed cell death.[90] Although most of the transplanted cells died, a minority of myogenic cells were able to survive and to regenerate the host muscle.[91] Additionally, a specific myogenic cell population was enriched based upon their adhesion capabilities. These cells were able to survive after transplantation and fused with host myofibers, thus demonstrating their regenerative capacity.[90] Two additional myogenic populations were found based upon M-cadherin and CD34 expression.[92] In this study, these two markers could not be co-localized in skeletal muscle of normal mice, but cells expressing M-cadherin or CD34 both reside between the basal lamina and the sarcolemma of the myofibers, which is also the niche of satellite cells. However, in another study, M-cadherin$^+$ satellite cells were also positive for CD34.[70] Cloned myogenic cells from the CD34$^+$ population enhanced muscle regeneration, and partially restored dystrophin expression.[92] In another study, three myogenic cell populations were found based upon their adherence capacity. Two of these cell populations represented the satellite cells and showed limited capacity to regenerate the host muscle. However, the third cell population showed a strong capacity to improve muscle regeneration. These cells were long-time proliferative and also called muscle derived cells (MDSCs).[93] Interestingly, these cells did not activate T cells, indicating that they were not rejected by the immune system of the host. However, it was also shown that, on the basis of the mass and functional properties of the muscle, transplantation of primary myoblasts or the same MDSCs as described before[93] did not induce muscle regeneration in mdx mice.[94]

These results suggest that myoblast transplantation is helpful for the treatment of DMD by the fusion of myoblasts with host muscle fibers and restoring dystrophin expression, but in human experiments this strategy was unsuccessful. The translation from mice to humans might have been made too easily. Human muscles might be too large to allow transplanted

myoblasts to migrate throughout the entire muscle. A discussion of these problems and the treatment of DMD in humans has been published elsewhere.[95] In a recent study in the golden retriever muscular dystrophy (GRMD) model, a new therapy modality was developed for the treatment of DMD. The transplantation of autologous mesangioblasts transfected with small dystrophin genes resulted in the expression of dystrophin in host muscle and the formation of functional muscle with a normal morphology.[96]

In conclusion, most of these studies show the potential of transplanting myoblasts and MDSCs into muscle tissue to produce new muscle fibers or to restore dystrophin expression in host muscle fibers for the treatment of DMD. Moreover, the transplanted myoblasts are also able to create a new satellite cell pool, necessary for muscle regeneration throughout life and normal tissue turnover. Therefore the combination of transplanted cells with suitable scaffolds might offer new strategies for the repair of major muscle damage.

2.4.3 Scaffold-based therapy

In contrast to the transplantation of myoblasts and the use of growth factors to heal minor muscle injuries, these methods might not be appropriate for large defects. The application of a three-dimensional scaffold, which fills up the defect and induces the formation of new muscle, seems more suitable. The scaffold can be seeded with myoblasts or other myogenic cells, and can be implanted into a large muscle defect to improve muscle regeneration. However, the transplantation of a collagen disc seeded with or without myoblasts into an abdominal wall muscle defect in rats did not induce muscle formation.[97] Similar results were obtained after the transplantation of an acellular muscle matrix to reconstruct an abdominal wall muscle defect in rabbits[98] and rats[99] and a dorsal muscle defect in rats.[100] Eventually these constructs were replaced by fibrous tissue.[98–100] However, seeding the matrix with autologous satellite cells reduced the inflammation, and fibrosis occurred at the edge of the implant. This indicates that satellite cell seeding improves the biocompatibility of the scaffold. *In vitro* studies showed that an acellular muscle matrix supports the growth and differentiation of satellite cells

isolated from rats, but *in vivo* studies showed no convincing evidence of skeletal muscle formation inside the matrix after transplantation into a dorsal muscle defect in rats.[100] However, electric activity was detected, indicating that skeletal muscle fibers were present within the matrix (99). This result shows the potential of using constructs seeded with myoblasts to reconstruct injured muscle. Additionally, transplantation of a muscle matrix seeded with male rat myoblasts into full-thickness abdominal wall defects of female rats resulted in the formation of a dense capillary network, skeletal muscle fibers, and evidence for nerve formation.[101] This matrix stained positively for FGF-2 and TGF-β, which could play a role in the regeneration of injured muscle. It has also been shown that muscle formation occurred only at the border of the matrix after the transplantation of an autologous myoblast-seeded muscle matrix construct into the abdominal muscle of rats.[102]

Thus, it seems that growth factors inside the matrix have a positive effect on seeded myoblasts and improve the formation of muscle fibers. The addition of HGF and FGF-2 to alginate scaffolds seeded with myoblasts greatly increased the viability of these cells.[103] Transplantation of such a scaffold seeded with myoblasts resulted in enhanced muscle regeneration by the engrafted donor cells.[104] Also other biomaterials were used as a three-dimensional matrix to reconstruct muscle tissue. The implantation of degradable polyglycolic acid (PGA) meshes,[105,106] alginate, and hyaluronic acid constructs,[107] all seeded with myoblasts, into a nonmuscle environment resulted in vascularization and muscle formation. Using fibrin as a three-dimensional matrix, *in vitro* studies showed that myoblasts can fuse into myotubes with physiological functions, such as force production.[108] After injecting fibrinogen with male myoblasts into a muscle defect of female rats, the fibrin matrix was eventually dissolved and the myoblasts fused with the host muscle.[109] More importantly, no inflammation was observed and fibrosis was absent.[109] However, full integration of the scaffolds seeded with myoblasts into the host muscle and total functional recovery without scarring still remain to be realized.

In summary, three-dimensional scaffolds might have advantages in the treatment of large muscle defects. Seeding of the scaffold with

myoblasts appears to be essential to induce the formation of new muscle mass. Furthermore, the addition of growth factors might improve the proliferation, migration and fusion of these myoblasts into new myofibers. A crucial aspect is the vascularization and innervation of the construct. It is important to keep in mind that, in line with the problems of the treatment of DMD in humans, these studies were carried out in the muscles of small animals. Thus, caution must be taken in the translation of the data to humans with larger muscles. The migration of myoblasts out of the scaffold, and the diffusion of signaling molecules and nutrients might be different.

2.5 Conclusion

Several strategies are being investigated to improve muscle regeneration after muscle trauma. For small defects, the administration of appropriate growth factors might increase satellite cell activation and improve muscle regeneration, while inhibiting fibrosis. The application of satellite cells or other myogenic cells capable of forming new muscle tissue might also improve muscle regeneration. These cells, as well as genetically corrected satellite cells, might also be used to restore intrinsic molecular defects such as DMD. However, for large muscle defects, suitable scaffolds must be used to induce muscle regeneration. These scaffolds should act as a temporary guide for host muscle cells or seeded satellite cells. Additionally, growth factors can be introduced into the scaffolds to create a suitable microenvironment for satellite cells. It is essential that these scaffolds: (1) initially provide mechanical stability to the defect, (2) induce satellite cell proliferation and differentiation into mature muscle, (3) induce fusion and alignment of myofibers with host myofibers, and (4) provide a niche to harbor satellite cells needed for normal tissue turnover and for future muscle regeneration. Furthermore, for a complete and functional recovery of damaged muscle the new tissue must be well vascularized and innervated. The ultimate goal of these strategies is to induce rapid muscle regeneration, leading to a functional muscle without the formation of dysfunctional scar tissue.

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Chapter 3

Regulatory factors and cell populations involved in skeletal muscle regeneration

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Abstract

Skeletal muscle regeneration is a complex process, which is not yet completely understood. Satellite cells, the skeletal muscle stem cells, become activated after trauma, proliferate, and migrate to the site of injury. Depending on the severity of the myotrauma, activated satellite cells form new multinucleated myofibers or fuse to damaged myofibers. The specific microenvironment of the satellite cells, the niche, controls their behavior. The niche contains several components that maintain satellite cells quiescence until they are activated. In addition, a great diversity of stimulatory and inhibitory growth factors such as IGF-1 and TGF-β1 regulate their activity. Donor-derived satellite cells are able to improve muscle regeneration, but their migration through the muscle tissue and across endothelial layers is limited. Less than 1% of their progeny, the myoblasts, survive the first days upon intra-muscular injection. However, a range of other multipotent muscle- and non-muscle-derived stem cells are involved in skeletal muscle regeneration. These stem cells can occupy the satellite cell niche and show great potential for the treatment of skeletal muscle injuries and diseases. The aim of this review is to discuss the niche factors, growth factors, and other stem cells, which are involved in skeletal muscle regeneration. Knowledge about the factors regulating satellite cell activity and skeletal muscle regeneration can be used to improve the treatment of muscle injuries and diseases.

3.1 Introduction

Skeletal muscle is the largest tissue in the human body, composing 40-50% of total human body mass.[1] The functions of skeletal muscles include movement, breathing, and posture maintenance.[2] Skeletal muscles consist of muscle cells, networks of nerves and blood vessels, and connective tissues that connect individual fibers into bundles, which form the muscle. The epimysium is the fibrous outer layer that surrounds the complete muscle, the perimysium surrounds the bundles of myofibers, and the endomysium (also called the basement membrane) surrounds individual myofibers.[1,3] Myofibers are the basic structural elements of skeletal muscle and are composed of multiple fused myoblasts. Newly formed multinucleated fibers exhibit central nucleation, and once the nuclei move to a subsarcolemmal position they are called myofibers.[1,3,4] The interior of a myofiber contains the sarcomeres, which are the basic functional units of skeletal muscle. The sarcomere consists of thick myosin filaments that interdigitate with thin actin filaments and is specialized to respond to neuromuscular signals. As a response to these signals (an acetylcholine-induced action potential), the cell depolarizes resulting in calcium release from the sarcoplasmatic reticulum (SR). The released calcium induces ATP-driven interactions between myosin and actin leading to sarcomere shortening and muscle contraction.[5,6]

Most skeletal muscles contain a mixture of 3 different types of myofibers. Type 1 myofibers are slow twitch and fatigue-resistant, type 2A myofibers are fast twitch and moderately fatigue-resistant, and type 2B myofibers are fast twitch and not-fatigue resistant. These different fiber types contain either slow myosin heavy chain (MyHC) or fast MyHC. These two isoforms have the same subunit structure, but differ in the rate of ATPase activity.[7,8] The composition of myofibers in skeletal muscle is dynamic and can change throughout life.[1,3-5]

In addition, skeletal muscles contain stem cells, which are also known as the satellite cells (SCs). SCs are located between the plasma membrane (sarcolemma) of the myofiber and the basal membrane (BM).[9,10] These cells are normally quiescent and will be activated after myotrauma, proliferate, self-renew, and finally differentiate into

multinucleated myofibers.[4,11-13] Since the original identification of the SC in 1961[9] it has been hypothesized that SCs are remaining embryonic myoblasts from the developing somites. Several studies[14-17] have demonstrated that progenitor cells from the dermomyotome give rise to SCs, but it remains unclear whether these cells are the only precursors of SCs.[18] After birth, the SCs proliferate extensively and play a major role in skeletal muscle growth and regeneration.[15-17]

Many regulatory processes are involved in skeletal muscle regeneration. Mainly the specific microenvironment of the SCs, the niche, and many growth factors, play a major role. In addition, a wide range of other multipotent stem cells, seem to be involved. The aim of this review is to discuss the factors that regulate SC activity and skeletal muscle regeneration, and their promising role in the improvement of skeletal muscle diseases in the future. In the next section, an overview of skeletal muscle regeneration is presented.

3.2 Skeletal muscle regeneration

The healing of skeletal muscle in response to trauma depends on the type of injury such as contusion, strain, and laceration, and on the severity. However, in general, the healing process consists of three phases: the destruction phase, the repair phase, and the remodeling phase.[1,3,4,19] The destruction phase is characterized by necrosis, hematoma formation, and the influx of inflammatory cells. During the repair phase, the necrotic debris is phagocytosed, and regeneration of myofibers occur through the action of SCs.[20,21] Firstly, quiescent SCs expressing Pax7 migrate to the site of injury, up-regulate the myogenic regulatory factors (MRFs) MyoD and Myf5, and become proliferative.[22-26] From now on, the SCs are also known as myoblasts. Subsequent differentiation of the myoblasts is marked by the down-regulation of Pax7[27,28] and up-regulation of the MRFs Mrf4 and Myogenin.[22-24] Ultimately, these differentiated myoblasts form multinucleated myofibers (hyperplasia) or fuse to damaged myofibers (hypertrophy) for muscle regeneration.[4,29] However, some of the activated SCs do not proliferate or differentiate, but self-renew and

replenish the satellite cell pool, which is a unique and crucial property of all stem cells.[28,30-32] Two different mechanisms have been proposed for self-renewal, asymmetric and symmetric division of SCs.[30,33,34] Asymmetric division results in two different daughters cells, one is beginning to differentiate while the other will remain quiescence and self-renew. Recent research demonstrated that asymmetric self-renewal of SCs occurs in skeletal muscles.[31,35,36] The study of Kuang et al. also suggests that 10% of the SCs have never expressed Myf5 suggesting that they did not proliferated and self-renew using asymmetric cell division.[31] In contrast, symmetric division results in the activation and proliferation of all daughter SCs. A minority of these cells will self-renew by maintaining Pax7 expression while down-regulating MyoD expression. However, most of these activated and proliferating cells down-regulate Pax7 expression and then differentiate.[27,28] During the last phase, the remodeling phase, the regenerated myofibers mature and contract. However, in some cases, reorganization and contraction of unstructured connective tissue occur, resulting in scar tissue and subsequent incomplete skeletal muscle regeneration.[3,37,38]

Next to skeletal muscle injury, diseases such as muscular dystrophy also lead to impaired muscle function.[39] Muscular dystrophy is characterized by muscle weakness and wasting. Many different forms of this disease have been identified.[39,40] The most severe form is Duchenne muscular dystrophy (DMD), which is characterized by the absence of dystrophin.[41] Dystrophin is, together with other membrane-associated proteins, required for the structural integrity of the muscle fibers. The lack of dystrophin leads to membrane instability and tears in the sarcolemma of the muscle fibers.[42-44] This results in repeated cycles of muscle fiber necrosis and regeneration until the regenerative capacity is exhausted. Eventually the muscle fibers are mostly replaced by adipose and fibrous tissue.[45-47] The mdx mouse is an animal model for DMD that also lacks dystrophin in skeletal muscle fibers.[48] However, the utrophin-dystrophin double-mutant mice may represent DMD in patients more accurately.[49]

In general, the direct environment of the SC, which is called the niche, and many regulatory factors, play a major role in muscle regeneration. These factors will be discussed in the following sections

3.3 Regulation of skeletal muscle regeneration

As mentioned before, SCs are normally quiescent, but become activated in response to injury, proliferate, differentiate, and fuse to repair or replace damaged myofibers. In these processes the stem cell niche, growth factors, cytokines, and neurotrophic factors play a prominent role.

3.3.1 The satellite cell niche

Next to soluble factors, the functioning of SCs is governed by their specific niche (figure 1). The most common definition of a stem cell niche is "a specific location in a tissue where stem cells can reside for an indefinite period of time and produce progeny cells while self-renewing".[50] The most obvious difference of the SC niche compared to other niches, is that the SCs are kept quiescent most of the time.[51]

In the SC niche many factors influence SC behavior. Structural elements of the niche are the BM and the myofiber.[33] SC behavior is also influenced by secreted products from local cells, such as the interstitial cells, microvasculature, neuromuscular junction, and immune cells.[52] Additionally, experiments using parabiotic pairing demonstrated that systemic factors in serum have a major effect on SC activity.[53,54] However, it still remains unclear how all these factors cooperatively regulate quiescence and activation of the SCs. In the next section we describe some of the niche elements, which are in direct contact with, or in the proximity of the SCs.

The SC niche is directly surrounded by the BM and the adjacent differentiated myofiber. It has been shown, that mechanical-, electrical-, and chemical signals from the adjacent myofiber and the BM, which is a component of the extracellular matrix (ECM), are involved in SC regulation.[4,34] Also the microvasculature seems to play a major role. In humans and mice, respectively 68% and 82% of the SCs are located

within 5 µm from neighboring capillaries or vascular endothelial cells (EC).[55] In addition, there is also correlation between the number of capillaries per muscle fiber and the number of SCs.[55] This strongly suggests a correlation between SC and ECs during myogenesis.

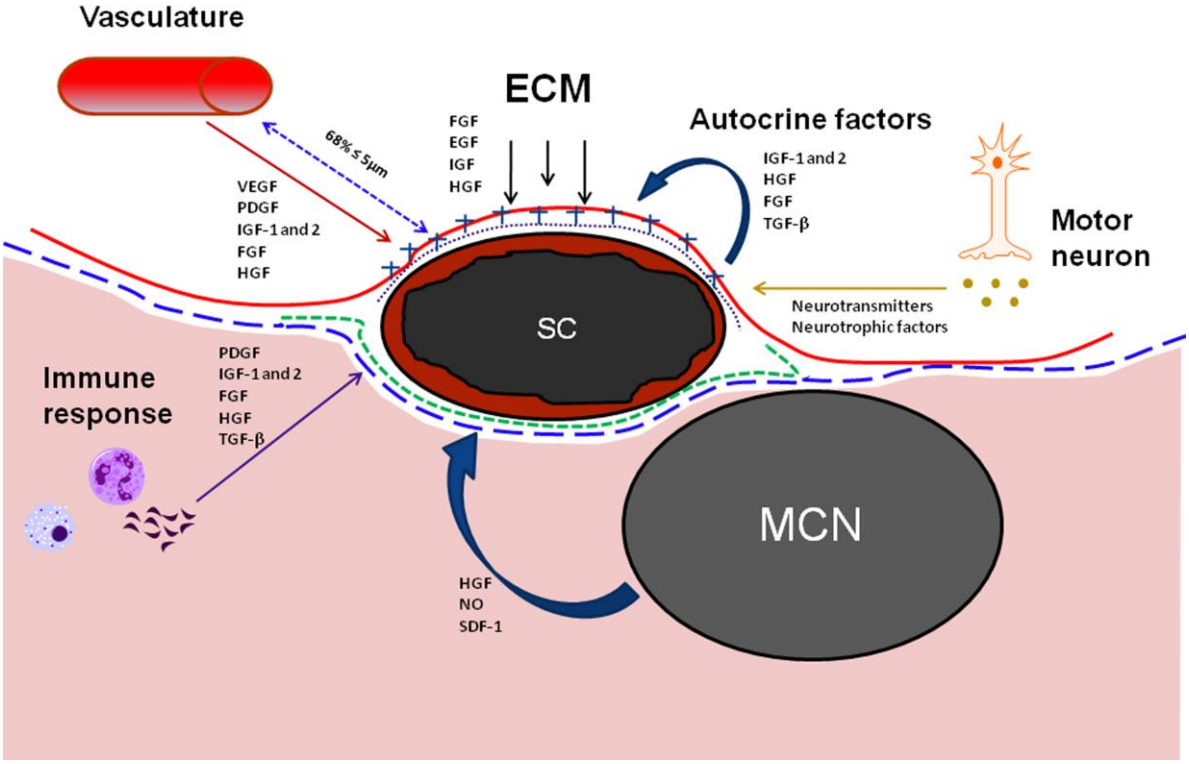

Figure 1. ***The satellite cell niche and regulatory factors.*** *SCs are located in a specific niche between the sarcolemma (blue long dashed line) and the basal lamina (red solid line). The basal side of the SC expresses integrin α7β1 (purple dotted line), which links the SC with the laminin (blue crosses) in the basal membrane. The apical side expresses M-cadherin (green dashed line), which attaches the SC to the adjacent myofiber. These areas are essential for signal transduction between the SC and the adjacent structures. SCs are normally quiescent and are activated after myotrauma, through the action of many regulatory factors. The proximity of the microvasculature suggests a reciprocal interaction between SCs and these vessels. ECM, extracellular matrix; MCN, myocyte nucleus; SC, satellite cell*

In addition, recent studies showed that macrophages, which are attracted upon injury, play a crucial role in skeletal muscle regeneration. *In vivo*, macrophage suppression leads to incomplete skeletal muscle regeneration.[56] Furthermore, the prevention of monocyte recruitment to the site of injury completely inhibits skeletal muscle regeneration.[57] Malerba[58] and Segawa[56] suggest that macrophages directly affect SCs by

two different mechanisms.[59] First, the macrophages can secrete soluble factors affecting SCs, and second, macrophages can interact with SCs by cell-cell contact, and thereby protect them from apoptosis.[60] However, macrophages play a dual role depending on their activity.[57,59,61] Pro-inflammatory macrophages induce myogenic precursor cell proliferation, while anti-inflammatory macrophages induce differentiation and fusion of these cells.[61] A switch between the pro- and anti-inflammatory macrophages has been observed *in vivo* after injury, and during the course of muscular dystrophy.[57,61] Depletion of the anti-inflammatory macrophages reduces the diameter of regenerating myofibers.[59] In addition, a combination of autocrine factors, factors from infiltrating inflammatory cells, and to a lesser extent innervating motor neuron-derived factors, seem to govern the behavior of the SCs.[29]

The basis for the regulation of SC behavior is the attachment within their specific niche, which is established through cell-BM and cell-cell interactions. The basal side of the SCs expresses integrin α7β1, which links the cytoskeleton with laminin in the BM.[62,63] It plays a major role in the transduction of strain-induced mechanical forces into chemical signals, which are involved in the regulation of myogenesis.[64] The apical side expresses M-cadherin that attaches the SC to the adjacent myofiber.[22,34] Both attachment sites are essential for signal transduction between the SC and the two flanking structures.[22,62] In addition, it has been suggested that M-cadherin plays a significant role in the attachment and fusion of myoblasts to form new and regenerate damaged myotubes.[65] This is supported by a significant increase of M-cadherin in activated SCs during skeletal muscle regeneration.[66]

The main constituents of the BM are type IV collagen, laminin, and heparan sulfate proteoglycans (HSPGs).[51] Laminin connects the collagen via the linker protein entactin-1 (or also called nidogen-1) with the integrins on the SCs, which in turn anchore the BM with the intracellular cytoskeleton.[51,67] Upon binding, integrins may influence cell migration, cell shape, and cell-cell interaction and thus play a major role in SC physiology.[68] The differential expression of integrins and tissue-specific laminin regulates homing and activation of stem cells.[69] A recent study showed that integrin α7β1 is required for SC migration and that

hepatocyte growth factor (HGF) plays a crucial role in the guidance of SCs. Furthermore, the results suggests that unrelated and divided SCs stay in long contact with each other and co-migrate along the myofiber.[70] However, specific SC staining should be performed to confirm the SC origin. These results indicate that many factors are involved in SC migration.

In addition, the ECM is capable to capture growth factors such as HGF. In the ECM, HGF is bound to HSPGs preventing SC proliferation.[71,72] The binding of HSPGs to laminin and collagen IV probably integrates these proteins into the BM.[73] HSPGs are not only found in the BM but also on the surface of the SCs. These HSPGs (e.g. syndecan) differ in their extracellular domains and play a role in signal transduction.[74,75] They become upregulated upon SC activation and can transduce signals directly through binding of signaling molecules, or by presenting them to their specific receptors.[76] *In vivo* experiments show that both fibroblast growth factor (FGF) and HGF, which are critical growth factors in skeletal muscle regeneration, require HSPGs for proper signaling.[74] These data suggest HSPGs play a significant role in the regulation of skeletal muscle development and regeneration. Finally, the integrity of the BM is essential to prevent movement of the cells through the tissue.[77]

In vitro studies, showed that the frequency of asymmetric cell division of SC-derived myoblasts diminishes in time,[35,36] and that SC-derived myoblasts have a limited proliferation capacity compared to *in vivo*.[78] This might be caused by sub-optimal levels of growth factors in the culture medium. However, we and others suggest that it could also be due to the loss of specific niche factors *in vitro*.[51] Although this has not been directly demonstrated, knocking out laminin-2 in mice, results in an almost complete absence of skeletal muscle BM[79] and a decrease in the total number of SCs.[80] Thus the integrity of the BM and the ability of SCs to bind to it seems to be essential for SC quiescence and proper functioning upon activation. Furthermore a depleted SC niche, is able to house another stem cell,[81,82] which may then contribute to skeletal muscle regeneration.

Recent studies on the molecular signals regulating SC functioning, were focused mainly on maintenance of SC quiescence. Caveolin-1 and sphingomyelin, which are specifically expressed in the membrane invaginations (caveolae) of quiescent SCs,[83] seem to play a major role in this process. Caveolin-1 regulates caveolae formation and seems to trigger sphingomyelin, which is a lipid in the plasma membrane that facilitates cytoplasmic signaling by concentrating signaling molecules in the caveolae.[84,85] Calcitonin receptors (CTRs) are specifically present in quiescent SCs,[83] suggesting that they are also involved in this process. However, the exact molecular processes of are still unknown.[84,86] Beside factors involved in the regulation of SC quiescence, there are also factors that regulate SC activation. Recent research suggests that Megf10, a transmembrane protein, belongs to this group of factors. Megf10 gene silencing induces differentiation and decreases proliferation, while overexpression enhances proliferation.[87] Overall, many molecular processes and signals from the adjacent myofiber, microvasculature, BM, the SC itself, inflammatory cells, and motor neurons are involved in maintaining SC quiescence, activation, and the subsequent choice between self-renewal, proliferation, and differentiation.

The next section gives an overview of the stimulatory and inhibitory growth factors, which are involved in the regulation of skeletal muscle regeneration.

3.3.2 Growth factors

Growth factors are crucial in SC regulation (table 1). Due to growth factor-activated intracellular signaling pathways e.g. insulin growth factor-1 (IGF-1),[4,88] both controlled up- and down-regulation of muscle-specific genes occur.[4,29] Next to that, the sequence of their release and their cooperation seems also to be important.[89] Growth factors are mostly secreted by active immune cells and by muscle cells after injury. In addition, the vasculature, the SCs themselves, and motor neurons are also responsible for growth factor production[29,90] (figure 1). The ‘indirect’ growth factors are stored in the ECM by binding to proteoglycans[91] and are released from the ECM after skeletal muscle injury. To make this possible, SCs may increase matrix metalloproteinases-2 and -9 (MMP-2

and -9) release after injury.[92,96] These MMPs are involved in ECM degradation that liberates growth factors and cytokines. In this way, SCs can activate themselves indirectly. In addition, MMPs are involved in myoblast migration during regeneration.[93,97,98]

In particular the active neutrophils and macrophages, which infiltrate the necrotic area are responsible for growth factor secretion, but also T-cells and platelets are involved.[29,90] These secreted growth factors, together with the growth factors released from the ECM, attract, activate, and induce differentiation the SCs.[1,19] The activated immune cells also produce adhesion molecules, such as selectins[99] and cytokines, such as IL-6 and TNF-α. The latter influences the local blood flow and vascular permeability, which accelerates the inflammatory response.[1,51]

Table 1. Key growth factors regulating skeletal muscle regeneration.

Growth Factor	Producing cell type	Proliferation/ differentiation	Function	References
HGF	Active immune cells + vasculature + ECM	+/+	Induces quiescent SC activation	Allen et al., 1995; Suzuki et al., 2002; Tatsumi et al., 1998; Tatsumi et al., 2001
Basic FGF	Active immune cells + vasculature + autocrine + ECM	+/+	Upregulated during regeneration, specific role is unclear	Allen and Boxhorn, 1989; Doumit et al., 1993; Haugk et al., 1995; Robertson et al., 1993
IGF-1	Active immune cells + vasculature + autocrine + ECM	++/+	Highly mitogenic for myoblasts and promotes cell survival	Adams and McCue, 1998; Allen and Boxhorn, 1989; Doumit et al., 1993; Haugk et al., 1995; Menetrey et al., 2000; Sato et al., 2003
IGF-2	Active immune cells + vasculature + autocrine	+/+	Upregulated after IGF-1 upregulation, and has a small contribution in myoblast proliferation/differentiation	Doumit et al., 1993; Haugk et al., 1995
VEGF	Variety of cell types, up-regulated during hypoxia	+/?	Stimulates angiogenesis	Doumit et al., 1993; Gowdak et al., 2000; Springer et al., 1998
PDGF-AA PDGF-BB	Active immune cells + Endothelial cells	-/+ +/-	Regulate proliferation/differentiation in opposite ways and support ngiogenesis	Doumit et al., 1993; Robertson et al., 1993
Myostatin	Circulation + autocrine	-/-	Maintains SC quiescence	Amthor et al., 2002; McCroskery et al., 2003; McPherron and Lee, 1997
TGF-β1 & TGF- α	Active immune cells + autocrine	-/-	Prevents myoblast differentiation and recruitment	Allen and Boxhorn, 1989; Haugk et al., 1995; Robertson et al., 1993

3.3.3 Stimulatory growth factors

Many growth factors, such as HGF, FGF-2 and -6, vascular endothelial growth factor (VEGF), platelet-derived growth factor-AA and -BB (PDGF-AA and -BB), stromal derived factor-1 (SDF-1) and IGF-1 and -2 play a major role in myogenic proliferation and differentiation[4,29,51,100-104] (figure 2).

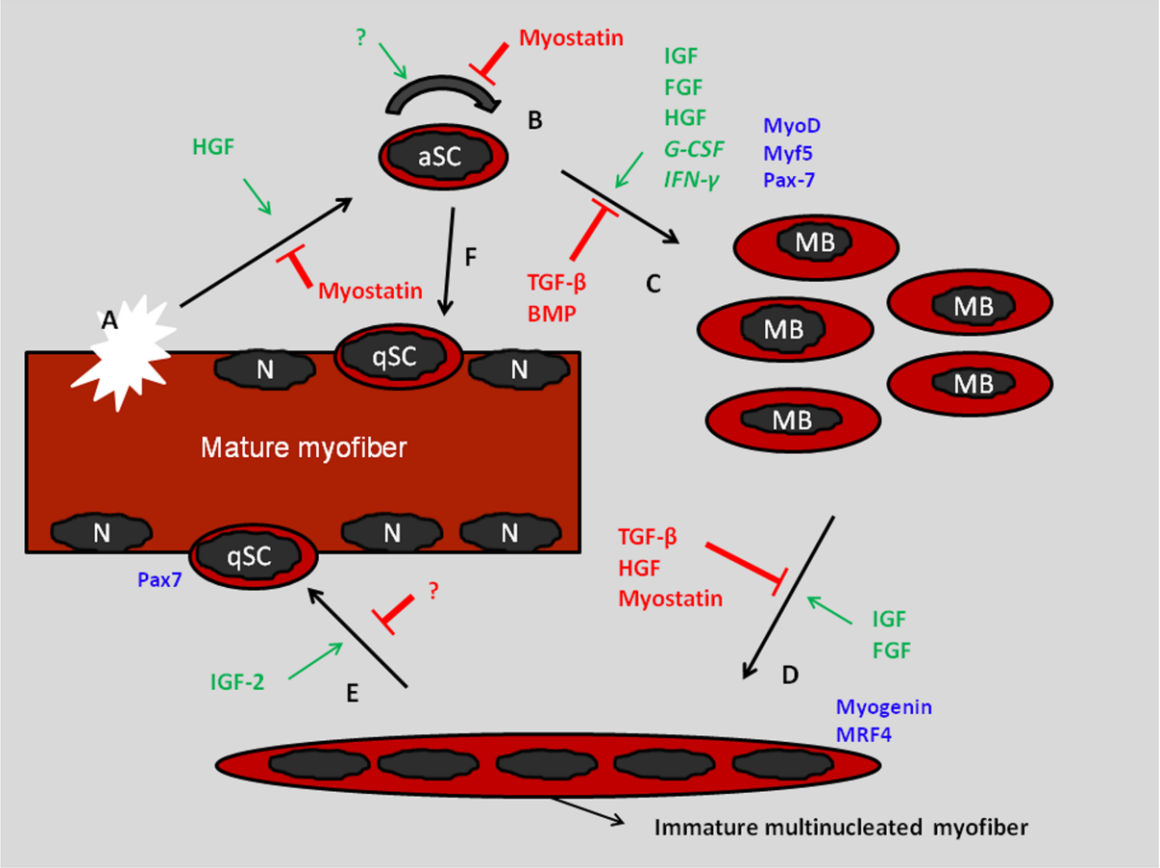

Figure 2. Regulatory growth in satellite cell behavior. *Following myotrauma (A), quiescent satellite cells are activated (by HGF) to enter the cell-cycle for self-renewal (B) and proliferation (C). Activated satellite cells are characterized by a high expression of Pax7, MyoD, and/or Myf5. Subsequent differentiation is marked by the down-regulation of Pax7 and up regulation of Mrf4 and Myogenin. The differentiated myoblasts form new immature multinucleated myofibers (D) or fuse to damaged myofibers (not shown). Finally, the central SC nuclei migrate to a subsarcolemmal position in mature myofibers (E). After SC activation, a subset of activated SCs re-enters the quiescent state to replenish the satellite cell pool (F). aSC, activated satellite cell; qSC, quiescent satellite cell; MB, myoblast; N, nucleus*

In particular IGF-1 is critical for skeletal muscle growth.[105,106] *In vitro*, IGF-1, and in a later phase IGF-2, are both able to alter the expression of myogenic regulatory factors and promote the proliferation and the

differentiation of SC-derived myoblasts.[4,107] Systemic administration of IGF-1 results in increased DNA and protein content in muscle.[108] This was confirmed by using transgenic mice demonstrating that overexpression of human IGF-1 induces muscle hypertrophy.[108] In addition, direct injection of IGF-1 improves muscle regeneration.[38,105,106] *In vitro* studies showed that this is regulated primarily through the phosphatidylinositol 3-kinase (PI3K) pathway and subsequent anti-apoptotic Akt activation.[67] PI3K is also involved in the IGF-1-related increase in protein production.[109,110]

Besides IGF-1, also HGF and VEGF are involved.[1] HGF is the primary factor to induce SC proliferation by binding to c-met.[101,111,112] Correlating with this property, HGF expression is increased in proportion with the degree of injury, during the early proliferation phase of muscle regeneration.[112-114] In correlation with these findings, direct injection of HGF in later stages of muscle regeneration does not promote skeletal muscle repair.[112,115] In addition, HGF plays a role in the migration of SCs to the site of injury.[116,117] In contrast to these stimulatory effects, the inhibitory effect of HGF on the formation of multinuclear myotubes, indicates the pleiotrophic effect of this growth factor.[89] Finally, it has been demonstrated that stretching muscles secrete HGF in a nitric oxide (NO)-dependent way, which might also have a role in SC activation.[118]

VEGF can improve muscle healing by stimulating angiogenesis to increase the nutrient and oxygen supply, which is essential for the healing process.[119,120] VEGF acts together with PDGF, which is also involved in SC regulation. There are some indications that suggest a role for FGFs. FGF-6 expression for example, is muscle specific and is upregulated during muscle regeneration.[121] However, the specific role of these growth factors remains unclear. SDF-1, which is secreted by the adjacent myofiber as well as by the bone marrow, is mainly functioning as a chemoattractant.[103] Recent research suggests that after injury, granulocyte-colony stimulating factor (G-CSF)[122] and interferon-γ (IFN-γ)[123] enhance skeletal muscle cell proliferation. Finally it is important to know that some combinations of growth factors, e.g. HGF and either FGF-2 or -6, have synergistic effects on SC proliferation.[124]

Overall, highly mitogenic IGF-1 seems to be the main growth factor in skeletal muscle regeneration. Also HGF, which is the primary factor that induces proliferation of quiescent SCs, and VEGF, which stimulates angiogenesis, play a major role.

3.3.4 Inhibitory growth factors

The major inhibitory factors in skeletal muscle regeneration are myostatin, transforming growth factor-α and -β1 (TGF-α and -β1), and bone morphogenetic proteins (BMPs), which are all members of the TGF-β superfamily.[125] This family contains many regulatory factors, which depending on the tissue, affect cellular behavior. In skeletal muscle, TGF-β superfamily members have potent inhibitory effects on both muscle development and postnatal regeneration of skeletal muscle.[126]

The TGF-β signaling pathway consists of three main components: the ligand, the receptor, and the intracellular mediators. After ligand binding, receptor dimerization occurs between receptor type I and type II, which transphosphorylates the type I receptor. This activates the latent kinase activity of the receptor complex, which then phosphorylates a receptor-regulated Smad protein that oligomerizes with a common-Smad (also called co-Smad) termed Smad 4. This oligomer translocates into the nucleus where it can interact with Smad-binding elements to regulate transcription of target genes in a cell type-specific manner.[126]

Myostatin is expressed in SCs and myoblasts. Myostatin release results in a down-regulation of Pax3 and Myf5, and prevents the expression of MyoD.[127] Knock-out mice that lack myostatin have extensive muscle hypertrophy.[128] Myostatin may maintain SC quiescence and repress self-renewal through the induction of p21CIP,[13,129] which is a universal inhibitor of cyclin-dependent protein kinase and thus a cell cycle inhibitor.[130] In addition, myostatin and TGF-β1 may reduce myoblast recruitment and differentiation.[131] TGF-β1 also induces remodeling and repair of the ECM and the BM by stimulation of fibroblasts, which results in collagen and fibronectin production.[131] This can result in the formation of scar tissue. It has been demonstrated that decorin, which is an inhibitor of TGF-β, prevents muscle fibrosis and enhances skeletal muscle regeneration.[106] BMPs prevent stem cell

proliferation in a number of stem cell niches (e.g. neural crest), which can be counteracted by the upregulation of Noggin.[77,132,133]

In conclusion, TGF-β1 has the most obvious effect on proliferation and differentiation of SCs, and seems to be the major inhibitor of skeletal muscle regeneration. In addition, other growth factors are involved in skeletal muscle development and regeneration, but a lot of research is needed to define the exact mechanisms.

3.4 Other stem cells in skeletal muscle regeneration

Next to the SCs, recent research also suggests that other precursor cells might play a role in skeletal muscle regeneration (table 2).[4,134] These cells can be divided into muscle- and non muscle-derived stem cells.[135] They might also be isolated and subsequently used to treat muscle injuries or diseases by systemic injection. From a practical point of view, the ideal stem cell population for the treatment of muscle defects should be present in easily accessible postnatal tissues, expandable *in vitro*, able to differentiate into skeletal muscle cells *in vivo*, and should be able to reach skeletal muscle through a systemic route.[136]

Satellite cells are the primary cells involved in skeletal muscle regeneration and are therefore a good candidate for the therapy of injured or diseased muscle.[21] Recent studies showed that transplantation of freshly isolated SCs or myofibers containing SCs can efficiently regenerate skeletal muscles.[30,32,137,138] In addition, new SCs are found in the host muscle.[30-32] In contrast, cultured SC-derived myoblasts gradually lose their myogenic potential, and the transplantation of these cells induces regeneration with much lower efficiency.[32,78] Furthermore, less than 1% of these SC-derived myoblasts survive the first days after transplantation.[139,140] Thus, it appears crucial to use freshly isolated SCs to treat muscle injuries or diseases. However, SCs lack the ability to cross the endothelial lining of the blood vessels in skeletal muscle. Therefore the cells must be injected many times intra-muscularly (IM), which make them less suitable for systemic delivery.[136,141] For these reasons, alternative stem- and precursor cells that are capable to become myogenic

precursor cells were investigated. In the next section, recent developments into the quest for precursor cells with myogenic potential are discussed.

Table 2. Properties of different stem cells involved in skeletal muscle regeneration.

Cell type	Developmental derivation (origin)	Anatomical localization	Lineage potential	Physiological function	References
Satellite cells (Myoblasts)	Mesoderm	Attached to the muscle fiber under the basal lamina	Myogenic	Regeneration of skeletal muscle fibers in injured- and diseased muscle	Boldrin et al., 2009; Collins et al., 2005; Montarras et al., 2005; Sacco et al., 2008; Zammit et al., 2006
SP cells	60% somatic 40% unknown	Interstitial; associated to blood vessels?	Myogenic, hematopoietic	Unknown	Asakura et al., 2002; Uezumi et al., 2006
MDSCs	Unknown	Myofiber periphery closely associated to blood vessels	Myogenic, osteogenic, hematopoietic, cardiogenic, chondrogenic	Unknown	Qu-Petersen et al., 2002; Torrente et al., 2003
Pericytes	Mesectoderm in the head; mesoderm in the body	Periphery of capillaries and microvessels	Myogenic, osteogenic, adipogenic, chondrogenic	Blood flow regulation, control of angiogenesis	Dellavalle et al., 2007
Mesangioblasts	Mesoderm; walls of blood vessels	Associated to microvessel walls	Myogenic, adipogenic, cardiogenic, osteogenic	Unknown	Galvez et al., 2006; Sampaolesi et al., 2006; Sampaolesi et al., 2003
Hematopoietic stem cells	Embryonic vessel endothelium	Bone marrow	Myogenic, myelogenic, lymphogenic	Production of blood cells	Bittner et al., 1999; Ferrari et al., 1998; LaBarge and Blau, 2002
$CD133^+$ cells	Mesoderm	Myofiber close to blood vessels	Myogenic, hematopoietic, endothelial	Angiogenesis after injury, hematopoiesis	Negroni et al., 2009; Torrente et al., 2004

3.4.1 Stem cells in muscle tissue

In addition to SCs, several other stem cells in skeletal muscle show myogenic potential depending on the environmental cues.[4,134] They can be divided into mesoangioblasts (vessel-associated stem cells), side population cells (SP cells), muscle-derived stem cells (MDSCs), pericytes, and $CD133^+$ stem cells.[134,136,142-145] Although the origin, identity, and localization of these cells remains speculative, recent studies suggest that mesoangioblasts and pericytes originate from the walls of blood vessels.[134]

Mesoangioblasts are vessel-associated stem cells derived from the embryonic dorsal aorta and are able to differentiate into several mesodermal cell types including skeletal muscle.[146,147] As an alternative to myoblast transplantation, allogenic transplantation of mesoangioblasts into the blood circulation of dystrophic mice have recently shown great potential for skeletal muscle regeneration.[145,148] One disadvantage, which explains the moderate effect after transplantation,[148] is their limited ability to colonize the muscle. This is caused by incomplete adhesion and extravasation of these cells.[145,148] Furthermore, transplantation of autologous mesoangioblasts transfected with small dystrophin genes into golden retriever dystrophic dogs seems to enhance the formation of functional muscle and dystrophin expression in host muscle tissue.[149] So they have potential, but further research on the role of mesoangioblasts in tissue skeletal muscle regeneration is required.

The heterogeneous muscle SP cells, are a rare, poorly-defined population in skeletal muscle, but they have the potential to give rise to both myocytes and SCs after IM injection.[134,144,150] Muscle SP cells are still present in Pax7-/- mice, which exhibit a severe deficiency in SCs.[144] Secondly, *in vitro* cultured SP cells with a myogenic fate express markers, which are also present both on quiescent and activated SCs upon a myogenic cell culture.[134] Finally, unlike SCs, SP cells also possess hematopoietic potential.[151] These data indicate that SP cells and SCs are distinct populations with similar properties for skeletal muscle regeneration.[144]

MDSCs are a population of early myogenic progenitor cells which have, in contrast to SCs, multi-lineage potential.[134] The transplantation of MDSCs into the skeletal muscle of mice gives better results compared to SCs.[152] One of the advantages of MDSCs, is their prolonged proliferation *in vivo*.[153] This capacity, combined with their strong tendency for self-renewal, multi-lineage differentiation, and immune tolerance, explains the improvements observed after systemic transplantation of MDSCs .[134,153] However, there is a lack of evidence for their long-term self-renewal capacity and their efficacy in dystrophic mice.[2,154] Everything considered, MDSCs are possible candidates to treat skeletal muscle injuries or disorders such as DMD.

Pericytes are localized underneath the basal lamina of the microvasculature and interdigitate with the endothelial cells. They give stability to the microvessels and also regulate blood flow and permeability of the vessels.[155] It has been suggested that pericytes are developmentally derived from mesoangioblasts.[136,146,147] They become myogenic *in vitro* when differentiation is induced and contribute to muscle regeneration in dystrophic mice after intra-arterial injection.[2,136] Unlike SCs, pericyte-derived myogenic cells express myogenic markers only in differentiated myotubes. When pericytes are injected systemically into immune-deficient mice with severe muscular dystrophy (scid-mdx mice), they colonize host skeletal muscle and generate many (dystrophin-rich) muscle fibers.[136] Furthermore, some pericytes were localized in a satellite cell position suggesting that these cells, although at a low efficiency, are able to replenish the satellite cells pool.[136] This make them an interesting potential candidate for future cell therapy in (e.g. DMD) patients.

$CD133^+$ cells circulate in the blood stream and they are able to differentiate, *in vitro*, into endothelial, hematopoietic, and muscle cell types.[143] $CD133^+$ cells express adhesion molecules such as very late antigen-4 (VLA-4), which renders them capable to migrate through blood vessel walls.[134] Injection of human $CD133^+$ cells into the circulation of scid/mdx mice improves skeletal muscle structure and function, and replenishes the SC pool.[143] Muscle exercise 24 hours prior to the injection of $CD133^+$ cells significantly increases human dystrophin expression.[156] The exercise results in an increase of vascular adhesion molecule-1 (VCAM-1) on the endothelium, which improves recruitment of these cells.[156] Recently, IM injected human $CD133^+$ cells showed greater regenerative capacity and increased repopulation of the SC pool compared to injected human myoblasts.[142] These results indicate that $CD133^+$ cells have also a high potential for the treatment of skeletal muscle injuries and diseases.

3.4.2 Other stem cells

The only relevant non muscle-derived stem cell, the hematopoietic stem cell (HSC), is also the most important multipotent stem cell participating

in skeletal muscle regeneration after the SC.[4,157,158] Due to their developmental plasticity in response to injury, transplantation of HSC resulted in the formation of 3.5% GFP^+ myofibers, and they also contribute to the satellite cell pool.[159] This percentage indicates that other stem cells play a marginal role in skeletal muscle regeneration. However, IM- or intra-venously injected donor bone marrow cells were clearly identified within both muscle connective tissue and SC niches of the host musculature.[158] Other studies also observed the incorporation of donor-derived HSC cells.[160,161] Another study,[159] has demonstrated that HSCs also contribute to the muscle SC pool.

In general stem cells have a high proliferative capacity, which might lead to neoplastic transformations. Although there is no direct evidence for this, we should keep in mind that systemic stem cell delivery could be potentially dangerous.[2,162]

In summary, many types of stem cells are currently being studied for their potential in the treatment of skeletal muscle diseases. Many different populations of stem cells might be involved in muscle regeneration and can be used in the treatment of diseased skeletal muscle. However, there is still a long way to go before skeletal muscle regeneration can be routinely induced by injecting stem cells.

3.5 Conclusion

Skeletal muscle regeneration is governed by SCs and their niche, a wide range of growth factors, and probably also by other stem cells. The role of the SC niche factors has become increasingly clear in recent years. The basis for the maintenance of SC quiescence is the attachment within their specific niche by integrin α7β1, which links to cytoskeleton with laminin in the BM, and M-cadherin that attaches the SC to the adjacent myofiber. The niche ECM contributes to satellite cell quiescence by capturing stimulatory HGF. After injury, the ECM releases HGF, and the microvasculature and inflammatory cells release additional activating growth factors such as IGF-1. HGF initiates SC proliferation, while IGF-1 stimulates both proliferation and differentiation of SCs. TGF-β1

negatively influences these processes, and induces the formation of scar tissue. Intra-muscular injection of HGF, IGF, or decorin, seem to improve muscle regeneration. The latter by binding and inactivating TGF-β1. Correct timing of injection is essential for improvement of muscle regeneration. Besides SCs, several populations of other stem cells in muscle might be involved in the muscle regeneration process. For therapy, SCs are unsuitable at the present, because they lack the ability to cross endothelial layers, and less than 1% of the SC-derived myoblasts survives the first days after injection. Due to the limitations of SCs, the MDSCs, mesoangioblasts, pericytes, $CD133^+$ cells, and the non muscle-derived HSCs may prove to be more suitable for the treatment of skeletal muscle injuries and diseases. However, their contribution to the satellite cell pool and future regeneration cycles remains to be established. In particular MDSCs might be promising, because of their prolonged proliferation time *in vivo*. Pericytes, which are able to colonize skeletal muscle after systemic injection, may also be suitable. Future research should focus on optimizing the homing of these cells to the muscle defect after local or systemic injection. Additionally, long-term research into the treatment of DMD should be performed to investigate whether these cells are able to home to the SC niche, and to participate in future regeneration cycles. Ultimately, knowledge about the factors that regulate SC activity, and the potential of other stem cells during muscle regeneration will lead to new therapies for skeletal muscle diseases.

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Chapter 4

A model for muscle regeneration around fibrotic lesions in recurrent strain injuries

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Abstract

Purpose: To establish an *in vivo* model for muscle regeneration after strain injury in the presence of a fibrotic discontinuity.

Methods: The *musculus soleus* of 5-week-old male rats was exposed, completely lacerated, and sutured together with or without a collagen scaffold in between the muscle ends. The scaffold represents a fibrotic discontinuity in the muscle. Muscle healing was evaluated after 14 days by general histology and staining for myofibroblasts, (activated) satellite cells, and inflammatory cells.

Results: Around all wounds satellite cells were activated. Inside the collagen scaffolds satellite cells were absent indicating that muscle regeneration was impaired. In the wounds without a collagen scaffold, the lacerated and sutured myofibers contacted and had already started to regenerate, while this did not occur with an implanted scaffold.

Conclusion: A fibrotic discontinuity, such as an implanted collagen scaffold delays muscle regeneration in skeletal muscle. This model is suitable to study skeletal muscle regeneration in the presence of a fibrotic lesion, and to evaluate new treatment modalities for muscle strain injuries

4.1 Introduction

Muscle strain injuries occur regularly in professional athletes as well as in the general population.[1] The hamstring is the most common muscle group affected and is characterized by a recurrence rate of 30% within the first year after injury. This indicates that full recovery of a hamstring strain injury is often not obtained.[2-4] MRI analysis shows that during the healing of a hamstring injury fibrotic tissue is formed preventing full recovery.[5,6]

In muscle strain injuries, the muscle is sheared, which results in a total rupture of the myofibers and their plasma membrane.[7,8] At this site necrosis of the myofibers begins, but is restricted to the injury site by contraction bands inside the myofibers.[9] After injury, satellite cells, which are located between the sarcolemma and the basal lamina of the muscle fibers,[10,11] are released, activated, and migrate to the site of injury. There they proliferate, differentiate, and fuse to each other or to damaged myofibers to regenerate the skeletal muscle.[12,13] However, blood vessels are also torn and a hematoma is formed filling the gap between the damaged muscle ends. This forms a primary matrix for inflammatory cells, but also for fibroblasts, which synthesize extracellular matrix components.[7,8] These fibroblasts firstly produce fibronectin, followed by collagen type III and finally collagen type I.[14] This might lead to a fibrotic tissue that inhibits growth of muscle fibers and thus impairs regeneration and muscle function.[8,15-17] It has been shown that recurrent muscle strains occur in proximity of this fibrotic discontinuity probably due to its different stiffness and contractility properties.[3,6] Furthermore, recurrent injuries are also more severe and take a longer time to heal than primary strain injuries.[2,18] It is therefore important to prevent or minimize the formation of such a fibrotic discontinuity in order to reduce the risk of recurrence.

In order to reduce fibrosis, and to optimize muscle regeneration, several strategies have been evaluated. The injection of growth factors such as insulin-like growth factor (IGF), fibroblast growth factor-2 (FGF-2), nerve growth factor (NGF), and granulocyte colony-stimulating factor (G-CSF) improves muscle regeneration.[17,19,20] More importantly, the

administration of decorin, which is an inhibitor of transforming growth factor-β (TGF-β), reduces fibrosis.[19,21] The direct delivery of isolated muscle cells is another approach.[22-24] Although the latter yields promising results, a major problem is the poor cell survival and limited migration of the injected cells.[25,26] Alternatively, several different scaffold materials have been used for improving muscle regeneration, but with varying results.[27-30] However, a model to study impaired healing in the presence of a fibrotic lesion is not yet available. Therefore, the aim of this study is to establish an *in vivo* model for a fibrotic discontinuity in healing skeletal muscle by implanting a collagen scaffold.

4.2 Materials and methods

4.2.1 Animals

All animal experiments were approved by the Animal Experiments Committee of the Radboud University Nijmegen Medical Centre (RUNMC) in accordance to the Dutch laws and regulations on animal experiments, which conforms to the ACSM animal care standards. Twenty-four 5-week-old male Sprague-Dawley rats (Janvier, Le Genest, France) were used for the experiments. The rats were housed under normal laboratory conditions, but in the first week after the experimental procedure they were housed individually. All the rats were fed normal rat chow and water ad libitum. Before the start of the experiments the rats had been acclimatized to the animal facility for one week.

4.2.2 Preparation of the collagen scaffolds

The collagen scaffolds were prepared and chemically crosslinked as previously described.[31] Briefly, a 1% (w/v) homogenized collagen suspension was prepared using insoluble type I collagen from bovine achilles tendon (Sigma Chemical CO, St. Louis, MO, USA). The collagen suspension was degassed to remove air bubbles, frozen overnight at -25°C in aluminum trays, and lyophilized. The dried collagen scaffolds were crosslinked using 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride and n-hydroxy-succinimide.[32]

4.2.3 Experimental procedures

At the day of surgery, the rats received 0.02 mg/kg body weight buprenorfine (Temgesic; Schering Plough, Brussels, Belgium) subcutaneously as an analgesic and also at the next two days with a twelve hour interval. Under 5% (induction) followed by 2-3% (continuation) isoflurane anesthesia (Pharmachemie BV, Haarlem, the Netherlands), the left lower limb of the rats was shaved. After a longitudinal incision in the skin and underlying fascia, the M. soleus was gently exposed and transversally lacerated. The two ends were sutured together using a 7-0 polysorb suture (Tyco Healthcare UK, Gosport, UK) with or without the collagen scaffold in between. Before implantation, the collagen scaffolds were sterilized by immersion in 70% ethanol for 1 hour, and then washed three times with sterile phosphate-buffered saline (PBS). The animals were divided into four groups of six rats according to the suturing method and the presence of a collagen scaffold: A) knot-suturing without collagen scaffold, B) knot-suturing with collagen scaffold, C) continuous-suturing without collagen scaffold, and D) continuous-suturing with collagen scaffold. The easiest method for suturing is with one continuous suture around the muscle. However, if this one suture breaks, the wound opens and the scaffold might be lost. To be sure, we also used a method with multiple sutures. However, none of the sutures had broken and there was no different response between the two suturing methods. We therefore decided to group the animals together (A+C and B+D). The fascia and skin were closed with 5-0 polysorb and 5-0 Vicryl sutures (Johnson-Johnson, Langhorne, PA, USA), respectively. To minimize muscle tension, the paw was splinted with an aluminum strip at an angle approximately 45° with respect to the tibia for one week. In group B the paws were swollen and reddish when the aluminum strips were removed. These rats therefore received 1 mg/kg enrofloxacin two times a day (Bayer Healthcare, Brussels, Belgium) for seven days. After fourteen days, the rats were sacrificed according to the standard CO_2/O_2 protocol.

4.2.4 Histology and immunohistochemistry

After sacrifice, the left (wound) and right (internal control) M. soleus of three rats of each group were fixed in freshly prepared 4% paraformaldehyde (PFA) in PBS for 4-6 hours and processed for paraffin embedding. The left and right M. soleus of the other three rats of each group were immediately frozen in OCT embedding compound (CellPath, Newtown, UK) using isopentane precooled in liquid nitrogen. The muscles were cut longitudinally and 5 µm-sections were collected on superfrost plus slides (Menzel-Gläser, Braunschweig, Germany). For general morphology, paraffin sections were stained with hematoxilin and eosin (H&E).

Paraffin sections were also stained with the following antibodies: mouse anti-alpha-smooth muscle actin (α-SMA; Sigma), rabbit anti-Ki67 (Research Diagnostics Inc, Flanders, NJ, USA), mouse anti-ED1 (CD68, Serotec, DPC, Breda, the Netherlands), and mouse anti-MyoD (DAKO, Dakopatts, Glostrup, Denmark). Briefly, the sections were deparaffinated, rehydrated, treated with 3% H_2O_2 for 20 minutes to inactivate endogenous peroxidase, and post-fixed with 4% formaldehyde in PBS. For α-SMA and ED1 staining, the sections were heated in citrate buffer (pH 6.0) for 10 minutes at 70°C. For Ki67 and MyoD staining the sections were heated to 100°C for 10 and 40 minutes, respectively. After rinsing with 0.075% glycine in PBS, the sections were pre-incubated with 10% normal donkey serum (NDS; Chemicon, Temecula, CA, USA) followed by the antibodies against α-SMA (1:1600), ED1 (1:100), Ki67 (1:50) or MyoD (1:25) for 60 minutes. Subsequently, the biotinylated secondary antibodies goat-anti-mouse IgG (H+L) (1:500; Jackson Labs, West Grove, PA, USA) for α-SMA, ED1, and MyoD, and goat-anti-rabbit IgG (H+L) (1:500; Jackson Labs) for Ki67 were added. The bound antibodies were visualized using a preformed biotinylated horse radish peroxidase and avidin complex (Vector Laboratories, Burlingame, CA, USA).

The frozen sections were double-stained with the antibodies rabbit anti-collagen IV (Euro-Diagnostica BV, Arnhem, the Netherlands) and mouse anti-Pax7 (Developmental Studies Hybridoma Bank, Iowa City, CA, USA). Briefly, the sections were dried in air overnight en post-fixed

with 1% PFA in PBS for 10 minutes. After rinsing with 0.05% Triton-X100 in PBS, the sections were pre-incubated with 10% NDS followed by rabbit anti-collagen IV (1:100) for 60 minutes. Collagen IV was then detected using the biotinylated donkey anti-rabbit IgG (H+L) (1:500; Jackson Labs) for 60 minutes and an AlexaFluor-488-labeled avidin (1:500; Molecular Probes, Eugene, OR, USA) for 60 minutes. Thereafter, the sections were again pre-incubated with 10% NDS and then incubated with mouse anti-Pax7 (1:100) overnight at 4°C. Pax7 was detected using an AlexaFluor-594-labeled goat anti-mouse IgG (H+L) (1:200; Molecular Probes). All sections were photographed with the Zeiss Imager.Z1 together with the AxioCam MRc5 camera using the AxioVision 4.6.3 software (Carl Zeiss Microimaging GmbH, Jena, Germany).

4.2.5 Statistical analysis

The numbers of Pax7- and MyoD-positive cells were counted in 1) the control muscle (C), 2) the cutting zone without the collagen scaffold (W), 3) the cutting zone with the collagen scaffold (W+S), 4) inside the collagen scaffold (S), and 5) in the non-injured muscle tissue of the wounded M. soleus (NI). To count the Pax7-positive cells the images were divided into 50 squares. In 5 random squares the total number of Pax7-positive cells and DAPI-stained nuclei was counted. The total number of MyoD-positive cells and nuclei of every group was determined in three different fields of an overview image. The numbers of Pax7- and MyoD-positive cells were expressed as a percentage ± SD of the total number of cells. The differences in the percentages of Pax7- and MyoD-positive cells were tested for significance using a Kruskal-Wallis One-Way ANOVA on Ranks followed by Dunn's method. A value of $p < 0.05$ was considered to be significant.

4.3 Results

Out of the twenty-four rats, one animal in group A without a collagen scaffold did not survive the surgery. After an initial growth arrest, all rats in every group had gained about 25% body weight at the tenth day. The

groups were not significantly different. The immobilization of the left hind leg did not affect the growth of the rats. Macroscopically, the wounded muscle adhered partly to the surrounding tissues. Furthermore, the collagen scaffolds were not visible anymore and appeared to be integrated into the muscle tissue. The sutures did not break and the different suturing methods had no effect on muscle morphology and gave the same results regarding muscle regeneration. Therefore, the animals of group A and C, and B and D were grouped together.

4.3.1 General histology

H&E staining (figure 1A) revealed properly arranged longitudinal myofibers in the controls (C), but not in the wounded muscles. Within the wounds, regenerating myofibers were present indicated by centrally located nuclei (figure 1A, magnification). Some myofibers in the cutting zone had fused properly in the group without the collagen scaffold. On the contrary, the implantation of a collagen scaffold prevented fusion of the myofibers (W+S). The collagen scaffolds were surrounded by giant cells and a cell layer (an interphase).

4.3.2 Immunostainings

Paraffin sections were stained with antibodies against α-SMA, ED1 (CD68), Ki67, and MyoD to identify blood vessels and myofibroblasts, inflammatory cells, proliferating cells, and activated satellite cells, respectively (figure 1B). In the controls (C), ED1-positive inflammatory cells and Ki67-positive proliferating cells were present. However, the controls hardly contained any MyoD-positive nuclei (indicated by arrows). As expected, α-SMA-positive cells were not present in the muscle tissue of the controls, but only in blood vessels. Without a collagen scaffold (W) there was an increase in the number of ED1- and Ki67-positive cells of which the majority surrounded the sutures. More importantly, many MyoD-positive nuclei were present (a few are indicated by arrows). The number of α-SMA-positive blood vessels was also increased, and there were also α-SMA-positive cells in the muscle tissue. The implantation of a collagen scaffold (W+S) caused an infiltration of ED1-positive giant cells and other inflammatory cells,

which surrounded the scaffold. Even inside the scaffolds inflammatory cells were present. Proliferating Ki67-positive cells were present in the muscle tissue, the interphase, and also inside the collagen. Again, the muscle tissue around the scaffold (W+S) contained many MyoD-positive nuclei (a few are indicated by arrows), but all the cells inside the scaffolds were negative for MyoD. The expression pattern of α-SMA was similar to the wounds without the scaffold (W). Inside the scaffolds α-SMA-positive blood vessels were also found.

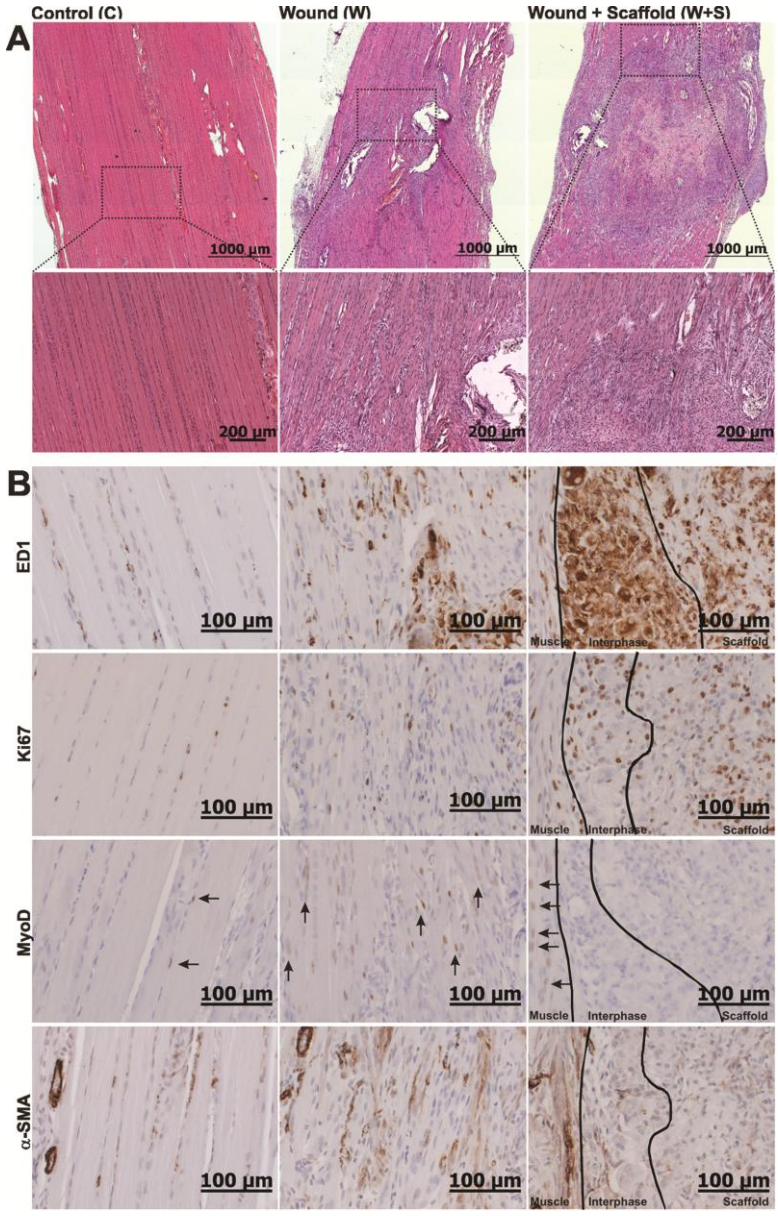

Figure 1. ***Histology of the M. soleus at 14 days post-surgery.*** *A) H&E staining of the control (C), wound without the collagen scaffold (W; group C), and wound with the collagen scaffold (W+S; group D) revealed the disruption of the aligned myofibers at the*

cutting zone after laceration. The implanted collagen scaffold is surrounded by an interphase and prevented myofiber fusion. B) Immunohistochemistry of the control (C), wound without the collagen scaffold (W; group C), and wound with the collagen scaffold (W+S; group D) with antibodies directed against ED1, Ki67, MyoD, and α-SMA. Only a few ED1-, Ki67-, and MyoD-positive cells (indicated by arrows) and α-SMA-positive blood vessels are present in the control (C). In the wound (W) the number of these cells are higher and α-SMA-positive cells are present. The collagen scaffold (W+S) is surrounded by an ED1-positive interphase. In the wounded muscles and around the scaffold many Ki67-, MyoD- (a few are indicated by arrows) and α-SMA positive cells are present. The scaffold also contains ED1-, Ki67, and α-SMA-positive cells and blood vessels, but no MyoD-positive cells.

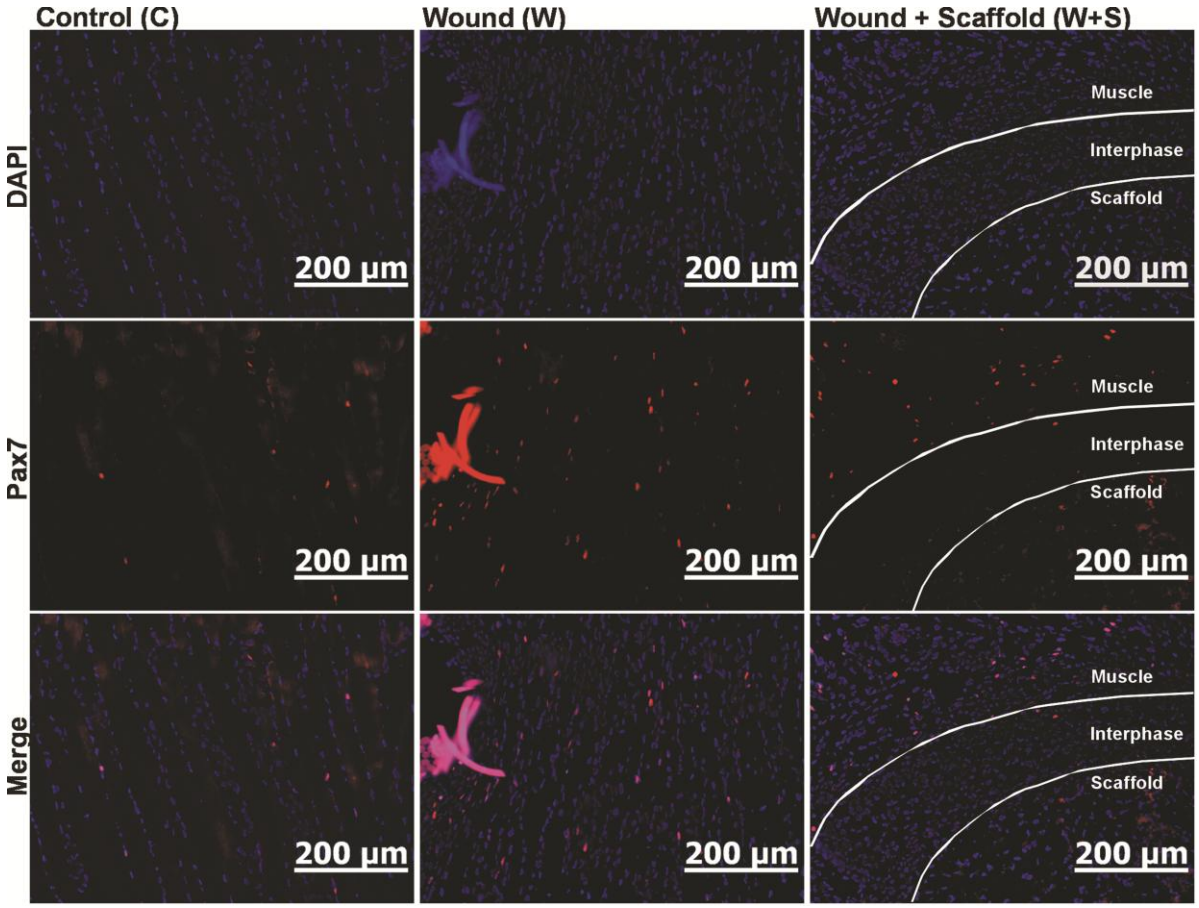

Figure 2. Fluorescent immunohistochemistry of the M. soleus at 14 days post-surgery. *The control (C), wound without the collagen scaffold (W; group A), and wound with the collagen scaffold (W+S; group B) were stained with the antibody directed against Pax7. In the control only a few Pax7-positive cells are present, while in the wound (W) the number of these cells is increased. In the wounded muscle tissue around the collagen scaffold (W+S) the number of Pax7-positive cells is also increased. On the contrary, these cells are absent in the interphase and the collagen scaffold.*

To identify the resident satellite cells, cryosections were stained with the Pax7 antibody (figure 2). In the controls (C) only a few satellite cells were present, but around the cutting zone in the wounded muscle tissue with (W+S) or without (W) a collagen scaffold the number of satellite cells was increased. However, no satellite cells were present within the collagen scaffolds.

4.3.3 Quantifications

The percentage of MyoD- and Pax7-positive cells were determined on the paraffin (figure 1B) and cryosections (figure 2), respectively (figure 3).

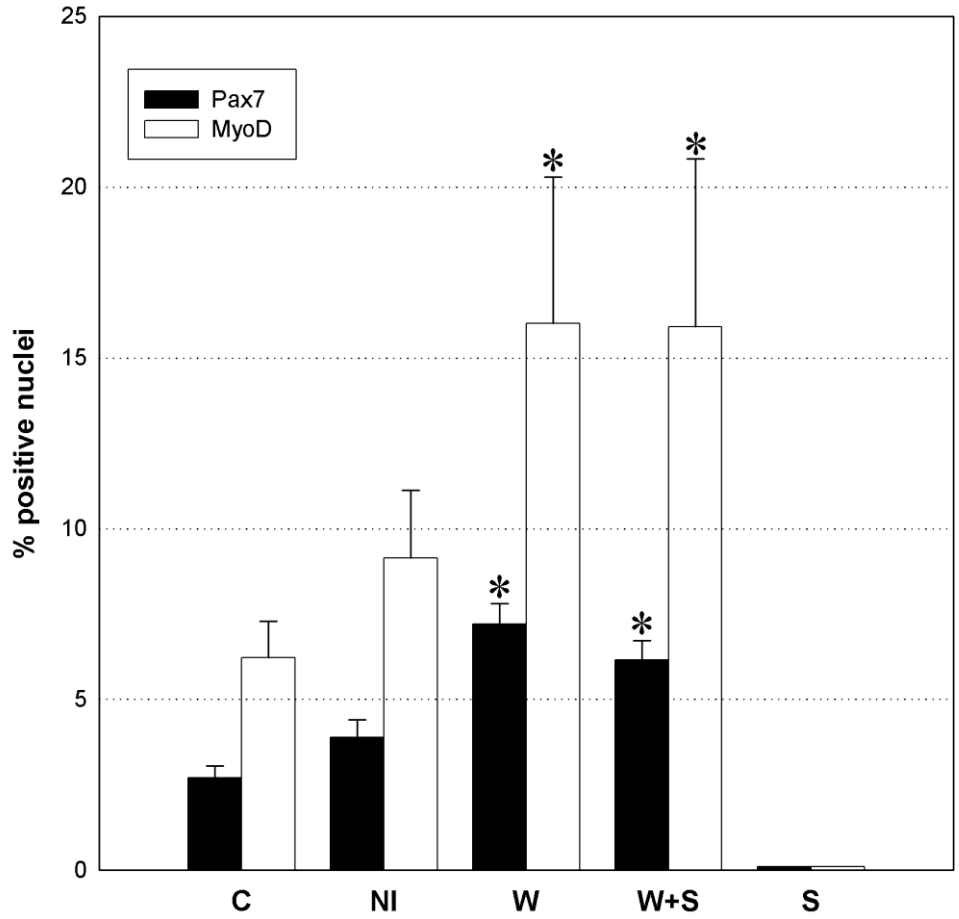

Figure 3. Quantification of Pax7- and MyoD-positive (activated) satellite cells. *The number of Pax7- and MyoD-positive cells are expressed as a mean percentage ± SD of the total number of cells. Compared to the controls (C; N=11), the number of Pax7- and MyoD-positive cells is significantly increased in the wounded muscle tissue of wounds with (W+S; N=6) or without (W; N=5) the collagen scaffold. In the non-injured area of the wounded muscles (NI; N=11) the number of Pax7- and MyoD-positive cells is also, but not significant, increased. In the scaffolds (S; N=6) no Pax7- and MyoD-positive cells are found. * significantly increased (P < 0,05) compared to the control.*

The controls contained only a low number of Pax7-positive satellite cells (2.7 ± 0.4%), which significantly ($p < 0.05$) increased to 7.2 ± 0.6% and 6.2 ± 0.6% in the wounded tissue without (W) or with (W+S) the collagen scaffold, respectively. The number of MyoD-positive cells also significantly ($p < 0.05$) increased from 6.2 ± 1.1% in the controls to 16 ± 4.3% and 15.9 ± 4.9% in the wounds without (W) or with (W+S) the collagen scaffold, respectively. Furthermore, there was a slight but non-significant increase of Pax7- (3.9 ± 0.5%) and MyoD-positive cells (9.1 ± 1.9%) in the non-injured area (NI) of the wounded muscles compared to the controls. However, no Pax7- and MyoD-positive cells were found inside the collagen scaffolds.

4.4 Discussion

The successful treatment of muscle strains in sports medicine is still a problem. Fibrotic lesions are often formed during muscle regeneration causing incomplete functional recovery. More importantly, recurrent muscle injuries may occur near this fibrotic tissue.[3,5,6] Since fibrotic tissue consist mainly of collagen type I,[33,34] we developed an *in vivo* model for a fibrotic discontinuity by implanting a type I collagen scaffold between the lacerated muscle ends. Using this method it is possible to standardize the wounds with a collagen scaffold, but it is important to be aware that this is an extreme version of a muscle strain. In this model we evaluated muscle regeneration after a two-week healing period. The numbers of Pax7- and MyoD-positive (activated) satellite cells or myoblasts were increased about two-fold in the wounded muscle tissue and around the collagen scaffolds compared to the control muscle. This indicates that the muscle fibers were regenerating, and that the scaffold did not inhibit the activation of satellite cells in the adjacent muscle tissue. However, inside the collagen scaffold these cells were absent. Thus, in the presence of a fibrotic discontinuity the skeletal muscle cannot regenerate properly since activated satellite cells do not migrate into the fibrotic tissue. Similar to our results, others have also shown that after a strain injury, inflammation occurs, followed by the production of

fibrous tissue, which could eventually develop into a fibrotic lesion.[1,35] Another study on rectus femoris strain in humans showed a chronic inflammation and a mixture of regenerating muscle fibers and fibrotic tissue in the wound.[36] Although muscle regeneration was only evaluated after two weeks in this initial study, collagen scaffolds can persist in the muscle tissue for up to 50 days.[37] Therefore, our model can be used to evaluate treatment strategies for recurrent muscle strains.

Optimal treatment should diminish or prevent the formation of fibrotic tissue, and reduce the risk of recurrence. We and others[38,39] observed that suturing the lacerated muscle ends directly together allows full regeneration of the muscle. Currently, the treatment principle of muscle strains consist of rest, ice, compression, and elevation (RICE).[14] With specific compression, which could serve as a splint, it may also be possible to bring the muscle ends to each other and diminish the onset of fibrosis. Surgical treatment to suture the muscle ends together, is only indicated in cases with extensive injury to the muscle.[14] If a fibrotic tissue from a previous injury is already present, additional treatment with matrix metalloproteinase-1 (MMP-1) might offer a solution. Previous research has shown that treatment with injection of MMP-1 improves muscle regeneration and that a fibrotic lesion can be partially resolved.[33,40] Thus, combining the injection of MMP-1 with specific compression therapy might diminish a pre-existing fibrotic discontinuity or minimize the risk of a secondary fibrosis.

In this study the M. soleus in rats is used as a wound model, because all the myofibers run parallel. However, the M. soleus consist mainly of type I (slow) fibers,[41] while the hamstring, which is the most common muscle group affected in muscle strains, consist of type II (fast) fibers.[42] It has been shown that type II muscles regenerate better than type I muscles, which more often develop fibrotic lesions.[43] This indicates that the results obtained in this study may differ from a hamstring injury, in which the regeneration proces could be more efficient. However, it also demonstrates that the M. soleus is a good model to study the effects of the presence of a fibrotic discontinuity on muscle regeneration.

In this study we only analyzed 14 days post-surgery because satellite cell activation is a relatively early event in muscle healing.[12,13] In future

studies, analysis at later time-points is necessary to exclude the possibility that implantation of a collagen scaffold only delays muscle regeneration. In addition, it is important that functional studies are performed to further evaluate this model.

In conclusion, we generated a model for the regeneration of skeletal muscle in the presence of a fibrotic discontinuity. This model can be used to evaluate new treatment strategies for recurrent muscle strains.

4.5 Acknowledgments

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Chapter 5

Skeletal muscle fibrosis: the effect of stromal-derived factor-1α-loaded collagen scaffolds

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Abstract

Aim: To develop a model for muscle fibrosis based on full-thickness muscle defects, and to evaluate the effects of implanted stromal-derived factor (SDF)-1α-loaded collagen scaffolds.

Methods: Full-thickness defects 2 mm in diameter were made in the *musculus soleus* of 48 rats and either left alone or filled with SDF-1α-loaded collagen scaffolds. At 3, 10, 28 and 56 days post-surgery, muscles were analyzed for collagen deposition, satellite cells, myofibroblasts and macrophages.

Results: A significant amount of collagen-rich fibrotic tissue was formed, which persisted over time. Increased numbers of satellite cells were present around, but not within, the wounds. Satellite cells were further upregulated in regenerating tissue when SDF-1α-loaded collagen scaffolds were implanted. The scaffolds also attracted macrophages, but collagen deposition and myofibroblast numbers were not affected.

Conclusion: Persistent muscle fibrosis is induced by full-thickness defects 2 mm in diameter. SDF-1α-loaded collagen scaffolds accelerated muscle regeneration around the wounds, but did not reduce muscle fibrosis.

5.1 Introduction

Skeletal muscle tissue repairs itself by the activation of satellite cells, which are associated with the myofibers.[1,2] Activated satellite cells migrate to the site of injury and generate myoblasts, which eventually differentiate and fuse to each other or to damaged myofibers to restore muscle structure and function.[3-5] The satellite cells also have the capacity to replenish their numbers by self-renewal for future regeneration cycles.[6-8] However, fibrosis can also occur, which prevents full functional recovery of the muscle.[3,4,9,10] Many approaches have been developed to improve muscle regeneration. The application of growth factors such as insulin growth factor (IGF)-I, fibroblast growth factor (FGF)-II, hepatocyte growth factor (HGF) nerve growth factor (NGF), or granulocyte-colony stimulating factor (G-CSF), has been shown to improve muscle regeneration by inducing satellite cell proliferation and differentiation.[11-15] Inhibition of transforming growth factor (TGF)-β can reduce the extent of fibrosis, and promotes muscle regeneration.[11,16] The injection of cell types such as satellite cell-derived myoblasts, side-population (SP) cells, muscle-derived stem cells (MDSC), mesoangioblasts, pericytes, and $CD133^+$ stem cells improves muscle regeneration after muscle injury, but also in muscle diseases, like Duchene Muscular Dystrophy (DMD).[8,17-22] Many muscle injury models exist such as crush injury, freeze injury, toxin-induced injury, strains, contusions, lacerations, and muscle disease models, which can induce minor muscle fibrosis.[3,4,23] However, full-thickness defects, which result in the loss of muscle tissue and the formation of large fibrotic lesions, are not widely studied. Such a model represents muscle resection after tumor ablation or other surgical muscle traumas. It can be used to develop methods to (re)generate skeletal muscle tissue and inhibit the formation of fibrotic tissue by implantation of a regenerative scaffold. To achieve this, the addition of growth factors or cells alone is not sufficient. Tissue-engineered constructs are required to fill up the defect and provide the necessary structural cues for the satellite cells. Several studies using such scaffolds have been performed with varying results, but loading of the scaffolds with growth factors and/or cells generally improves muscle

regeneration.[24-27] However, the myogenic potential of satellite cells is readily lost during culture.[8,28] Furthermore, transplanted satellite cells and myoblasts hardly survive and their migration into the muscle tissue is limited.[17,29] Since satellite cells are the primary cells for muscle regeneration, we loaded collagen scaffolds with stromal-derived factor (SDF)-1α to attract resident sattelite cells into the defect. SDF-1α is a CXC chemokine, which controls processes such as trafficking and transendothelial migration of hematopoietic cells.[30] SDF-1α binds to CXCR4, which is also present on satellite cells.[31] During embryogenesis, SDF-1α regulates the migration of muscle precursor cells.[32,33] Moreover, in adulthood, SDF-1α is expressed by myofibers and induces migration of satellite cells.[34,35] Therefore, the aim of this study was to induce muscle fibrosis with a full-thickness muscle defect, and to evaluate the effects of SDF-1α-loaded non-crosslinked collagen scaffolds.

5.2 Materials and methods

5.2.1 Rats

All animal experiments were approved by the Animal Experiments Committee of the Radboud University Nijmegen Medical Centre (RUNMC) in accordance to the Dutch laws and regulations on animal experiments. Forty-eight 5-week-old male Sprague-Dawley rats (Janvier, Le Genest, France) were used for the experiments. The rats were housed under normal laboratory conditions and fed normal rat chow and water ad libitum. Before the start of the experiments the rats had been acclimatized to the animal facility for one week.

5.2.2 Experimental procedures

At the day of surgery, the rats received 0.02 mg/kg body weight buprenorfine (Temgesic; Schering Plough, Brussels, Belgium) subcutaneously as an analgesic, and also at the next two days with a twelve hour interval. Under 5% (induction) followed by 2-3% (continuation) isoflurane anesthesia (Pharmachemie BV, Haarlem, the Netherlands), the M. soleus of the left lower limb of the rats was gently

exposed. Using a 2mm biopsy punch, a full-thickness defect was made in the centre of the M. soleus. The rats were divided into two groups of 24 animals: 1) Ø 2mm wounds without a scaffold and 2) Ø 2mm wounds with collagen scaffold + SDF-1α.

The collagen scaffolds were prepared with a 1% (w/v) homogenized collagen suspension with insoluble type I collagen from bovine achilles tendon (Sigma Chemical CO, St. Louis, MO, USA). The collagen suspension was degassed, frozen overnight at -25°C in aluminum trays, and lyophilized. Before implantation, the collagen scaffolds were sterilized by immersion in 70% EtOH for 30 min, washed three times with sterile phosphate-buffered-saline (PBS), and then incubated in PBS containing 0,1 % (w/v) bovine serum albumin (BSA) and 2 μg/ml SDF-1α (R&D Systems, Minneapolis, MN, USA) at room temperature for 48 hours. Then the scaffold were directly implanted into the muscle defects. The fascia and skin were closed with 5-0 polysorb and 5-0 Vicryl sutures (Johnson-Johnson, Langhorne, PA, USA), respectively. Rats were sacrificed according to the standard CO_2/O_2 protocol at 3, 10, 28, and 56 days post-surgery (6 rats per group for every time point).

5.2.3 Histology

The left (wound) and right (internal control) M. soleus of the rats were excised and fixed in freshly prepared 4% paraformaldehyde (PFA) in PBS for 24 hours, and processed for paraffin embedding. Longitudinal muscle sections (5μm) were collected on superfrost plus slides (Menzel-Gläser, Braunschweig, Germany). For general morphology, paraffin sections were stained with hematoxiline and eosin (H&E according to Delafield, not shown). To detect collagen fibers, the sections were stained with azocarmine G and aniline blue (AZAN) according to standard protocols.

5.2.4 Immunohistochemistry

Sections were deparaffinated, rehydrated, treated with 3% H_2O_2 for 20 minutes to inactivate endogenous peroxidase, and post-fixed with 4% formaldehyde in PBS. The sections were incubated with mouse anti-alpha-smooth muscle actin (α-SMA, 1:1600; Sigma), mouse anti-ED1

(CD68, 1:100; Serotec, DPC, Breda, the Netherlands), and mouse anti-MyoD (1:25; DAKO, Dakopatts, Glostrup, Denmark) overnight at 4°C as described previously.[36] Paraffin sections were also incubated with mouse anti-Pax7 (1:100; Developmental Studies Hybridoma Bank, Iowa City, CA, USA), mouse anti-collagen type I (1:1000; Sigma), rabbit anti-collagen type III (1:1600; Chemicon International, Temecula, USA), mouse anti-Hsp47 (1:24000; Assay Design, Ann Harbor, MI, USA), and mouse anti-Myogenin (F5D, 1:100; Developmental Studies Hybridoma Bank). For collagen type I, Hsp47, and Myogenin staining, the sections were heated in citrate buffer (pH 6,0) for 10 min at 70°C, and subsequently treated with 0,075% trypsin in PBS (pH 7.4) for 15 minutes. For Pax7 and collagen type III staining, the sections were first heated in 0.25mM EDTA/10mM TRIS buffer (pH 9,0) at 100°C for 10 minutes. After rinsing with 0.075% glycine in PBS, all sections were pre-incubated with 10% normal donkey serum (NDS; Chemicon, Temecula, CA, USA), then followed by the primary antibodies overnight at 4°C. Subsequently, the bound antibodies were visualized using the biotinylated secondary antibodies donkey-anti-mouse IgG (H+L) (1:500; Jackson Labs, West Grove, PA, USA) for Pax7 and collagen type I, and donkey-anti-rabbit IgG (H+L) (1:500; Jackson Labs) for collagen type III, and a preformed biotinylated horse radish peroxidase and avidin complex (Vector Laboratories, Burlingame, CA, USA). The sections were stained with 3,3-diaminobenzidine (DAB) and photographed with a Zeiss Imager.Z1 together with an AxioCam MRc5 camera using AxioVision 4.6.3 software (Carl Zeiss Microimaging GmbH, Jena, Germany).

5.2.5 Quantification

The relative amount of collagen was analyzed on the AZAN-stained muscle sections in 1) the control muscle (C), 2) wounded muscle without the scaffold (W), and 3) wounded muscle with the collagen scaffold + SDF-1α (W+SDF-1α) at every time-point. The amounts of collagen (blue) and muscle tissue (red) were analyzed in a fixed span of 0.5 cm muscle tissue containing the wound area, and quantified using Qwin software (Leica Imaging Systems, Cambridge, UK). The amount of

collagen was expressed as the mean area percentage ± SD of the total area. The number of $Pax7^+$ and $MyoD^+$ cells was counted in the C and in the regenerative zone (regenerating muscle tissue at the border of the 2mm wound) of the groups W and W+SDF-1α at every time-point. The numbers of $Pax7^+$ and $MyoD^+$ cells were expressed as a mean percentage ± SD of the total number of cells.

5.2.6 Statistics

The differences in the percentages of collagenous tissue and $Pax7^+$ cells were tested for significance using a Two-Way ANOVA with Holm-Sidak post-hoc analysis. A value of $p < 0.05$ was considered to be significant. The percentages of $MyoD^+$ cells were not normally distributed and therefore the One-Way ANOVA with post-hoc Holm-Sidak analysis was used to test significance at individual time points. A value of $p < 0.01$ was then considered to be significant (Bonferroni correction).

5.3 Results

In the experimental group without the collagen scaffold (W) one rat died during surgery. All the other rats showed an initial growth arrest, but all had gained about 25% body weight at the tenth day post-surgery. Macroscopically, the collagen scaffolds were only visible at day three post-surgery and remained inside the defect.

5.3.1 AZAN staining

Paraffin sections were stained with AZAN to identify muscle tissue (red) en collagen (blue) (figure 1A). In the control (C)-group only little collagen was present. At three days post-surgery, the defect is still visible in the wound (W)-group, and some collagen is already deposited at the borders of the defects. At 10 days, large collagen deposits are present in and around the wounds, which persist at 28 and 56 days. In the wound with SDF-1α-loaded collagen scaffold (W+SDF-1α)-group, the scaffolds are clearly visible and surrounded by a fibrin blood clot at three days. Collagen is also visible at the borders of the defects. At 10 days, the

scaffold has become smaller and has completely disappeared at 28 and 56 days, but extensive collagen depositions are still present in these wounds. Few regenerating myofibers are found within the collagen depositions.

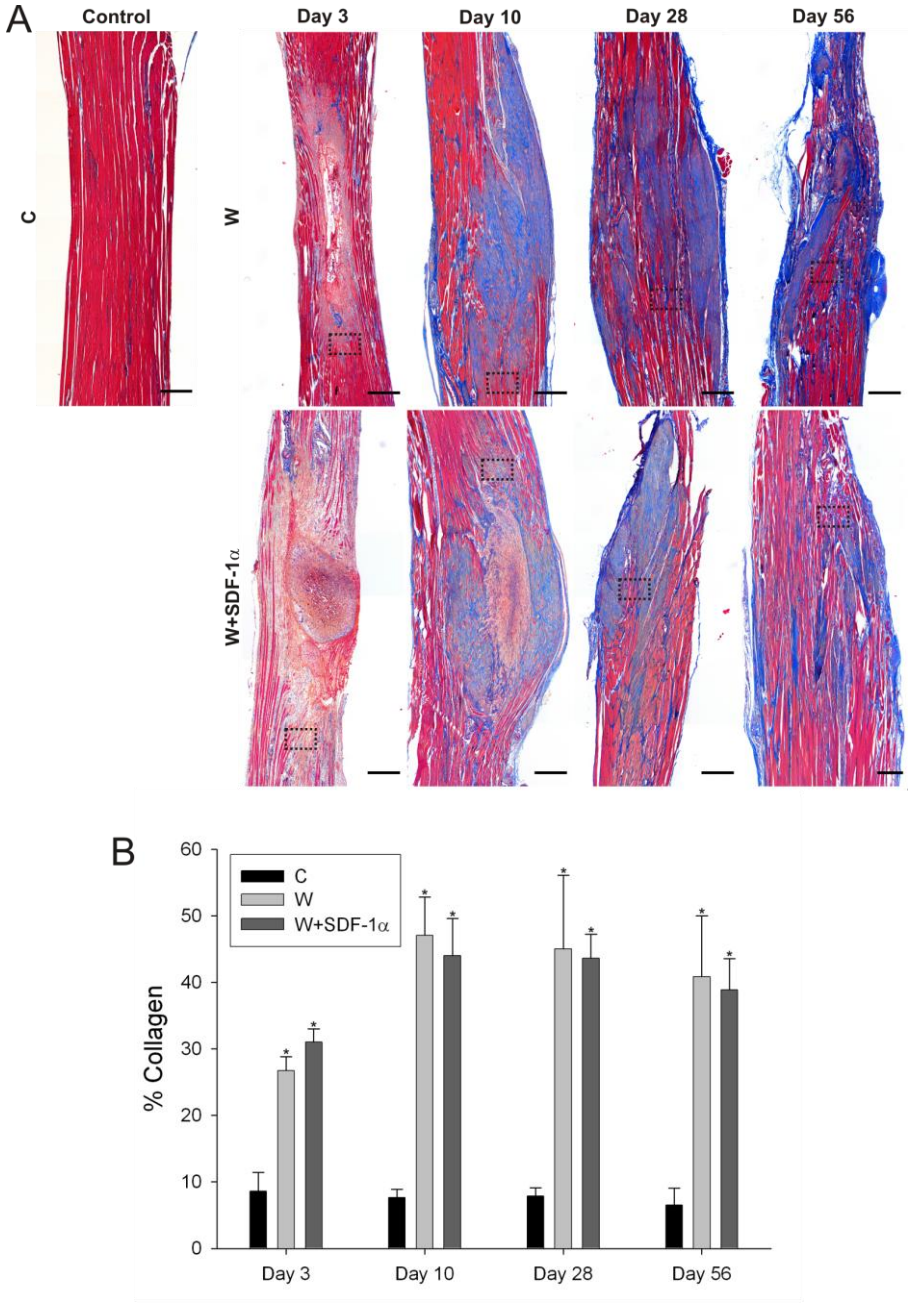


Figure 1. ***AZAN staining and quantification.*** *A) The wounds without (W) and with SDF-1α-loaded collagen scaffolds (W+ SDF-1α) were stained with AZAN (muscle tissue red, collagen blue). B) Quantification of collagen in the controls (C), wounds without (W) and with SDF-1α-loaded collagen scaffolds (W+SDF-1α). The area of collagen is expressed as a mean percentage ± SD compared to the total tissue area. * significantly different from C ($p < 0.05$).The scale bar represents 500 μm and the marked area represents the regenerative zone used for Pax7 and MyoD quantification.*

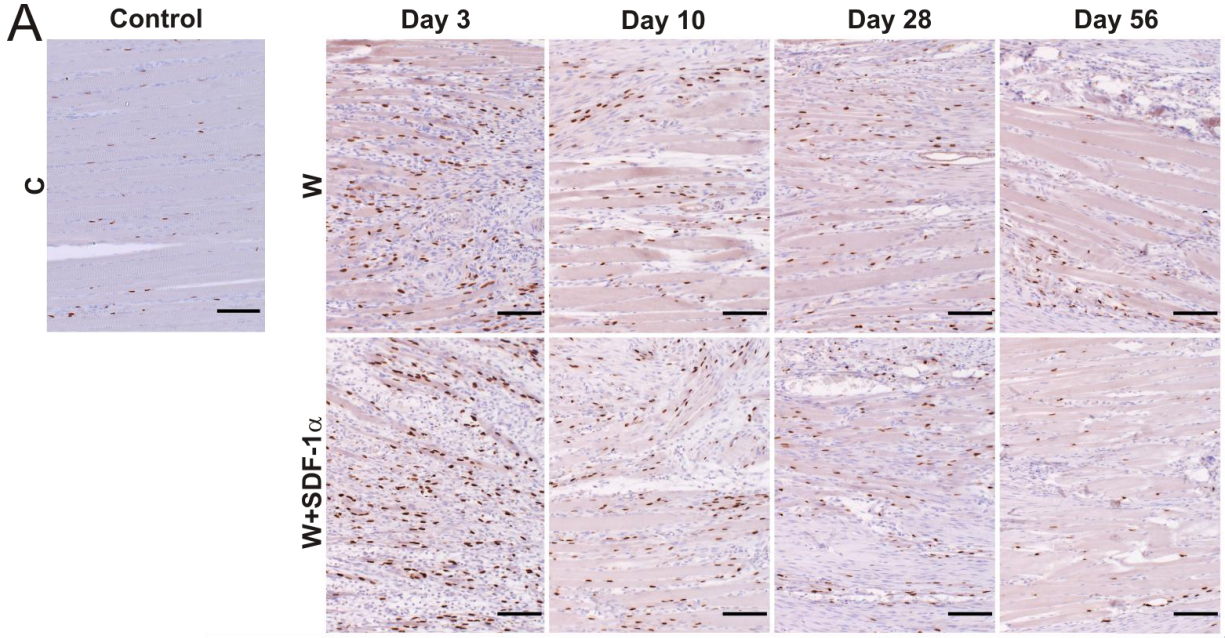
Quantification of the relative amount of collagen is presented in figure 1B. At three days post-surgery, the amount of collagen is significantly increased from 8.6 ± 2.9% in the C-group to 26.7 ± 2.1% and 31.1 ± 1.9 % in the W- and W+SDF-1α-groups, respectively. At 10 days, the amount of collagen in both wounds is further increased to approximately 45% and remains constant up to 56 days post-surgery. The amount of collagen between the W- and W+SDF-1α-group is not significantly different at any time-point.

5.3.2 Pax7 and MyoD immunostaining

Paraffin sections are stained with antibodies against Pax7 (figure 2A) and MyoD (figure 3A) to identify activated satellite cells. In the C-group only few $Pax7^+$ cells are found in the muscle tissue. In both the W- and W+SDF-1α-group many $Pax7^+$ cells are present after three and ten days at the border of the wounds (indicated in figure 1A) in the regenerative zone. The number of these cells is clearly diminished at 28 and 56 days. Within the wounds no $Pax7^+$ cells are found at any time point. The relative numbers of $Pax7^+$ cells are presented in figure 2B. At three days, the numbers of $Pax7^+$ cells are significantly increased from 7.8 ± 0.9% in the C-group to 14.5 ± 1.2% in the W-group. In the W+SDF-1α-group it is further increased to 18.6 ± 1.4%. At ten days, the numbers of $Pax7^+$ cells are compared to the C-group significantly increased to 12.7 ± 1.1% in the W-group, and in the W+SDF-1α-group even further to 16.7 ± 1.4%. At 28 days the numbers of $Pax7^+$ cells are still significantly increased to approximately 11% in both the W- and W+SDF-1α-groups. At 56 days it is normalized to control levels in both groups. In the C-group the numbers of $Pax7^+$ cells are diminished significantly in time, and in the wounds their numbers are upregulated in the first 10 days and then gradually diminish to control levels.

In the C-group only a few numbers of $MyoD^+$ cells are found, but in the W- and W+SDF-1α-group many $MyoD^+$ cells and myofibers are present in the regenerative zone around the wounds. In the W- and W+SDF-1α-group, the numbers of $MyoD^+$ cells seem to have increased at 3 and 10 days and then diminish again. The quantification of the relative numbers of $MyoD^+$ cells and myofibers is presented in figure 3B.

The numbers of MyoD$^+$ cells in the C-group diminish in time and is always significantly lower than in the W- and W+ SDF-1α-group.



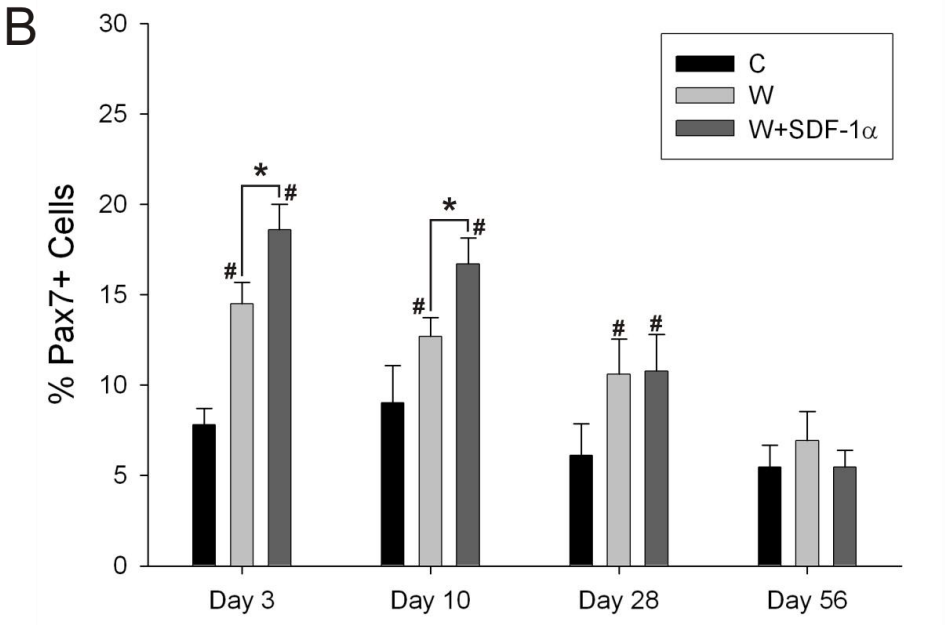


Figure 2. Pax7 immunohistochemistry and quantification. *A) Sections of the wounds without (W) and with SDF-1α-loaded collagen scaffolds (W+SDF-1α) are shown. Pictures were taken at the border of the wound in the regenerative zone as indicated in figure 1A. B) Quantification of Pax7$^+$ cells in the controls (C), wounds without (W) and with SDF-1α-loaded collagen scaffolds (W+SDF-1α). The numbers of Pax7$^+$ cells are expressed as a mean percentage ± SD of the total numbers of cells. # significant difference with C ($p < 0.05$). * significant difference between W and W+ SDF-1α ($p < 0.05$). The scale bar represents 100 μm.*

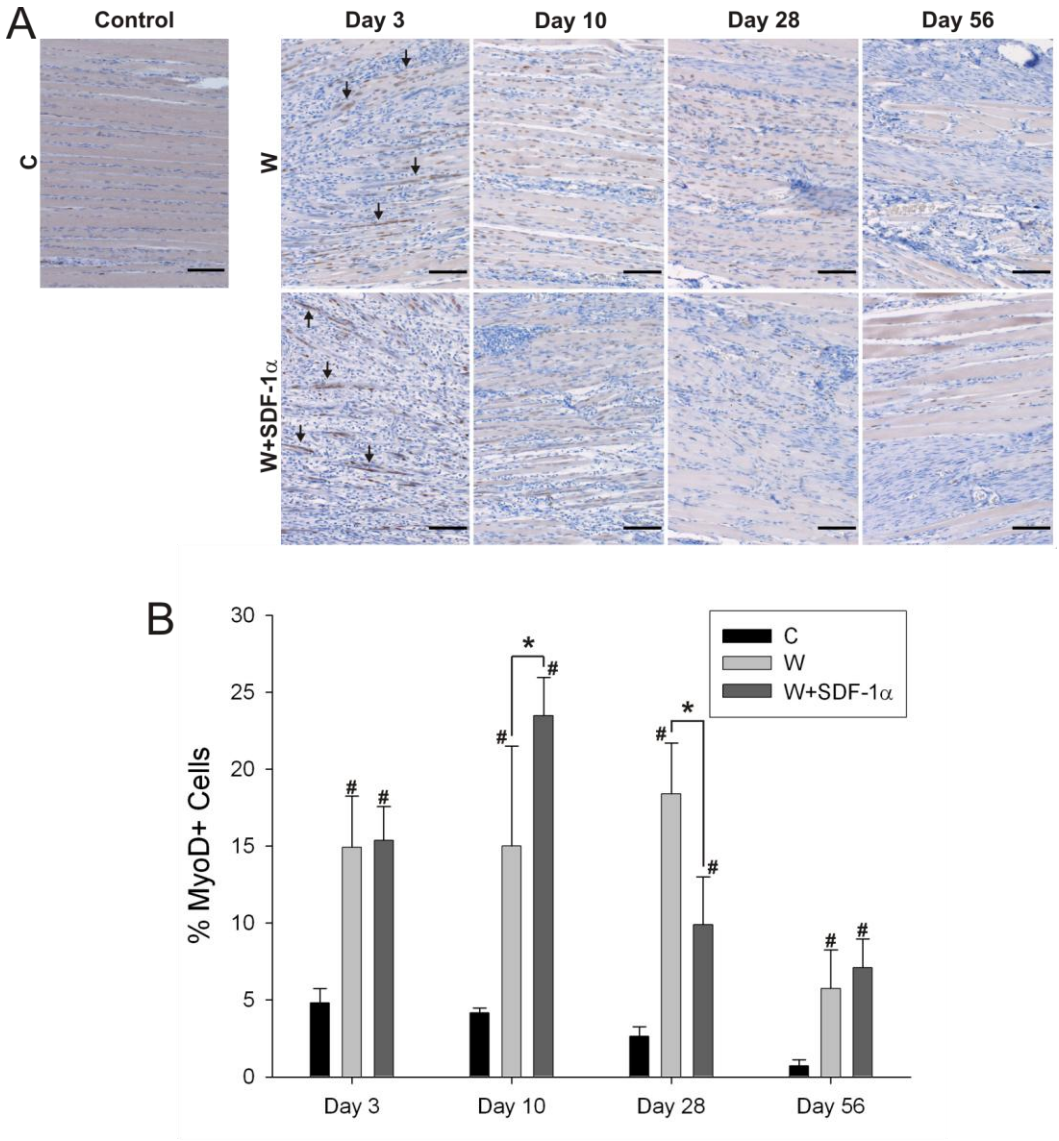


Figure 3. ***MyoD immunohistochemistry and quantification.*** *A) Sections of the wounds without (W) and with SDF-1α–loaded collagen scaffolds (W+SDF-1α) are shown. Pictures were taken at the border of the wounds in the regenerative zone as indicated in figure 1A. B) Quantification of MyoD$^+$ cells in the controls (C), wounds without (W) and with SDF-1α-loaded collagen scaffolds (W+SDF-1α). The numbers of MyoD$^+$ cells are expressed as a mean percentage ± SD of the total numbers of cells. # significant difference with C ($p < 0.01$).* significant difference between W and W+ SDF-1α ($p < 0.01$). The scale bar represents 100 μm.*

At three days post-surgery, the numbers of MyoD$^+$ cells and myofibers in the W-group (14.9 ± 3.3%) and in the W+SDF-1α-group (15.4 ± 2.2)% are not significantly different. At 10 days the numbers of MyoD$^+$ cells and myofibers are significantly higher in the W+SDF-1α-group (23.5 ± 2.5%) than in the W-group (17.7 ± 2.8%), but at 28 days the number of

MyoD^{+}-cells and myofibers is significantly lower in the W+SDF-1α-group (9.9 ± 3.1%) than in the W-group (18.4 ± 3.3%). At day 56 post-surgery, there are no differences found between the W- and W+SDF-1α-group.

5.3.3 Collagen I and III immunostaining

Paraffin sections are stained with antibodies against collagen I and III (figure 4). In the C-group collagen I expression is only found near blood-vessels.

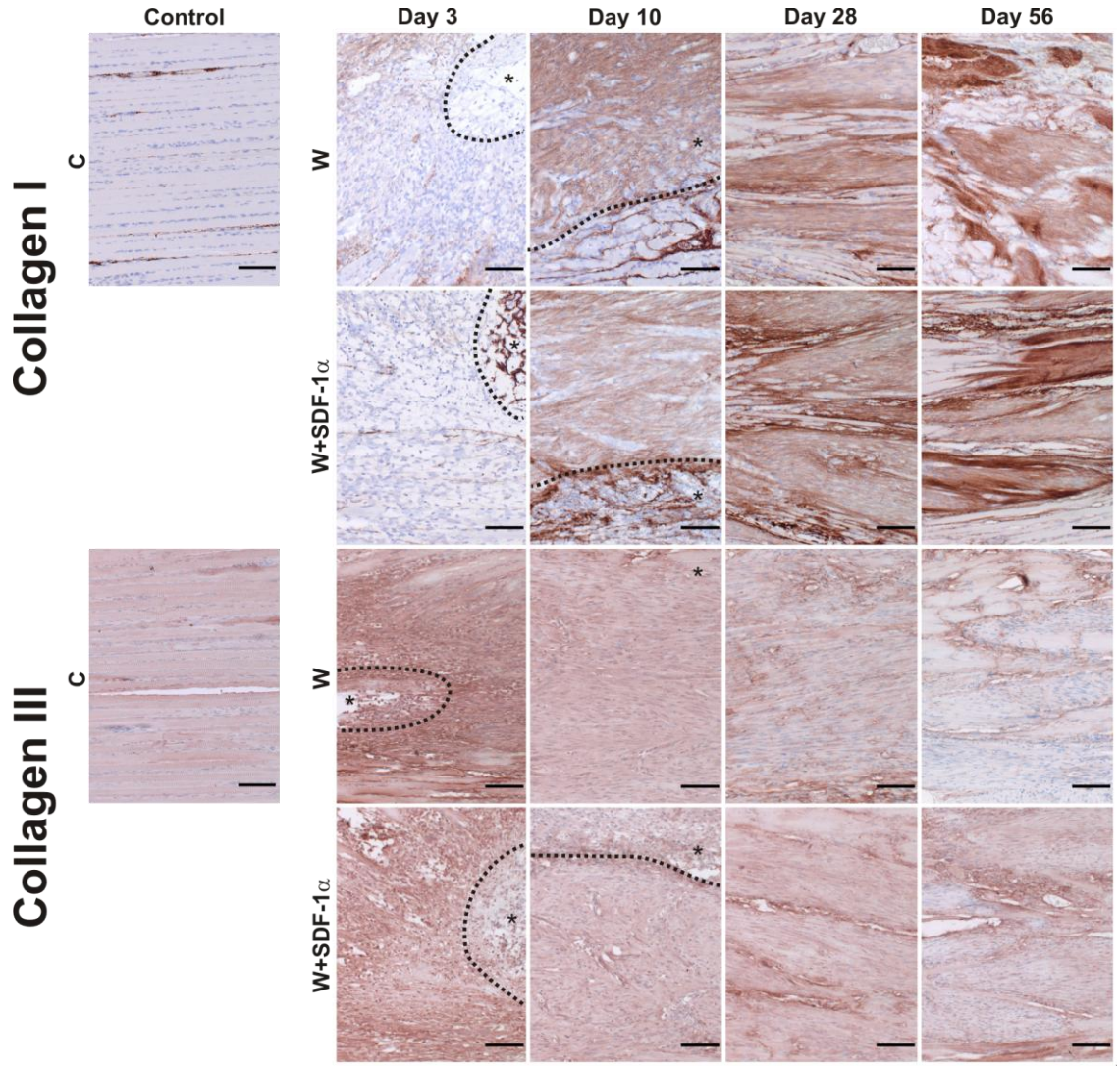


Figure 4. Collagen I and III immunohistochemistry*. Sections of the wounds without (W) and with SDF-1α-loaded collagen scaffolds (W+SDF-1α) are shown. Pictures were taken around the wound, which is marked by an asterisk at days 3 and 10. The scale bar represents 100 μm.*

In time, the expression pattern of collagen I is not different between the W- and W+SDF-1α-group. At three days post-surgery there is no expression of collagen I around the wounds in both the W- and W+SDF-1α-group. At 10 days, collagen I is expressed around the wounds in both groups. At these time points, the collagen scaffold is also positive for collagen I. At 28 and 56 days more collagen I is expressed and bundles of collagen is formed, but the expression varies within the wounds in both groups.

In the C-group collagen III is only expressed around blood-vessels. Between the W- and W+SDF-1α-group no differences are found in collagen III expression. At 3 days post-surgery, collagen III is expressed around the wounds in the W- and W+SDF-1α-group. In time, the expression of collagen III diminishes in both groups.

5.3.4 α-SMA and ED1 immunostaining

Sections are also stained with antibodies against α-SMA to identify myofibroblasts and blood vessels, and ED1 to identify macrophages (figure 5). In the C-group only α-SMA$^+$ blood vessels are found. In both the W- and W+SDF-1α-group the expression pattern of α-SMA is similar in time. At three days, α-SMA$^+$ myofibroblasts are found around the wounds in the W- and W+SDF-1α-group. Inside the scaffold α-SMA expression is absent. At 10 days, the wound in the W-group is filled with α-SMA$^+$ myofibroblasts. In the W+SDF-1α-group, the scaffold is surrounded by α-SMA$^+$ myofibroblasts. Within the scaffolds some α-SMA$^+$ blood vessels are also found. At 28 days, the wounds in both groups still contain α-SMA$^+$ myofibroblasts, which is diminished after 56 days.

Only a small number of ED1$^+$ cells are found in the C-group. In both the W- and W+SDF1α-group, the tissue around the wounds is infiltrated by many ED1$^+$ cells at three days. In the W+SDF-1α group, it seems that more ED1$^+$ cells have infiltrated the tissue around and inside the scaffold. At 10 days, the numbers of ED1$^+$ cells are greatly reduced in the tissue around the wounds in both groups. The scaffold itself is still completely filled with ED1$^+$ cells. At 28 and 56 days the numbers of ED1$^+$ cells have decreased further and seem to be equal in the W- and W+SDF-1α-group.

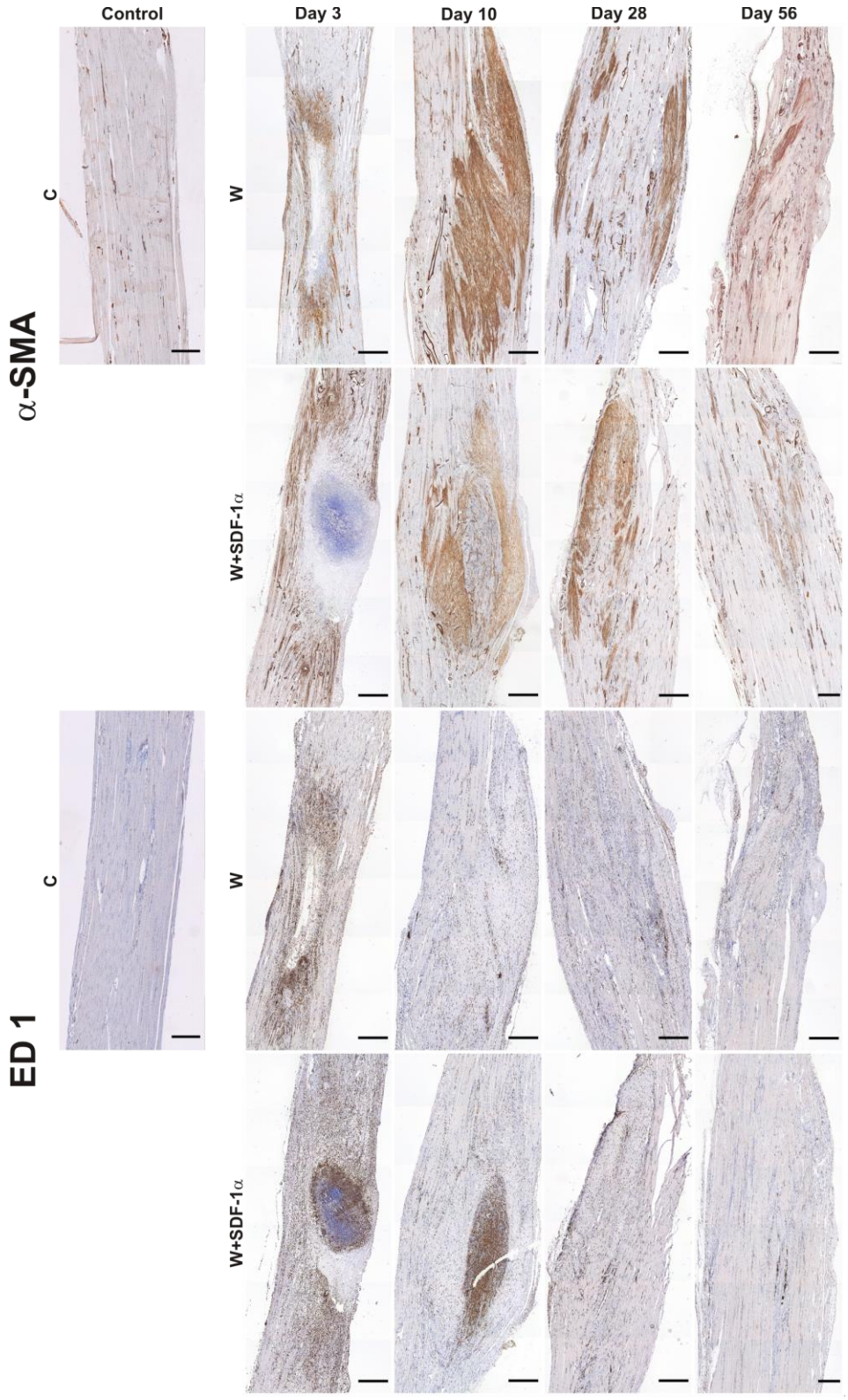


Figure 5. α–SMA and ED1 immunohistochemistry. *Sections of the wounds without (W) and with SDF-1α-loaded collagen scaffolds (W+SDF-1α) are shown. The scale bar represents 500 μm.*

5.4 Discussion

Full-thickness defects in the M. soleus impair muscle regeneration. Histology showed that regenerating fibers are present at the border of the defects, and only small numbers of regenerating myofibers are present within the defect, but these do not cross the wound. The defect is replaced by fibrotic tissue at 10 days, which persists for up to 56 days, and it is unlikely that complete regeneration of the M. soleus will occur. We assume that the function of these muscles will be severely impaired although this was not studied. It has recently been reported that large defects in the M. gastrocnemius also show impaired regeneration and significant loss of function.[37] Therefore, the full-thickness wound model presented here provides a solid basis to develop tissue engineering therapies to improve muscle regeneration, prevent fibrosis, and restore muscle function. Nevertheless, future studies should also include functional testing.

Tissue engineering is a powerful and promising strategy to repair full-thickness tissue defects.[25-27] The constructs should provide the necessary cues for the cells to regenerate the muscle tissue. In this study, SDF-1α-loaded collagen scaffolds are used for the first time to attract resident sattelite cells to induce muscle regeneration. It has been previously demonstrated that only part of the SDF-1α is released from the scaffold *in vitro* in time.[38] Therefore, SDF-1α is probably gradually released into the muscle tissue and provides a migratory gradient for satellite cells directed towards the defect. Although SDF-1α has the potential to attract satellite cells by binding to the CXCR4 receptor,[34,35] these cells are absent within the scaffold, and regeneration of the defect does not occur. In contrast, during the first 10 days, the number of satellite cells, myoblasts and myofibers are significantly increased in the regenerative zone around the SDF-1α-loaded collagen scaffolds. At 28 days, the number of satellite cells is equal in the W- and W+SDF-1α-group. However, the number of $MyoD^+$ myoblasts and myofibers is decreased. Therefore it is likely that SDF-1α creates an influx of satellite cells towards the regenerative zone in the first days, which will produce $MyoD^+$ myoblasts and myotubes in time, and thereby accelerate muscle

regeneration around the scaffolds. According to myogenin expression these satellite cells and myoblasts do not migrate into the scaffold to form new myofibers. This indicates that internal muscle regeneration does not occur. Since we found many other cells, and also blood-vessels inside the scaffold, it remains unclear why the satellite cells did not migrate into the scaffold. Collagen type I might not be a suitable substrate for satellite cells.[39]

Implanting SDF-1α-loaded collagen scaffolds also creates a larger influx of macrophages around and inside the scaffolds. It might be possible that the collagen scaffold exerts an immune response, but immune cells also express CXCR4,[40] which could also explain this. It has been shown that macrophages and monocytes play a crucial role in muscle regeneration.[41-43] Macrophages secrete soluble factors regulating satellite cell activity, and they protect the satellite cells from apoptosis through cell-cell contact.[44] In the initial inflammatory phase of muscle regeneration, M1 (early) macrophages are present, which stimulate the proliferation of satellite cells. Later, M2 (late) macrophages appear and stimulate the differentiation of satellite cells.[45] Therefore, the influx of macrophages might be beneficial for muscle regeneration. It is possible that macrophages are attracted by the collagen scaffold itself, and induce the increase of $Pax7^+$ cells, $MyoD^+$ cells and myotubes in the regenerative zone. However, in a previous laceration wound model, we implanted an empty cross-linked collagen scaffold, which induced a similar inflammatory response and influx of macrophages.[36] This did not increase the number of $Pax7^+$ cells (figure 6). Therefore, we conclude that the increased influx of $Pax7^+$ cells, and $MyoD^+$ cells and myotubes in the regenerative zone is a specific effect of the SDF-1α.

Furthermore, macrophages are probably responsible for the degradation of the collagen scaffold. This is necessary before the defect can be replaced with functional muscle tissue. However, in this study the SDF-1α-loaded collagen scaffold seems to be replaced by de novo collagen deposition, as in the wound only group (W). In the first days collagen III is predominantly expressed, which diminish in time. In contrast, collagen I deposition is increased in time. The same results are also found in other studies.[46,47] However, the expression of collagen I

varies within the wound, and is not as strong as in skin wounds (own results).

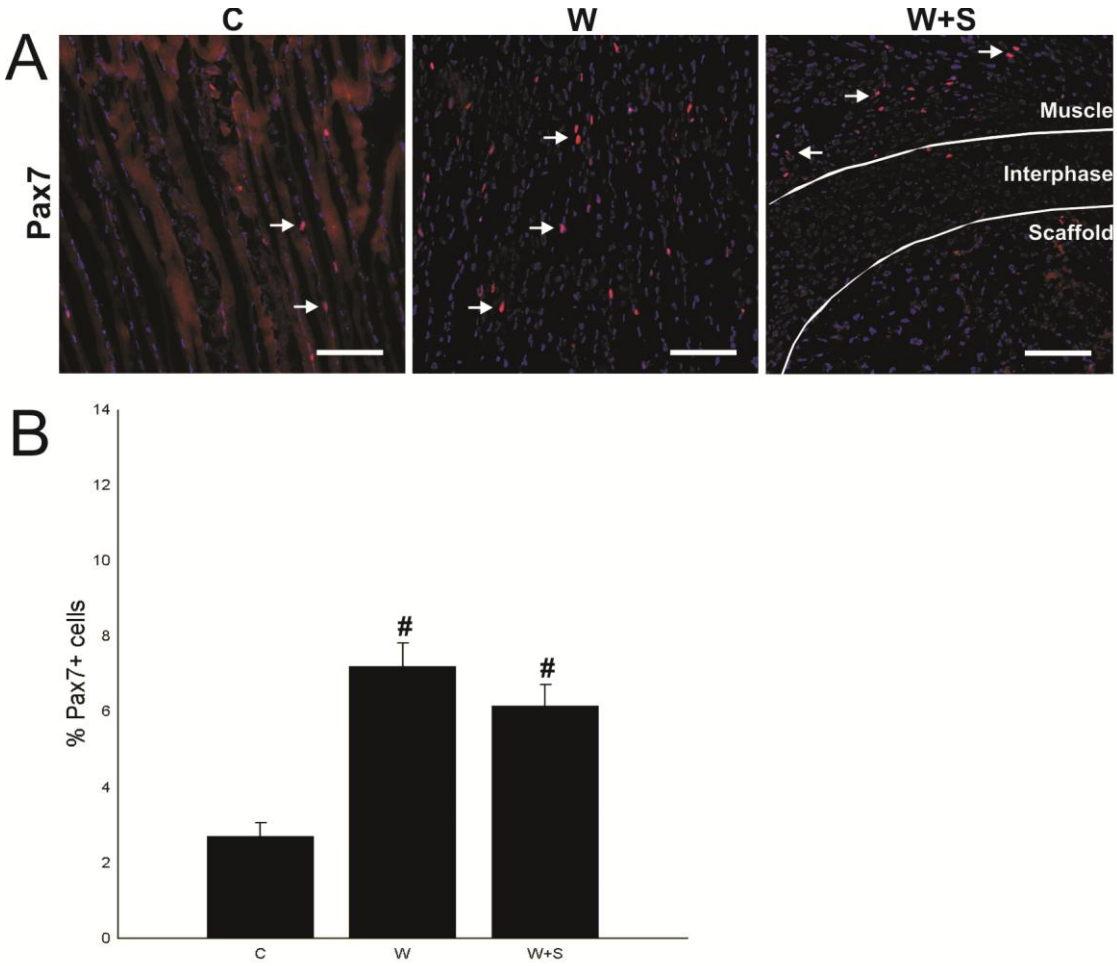


Figure 6. Pax7 fluorescent immunohistochemistry. *A) Sections of the control (C), wound without the collagen scaffold (W), and wound with the collagen scaffold (W+S) are shown. Pax7 is stained in red (indicated by arrows) and nuclei in blue. B) Quantification of Pax7$^+$ cells in the controls (C), wounds without (W) and with the collagen scaffold (W+S). The numbers of Pax7$^+$ cells are expressed as a mean percentage ± SD of the total numbers of cells. # significant difference with C ($p < 0.05$). The scale bar represents 100 μm. Adapted from.*[36]

Myofibroblasts, generally identified by the expression of α-SMA, produce large amounts of collagen, and play an important role in fibrosis.[48] They rapidly appear in both wound groups, and persist up to 56 days. However, it has been shown that myoblasts can also express α-SMA.[49] The fibrotic areas are also positive for Hsp47 (figure 7), which is related to collagen production in (myo)fibroblasts, and thus further

identifies these cells.[50] Unfortunately, myoblasts and regenerating myofibers can also express Hsp47.[51] The fact that all α-SMA$^+$ and Hsp47$^+$ cells do not show Pax7, MyoD, and Myogenin staining (figure 7), indicates that the vast majority of these cells are myofibroblasts and not myoblasts. The expression pattern of α-SMA and Hsp47 is also mirrored by collagen type I and III expression, which confirms the role of myofibroblasts in muscle fibrosis. In both groups, the area of myofibroblasts and collagen expands outside the defect. Factors from the wound might attract myofibroblasts into the surrounding muscle tissue, which produce collagen and enlarge the fibrotic area. The remodeling of the fibrotic tissue seems to go on up to 56 days as myofibroblasts are still present. Within the scaffold α-SMA$^+$ blood vessels are also present indicating that they are well-vascularized and support cell survival.

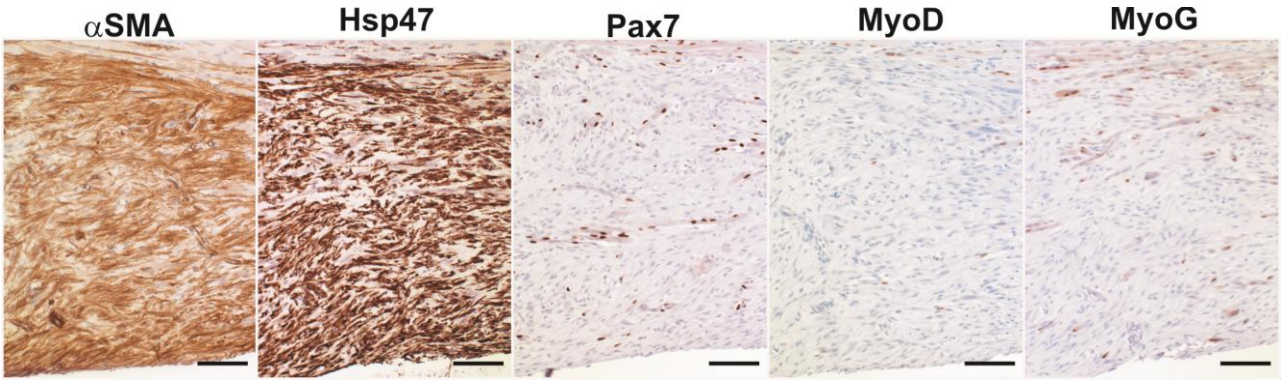


Figure 7. α-SMA, Hsp47, Pax7, MyoD and Myogenin (MyoG) immunohistochemistry. *Representative consecutive sections of the wounds without scaffold at 10 days post-surgery are shown. The scale bar represents 100 μm.*

In order to reduce the formation of fibrotic tissue, other scaffolds might be used. It is possible that collagen type I scaffolds trigger degradation and replacement with de novo collagen. Other scaffolds, such as alginate hydrogels, fibrin gels, and synthetic scaffolds could be used.[25,27,52] Furthermore, the incorporation of anti-fibrotic components in the scaffold might further inhibit the formation of fibrotic tissue. Decorin is a proteoglycan that can bind TGF-β, which plays a major role in tissue fibrosis.[53] Several studies show that decorin reduces fibrosis, leading to improved functional muscle regeneration.[11,16] However, in these studies, decorin was injected into lacerated muscle, but no studies have been

performed on the implantation of scaffolds loaded with decorin into full-thickness muscle defects. Additional growth factors such as SDF-1α, HGF, IGF-I, FGF-II, NGF, or G-CSF can be incorporated into the scaffold. This type of approach might be promising to induce functional muscle regeneration in full-thickness muscle defects.

5.5 Conclusion

Taken together, these data show that full-thickness defects impair muscle regeneration leading to muscle fibrosis. This model can be used to study therapeutic modalities to improve muscle regeneration. Implantation of an SDF-1α-loaded collagen scaffold into the defect increases the number of $Pax7^+$ satellite cells, and $MyoD^+$ myoblasts and myofibers in the regenerative zone around, but not within the scaffolds. In time, the scaffold is replaced by fibrotic tissue. Future experiments should focus on growth factor-loaded scaffolds to accelerate muscle regeneration in combination with anti-fibrotic components to inhibit the formation of fibrotic tissue.

5.6 Acknowledgements

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5.7 Financial & competing interests disclosure

This study was supported by a grant from the Radboud University Nijmegen Medical Centre, The Netherlands. The authors have no other relevant affiliations or financial involvement with any organization or

entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

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Chapter 6

Decorin/SDF-1α-loaded collagen scaffolds in skeletal muscle regeneration

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Submitted

Abstract

Many approaches have been used to improve skeletal muscle regeneration, but constructs that prevent the formation of fibrotic tissue in large muscle defects are not yet available. It has been shown that decorin, a small leucine-rich proteoglycan, binds and inactivates TGF-β1 leading to less fibrosis. In addition, SDF-1α induces the migration of muscle satellite cells towards the defect. Therefore, decorin-loaded collagen scaffolds with or without SDF-1α were implanted into a muscle fibrosis model to reduce fibrosis and to improve muscle regeneration.

In vitro studies showed that the bulk of decorin was released within the first 3 days, inducing a short-term release of decorin into the muscle defect. Circular (2 mm) full-thickness defects were made in the *musculus soleus* of 20 rats and filled with an empty collagen scaffold, a decorin-loaded collagen scaffold, or a decorin- and SDF1-α-loaded collagen scaffold.

Immunohistochemistry was performed at 56 days post-surgery to identify myofibroblasts, activated fibroblasts, satellite cells, and fused myoblasts. Histology revealed that fibrosis, measured as collagen deposition, was the same in all treatment groups. The group with the empty scaffolds showed large numbers of (myo)fibroblasts, but low numbers of satellite cells and fused myoblasts. The SDF-1α and/or decorin groups showed virtually the same frequency of these cells.

Therefore, we conclude that the release window of decorin was probably too short to prevent fibrosis. Future studies should aim to develop scaffolds resulting in a timely-tuned delivery of factors out of the scaffold and thereby inhibit fibrosis and improve muscle regeneration.

6.1 Introduction

Regenerative medicine deals with the treatment of skeletal muscle diseases and injuries. In muscle healing the myofiber-associated satellite cells play a central role.[1,2] After injury, these cells regenerate the muscle tissue by generating myoblasts, which then differentiate and fuse to damaged myofibers or form new ones.[3-5] More importantly, satellite cells self-renew and therefore form a continuous cell source for regeneration.[6,7] Unfortunately, muscle healing can lead to fibrosis, which prevents full functional recovery.[3,4,8]

Over the years, satellite cell functioning has been associated with many growth factors such as insulin growth factor (IGF)-I, fibroblast growth factor (FGF)-II, hepatocyte growth factor (HGF), nerve growth factor (NGF), or granulocyte-colony stimulating factor (G-CSF).[9-12] These growth factors together with satellite cells are the main tools for developing strategies to repair skeletal muscle injuries. Injection of growth factors or satellite cells enhances muscle regeneration up to a certain extent.[6,9-17] However, in full-thickness defects leading to fibrosis, tissue engineered scaffolds are required to provide structural cues for the satellite cells and regenerating myofibers. Such scaffolds have been used, but with varying results.[18-22] Loading the scaffolds with cells and/or growth factors usually improves their efficacy.

To obtain sufficient satellite cells for seeding the scaffolds, *in vitro* expansion is required. Unfortunately, this often results in the loss of myogenic potential.[14,23] Furthermore, satellite cells and myoblasts hardly survive and do not migrate into the defect after transplantation.[15,24] Loading the scaffolds with appropriate growth factors to attract resident satellite cells, and to induce their proliferation and differentiation might eliminate the requirement of cultured satellite cells. We already showed that collagen scaffolds loaded with stromal-derived factor (SDF)-1α attract satellite cells towards the border of the defect, but does not prevent fibrosis.[22]

One of the major factors involved in tissue fibrosis is transforming growth factor (TGF)-β1,[25] which is also a key factor in muscle fibrosis.[9,13,26] The small leucine-rich proteoglycan decorin can bind and

inactivate TGF-β1, and therefore inhibit fibrosis and improve muscle regeneration.[9,13,26] A sustained delivery of decorin induce by gene transfection, showed an increased differentiation rate of myoblasts, which improved muscle regeneration.[27]

Previously, we showed that in full-thickness defects fibrosis starts already within 3 days according to the presence of myofibroblasts and collagen depositions at the border of the wounds.[22] This strongly suggests that the fibrotic process already starts early after injury. It has also been shown that the reduction of TGF-β1 activity directly after skin wounding reduces fibrosis.[28,29] Therefore, we studied the development of fibrosis after a short-term release of decorin by implanting decorin-loaded non-crosslinked collagen scaffolds with or without SDF-1α in our fibrosis model in the M. soleus of the rat.

6.2 Materials and methods

6.2.1 Rats

Twenty 5-week-old male Sprague-Dawley rats (Janvier, Le Genest, France) were used for the experiments. The rats were housed under normal laboratory conditions and received normal rat chow and water ad libitum. Before the start of the experiments the rats had been acclimatized to the animal facility for one week. All animal experiments were approved by the Animal Experiments Committee of the Radboud University Nijmegen Medical Centre (RUNMC) in accordance to the Dutch laws and regulations on animal experiments.

6.2.2 Scaffold preparation and characterization

Insoluble type I collagen from bovine achilles tendon (Sigma Chemical CO, St. Louis, MO, USA) was homogenized to a 1% (w/v) collagen suspension. To obtain a scaffold, the collagen suspension was degassed, frozen overnight at -25°C in aluminum trays, and lyophilized. For characterization, small pieces of the scaffold were fixed with 2% glutaraldehyde (v/v) and 50% (v/v) osmium oxide and evaluated using scanning electron microscopy.

6.2.3 Decorin release from the scaffolds

Round (2mm) collagen scaffolds were incubated in PBS containing 0.1% (w/v) bovine serum albumin (BSA) and 1.6 mg/ml decorin (from bovine cartilage; Sigma Chemical CO, St Louis, MO, USA) at room temperature for 48 hours. Then, scaffolds were incubated in PBS for 0, 3, 6, and 10 days. At the indicated time points, scaffolds were fixed in freshly prepared 4% paraformaldehyde (PFA) in PBS for 2 hours and processed for paraffin embedding and histological analyses.

6.2.4 Experimental procedures

At the day of surgery, the rats received 0.02 mg/kg body weight buprenorfine (Temgesic; Schering Plough, Brussels, Belgium) subcutaneously as an analgesic and also at the next two days with a twelve hour interval. Under 5% (induction) followed by 2-3% (continuation) isoflurane anesthesia (Pharmachemie BV, Haarlem, the Netherlands), the M. soleus of the left lower limb of the rats was gently exposed. Using a 2 mm round biopsy punch, a full-thickness defect was made in de middle of the M. soleus. The rats were divided into three groups according to the presence of the different collagen scaffolds: 1) an empty scaffold (W; N = 6), 2) decorin-loaded collagen scaffold (DEC; N = 7), and 3) decorin + SDF-1α-loaded collagen scaffold (DEC/SDF-1α; N = 7). These abbreviations are used further in this article.

Before implantation, twenty scaffolds were sterilized in 70% EtOH for 30 minutes and washed in PBS. Then six scaffold were incubated in PBS containing 0.1 % (w/v) (BSA), seven scaffolds were incubated in PBS containing 0.1 % (w/v) BSA and 1.6 mg/ml decorin, and seven scaffolds were incubated in PBS containing 0.1 % (w/v) BSA, 2 μg/ml SDF-1α, and 1.6 mg/ml decorin at room temperature for 48 hours and then directly implanted into the muscle wounds. After implantation, the fascia and skin were both closed with 5-0 Vicryl sutures (Johnson-Johnson, Langhorne, PA, USA). In every group, the rats were sacrificed according to the standard CO_2/O_2 protocol at 56 days post-surgery.

6.2.5 Histology and immunohistochemistry

The left (wound) and right (internal control) M. soleus of the rats were fixed in freshly prepared 4% paraformaldehyde (PFA) in PBS for 22-23 hours and processed for paraffin embedding. Longitudinal muscle sections (5µm) were collected on superfrost plus slides (Menzel-Gläser, Braunschweig, Germany). To discriminate collagen fibers (blue color) from the muscle tissue (red color), the sections were stained with azocarmine G and aniline blue (AZAN) according to standard protocols. For immunohistochemistry, the sections were treated and stained as described previously.[22] The primary antibodies, mouse anti-alpha-smooth muscle actin (α-SMA, 1:1600; Sigma), mouse anti-Hsp47 (1:24000; Assay Design, Ann Harbor, MI, USA), mouse anti-Pax7 (1:100; Developmental Studies Hybridoma Bank, Iowa City, CA, USA), mouse anti-Myogenin (F5D, 1:100; Developmental Studies Hybridoma Bank), mouse anti-decorin (3B3, 1:1; Developmental Studies Hybridoma Bank) were visualized using the biotinylated secondary antibody donkey-anti-mouse IgG (H+L) (1:500; Jackson Labs, West Grove, PA, USA), and a preformed biotinylated horse radish peroxidase and avidin complex (Vector Laboratories, Burlingame, CA, USA). The sections were colored with 3,3-diaminobenzidine (DAB) and photographed with the Zeiss Imager.Z1 together with the AxioCam MRc5 camera using the AxioVision 4.6.3 software (Carl Zeiss Microimaging GmbH, Jena, Germany).

6.2.6 Quantification

The relative area of fibrosis was quantified on the AZAN-stained muscle sections, and the relative area of α-SMA and Hsp47 was quantified in the immunostained muscle sections of the control muscle (C) and the experimental groups W, DEC, and DEC/SDF-1α using the Qwin software (Leica Imaging Systems, Cambridge, UK). The area of fibrotic tissue (blue) and muscle tissue (red) and the α-SMA$^+$ and Hsp47$^+$ area (brown) and muscle tissue (blue) were quantified on a fixed length of 0.9 cm muscle tissue containing the wound area. The results were expressed as a mean percentage ± SD of the total amount of tissue.

The number of $Pax7^+$ and $Myogenin^+$ cells was counted in the C and in the regenerative zone (regenerating muscle tissue at the border of the 2 mm wound) of the groups W, DEC, and DEC/SDF-1-α. The numbers of $Pax7^+$ and $Myogenin^+$ cells were expressed as a mean percentage ± SD of the total number of cells.

The differences in the percentages of AZAN, α-SMA^+ and $Hsp47^+$ tissue and $Pax7^+$ and $Myogenin^+$ cells were tested for significance using a Two-Way ANOVA with Holm-Sidak post-hoc analysis. A value of $p < 0.05$ was considered to be significant.

6.3 Results

6.3.1 Scaffold characterization

Scanning electron microscopy shows that the scaffolds have a porous structure (figure 1A) with a pore size of approximately 100 µm.

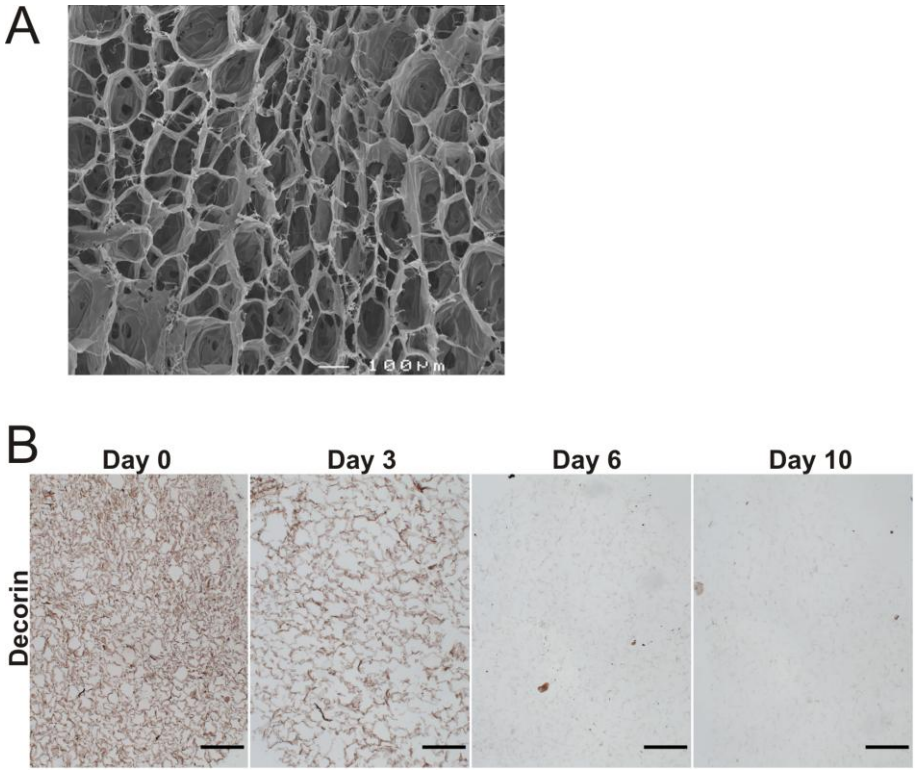

Figure 1. Scaffold characterization and decorin immunohistochemistry. *A) Scanning electron microscopy of the scaffold. B) Sections of decorin-loaded scaffolds are incubated in PBS for 0, 3, 6, and 10 days and subsequently stained for decorin. The scale bar represents 200 µm.*

The binding of decorin to the scaffold is shown in figure 1B. Directly after loading, the scaffolds are filled with decorin. After 3 days in PBS, the scaffolds still retain some decorin although less than directly after loading. The scaffolds do not contain decorin anymore when incubated in the PBS for longer than 6 days.

6.3.2 AZAN, α-SMA, and Hsp47 staining

All rats survived the experimental procedures and gained weight after a short period of growth arrest. Macroscopically, all scaffolds had disappeared, and the wounded muscles showed some adherence to the surrounding tissue.

The AZAN-staining (figure 2A) reveals proper aligned muscle fibers and little collagenous tissue in the C-group. In the W-, DEC-, and DEC/SDF-1α-groups, large collagen depositions are present. Regenerating muscle fibers seem to be disorganized and do not penetrate through the collagenous tissue. To identify myofibroblasts, sections are stained with α-SMA (figure 2A). In the C-group myofibroblasts are absent and only α-SMA$^+$ blood vessels are found. In the W-, DEC-, and DEC/SDF-1α-groups large areas of α-SMA$^+$ myofibroblasts are found within the collagenous tissue. Sections are also stained with Hsp47 to identify activated fibroblasts (figure 2A). In the C-group only few activated fibroblasts are present throughout the muscle tissue. In the W-, DEC-, and DEC/SDF-1α-groups large areas with activated fibroblasts are present within collagenous tissue.

The quantification of these areas is presented in figure 2B. The relative amount of collagenous tissue significantly increased from 6.3 ± 2.0% in the C-group to 37.8 ± 6.6%, 37.5 ± 4.4%, and 41.8 ± 6.1% in the W-, DEC-, DEC/SDF-1α-groups, respectively. Although the largest amount of collagenous tissue is found in the DEC/SDF-1α-group, there are no significant differences between the three experimental groups. In the C-group the relative amount of α-SMA$^+$ and Hsp47$^+$ areas are 2.6 ± 0.4% and 4.6 ± 2.3%, respectively. These areas are significantly increased ($p < 0.05$) to 19.3 ± 4.7% (α-SMA) and 15.9 ± 3.8% (Hsp47) in the W-group, 17.3 ± 3.9% (α-SMA) and 16.1 ± 5.1% (Hsp47) in the DEC-group, and 18.5 ± 6.1 (α-SMA) and 17.4 ± 5.4 (Hsp47) in the

DEC/SDF-1α-group. Between the three experimental groups no significant differences are found.

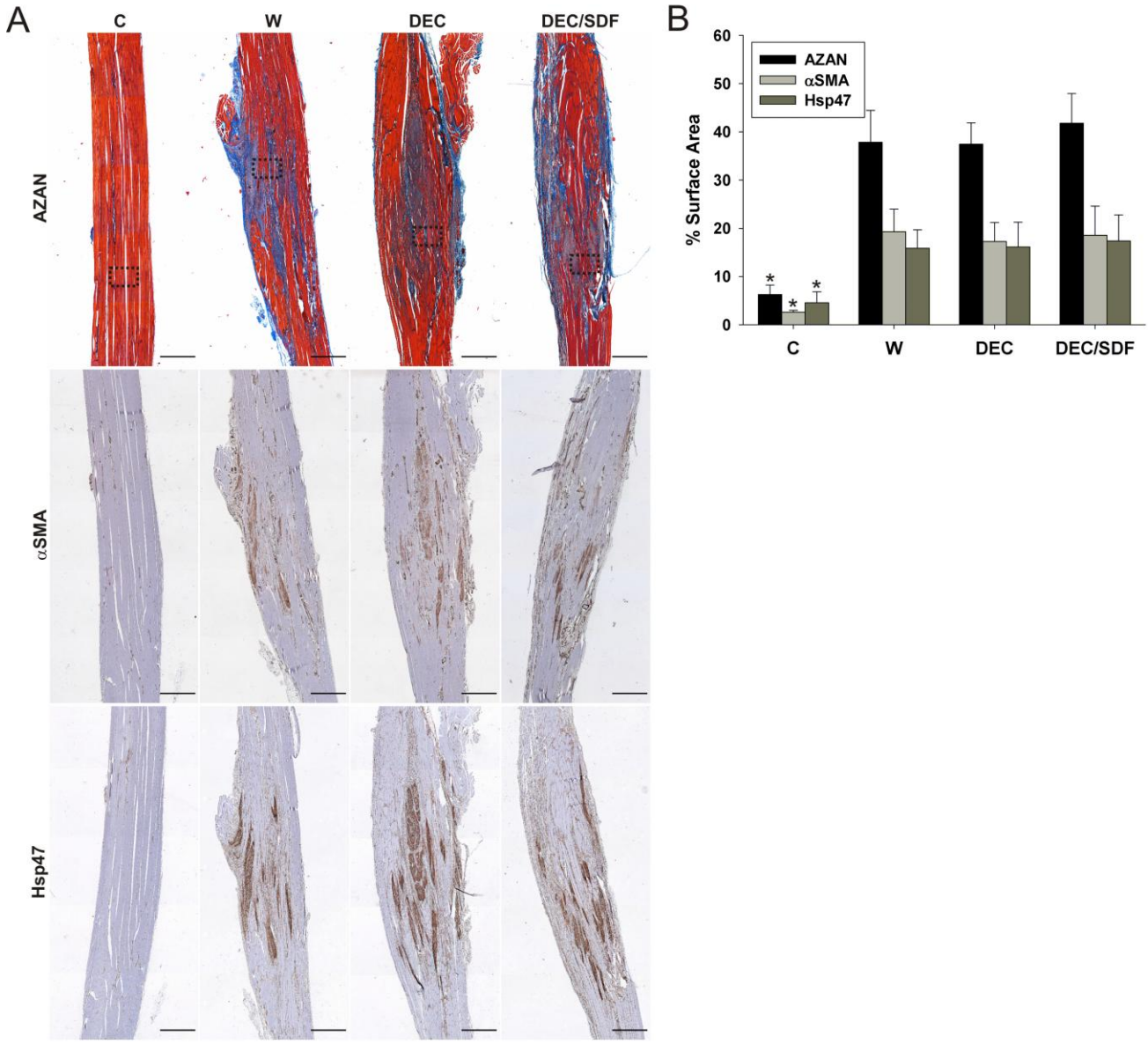

Figure 2. AZAN staining, α-SMA and Hsp47 immunohistochemistry, and quantification. *A) Sections of control (C) and wounds with empty collagen scaffolds (W), decorin-loaded collagen scaffolds (DEC), and decorin and SDF-1α-loaded collagen scaffolds (DEC/SDF) were stained to identify collagen (AZAN; muscle tissue red, collagen blue), α-SMA, and Hsp47 (both brown staining). B) Quantification of the surface area of collagen, α-SMA, and Hsp47 in the controls (C), wounds with empty collagen scaffolds (W), decorin-loaded collagen scaffolds (DEC), and decorin and SDF-1α-loaded collagen scaffolds (DEC/SDF). The area of collagen, α-SMA, and Hsp47 is expressed as a mean percentage ± SD compared to the total tissue area. * all wounds are significantly different from C ($p < 0.05$).The scale bar represents 1000 μm and the boxed area represents the regenerative zone used for Pax7 and Myogenin quantification.*

6.3.3 Pax7 and Myogenin staining

Paraffin sections are stained with antibodies against Pax7 and Myogenin to identify satellite cells and differentiating myoblasts (figure 3A).

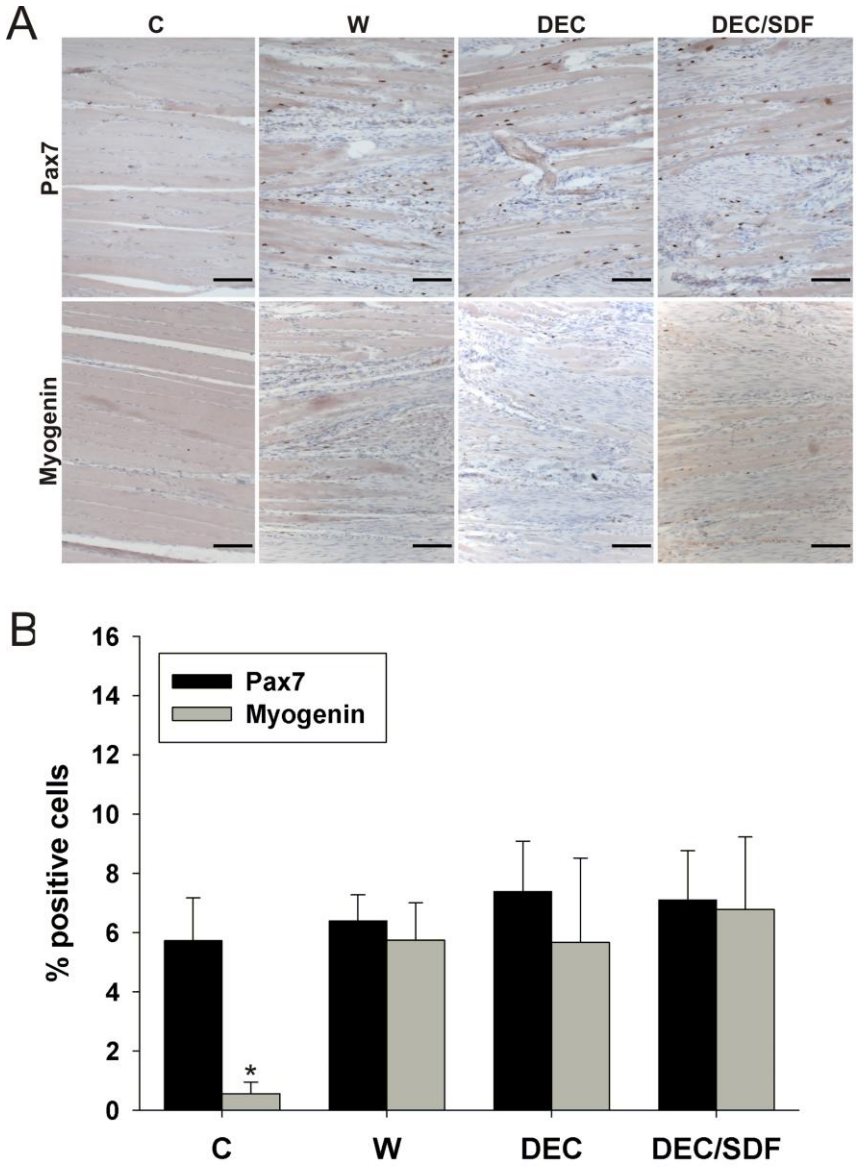

Figure 3. Pax7 and Myogenin immunohistochemistry and quantification. *A) Sections of control (C) and wounds with empty collagen scaffolds (W), decorin-loaded collagen scaffolds (DEC), and decorin and SDF-1α-loaded collagen scaffolds (DEC/SDF) are shown. Pictures were taken at the border of the wound in the regenerative zone as indicated in figure 1A. B) Quantification of Pax7$^+$ and Myogenin$^+$ cells in the controls (C), wounds with empty collagen scaffolds (W), decorin-loaded collagen scaffolds (DEC), and decorin and SDF-1α-loaded collagen scaffolds (DEC/SDF). The numbers of Pax7$^+$ and Myogenin$^+$ cells are expressed as a mean percentage ± SD of the total numbers of cells.* the number of Myogenin$^+$ cells of all wounds are significantly different from C ($p < 0.05$). The scale bar represents 100 μm.*

In the C-group few $Pax7^+$ and almost no $Myogenin^+$ cells are found. In the regenerative zone around the wounds, more $Pax7^+$ and $Myogenin^+$ cells seem to be present in all the groups. Quantification of the relative numbers of these cells is shown in figure 3B.

The number of $Pax7^+$ cells is around 6% in every experimental group and no significant differences were present. The number of $Myogenin^+$ cells is significantly increased ($p < 0.05$) from 0.6 ± 0.4% in the C-group to 5.7 ± 1.3% in the W-group, 5.7 ± 2.8% in the DEC-group, and 6.8 ± 2.5% in the DEC/SDF-1α-group. The number of $Myogenin^+$ cells in the three experimental groups was not significantly different.

6.4 Discussion and conclusion

One of the major factors involved in fibrosis is TGF-β1,[25] and inhibition of this growth factor by injecting decorin reduced fibrosis and improved muscle regeneration in laceration wounds.[9,13,26] However, the effects of decorin in full-thickness muscle defects that lead to large fibrotic tissue have not been studied. In our previous study using the fibrosis model, muscle regeneration and fibrosis already started within 3 days post-surgery.[22] Others have shown that reducing TGF-β1 activity directly after skin wounding prevented fibrosis.[28,29] Therefore we hypothesized a beneficial effect of the application of decorin directly after injury.

In this study, we loaded collagen scaffolds with decorin alone, or together with SDF-1α to induce a short-term release of decorin and SDF-1α at the start of muscle regeneration to attract resident satellite cells towards the defect and to prevent fibrosis. The scaffolds are highly porous allowing cell ingrowth, and decorin is released for up to 3 days. The implantation of the decorin-loaded collagen scaffolds did not reduce the amount of fibrotic tissue, $\alpha\text{-SMA}^+$ myofibroblasts, and $Hsp47^+$ fibroblasts. As also found in the previous study,[22] the number of $Pax7^+$ and $Myogenin^+$ cells was not different between the W-, DEC-, DEC/SDF-groups. This indicates that the regeneration phase had already ended at 56 days. Since decorin is rapidly released, it is possible that the effects are

only visible during the first days of regeneration. However, as shown in the present study, the final result still is the formation of fibrotic tissue.

In laceration studies, the injection of decorin decreased the amount of fibrotic tissue. In these studies, similar amounts of decorin (50 µg) were injected into the muscle at 14 days post-surgery.[9,13] Inhibiting TGF-β1 activity at day 14, by injecting gamma interferon (γIFN) or suramin, an antiparasitic and antitumor drug, showed similar results.[30-33] Furthermore, it has been shown that transplantation of myoblasts transfected with decorin improves myoblast differentiation and inhibited fibrosis.[27] Conversely, myoblasts transfected with TGF-β1 induce their differentiation into myofibroblasts, which play a role in muscle fibrosis. When decorin was injected 14 days after cell transplantation, the differentiation of myoblasts to myofibroblasts was blocked.[26] Together with the present findings these data suggest that inhibition of TGF-β1 activity might only be effective in a window between 3 and 14 days post-surgery. Therefore, timely-tuned release scaffolds need to be developed to provide the release of decorin during a specific time window. Recently, it has been shown that by modifying the scaffold or by introducing microspheres a sustained release of growth factors can be obtained.[34-36] Thus, by using such release systems for decorin and SDF-1α improved muscle regeneration and reduced fibrosis might be achieved.

6.5 Acknowledgements

The Pax7 antibody developed by Atsushi Kawakami, the Myogenin (F5D) antibody developed by Woodring E. Wright, and the decorin (3B3) antibody developed by G. A. Pringle, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

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Chapter 7

Niche factors maintain satellite cell proliferation and differentiation in 2D and 3D cultures

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Submitted

Abstract

Satellite cells are key cells for post-natal muscle growth and regeneration and they play a central role in the search for therapies to treat muscle injuries. In this study the myogenic potential of muscle stem cells was studied in 2D- and 3D-cultures with collagen type I and Matrigel, which contains the niche factors laminin and collagen type IV.

Muscle stem cells were cultured to induce proliferation and differentiation on collagen- or Matrigel-coated surfaces (2D) or in gels (3D).

In the 2D-cultures, muscle stem cells proliferated faster on Matrigel than on collagen. The numbers of $Pax7^+$ and $MyoD^+$ cells were also significantly higher on Matrigel than on collagen. During differentiation, muscle stem cells formed more and larger $MyoD^+$ and $Myogenin^+$ myotubes on Matrigel. In the 3D-cultures, muscle stem cells in Matrigel expressed higher mRNA levels of MyoD and Myogenin, and formed elongated myotubes expressing Myogenin and myosin. In collagen gels, the myotubes were short, rounded, and expressed only Myogenin.

In conclusion, muscle stem cells, both in 2D and 3D, lose their differentiation capacity in collagen but not in Matrigel. This underscores the importance of niche factors for maintaining the myogenic potential of muscle stem cells, and for tissue engineered constructs aiming to restore skeletal muscle defects.

7.1 Introduction

Satellite cells play a central role in the homeostasis and regeneration of skeletal muscle tissue. Upon injury, quiescent satellite cells, which express Pax7, up-regulate MyoD and Myf5 expression, and become proliferating myoblasts.[1-5] Subsequent down-regulation of Pax7,[6,7] and up-regulation of Mrf4 and Myogenin mark the onset of myoblast differentiation[3-5] to form multinucleated myofibers or repair damaged myofibers by fusion.[8,9] However, a fraction of the satellite cells does not proliferate or differentiate, but self-renews to maintain the satellite cell pool.[7,10-12] Although muscle regeneration is an efficient process, scar tissue is often formed, which hampers muscle function.[13-15]

The ability of satellite cells to self-renew makes them promising candidates for regenerative medicine approaches for muscle injury.[10,11] Minor muscle injuries only require the injection of satellite cell-derived myoblasts into the defect, but larger defects require tissue-engineered constructs seeded with myoblasts and/or growth factors.[16] The injection of myoblasts improves muscle regeneration after injury, but this approach still faces major limitations. To obtain large quantities of myoblasts, satellite cells need to be isolated and expanded *in vitro*. During this process, satellite cells lose their myogenic potential and will therefore be less efficient in regenerating the injured muscle.[12,17] Furthermore, massive cell death occurs directly after injection.[18]

In vivo, satellite cells are located between, and in direct contact with the adjacent myofiber and the basal membrane.[19,20] Asymmetrical cell division results in two daughter cells of which one remains in contact with the basal membrane and preserves stem cell properties, while the other loses contact and differentiates.[11] Thus, the loss of their niche during isolation might cause the reduction of myogenic potential due to premature differentiation. The basal membrane consists mainly of laminin, collagen type IV, and heparin sulphate proteoglycans (HSPGs). It provides growth factors and attachment sites for the satellite cells.[21,22] In addition secreted products from the microvasculature, neuromuscular junction and immune cells also influence satellite cell behavior.[22,23]

Collagen type I, although not present in the niche, is widely used for culturing satellite cells.[24-27] Matrigel, which consist mainly of laminin, collagen type IV and HSPGs, is also being used and improved the myogenic capacity of isolated satellite cells, now called muscle progenitor cells (MPCs).[28-30] Collagen type I and other materials, such as fibrin and alginate have also been used as a scaffold to repair muscle defects. Although some studies show favorable results, none of these materials mimic the satellite cell niche.[13,31-35] Better results might be achieved by using materials that mimic the satellite cell niche. Up to now, no data are available on the myogenic capacity of MPCs in a 3D environment, although this is critical to develop tissue engineered constructs for regenerative medicine purposes. Therefore, the aim of this study was to investigate the myogenic potential of MPCs in 2D and 3D cultures with either collagen type I or Matrigel.

7.2 Materials and methods

7.2.1 Muscle progenitor cell isolation

MPCs were isolated from the hind limb muscles of 5-week-old male Sprague-Dawley rats (Janvier, Le Genest, France). The dissected muscle were minced in phosphate-buffered-saline (PBS) containing 5 µg/ml Amphotericine B (Sigma Chemical CO, St Louis, MO, USA) using scissors, and excessive fat and tendon were removed. Minced tissue fragments were incubated in 2% (w/v) Collagenase type II (Invitrogen HQ, San Diego, CA, USA) in Dulbecco's Modified Eagle's Medium-High Glucose (DMEM-HG; Invitrogen) for 1 hour at 37°C. Tissues were further homogenized by trituration using 10 ml pipettes and filtered through a 100 µm cell strainer to obtain single cell suspensions. The cell suspensions were incubated in hypotonic buffer (0.1 mM EDTA; 0.15 M NH_4Cl; 10 mM $KHCO_3$, pH 7.4) for 1.5 minutes to lyse the erythrocytes. The remaining cells were centrifuged (5 minutes at 300g) and resuspended in 15 ml proliferation medium: DMEM-HG containing 20% fetal bovine serum (FBS), 2% penicillin-streptomycin (p/s), 1 mM pyruvate, and 5 ng/ml bFGF (all from Invitrogen). To remove fast-

adhering fibroblasts the cells were incubated in uncoated culture flasks for 1 hour (37°C, 5% CO_2). Non-adhering cells were then transferred to tissue culture flasks coated with 1 mg/ml Matrigel (Matrigel^{TM} Basement Membrane Matrix, BD Bioscience, Bedbord, MA, USA), and cultured for 4 days (37°C, 5% CO_2). Proliferation medium was refreshed every day. After 4 days, MPCs were significantly enriched up to 60% according to Pax7 expression.

7.2.2 Proliferation and differentiation of muscle progenitor cells

Three batches of enriched MPCs from different rats were used for the 2D- and 3D-experiments. For the 2D-experiments, 24-wells plates and Lumox dishes (Ø 35 mm; both Greiner Bio-One, Frickenhausen, Germany) were coated with 0.2% (w/v) collagen type I (rat tail tendon; Serva Electrophoresis, Heidelberg, Germany) or 1 mg/ml Matrigel for 1 hour at 37°C. In the 24-wells plate, enriched MPCs were plated at a density of 10.000 cells/well and cultured in proliferation medium for 1, 2, and 3 days. In the collagen- and Matrigel-coated Lumox dishes, 40.000 enriched MPCs were seeded and cultured in proliferation medium for 2 days to induce proliferation. Enriched MPCs were also seeded at a density of 500.000 cells in the collagen- and Matrigel-coated Lumox dishes. Cells were then cultured for 1 day in proliferation medium and then 2 additional days in differentiation medium: DMEM-HG containing 2% fetal FBS, 1 mM pyruvate, and 2% p/s to induce fusion. For the 3D-experiments, 24-wells culture plates were pre-coated with 1% (w/v) bovine serum albumin (BSA) in PBS. Enriched MPCs were mixed with collagen type I solution containing 1.2 mg/ml collagen type I from rat tail, 10% (v/v) minimal essential medium (10x), 0.1 M 4-(20hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), 30 mM $NaHCO_3$ (all from Invitrogen), and 2 mM NaOH, or with Matrigel (1:1) on ice at a density of $1*10^6$ cells/ml. In each well, 0.5 ml cell/gel suspension was carefully dispensed and incubated at 37°C to gelate for 45 minutes. Then, 0.5 ml proliferation medium was added to each well to obtain final concentrations of 20% FBS, 1 mM pyruvate, and 2% p/s. For the proliferation experiments, cells were cultured for 2 days with proliferation medium. For the differentiation experiments, the

proliferation medium was replaced after 1 day and cells were cultured for 2 additional days with differentiation medium. After the experiments, the cells were analyzed using DNA quantification, immunofluorescence staining, and quantitative PCR.

7.2.3 DNA quantification

Cell proliferation of the enriched MPCs was measured using the PicoGreen dsDNA Quantification assay (Molecular Probes, Eugene, OR, USA). At the appropriate time points, the cells were lysed in 0.1% (v/v) Triton X-100 in PBS and three times freeze and thaw cycles. The assay was performed according to the manufactures protocol. Fluorescence was measured in a FL600 Microplate Fluorescent Reader (Bio-Tek Instruments Inc., Winooski, VT, USA) at excitation 485 nm and emission 520 nm. The amount of DNA was calculated using a standard curve ranging from 0 to 15000 cells.

7.2.4 Immunofluorescence staining

After the experiments, the enriched MPCs were washed with PBS and fixed in 4% formaldehyde in PBS for 15 minutes. After washing with PBS, the membranes of the Lumox dishes were cut into 5 pieces and the cells were permeabilized in 0.5% Triton X-100 in PBS for 20 minutes. The cells were washed with 0.05% (v/v) Tween-20 in PBS, and then incubated in blocking buffer containing 2% (w/v) bovine serum albumin (BSA), 2% (v/v) normal goat serum (NGS), 0.1% (v/v) Triton X-100, 0.05% (v/v) Tween-20, and 100 mM glycine in PBS for 30 minutes. Then, the cells were incubated with a mouse anti-Pax7 (1:25; Developmental Studies Hybridoma Bank, Iowa City, CA, USA), mouse anti-MyoD (1:25; DAKO, Dakopatts, Glostrup, Denmark), mouse anti-Myogenin (F5D, 1:100; Developmental Studies Hybridoma Bank), and mouse anti-fast myosin heavy chain (FMHC, 1:1600; Sigma Chemical CO, St Louis, MO, USA) in blocking buffer without glycine for 1 hour. For the proliferation experiments, the bound antibodies were visualized with AlexaFluor-488-labeled goat anti-mouse IgG (H+L) (1:200; Molecular Probes). For the differentiation experiments, the bound antibodies were visualized with AlexaFluor-488-labeled goat anti-mouse

IgG (H+L) (1:200; Molecular Probes) combined with Texas Red-X Phalloidin (1:250; Molecular Probes) to stain the actin filaments. The cells were visualized with a Zeiss Imager.Z1 microscope and photographed.

7.2.5 Quantification

The fusion index, the number of myofibers containing 3-6, 7-10 or >10 nuclei, and the number of $Pax7^+$, $MyoD^+$, and $Mygogenin^+$ cells in the enriched muscle progenitor cells cultures were calculated using 8 different representative fields of an overview image for each coating condition after all immunostainings (N=3 batches). All the results were expressed as a mean percentage ± SD of the total number of cells.

7.2.6 Whole mount immunofluorescence staining

The gels were fixed in 4% formaldehyde in PBS for 15 minutes, permeabilized in 0.5% Triton X-100 in PBS for 30 minutes, and incubated in blocking buffer for 1 hour. Then, the gels were incubated with the antibodies mouse anti-MyoD (1:25), mouse anti-Myogenin (1:100), and mouse anti-FMHC (1:1600) for 2 hours. The bound antibodies and actin filaments were visualized using AlexaFluor-488-labeled goat anti-mouse IgG (H+L) (1:200) combined with Texas Red-X Phalloidin (1:250). The structure of the whole gels was maintained during sealing. The cells were analyzed using an Olympus FV1000 Confocal Laser Scanning Microscope (CLSM, Olympus, Center Valley, PA, USA). In the pictures, artifacts were removed with the NIH ImageJ software using the Remove Outliers and Despeckle options.

7.2.7 Quantitative PCR

The gels were washed with PBS and homogenized with 1 ml Trizol (Invitrogen). Then 200 µl chloroform (Sigma) was added, mixed, and centrifuged for 20 minutes at 4ºC. The water phase containing the RNA was mixed with 70% ethanol (1:1), and RNA was extracted according to the manufacturer's protocol (Qiagen, Hilden, Germany) including a DNase I treatment. cDNA was generated with 0,5-1 µg RNA using the SuperScript™ II system (Invitrogen) according to the manufacturer's

protocol. Quantitative real-time PCR was performed in a final volume of 25 µl containing 12.5 µl SYBR®Green Supermix (Bio-Rad, Hercules, CA, USA), 5 µl (40x diluted) cDNA, 4.5 µl RNAse-free water, 1.5 µl 2.5M forward primer and 1.5 µl 2.5M reverse primer. The primers for β-actin, Pax7, MyoD, Myogenin, and Myh-1 were obtained from Biolegio (Nijmegen, The Netherlands) and the primer sequences are provided in table 1. The cDNA was amplified in the C1000 Thermal Cycler (Bio-Rad) and fluorescence was analyzed using the CFX96™ Real-Time System (Bio-Rad). The PCR conditions were 95ºC for 3 minutes (1 cycle), 95ºC for 15 seconds and 60ºC for 30 seconds (39 cycles), and finally a temperature increase starting at 65ºC to 95ºC with 0.5ºC intervals. RNA expression was normalized against the mRNA level of β-actin (ΔCt) and represented as $2^{-\Delta Ct}$.

Table 1. ***Primer sequences.***

Gene	Forward primer	Reverse primer
β-actin	TTCAACACCCCAGCCATGT	TGTGGTACGACCAGAGGCATAC
Pax7	AGCCGAGTGCTCAGAATCAA	TCCTCTCGAAAGCCTTCTCC
MyoD	CGACTGCCTGTCCAGCATAG	GGACACTGAGGGGTGGAGTC
Myogenin	AACCCAGGAGATCATTTGCT	GGTGACAGACATATCCTCCA
Myh-1	CCTGGATGATCTACACCTACTC	GTCAGAGATAGAGAAGATGTGGG

7.2.8 Statistical analysis

All results were tested for significance ($p < 0.05$) using a Two-Way ANOVA with Holm-Sidak post-hoc analysis, except for the results of the DNA quantification and Quantitative real-time PCR, which were not normally distributed. For the DNA quantification a One-Way ANOVA with post-hoc Holm-Sidak analysis was used (Bonferroni correction) at the individual time points ($p < 0.01$). The results of the Quantitative real-time PCR were normally distributed after log transformation and a Two-Way ANOVA with Holm-Sidak post-hoc analysis was used.

7.3 Results

7.3.1 2D-proliferation

After processing the muscle and initial enrichment, up to 60% of the MPCs expressed Pax7 and MyoD. These cells proliferated on collagen and Matrigel coatings for an additional two days, and were then stained for Pax7 and MyoD (figure 1A).

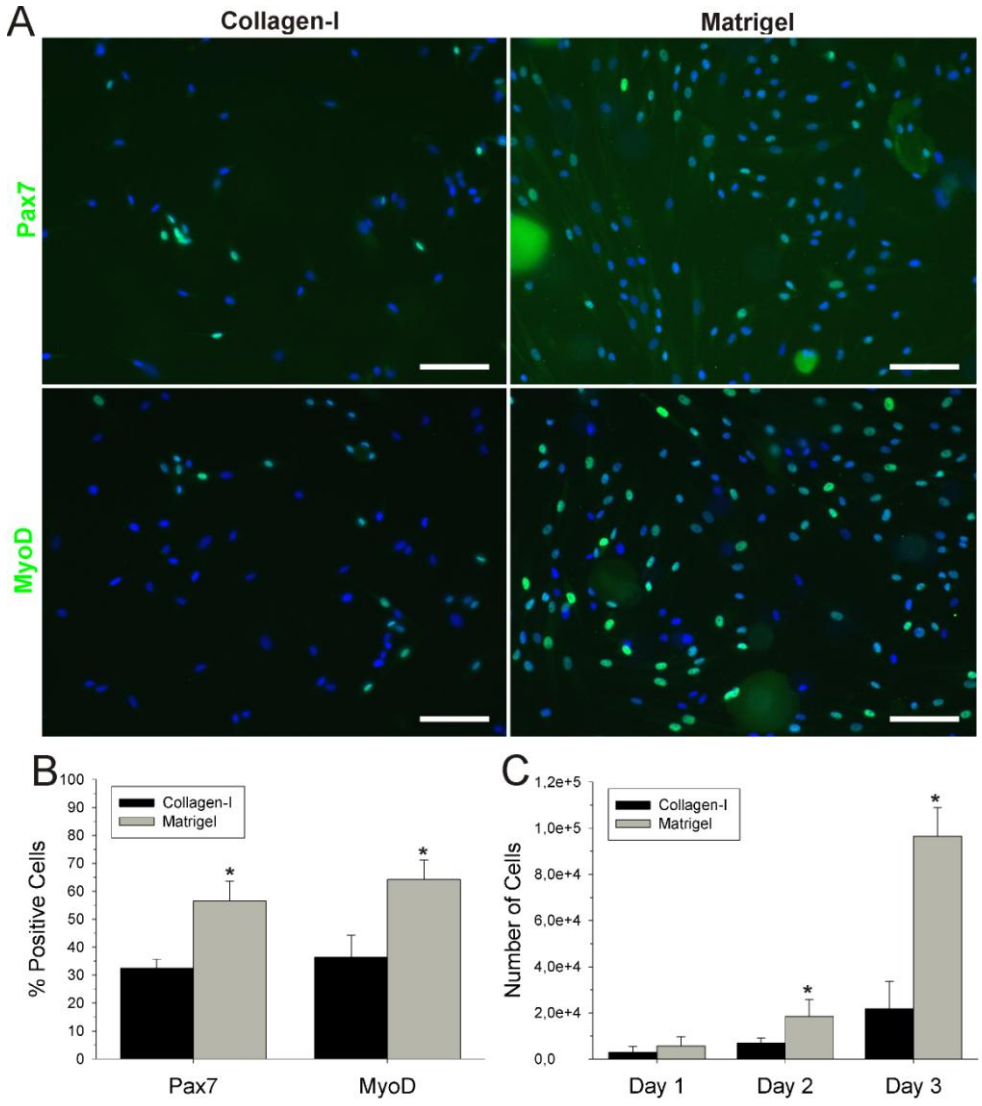

Figure 1. Pax7 and MyoD fluorescent immunohistochemistry and quantification. *A) Proliferating MPCs cultured on collagen-I and Matrigel coatings were stained for Pax7 or MyoD (both green) and DAPI (blue). B) Quantification of Pax7$^+$ and MyoD$^+$ cells (expressed as a mean ± SD) on collagen-I and Matrigel coatings. C) Quantification of the number of cells (expressed as mean ± SD) on collagen-I and Matrigel coatings after 1, 2, and 3 days of culture. Scalebar represents 100 µm. * significant difference between collagen-I and Matrigel.*

On Matrigel, many MPCs still express Pax7 and MyoD, whereas on collagen only a small fraction is positive for Pax7 and MyoD expression. Quantification (figure 1B) shows that, 56.5 ± 7.2% and 64.2 ± 7.0% of the cells are $Pax7^+$ and $MyoD^+$, respectively. On collagen, these numbers are significantly decreased to 32.5 ± 3.3% for Pax7 and 36.5 ± 7.9% for MyoD. Furthermore, DNA quantification shows (figure 1C) a 5-fold higher cell number on Matrigel than on collagen after three days of culture.

7.3.2 2D-differentiation

Differentiating and fused MPCs were stained for Pax7, MyoD, Myogenin, and actin filaments (figure 2A). According to the actin expression, multinucleated myotubes are formed on both collagen and Matrigel after two days of differentiation. However, on Matrigel, more and larger myotubes are found of which some showed cross-striations. Pax7 expression is diminished on both collagen and Matrigel, and only found in non-fused MPCs. On Matrigel, many $MyoD^+$ and $Myogenin^+$ muscle cells are present, but on collagen only few cells express MyoD and Myogenin. Almost all of the $MyoD^+$ and $Myogenin^+$ cells are within the myotubes on both substrates. After quantification, only 3.5 ± 0.8% and 7.5 ± 0.5% $Pax7^+$ cells are present on collagen and Matrigel, respectively (figure 2B). However, this difference was not significant. The fraction of $MyoD^+$ cells is around 3-fold larger on Matrigel (52.9 ± 2.5%) than on collagen (16.2 ± 4.9%). On Matrigel, the fraction of $Myogenin^+$ cells (55.0 ± 2.4%) is around 2-fold larger than on collagen (24.4 ± 7.1%).

More and larger $FMHC^+$ myotubes are formed on Matrigel than on collagen (figure 3A), which confirms the results of the actin staining. Quantification of the number of fused nuclei and the size of the myotubes is presented in figure 3B. On Matrigel, 49.1 ± 1.6% of the nuclei are fused, while this is only 16.1 ± 6.3% on collagen. The number of large myotubes formed on Matrigel is up to 4-fold higher than on collagen (figure 3C).

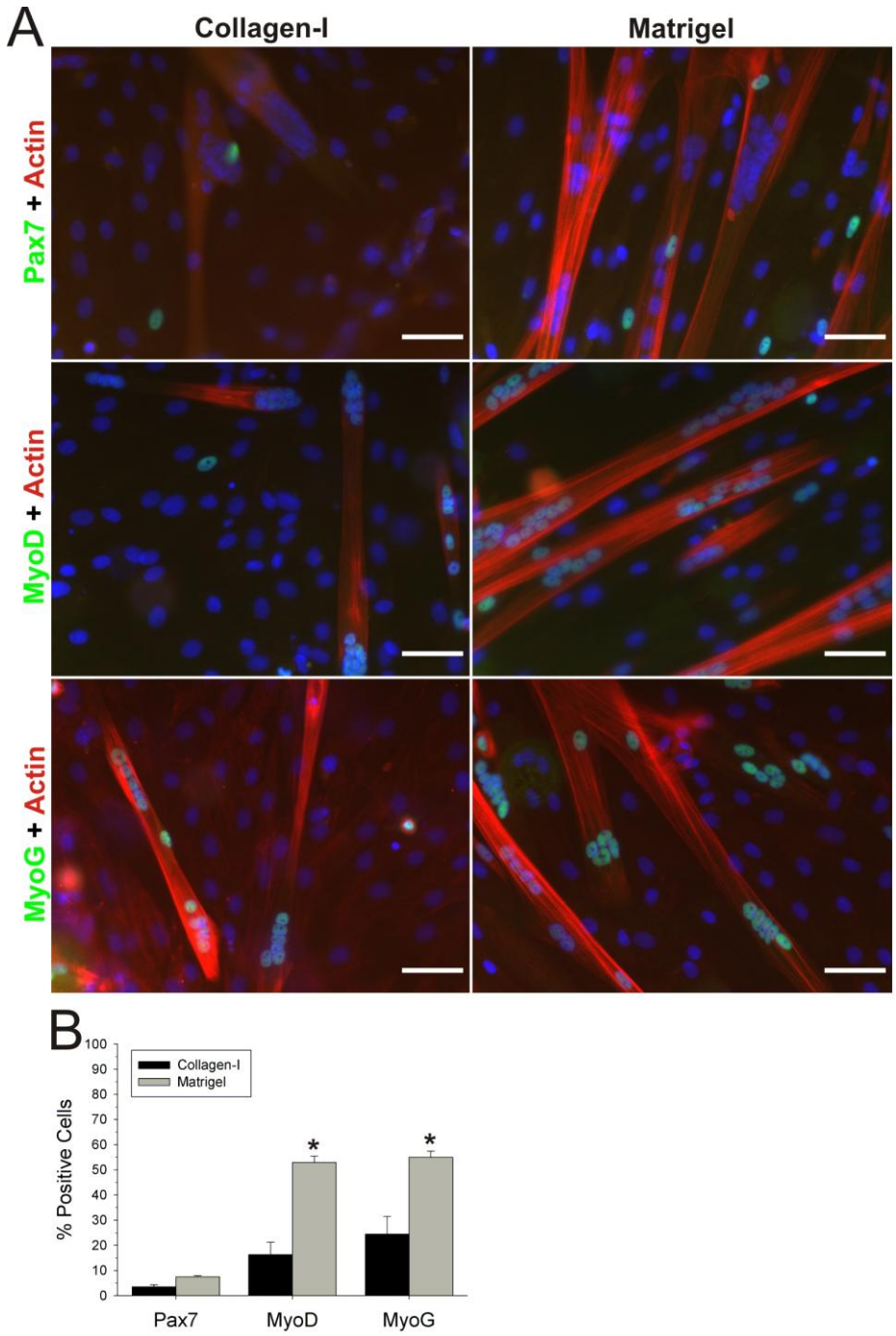

Figure 2. Pax7, MyoD, and Myogenin fluorescent immunohistochemistry and quantification. *A) Differentiating MPCs cultured on collagen-I and Matrigel coatings were stained for Pax7, MyoD, or Myogenin (all green) together with Actin (red) and DAPI (blue). B) Quantification of Pax7^{+}, MyoD^{+}, and Myogenin^{+} cells (expressed as a mean ± SD) on collagen-I and Matrigel coatings. Scalebar represents 50 µm. * significant difference between the collagen-I and Matrigel.*

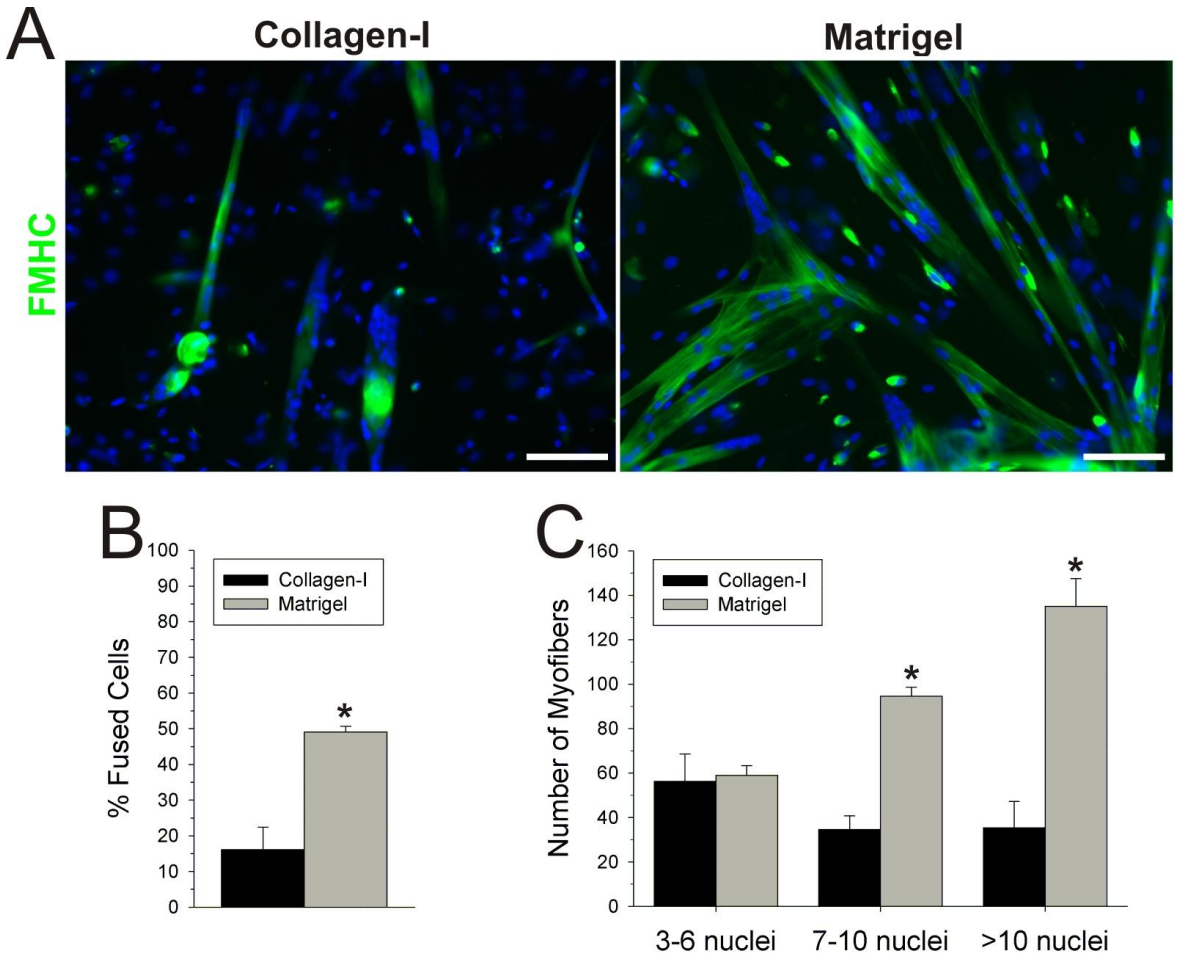

Figure 3. Fast Myosin Heavy Chain (FMHC) fluorescent immunohistochemistry and quantification. *A) Differentiating MPCs cultured on collagen-I and Matrigel coatings were stained for FMHC (green) and DAPI (blue). B) Quantification of the number of fused cells (expressed as a mean ± SD) on collagen-I and Matrigel coatings. C) Quantification of the number of myofibers containing 3-6, 7-10, or >10 nuclei (expressed as a mean ± SD) on collagen-I and Matrigel coatings. Scalebar represents 50 µm. * significant difference between the collagen-I and Matrigel.*

7.3.3 3D-proliferation and differentiation

Proliferating MPCs in the gels are stained for MyoD and actin, and the differentiating MPCs are stained for Myogenin, FMHC, and actin (figure 4A). The gels of collagen contain only few proliferating $MyoD^+$ cells whereas in the gels of Matrigel many $MyoD^+$ cells are found. After differentiation, $Myogenin^+$ cells are present in both gels and few of these cells have fused (arrows). In the gels of Matrigel more myotubes are found than in collagen, but in both gels they do express FMHC.

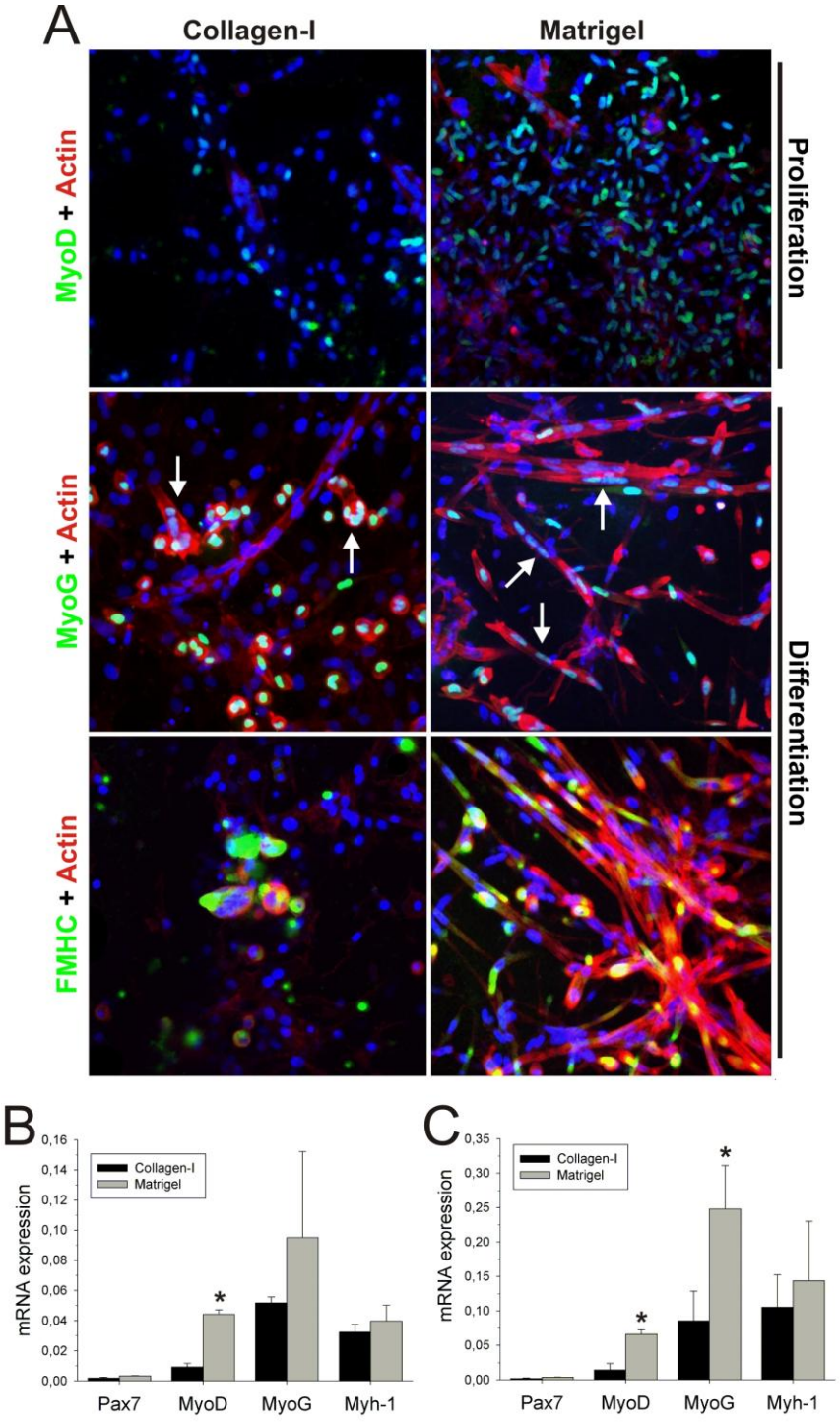

Figure 4. Fluorescent immunohistochemistry and qPCR of proliferating and differentiating MPCs in gels of collagen-I and Matrigel. *A) Proliferating MPCs were stained for MyoD (green), Actin (red) and DAPI (blue). Differentiating MPCs were stained for MyoG or Myogenin together with Actin (red) and DAPI (blue). Gene expression of Pax7, MyoD, Myogenin, and Myosin Heave Chain (Myh)-1 of proliferating (B) and differentiating (C) MPCs in gels of collagen-I and Matrigel. Gene expression was expressed as* $2^{-\Delta Ct}$*. * significant difference between the collagen-I and Matrigel. Pictures were taken at a final magnification of 400x.*

Morphologically, all the muscle cells and myotubes are rounded in the gels of collagen and more elongated in Matrigel. Since quantification of the immunostaining is very complicated, the mRNA levels of Pax7, MyoD, Myogenin, and Myh-1 was analyzed. When proliferating (figure 4 B), low expression levels of Pax7 are found in both gels. In the gels of Matrigel, the expression of MyoD is 4-fold higher than in the collagen gels. Although Myogenin expression seems to be higher in the gels of Matrigel than in the collagen gels, this is not significant. Proliferating MPCs also express equal levels of Myh-1 in both gels. When differentiating (figure 4C), the muscle cells express very low levels of Pax7. The levels of MyoD and Myogenin expression are 3-4 times significant higher in the gels of Matrigel than in collagen. Myh-1 expression is not significantly different between MPCs cultured in both gels.

7.4 Discussion

We showed that the presence of niche elements is crucial for the myogenic potential of MPCs. However, constructs for muscle regeneration that mimic the satellite cell niche are still lacking. On Matrigel coatings, Pax7 and MyoD are persistently expressed during proliferation, while this is reduced by half on collagen. Since Myogenin expression remains low on both coatings, the low number of $Pax7^{+}$ and $MyoD^{+}$ cells is not caused by early differentiation of the MPCs on collagen. On Matrigel, MPCs proliferate faster, but the percentage stays the same meaning that both cell populations divide evenly. Furthermore, differentiation of MPCs leads to more and larger myotubes on Matrigel than on collagen. These results indicate that MPCs lose their myogenic potential on collagen.

In literature, such differences have not been reported up to now, and inconsistency exists in the effect of different ECM molecules on the behaviour of primary MPCs. Varying effects of ECM molecules on the myogenic capacity of MPCs have been reported.[28,30,36-38] Overall, it seems that Matrigel and laminin have a positive effect. The dissimilarities

between these studies could be due to the fact that different animals, strains, isolation and culture protocols, and muscle cell-lines have been used. Two different mouse strains show a different fusion capacity of MPCs on Matrigel.[38] However, we found no differences between MPCs isolated from Wistar or Sprague-Dawley rats (data not shown). With C2C12 myoblasts, hardly any differences were found in the myogenic capacity of these cells on collagen and Matrigel. Furthermore they were less efficient compared to MPCs (data not shown). Since earlier reports also show that differences exist in the response of myoblast cell-lines and MPCs,[38,39] the results obtained with myoblast cell-lines should be carefully interpreted. Furthermore, different isolation protocols are used, probably yielding different populations of MPCs. Overall, this makes it difficult to compare the results.

In none of these studies, the differentiation of MPCs in 3D gels of collagen and Matrigel were examined. We showed in the present study that MPCs have a higher myogenic capacity in the gels of Matrigel. Fluorescence immunostaining of whole gels shows that in the gels of Matrigel more $MyoD^+$ and $Myogenin^+$ cells are present during proliferation and differentiation, respectively. Quantification, as in the 2D experiments was not possible due to difficulties in cell counting in 3D. However, qPCR revealed that in Matrigel significantly higher MyoD and Myogenin levels are expressed during proliferation and differentiation, respectively. Although no differences were found in Myh-1 expression, in the gels of Matrigel more myotubes are formed based on FMHC expression. Overall, MPCs show better myogenic potential when cultured with Matrigel than with collagen on both 2D-coatings and in 3D-gels.

This might be explained by the presence of several growth factors such as basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF-I), platelet-derived growth factor (PDGF), and transforming growth factor (TGF)-β in Matrigel, which all exert effects on skeletal muscle regeneration *in vivo*.[15,40-42] However, when growth factor-reduced Matrigel was used as a coating, similar results were obtained (data not shown). Furthermore, other *in vitro* studies showed that TGF-β, PDGF, IGF-I did not have any effect on the proliferation and differentiation

capacity of muscle cells.[38,43] bFGF did have a positive effect on the proliferation, which was independent from the ECM.[38] In the present study bFGF was already added to the culture medium of MPCs on both collagen and Matrigel, making the culturing conditions similar. Additionally, Matrigel contains very little PDGF and bFGF, and is probably not significant compared to literature. Thus the superior proliferation and differentiation of MPCs in Matrigel is probably not due to the growth factors, but to the ECM molecules present in Matrigel.

The morphology of the myotubes on the 2D coatings, is more rounded without branching on collagen, while they are more elongated, wider, and branched on Matrigel. In the 3D gels, the myotubes were also small and rounded in collagen but elongated in Matrigel. These morphological differences suggest that the (fused) MPCs can attach to Matrigel, but not to collagen. Matrigel consist mainly of laminin-111 (composed of α1, β1, γ1 chains) and collagen type IV. On the contrary, the basal membrane in the satellite cell niche contains laminin-211 and collagen type IV. Satellite cells attach to laminin-211 via the integrin α7β1 receptor, which is important for satellite cell functioning.[44] The absence of integrin α7β1 and mutations in the laminin α2 chain also lead to congenital muscular dystrophy, which support their crucial role.[44,45] Integrin α7β1 can also bind to laminin-111 (present in Matrigel), and blocking it inhibits cell adhesion and migration.[46] Furthermore, porcine MPCs express lower numbers of integrin α7β1 on collagen than on Matrigel.[30] It has been suggested that after asymmetric division of the satellite cells, the daughter cell that stays in contact with the basal membrane remains quiescent, while the cell that loses contact differentiates and fuses with the existing myofiber.[11] Thus the binding of integrin α7β1 to sites in Matrigel might preserve satellite cell properties of MPCs, but these are lost in collagen. The fact that laminin and collagen type IV alone are not as efficient as Matrigel[30] suggest that combinations of niche factors might give better results. Furthermore, in the niche, the satellite cell binds to both the basal membrane via integrin α7β1 and to the myofiber (opposite to the basal membrane) via M-cadherin expressed by both the satellite cell and the myofiber, which allows mutual binding.[3,44] Thus, the addition of attachment sites for M-

cadherin to the substrate might improve proliferation, differentiation and self-renewal capacities of isolated MPCs.

In conclusion, our study shows that MPCs have a larger myogenic capacity with Matrigel than with collagen in both 2D-coatings and 3D-gels. This might be related to the presence of integrin α7β1 binding sites in Matrigel but not in collagen. Matrigel contains elements of the satellite cell niche, which underscores the importance of including niche factors for culturing MPCs. For regenerative medicine purposes, constructs adequately mimicking the niche might improve muscle regeneration. Polymer constructs that contain laminin and collagen type IV and thus mimic the satellite cells niche might also offer new opportunities to treat skeletal muscle defects.

7.5 Acknowledgements

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Chapter 8

The myogenic capacity of muscle progenitor cells from head and limb muscles

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Submitted

Abstract

The restoration of muscles in the soft palate in cleft lip and/or palate patients is accompanied by fibrosis, which leads to speech and feeding problems. Treatment strategies that improve muscle regeneration and inhibit fibrosis have only been tested in limb muscles. Since differences exist between muscles from the head and limb, translation of these treatment strategies for head muscle injuries is needed. Therefore, the myogenic potential of muscle progenitor cells (MPCs) isolated from head and limb muscles is compared.

MPCs were isolated from head and limb muscle of rats and cultured to induce proliferation and differentiation. The proliferation of MPCs was analysed by DNA quantification. The differentiation capacity was analysed by quantifying the numbers of fused cells, and by measuring the mRNA levels of several differentiation markers. Proliferating and differentiating MPCs were also stained to quantify Pax7, MyoD, and Myogenin expression.

During proliferation the amount of DNA was similar in the head and limb MPC cultures indicating equal proliferation capabilities. Differentiating head and limb MPCs show a comparable number of fused cells and mRNA expression levels of Myh-1, -3 and -4. During proliferation and differentiation, the number of $Pax7^+$, $MyoD^+$, and $Myogenin^+$ cells in head and limb MPCs did not differ.

In conclusion, the head and limb MPCs show similar myogenic capacities. Therefore, the differences between those muscle groups rely on the local micro-environment and are not due to intrinsic differences in MPCs. The results of the treatment strategies for limb muscle injuries can also be used for head muscles.

8.1 Introduction

Cleft lip and/or palate (CLP) is one of the most frequent congenital malformations in the facial area of man.[1] About 45% of the CLP patients have a cleft in the soft palate, which consists mainly of muscle tissue.[2] Normal functioning of these muscles is crucial for proper speech and feeding. The levator veli palatini (LVP) is the main muscle component of the soft palate required for speech and feeding.[3-5] Surgical closure of the cleft in the soft palate aims to improve speech and feeding, but often these problems persist after surgery. The formation of scar tissue in the muscles of the soft palate, which also occurs after muscle injury in other muscles, is the most plausible cause of these problems.[6-8] Scar tissue is also found after the repair of a cleft lip decreasing the function of the orbicular oris muscle.[9]

The ability of skeletal muscle to regenerate itself through the action of satellite cells, is well established. Satellite cells are located between the sarcolemma and the basal lamina of the muscle fiber.[10,11] After injury, these cells become activated and migrate to the site of injury, proliferate, differentiate, and fuse to form new myofibers or repair damaged ones. Eventually, the formation of scar tissue prevents complete muscle regeneration.[6-8,12]

Several strategies have been evaluated to optimize and improve muscle regeneration. The injection of growth factors and transplantation of satellite cells or tissue engineered scaffolds have been used with varying results.[8,13-29] These three approaches can also be combined to optimize treatment of muscle injuries. However, treatment with satellite cells still faces problems regarding their isolation and cell culture before and poor cell survival and limited migration after transplantation.[19,20,30,31] Eventually, these results can lead to optimized therapies for the regeneration of skeletal muscle, for example of the soft palate and lip after surgical closure.

However, all studies were performed in muscles of the limb and not in muscles of the head, such as the soft palate. This is of importance since it was shown that muscles from the limbs and from the head differ. For example, limb muscles are derived from the somites, while the

brachiomeric muscles that control jaw movement, facial expression and pharyngeal and laryngeal function, are derived from the cranial paraxial mesoderm.[12,32] During early myogenesis, Pax3, a marker for embryonic muscle stem cells, is only expressed in the limb muscles but not in the head muscles.[33] Additionally, knocking out Pax3 in mice results only in the absence of limb muscles.[34,35] It has also been shown that the associated satellite cells derive from separate genetic lineages and follows different genetic programs.[36,37] These difference are retained in the myoblast progeny[38] and also into adulthood.[39] These developmental differences explain at least in part the unequal occurrence of muscle myopathies in different head and limb muscles.[40-44] This might also explain that head muscles such as the musculus masseter regenerate worse than limb muscles.[45]

All these data indicate that dissimilarities exist in the satellite cells from head and limb muscles, and that they may react differently to muscle injury and disease. This makes it important to characterize the myogenic potential of satellite cells from head muscles after isolation. This will provide a basis for proper translation of the results from the limb muscle regeneration studies to specific treatment strategies for regenerating head muscles such as the soft palate. Therefore, the aim of this study is to compare the myogenic potential of muscle progenitor cells (MPCs) isolated from head and limb muscles.

8.2 Materials and methods

8.2.1 Muscle progenitor cell isolation

MPCs were isolated from the hind limb muscles and musculus masseter of 5-week-old male Sprague-Dawley rats (Janvier, Le Genest, France). The dissected muscles were minced in phosphate-buffered-saline (PBS) containing 5 µg/ml Amphotericine B (Sigma Chemical CO, St Louis, MO, USA) using scissors, and excessive fat and tendon were removed. Minced tissue fragments were incubated in 2% (w/v) Collagenase type II (Invitrogen HQ, San Diego, CA, USA) in Dulbecco's Modified Eagle's Medium-High Glucose (DMEM-HG; Invitrogen) for 1 hour at 37°C.

Tissues were further homogenized by trituration using 10 ml pipettes and filtered through a 100 µm cell strainer to obtain single cell suspensions. The cell suspensions were incubated in hypotonic buffer (0.1 mM EDTA; 0.15 M NH4Cl; 10 mM KHCO3, pH 7.4) for 1.5 minutes to lyse the erythrocytes. The remaining cells were centrifuged (5 minutes at 300g) and resuspended in 15 ml proliferation medium: DMEM-HG containing 20% fetal bovine serum (FBS), 2% penicillin-streptomycin (p/s), 1 mM pyruvate, and 5 ng/ml bFGF (all from Invitrogen). To remove fast-adhering fibroblasts, the cells were incubated in uncoated culture flasks for 1 hour (37°C, 5% CO2). Non-adhering cells were then transferred to tissue cultured flasks coated with 1 mg/ml Matrigel (Matrigel™ Basement Membrane Matrix, BD Bioscience, Bedbord, MA, USA), and cultured for 4 days (37°C, 5% CO2). Proliferation medium was refreshed every day. After 4 days, MPCs were significantly enriched up to 50% according to Pax7 expression. MPCs from 4 rats were pooled to create one batch.

8.2.2 Proliferation and differentiation of muscle progenitor cells

Three batches of enriched MPCs were used for the proliferation and differentiation experiments. For the proliferation experiments, 24-wells plates and Lumox dishes (Ø 35 mm; both Greiner Bio-One, Frickenhausen, Germany) were coated with 1 ml (1 mg/ml) Matrigel for 1 hour at 37°C. In the 24-wells plate, enriched MPCs were plated at a density of 10.000 cells/well and cultured in proliferation medium for 1, 2 and 3 days. In the Matrigel-coated Lumox dishes, 50.000 enriched MPCs were seeded and cultured in proliferation medium for one and three days. For the differentiation experiments, enriched MPCs were seeded at a density of 500.000 cells in the Matrigel-coated Lumox dishes. Cells were cultured for 1 day in proliferation medium and then in differentiation medium: DMEM-HG containing 2% fetal FBS, 1 mM pyruvate, and 2% p/s to induce fusion for 1 and 2 additional days. After the experiments, the MPCs were analyzed by DNA quantification, immunofluorescence staining, and quantitative PCR.

8.2.3 DNA quantification

Cell proliferation of the enriched MPCs was measured using the PicoGreen dsDNA Quantification assay (Molecular Probes, Eugene, OR, USA). At the appropriate time points, the cells were lysed in 0.1% (v/v) Triton X-100 in PBS and subjected to three freeze and thaw cycles. Lysed cells were processed according to the manufactures protocol. Fluorescence was measured in a FL600 Microplate Fluorescent Reader (Bio-Tek Instruments Inc., Winooski, VT, USA) at excitation 485 nm and emission 520 nm. The amount of DNA was calculated using a standard curve ranging from 0 to 15000 cells.

8.2.4 Immunofluorescence staining

After the experiments, the enriched MPCs were washed with PBS and fixed in 4% formaldehyde in PBS for 15 minutes. After washing with PBS, the membranes of the Lumox dishes were cut into 5 pieces and the attached cells were permeabilized with 0.5% Triton X-100 in PBS for 20 minutes. The cells were washed with 0.05% (v/v) Tween-20 in PBS, and then incubated in blocking buffer containing 2% (w/v) bovine serum albumin (BSA), 2% (v/v) normal goat serum (NGS), 0.1% (v/v) Triton X-100, 0.05% (v/v) Tween-20, and 100 mM glycine in PBS for 30 minutes. Then, the cells were incubated with a mouse anti-Pax7 (1:25; Developmental Studies Hybridoma Bank, Iowa City, CA, USA), mouse anti-MyoD (1:25; DAKO, Dakopatts, Glostrup, Denmark), and mouse anti-Myogenin (F5D, 1:100; Developmental Studies Hybridoma Bank) in blocking buffer without glycine for 1 hour. For the proliferation experiments, the bound antibodies were visualized with AlexaFluor-488-labeled goat anti-mouse IgG (H+L) (1:200; Molecular Probes). For the differentiation experiments, the bound antibodies were visualized with AlexaFluor-488-labeled goat anti-mouse IgG (H+L) (1:200; Molecular Probes) combined with Texas Red-X Phalloidin (1:250; Molecular Probes) to stain the actin filaments. The cells were visualized with a Zeiss Imager.Z1 microscope and photographed.

8.2.5 Quantification

The number of $Pax7^+$, $MyoD^+$, and $Myogenin^+$, and fused (DAPI) cells in the enriched MPC cultures were calculated using 8 different representative fields of all immunostainings (N=3 batches). All the results were expressed as a mean percentage ± SD of the total number of cells.

8.2.6 Quantitative PCR

The cells were washed with PBS and homogenized with 1 ml Trizol (Invitrogen). Then 200 µl chloroform (Sigma) was added, mixed, and centrifuged for 20 minutes at 4ºC. The water phase containing the RNA was mixed with 70% ethanol (1:1), and RNA was extracted according to the manufacturer's protocol (Qiagen, Hilden, Germany) including a DNase I treatment. cDNA was generated with 1 µg RNA using the iScriptTM Reverse Transcriptase system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Quantitative real-time PCR was performed in a final volume of 25 µl containing 12.5 µl SYBR®Green Supermix (Bio-Rad), 5 µl (40x diluted) cDNA, 4.5 µl RNAse-free water, 1.5 µl 2.5M forward primer, and 1.5 µl 2.5M reverse primer. The primers for β-actin, Pax3, Pax7, MyoD, Myogenin, Myh-1, Myh-2, Myh-3, Myh-4, and Myh-8 were obtained from Biolegio (Nijmegen, The Netherlands) and the primer sequences are provided in table 1.

Table 1. Primer sequences.

Gene	Forward primer	Reverse primer
β-actin	TTCAACACCCCAGCCATGT	TGTGGTACGACCAGAGGCATAC
Pax3	CTTTCACCTCAGGTAATGGGA	TCTTCATGTGCTCCAATCTC
Pax7	AGCCGAGTGCTCAGAATCAA	TCCTCTCGAAAGCCTTCTCC
MyoD	CGACTGCCTGTCCAGCATAG	GGACACTGAGGGGTGGAGTC
Myogenin	AACCCAGGAGATCATTTGCT	GGTGACAGACATATCCTCCA
Myh-1	CCTGGATGATCTACACCTACTC	GTCAGAGATAGAGAAGATGTGGG
Myh-2	CGAGACATATCTGCTAGAGAAG	GTAATCGTATGGGTTTGTGGT
Myh-3	CTGGATGATCTACACCTATTCAG	CAGAGATGGAGAAGATGTGG
Myh-4	GTCTTCTCCATGAACCCTCC	CCCGAATAAGTGTAGATCATCC
Myh-8	GAAACCTTGAGAAGATGTGCC	AATACTCTCCTGCTTCTGTCTG

The cDNA was amplified in the C1000 Thermal Cycler (Bio-Rad) and fluorescence was analyzed using the CFX96TM Real-Time System (Bio-Rad). The PCR conditions were 95ºC for 3 minutes (1 cycle), 95ºC for 15 seconds and 60ºC for 30 seconds (39 cycles), and finally a temperature increase starting at 65ºC to 95ºC with 0.5ºC intervals. RNA expression was normalized against the mRNA level of β-actin (ΔCt) and presented as $2^{-\Delta Ct}$.

8.2.7 Statistical analysis

All the results were tested for significance at every single time-point ($p < 0.05$) using a Two-Way ANOVA with Holm-Sidak post-hoc analysis. The results of the Quantitative PCR were only normally distributed after log transformation.

8.3 Results

8.3.1 Cell density of proliferating head and limb muscle progenitor cells (MPC)

Enriched MPCs isolated from M. masseter (head MPCs) and hindlimb muscles (limb MPCs) were cultured for three days. In time, head and limb MPCs proliferated, and quantification of their numbers reveal a significant 5-fold increase of both head and limb MPCs within three days (figure 1). Overall, the numbers of head MPCs are always higher than the limb MPCs and the main differences are found at the first two days, but this is not statistically significant.

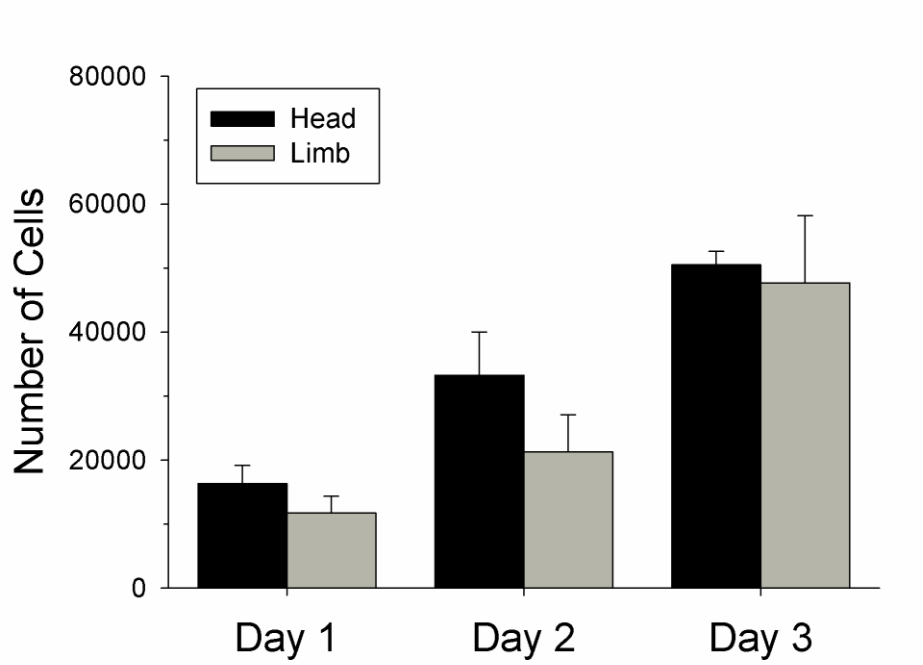

Figure 1. ***DNA quantification.*** *Quantification of the number (B; expressed as mean ± SD) of proliferating head and limb MPCs after 1, 2, and 3 days.*

8.3.2 Pax7, MyoD, and Myogenin in proliferating head and limb MPCs

After an enrichment step, the head and limb MPCs proliferated for one and three additional days and were stained to identify Pax7, MyoD and Myogenin expression (figure 2A). In time the total number of cells together with the numbers of $Pax7^+$ and $MyoD^+$ cells increased in both MPC cultures. Only low numbers of $Myogenin^+$ cells are found within the cultures of both the head and limb MPCs.

Quantification of the relative numbers of $Pax7^+$, $MyoD^+$, and $Myogenin^+$ cells are shown in figure 2B and C. After one day of proliferation, 62.2 ± 4.2% and 63.6 ± 8.2% of the cells in the limb MPC cultures are positive for Pax7 and MyoD, respectively. In the head MPC cultures, these numbers are slightly higher for Pax7 at 67.7 ± 4.0% and for MyoD at 68.9 ± 4.3%. The number of $Myogenin^+$ cells is significantly lower in both the head MPC (4.9 ± 1.9%) and limb MPC (3.2 ± 0.5%) cultures.

After three days of culture the numbers of $Pax7^+$ and $MyoD^+$ cells are decreased, but the number of $Myogenin^+$ cells is increased. The number of $Pax7^+$ cells decreased in the head and limb MPC cultures to

36.7 ± 5.6% and 40.2 ± 10.9%, respectively. The number of MyoD$^+$ cells also decreased in the limb MPC cultures to 44.9 ± 6.9%, but only slightly in the head MPC cultures to 61.0 + 14.1%. The number of Myogenin$^+$ cells increased in the limb MPC cultures to 8.8 ± 3.3% and even more in the head MPC cultures to 21.5 ± 8.6%. Although the numbers of Pax7$^+$, MyoD$^+$, and Myogenin$^+$ cells are always higher in the head MPC cultures, this was not significant.

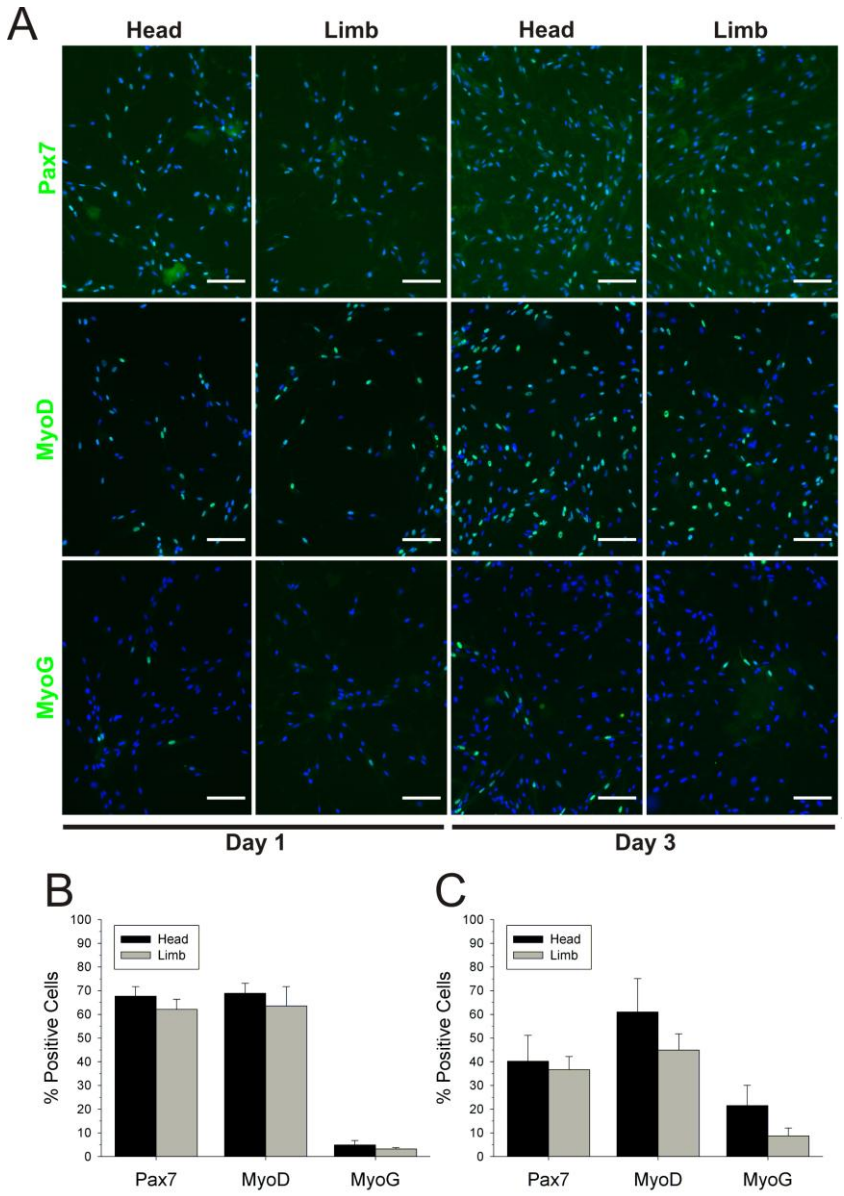

Figure 2. Pax7, MyoD, and Myogenin fluorescent immunohistochemistry and quantification. *A) Proliferating head and limb MPCs were stained for Pax7, MyoD, or Myogenin (all green) and DAPI (blue). Quantification of Pax7$^+$, MyoD$^+$, and Myogenin$^+$ cells (expressed as a mean ± SD) in the head and limb MPC cultures after one (B) and three (C) days of proliferation. Scalebar represents 100 µm.*

8.3.3 Pax7, MyoD, and Myogenin in differentiating head and limb MPCs

MPCs were stained for Pax7, MyoD, Myogenin, and actin filaments after one and two days of differentiation (figure 3A).

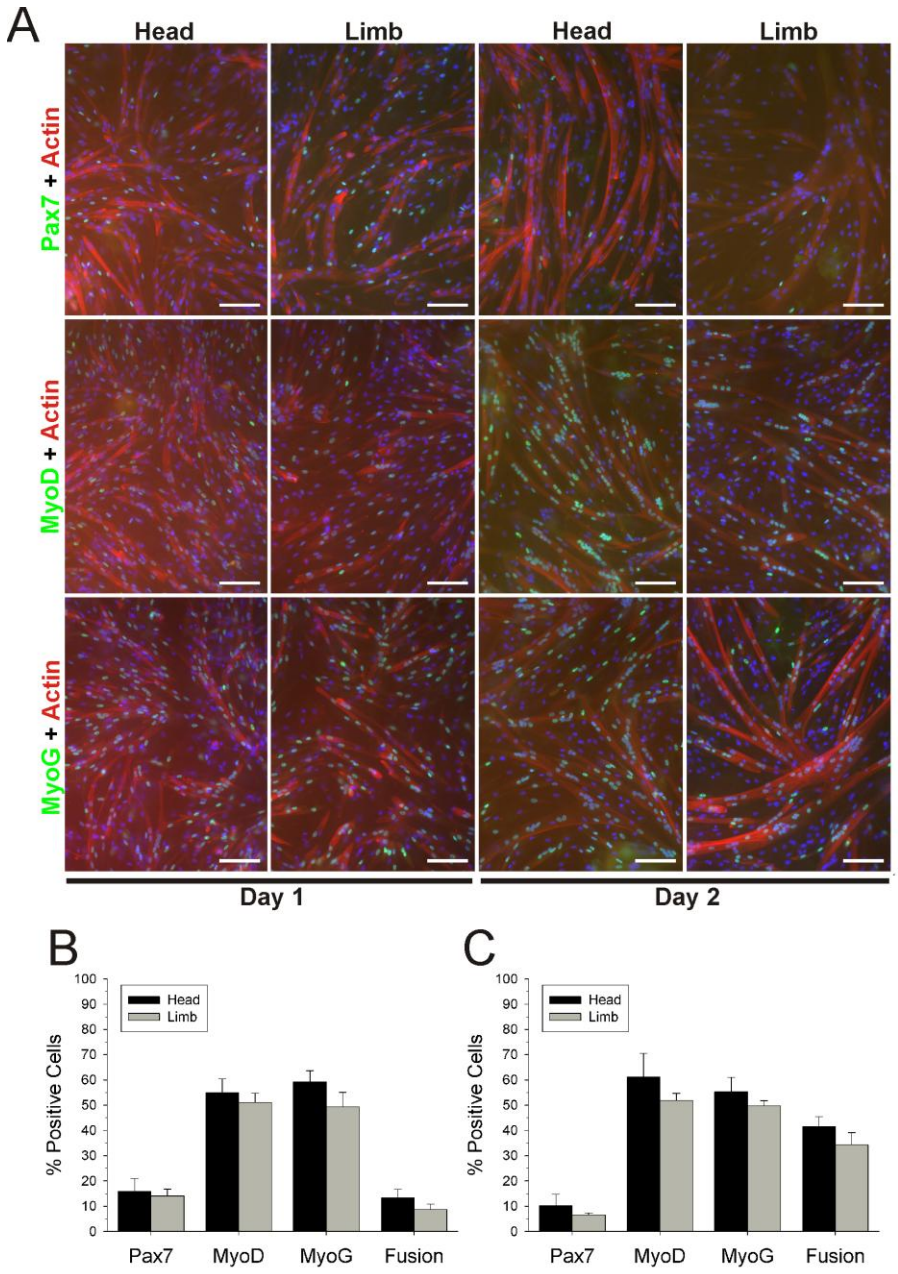

Figure 3. Pax7, MyoD, and Myogenin fluorescent immunohistochemistry and quantification. *A) Differentiating head and limb MPCs were stained for Pax7, MyoD, or Myogenin (all green) together with Actin (red) and DAPI (blue). Quantification of Pax7$^+$, MyoD$^+$, Myogenin$^+$, and fused cells (expressed as a mean ± SD) in the head and limb MPC cultures after one (B) and two (C) days of differentiation. Scalebar represents 100 µm.*

The head and limb MPCs already form myotubes after one day of differentiation, which increased in time. In comparison to the proliferation phase, the number of $Pax7^+$ cells dropped, the number $MyoD^+$ cells maintained, and the number of $Myogenin^+$ cells increased during the differentiation of both the head and limb MPCs. None of the $Pax7^+$ cells seem to be within the myotubes, but most of the fused cells are positive for MyoD and Myogenin.

Quantification of the relative numbers of $Pax7^+$, $MyoD^+$, $Myogenin^+$, and fused cells are presented in figure 3B and C. Also during differentiation the head MPCs show more $Pax7^+$, $MyoD^+$, $Myogenin^+$, and fused cells, but also now this is not significant. Specifically, after one day of differentiation the numbers of $Pax7^+$, $MyoD^+$, and $Myogenin^+$ cells are 14.0 ± 2.7%, 51.1 ± 3.6%, and 49.4 ± 5.7%, respectively, for the limb MPCs. These numbers are higher in the head MPCs for Pax7 (15.9 ± 5.0%), MyoD (54.9 ± 5.5%), and Myogenin (59.3 ± 4.4%). After two days of differentiation, the number of $Pax7^+$ cells decreased to 6.6 ± 0.8%, while the numbers of MyoD (51.9 ± 2.8%) and Myogenin 49.8 ± 2.1%) did not change in the limb MPCs. In the head MPCs, the numbers of $Pax7^+$ and $Myogenin^+$ cells decreased to 10.2 ± 4.5% and 55.3 ± 5.7% , while the number $MyoD^+$ cells increased to 61.2 ± 5.7%.

The numbers of fused cells are 8.7 ± 2.1% for limb MPCs and slightly higher at 13.4 ± 3.2% for head MPCs after one day of differentiation. After two days of differentiation, these numbers significantly increased to 34.3 ± 4.9% for limb MPCs and again slightly higher to 41.6 ± 3.8% for head MPCs.

8.3.4 Quantitative PCR in differentiating head and limb MPCs

Of the differentiating MPCs, the mRNA levels of Pax3, Pax7, MyoD, Myogenin, Myh-1, Myh-2, Myh-3, Myh-4, and Myh-8 were also analyzed (figure 4). The expression levels of Pax3, Pax7, Myh-2, and Myh-8 are extremely low and can therefore not be quantified. After one day, low levels of MyoD and high levels of Myogenin are expressed. In the limb MPCs the expression of MyoD is higher while the expression of Myogenin is lower compared to the head MPCs. The fusion markers, Myh-1, -3, and -4 are expressed in both head and limb MPCs, but there is

a trend of higher expression in the limb MPCs. After two days, MyoD expression diminished in the limb MPCs while it did not change in the head MPCs. Myogenin expression decreased, but all the fusion markers Myh-1, -3, -4, -8 increased two- or three-fold in both the head and limb MPCs. Now there is a trend of higher expression of these markers in the head MPCs.

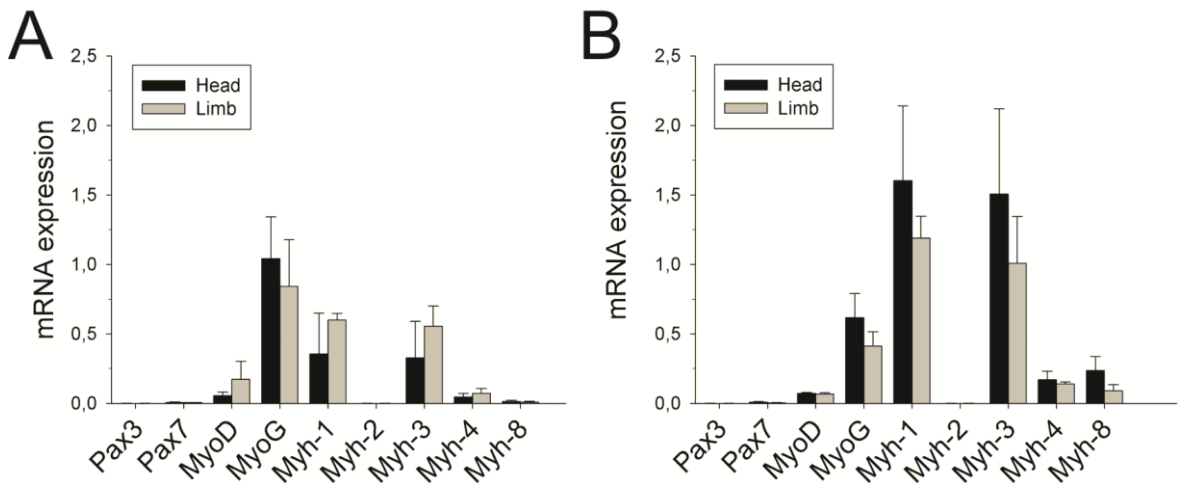

Figure 4. qPCR of differentiating head and limb MPCs. *Gene expression of Pax3, Pax7, MyoD, Myogenin, Myh-1, Myh-2, Myh-3, Myh-4, Myh-8 in head and limb MPC cultures after one (A) and two (B) days of differentiation. Gene expression was expressed as* $2^{-\Delta Ct}$.

8.4 Discussion

The aim of this study was to provide a basis for translating the results of regeneration studies in limb muscles towards a therapy for head muscle injuries. Since it has been shown that satellite cells from these muscle groups originate from separate genetic lineages, and follow different genetic programs *in vivo*,[36,37] we investigated their myogenic potential after isolation. We show that enriched MPCs from head and limb muscles are similarly efficient in their proliferation and differentiation capacity. During proliferation and differentiation the numbers of $Pax7^+$, $MyoD^+$, $Myogenin^+$, and fused cells are not signifanctly different in the head and limb MPC cultures. Furthermore, the expression of fusion genes Myh-1, -3 and -4 are also comparable between MPCs from head and limb muscles.

Therefore, the proliferation and differentiation potential is very similar for both types of muscle satellite cells.

In contrast, recent research showed that, after isolation, MPCs of head and limb muscles do differ in their myogenic capacity.[38] They found that head MPCs form myofibers at a slower rate than limb MPCs. This reflects the observation that the M. masseter regenerates worse than limb muscles.[45] The difference between the results of this study and our results might be due to the different isolation protocol. Our protocol leads to a bulk of enriched MPCs, while Ono et al.[38] first isolate single myofibers before liberating the MPCs. This might result in a higher purity of MPC cultures that are isolated directly from the satellite cell niche, which resembles the *in vivo* situation more closely. Our MPCs were isolated earlier from their niche and probably already started to adapt to the culture conditions.

Muscle regeneration is different between different muscle groups. For example, the M. masseter regenerates worse than tibialis anterior muscles.[45] Since muscle regeneration depends on many factors in the micro-environment such as the inflammatory response and growth factors,[7,46] this might be different in head and limb muscles. For example, a mouse strain showing slower muscle regeneration has less FGF-2 expression and a reduced inflammatory response.[47,48]

This indicates that the micro-environment, and specifically the niche of the (activated) satellite cells plays a crucial role in their regeneration capacity. In our protocol, MPCs are immediately taken out of their niche and are therefore lack these instructing factors. Furthermore, it has been shown that differentiating MPCs isolated from extraocular muscles also do not express their specific myosin heavy chain markers *in vitro*. When injected into a hind limb muscle, they formed new muscle tissue and generated satellite cells, but also without their original specific markers (36). Additionally, transplantation of head satellite cells into a limb host muscle showed muscle regeneration with the same efficiency as transplanted limb satellite cells.[38] It has also been found that in both head and limb muscles, $Pax7^+$ satellite cells appear after embryogenesis.[49] All these data imply that satellite cells throughout the entire body can follow a similar differentiation program, depending on the micro-environment.

The variation in the micro-environment might explain the different regeneration capacities of the various muscle groups.

8.5 Conclusion

Our study shows that MPCs isolated from head and limb muscles have similar myogenic capacities. This shows that in the absence of niche and micro-environmental factors *in vitro*, MPCs follow a similar differentiation program. We conclude that regeneration strategies for limb muscles can also be used for head muscles. However, the modulation of the micro-environment in the head area is of utmost importance for proper regeneration. Those micro-environmental cues that apparently differ between head and limb require further investigation.

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Chapter 9

General discussion and future perspectives

9.1 Introduction

The main goal of skeletal muscle engineering is the treatment of a wide variety of muscle defects and diseases. Three different approaches have been used to improve muscle regeneration; growth factor-, cell-, and scaffold-based therapies, or combinations of these. For the treatment of large muscle defects such as the soft palate in cleft palate (CLP) patients, three-dimensional scaffolds are needed. Therefore, we focused on improving muscle regeneration using scaffold-based approaches in full-thickness muscle defects. Specifically, our aims were:

1. To develop new fibrosis models to study the effect of implanted scaffolds on muscle regeneration (chapters 4 & 5).
2. To test scaffolds loaded with growth factors for their ability to improve muscle regeneration and to inhibit fibrosis (chapters 5 & 6).
3. To develop a 3D culture system for satellite cells to analyze the effects of ECM components on their myogenic capacity (chapter 7).
4. To compare the myogenic capacity of satellite cells derived from a limb and a craniofacial muscle (chapter 8).

In the next sections, the results of these studies are discussed in a wider perspective. Specifically, the focus is on the requirements of the scaffolds and on the use of *in vitro* culture systems for satellite cells to improve muscle regeneration.

9.2 Scaffolds in muscle regeneration

In order to test scaffolds in full-thickness defects leading to fibrotic lesions such as in muscle trauma and in clefts of the soft palate, a new fibrosis model was required. The M. soleus in rats was used since the myofibers are perfectly aligned, which allows the study of the orientation and continuity of regenerating myofibers (chapter 4).[1] Our full-thickness wound model represents large muscle defects that spontaneously develop

fibrotic lesions (chapter 5). Furthermore, this model enabled us to implant scaffolds into the defect without sutures. In conclusion, the M. soleus model presented in this study provides a solid base to test scaffolds that aim to improve muscle regeneration and to inhibit fibrosis. A limitation of our study is that we were unable to perform functional testing of the regenerated muscle because of lacking equipment. It has been shown recently that muscle defects with large fibrotic lesions significantly reduce muscle function.[2] Thus, we infer that, in our fibrosis model, muscle function is also severely impaired. Still, this should be confirmed in future studies. When experimental therapeutic interventions show improvement on histological basis, it becomes crucial to test muscle function to draw definite conclusions.

Scaffolds that are being used in tissue engineering in general and specifically for muscle regeneration require certain characteristics. They should be biocompatible to prevent an extensive immune response and biodegradable in order to be replaced by host muscle tissue. Furthermore, the scaffolds should stimulate adhesion, proliferation, and differentiation of satellite cells. Collagens are the main extracellular matrix (ECM) molecules in the human body, and collagen type I is used widely in muscle engineering.[3-6] More importantly, collagen type I can be used to generate biodegradable and porous scaffolds, which provide structural integrity and can serve as a reservoir for growth factors to attract cells (this thesis).[7] Such collagen scaffolds can also be crosslinked to reduce degradation. These crosslinked collagen scaffolds can be used to mimic fibrotic tissue after implantation into the muscle, and enables us to investigate treatment modalities aimed at reducing existing fibrosis as in recurrent muscle strain injuries (chapter 4). However, we subsequently focused on a muscle model with spontaneous fibrosis to test non-crosslinked collagen type I scaffolds for the prevention of fibrosis (chapters 5 and 6).

Two different approaches with such non-crosslinked collagen scaffolds can be followed. They can either act as a carrier for isolated satellite cells, or they can be modified to stimulate the endogenous satellite cells. It is still uncertain whether the first approach is feasible, because of major drawbacks. Firstly, muscle biopsies, creating new

injuries, have to be made for the isolation of autologous satellite cells. Secondly, to obtain sufficient cell numbers, satellite cells need to be cultured, which is expensive and laborious. More importantly, *in vitro* culture is detrimental for the myogenic capacity of satellite cells.[8,9] Thirdly, their migration and survival after transplantation is low.[10,11] This makes the use of satellite cells inefficient, and research should be aimed to overcome these problems. The migration and survival of transplanted satellite cells can be improved by loading the scaffolds with growth factors.[12] Growth factors can also be used to attract resident satellite cells to avoid the problems associated with cultured satellite cells. Furthermore, growth factor-loaded scaffolds can be used off-the-shelf, and implanted directly into the defect during the surgery of for example CLP patients.

In our muscle fibrosis model, we showed that collagen scaffolds loaded with SDF-1α stimulates the migration of satellite cells towards the regenerative zone around the defect (chapter 5). However, the satellite cells were unable to migrate into the scaffold. Possibly, the SDF-1α is already lost when the satellite cells reach the regenerative zone or the scaffold does not allow attachment and migration of these cells. These problems need to be overcome, which is discussed in the following sections. Eventually, the regeneration process may further be improved when SDF-1α is used in combination with other growth factors such as HGF and IGF-I that stimulate the proliferation and differentiation of the migrated satellite cells. However, loading the scaffolds with SDF-1α did not reduce fibrosis. Thus, increasing the numbers and the function of satellite cells is not enough to prevent fibrosis. TGF-β is the main factor involved in fibrosis in many tissues including skeletal muscle,[13-15] and inhibition of its activity is supposed to diminish fibrosis. We used decorin, which consist of a core protein, containing two binding sites for TGF-β, and a dermatan/chondroitin sulfate chain.[16,17] Inhibition of TGF-β activity by decorin reduced fibrosis in many organ systems, but also in skeletal muscle.[14,18-21] However, in our model decorin alone or together with SDF-1α did not reduce fibrosis (chapter 6). Possibly, decorin is already released before the anti-fibrotic effect can take place. As mentioned, SDF-1α did not induce the migration of satellite cells into the

scaffolds. These studies indicate that the release profile of (growth) factors is crucial. New strategies are being investigated to develop timely-tuned release of factors from the scaffolds. Using microspheres it is possible to control the release of such factors.[22,23] With specific microspheres for each (growth) factor, the release of factors in a specific order and at the appropriate time points during muscle regeneration can be achieved. Furthermore, alginate-, gelatin-, and fibrin-based hydrogels can also induce a sustained release of factors without the use of microspheres, and might be combined with synthetic polymers to provide more mechanical strength.[24-28]

The inability of satellite cells to migrate into the scaffold could also be caused by the type of scaffold material. *In vivo*, satellite cells attach to the basal lamina via the laminin receptor integrin α7β1, and to the myofiber via M-cadherin. Isolated satellite cells cultured in collagen type I gels lose their myogenic potential (chapter 7). More importantly, fused myofibers show a rounded phenotype indicating that collagen type I does not contain appropriate binding sites. This may cause the inability of satellite cells to migrate into the collagen scaffold after implantation. In matrigel, which contains laminin, satellite cells retain their differentiation capacity and form elongated myofibers. *In vivo*, the laminin and myofiber binding sites in the niche are on opposite sides of the satellite cell, which appears to be important for asymmetric self-renewal.[29,30] This indicates that niche factors, and their bipolar orientation is crucial for satellite cell adhesion, proliferation, differentiation, and self-renewal. Thus, scaffolds containing binding sites for M-cadherin and integrin α7β1 should be created for proper satellite cell functioning and self-renewal. The binding site for integrin α7β1 lies within the G1-G3 domain of the E8 region of laminin.[31] Although in muscle laminin-2 and -4 are predominantly present, satellite cells are also able to bind to laminin-1.[32] Recently, it has been shown that laminin-1 can be incorporated into polymer substrates, which could provide binding sites for the satellite cells.[33]

In our muscle fibrosis model, regenerating muscle fibers are also not able to align with the existing myofibers. Such alignment is crucial for coordinated contraction of all myofibers and thus for full muscle function. Therefore, the scaffold should also contain structural cues to

induce proper alignment of the regenerating myofibers. The use of polymers offers new opportunities to generate reproducible scaffolds with specific porosity, and fiber thickness and orientation that could be promising for skeletal muscle tissue engineering.[34,35] Furthermore, biomechanical conditioning of cell-seeded scaffolds might improve the alignment of myofibers even more.[36,37]

The translation of these results towards a therapy for large muscle defects and clefts in the soft palate will require much more research. In the study described here, we report that satellite cells derived from a limb and a craniofacial muscle show similar differentiation capacities, and that the environment is crucial for satellite cell functioning. New muscle fibrosis models should be developed for specific conditions such as clefts of the soft palate in rats to further optimize the scaffold-based approach.

In conclusion, bioactive scaffolds that stimulate endogenous satellite cells abolish the need for cultured satellite cells, and might be the best solution for skeletal muscle tissue engineering. However, it will be challenging to develop appropriate bioactive scaffolds that 1) mimic the bipolar satellite cell niche, 2) induce timely-tuned release of factors that stimulate satellite cells and inhibit fibrosis, and 3) contain structural cues forcing the regenerating myofibers into alignment.

9.3 Cultured muscle progenitor cells in muscle regeneration

To develop bioactive scaffolds for *in vivo* implantation, specific *in vitro* cell culture systems are required to identify suitable niche factors and growth factors. In addition, isolated muscle cells can also be included in the scaffolds. First satellite cells, or muscle progenitor cells (MPCs), need to be isolated. Since many different protocols are being used that lead to different cell populations, it is difficult to compare the results. Enzymatic digestion and trituration of skeletal muscle tissue will result in single cell suspensions containing the satellite cells, and other cell types such as fibroblasts and endothelial cells. Based upon their adhesive properties, fibroblasts can be removed, and satellite cells can then be

enriched by pre-plating (chapter 7).[5,38,39] Furthermore, different populations of muscle stem cells such as satellite cells and a more enriched population of late-adherent muscle-derived stem cells can be obtained using the pre-plating technique.[5] Alternatively, FACS isolation can produce a highly enriched population of satellite cells, but with a low yield. Two different satellite cell populations have been isolated according to CXCR4 and integrin β1 expression,[40,41] and integrin α7 and CD^{34} expression.[42] A disadvantage of generating single cell suspensions is the absence of the satellite cell niche during isolation. This could lead to the partial loss of their myogenic capacity. Isolated MPCs from limb and craniofacial muscle show a similar differentiation capacity *in vitro* although clear differences in regeneration exist *in vivo* (chapter 8). Therefore the presence of niche factors is important to maintain satellite cell properties. Single myofibers with satellite cells residing in their physiological niche can also be isolated from muscle tissue. From these myofibers, satellite cells can be liberated by trituration or by culture.[43]

For our purpose, the optimization of scaffolds for large tissue defects, two isolation methods might be useful 1) enriched MPCs from which fast-adhering fibroblast are removed (chapter 7) and 2) single myofibers with satellite cells. High numbers of satellite cells are needed to optimize the scaffolds regarding the addition of appropriate ECM molecules and structural cues to induce myofiber alignment. Therefore, enriched MPCs, as studied in this thesis, are the cells of choice. This results in large numbers of 50-60% $Pax7^{+}$ MPCs that form functional myofibers within two days. Our study also showed that laminin is crucial for adhesion and maintenance of the differentiation capacity (chapter 7). To identify growth factors that are able to induce proliferation and differentiation of resident satellite cells, and migration into the scaffolds, satellite cells need to be in their physiological niche after isolation. For these experiments, single myofibers should therefore be used. Furthermore, isolated myofibers are also crucial to study satellite cell biology during migration, activation, proliferation, and differentiation.[44,45] Eventually, these experiments should lead to optimal scaffolds for *in vivo* implantation into muscle defects.

Another lack in the current knowledge is the specific *in vitro* conditions that maintain satellite cell self-renewal to obtain large numbers of cells. Further, the techniques to obtain functionally mature and aligned myofibers to construct muscle substitutes are also lacking. Future research should aim to identify specific ECM molecules that regulate satellite cell functioning, and to develop constructs that mimic the *in vivo* bipolar satellite cell niche. Furthermore, substrate stiffness and mechanical force have been shown to influence satellite cell behavior, and mature myofiber formation and orientation.[36,37,46-50] A practical problem is that for transplantation, the cells must be cultured in serum-free medium. The identification of specific growth factors is essential to optimize satellite cell cultures without using serum.[51] For the above, pure populations of satellite cells, isolated based on specific marker expression, are needed.

In summary, two crucial protocols should be developed that 1) yield large numbers of satellite cells maintaining their myogenic potential, and 2) generate muscle substitutes with mature and functional aligned muscle fibers. For the latter, however, blood vessels also need to be engineered to provide nutrients and oxygen after transplantation. This is an additional challenge to the field of muscle engineering.

9.4 Conclusions

Skeletal muscle engineering will significantly contribute to the repair of muscle defects by developing suitable scaffolds and *in vitro* culture systems for satellite cells. We developed a suitable model for spontaneous muscle fibrosis to test such scaffolds. In the studies described here, we show that scaffolds with growth factors are promising to attract endogenous satellite cells towards the defect. However, it also became clear that inhibition of fibrosis is crucial to obtain functional muscle regeneration. Using isolated satellite cells we showed that elements of the satellite cell niche should be incorporated into the scaffolds to maintain their stem cell properties. In the future, the use of isolated satellite cells and/or myofibers will lead to the development of

smart scaffolds. Eventually, these smart scaffolds will eliminate the use of isolated satellite cells because they induce the endogenous satellite cells to migrate into the defect and form aligned myofibers.

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Chapter 10

Summary

In **chapter 1**, the background and rationale of the study is explained. The field of skeletal muscle engineering together with strategies to improve muscle regeneration is introduced and the outline of the study is presented. The aim of the present study was to improve muscle regeneration and to inhibit fibrosis in full-thickness muscle defects using scaffold-based approaches.

Chapters 2 and 3 present an overview of the biological aspects of skeletal muscle development and regeneration with the main focus on satellite cells. Satellite cells regenerate the muscle tissue by migrating to the site of injury where they proliferate, differentiate, and form myofibers. The specific micro-environment of the satellite cells, the niche, controls satellite cell behavior. In addition, a large diversity of growth factors regulates satellite cell activity after injury. Since the formation of scar tissue can prevent the recovery of full muscle function, three different approaches to improve muscle regeneration and to inhibit fibrosis are discussed: growth factor-, cell-, and scaffold-based therapies. For large muscle defects mainly the scaffolds-based approach is suitable, which is the focus in the next studies.

In **chapter 4** an *in vivo* model for muscle regeneration in recurrent strain injury is established. The results showed that satellite cell activation around the defect, revealed by Pax7 and MyoD expression, was not affected by the implantation of a cross-linked collagen scaffold in the lacerated M. soleus. However, these cells were absent inside the scaffold and muscle regeneration inside the defect was impaired. It was concluded that the implantation of a cross-linked collagen scaffold into the lacerated M. soleus mimics a muscle discontinuity caused by a fibrotic wedge and can be used to evaluate new treatment modalities for recurrent strain injuries.

A new wound model that mimics full-thickness muscle defects and induces spontaneous fibrosis is described in **chapter 5**. By loading non cross-linked collagen scaffolds with SDF-1α, an attempt was made to improve muscle regeneration. The results showed that in this model a significant amount of fibrotic tissue was formed. The implantation of SDF-1α-loaded collagen scaffolds induced migration of $Pax7^+$ satellite cells towards the regenerative zone around the wounds within the first ten

days post-surgery. However, these cells did not enter the scaffold and the numbers of myofibroblasts and collagen deposition were not affected after 56 days. In conclusion, this spontaneous muscle fibrosis model can be used to test scaffold-based therapies. Loading scaffolds with SDF-1α induced satellite cell migration but did not reduce fibrosis.

In **chapter 6** the putative inhibition of fibrosis using decorin-loaded collagen scaffolds with or without SDF-1α in the spontaneous muscle fibrosis model is described. *In vitro* studies showed that the decorin-loaded collagen scaffolds induced a short-term release of decorin within the first 3 days. *In vivo*, the SDF-1α and/or decorin-loaded collagen scaffolds did not affect the numbers of myofibroblasts, activated fibroblasts, satellite cells, and fused myoblasts at 56 days post-surgery. Moreover, fibrosis was not reduced. It is concluded that the release window of decorin was probably too short to prevent fibrosis.

In **chapter 7**, the myogenic potential of muscle stem cells is studied in 2D- and 3D-cultures with collagen type I and Matrigel. The latter contains satellite cell niche factors. In the 2D-cultures, higher numbers of proliferating $Pax7^+$ and $MyoD^+$ cells were found on Matrigel than on collagen. In addition, differentiating muscle stem cells formed more and larger $MyoD^+$ and $Myogenin^+$ myotubes on Matrigel. In the 3D-cultures, myofibers were also longer in Matrigel, but short and rounded in collagen. MyoD and Myogenin mRNA levels were also higher in muscle stem cells cultured in Matrigel. It was concluded that muscle stem cells, both in 2D and 3D, lose their differentiation capacity in collagen but not in Matrigel, which might be caused by the presence of niche factors.

Because differences were described for head and limb muscles, the myogenic potential of these muscle progenitor cells is compared in **chapter 8**. The muscle progenitor cells derived from head and limb muscles showed equal proliferation capabilities *in vitro*. During differentiation, head and limb muscle progenitor cells formed equal numbers of fused myotubes and showed comparable mRNA expression levels of several Myh-isoforms. The number of $Pax7^+$, $MyoD^+$, and $Myogenin^+$ cells in head and limb muscle progenitor cells also did not differ during proliferation and differentiation. Thus, head and limb muscle progenitor cells show similar myogenic capacities *in vitro*. The

reported differences must therefore be due to the different micro-environments of the muscles.

In **chapter 9**, the results of the previous chapters are discussed in the wider perspective of skeletal muscle engineering. Suggestions for future research are; the further development of smart scaffolds that induce the migration and attachment of satellite cells. The alignment of regenerating myofibers should also be stimulated in the scaffolds. Furthermore, *in vitro* culture conditions need to be optimized to maintain the stem cell status of satellite cells, and to generate aligned functional muscle tissue.

Chapter 11

Samenvatting

In **hoofdstuk 1** wordt de achtergrond en het belang van de studie beschreven. De verschillende strategieën voor de tissue engineering van skeletspieren wordt geschetst, waarna een overzicht van alle studies wordt gegeven. Het doel van deze studie is het verbeteren van de spierregeneratie en het verminderen van littekenweefsel door het implanteren van driedimensionale constructen.

In de **hoofdstukken 2 en 3** wordt een overzicht van de biologische aspecten van de ontwikkeling en regeneratie van skeletspieren gegeven. De focus ligt hierbij op de satellietcellen, de stamcellen van spieren, en op de factoren die hun activiteit reguleren. Satellietcellen migreren naar de wond, waar ze zich vermenigvuldigen en spiervezels vormen voor de spierregeneratie. De specifieke micro-omgeving van de satellietcellen, de niche, en de groeifactoren die vrijkomen tijdens de spierregeneratie, reguleren de activiteit van deze cellen. De vorming van littekenweefsel tijdens de spierregeneratie leidt vaak tot onvolledige spierfunctie. Het aanbrengen van groeifactoren, cellen, of constructen zijn drie verschillende methodes om de spierregeneratie te bevorderen en de vorming van littekenweefsel te remmen. Voor grote spierdefecten is voornamelijk de implantatie van constructen zinvol en dit is dus de focus van de beschreven studies.

In **hoofdstuk 4** wordt een *in vivo* model voor spierregeneratie ontwikkeld dat het litteken na spierscheuring nabootst. De resultaten tonen aan dat de activiteit van de satellietcellen, op basis van Pax7 en MyoD expressie, niet veranderd na de implantatie van gecrosslinkte collageenconstructen in een doorgesneden M. soleus. Echter, deze cellen zijn afwezig binnenin de constructen en de spierregeneratie is incompleet. Geconcludeerd wordt dat een spierscheuring met littekenweefsel kan worden nagebootst door een gecrosslinkte collageenconstruct in de doorgesneden M. soleus te implanteren. Dit model kan gebruikt worden om nieuwe behandelmethoden voor spierscheuringen te ontwikkelen.

In **hoofdstuk 5** wordt een nieuw wondmodel ontwikkeld dat spontaan littekenweefsel vormt door het maken van een groot spierdefect. De resultaten geven aan dat in deze spierdefecten grote hoeveelheden littekenweefsel worden gevormd. Implantatie van ongecrosslinkte

collageenconstructen met SDF-1α induceert de migratie van $Pax7^+$ satellietcellen naar het regeneratieve gebied rondom het defect in de eerste 10 dagen tijdens de spierwondgenezing. Echter, de satellietcellen migreren niet de constructen in. Verder is het aantal myofibroblasten en de hoeveelheid littekenweefsel na 56 dagen niet veranderd. Dit wondmodel induceert dus littekenweefsel en kan gebruikt worden om nieuwe constructen te testen om de spierwondgenezing te verbeteren. Ongecrosslinkte collageenconstructen met SDF-1α induceren de migratie van satellietcellen, maar verminderen niet de vorming van littekenweefsel.

In **hoofdstuk 6** wordt het effect van ongecrosslinkte collageenconstructen met decorine en met of zonder SDF-1α op de spierwondgenezing beschreven. Uit *in vitro* studies blijkt dat decorine binnen 3 dagen vrijkomt uit de ongecrosslinkte collageen constructen. Uit de *in vivo* studies blijkt vervolgens dat de ongecrosslinkte collageenconstructen met SDF-1α en/of decorin geen invloed hebben op het aantal myofibroblasten, geactiveerde fibroblasten, satellietcellen en gefuseerde myoblasten na 56 dagen. Bovendien is de vorming van littekenweefsel ook niet verminderd. Geconcludeerd wordt dat het tijdsbestek waarin decorine vrijkomt uit de ongecrosslinkte collageen constructen waarschijnlijk te kort is om de vorming littekenweefsel te voorkomen.

In **hoofdstuk 7** wordt het effect van collageen type I en Matrigel, dat satellietcel nichefactoren bezit, op de functie van satellietcellen in 2D- en 3D-kweeksystemen onderzocht. De resultaten tonen aan dat tijdens de proliferatie het aantal $Pax7^+$ en $MyoD^+$ cellen hoger is met Matrigel dan met collageen type I. Verder worden er meer en grotere $MyoD^+$ en $Myogenin^+$ spiervezels gevormd met Matrigel. In het 3D-kweeksysteem zijn de gevormde spiervezels ook langgerekt in Matrigel, terwijl de spiervezels kort en rond zijn in collageen type I. De mRNA expressieniveaus van MyoD en Myogenin zijn ook hoger in de spierstamcellen gekweekt in Matrigel. De spierstamcellen verliezen dus het vermogen om te differentiëren in de 2D- en 3D-kweeksystemen met collageen type I, maar niet met Matrigel.

In **hoofdstuk 8** wordt het differentiatievermogen van satellietcellen die verkregen zijn van spieren uit het hoofd of het onderbeen met elkaar vergeleken. Beide soorten spierstamcellen hebben dezelfde capaciteit om te prolifereren. Differentiatie van deze spierstamcellen leidt tot dezelfde aantallen spiervezels en vergelijkbare mRNA expressie niveaus van verschillende Myh-genen. Tijdens de proliferatie en differentiatie van de spierstamcellen, geïsoleerd uit beide type spieren, worden dezelfde aantallen $Pax7^+$, $MyoD^+$, en $Myogenin^+$ cellen gevonden. Spierstamcellen in de hoofd- en onderbeenspieren hebben dus dezelfde capaciteit om te prolifereren en differentiëren.

In **hoofdstuk 9** worden de resultaten van de vorige hoofdstukken in een breder perspectief van de tissue engineering van skeletspieren besproken. Verder worden er ook suggesties voor toekomstig onderzoek gegeven. Er moeten nieuwe constructen worden ontwikkeld die de migratie van satellietcellen en de uitlijning van de nieuwgevormde spiervezels binnen het construct induceren. Verder moeten de *in vitro* kweeksystemen worden geoptimaliseerd zodat de functie van satellietcellen behouden blijft en dat er parallelle functionele spiervezels gevormd worden in de spierconstructen.

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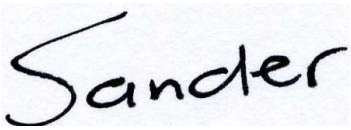

Curriculum vitae

Sander Grefte werd op 11 december 1980 geboren te Hengelo (O). In 1999 haalde hij zijn HAVO diploma en begon hij met de studie Medische Biochemie aan de Saxion Hogeschool Enschede. Na het behalen van zijn diploma in 2003 startte hij met de studie Biomedische Wetenschappen aan de Radboud Universiteit Nijmegen. Tijdens deze studie deed hij zijn onderzoeksstages op de afdeling Biochemie onder begeleiding van Dr. W. Koopman en bij de afdeling Tumor Immunologie onder leiding van Dr. R. Torensma. Dit onderzoek resulteerde in 3 publicaties. In 2006 behaalde hij zijn Master of Science diploma met Pathobiologie als afstudeerrichting. In april 2007 begon hij zijn promotieonderzoek "Improving the regeneration of injured muscle" op de afdeling Orthodontie en Craniofaciale Biologie (hoofd Prof. A.M. Kuijpers-Jagtman). De resultaten van dit onderzoek staan beschreven in dit proefschrift. In april 2011 werd gestart met vervolgonderzoek op de afdeling Orthodontie en Craniofaciale Biologie. In augustus 2011 heeft hij een bezoek gebracht aan de Randall Division and Molecular Biophysics van het King's College in Londen onder de supervisie van Dr. P. Zammit. Hij heeft hiervoor een subsidie gekregen van de European Molecular Biology Organization en van de Koninklijke Nederlandse Akademie van Wetenschappen (Ter Meulen Fonds).

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De uitgave van dit proefschrift is mede mogelijk gemaakt door