Essential environmental cues from the satellite cell niche unraveled

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Essential environmental cues from the satellite cell niche unraveled

Proefschrift

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Kristel Johanna Maria Boonen

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Dit proefschrift is goedgekeurd door de promotoren:

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Summary

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Tissue engineering of skeletal muscle can be used for numerous purposes. The most obvious purposes lie in the field of regenerative medicine: treatment of muscular dystrophies or reconstruction surgery after trauma. In addition, tissue engineered skeletal muscle tissue can be used as a model system to test new drugs or as a model for pressure ulcer research or muscle physiology. Less obvious is its use in the field of consumption as a meat replacement. Contemporary meat production is a heavy burden for the environment, because of an increasing demand of meat. It results in inefficient use of land and water, high emission of greenhouse gasses and risk of spreading of infectious diseases. On top of this, animals often live pitiful lives in bioindustry. Through large scale, industrial production of tissue engineered meat, some of these problems could be diminished.

To accomplish this kind of meat production, a number of requirements need to be met. First of all, a cell source is needed that is able to undergo many population doublings, thus produce much progeny, which retains the capacity to differentiate into skeletal muscle. Second, these cells will need to be exposed to the right signals in a three dimensional (3D) environment in order to enable differentiation into mature skeletal muscle tissue. Skeletal muscle cells themselves cannot be used as a cell source, since these cells are built up of many fused cells and are post-mitotic. We therefore make use of the skeletal muscle's endogenous stem cell population: satellite cells. Satellite cells are responsible for the remarkable regenerative capacity of skeletal muscle tissue; they can repair and regenerate large defects after injury and can respond to changes in load leading to hypertrophy. Unfortunately, satellite cells seem to lose much of their stem cell capacities when cultured *in vitro,* mostly resulting in a loss of proliferative ability caused by early differentiation. We hypothesized this phenomenon to be caused by loss of the specific environment that these cell usually find themselves in: the niche.

Several niche factors can play a role in the satellite cell functioning: growth factors, neighboring cells, extracellular matrix (ECM) proteins, electrical signals from nerves, stretch caused by movement and growth and the elasticity of the environment. In this thesis we investigated the effects of several of these niche factors separately or combined on the proliferation and differentiation capacity of murine satellite cells.

We have shown that the choice of ECM protein coating is crucial for all aspects of satellite cell functioning (proliferation, differentiation and maturation). We found maturation (characterized by the presence of cross-striations and spontaneous contractions) to be best on laminin-coated substrates. This seems logical, since the laminin network is the first part of the basement membrane connected to the satellite cells. The elasticity of the matrix influenced both proliferation and maturation of the cells. Proliferation was found to be highest on substrates with an elasticity close to *in vivo* elasticity of skeletal muscle and classic culturing substrates. For maturation into cross-striated myotubes, it was essential that the elasticity of the substrate was higher than a certain threshold value. Concerning electrical stimulation, we observed an advance in maturation, demonstrated by earlier presence of cross-striations and an upregulation in skeletal muscle differentiation markers. Moreover, electrical stimulation caused a switch in myosin isotype, establishing the possibility to tune the type of skeletal muscle tissue formed (fast or slow type) by electrical stimulation. In contrast, the stretching regime we used had negative effects on muscle maturation, demonstrated by a delay in the development of cross-striations and a downregulation of skeletal muscle differentiation markers. In addition, culturing in different systems has taught us that mere culturing in a 3D environment is much more beneficial for maturation than 2D culturing systems.

In conclusion, we have shown that several niche factors play an important role in satellite cell functioning. The results presented in this thesis have important implications for the development of a culturing system for tissue engineered meat.

Chapter 1

General introduction

1.1 Skeletal muscle

Skeletal muscle is the largest metabolic organ and the main storage site of proteins in the body. Most muscles are attached to bones with tendons and all of them receive input from the nervous system. They are responsible for voluntary movement, posture and balance, but are also involved in the protection of organs. In addition to its physiological importance in most multicellular animals, muscle (meat) serves as a major source of dietary protein in the western world.

1.1.1 Skeletal muscle anatomy

In the body, whole muscles are surrounded by a thick connective tissue sheet, called the epimysium. Each muscle is made up of bundles of fibers (fascicles) surrounded by a thinner connective tissue sheet, called the perimysium. Each single fiber (muscle cell) is encircled by a basement membrane. Inside a muscle cell, the cytoplasm is mostly made up of bundles of uniformly organized myofibrils (Figure 1.1). These myofibrils are the contractile elements of the cells and consist of repeating units of sarcomeres (composed mainly of actin and myosin), giving skeletal muscle its striated appearance (Cooper 2000).

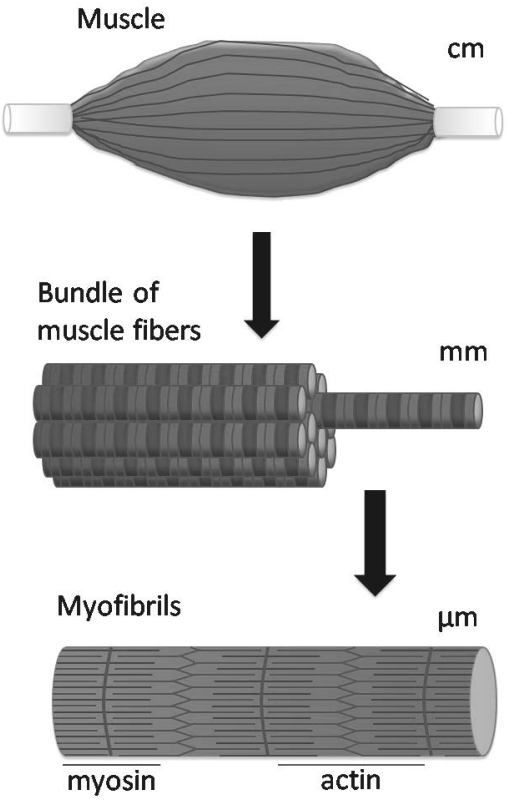

Figure 1.1: Organization of skeletal muscle.

Muscle fibers are created during development by fusion of several myoblasts (muscle progenitor cells), forming multinucleated fibers. These fibers are post-mitotic, hence have lost their ability to proliferate (divide) and create new fibers. Therefore, quiescent muscle stem cells (satellite cells) continue to populate adult skeletal muscle. They can be activated to proliferate and differentiate into new skeletal muscle when called for. These satellite cells reside in between the sarcolemma (cell membrane) and the basement membrane (Partridge 2004).

1.1.2 Skeletal muscle physiology

The main function of skeletal muscle is contraction. Contractions are produced by the movement of actin along myosin, causing the muscle fiber to decrease in length and thereby contract. This contraction is triggered by neurons that communicate with the muscle fiber at the motor end plate (a special part of the sarcolemma) by use of the neurotransmitter acetylcholine. One neuron can connect to multiple fibers, but each fiber is only connected to one neuron. Arrival of an action potential (signal) at the synaptic end of a motoneuron releases acetylcholine, which causes depolarization of the membrane of the muscle fiber. This depolarization elicits the release of calcium from the sarcoplasmic reticulum. Calcium then binds to troponin C, which is bound to actin, causing a conformational change in tropomyosin, which leads to myosin being able to bind to actin. Myosin shortens the myofibril by binding to and releasing actin and in this way trigger contraction, a process which requires energy in the form of adenosine triphosphate (ATP). Rapid energy sources for explosive muscle activity are stored in the muscle in the form of creatine phosphate, glycogen and fat (Cooper 2000).

1.2 Tissue engineering/*In vitro* meat production

Contemporary large-scale farming and transportation of livestock bring along a high risk of infectious animal diseases, environmental burden through greenhouse gas emission (Stamp Dawkins and Bonney 2008) and a certain degree of animal suffering. A new approach to produce meat and thereby reduce these disadvantages is found in tissue engineering of skeletal muscle (Van Eelen et al. 1999; Edelman et al. 2005). In tissue engineering approaches, a high number of cells is cultured in a three-dimensional (3D) carrier material and provided with biophysical and biochemical cues to form the desired tissue. The review that can be found in chapter 2 discusses the requirements that need to be met to increase the feasibility of meat production *in vitro.* These requirements include finding an appropriate stem cell source and being able to grow them in a 3D environment inside a bioreactor, providing essential cues for proliferation and differentiation. In addition, tissue engineered skeletal muscle can be used in the field of regenerative medicine, for the treatment of muscular defects and dystrophies.

1.3 Stem cells

To enable skeletal muscle tissue engineering, an appropriate cell source is necessary that is able to sustain proliferation to produce large numbers of cells, but retains the ability to differentiate into skeletal muscle when appropriately stimulated. As adult skeletal muscle is post-mitotic, we set out to find a precursor cell source in adult pig and mice muscle and induce myogenesis in these cells. To this end, several multipotent muscle progenitor cell populations were isolated from muscle biopsies using different methods. In order to select the most promising cell type, proliferation capacity was tested and differentiation towards myotubes was evaluated.

1.3.1 Muscle derived stem cells

Muscle derived stem cells (MDSCs) were first described by the group of Huard (Qu-Petersen et al. 2002). These cells show avid proliferation and differentiation into multiple lineages, making them a promising candidate for muscle tissue engineering. MDSCs are isolated and selected by preplating. Thus, the specific adhesion avidity of MDSCs to collagen is used as a selection criterion.

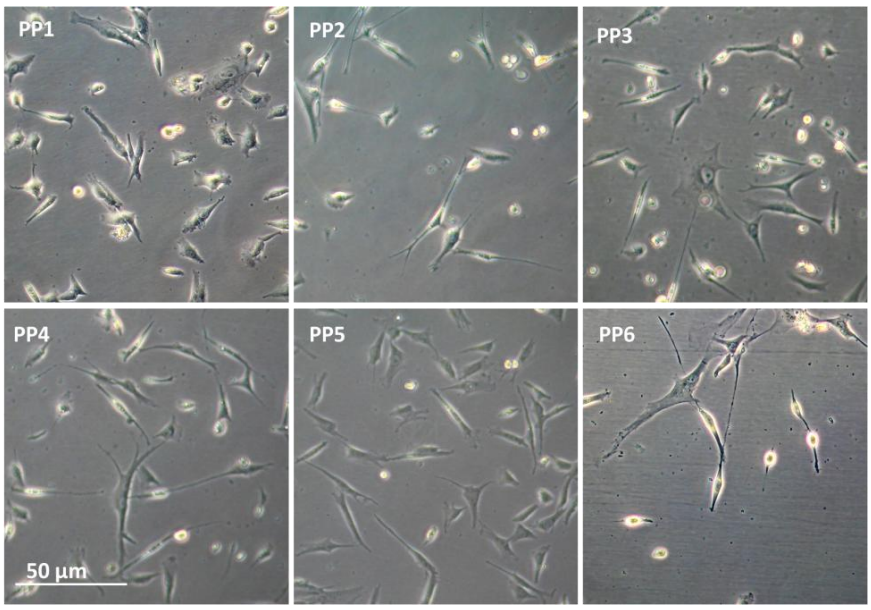

Figure 1.2: Pig muscle derived stem cells: Preplates (PP) 1 to 6 during culture (passage 0).

Using the described methods (Qu-Petersen et al. 2002), we were unable to reproduce their results. We performed several trials to isolate these cells from pig and mouse muscle. In our first trial, a pig muscle biopsy was coarsely cut and enzymatically digested (using collagenase type 1, proteinase K and trypsin). Afterwards, the slurry was sheared

through needles in order to generate a suspension containing all cells available in muscle. This suspension was plated out (preplate (PP) 1) and floating cells were replated after 2 hours (PP2). Consecutively, every 24 hours, floating cells were removed and replated (PP3, PP4 and PP5). After 96 hours, PP6 was left for another 72 hours and should then contain MDSCs, albeit very few. Cells from each preplate (PP) were isolated (Figure 1.2) and readily proliferated for more than 20 passages, but we were unable to induce differentiation (fusion) by serum reduction and insulin addition (Figure 1.3). The medium switches did terminate proliferation.

We also tried to isolate MDSCs from mice in a similar fashion, but were unable to sustain long term culture (Figure 1.4).

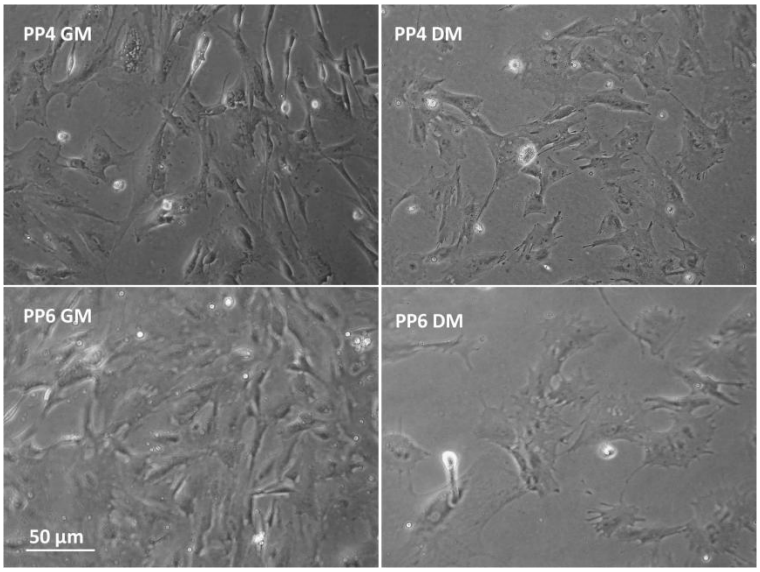

Figure 1.3: Pig muscle derived stem cells: Preplates 4 and 6 during culture in growth medium (GM) and differentiation medium (DM).

1.3.2 Satellite cells

Satellite cells are responsible for regeneration of muscle under physiological circumstances (section 1.1.1). Single fibers containing satellite cells can be isolated from mice by digesting whole muscles in collagenase type I, where after the muscle is repeatedly triturated though increasingly narrow pipettes, releasing single fibers. In an elegant study by Collins et al., (2005) in mice, it was shown that transplantation of single fibers gave much more repair in a disease model than enzymatically isolated satellite cells. In addition, culturing of satellite cells was shown to be deleterious for their regenerative potential.

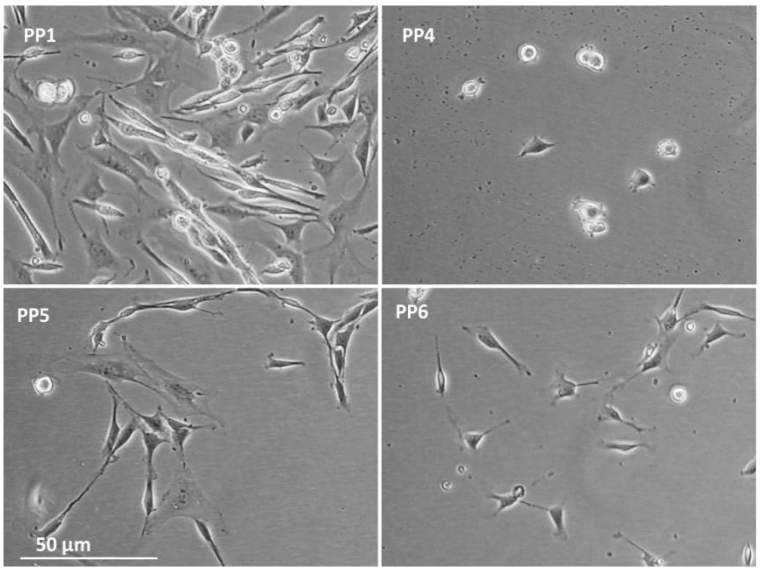

Figure 1.4: Mouse muscle derived stem cells: Preplates (PP) 1, 4, 5 and 6.

Single fibers can be plated directly on a 1 mg/ml Matrigel™ coating, after which satellite cells migrate out (Figure 1.5 panel A) or the fibers can be sheared through needles, liberating the satellite cells, which can then also be plated on 1 mg/ml Matrigel™ coated substrates. When they are proliferative, we call these cells muscle precursor cells (MPCs). These cells can be passaged for a maximum of 3-4 times (Figure 1.5, panel B), keeping them <30% confluent. However, these cells will easily differentiate, forming spontaneously contracting myotubes (Figure 1.5, panel C). We therefore decided to use these murine cells for subsequent experiments.

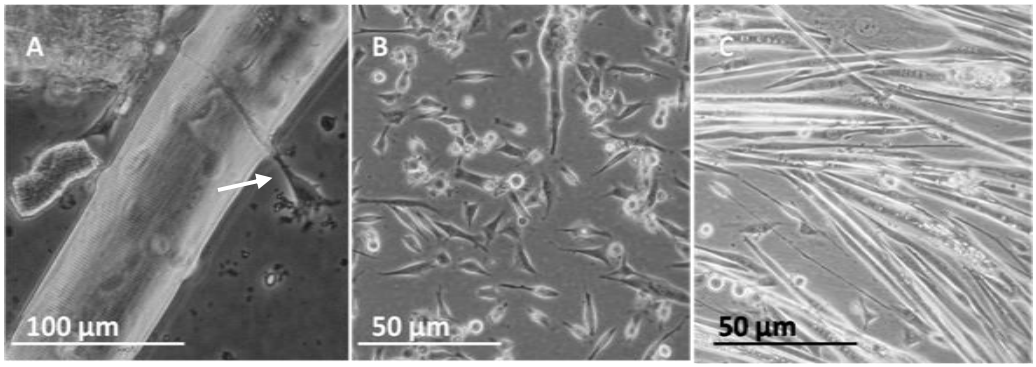

Figure 1.5: Single fiber derived murine satellite cells: isolation (A), proliferation (B) and differentiation (C).

1.4 Satellite cell niche

Satellite cells are considered to be adult skeletal muscle stem cells (Seale and Rudnicki 2000; Partridge 2004). Their ability to regenerate large muscle defects is highly dependent on their specific niche. When these cells are cultured *in vitro,* the loss of this niche leads to a loss of proliferative capacity and defective regeneration when implanted back into a muscle defect (Collins et al. 2005; Montarras et al. 2005). In chapter 3, we have reviewed the most important aspects of the niche, in particular the basement membrane, the niche's mechanical properties, its supporting cells and the influence these features have on satellite cell activation, proliferation and differentiation. Better understanding the role of the niche in these satellite cell activities will facilitate their recruitment and effective deployment for meat production and regenerative medicine.

1.5 Rationale and outline

Before satellite cells or muscle progenitor cells (MPCs) can be considered for tissue engineering purposes, a number of criteria need to be met (reviewed in chapter 2). First of all, cells need to be able to proliferate extensively, if not indefinitely, while retaining the capacity to differentiate into skeletal muscle. Furthermore, maturation into mature 3D skeletal muscle that contains characteristic cross-striations and exhibits contractions is necessary for its use in meat production or regenerative medicine.

In the present thesis, we set out to unravel the contribution of different satellite cell niche factors on the proliferation, differentiation and maturation of MPCs. Chapter 2 contains a review that deals with the system requirements to use skeletal muscle tissue engineering for *in vitro* meat production. In chapter 3, we reviewed the most important components of the satellite cell niche. We then investigated the effects of several of these components (substrate stiffness and extracellular matrix (ECM) protein coating) on the proliferative and differentiative behavior of MPCs. The results of this study can be found in chapter 4. Afterwards, we added electrical stimulation to this system of substrate stiffness and ECM protein coating and focused on the maturation of MPCs into myofibers (Chapter 5). This system was then translated to a 3D situation, in which a Matrigel^{TM}/collagen gel was used to produce bioartificial muscles (BAMs) that were electrically stimulated. MPCs were compared to C2C12 myoblasts to investigate the difference between a cell line and primary cells. The results of this study can be found in chapter 6. A different 3D system using a fibrin gel was later used for stretch experiments. Chapter 7 presents the results of the comparison between a 2D and 3D situation and C2C12 versus primary myoblasts in their response to stretch. Finally, the most important findings of this thesis are discussed in chapter 8, followed by their implications for future research.

Chapter 2

Meet the new meat: Tissue engineered skeletal muscle

This chapter is based on: Marloes L.P. Langelaan, Kristel J.M. Boonen, Roderick B. Polak, Frank P.T Baaijens, Mark J. Post, Daisy W.J. van der Schaft, *Meet the new meat: Tissue engineered skeletal muscle.*

2.1 Introduction

The demand for meat continues to grow worldwide. With this growing demand, the increasing production of meat leads to environmental problems as well as animal suffering. We propose *in vitro* meat production using stem cells as an appealing alternative for general meat production through livestock. Reasons for promoting *in vitro* meat production include animal well fare, process monitoring, environmental considerations as well as efficiency of food production in terms of feedstock. *In vitro* meat production through stem cell technology potentially leads to a dramatic reduction in livestock. In addition, the production process can be monitored in detail in a laboratory, which could result in the elimination of food borne illnesses, such as mad cow disease or salmonella infection. Furthermore, less livestock could lead to a decrease in intense land usage and greenhouse gas emissions (Stamp Dawkins and Bonney 2008).

The idea of culturing muscle tissue in a lab *ex vivo* already originates from the early nineteen hundreds. From 1912 until 1944, Alexis Carrel, surgeon and Nobel Prize laureate, managed to keep a piece of chick heart muscle alive and beating in a Petri dish, feeding it every other day. This experiment demonstrated that it was possible for muscle tissue to stay alive outside the body, provided that it was nourished with suitable nutrients.

This phenomenon of keeping muscle tissue alive *ex vivo* inspired many great thinkers and writers to reflect on futuristic perspectives of how meat would be produced in the future. Among them, Winston Churchill predicted that it would be possible to grow chicken breasts and wings more efficiently without having to keep an actual chicken (Churchill 1932). Although he predicted that it could be achieved within 50 years, his concept was not far off from reality today.

Some efforts have already been put into culturing artificial meat. SymbioticA, a laboratory at the University of West-Australia, where artists and scientists work together, dedicated an arts project to this subject. They harvested muscle biopsies from frogs and kept these tissues alive in culture dishes. The frog meat even grew slightly and at the exposition, it was shown next to the frogs that were still alive (Catts and Zurr 2002). In a NASA project exploring the possibilities to grow meat on space travels, gold fish muscle was studied. Similar to the SymbioticA project, biopsies were taken and using medium with bovine serum, tissue survived and even grew 14 percent. They also achieved keeping the tissues alive in a fungal medium, anticipating on the infection risk associated with serum-based media (Benjaminson et al. 2002). Although their results seemed promising, they did not observe tissue growth in the serum-free situation.

Obviously, these small biopsies will not be practical for large-scale meat production. Therefore, we propose to use tissue engineering as a technique to produce *in vitro* cultured meat. Tissue engineering is a powerful technique that is mainly being used for

regenerative medicine in a wide variety of tissues and organs (Bach et al. 2003; Mol et al. 2005). For additional research purposes, tissue engineering has also been employed to develop *in vitro* models (Vandenburgh et al. 2008). In particular, tissue engineering of skeletal muscle has many applications, ranging from *in vitro* model systems for drug-screening (Vandenburgh et al. 2008), pressure sores (Gawlitta et al. 2007) and physiology to *in vivo* transplantation to treat muscular dystrophy and muscular defects (Boldrin et al. 2008) (Figure 2.1) . Obviously, tissue engineering could also be employed to produce meat (van Eelen et al. 1999; Edelman et al. 2005).

For tissue engineering to be used for meat production, a number of demands need to be met. First of all, a cell source is required that can proliferate indefinitely, but can also differentiate into functional skeletal muscle tissue. Furthermore, these cells need to be embedded in a three dimensional (3D) matrix that allows for muscle growth, while keeping the delivery of nutrients and release of waste products undisturbed. Lastly, muscle cells need to be conditioned adequately in a bioreactor environment in order to get mature, functional muscle fibers.

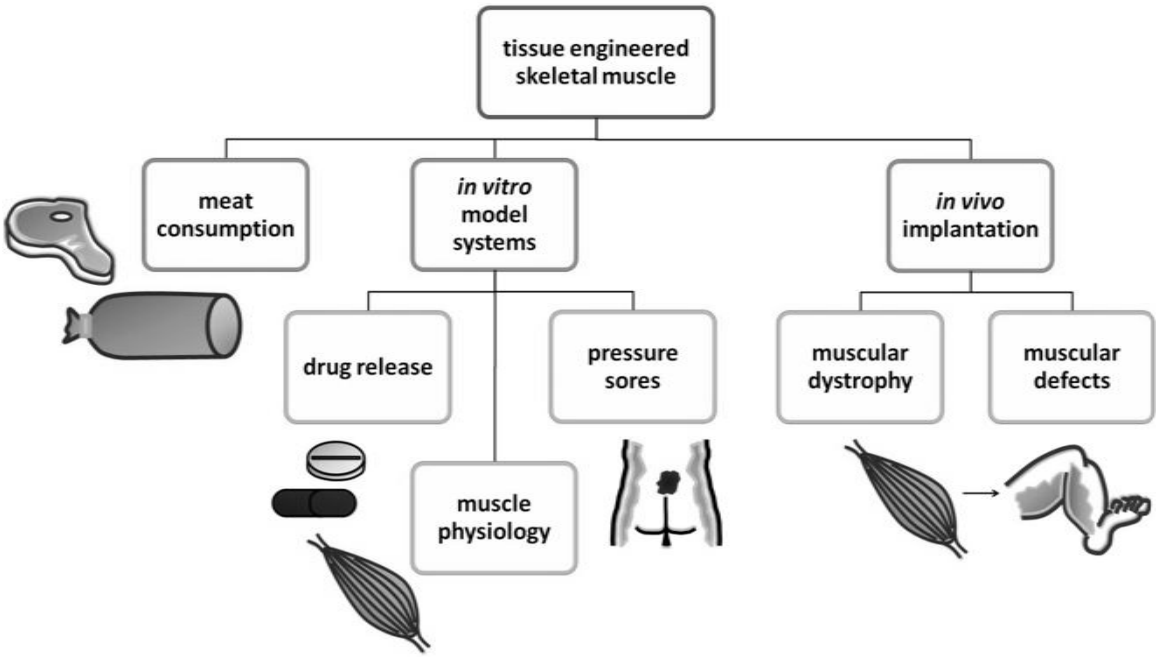

Figure 2.1: Applications for tissue engineered skeletal muscle, ranging from regenerative medicine purposes, being implantation and the development of a model system for different pathologies, to in vitro *cultured meat for consumption.*

2.2 Cell sources for tissue engineered meat

2.2.1 Stem cells for muscle tissue engineering

For tissue engineering of skeletal muscle, it is essential to choose the right cell source. Ideally, the cells should be easily accessible and able to proliferate indefinitely. Stem cells are considered the most promising cell source, since in theory, these cells can divide indefinitely while retaining the capacity to differentiate into the required phenotype. Different types of stem cells of embryonic and adult origin exist. We hypothesize that satellite cells, which are the natural muscle stem cells responsible for regeneration, are the best candidate for tissue engineering of skeletal muscle and consequently for *in vitro* meat production. However, other sources of stem cells are still under evaluation.

For instance, embryonic stem cells may also be a potential cell source for *in vitro* meat production. These stem cells are derived from the inner cell mass of an early stage embryo. Pluripotent embryonic stem cells show unlimited self-renewal and can differentiate into almost any desired cell type. For embryonic stem cells to become muscle fibers, the cells first need to differentiate into myogenic progenitor cells (MPCs). One of the major challenges when using embryonic stem cells is to direct differentiation into MPCs while avoiding development of other lineages. Only by transfection with the transcription factor MyoD for instance, does the percentage of differentiation into skeletal muscle increase to greater than 90% (Dinsmore et al. 1996). Interestingly, it seems to be more difficult to induce myogenesis in embryonic stem cells *in vitro* than *in vivo*; myogenic precursor progeny from human embryonic stem cells readily form myofibers and satellite cells when transplanted *in vivo* in mice after muscle damage. *In vitro* formation of myofibers from the same cells, however, has proven challenging (Zheng et al. 2006). Apparently, some important *in vivo* niche components are still missing in the *in vitro* system. Additional concerns with embryonic stem cells for replacement therapy include the risk of uncontrolled proliferation and differentiation leading to teratoma formation. In addition, there are ethical concerns about the use of this cell source.

In addition to embryonic stem cells, adult stem cells could be a potential source for muscle tissue engineering. Different types of adult muscle stem cells have been isolated from skeletal muscle: muscle derived stem cells (MDSCs) (Peng and Huard 2004), side population (SP) cells (Asakura et al. 2002; Tamaki et al. 2003) and satellite cells (SCs) (Asakura et al. 2001; Zammit et al. 2004). Satellite cells are resident muscle stem cells responsible for regeneration and repair in the adult and are already programmed to differentiate into skeletal muscle. Therefore, these cells are an appealing source for muscle tissue engineering. Activated satellite cells differentiate to MPCs, which then proliferate and migrate in order to repair defects. The function of the other types of muscle derived adult stem cells as well as bone marrow derived stem cells (Gussoni et

al. 1999; Gang et al. 2004) in physiological circumstances remains unclear. However, adult stem cells derived from either the muscle or the bone marrow, including hematopoietic and mesenchymal stem cells, appear to have conserved the capacity to differentiate into skeletal muscle and therefore remain potential candidates for muscle regeneration (Gussoni et al. 1999; Gang et al. 2004).

Unfortunately, at present, the proliferative capacity of adult stem cells does not match that of embryonic stem cells, mostly because they tend to differentiate spontaneously *in vitro*. It is anticipated that this issue will be tackled by optimizing the culture conditions, for example by mimicking the *in vivo* environment (niche) of the cells (Boonen and Post 2008). An advantage of using adult stem cells over embryonic stem cells is that pure adult stem cell populations, when stimulated to differentiate into skeletal muscle, will give rise to a homogeneous tissue.

2.2.2 Co-culturing

Once stem cells are differentiated into myoblasts, these cells are specialized to produce contractile proteins, but produce only little extracellular matrix. Therefore, other cells likely need to be introduced to engineer muscle, with a texture and taste that sufficiently resembles meat. Extracellular matrix is mainly produced by fibroblasts residing in the muscle, so it could be beneficial to add fibroblasts to the culture system (Brady et al. 2008). However, co-cultures of fibroblasts and myoblasts involve the risk of fibroblasts overgrowing the myoblasts, due to the difference in growth rate. Next to fibroblasts, regular consumption meat also contains fat and a vasculature. Possibly, co-culture with fat cells should also be considered (Edelman et al. 2005). The problem of vascularization is a general issue in tissue engineering. Tissue engineering is currently at the level in which we can only produce thin tissues because of passive diffusion limitations. To overcome the tissue thickness limit of 100-200 µm, functional blood vessels or a functioning tubular network mimicking the vasculature therefore need to be created (Jain et al. 2005; Levenberg et al. 2005). Proof of concept for endothelial networks within engineered tissues has been provided (Levenberg et al. 2005), but reproducible and routine incorporation of vascular networks in a co-culture system will pose a special challenge.

2.3 Cell matrices

2.3.1 *In vivo* cell niche

In vivo, stem cells occupy a cell specific niche, which directs the cellular behavior and comprises soluble factors such as growth factors, insoluble factors including extracellular matrix proteins, physiological factors such as neurological stimulation, and mechanical

features such as dynamic stretch and matrix elasticity (reviewed by Boonen and Post (2008)). It was hypothesized that these niche components are essential to mimic the regenerative process *in vitro*, which is necessary to produce mature, functional muscle. Extracellular matrix components to which cells attach include fibronectin, collagen and laminin. Myoblasts binding to different matrix molecules leads to induction of different pathways (Maley et al. 1995; Macfelda et al. 2007). In addition, Grossi et al. (2007) demonstrated that mechanical stimulation of myoblasts through laminin receptors, but not through fibronectin receptors, increased differentiation. For cells to be grown in a 3D structure, several of these niche factors should obviously be taken into account.

An important feature that has to be considered is the overall stiffness of the scaffold material. Engler et al. showed that it is possible to direct stem cell lineages by varying matrix stiffness, e.g. mesenchymal stem cells can be differentiated towards neuronal, muscular or osteogenic lineages when cultured on substrates with a stiffness of 0.1-1.0 kPa, 8-17 kPa and 25-40 kPa, respectively (Engler et al. 2006). Moreover, they found that the optimal substrate stiffness that gives rise to the characteristic striation of myosin/actin in C2C12 myoblasts is very delicate (Engler et al. 2004b). In addition, Boonen et al. showed that proliferation and differentiation of primary murine satellite cells were affected by the elasticity of the culture matrix. However, they found striations in cells cultured on all elasticities above a certain threshold elasticity (Boonen et al. 2009). Boontheekul et al. also showed that by varying matrix stiffness, gene expression was strongly regulated and the amount of adhesion, proliferation and differentiation of primary myoblasts differed significantly in cells cultured on different elasticities (Boontheekul et al. 2007). However, these results originate from 2D studies and still need translation to a 3D situation. One study using a PLLA/PLGA scaffold in different ratios indicates that the scaffold stiffness can be tailored in such a way that it directs myoblast differentiation and organization, but these elasticities are of a different order of magnitude compared to the 2D studies (Levy-Mishali et al. 2008). Additionally, in a 3D situation not only the stiffness seems important for cell behavior, but also cell forces and deformation of the scaffold will affect cell survival, organization and differentiation (Levy-Mishali et al. 2008).

2.3.2 Model systems for 3D tissue engineering

Potential 3D model systems, ideally incorporating the components of the *in vivo* cell niche, as mentioned above, need to meet certain requirements, as Bian and Bursac (Bian and Bursac 2008) reviewed. Broadly speaking, the fabrication of dense skeletal muscle tissue necessitates a uniform cell alignment and reproducible architecture. The options that are currently available for 3D muscle tissue engineering are illustrated in Figure 2.2. Bian and Bursac suggested the usage of biocompatible hydrogels as a promising approach for the design of engineered muscle to allow a spatially uniform and dense cell

entrapment (Bian and Bursac 2008). The advantage of using a gel system is that the stiffness is probably more comparable to the *in vivo* environment. In addition, the process of myotube alignment is relatively easy by the creation of intrinsic tension by compaction and active force generation by the cells. Gel systems that are currently employed for tissue engineering of skeletal muscle include fibrin gels of different concentrations, and a mixture of collagen and Matrigel™ (Bian and Bursac 2008; Gawlitta et al. 2008; Vandenburgh et al. 2008).

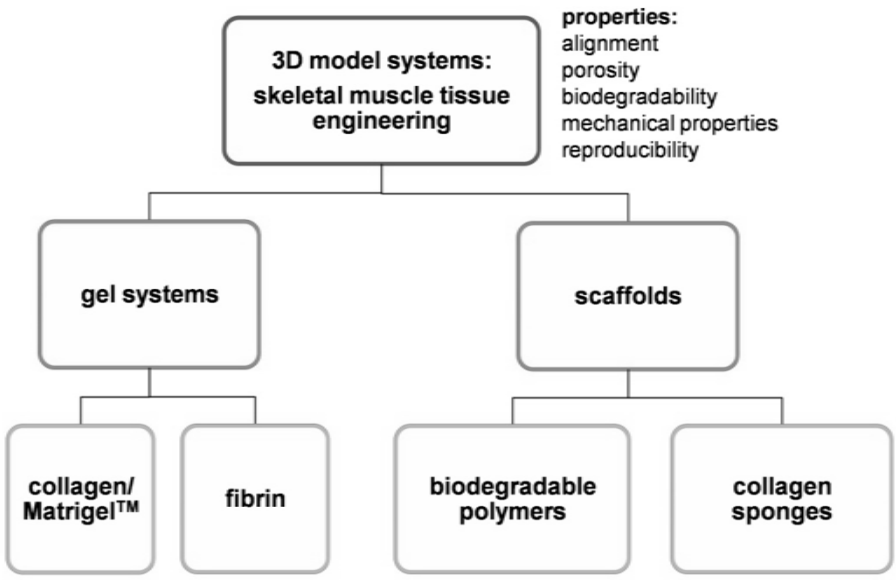

Figure 2.2: Examples of 3D model systems for skeletal muscle tissue engineering. Specific properties for optimal muscle development are listed. In addition, examples are given.

Scaffolds produced of synthetic biodegradable polymers are also a potential 3D model system that is suitable for *in vitro* cultured meat. Reproducibility and uniformity of scaffolds can be achieved by producing them with electrospinning techniques. However, this generally results in very dense structures, which are difficult for cells to enter and will preclude homogeneous cell distribution. A new approach to overcome this problem is the use of low temperature electrospinning (Simonet et al. 2007). With this technique one creates an open structure by the incorporation of water crystals between the electrospun polymers, which will be removed later in the process. These “cryospun” scaffolds can be produced from various polymers, e.g. poly-caprolactone (PCL), poly-lactic-acid (PLA), poly-glycolic-acid (PGA) (Boland et al. 2001) or combinations thereof, depending on the desired mechanical and degradation properties as well as cell attachment demands. Parameters such as fiber thickness and orientation can be adjusted and optimized in the electrospinning process in order to influence the

architecture and mechanical properties (Ayres et al. 2007). Orienting the fibers parallel in one direction is clearly beneficial in engineered muscle constructs, since this resembles the *in vivo* texture of a muscle, as was previously shown in a different scaffold system by Riboldi and colleagues (Riboldi et al. 2008). Control over the final scaffold architecture, for instance through electrospinning, also enables creation of tubular structures, which allow the delivery of oxygen and nutrients to large constructs.

When all parameters of the low temperature electrospinning process are optimized towards creating ideal scaffolds for tissue engineered muscle, this may result in very reproducible scaffolds with excellent material properties.

2.4 Conditioning

The creation of a native-like tissue architecture with the capacity of active force generation is crucial in the process towards tissue engineered muscle, and consequently also important for *in vitro* meat production. However, the advances made in culturing of engineered muscle constructs have not yet resulted in satisfactory products. An important hurdle that still has to be overcome is the inability of muscle cells to fully mature within these engineered muscle constructs. Although biochemical stimuli may be more important in the initial differentiation process, biophysical stimuli have proven to be crucial in the maturation towards functional tissue with native-like properties (Kosnik et al. 2003). Therefore, we hypothesize that for successful tissue engineering of skeletal muscle, the design of a bioreactor should also incorporate the ability to apply biophysical stimulation regimes that resemble the native *in vivo* environment regarding muscle regeneration. The effects of both biochemical and biophysical stimuli on muscle differentiation and maturation are summarized in Figure 2.3.

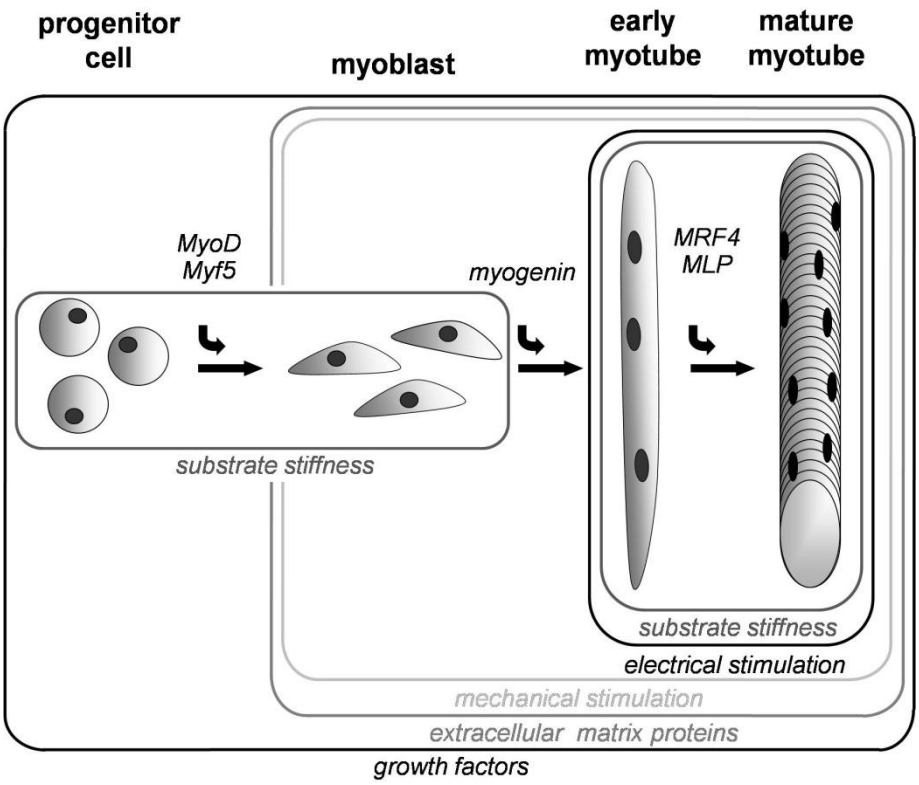

Figure 2.3: Factors affecting muscle cell proliferation, differentiation and maturation. Substrate stiffness is involved in both the proliferation of progenitor cells and the maturation of myotubes. Electrical stimulation results in enhanced maturation of myotubes, whereas mechanical stimulation is important for the alignment of myoblast and the maturation of myotubes. Extracellular matrix proteins and growth factors are involved in the overall process of differentiation and maturation of muscle progenitor cells towards mature myotubes.

2.4.1 Biochemical conditioning

Conventionally, application of a biochemical stimulus can induce the differentiation of muscle precursor cells. It is well known that for instance the C2C12 murine myoblast cell line differentiates into multinucleated myotubes by serum deprivation (Blau et al. 1985). In addition, growth factors have been identified that influence myoblast proliferation and differentiation to a great extent. In reaction to a certain stimulus muscle cells or cells in close proximity to the muscle cells, e.g. immune cells, can start producing growth factors. Alternatively, the stimulated cells can liberate growth factors from the extracellular matrix where they reside in an inactive form (Miura et al. 2006). Different members of the Transforming Growth Factor-β (TGF-β) superfamily, Fibroblast Growth Factors (FGFs) and Insulin-like Growth Factors (IGF) are crucial in this respect.

TGF-β, BMP4 and myostatin are members of the TGF-β superfamily and act as differentiation antagonists. TGF-β reduces myoblast recruitment and differentiation (Allen and Boxhorn 1987; Goetsch et al. 2003). During differentiation, TGF-β is bound by proteoglycans at increasing levels and stored in the extracellular matrix in an inactive form (Casar et al. 2004; Droguett et al. 2006). In addition, BMP4 in combination with functional Notch signaling blocks myogenic differentiation in C2C12 myoblasts and satellite cells (Droguett et al. 2006). Myostatin, which can be upregulated by TGF-β (Budasz-Rwiderska et al. 2005), inhibits muscle growth (Amthor et al. 2002) and satellite cell proliferation and differentiation (Wagner 2005a). FGFs are more stimulatory in their actions than TGF-β family members; FGFs 2, 4 and 6 increase myoblast proliferation *in vitro* and thereby inhibit differentiation (Hannon et al. 1996). Comparable to FGFs, a splice variant of IGF-1, called mechano growth factor (MGF) increases proliferation of myoblasts (Ates et al. 2007). In contrast, systemic IGF-1, which replaces MGF after an initial phase of activation, is more involved in accelerating differentiation in C2C12 myoblasts (Florini et al. 1996) and in inducing hypertrophy *in vitro* (Semsarian et al. 1999; Gawlitta et al. 2008).

2.4.2 Biophysical conditioning

Regarding the relatively poor development of sarcomeres *in vitro* (Engler et al. 2004b), indicated by a lack or limited level of maturation specific cross-striations, biochemical stimulation alone may not be sufficient in the maturation process towards fully functional engineered muscle constructs. When considering *in vivo* myogenesis and the niche in which satellite cells reside, it appears that in addition to biochemical stimuli, biophysical stimulation is required for full muscle maturation and function.

2.4.2.1 Electrical stimulation

Neuronal activity has proven to be pivotal in the development of mature muscle fibers (Wilson and Harris 1993). In *in vitro* cultures, electrical stimulation can mimic this nerve stimulation during myogenesis and regeneration of injured skeletal muscle as it occurs *in vivo* (Bach et al. 2004). As early as 1976, it was already described that repetitive electrical stimulation of chick embryo skeletal muscle cells *in vitro* resulted in increased rates of myosin synthesis (Brevet et al. 1976). More recent work has shown that induction of contractile activity promoted the differentiation of myotubes in culture. More precisely, chronic and long-term electrical stimulation of myoblasts affected myosin heavy chain expression of different isoforms and sarcomere development (Naumann and Pette 1994; Wehrle et al. 1994; Fujita et al. 2007). We also showed that early electrical stimulation affected maturation of myotubes with respect to sarcomere development in the C2C12 murine myoblast cell line (Langelaan et al., 2009). Within a relatively short differentiation period of 5 days, mature cross-striations developed in the

electrically stimulated cultures, whereas non-stimulated control cultures did not show these cross-striations. This effect was accompanied by upregulated expression levels of the muscle maturation inducer muscle LIM protein, and the sarcomere components perinatal myosin heavy chain, actin and α-actinin. In another study, Fujita et al. showed that short-term electrical stimulation applied at a later time point in the differentiation process of C2C12 also enhanced the development of striations and functionality of the myotubes (Fujita et al. 2007). This indicates that electrical stimulation leads to assembly of sarcomeres and therefore maturation of the myotubes.

Alternatively, electrical stimulation can provide a non-invasive, accurate tool to assess the functionality of the construct (Dennis and Dow 2007; Dennis et al. 2009). By generating a homogeneous electrical field inside the bioreactor, functional muscle constructs will exert a force due to active contractions of the muscle cells. So far, these forces generated by engineered muscle constructs only reach 2-8% of those generated by skeletal muscles of adult rodents (Dennis et al. 2001). Therefore, at this moment, functional properties of tissue engineered muscle constructs are still unsatisfactory.

2.4.2.2 Mechanical stimulation

Another important biophysical stimulus in myogenesis is mechanical stimulation (Vandenburgh and Karlisch 1989). Mechanotransduction, the process through which cells react to mechanical stimuli, is a complex, but increasingly understood mechanism (Hinz 2006; Burkholder 2007). Cells attach to the insoluble meshwork of extracellular matrix proteins mainly by means of the family of integrin receptors (Juliano and Haskill 1993). The force applied on these integrins is transmitted to the cytoskeleton consistent with the tensegrity model (Wang et al. 1993). The resulting series of events shows parallels to growth factor receptor signaling pathways, which ultimately lead to changes in cell behavior, such as proliferation and differentiation (Juliano and Haskill 1993; Burkholder 2007). An extensive overview of the role of integrins and cadherins in mechanotransduction and especially the intracellular signaling pathways involved can be found in a recent review by Schwartz and DeSimone (Schwartz and DeSimone 2008).

Other variables, such as the mechanical stimulation regime itself, also affect muscle growth and maturation. Vandenburgh and Karlisch, for example, showed that the application of static mechanical stretch to myoblasts *in vitro* resulted in a facilitated alignment and fusion of myotubes. Moreover, myofiber diameter increased and hypertrophy of the myotubes occurred (Vandenburgh et al. 1989). Cultured muscle constructs also remained more elastic by inhibition of collagen cross-linking as a result of the mechanical stretch (Powell et al. 2002). Positive effects on development and maturation of myotubes have also been noted by the application of cyclic strain. In addition, cyclic strain activates quiescent satellite cells (Tatsumi et al. 2001) and increases proliferation of myoblasts (Kook et al. 2008a). These results indicate that

mechanical stimulation protocols affect both proliferation and initial differentiation. The applied stimulation should be tuned very precisely to reach the desired effect. Percentage of applied stretch, frequency of the stimulus and timing in the differentiation process are all parameters that presumably influence the outcome of the given stimulus. As is the case for electrical stimulation, the *in vivo* situation during regeneration is an excellent starting point towards optimal mechanical stimulation protocols.

2.5 Discussion

2.5.1 Challenges in tissue engineering of meat

This review has dealt with the challenges of *in vitro* meat production. By taking the appropriate stem cells, proliferating them under the right conditions to reach sufficient numbers and providing them with the right stimulatory signals in a 3D environment, industrial meat production seems feasible (Figure 2.4). We described three major issues in skeletal muscle tissue engineering, being the proper cell source, the optimal 3D environment for cells to be cultured and differentiated in, and adequate conditioning protocols. Adult stem cells, i.e. satellite cells, seem a promising cell source. However, there still is room for improvement of the proliferative capacity as well as the differentiation protocol of these cells. Unfortunately, culturing of only muscle cells in a construct will not result in a tissue structure comparable to an *in vivo* muscle. Co-culturing with other cells, such as fibroblasts or adipocytes, is probably the solution to this problem.

The issue of the optimal matrix in which muscle precursor cells are cultured demands careful determination of the most favorable combination of biochemical and biophysical factors for the production of functional muscle tissue. Subsequently, the combination of stimuli must be incorporated into the design of a bioreactor.

The medical applications of *in vitro* cultured muscle tissue as an alternative for tissue replacement have been investigated extensively. Transplantations have been undertaken in several model systems. Serena and co-workers implanted collagen sponges seeded with primary myoblasts into damaged muscles of mice and reported survival of the grafts and even formation of new myotubes at the location of implantation (Serena et al. 2008). Comparable to this study, human muscle precursor cells (Boldrin et al. 2008) or mouse satellite cells (Boldrin and Morgan 2007) seeded in a polymeric scaffold were implanted into muscular defects in mice. These constructs survived and contributed to the regeneration of the host muscle. In addition, seeding C2C12 myoblasts in a collagen gel and subsequently transplanting this construct subcutaneously in nude mice resulted in survival, differentiation and even vascularization of the grafts (Okano and Matsuda 1998a). Furthermore, when constructs

were vascularized before implantation, further vessel formation and improved connection to the host vasculature was observed (Levenberg et al. 2005).

The examples described above, originating from the field of regenerative medicine, show that culturing of artificial meat is technologically feasible. Tissue engineering research has provided us with the possibility to culture tissues in a 3D environment while applying the right biochemical and biophysical stimuli to ensure stem cell differentiation and maturation.

The farm animal derived stem cells that are, in our view, required for the production of artificial meat as mentioned before, will be available over time. Adult stem cells, derived from skeletal muscle, have already been isolated from pigs (Wilschut et al. 2008). Until now, only embryonic stem cell lines originating from several model species and humans have been isolated and cultured successfully. Deriving a new embryonic stem cell line from livestock animals is a matter of time and continuous effort. After the discovery of murine embryonic stem cells in 1981 by Evans and Kaufman (Evans and Kaufman 1981), a breakthrough in human embryonic stem cell research came more than fifteen years later. At that time, Thomson and co-workers developed a technique to isolate and grow cells derived from human blastocysts (Thomson et al. 1998). This timeframe shows that, although it might take time to isolate and culture an embryonic stem cell line from a completely new origin, it is feasible.

For practical reasons, most research on skeletal muscle regeneration has been performed in mice (Fan et al. 1996b; Beauchamp et al. 1999). It remains questionable if these results can be translated to farm animals such as pigs and cows. For instance, it appears highly challenging to isolate and propagate embryonic stem cells of porcine or bovine origin (Keefer et al. 2007; Talbot and Blomberg 2008). Indeed, we study the scientific foundation of *in vitro* meat production also with satellite cells of murine origin, remaining aware that mouse meat will not appeal to projected consumers. In addition to the species differences, cell lines that are commonly used for the development of model systems may react differently from primary cells, even when they are derived from the same species (Maley et al. 1995; Boontheekul et al. 2007; Boonen and Post 2008).

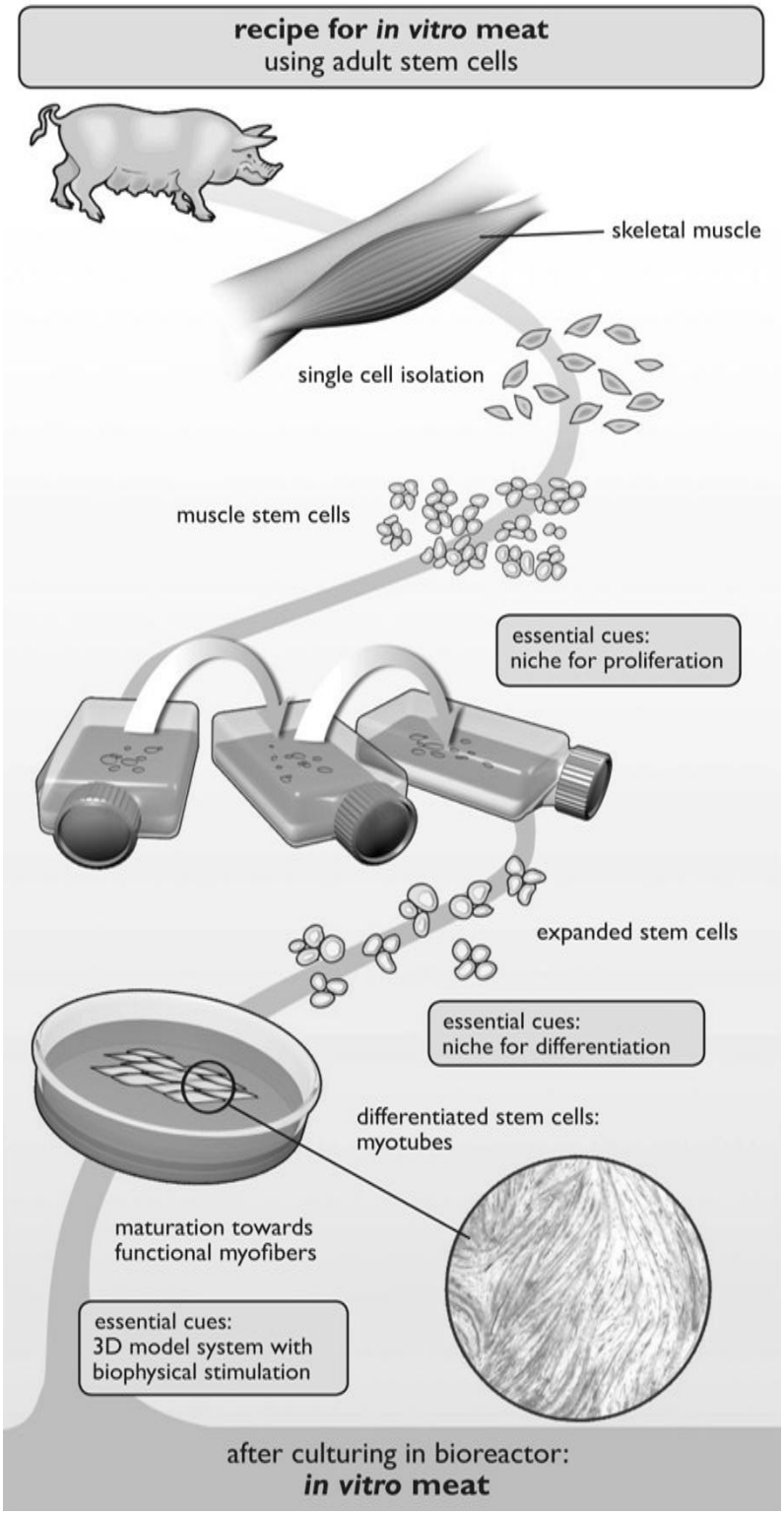

Figure 2.4: Recipe for in vitro *meat using adult stem cells. The essential cues indicate the challenges that have to be met in the distinctive processes (illustration Sebastiaan Donders).*

2.5.2 Future considerations

The next hurdle that has to be overcome in time is the size of the cultured meat. At the moment, skeletal muscle constructs of approximately 1.5 cm in length and 0.5 cm in width can be cultured (Gawlitta et al. 2008). These sizes of artificial meat can already be used as a supplement in sauces or pizzas, but the production of a steak, for example, demands for larger tissue sizes. Upscaling of the cell and tissue culturing processes is therefore necessary. In this process of upscaling, it should be guarded that no component other than the cell source itself originates from animal sources. Another aspect of up-scaling, automation of the processes, was already investigated by Kino-Oka et al. (2009) who developed an automated system to expand and passage myoblasts, while carefully monitoring the phenotype of the cells.

Since no further animal sources are wanted in the process of *in vitro* cultured meat, conventional culture medium, commonly supplemented with fetal bovine serum, has to be adjusted. For example, a cocktail of growth factors and other essential additives can be produced by bacteria or yeast cells (Halasz and Lasztity 1990) resulting in a defined culture medium. The technologies required for this quality controlled and reproducible production of additives are beyond the scope of this review. In addition, to enable the nutrients to penetrate larger tissues, blood vessels or a different kind of tubular system need to be incorporated. When all technological challenges regarding artificial meat production are overcome, the next step towards a successful substitute for authentic meat is product marketing. Introduction of artificially cultured meat is undoubtedly challenging, but potential negative connotations may be off-set by the impact of such a product on animal suffering, environment and world food supply. Therefore, the idea that people would eat meat originating from the lab does not seem so farfetched.

For artificial meat to compete with authentic meat, cost efficiency is very important. Considering the environmental burden, meat might even be more appealing by *in vitro* production. For example, livestock contribution to the emission of carbon dioxide, methane and particularly nitrous oxide is considerable (Koneswaran and Nierenberg 2008). In terms of CO_2 equivalents, the impact of the gaseous emissions from livestock production is more than that from the total transportation sector. Culturing meat *in vitro* is therefore an attractive alternative (Koneswaran and Nierenberg 2008). Of course, numerous meat replacements are already available for consumers, mostly produced from fungal, plant or milk proteins (Sadler 2004). However, these lack essential nutritional components for humans; meat consists of water (75%), protein (20-25%), fat (5%) and contains high amounts of iron, vitamin B12, zinc and phosphor. In addition, effort is put into making meat healthier by changing the lipid content (Jiménez-Colmenero 2007). Possibly, cells could also be manipulated to produce different lipids in the proposed system. Therefore, we believe that there is a realistic market for high quality *in vitro* produced meat.

Chapter 3

The muscle stem cell niche: Regulation of satellite cells during regeneration

This chapter is based on: Kristel J.M. Boonen, Mark J. Post, *The muscle stem cell niche: Regulation of satellite cells during regeneration*, Tissue Engineering Part B, 14(4), 419-431, (2008).

3.1 Introduction

Tissue resident adult stem cells play a crucial role in the maintenance of tissues that are subject to daily wear and tear. They have been identified in many different positions in the body, including bone marrow, brain, liver, intestine and heart (Alvarez-Buylla and Lim 2004; Bjerknes and Cheng 2005; Theise 2006; Wilson and Trumpp 2006; Leri et al. 2008). In general, a small population of stem cells is present in a highly specific environment that consists of extracellular matrix (ECM) and different types of surrounding cells, including organ specific and mesenchymal cells (Schofield 1978). As the need and capacity for repair differs widely among tissues, the interaction of stem cells with their direct environment has tissue specific characteristics in addition to common features. The presumed specificity of the environment has formed the basis for the "niche" concept in which structural and biochemical cues importantly determine stem cell behavior. As a common feature, niches contain a basement membrane (BM) as one of their most important components (Spradling et al. 2001). The key question therefore is how such a common feature confers specificity on the process of stem cell recruitment.

Adult stem cells have the capability to self-renew, with a seemingly indefinite number of cell doublings. While self-renewal is functional for regenerative purposes, it is clear that proliferation and differentiation of stem cells need to be tightly regulated to prevent uncontrolled growth. An important part of this regulation is probably provided by the stem cell niche which exerts this control through guidance of signals by the re-organizing ECM (Spradling et al. 2001; Fuchs et al. 2004; Naveiras and Daley 2006; Theise 2006).

Skeletal muscle is a type of tissue that typically experiences bouts of high levels of regeneration and repair and for this reason houses stem cells. There has been much debate about which cell type qualifies as the muscle adult stem cell, but the most prevalent notion is that the satellite cell or a subset of satellite cells most likely assumes this role (Seale and Rudnicki 2000; Asakura et al. 2001; Chen and Goldhamer 2003; Partridge 2004; Collins et al. 2005; Dhawan and Rando 2005; Wagers and Conboy 2005). The anatomical location of the satellite cell (in between the sarcolemma and the basement membrane, figure 3.1), its capability of self-renewal (Zammit et al. 2004; Collins et al. 2005; McKinnell et al. 2005; Collins 2006), its regenerative capacity *in vivo* (Collins et al. 2005) and plasticity *in vitro* (Asakura et al. 2001; Csete et al. 2001; Wada et al. 2002; Shefer et al. 2004) show that the satellite cell possesses all the requisite characteristics of a skeletal muscle stem cell (Rando 2005). In case of skeletal muscle injury or another type of stimulus, satellite cells are activated and become proliferating myoblasts. If necessary, they migrate to the designated site where they differentiate and fuse with existing or damaged fibers or form new fibers by fusing with other myoblasts.

However, an important and poorly understood limitation of the satellite cell is its *in vitro* proliferative capacity: After isolation, satellite cells can only divide a small number of

times *in vitro* (Machida et al. 2004). On the other hand, *in vivo*, a small amount of tissue-resident satellite cells is sufficient to regenerate large parts of muscle tissue (Collins et al. 2005; Montarras et al. 2005). The inability to recapitulate the proliferative capacity *in* vitro is probably due to loss of the highly specific niche that normally surrounds these cells (Blau et al).

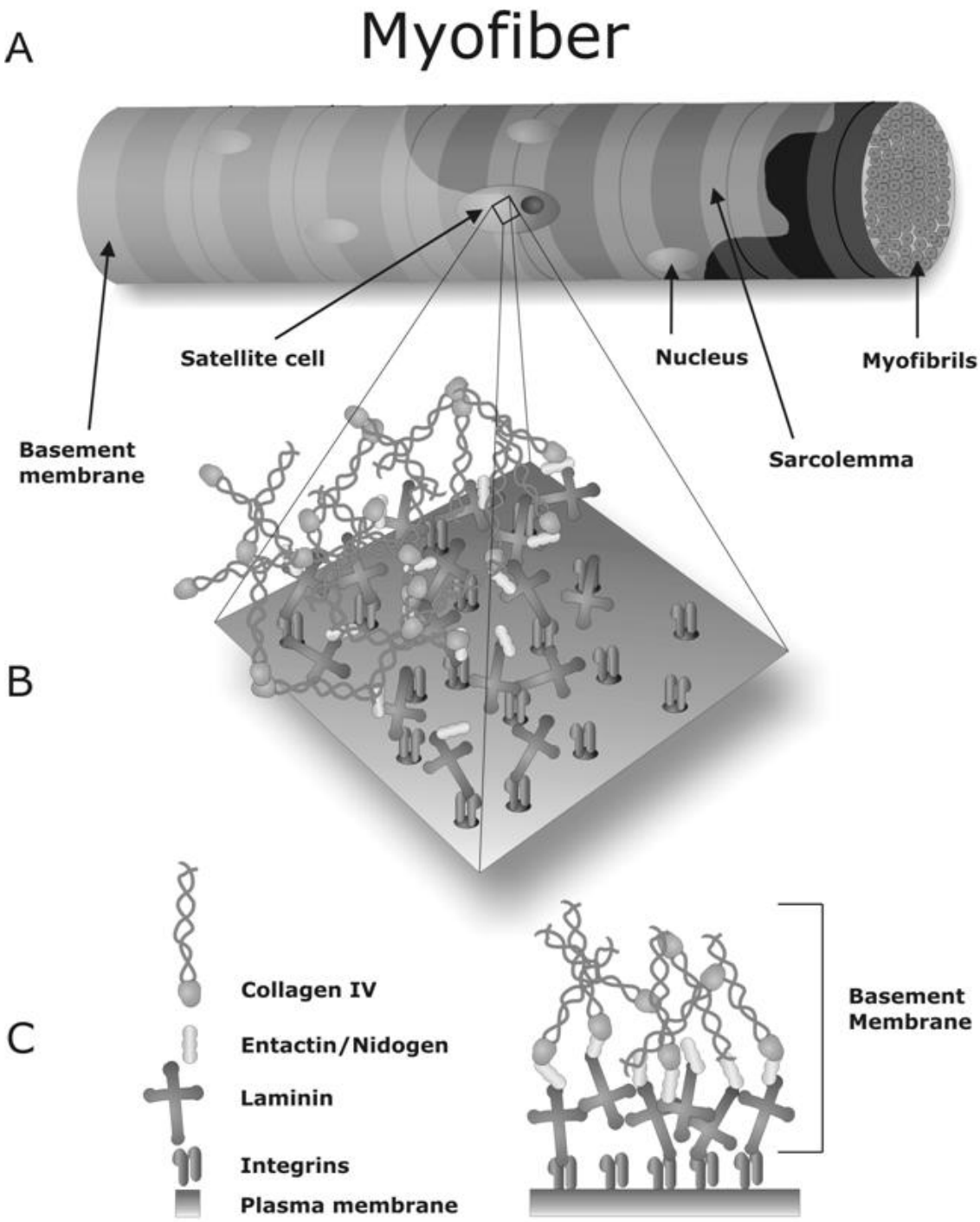

Figure 3.1: A) Location of the satellite cell in between the sarcolemma and the basement membrane. B) Close-up of the coupling of the basement membrane to the satellite cell plasma membrane. C) Cross section of B.

Satellite cells are commonly defined according to their anatomical location (Bischoff 1986). However, they have been shown to be a heterogeneous population according to their expression of molecular markers, suggesting varying roles for the different subpopulations in the regenerative process (Schultz 1996; Cornelison and Wold 1997; Beauchamp et al. 2000; Zammit and Beauchamp 2001; Conboy and Rando 2002; Sherwood et al. 2004; Holterman and Rudnicki 2005; Brzoska et al. 2006; Kuang et al. 2006). A number of markers have been proposed that should distinguish the entire population as such: M-cadherin (Cornelison and Wold 1997), CD34 (Beauchamp et al. 2000), c-met (Cornelison and Wold 1997) and Pax7 (Zammit et al. 2006). Evidence exists for the presence of a small stem cell-like population within the satellite cell compartment (Morgan and Partridge 2003; Collins et al. 2005; Collins 2006), indicated by the observation that some myoblasts do survive after injection into injured host muscle and are capable of robust regeneration, albeit only at the site of injection (Beauchamp et al. 1999).

Stem cells are the ideal candidates to boost regeneration after extensive injury or to substitute a defective repair mechanism. The oldest regenerative therapy proposed is myoblast transfer therapy (MTT) (Partridge 1991) in which isolated myoblasts are cultured *in vitro* and then injected into muscles of compromised living recipients (Rando and Blau 1994). The first MTT studies focused on the restoration of dystrophic muscle in a mouse (mdx) model of Duchenne Muscular Dystrophy (DMD) (Huard et al. 1994; Fan et al. 1996b; Beauchamp et al. 1999) and later on in DMD patients (Huard et al. 1991; Gussoni et al. 1992; Huard et al. 1992; Karpati et al. 1993; Tremblay et al. 1993). Unfortunately, most injected myoblasts do not even survive the first hour after injection and do not migrate from the site of injection, resulting in failure to restore function (Huard et al. 1994; Beauchamp et al. 1999; Skuk et al. 2002; Cao et al. 2005). When myoblasts are injected in a fibrin clot (Beauchamp et al. 1997), survival does not improve. However, when satellite cells are isolated without enzymatic digestion of the muscle fiber (Collins et al. 2005) and injected without further culturing *in vitro*, regeneration of damaged muscles is successful (DiMario and Stockdale 1995; Collins et al. 2005; Montarras et al. 2005; Sherwood and Wagers 2006).

More recently, tissue engineering using primary cells (Shansky et al. 1997; Powell et al. 1999; Dennis and Kosnik 2000; Dennis et al. 2001; Kosnik et al. 2001; Powell et al. 2002; Cronin et al. 2004) or cell lines (Okano and Matsuda 1997; Okano and Matsuda 1998b; Dennis et al. 2001; Cheema et al. 2003) and standard matrices such as collagen and PGA/PLLA has been studied for regenerative purposes, but with limited success (Vandenburgh et al. 1996; Okano and Matsuda 1998b; Levenberg et al. 2005; Bach et al. 2006; De Coppi et al. 2006). However, implantation of myoblasts seeded in a decellularized muscle matrix gives long term repair (De Coppi et al. 2006). Current studies on the optimal biochemical and biophysical conditions of the scaffolds to support myoblast survival and differentiation are aimed at improving this therapeutic

platform. The studies are based on the premise that the environment should resemble the natural recipient environment as much as possible in order to fully support differentiation and maturation of myoblasts into adult skeletal muscle. Finally, understanding these niche principles could also facilitate stimulating satellite cells *in situ* for skeletal muscle regeneration.

In this review, we provide an integrated view on the role of the most important aspects of the satellite cell niche (the basement membrane (BM), mechanical properties and supporting cells) and their putative pathways to control stem (satellite) cell activation, proliferation and migration in regenerating skeletal muscle (figure 3.2).

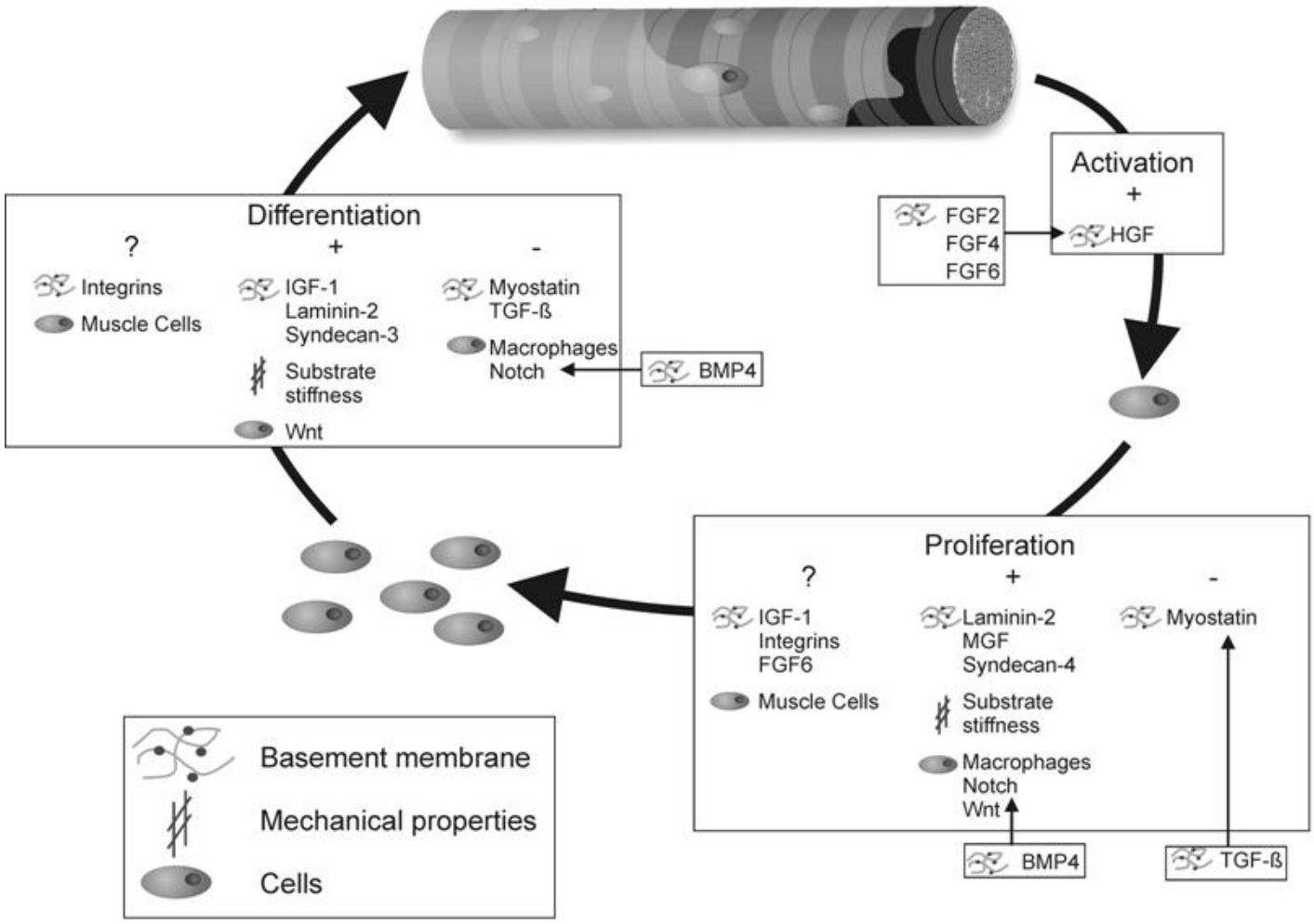

Figure 3.2: Overview of the most important components of the satellite cell niche and their influence on activation, proliferation and differentiation.

3.2 Satellite cell niche

A stem cell niche is commonly defined as "a specific location in a tissue where stem cells can reside for an indefinite period of time and produce progeny cells while self-renewing" (Ohlstein et al. 2004). Most niches contain a BM, to which the stem cells attach (Spradling et al. 2001). At either site of the BM, permanent so-called supporting cells can be localized that are of importance to stem cell functioning (Spradling et al. 2001). The satellite cell niche indeed contains a BM and muscle fibers (Dhawan and Rando 2005) and/or endothelial cells (Christov et al. 2007) are close by and could function as supporting cells. However, although they seem to have an effect on myoblasts proliferation *in vitro*, endothelial cells have not been shown to be in direct contact with satellite cells. In most stem cell niches (e.g. in the intestine) the supporting cells are small cells that are located at the opposite site of the BM, but cases exist (e.g. in the testis, reviewed in (Wong et al. 2005)) in which stem cells are supported by big cells located at the same site of the BM. Therefore, the muscle fiber is the most promising candidate as the satellite cell niche's supporting cell. Alternatively, in the Drosophila midgut, stem cell niches were described that do not rely on supporting cells for their function (Ohlstein and Spradling 2006), opening the possibility that the satellite cell niche exists without any supporting cells. This hypothesis is reinforced by the fact that satellite cells with or without the parent fiber regenerate defects similarly after transplantation, as long as the isolation methods are optimal (Collins et al. 2005).

Another prerequisite of a stem cell niche is that when it is depleted of stem cells, it should persist and be able to house new stem cells (Li and Xie 2005). Bone marrow stem cells, for example can enter and leave the circulation to occupy empty niches in a process called homing (reviewed in (Whetton and Graham 1999)). Satellite cells (Sherwood et al. 2004; Montarras et al. 2005) but also bone marrow cells (Sherwood et al. 2004; Li and Xie 2005; Christov et al. 2007) are able to occupy empty satellite cell niches, satisfying also this requirement.

When the satellite cell niche is compared to niches in other common stem cell systems such as skin and intestine, the most obvious difference is that satellite cells in their niche are quiescent, whereas in most systems stem cells are constantly active in order to replace cells that are lost due to daily wear and tear. Usually an intermediate cell called transit amplifying (TA) cell takes care of actual expansion, whereas the stem cell only continuously replenishes this TA population. In the intestine, for example, stem cells at the bottom of crypts constantly divide to give rise to TA cells that move up and differentiate to ensure a steady flow of cells that are shed at the surface (reviewed in Yen and Wright 2006). However, in other tissues, niches do exist that contain quiescent stem cells. In the heart, for example, cardiac stem cells (CSCs) are thought to take care of regeneration (reviewed in (Leri et al. 2008)). It is hypothesized that CSCs are quiescent cells, surrounded by a BM, that give rise to a TA population after activation, which is then responsible for actual repair (Leri et al. 2008).

Possibly, such a TA population also exists in the muscle stem cell system (Zammit and Beauchamp 2001). It has been shown that satellite cells are a heterogeneous population containing a small stem cell compartment (Cornelison and Wold 1997; Zammit and Beauchamp 2001; Dhawan and Rando 2005; Kuang et al. 2006; Kuang et al. 2007), which could mean that the rest of the satellite cells should be perceived as TA cells. Evidence for this theory comes from elegant studies showing a hierarchical lineage relationship of satellite cells (Kuang et al. 2008; Tamaki et al. 2008). Alternatively, all satellite cells should be considered TA cells that descend from a common precursor stem cell located elsewhere, for example in the interstitial spaces of skeletal muscle (Zammit and Beauchamp 2001; Tamaki et al. 2008). However, observations that satellite cells self-renew (Zammit et al. 2004; Collins et al. 2005; Collins and Partridge 2005) and regenerate muscle (Collins et al. 2005) clearly show that these cells are more than just TA cells (Rando 2005).

3.3 Basement membrane

The BM is a specialized sheet of connective tissue. Its most important constituents are type IV collagen, laminin and heparan sulfate carrying proteoglycans (HSPGs). A laminin-network faces the muscle fiber, complemented by a network of collagen IV. These proteins are linked by entactin-1 (also called nidogen-1) to form a complex structure containing a high number of binding sites for e.g. proteoglycans and integrins (figure 3.1). In skeletal muscle, the interstitial fibroblasts are responsible for the production and excretion of ECM molecules. During development and regeneration, mononuclear muscle cells also synthesize and remodel ECM, whereas the contribution of the adult muscle cell is relatively small (Kovanen 2002). Homing, residence and activation of stem cells can be regulated through differential expression of integrins and tissue specific laminin isoforms in the BM (Beaulieu 1997; Sanes 2003; Yurchenco et al. 2004; Givant-Horwitz et al. 2005). Integrity of the BM is crucial to keep the stem cells physically in place; defects or regulated gaps in the basement membrane will allow cells to move through the tissue (Brennan et al. 2004).

Integrins are involved in linking the BM to the intracellular cytoskeleton (Watt 2000) and consist of a large family of cell surface receptors composed of an alpha and a beta subunit. They act as signal transducers after binding to the appropriate ligand, influencing cell migration, cell shape and cell-cell interactions (Beaulieu 1997; Burkin and Kaufman 1999; Kovanen 2002). Integrin α7 is the main isoform in mature skeletal muscle (von der Mark et al. 1991; Huijbregts et al. 2001). It binds muscle fibers to laminin and dystroglycan and is upregulated during regeneration (Yao et al. 1996; Kaariainen et al. 2001). Myoblasts and newly formed myotubes *in vitro* express integrin α5 (Huijbregts et al. 2001) and together with integrin β1 it forms the main receptor complex for fibronectin, which is also present in the BM (Pytela et al. 1985; Zhao et al. 2004). Integrin

α3 is found on quiescent satellite cells and myoblasts and plays a role in migration and differentiation by forming complexes with the integrin β1 subunit and ADAM12 (see below) (Davis and Senger 2005; Brzoska et al. 2006). Integrin β1 plays a role in laminin-binding of myoblasts *in vitro* (von der Mark et al. 1991; Patton et al. 1999; Zhao et al. 2004). While these integrins are of obvious importance for mechanical coupling and deficient mice develop different types of muscular dystrophy, a role in regeneration remains speculative (Mayer et al. 1997; Taverna et al. 1998; Brzoska et al. 2006).

One of the BM components that is specific for muscle and therefore is likely to play an important role in the niche functioning is laminin-2. Laminins are heterotrimers composed of alpha, beta and gamma subunits that form a network-like structure in BMs (figure 3.1). Laminin-2 is composed of α2, β1 and γ1 proteins and is also referred to as merosin (Watchko et al. 2002). The laminin α2 subunit is the muscle specific part of laminin-2 and is connected to muscle fibers through integrin α7β1 and dystroglycan (Mayer 2003) in a large dystrophin-associated protein (DAP) complex (Ervasti 1993; Henry and Campbell 1996; Brennan et al. 2004). The significance of laminin α2 for satellite cell function is underscored by the phenotype of the laminin α2 knockout mouse. Not only is the skeletal muscle BM almost completely absent in these animals (Miyagoe et al. 1997) leading to a decrease in the relative amount of satellite cells (Girgenrath et al. 2005), during regeneration the amount of myoblasts is additionally decreased due to reduced proliferation rates (Girgenrath et al. 2005) and increased apoptosis (Miyagoe et al. 1997).

Multiple proteoglycans can be found in the ECM in skeletal muscle, but only a few seem to be important during regeneration. Proteoglycans consist of glycosylated core proteins with one or more covalently attached sulfated glycosaminoglycan (GAG) chains. Quiescent satellite cells express syndecan-3 and -4, which are transmembrane HSPGs that become upregulated upon activation. They only differ in their extracellular domains (Cornelison et al. 2001) and are required to transduce signals (Rapraeger 2000) directly through binding to signaling molecules or by presenting them to their specific receptors (Miller et al. 1991). Syndecan-4 has been implicated in Fibroblast Growth Factor (FGF) signaling (Horowitz et al. 2002; Rauch et al. 2005) and seems to be required for early satellite cell activation and proliferation. Satellite cells in knockout mice express reduced levels of syndecan-3 and c-met and the BM appears disorganized *in vivo.* When muscle damage is induced in these animals, regeneration fails and results in non-functional myofibers and scar tissue (Cornelison et al. 2004). In cell culture, proliferation of myoblasts is delayed and differentiation does not even take place (Cornelison et al. 2004). In syndecan-$3^{-/-}$ mice the number of satellite cells and myonuclei is increased in muscles in spite of normal size, location and gene expression profile of satellite cells and intact BM. When cells isolated from these mice are cultured, differentiation is abnormal, which is evident by aberrant fusion into syncytia instead of fibers (Cornelison et al.

2004). Therefore, it seems that integrity of the BM and the ability of cells to bind to the BM (for instance through HSPGs) is essential for regeneration.

In addition to laminins and HSPGs, growth factors play an important role in the regeneration process. They can be produced and secreted by muscle cells or immune cells and can be liberated from the ECM where they are bound to proteoglycans (Miura et al. 2006). Growth factors that are involved in the regeneration program include HGF, members of the Transforming Growth Factor- β (TGF-β) superfamily, Fibroblast Growth Factors (FGFs) and Insulin-like Growth Factor-1 (IGF-1) isoforms. HGF is a heparan-binding protein that activates quiescent satellite cells through its receptor c-met (Allen et al. 1995; Gal-Levi et al. 1998; Tatsumi et al. 1998) and is normally present in an active form in the ECM of uninjured muscle (Tatsumi and Allen 2004). HGF is released by matrix metalloproteinases (MMPs) (Tatsumi and Allen 2004; Yamada et al. 2006) after stretch or other types of injury (Tatsumi et al. 2001) and can be produced by satellite cells *in vitro* (Sheehan et al. 2000). Next to HGF, FGFs are involved in the regulation of regeneration. FGFs 2, 4 and 6 stimulate proliferation of myoblasts *in vitro* (Hannon et al. 1996), but only in the presence of HGF. FGF2 can be secreted by infiltrating macrophages (Merly et al. 1999) and is found to be upregulated in regenerating muscle (Anderson et al. 1991) together with FGF6, which is thought to be secreted by injured muscle fibers (Kastner et al. 2000; Zhao and Hoffman 2004). FGF6 knockout mice show a severe regeneration defect that is most likely caused by disturbed activation or proliferation of satellite cells (Floss et al. 1997). Migration of $FGF6^{-/-}$ myoblasts is also impaired (Neuhaus et al. 2003). IGF-1 isoforms resemble FGFs in their actions. At least four different isoforms exist, two of which play a role in muscle growth and repair (Mourkioti and Rosenthal 2005). Mechano growth factor (MGF), which is induced during the initial satellite cell activation (Yang and Goldspink 2002), especially after mechanical stimulation (Cheema et al. 2005) and damage (Hill and Goldspink 2003) stimulates proliferation of myoblasts (Ates et al. 2007). After this initial phase, MGF is replaced by systemic IGF-1, through alternative splicing (Hill and Goldspink 2003; Goldspink 2006). Systemic IGF-1 has been shown to induce faster differentiation in C2C12 myoblasts (Florini et al. 1996) and increased regeneration in mice (Menetrey et al. 2000). In IGF-1 transgenic mice, myoblast proliferation as well as differentiation is augmented (Rabinovsky et al. 2003), whereas IGF-1 knockout mice die at birth, because of severe muscular dystrophy (Powell-Braxton et al. 1993). In contrast to IGFs, several members of the TGF-β superfamily, including TGF-β, Bone Morphogenetic Proteins (BMPs) and myostatin, are involved in negative regulation of regeneration by inhibiting proliferation or differentiation. TGF-β (Allen and Boxhorn 1987; Goetsch et al. 2003) and myostatin (Amthor et al. 2002; Wagner et al. 2005b) reduce myoblast recruitment and differentiation and TGF-β also remodels and repairs ECM and BM (Massague et al. 1986). In a number of known stem cell niches (e.g. in neural crest (Shah et al. 1996)), BMPs prevent stem cell proliferation which can be counteracted by upregulation of

Noggin (Song et al. 2004; Moore and Lemischka 2006). In C2C12 myoblasts and satellite cells, BMP4 in combination with Notch signaling can block myogenic differentiation (Dahlqvist et al. 2003).

In order to get to the site of injury for repair, myoblasts have to migrate through the ECM. Matrix metalloproteinases (MMPs) are involved in degrading ECM components, which not only enables myoblast migration, but also leads to release of and exposure to cytokines and growth factors that target myoblast proliferation and differentiation (Hedin et al. 1999; Torrente et al. 2000; Davis and Senger 2005; Debnath and Brugge 2005; Irving-Rodgers and Rodgers 2005). MMP2 and 9 can be secreted by satellite cells (Guerin and Holland 1995; Kherif et al. 1999) and are upregulated in muscle during injury and regeneration (Lewis et al. 2000; Koskinen et al. 2001; Koskinen et al. 2002). They degrade collagen IV, but also other BM components such as dystroglycan (Brennan et al. 2004), and are secreted into the ECM in an inactive form that is activated after cleavage. Inhibition of these MMPs prevents migration of myoblasts *in vitro* (Kherif et al. 1999; El Fahime et al. 2000) and when injected *in vivo*, migration of myoblasts is triggered (Torrente et al. 2000). A Disintegrin And Metalloproteinases (ADAMs) are cell surface receptors involved in regeneration by mediating adhesion and transmembrane signaling. ADAM12, also called meltrin-α, is expressed during muscle development and regeneration (Yagami-Hiromasa et al. 1995; Galliano et al. 2000; Brzoska et al. 2006). It is also expressed in activated satellite cells (Borneman et al. 2000) and binds to integrin α7β1 (Zhao et al. 2004), integrin α9β1 (Eto et al. 2000; Eto et al. 2002; Lafuste et al. 2005), integrin α3β1 (Brzoska et al. 2006) and cell surface syndecans (probably syndecan-4), leading to integrin-dependent spreading of cells (Iba et al. 2000).

Obviously, the basement membrane plays a crucial role in maintaining the stem cell function of satellite cells. However, it is hard to consider the effects of integrins, different matrix proteins, growth factors and MMPs individually, because they all play interconnected roles in the complex signaling pathways between the satellite cell and its environment.

3.4 Mechanical properties

It is clear, although under-appreciated that the mechanical properties of the matrix greatly affect the cellular phenotype. Examples are surface tension induced cellular organization during embryonic development (Foty et al. 1996) and locomotion (Pelham and Wang 1997). In muscle cells, (cyclic) stretch has been shown to induce hypertrophy (Vandenburgh 1987) and protein expression of myogenic regulatory factors (MRFs) (Grossi et al. 2007) and IGF-1 splice variants (Cheema et al. 2005), activate satellite cells (Tatsumi et al. 2001; Tatsumi et al. 2002; Wozniak et al. 2005) and improve tissue

engineered muscle constructs (Vandenburgh et al. 1991; Powell et al. 2002; Auluck et al. 2005; Moon du et al. 2008).

Cells can feel their surroundings by anchoring and pulling with their cytoskeletal proteins, integrins and other molecules that mediate adhesion to the ECM (Discher et al. 2005). Recently, the stiffness of the substratum that cells are cultured on has gained interest. Mesenchymal stem cells, for example, differentiate either into the neuronal, muscular or osteogenic lineage when cultured on substrates with a stiffness of 0.1-1 kPa, 8-17 kPa and 25-40 kPa, respectively. After one week on such a matrix, differentiation pathways seem to be fixed and can no longer be reprogrammed with specialized media (Engler et al. 2006). For skeletal muscle cell differentiation and especially maturation into mature, striated skeletal muscle, the stiffness of the environment is equally important (Engler et al. 2004a; Engler et al. 2004b) (Chapter 4). Muscle has been shown to possess a Young's modulus of about 12 kPa in rest, when measured in the transverse direction (the direction of adhesion of cells), which is the same as differentiating skeletal muscle cells *in vitro* (Collinsworth et al. 2002). Differentiation and maturation of myoblasts have been shown to be optimal with this stiffness: When C2C12 murine myoblasts were cultured on gels with different Young's moduli, cross-striation, which is an indicator of muscle maturation, only occurred on gels of intermediate stiffness (8 and 11 kPa) (Engler et al. 2004b) (figure 3.3) or on top of a layer of myotubes (Young's modulus: 12-15 kPa) (Engler et al. 2004b). Probably this is also true for the proliferative capacity of cells (Boontheekul et al. 2007) (Chapter 4), which could partly explain the discrepancy between the *in vivo* and *in vitro* proliferative behavior of satellite cells. Primary myoblasts were shown to proliferate at a higher rate on stiffer gels (12 and 45 kPa) compared to softer gels (1 kPa) (Boontheekul et al. 2007).

This aspect deserves further exploration as it is likely to be of importance for standardization of cell culture experiments, but also for the design of matrices that support cell therapies in regenerative medicine. In terms of mechanical properties, for example, the recipient environment in patients that qualify for MTT is very different from the required environment for myoblast proliferation and differentiation. In mdx mice and Duchenne patients, extensive scar formation replaces the affected muscles and this fibrotic tissue might prevent myoblasts from surviving and regenerating the diseased muscle, not only by interfering positionally, but also by increasing stiffness beyond the optimal parameters for myoblast proliferation and differentiation (Partridge 1991).

The BM, and most importantly its collagen/laminin network, is probably responsible for determining the stiffness of muscle fibers (Kovanen et al. 1984; Koskinen et al. 2001; Kovanen 2002). However, up till now, no mechanical tests have been performed on muscle BM. Nonetheless, not only the mechanical properties themselves, but also the combination of stimulatory effects with the different BM components can have

synergistic, complementary or opposing effects on myogenesis. For example, mechanical stimulation through the laminin receptor results in differentiation of C2C12 murine myoblasts and has been shown to be mediated through β1 integrins, whereas mechanical stimulation through the fibronectin receptor encourages proliferation (Grossi et al. 2007).

In summary, the mechanical properties of the satellite cell niche are almost certainly determined by the BM and are pivotal for proper stem cell function of satellite cells. However, it is not likely that the stiffness of the environment is actively regulated in order to trigger different processes in the regenerative process; it could therefore be considered to be an essential requirement.

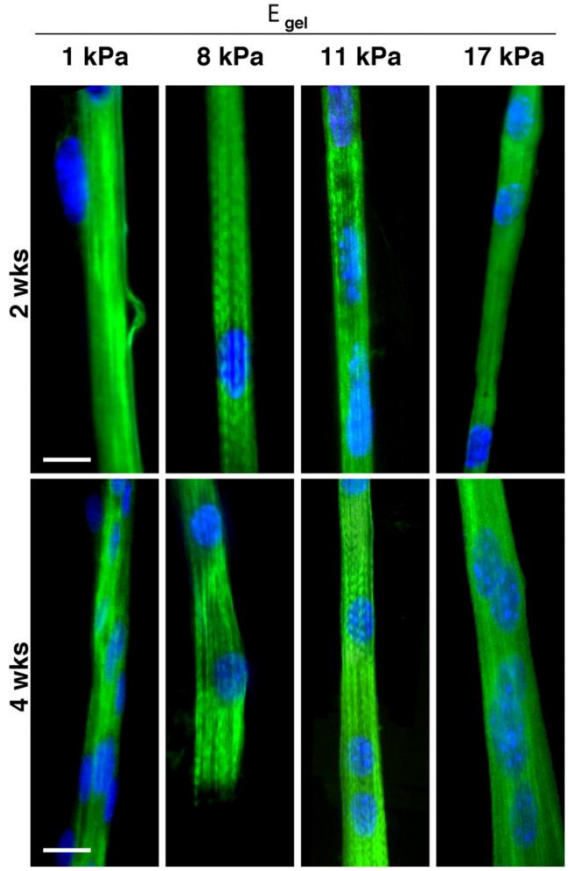

Figure 3.3: Cross-striations in myotubes depend on substrate stiffness. Two or four weeks after plating cells on collagen-coated polyacrylamide (PA) gels of different elasticities, only myotubes on gels of intermediate stiffness showed cross-striations of myosin (green). Nuclei are in blue. Bars: 20 µm.

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3.5 Cells

Another important niche component is the cells other than the stem cells. For bone marrow for instance, these supporting cells, most notably osteoblasts, play an important role in stem cell maintenance and regulation (reviewed in (Wilson and Trumpp 2006)). In the muscle stem cell niche, mature muscle cells, other mesenchymal cells and cells of the specific and innate immune system connect with the satellite cells.

In the normal situation, satellite cells are only in direct contact with the adjacent muscle cell. The importance of this connection is underscored by the fact that the result of myoblast transplantation is better when fibers containing satellite cells are transplanted as a whole compared to transplantation of cells liberated from the fiber by enzymatic digestion (Collins et al. 2005). However, when satellite cells are dissociated from the muscle fiber by physical trituration, their regenerative potential *in vivo* is comparable to transplantation of single fibers. Therefore, although the parent fiber itself does not seem to be required for satellite cell function, the ability of the satellite cell to bind and communicate to the fibers or the niche present in the recipient muscle seems to be essential and might be impaired by enzymatic digestion (Collins et al. 2005).

In the event of injury and muscle damage, cells of the immune system are also able to come into contact with the satellite cell and convey signals directing proliferation, migration and differentiation, for example through the production and excretion of growth factors. Macrophages are important for removal of dead cells and the dead parts of muscle fibers. They have also been shown to be able to directly stimulate satellite cell proliferation and delay their differentiation. This effect is probably mediated by FGF2, PDGF or LIF.

More than 60% of the satellite cells are located close to capillaries and are thought to receive signals from endothelial cells, although they are not in direct contact with them. The number of capillaries per muscle fiber has been shown to correlate to the number of satellite cells and loss of capillaries leads to loss of satellite cells, pointing to some sort of interaction (Christov et al. 2007). In addition, transwell experiments showed that endothelial cells have a positive effect on proliferation of myoblasts which is mediated by growth factors secreted by the endothelial cells (Christov et al. 2007).

Cell-cell interactions have been shown to be pivotal for muscle regeneration and can be mediated by components of the cell-bound receptor-ligand complexes of the Notch and Wnt family. Wnt signaling plays a role in stem cell determination (Sato et al. 2004) in different stem cell niches (e.g. the intestinal stem cell niche (Moore and Lemischka 2006)) and is also thought to activate stem cells during muscle regeneration (Fuchs et al. 2004; Grenier and Rudnicki 2006). The canonical Wnt pathway is mediated by β-catenin, which translocates to the nucleus where it induces transcription of specific genes involved in cell proliferation and survival together with transcription factors of the

Tcf/Lef family. Overexpression of β-catenin increases proliferation and induces hypertrophy *in vitro* in C2C12 myoblasts and increases regeneration and the number of satellite cells *in vivo* in mice (Kim et al. 2006).

Notch is an evolutionary conserved heterodimeric transmembrane receptor that is involved in cell fate control by local cell interactions. Most of the ligands involved in Notch signaling are also membrane-bound and interact with Notch receptors on adjacent cells, leading to a signaling cascade that regulates the transcription of specific genes involved in self-renewal, proliferation and differentiation of stem cells together with functional Wnt signaling (Artavanis-Tsakonas 1999; Fre et al. 2005; Radtke and Clevers 2005; van Es et al. 2005). During muscle regeneration, satellite cells are activated and start proliferating due to Notch signaling, through the ligand Delta-1 (Conboy and Rando 2002; Conboy et al. 2003; Luo et al. 2005; Shinin et al. 2006; Kuang et al. 2007).

Different types of coculture experiments have been performed to investigate the effect of contact with a muscle environment on different types of cells. In general, it seems to be the case that direct contact with either muscle fibers or myoblasts is essential for myogenic conversion of different types of cells (Salvatori et al. 1995; Lee et al. 2005; Di Rocco et al. 2006; Carlson and Conboy 2007; Nunes et al. 2007).

Overall, the ability to bind to and be in direct contact with muscle fibers seems to be essential for the regenerative potential of satellite cells. Different cell cell interaction and signaling molecules seem to play an important role in this process, although we need more direct results to explain some of the contradictory roles attributed to these molecules.

3.6 Discussion

Satellite cells from skeletal muscle are identified as correlate of a tissue resident stem cell, i.e. they are capable of *in vivo* tissue regeneration (Asakura et al. 2001; Csete et al. 2001; Zammit et al. 2004; Collins et al. 2005; Dhawan and Rando 2005). Since satellite cells are easily identifiable and can be harvested from intact muscle, therapeutic applications for MTT or for tissue engineering have been envisaged. However, it appears that the myoblast population that is harvested from adult skeletal muscle quickly loses its self-renewal capacity during in vitro expansion (Bischoff 1986; Yablonka-Reuveni and Rivera 1994; Rosenblatt et al. 1995).

Crucial evidence suggests that the isolation procedure and/or *in vitro* expansion causes this fate change; enzymatically isolated or cultured myoblasts have been shown to lose their regenerative capacity, whereas direct implantation of non-enzymatically isolated myoblasts or single fibers with resident satellite cells is extremely effective to regenerate damaged muscle tissue (Collins et al. 2005). In addition, when sliced muscle

grafts are used in muscle transplantation, cells survive for up to a year (Partridge et al. 1978; Fan et al. 1996a; Smythe et al. 2000).

We suggest that preservation of critical stem cell niche components is important for maintaining the regenerative capacity of satellite cells. We have reviewed the BM contribution to this niche. Appealing candidates are muscle specific laminin isoforms (Yurchenco et al. 2004) and syndecans 3 and 4 (Cornelison et al. 2004). However, systematic studies addressing the essential components for the satellite stem cell niche are needed. Once identified, reconstitution of these components during cell culture might improve the applicability of satellite cells in regenerative medicine. An under-appreciated aspect of the stem cell niche is the mechanical framework it provides. There is now increasing evidence that cells sense the mechanical properties of their matrix and respond by phenotypic change (Engler et al. 2004a; Engler et al. 2006), possibly by differentiating away from their precursor state (e.g. in the case of rigid culture plastic). Support for the importance of the matrix in the stem cell niche also comes from observations that BM integrity during injury accelerates natural healing (Caldwell et al. 1990; Sanes 2003). Not only loss of the matrix context, but also loss of the cellular context may cause fate change of the satellite cell. Experience with co-cultures of cells of different origins with adult muscle fibers or myoblasts strongly suggests that physical contact of stem cells with differentiated muscle favors muscular differentiation (Salvatori et al. 1995; Lee et al. 2005; Di Rocco et al. 2006; Carlson and Conboy 2007; Nunes et al. 2007).

In addition, the isolation procedure might select non-regenerative subpopulations of satellite cells (Collins and Partridge 2005). Different subpopulations of stem cells in skeletal muscle appear to exist and it is very likely that some have more regenerative capacity than others (Morgan and Partridge 2003; Collins et al. 2005; Collins 2006). Clearly, detailed marker studies complemented by clonal analyses are required to dissect the importance of these subpopulations and the extent to which the isolation procedure affects their presence and performance.

Elucidating the micro-environmental needs of satellite cells will have considerable implications for the use of these cells for regenerative medicine. In addition, the two approaches, MTT and tissue engineering, will likely differ in their requirements concerning proliferative and migratory capacity. The indications and therefore the recipient characteristics, for instance large defects in tissue engineering versus generalized dystrophy for MTT, will be different as well.

For both therapies to advance, it is therefore essential that we understand the biochemical, cellular and mechanical cues that promote satellite cell proliferation and differentiation *in vitro* and *in vivo*. The current evidence provides a sound basis for systematic studies of these cues in the setting of regeneration of skeletal muscle.

Chapter 4

Essential environmental cues from the satellite cell niche: Optimizing proliferation and differentiation

This chapter is based on: Kristel J.M. Boonen, Kang Yuen Rosaria-Chak, Frank P.T. Baaijens, Daisy W.J. van der Schaft, Mark J. Post, *Essential environmental cues from the satellite cell niche: Optimizing proliferation and differentiation*, The American Journal of Physiology-Cell Physiology, 296(6): C1338-1345, (2009).

4.1 Introduction

Satellite cells (SCs) can be considered adult muscle stem cells and are the most promising candidate for regenerative medicine in skeletal muscle disorders (Seale and Rudnicki 2000; Asakura et al. 2001; Partridge 2004; Collins et al. 2005; Dhawan and Rando 2005). In the body, when muscles are damaged or otherwise compromised, these ordinarily quiescent cells are activated and become proliferating myoblasts. Myoblasts then migrate to the designated site to form new myofibers by fusing with each other or with damaged fibers. In addition, they can give rise to new SCs by self-renewal (Zammit et al. 2004). Thus, in theory these cells are an inexhaustible source of cells for muscle regeneration throughout life. It is therefore not surprising that they have been used for regenerative medicine purposes, such as myoblast transfer therapy (MTT) for muscular dystrophies (Gussoni et al. 1992; Huard et al. 1992; Karpati et al. 1993; Tremblay et al. 1993; Rando and Blau 1994; Fan et al. 1996b) and tissue engineering for replacement therapy (Shansky et al. 1997; Okano and Matsuda 1998a; Okano and Matsuda 1998b; Dennis and Kosnik 2000; Dennis et al. 2001; Levenberg et al. 2005; Bach et al. 2006). For both types of therapies, cells will need to be expanded in order to reach required cell numbers.

In the case of the SC and its proliferating progeny, the myoblast or muscle progenitor cell (MPC), it has been shown that *in vitro* culturing is deleterious to its performance as a stem cell (Collins et al. 2005), leading to a loss of proliferative capacity and failure to differentiate into functional muscle tissue. It has been hypothesized that this phenotypical change is caused by loss of the 'niche' environment that surrounds these cells in an *in vivo* situation (Boonen and Post 2008). Clearly, cultured cells encounter a two-dimensional environment, which is very different from the *in vivo* environment. First, *in vivo*, the SC is located in between the sarcolemma of a muscle fiber and a basement membrane. The importance of the close contact of the SC with its parent fiber is underscored by the fact that implantation of SCs together with their parent fiber results in higher regenerative capacity than implantation of enzymatically isolated 'single' SCs, suggesting that either the parent fiber or the ability of the SC to bind to the parent fiber is pivotal to its stem cell nature and that this is compromised by *in vitro* culturing or enzymatic isolation (Collins et al. 2005). The basement membrane (BM) is made up of networks of laminin and collagen IV, linked by entactin, which provide attachment sites, but also serve as a reservoir and regulator of growth factor supply to SCs. *In vitro* experiments have shown that several matrix proteins including those present in the BM affect myoblast proliferation and differentiation (Maley et al. 1995; Grossi et al. 2007; Macfelda et al. 2007). Second, the elasticity of an *in vitro* environment (tissue culture plastic) is very different from the *in vivo* environment, which could also affect stem cell phenotype. Several observations suggest that muscle differentiation is strongly influenced by substrate stiffness: C2C12 myoblasts only showed striations characteristic of maturation on a small range of soft gels or on top of a layer of

myotubes of approximately equal stiffness (Engler et al. 2004b). In addition, proliferation of myoblasts was shown to be influenced by substrate stiffness, with higher stiffness leading to higher proliferation rates (Boontheekul et al. 2007).

In this study we investigated the influence of substrate elasticity and protein coating, alone or in combination, on primary myoblast proliferation and differentiation. For this reason we isolated murine MPCs and cultured them without prior expansion on coverslips or polyacrylamide (PA) gels with different elastic properties and BM protein coatings after which the proliferation and differentiation capacities of these cells were analyzed.

4.2 Materials & methods

4.2.1 MPC isolation and culture

MPCs were prepared from single fibers from intact hindlimb muscles (EDL, TP, TA and soleus) as described (Shefer et al. 2004; Collins et al. 2005). Briefly, whole muscles were dissected out of 10 to 12-week-old male C57BL/6 mice and were digested for 1 hour with 2 mg/ml (567 U/ml) collagenase type I (Sigma-Aldrich, St. Louis, USA). Afterwards, the muscles were passed through pipettes of decreasing diameter in culture medium consisting of Dulbecco's modified Eagle medium (DMEM) Advanced (Invitrogen, Carlsbad, USA) containing 20% Fetal Bovine Serum (FBS; Greiner Bio-One, Frickenhausen, Germany), 10% Horse Serum (HS; Invitrogen), 100,000 IU/l penicillin and 100 mg/l streptomycin (Lonza, Walkersville, USA) and 4 mM L-Glutamine (Lonza), resulting in a single fiber suspension. Single fibers were then triturated with a 19G needle for 5 minutes and passed through a 40 µm cell sieve. The resulting single cell suspension was frozen in culture medium containing 10% DMSO (Merck, Schiphol-Rijk, The Netherlands) in liquid nitrogen until use. This animal study with number 2007-152 was authorized by the Dutch institutional animal care and usage committee.

4.2.2 Coated PA gels and coverslips

PA gels were created using a method adapted from Pelham and Wang (1997). *N,N′,N′*-methylene-bis-acrylamide (0.03%, 0.13%, 0.26% or 0.3%) (Sigma-Aldrich) was mixed with acrylamide (5% or 10%) (Sigma-Aldrich) and cross-linked with 10% ammonium persulfate (APS) (1/200 vol/vol; Fisher, Pittsburgh, USA) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) (1/2000 vol/vol; Merck). 100 µl Droplets of the solution were placed on a teflon surface and covered by ø 13 mm aminosilanized (with (3-aminopropyl)trimethoxysilane, Sigma-Aldrich) glass coverslips (Menzel, Braunschweig, Germany) for polymerization. Protein coatings of growth factor reduced Matrigel^{TM} (15 µg/cm^2) (BD Biosciences, Bedford, USA), Entactin-Collagen-Laminin (ECL)-

gel (15 μg/cm^2) (Millipore, Billerica, USA), Collagen IV (15 μg/cm^2) (BD Biosciences) or Poly-D-lysine (180 μg/cm^2) (Sigma-Aldrich) were cross-linked to the inert surface of the gels using heterobifunctional sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH) (Pierce Biotechnology, Rockford, USA). Laminin (15 μg/cm^2) (BD Biosciences) was adsorbed to poly-D-lysine (Sigma-Aldrich), because it was not suitable for cross-linking. Adsorption was also used for coating coverslips without gels. All proteins were added in excess to ensure equal coating.

4.2.3 Indentation tests of PA gels

The elastic modulus of the PA gels was determined by indentation tests (Cox et al. 2008). Indentation was applied to the center of the gels with a spherical indenter while measuring force and depth. Afterwards, a numerical model was iteratively fitted to these experimental results using a parameter estimation algorithm.

4.2.4 BrdU assay

Cells were labeled for 16 hours with BrdU and treated according to manufacturer's protocol (Roche Diagnostics GmbH, Penzberg, Germany). In short, cells were fixed with ethanol fixative (15 mM glycine (Sigma-Aldrich) in 70% ethanol) for 20 minutes at -30°C, washed with manufacturer's washing buffer (Roche) and incubated with an anti-BrdU antibody in incubation buffer (1:10) (Roche) for 30 minutes at 37 °C. Afterwards, cells were washed and incubated with an Alexa Fluor 488-conjugated goat anti-mouse IgG1 secondary antibody (1:300) (Invitrogen, A21121) for 30 minutes at 37 °C. Subsequently, cells were washed, treated with 4', 6-diamidino-2-phenylindole (DAPI) (0.1 mg/ml) (Sigma-Aldrich) for 5 minutes and mounted with mowiol (Calbiochem, San Diego, USA) on rectangular coverslips. Cells were evaluated using fluorescence microscopy at room temperature (Axiovert 200M, Zeiss, Göttingen, Germany) with a 20x objective (Zeiss LD ACHROPLAN) with an NA of 0.4. A Zeiss AxioCam HRM camera was used together with Zeiss AxioVision Rel. 4.4 acquisition software to take pictures. Nuclei of cells that had divided during BrdU treatment were positive for BrdU. The ratio was calculated using DAPI, which stained all nuclei.

4.2.5 Immunocytochemistry

Cells were washed with PBS (Sigma-Aldrich), fixed with 10% formalin (Sigma-Aldrich) for 10 minutes, and permeabilized with 1% Triton-X-100 (Merck) in PBS for 30 minutes. Subsequently, cells were incubated twice for 15 minutes with 1% HS in PBS to block non-specific binding and washed 2 times 15 minutes with NET-gel (50mM Tris pH 7.4, 150 mM NaCl, 5mM EDTA, 0.05% NP40, 0.25% gelatin). Afterwards, they were incubated for

2 hours with antibodies against myosin heavy chain (1:300) (MF20, developed by D.A. Fischman and obtained from the Developmental Studies Hybridoma Bank, maintained by The University of Iowa) and MyoD (1:50) (DakoCytomation, Glostrup, Denmark) in NET-gel with 10% horse serum. Next, cells were washed 6 times 5 minutes in NET-gel and incubated for one hour with Alexa Fluor 488-conjugated goat anti-mouse IgG2b (1:300) and Alexa Fluor 555-conjugated goat anti-mouse IgG1 (1:200) secondary antibodies (A21141, A21127; Invitrogen). Subsequently, cells were washed 2 times for 5 minutes with NET-gel and stained for an additional 5 minutes with DAPI. Afterwards, cells were washed 4 times 5 minutes in PBS and mounted on slides with mowiol. Cells were evaluated using fluorescence microscopy (Axiovert 200M, Zeiss) at room temperature with a 20x objective (Zeiss LD ACHROPLAN) with an NA of 0.4 and a 40x objective (Zeiss LD ACHROPLAN) with an NA of 0.6. A Zeiss AxioCam HRM camera was used together with Zeiss AxioVision Rel. 4.4 acquisition software to take pictures. WCIF ImageJ software was used to convert .zvi files to .jpeg files.

4.2.6 Statistical analysis

All data are expressed as mean and their standard deviation (SD). BrdU measurements on Matrigel™ coating are n=5, on all other coatings n=3. All immunofluorescence analyses are n=3. Two-way ANOVA was used to compare BrdU ratios using SPSS 15.0 software. The Bonferroni criterion was used for post-hoc testing. Differences were considered significant at P values of <0.05.

Acrylamide	Bisacrylamide	Stiffness
5%	0.03%	3 kPa
10%	0.03%	14 kPa
5%	0.3%	21 kPa
10%	0.13%	48 kPa
10%	0.26%	80 kPa

Table 4.1: Stiffness of gels measured and calculated by indentation tests.

4.3 Results

MPCs (p0) were seeded on gels with a stiffness of 3, 14, 21, 48, or 80 kPa or on coverslips. Substrates were coated with Matrigel™, Entactin-Collagen-Laminin–gel (ECL-gel), collagen IV, poly-lysine and laminin. Cells were analyzed after 1, 4, 6, 8, 11 or 15 days. Gels were created at least 1 week in advance to stabilize swelling. After stabilization of hydrogel swelling, stiffness of the gels was measured and calculated using indentation tests (Table 4.1).

4.3.1 Matrigel™ coating - myoblast proliferation

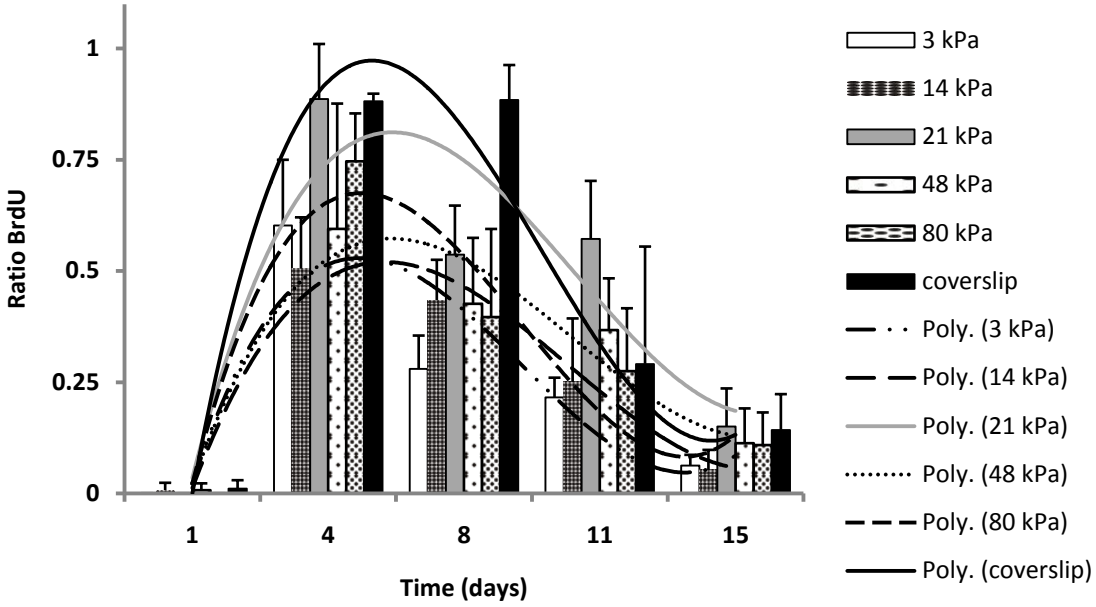
Proliferation was assessed by a BrdU assay in cells cultured on Matrigel™-coated coverslips or gels of 3, 14, 21, 48 or 80 kPa after 1, 4, 8, 11 or 15 days. Sixteen hours before each time point, BrdU was added to the medium. Proliferation did not start immediately after seeding. However, on day 4 MPCs were proliferating on all substrates, with BrdU ratios varying between 0.5 and 0.9, meaning that 50% - 90% of the cells were proliferating. Over time, proliferation gradually decreased, with a BrdU ratio between 0.1 and 0.2 at the end of the culturing period (day 15). Proliferation was found to be significantly higher and lasted longer ($P<0.01$) on glass and 21 kPa gels (close to physiological elasticity of skeletal muscle) compared to 3, 14, 48 and 80 kPa gels (Figure 4.1).

4.3.2 Matrigel™ coating - differentiation

Spontaneous differentiation of MPCs into muscle was analyzed on Matrigel™-coated 3, 21 or 80 kPa gels or coverslips by examining expression of differentiation markers (MyoD and Myosin heavy chain (MHC)). As cells started to differentiate from day 4 onward, we evaluated differentiation 4, 6, 8, 11 or 15 days after plating. MyoD is a transcription factor involved in early differentiation, but early MyoD expressing myoblasts are still capable of proliferation. After expression of MHC and fusion into myotubes, MyoD expression continues for a short period of time. However, fused cells can no longer proliferate.

In the present study, differentiation had already started on day 4, with expression of the differentiation markers clearly visible, although not in all cells. At this point in time, only a small number of cells was MyoD+/ MHC+, but in time the proportion of double positive cells increased as differentiation and maturation progressed (Figure 4.2). In addition, a large proportion of single cells was MyoD+, representing active myoblasts. Because of low seeding density, cells grew in colonies. Only few colonies were completely MyoD negative, whereas the majority of the colonies contained both MyoD+ and MyoD- cells or only MyoD+ cells. No difference was found in the percentage of MyoD+ colonies across the different elasticities.

Maturation was analyzed by assessing presence of cross-striations and spontaneous contractions. Myotubes cultured on gels never demonstrated spontaneous contractions and myotubes cultured on coverslips only sporadically contracted. Occasionally, contractions caused myotubes to detach, but only from coverslips. Cross-striations were almost always present in myotubes grown on coverslips, albeit never in myotubes grown on gels (Table 4.2).



	3 kPa	14 kPa	21 kPa	48 kPa	80 kPa	coverslip
3 kPa			P < 0.01			P < 0.01
14 kPa			P < 0.01			P < 0.01
21 kPa	P < 0.01	P < 0.01		P < 0.05	P < 0.01	
48 kPa			P < 0.05			P < 0.01
80 kPa			P < 0.01			P < 0.01
coverslip	P < 0.01	P < 0.01		P < 0.01	P < 0.01	

Figure 4.1: A) Proliferation of MPCs on Matrigel™ coated gels (3 kPa, 14 kPa, 21 kPa, 48 kPa, 80 kPa) or coverslips. BrdU ratio was calculated in cells cultured on different elasticities at several time points (1, 4, 8, 11, 15 days). Curves are fitted through the data to visualize progress of proliferation over time (Poly). B) Statistical analyses of differences in both level and duration of proliferation of MPCs cultured on different elasticities. N=5 for all groups. Data were analyzed with two-way ANOVA.

	Matrigel™		ECL-gel		Collagen IV		Poly-lysine		Laminin	
3 kPa	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
21 kPa	0%	0%	33%	17%	0%	0%	66%	33%	66%	33%
80 kPa	0%	0%	33%	17%	0%	0%	33%	17%	33%	33%
coverslip	66%	17%	100%	17%	100%	33%	100%	100%	100%	100%

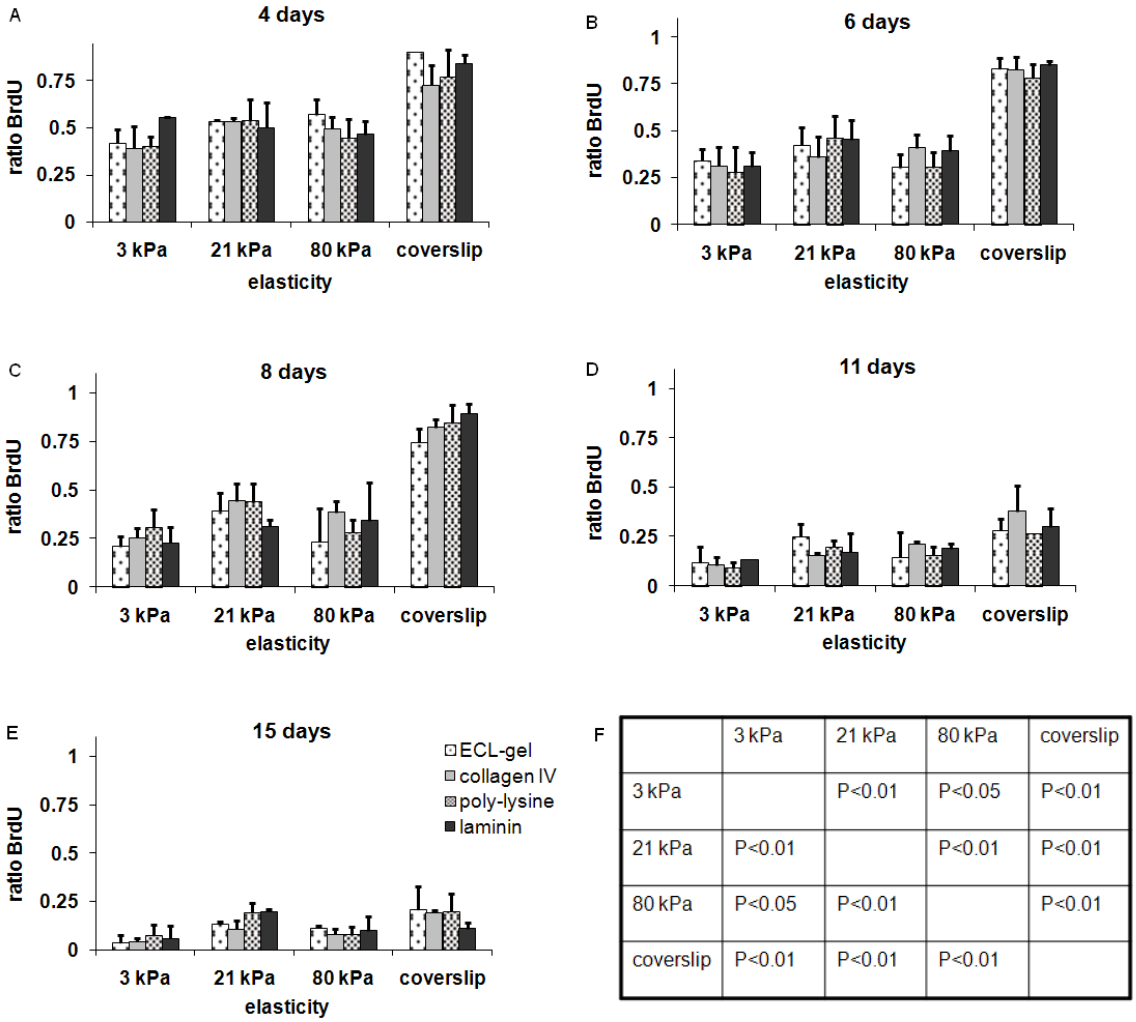
Table 4.2: Percentage of samples showing cross-striations (n=3; left cell) and spontaneous contractions (n=6; right cell) of myotubes on gels and coverslips with different coatings on day 15.

4.3.3 Basement membrane components - myoblast proliferation

MPCs were cultured on gels with a stiffness of 3, 21 or 80 kPa or on coverslips. Gels and coverslips were coated with ECL-gel (similar to Matrigel™, but purified for specific proteins), collagen IV, poly-lysine or laminin and cultures were analyzed after 4, 6, 8, 11 or 15 days. Proliferation of MPCs significantly differed between all elasticities, both in level and duration. Proliferation was highest on coverslips, followed by 21 kPa, and then 80 kPa and 3 kPa. No differences were found between the different coatings (Figure 4.3). However, proliferation ratios on these purified basement membrane components were lower than on Matrigel™ coating (0.5 versus 0.8 for Matrigel™, Figures 4.1 and 4.3).

4.3.4 Basement membrane components - myoblast differentiation

Differentiation of MPCs into myotubes was analyzed on 3, 21 or 80 kPa gels and coverslips and after 4, 6, 8, 11 or 15 days by MyoD and MHC expression. In agreement with cells growing on Matrigel™, differentiation of MPCs on the different coatings also started on day 4, with detectable expression of MyoD in the nucleus and MHC in the cytoplasm. Cells grew in colonies as a consequence of low density seeding. The proportion of MyoD+ and MyoD- cells was similar for different coatings and elasticities and comparable to the results with Matrigel™ coating. In addition, no differences in timing of differentiation were seen between different coatings and elasticities. However, at later time points, the number of myotubes formed was higher on laminin and poly-lysine coated substrates as compared to cells grown on ECL-gel and collagen IV coated substrates (Figure 4.4). Formation of more myotubes resulted in smaller myotubes (Figure 4.4).



F

	3 kPa	21 kPa	80 kPa	coverslip
3 kPa		P<0.01	P<0.05	P<0.01
21 kPa	P<0.01		P<0.01	P<0.01
80 kPa	P<0.05	P<0.01		P<0.01
coverslip	P<0.01	P<0.01	P<0.01	

Figure 4.3: Proliferation after 4 (A), 6 (B), 8 (C), 11 (D) or 15 (E) days of MPCs on 3, 21 or 80 kPa gels and coverslips with coatings of ECL-gel, collagen IV, poly-lysine and laminin. F) Statistical analysis of differences in both level and duration of MPC proliferation on different elasticities. Data were analyzed with two-way ANOVA. N=3 for all groups. No significant differences were found between coatings.

Maturation, as analyzed by MHC cross-striations and spontaneous contractions depended on substrate elasticity. Irrespective of coating, spontaneous contractions were observed in myotubes cultured on coverslips, sometimes in myotubes on gels of 80 and 21 kPa and were never observed in myotubes on 3 kPa gels (Table 4.2). The incidence of cross-striations and contractions was noticeably affected by coating. Myotubes grown on laminin- and poly-lysine-coated substrates had the highest presence of spontaneous contractions, whereas myotubes on ECL-gel-, collagen IV- and

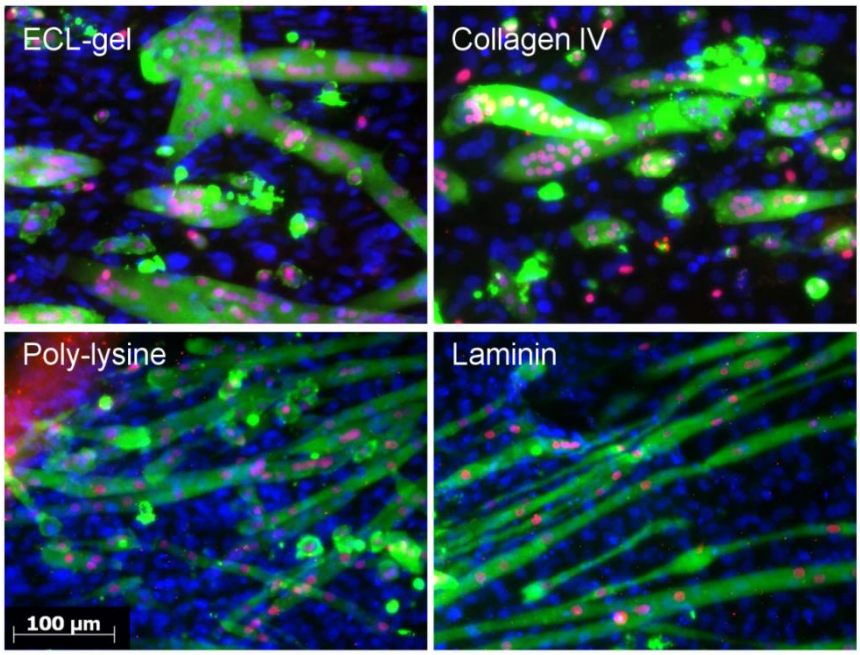


Figure 4.4: Staining of myotubes on coverslips with different coatings, cultured for 11 days. Myosin (green), MyoD (red) and nuclei (blue) are visualized. Magnification was 200x and the bar indicates 100 µm.

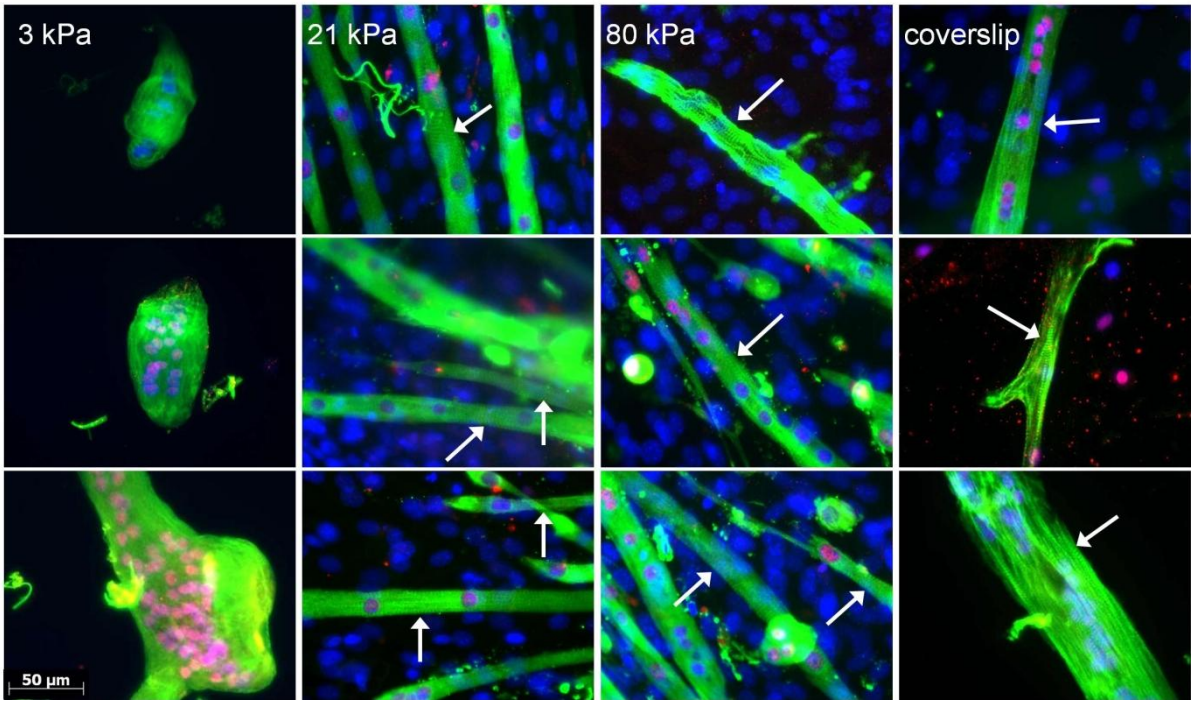


Figure 4.5: Cross-striations in myotubes cultured on different elasticities. SCs cultured on different elasticities were stained after 11 or 15 days for MHC (green) MyoD (red) and nuclei (blue). Pictures were taken at 400x magnification and the bar indicates 50 µm. Arrows point to cross-striations.

Matrigel™-coated substrates only incidentally demonstrated spontaneous contractions (Table 4.2). In addition, none of the myotubes on gels coated with collagen IV or Matrigel™ spontaneously contracted (Table 4.2). Spontaneous contractions also incidentally caused cell sheets to detach from coated coverslips, which did not occur on gels. Cross-striations were observed from day 11 onward in cells cultured at elasticities > 3 kPa, and never at 3 kPa (Figure 4.5). On coverslips cross-striations were invariably present, regardless of protein coating. On 21 and 80 kPa gels their presence depended on the coating. On these elasticities, Matrigel™ or collagen IV coating never resulted in cross-striations (Table 4.2).

4.4 Discussion

MPCs are a promising cell source for regenerative medicine. However, *in vitro* they have shown decreased proliferation and differentiation potential after passaging (Machida et al. 2004). In addition, repair of damaged muscles is much less successful when implanted cells have been cultured *in vitro* compared to implantation directly after isolation (Collins et al. 2005). It was hypothesized that changes in these cells occur because of loss of crucial stem cell niche components (Boonen and Post 2008). For applicability of MPCs in regenerative medicine both proliferation and differentiation need to be improved. To assess the role of biochemical and biophysical cues from the satellite cell niche in MPC proliferation and differentiation, the effects of substrate elasticity and matrix proteins were investigated separately and combined.

Proliferation was primarily influenced by substrate elasticity, and not by protein coating. Proliferation of MPCs was significantly higher and for a longer duration on coverslips and 21 kPa gels compared to 3, 14, 48 and 80 kPa gels. The optimum elasticity of approximately 21 kPa corresponds nicely to the physiological elasticity of skeletal muscle (Collinsworth et al. 2002). Substrate elasticity has previously been shown to influence proliferation of MPCs (Boontheekul et al. 2007; Moreo et al.). Boontheekul and co-workers compared proliferation of MPCs on 1, 13 and 45 kPa and showed increasing proliferation with increasing stiffness (Boontheekul et al. 2007), whereas we found the optimum to be 21 kPa. Our data suggest that the temporal profile of proliferation might vary between surfaces of different elasticity. In the experiment by Boontheekul et al. the cell number on 13 kPa gels was still increasing on day 8, whereas it had already stabilized on 45 kPa gels. We observed similar results on day 8 after which the curves for 14 and 48 kPa crossed each other, eventually ending up with higher proliferation in the 14 kPa gels. Although an optimal elasticity can be observed, cells on coverslips grow equally well or even better. For cell expansion purposes culturing on classic surfaces therefore seems optimal.

In our hands, MPC proliferation was independent of coating. Reported effects of coating on MPC proliferation are controversial. Similar to our findings, mouse myoblasts of passage 0 had a proliferation rate that was independent of protein coating on tissue culture plastic (collagen IV, laminin, Matrigel^{TM}) (Maley et al. 1995). In contrast, it has also been reported that proliferation was stimulated by different laminin isoforms (Yao et al. 1996; Grossi et al. 2007; Macfelda et al. 2007; Silva-Barbosa et al. 2008) and collagen IV (Macfelda et al. 2007) compared to BSA (Silva-Barbosa et al. 2008) or tissue culture plastic (Macfelda et al. 2007). The reason for this discrepancy is currently not clear.

Once expanded, the MPCs need to differentiate into myotubes, develop typical cross–striation and show contraction patterns. Early effectors in this differentiation track are MyoD and MHC, which are expressed when myoblasts start to differentiate, although MyoD+ cells can stay in this phase and proliferate for some time. We observed no difference in temporal profiles of MyoD and MHC expression and fusion of myoblasts into myotubes by MPCs cultured on varying elasticities and coatings. As a consequence of growth in colonies, we could distinguish MyoD/MHC positive cells form populations that were MyoD-/MHC-, suggesting differentiation of MPCs along different lineages or some level of heterogeneity in these primary cells. Obviously, a certain degree of heterogeneity already existed after isolation of the cells. Although the type of isolation efficiently gives rise to a relatively pure MPC population, one cannot rule out other types of cells (e.g. fibroblasts) being co-isolated. In a clinical study for instance, a “pure” myoblast population still consisted of 20% cells that were negative for CD56 or desmin (Vilquin et al. 2005). These cells are probably responsible for part of the MyoD$^-$/MHC$^-$ colonies. In addition, myoblast from different muscle groups may consist of populations with distinct phenotypes (Petersen and Huard 2000). However, because of the existence of mixed colonies, differentiation of MPCs into MyoD$^-$/MHC$^-$ cells cannot be excluded.

Spontaneous contractions and typical myosin cross-striations (necessary for contractions and indicative of maturation of myotubes) occurred in myotubes on gels independent of elasticity, but not in myotubes on 3 kPa gels. On coverslips, cross-striations and contractions were most prominent, although contractions sometimes caused cell sheets to detach from coated coverslips. Since this never happened on gels, this is an advantage of culturing cells on gels. As myoblasts differentiate by confluency, the higher proliferation rates on coverslips might have accounted for the difference in maturation. However, local confluency in colonies is just as high, suggesting other mechanisms to be more important. Maturation of C2C12 myoblasts (Engler et al. 2004b) has previously been shown to be influenced by substrate elasticity. Comparable to our results, Engler and co-workers found that although early fusing of myoblasts into myotubes took place regardless of the stiffness, differentiation and especially maturation of C2C12 myoblasts depended on the stiffness of the substrate. However, they demonstrated that cross-striations were only formed in myotubes cultured on 8 and 11 kPa gels, and not in

myotubes on 1 and 17 kPa gels, suggesting a very narrow elasticity window. One potentially important difference with our setup is the method of measuring the E-modulus of gels. With similar ratios of acrylamide/bisacrylamide, our tests show different E-moduli. For example, 5% acrylamide mixed with 0.3% bisacrylamide, results in 8.1 kPa in their experimental setup, whereas in our hands, an E-modulus of 21 kPa was measured after stabilization of hydrogel swelling. At these apparent higher stiffness values we observed cross-striations. From both studies it has become clear that a minimum stiffness is required to mature myotubes into cross striated muscle.

For different coatings, we demonstrated that MPCs grown on laminin and poly-lysine coated substrates resulted in more and thinner myotubes compared to myotubes on ECL-gel, collagen IV and Matrigel^{TM} coating. Previously, the opposite has been shown in mouse primary cultures, where Matrigel^{TM} gave rise to more myotubes than laminin and collagen IV after 4 days of culture, whereas a myogenic cell line formed equal amounts of myotubes on all coatings (Maley et al. 1995). Direct comparison with our experiments is confounded by the difference in culture time and the use of non growth factor reduced Matrigel^{TM}. More recently and in line with our results, it was shown that mechanical stimulation through the laminin-receptor increased differentiation compared to stimulation through the fibronectin receptor, inducing more and smaller myotubes in C2C12 myoblasts (Grossi et al. 2007). The positive influence of mechanical stimulation indicates a role for mechano-transduction in this part of the differentiation process.

However, the most important difference between these studies is passage number. We used cells without prior expansion, hence passage 0 at the time of seeding. Apparent from implantation experiments, even short periods of culturing on tissue culture plastic changes MPC phenotype and causes loss of regenerative capacity (Collins et al. 2005). In addition, when C2C12 cells were compared to primary (precultured) cells during culture on substrates of different elasticities, the effect of substrate elasticity was found to be much larger on primary cells compared to C2C12 myoblasts (Boontheekul et al. 2007). Moreover, in another experiment it was shown that a discrepancy exists between primary cells and cell lines in effects of coating on proliferation and differentiation (Maley et al. 1995). These results indicate that a significant difference possibly exists between primary cultures and cell lines that could also explain the difference between the results of Engler and co-workers and ours.

The effects of various coating and elasticity conditions were mainly temporal differences, with the exception of the lack of maturation at very low elasticity. For instance, on day 11, it became clear that laminin and poly-lysine coating gave rise to more and smaller myotubes and that ECL-gel, Matrigel^{TM} and collagen IV resulted in bigger but less myotubes. In addition, on gels coated with Matrigel^{TM} and collagen IV myotubes never showed any cross-striations, whereas myotubes on coated coverslips

did. However, contractions were seen on Matrigel™ coated 21 kPa gels that were cultured longer (unpublished data), so we believe that progression and speed of maturation is the most important distinction between the different conditions. Overall, we provided evidence that laminin or poly-lysine coated coverslips and 21 and 80 kPa gels are the best stimulus for differentiation of myotubes.

In conclusion, substrate elasticity influences both myoblast proliferation and differentiation, whereas basement membrane protein coating of these substrates only has an additive effect on the differentiation/maturation of these cells. The data presented here can be used to optimize culture conditions for future regenerative medicine applications.

4.5 Acknowledgements

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Chapter 5

Interaction between electrical stimulation, protein coating and matrix elasticity: A complex effect on muscle maturation

This chapter is based on: Kristel J.M. Boonen, Frank P.T. Baaijens, Daisy W.J. van der Schaft, Mark J. Post, *Interaction between electrical stimulation, protein coating and matrix elasticity: A complex effect on muscle maturation*.

5.1 Introduction

The success of tissue engineering of skeletal muscle so far has been limited. Although researchers have succeeded in producing 3D constructs (Shansky et al. 1997; Okano and Matsuda 1998b; Dennis and Kosnik 2000) and have achieved vascularization (Levenberg et al. 2005; Bach et al. 2006) and partial repair in defects (De Coppi et al. 2006; Boldrin et al. 2007; Boldrin et al. 2008), the formation of functional, mature skeletal muscle tissue remains a challenge (Bach et al. 2004; Koning et al. 2009).

To enhance the differentiation of muscle progenitor cells (MPCs) into functional muscle tissue, we focus on mimicking the niche, which means reproducing the natural *in vivo* environment of these cells *in vitro* (Boonen and Post 2008). Previously, we have demonstrated the influence of extracellular matrix protein coating and substrate stiffness on proliferation, differentiation and maturation of MPCs (Boonen et al. 2009). Maturation (characterized by cross-striations and spontaneous contractions), only occurred on elasticities higher than or equal to 10 kPa and was better in myotubes cultured on laminin- and poly-lysine-coated substrates, than on Matrigel^{TM}-, Collagen IV- and ECL-gel-coated substrates. In addition, culturing on coverslips and 21 kPa gels, which is close to physiological elasticity of muscle (Collinsworth et al. 2002), increased proliferation most.

Another important environmental signal during embryogenesis (Wilson and Harris 1993) as well as in the adult muscle (Midrio 2006) is electrical stimulation by nerves. Obviously, introduction of electrical stimulation under culture conditions can be used to simulate part of the natural environment in muscle and thereby stimulate formation and maturation of myotubes. Indeed, it has been shown that electrical stimulation can lead to enhanced sarcomere formation (Dusterhoft et al. 1999; Kawahara et al. 2006; Fujita et al. 2007) and myosin isotype switches to more adult forms (Dusterhoft et al. 1999; Bayol et al. 2005).

Numerous tissue engineering strategies use the C2C12 myoblast cell line or other immortalized cells as a model system. However, it has become clear that large differences exist between cells of primary origin and cell lines (Maley et al. 1995; Boontheekul et al. 2007; Boonen et al. 2009) in their response to environmental cues and their level of differentiation and maturation. We therefore used MPCs isolated from mice, with low passage numbers, since passaging has also been shown to have a detrimental effect on myoblast performance (Machida et al. 2004; Collins et al. 2005).

In this study, we combined several important environmental cues and cultured MPCs on soft gels and rigid glass coverslips coated with either Matrigel^{TM} or laminin. In addition, cells were electrically stimulated. We hypothesized synergistic effects between coating, elasticity and electrical stimulation as they might stimulate differentiation through different pathways.

5.2 Materials & methods

5.2.1 MPC isolation and culture

MPCs were prepared from single fibers from intact hindlimb muscles (EDL, TP, TA and soleus) as described (Shefer et al. 2004; Collins et al. 2005). Briefly, whole muscles were dissected from 8 to 10-week-old male C57BL/6 mice and were digested for 1 hour with 2 mg/ml (567 U/ml) collagenase type I (Sigma-Aldrich, St. Louis, USA). Afterwards, the muscles were passed through pipettes of decreasing diameter in culture medium consisting of Dulbecco's modified Eagle medium (DMEM) Advanced (Invitrogen, Carlsbad, USA) containing 20% Fetal Bovine Serum (FBS; Greiner Bio-One, Frickenhausen, Germany), 10% Horse Serum (HS; Invitrogen, Carlsbad, USA), 100,000 IU/l penicillin and 100 mg/l streptomycin (Lonza, Walkersville, USA) and 4 mM L-Glutamine (Lonza), resulting in a single fiber suspension. Single fibers were then triturated with a 19G needle for 5 minutes and passed through a 40 µm cell sieve. The resulting single cell suspension was frozen in culture medium containing 10% DMSO (Merck, Schiphol-Rijk, The Netherlands) in liquid nitrogen until use. 1000 cells/cm^2 were cultured on top of gels in culture medium, which was changed every 2/3 days. This animal study was authorized by the Dutch institutional animal care and usage committee.

5.2.2 Coated polyacrylamide (PA) gels and coverslips

PA gels were created using a method adapted from Pelham and Wang 1997. In short, *N,N',N'*-methylene-bis-acrylamide (0.03%, or 0.3%) (Sigma-Aldrich) was mixed with acrylamide (5%) (Sigma-Aldrich) and cross-linked with 10% ammonium persulfate (APS) (1/200 vol/vol; Fisher, Pittsburgh, USA) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) (1/2000 vol/vol; Merck) to produce 3 kPa and 21 kPa gels respectively (Boonen et al. 2009). 100 µl Droplets of the solution were placed on a teflon surface and covered by ø 13 mm aminosilanized (with (3-aminopropyl)trimethoxysilane, Sigma-Aldrich) glass coverslips (Menzel, Braunschweig, Germany) for polymerization. Protein coatings of growth factor reduced Matrigel^{TM} (15 µg/cm^2) or Poly-D-lysine (180 µg/cm^2) (Sigma-Aldrich) were cross-linked to the inert surface of the gels with heterobifunctional sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH) (Pierce Biotechnology, Rockford, USA). Laminin (15 µg/cm^2) (BD Biosciences) was adsorbed to poly-D-lysine (Sigma-Aldrich), because it was not suitable for cross-linking. Adsorption was also used for coating coverslips without gels. All proteins were added in excess to ensure equal coating. Cells were seeded at low density (approximately 1000 cells/cm^2).

5.2.3 Electrical stimulation

The C-Pace Culture Pacer (IonOptix Corporation, Milton, USA) was used for bipolar field stimulation. Cells underwent 48 hours of continuous pacing with 10 V pulses of 6 ms at a frequency of 2 Hz. Culture medium was changed every 24 hours during stimulation. The electrical stimulation protocol was introduced at 11 or 13 days after cell seeding, when small myotubes have been formed. After 48 hours of stimulation, cells were allowed to recover for another 48 hours.

5.2.4 Immunocytochemistry

Cells were washed with PBS (Sigma-Aldrich), fixed with 10% formalin (Sigma-Aldrich) for 10 minutes, and permeabilized with 1% Triton-X-100 (Merck) in PBS for 30 minutes. Subsequently, cells were incubated twice for 15 minutes with 1% horse serum (HS, Invitrogen) in PBS to block non-specific binding and washed 2 times 15 minutes with NET-gel (50mM Tris pH 7.4, 150 mM NaCl, 5mM EDTA, 0.05% NP40, 0.25% gelatin). Afterwards, they were incubated for 2 hours with antibodies against myosin heavy chain (1:300) (MF20, developed by D.A. Fischman and obtained from the Developmental Studies Hybridoma Bank, maintained by The University of Iowa) and sarcomeric α-actinin (1:800) (clone EA-53, Sigma-Aldrich) in NET-gel with 10% HS. Next, cells were washed 6 times for 5 minutes in NET-gel and incubated for one hour with Alexa Fluor 488-conjugated goat anti-mouse IgG2b (1:300) and Alexa Fluor 555-conjugated goat anti-mouse IgG1 (1:200) secondary antibodies (A21141, A21127; Invitrogen). Subsequently, cells were washed 2 times for 5 minutes with NET-gel and stained for an additional 5 minutes with 4', 6-diamidino-2-phenylindole (DAPI) (0.1 mg/ml) (Sigma-Aldrich). Afterwards, cells were washed 4 times 5 minutes in PBS and mounted on slides with mowiol (Calbiochem, San Diego, USA). Cells were evaluated using fluorescence microscopy (Axiovert 200M, Zeiss).

5.2.5 Quantification of cross-striations

Cross-striations were quantified by scoring the total number of tubes and the number of myosin and α-actinin cross-striated tubes at 5 different, independent locations in each sample. The observer was blinded to the conditions and treatments of the samples. Percentages of myotubes containing cross-striations were calculated and normalized to control (non-stimulated) samples.

5.2.6 Quantitative PCR

Total RNA was isolated using an RNeasy™ midi kit (Qiagen, Venlo, The Netherlands) according to manufacturer's protocol. RNA was amplified and converted to cDNA using

the QuantiTect™ whole transcriptome amplification (Qiagen) consistent with the handbook. Linear amplification was validated. cDNA samples were stored at -30°C until use for quantitative PCR (qPCR). Stability of reference genes was investigated using a geNorm reference gene selection kit (PrimerDesign, Southampton, UK). The two most stable reference genes were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB). Primer sets for MyoD, myogenin, sarcomeric actin, α-actinin (Sigma-Aldrich, St. Louis, USA) and perinatal myosin heavy chain (MHC-pn, PrimerDesign, Southampton, UK) were developed and optimized for qPCR (Table 6.1). The qPCR reaction (MyiQ, Bio-Rad, Hercules, USA) was performed using SYBR Green Supermix (Bio-Rad, Hercules, USA), primers (0.5 mM), ddH_2O, and 2 µl 50x diluted cDNA. The temperature profile was as follows: 3 min 95°C, 40x (20s 95°C, 20s 60°C, 30s 72°C), 1 min 95°C, 1 min 65°C, followed by a melt curve analysis. Target gene expression was evaluated using the ΔCT method.

5.2.7 Statistical analysis

All data are expressed as mean + standard error of the mean (SEM). Immunocytochemical and qPCR analyses were performed in 3 independent experiments. A two-tailed, unpaired student's t-test was used to compare cross-striations in treated samples to controls and to compare ΔCT values of treated samples to controls. Contractions were evaluated 6 times (2 samples per group). A Pearson Chi-Square test was performed to check for differences between coatings and elasticities. Differences were considered significant at P values of <0.05.

5.3 Results

Electrical stimulation of samples was started after 11 (ES1) or 13 (ES2) days of culture, since we expected electrical stimulation to play the most important role in myotube maturation (characterized by cross-striations and spontaneous contractions). Cells were stimulated for 48 hours and allowed to recover for another 48 hours to evaluate whether differences remained present after ceasing stimulation.

5.3.1 Spontaneous contractions

Samples were carefully examined for the presence of spontaneous contractions, approximately half an hour after switching off electrical stimulation. Spontaneous contractions are often seen in MPC cultures and are defined as contractions not induced during electrical stimulation. The number of samples exhibiting contractions at the time of evaluation in both control (non-stimulated) and stimulated cultures were expressed as the percentage of the total number per experimental group (3x n=2, table 5.1).

Spontaneous contractions were never observed in myotubes cultured on 3 kPa samples and were more frequent on coverslips compared to 21 kPa gels (χ^2; P<0.001). After 11 days of culture, when the first stimulation protocol was started, no spontaneous contractions were observed. For coverslips and 21 kPa gels and for both coatings, the incidence of contractions by electrical stimulation increased directly after the first stimulation period. However, this effect was not sustained.

21 kPa	Day 0	Day 2	Day 4
Matrigel™ ES1	0	50	0
Matrigel™ ES2	66.7	16.7	33.3
Laminin ES1	0	33.3	33.3
Laminin ES2	0	16.7	33.3

coverslip	Day 0	Day 2	Day 4
Matrigel™ ES1	16.7	100	100
Matrigel™ ES2	100	100	100
Laminin ES1	50	100	100
Laminin ES2	100	100	100

*Table 5.1: Percentage of samples (3*n=2) showing spontaneous contractions at different time points. ES1 denotes electrical stimulation started on day 11 for 48 hours until day 13, after which samples were allowed to recover for 48 hours until day 15. ES2 denotes electrical stimulation started on day 11 for 48 hours until day 15, after which samples were allowed to recover for 48 hours until day 17. (For statistics see text). In the table, day 0 denotes the start of stimulation, day 2 after 48 hours of stimulation and day 4 after 48 hours recovery.*

5.3.2 Cross-striations

Myosin and α-actinin cross-striations were evaluated in stimulated and unstimulated samples on day 11, 13, 15 or 17. Cross-striations were found in myotubes cultured on all substrates, although both myosin and α-actinin cross-striations were rare and not quantifiable in myotubes cultured on 3 kPa gels. In addition, myosin cross-striations were rare in myotubes cultured on 21 kPa gels and were therefore not quantifiable either, in contrast to α-actinin cross-striations. Typical examples of α-actinin and myosin cross-striations in myotubes cultured under different experimental conditions are shown in Figure 5.1. Numbers of α-actinin cross-striated tubes in stimulated samples were expressed as percentage of the numbers in control samples for each time point (Figure

5.2). The percentage of α-actinin cross-striated tubes was approximately 50% in control samples cultured on coverslips, independent of coating and time (data not shown). On 21 kPa gels, α-actinin cross-striations were also constant over time, but were higher on Matrigel™- than on laminin-coating with 20% and 10% cross-striated myotubes respectively (data not shown). The effect of electrical stimulation on cross-striations was complex. When started late, on day 13 of culture, electrical stimulation did not affect the number of cross-striations. The effect of early stimulation, after 11 days, depended on an interaction between coating and stiffness of the substrate. On Matrigel™-coating at 21 kPa, electrical stimulation induced an early but transient enhancement of cross-striations (Figure 5.2, panel A) whereas a late enhancement (after the recovery period) was observed on coverslips (Figure 5.2, panel B). In contrast, on laminin-coated substrates, cross-striations were transiently reduced early after stimulation at 21 kPa (Figure 5.2, panel C) and late after stimulation on coverslips (Figure 5.2, panel D). In addition to α-actinin cross-striations, myosin cross-striations were also quantified in samples cultured on coverslips. The trend that was found in these percentages was comparable to the percentages found in α-actinin cross-striations (data not shown).

5.3.3 qPCR analysis

For qPCR analysis, only the samples cultured on 21 kPa gels and coverslips that had received electrical stimulation from day 11 until day 13 (ES1) were used, because only in those samples, electrical stimulation had an effect on contractions as well as cross-striations. The transcription factors myoD and myogenin and the sarcomere proteins perinatal myosin heavy chain (MHC 8), sarcomeric actin and α-actinin were examined (Figure 5.3). Gene expression on 21 kPa gels was higher than on coverslips. All genes, except for α-actinin, showed a decrease in expression immediately after 48 hours of stimulation in myotubes cultured on Matrigel™-coated coverslips compared to non-stimulated samples (Figure 5.3, panel B,D,F,J) whereas on laminin-coated coverslips, no differences were observed. In myotubes cultured on Matrigel™-coated 21 kPa gels, sarcomeric actin was upregulated after the recovery period (Figure 5.3, panel I), whereas in myotubes cultured on laminin-coated 21 kPa gels, an upregulation was found in myoD and α-actinin immediately after stimulation (Figure 5.3, panel A and G).

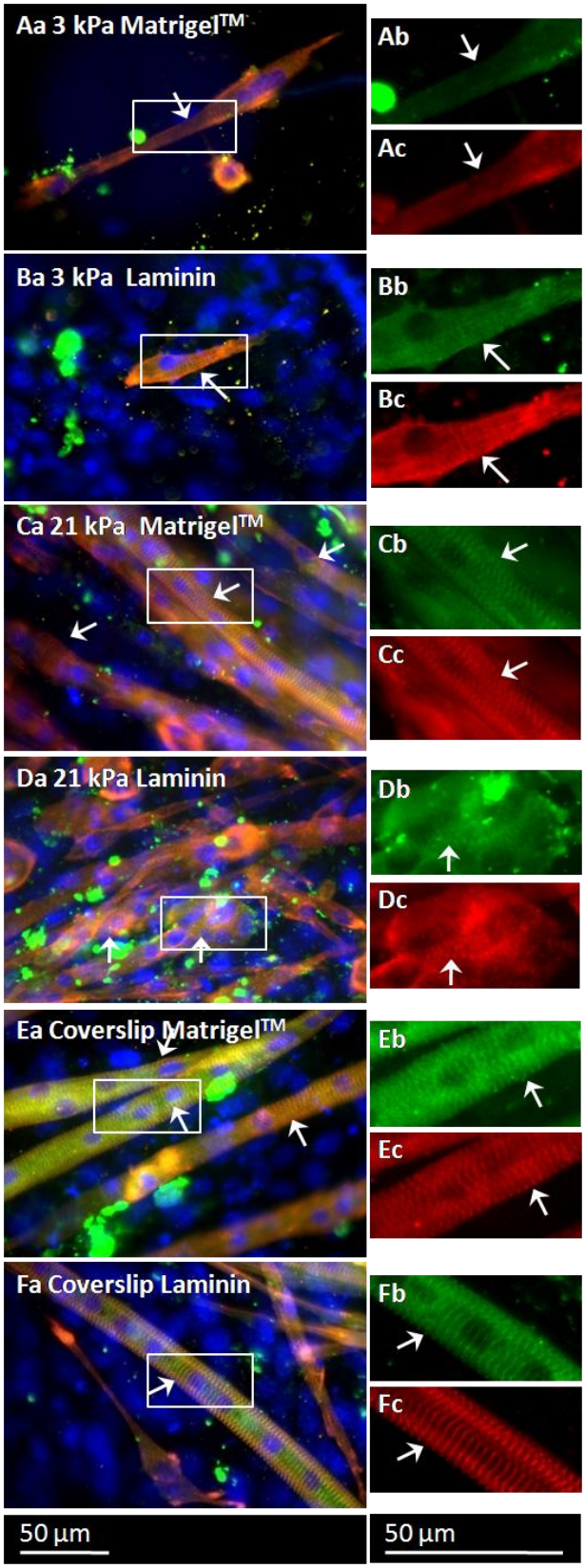

Figure 5.1: Examples of cross-striations in myotubes cultured under different experimental conditions. Panels Aa-Fa show combined pictures of samples stained for myosin, α-actinin and DAPI. Panels Ab-Fb show details of myosin cross-striations and panels Ac-Fc show details of α-actinin cross-striations.

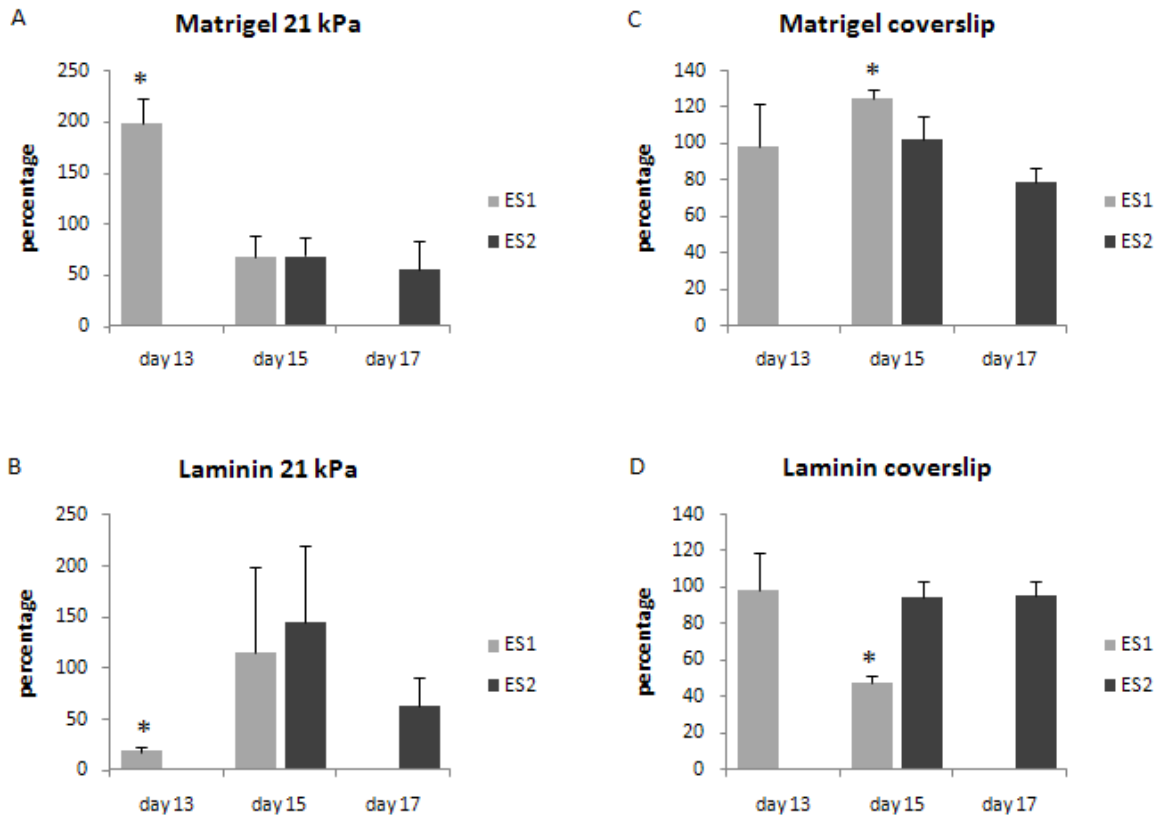

*Figure 5.2: Percentages of α-actinin cross-striations, normalized to control (non-stimulated) samples from the same time point. Panel A shows Matrigel™-coated 21 kPa gels, panel B shows Matrigel™-coated coverslips, panel C shows laminin-coated 21 kPa gels and panel D shows laminin-coated coverslips. * denotes a significant difference compared to non-stimulated controls (P<0.05) at the same time point.*

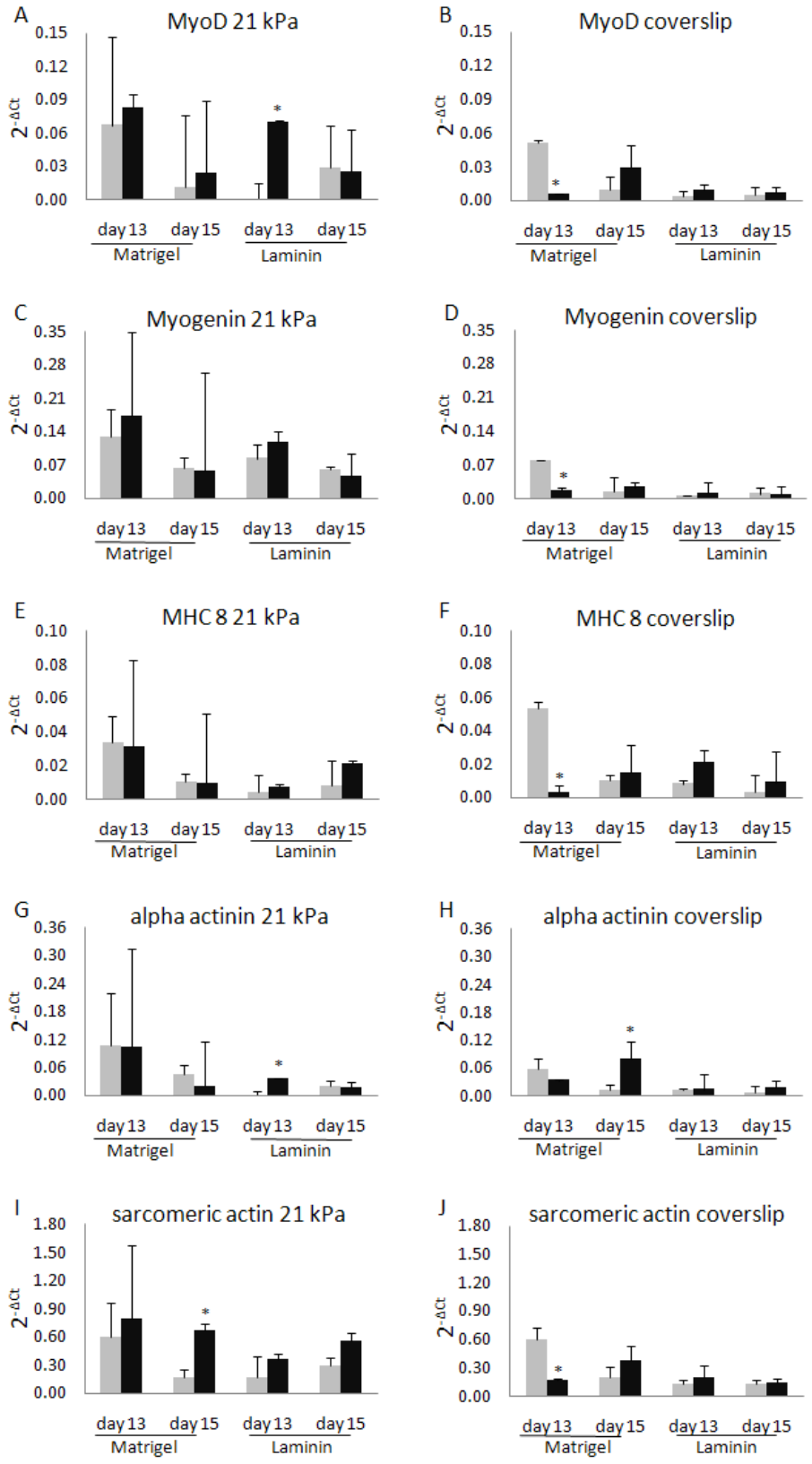

*Figure 5.3: Quantitative gene expression of different myogenic transcription factors and sarcomere proteins in myotubes after early electrical stimulation. All data are normalized to GAPDH. * denotes a significant difference compared to non-stimulated controls (P<0.05).*

5.4 Discussion

In a quest to uncover signals important for mature myofiber formation, which is indispensable in the creation of functional tissue engineered muscle, we investigated the effect of electrical stimulation on myotubes cultured on substrates with different elasticities and coatings. The most important advantage of culturing myotubes on soft substrates is that contracting tubes do not have the tendency to tear from the surface, a phenomenon we often observed in myotubes cultured on rigid coverslips. Overall, we found that a complex interaction existed between electrical stimulation, coating and substrate stiffness.

In this study, we used primary MPCs for myotube cultures as they are the designated cells to repair and regenerate skeletal muscle. A disadvantage of primary cells over well-established cell lines is the potential heterogeneity of the cell batch. We have previously shown that the proportion of myogenic cells was not influenced by substrate elasticity or protein coating (Boonen et al. 2009). The contribution of non-myogenic cells to the outcome of the different analysis was therefore not considered a confounder.

Although stimulation did not increase the ability of myofibers to contract on 3 kPa gels, on coverslips and 21 kPa gels, an advance of spontaneous contractions was caused by early stimulation, independent of coating. Spontaneous contractions were already 100% prevalent on coverslip, leaving no window for further improvement by coating or stimulation. It seemed that the beneficial effects of electrical stimulation were only temporal, which may be related with a short stimulation phase. In cell line cultures, spontaneous contractions are rarely seen in such a short culture period (Bayol et al. 2005; Fujita et al. 2007; Yamasaki et al. 2009). For example, Kawahara and co-workers found these contractions to be induced only by electrical stimulation in L6 myotubes after 18 days (Kawahara et al. 2006). However, synchronous contractions during electrical stimulation are common (Wehrle et al. 1994; Thelen et al. 1997; Bayol et al. 2005; Fujita et al. 2007; Yamasaki et al. 2009). Similar to our experiments, Cimetta and co-workers (2009) found presence of spontaneous contractions in satellite cell derived myotubes, cultured on laminin-coated soft gels after 7 days.

Appearance of cross-striations has been described as a result of electrical stimulation (Wehrle et al. 1994; Kawahara et al. 2006; Park et al. 2008). Indeed, we also observed an increase in both α-actinin and myosin cross-striations in myotubes cultured on Matrigel^{TM}-coated substrates. However, this phenomenon was shown to be dependent on coating: on laminin-coated substrates, electrical stimulation resulted in a decrease in cross-striated myotubes. In non-stimulated cultures, the number of cross-striated myotubes remained constant for both laminin and Matrigel^{TM}, suggesting an interaction between coating and electrical stimulation. This interaction was further affected by substrate stiffness. On 21 kPa gels, an initial difference in the amount of cross-striations existed between coatings, and this difference persisted during stimulation. It therefore

appears that the combination of Matrigel™ coating on coverslips is best. Regardless of electrical stimulation, no cross-striations were observed on 3 kPa gels. Hence, we were unable to change the elasticity threshold for maturation as previously found (Boonen et al. 2009) by electrical stimulation.

In these primary cultures, even without stimulation, formation of sarcomeres is much faster than in myoblast cell lines, which complicates the comparison of our cross-striation data to other studies. Whereas MPCs readily form α-actinin cross-striations and form myosin cross-striations relatively easily (Cimetta et al. 2009; Boonen et al. 2009), C2C12 myoblasts normally only show rather immature α-actinin cross-striations (Fujita et al. 2007) and myosin cross-striations only under special circumstances (Engler et al. 2004b). The increase observed in cross-striations in C2C12 myoblasts under the influence of electrical stimulation can therefore be quite large (Kawahara et al. 2006; Fujita et al. 2007; Park et al. 2008), whereas the improvement we found in MPC maturation was modest. Similar to our observations, Wehrle and co-workers also used primary myoblasts and found not more, but stronger myosin cross-striations in stimulated myotubes compared to control samples (Wehrle et al. 1994). In their experiments, the effect of electrical stimulation was shown to be temporal, similar to our observation of myotubes cultured on 21 kPa gels, where the changes faded out during the recovery period. The most striking difference between myotubes cultured on coverslips and 21 kPa gels is the difference in timing of the effect of electrical stimulation on cross-striations. The magnitude of the effect is similar, but on 21 kPa gels the effect was manifest directly after stimulation, while on coverslips it only became apparent after the 48 hour recovery period. No obvious explanation exists for this phenomenon, especially because this difference was not observed in any of the other analyses. Thus, it appears that the response to electrical stimulation is most efficient on substrates with an elasticity of 21 kPa, which is close to physiological stiffness of muscle (Collinsworth et al. 2002). A similar effect was shown for cell motion (Reinhart-King et al. 2008).

Similar to the cross-striations, we only found differences in gene expression level of MRFs and sarcomeric proteins caused by electrical stimulation on Matrigel™ coated substrates. The decrease we found in MHC8 expression due to electrical stimulation of samples cultured on Matrigel™-coated coverslips suggests a maturation in MHC isoforms, a result which is found more often in myotubes stimulated by electrical stimulation (Wehrle et al. 1994; Bayol et al. 2005). The decrease we found in myoD and myogenin was also found by Stern-Straeter and co-workers in primary myoblasts stimulated while cultured in fibrin constructs (Stern-Straeter et al. 2005) and could point to an increase in differentiation. All the observed decreases in transcription factors were transient, suggesting a reversibility of electrical stimulation induced maturation, with the setting that we used.

When Matrigel™-coated samples were compared to laminin-coated samples, a remarkable difference was found in the response to electrical stimulation. Except for the presence of spontaneous contractions, results show opposite effects of electrical stimulation in myotubes cultured on laminin versus Matrigel™-coated substrates. Whereas stimulated samples cultured on Matrigel™, showed an increase in maturation (augmented percentage of cross-striations and diminished expression of MHC8, myoD and myogenin), stimulated samples cultured on laminin showed a decrease in maturation (loss of cross-striations and no change in gene expression profiles). This is even more remarkable, since laminin makes up 80% of the protein fraction of Matrigel™. As far as the authors are aware, this is the first study that compares the effects of electrical stimulation in myotubes cultured on different elasticities and coatings. In stretch experiments performed by Grossi and co-workers, it was demonstrated that stimulation through laminin receptors led to an increase in differentiation when compared to stretch through fibronectin receptors (Grossi et al. 2007). Clearly, electrical stimulation-induced contractions of cells on different coatings can lead to different pathways being activated. Without electrical stimulation, Matrigel™ is found to be superior to other coatings or no coating (Maley et al. 1995; Langen et al. 2003) for induction of muscle maturation. However, we (Boonen et al. 2009) and others (Cimetta et al. 2009) have found that in standard culture conditions, maturation (observed cross-striations and contractions) is best on laminin-coated substrates. In general, it has been shown that at least some form of extracellular matrix (ECM) is indispensible for differentiation (Melo et al. 1996; Osses and Brandan 2002), although the separate roles of different ECM proteins remain unclear. Possibly, attachment to laminin is worse than to other ECM components, such as collagen IV -also present in Matrigel™- leading to problems during stimulation. Indeed, it was shown that when the expression of α7 integrin, which in combination with integrin β1 is the most important linker between laminin and the cell, was enhanced in C2C12 myoblasts, adhesion to laminin was improved (Liu et al. 2008). Stimulation of α7β1-integrin by matrix binding also protected against muscle damage, likely by inhibiting apoptosis signaling (Boppart et al. 2006). In all studies, however, the laminin-1 isoform has been used, whereas the most common isoform in skeletal muscle is laminin-2. Although primary myoblasts are shown to bind with similar affinities to both isoforms (Schuler and Sorokin 1995), signaling through stimulation may very well differ between the two.

In conclusion, we have shown that the effects of electrical stimulation on the maturation of MPC-derived myotubes are coating- and elasticity-dependent. However, the beneficial effects are modest and transient, so that with the current regime, electrical stimulation does not seem to be essential for full maturation of tissue engineered skeletal muscle from MPCs.

5.5. Acknowledgements

This work was financially supported by SenterNovem, grant ISO 42022. The authors would like to thank Leonie B.P. Niesen and Jo W. Habets for taking care of the animals. The MF20 antibody developed by Donald A. Fishman 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.

Chapter 6

Electrical stimulation of muscle constructs: Remarkable differences in response between C2C12 and primary muscle progenitor cells

This chapter is based on: Marloes L.P. Langelaan, Kristel J.M. Boonen, Kang Yuen Rosaria-Chak, Daisy W.J. van der Schaft, Frank P.T. Baaijens, Mark J. Post. *Electrical stimulation of muscle constructs: Remarkable differences in response between C2C12 and primary muscle progenitor cells.*

6.1 Introduction

Tissue engineering of skeletal muscle has many applications in the field of regenerative medicine, for example transplantation purposes (Boldrin et al. 2008) and gene therapy (Vandenburgh et al. 1996). Less evident is the possible use of tissue engineered muscle for *in vitro* meat production (Edelman et al. 2005). Although this type of meat production was already envisioned by Winston Churchill as early as 1932 (Churchill 1932), the first actual tissue engineering approaches have only started at the end of last century (Van Eelen et al. 1999).

The prerequisites for engineered skeletal muscle to be suitable for implantation or consumption are surprisingly similar. For both applications, muscle fibers need to be densely packed and aligned. This architecture of muscle cells provides the tissue with a structure that can either resist forces and manipulation during implantation, but also offers the desired texture for consumption meat. Skeletal muscle matures and organizes into bundles of sarcomeres, which is inevitably required for regenerative medicine, but presumably also of importance for meat texture.

Three dimensional (3D) tissue engineering strategies are typically based either on carriers made up of biodegradable polymer scaffolds or gels. Scaffolds can be modified to possess excellent mechanical properties and architecture which can be modulated to obtain the desired characteristics (Saxena et al. 2001; Fuchs et al. 2003; Levenberg et al. 2005). The gel systems are mostly based on collagen (Okano and Matsuda 1998b; Vandenburgh et al. 1998; Cheema et al. 2003; Gawlitta et al. 2008) or fibrin (Bach et al. 2003; Matsumoto et al. 2007; Bian and Bursac 2009). In our opinion, gel systems are more suitable for tissue engineering of skeletal muscle for consumption purposes compared to scaffolds, because they allow for dense cell seeding and easy alignment (Bian and Bursac 2008), without remains of synthetic degradation products that would be present in a scaffold system. However, the creation of an open architecture is more challenging in these gel systems and would be easier to realize in scaffolds, for example by low-temperature electrospinning methods (Simonet et al. 2007).

To approach our tissue engineering goals, we aim at mimicking the *in vivo* niche to improve tissue engineered skeletal muscle (Boonen and Post 2008). We have previously shown that substrate stiffness and coating play an important role in myogenic progenitor cell (MPC) proliferation, differentiation, and maturation in a 2D monolayer of cells (Boonen et al. 2009). Another important part of the niche environment, during development (Wilson and Harris 1993) as well as in adult muscle (Midrio 2006), is electrical stimulation by nerves. In fact, we (Langelaan et al. 2009, Chapter 5) and others (Dusterhoft et al. 1999; Kawahara et al. 2006; Fujita et al. 2007) have shown that electrical stimulation used in 2D culture leads to an advance in differentiation and maturation, most notable by more and earlier presence of cross-striations and

contractions and a switch into more mature isoforms of myosin heavy chain (MHC) (Naumann and Pette 1994).

In the 3D situation, electrical stimulation has also been shown to affect maturation (Pedrotty et al. 2005; Park et al. 2008; Serena et al. 2008; Yamasaki et al. 2009). However, these approaches were either scaffold based and/or made use of the C2C12 cell line. The advantages of using C2C12 myoblasts are obvious: easy expansion and culture conditions. However, the translation to primary cells is questionable as differences have been shown between cell lines and primary cells in their response to environmental cues (Maley et al. 1995; Boontheekul et al. 2007). Although the expansion of MPCs remains problematic (Machida et al. 2004; Collins et al. 2005; Montarras et al. 2005), for either implantation or consumption purposes, the usage of these cells is preferred, because of rejection problems and infection risks associated with cell lines.

In this study, we therefore compared C2C12 myoblasts and primary MPCs with respect to their responses to established electrical stimulation protocols that were previously shown to improve muscle maturation. The cells were seeded in a gel-based 3D model system consisting of collagen and Matrigel^{TM} to create murine bioartificial muscles (mBAMs) and the maturation level of the constructs in terms of cross-striations, contractility and expression of muscle differentiation markers was analyzed over time and upon electrical stimulation.

6.2 Materials & methods

6.2.1 Cell culturing and mBAM engineering

C2C12 (ECACC, Porton Down, UK) murine myoblasts were cultured in an incubator at 37°C and 5% CO_2. Growth medium (GM) consisted of high glucose Dulbecco's modified Eagle medium, with L-glutamine, 15% fetal bovine serum (Greiner Bio-One, Frickenhausen, Germany), 20mM HEPES buffer (Lonza, Walkersville, USA), 0.1mM non-essential amino acids (Lonza) 100,000 IU/l media of penicillin and 100 mg/l streptomycin (Lonza). Cells were passaged every 2-3 days at 75% confluency.

MPCs were prepared from single fibers from intact mouse hindlimb muscles (EDL, TP, TA, and soleus) with a method adapted from Shefer and co-workers (Shefer et al. 2004; Collins et al. 2005) as described by Boonen and co-workers (Boonen et al. 2009). Growth medium consisted of Dulbecco's modified Eagle medium (DMEM) Advanced (Invitrogen, Carlsbad, USA) containing 20% Fetal Bovine Serum (FBS; Greiner Bio-One), 10% Horse Serum (HS; Invitrogen), 1% chicken embryo extract (CEE, United States Biological, Massachusetts, USA) 100,000 IU/l penicillin and 100 mg/l streptomycin (Lonza) and 4

mM L-Glutamine (Lonza). Cells were passaged every 3 days at 30% confluency and grown in 1 mg/ml Matrigel^{TM}-coated (Sigma-Aldrich, St. Louis, USA) flasks.

C2C12 (passage 20) and MPCs (passage 3) were used to create mBAMs according to the protocol of Gawlitta and co-workers (Gawlitta et al. 2008) and previously described by Vandenburgh and co-workers (Vandenburgh et al. 1996). Briefly, cells were harvested and seeded into gel constructs at $4 \cdot 10^6$ cells/ml. The gel mixture consisted of collagen type I and Matrigel^{TM} (both BD Biosciences, Alphen a/d Rijn, The Netherlands). Cells were centrifuged and resuspended in a mixture consisting of 39.5% GM, 50% collagen type I (3.2 mg/ml), 2.5% NaOH (0.25 M; Sigma-Aldrich), and 8% Matrigel^{TM} (Sigma-Aldrich).

Before addition of the cell-gel suspension, house-shaped pieces (approximately 5 x 7 mm) of Velcro with 'rooftops' facing each other, functioning as anchoring points for the muscle constructs, were glued (Silastic MDX4-4210, Dow Corning, USA) onto the silicone membrane of each of the wells of a 6-well BioFlex culture plate (Flexcell International, Hillsborough, USA). Well plates were sterilized by dipping in 70% ethanol and exposure to UV for 30 minutes.

After preparation of the well plates, 375 µl of the cell/gel mixture was molded into shape between the anchoring points (12 mm). One hour after molding the mBAMs into and between the anchoring points, 5 ml of growth medium was added to the constructs. C2C12 mBAMs were kept in growth medium for 24 hours, after which medium was replaced by differentiation medium (DM). C2C12 DM consisted of growth medium in which FBS was replaced by 2% HS (Invitrogen). MPC mBAMs were cultured in DM directly after molding. MPC DM consisted of growth medium without CEE.

6.2.2 Electrical stimulation

The C-Pace Culture Pacer (IonOptix Corporation, Milton, USA) was used for bipolar field stimulation. Cells underwent a 48-hour pulsed electrical stimulation protocol consisting of 10 V, 6 ms pulses at a frequency of 2 Hz. Before use, electrodes were sterilized with 70% EtOH and subsequently dried under UV in a safety cabinet. The distance between the two electrodes was 2 cm. They were positioned in the wells parallel to the mBAMs for the electrical stimulation regimes. Culture medium was changed every 24 hours during stimulation. Two electrical stimulation regimes were applied to different experimental groups of mBAMs, introducing the electrical stimulation on day 2 and 4 after switching to DM. After 48 hours of stimulation, cells were allowed to recover for another 48 hours. Control cultures did not receive electrical stimulation and were cultured in DM throughout the experiment.

6.2.3 Immunocytochemistry

To prepare mBAMs for cryosectioning, they were washed 3 times with PBS and fixed for 1 hour in 10% formalin (Sigma-Aldrich). Afterwards, they were washed with PBS and incubated overnight in 30% sucrose (Merck, Schiphol-Rijk, The Netherlands) in PBS. Subsequently, mBAMs were dissected from their anchoring points and placed in cryomolds (Sakura Finetek Europe BV., Zoeterwoude, The Netherlands), after which they were snap-frozen in Tissue-Tek OCT compound (Sakura Finetek Europe BV) using ice-cold 2-methylbutane (Sigma-Aldrich) and sectioned into 10 µm slices, attached to poly-lysine coated glass slides. For the immunohistochemical staining, samples were permeabilized with 1% Triton-X-100 (Merck) in PBS for 20 minutes. Subsequently, they were incubated twice for 10 minutes with 1% HS in PBS to block non-specific binding and washed 2 times for 10 minutes with NET-gel (50mM Tris pH 7.4, 150 mM NaCl, 5mM EDTA, 0.05% NP40, 0.25% gelatin). Afterwards, they were incubated for 2 hours with antibodies against myosin heavy chain (1:300) (MF20; developed by D.A. Fischman and obtained from the Developmental Studies Hybridoma Bank, maintained by The University of Iowa) and sarcomeric α-actinin (1:800) (clone EA-53; Sigma-Aldrich) in NET-gel with 10% horse serum. Next, sections were washed 6 times for 5 minutes in NET-gel and incubated for one hour with Alexa Fluor 488-conjugated goat anti-mouse IgG2b (1:300) and Alexa Fluor 555-conjugated goat anti-mouse IgG1 (1:200) secondary antibodies (A21141, A21127; Invitrogen). Subsequently, they were washed 2 times for 5 minutes with NET-gel and stained for an additional 5 minutes with DAPI in PBS. Afterwards, sections were washed 4 times for 5 minutes in PBS and mounted on slides with mowiol (4-88; Calbiochem, San Diego, USA). Sections were evaluated using fluorescence microscopy (Axiovert 200M, Zeiss, Göttingen, Germany).

6.2.4 Histology

Frozen sections were thawed for 60 minutes at room temperature. Afterwards, they were fixed with ice-cold acetone for 5 minutes. Next, samples were incubated in Mayer's hematoxylin solution (Sigma-Aldrich) for 10 minutes and washed for 15 minutes in slowly running tap water. Subsequently, sections were stained with acidified aqueous eosin Y solution (Sigma-Aldrich) for 30 seconds and washed for 5 minutes in slowly running tap water. Next, sections were dehydrated by 10 dips in 70% ethanol, 10 dips in 96% ethanol and 3 times 10 dips in 100% ethanol, followed by 2 times 3 minutes xylol immersion (Merck). Finally, sections were mounted on slides with entallan, covered and left to dry. Sections were evaluated by light microscopy (Observer Z1, Zeiss).

6.2.5 Quantitative PCR

Total RNA of 6 samples per experimental treatment was isolated according to the manufacturer's instructions (RNeasy™, Qiagen, Venlo, The Netherlands). mBAMs were ground in RLT buffer containing 1% β-mercapto-ethanol using the Ultra-Turrax® T8 (IKA® Werke GmbH & Co. KG, Staufen, Germany). Synthesis of cDNA was carried out using 0.5 mM dNTPs (Invitrogen), 2 µg/ml random primers (Promega, Madison, USA), 10 mM DTT (Invitrogen), 4 IU/µl M-MLV (Invitrogen), M-MLV buffer (Invitrogen), and ddH_2O. Control reactions without M-MLV (-RT) were performed to screen for genomic DNA contaminations. The temperature profile of the cDNA synthesis protocol was as follows: 6 min at 72°C, 5 min at 37°C (subsequent addition of M-MLV), 60 min at 37°C, and 5 min at 95°C. Samples were stored at 4°C until use for quantitative PCR (qPCR). Stability of reference genes was investigated using a geNorm reference gene selection kit (PrimerDesign, Southampton, UK). The two most stable reference genes were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-2 microglobulin (B2M). Primer sets for MyoD, myogenin, MRF4, MLP, sarcomeric actin, α-actinin, MHC-IId/x, MHC-IIa, MHC-IIb (Sigma-Aldrich), and perinatal MHC (MHC-pn; PrimerDesign) were developed and validated for qPCR (Table 6.1). The qPCR reaction (MyiQ; Bio-Rad, Hercules, USA) was performed using SYBR Green Supermix (Bio-Rad), primers (0.5 mM), ddH_2O, and 1 µl cDNA. The temperature profile was as follows: 3 min 95°C, 40x (20s 95°C, 20s 60°C, 30s 72°C), 1 min 95°C, 1 min 65°C, followed by a melt curve analysis. Target gene expression was evaluated using the comparative method (ΔCT). Results were normalized for reference genes and compared to corresponding control cultures. Values for ΔCT of target genes were subjected to statistical analyses comparing electrically stimulated cultures with their corresponding controls and control cultures over time.

6.2.6 Statistical analysis

All data are presented as means and their standard deviations. Quantitative PCR analyses are n=6 and immunocytochemical analyses were performed for n=2. A two-tailed, unpaired Student's t-test was used to compare gene expression levels. One-way ANOVA was performed to compare differences over time in the non-stimulated controls. Differences were considered significant for P values <0.05.

Target gene	Gene symbol	Accession number	Primer sequences	Amplicon size
MyoD	*MyoD*	NM_010866	Sense: 5′-GGCTACGACACCGCCTACTA-3′ Antisense: 5′-CTGGGTTCCCTGTTCTGTGT-3′	216 bp
Myogenin	*Myogenin*	NM_031189	Sense: 5′-TGTCTGTCAGGCTGGGTGTG-3′ Antisense: 5′-TCGCTGGGCTGGGTGTTAG-3′	173 bp
MRF4 (Myf6)	*MRF4*	NM_008657	Sense: 5′-CGAAAGGAGGAGACTAAAG-3′ Antisense: 5′-CTGTAGACGCTCAATGTAG-3′	133 bp
Muscle LIM Protein	*MLP*	Z49883	Sense: 5′-TGGGTTTGGAGGGCTTAC-3′ Antisense: 5′-CACTGCTGTTGACTGATAGG-3′	84 bp
Sarcomeric actin	*Acta1*	NM_009606	Sense: 5′-ATGGTAGGTATGGGTCAG-3′ Antisense: 5′-GATCTTCTCCATGTCGTC-3′	126 bp
α-actinin	*Actn*	NM_033268	Sense: 5′-TCATCCTCCGCTTCGCCATTC-3′ Antisense: 5′-CTTCAGCATCCAACATCTTAGG-3′	288 bp
MHC-IId/x	*MYH1*	NM_030679	Sense: 5′-GCGACAGACACCTCCTTCAAG-3′ Antisense: 5′-TCCAGCCAGCCAGCGATG-3′	158 bp
MHC-IIa	*MYH2*	NM_001039545	Sense: 5′-GCAGAGACCGAGAAGGAG-3′ Antisense: 5′-CTTTCAAGAGGGACACCATC-3′	121 bp
MHC-IIb	*MYH4*	NM_010855	Sense: 5′-GAAGGAGGGCATTGATTGG-3′ Antisense: 5′-TGAAGGAGGTGTCTGTCG-3′	140 bp
Perinatal MHC	*MYH8*	NM_177369	Sense: 5′-ACTGAGGAAGACCGCAAGAA-3′ Antisense: 5′-CAGGTTGGCATTGGATTGTTC-3′	120 bp

Table 6.1: Primer sequences of investigated genes.

6.3 Results

The maturation level of both the MPC and C2C12 mBAMs was analyzed by examining induced, synchronous contractions during electrical stimulation, myotube alignment and cross-striations in frozen sections, and RNA expression of myogenic regulatory factors (MRFs) and sarcomere proteins.

6.3.1 Spontaneous alignment of myotubes as a result of mere culturing in a 3D model system

The 3D model system we used to create mBAMs proved to be very successful in the creation of mature muscle tissue by mere culturing of the cells in the gels using Velcro anchoring points attached to a flexible membrane. In this culture system, internal tension was generated by the cells which resulted in compaction of the tissue, and

subsequent alignment of myotubes. As early as 24 hours after creation of mBAMs, compaction was obvious in macroscopic observations, as mBAMs became thinner, especially in the center of the constructs (Figure 6.1, panel A). Myotubes were homogeneously localized throughout the mBAM, showing alignment in only one direction, as can be seen in Figure 6.1, panels Ba and Bb.

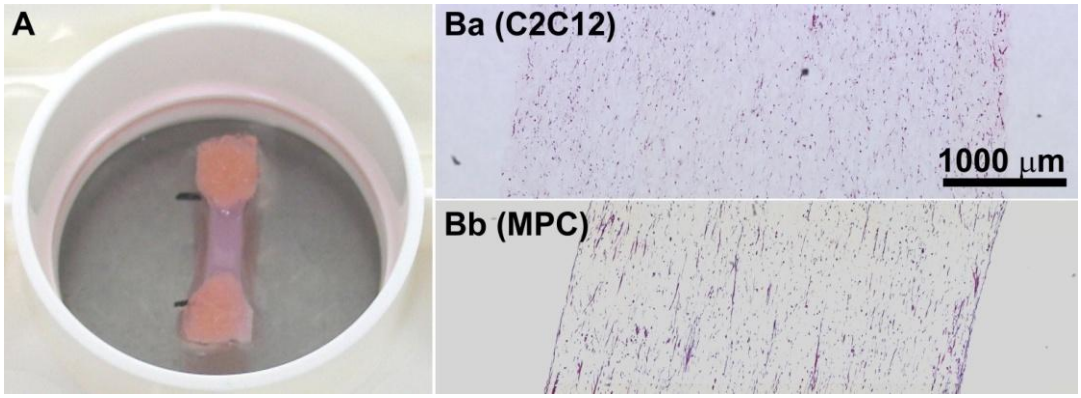

Figure 6.1: Macroscopic image of mBAMs cultured in a 6-well Flexwell dish for 24h (A) and typical examples of histological analysis (hematoxylin & eosin (H&E)) on frozen sections of C2C12 (Ba) and MPC (Bb) mBAMs.

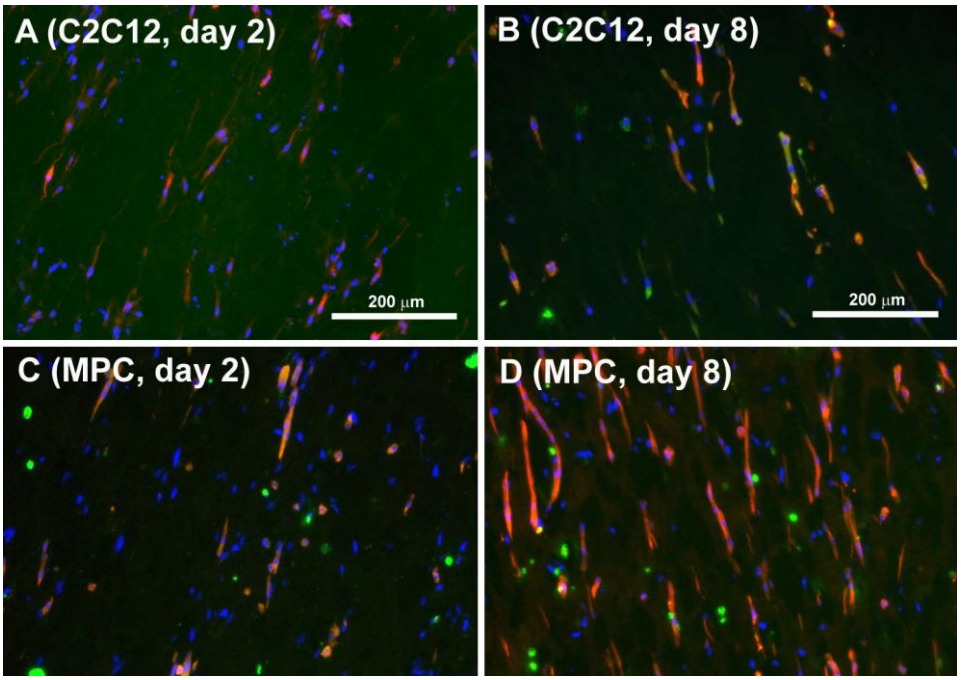

Figure 6.2: Immunohistochemical analyses of frozen sections showing muscle development in C2C12 and MPC mBAMs after 2 days of DM (panels A and C) and 8 days of DM (panels B and D) in non-stimulated control samples. Sections were stained for sarcomeric α-actinin (red), sarcomeric myosin (green), and nuclei (blue).

Electrical stimulation was started after 2 (ES1) or 4 (ES2) days after changing to DM, since pilot experiments showed that myotube development is a prerequisite for electrical stimulation to have enhancing effects on muscle maturation. The mBAMs were stimulated for 48 hours and allowed to recover for another 48 hours, to examine whether effects persisted after stimulation.

Over time and upon electrical stimulation, synchronous contractions of regions within the MPC mBAMs were observed under the microscope. In addition, spontaneous contractions were always observed in control constructs and stimulated constructs after switching off stimulation. However, these spontaneous contractions were much more irregular and only small parts of the constructs contracted. By contrast, no contractions were observed within any of the C2C12 mBAMs, demonstrating a lower maturation level compared to MPC mBAMs.

6.3.2 More mature cross-striations in MPC mBAMs over C2C12

Muscle tissue maturation was analyzed in frozen sections by immunofluorescent staining for the sarcomere components α-actinin and myosin. The samples, electrically stimulated and their corresponding controls, were scanned for characteristic cross-striations. On day 2, the non-stimulated samples of C2C12 and MPC mBAMs were similar. By contrast, a clear difference existed between the two cell sources at the end of the culturing period (day 8): non-stimulated C2C12 mBAMs displayed only a slight increase in amount and length of myotubes on day 2 versus day 8 (Figure 6.2, panels A and B), while in MPC mBAMs, length and number of myotubes had noticeably increased (Figure 6.2, panels C and D).

Typical examples of cross-striated myotubes in C2C12 and MPC mBAMs can be found in Figure 6.3. Myotubes in C2C12 mBAMs never showed myosin cross-striations and displayed α-actinin cross-striations from differentiation day 4 onwards. At that time, the amount of α-actinin cross-striations was clearly higher in electrically stimulated samples than in non-stimulated controls, suggesting advancement of maturation. In MPC mBAMs, α-actinin cross-striations were invariably present in high amounts in all samples, whereas myosin cross-striations showed a pattern that was similar to α-actinin cross-striations in C2C12 mBAMs; myosin cross-striations were visible from differentiation day 4 onwards, and were more abundant in stimulated samples.

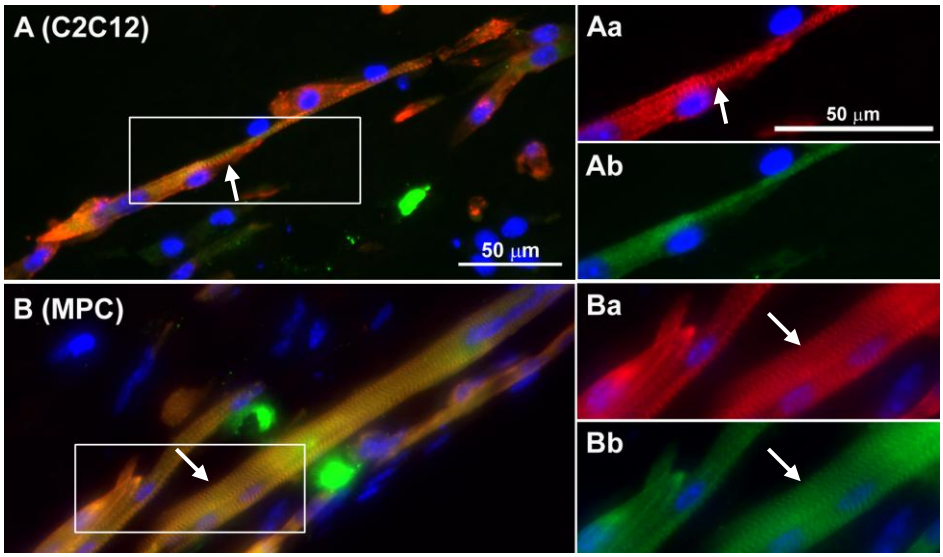

Figure 6.3: Cross-striations in C2C12 (A) and MPC (B) mBAMs. Frozen sections were stained for sarcomeric α-actinin (red), sarcomeric myosin (green), and nuclei (blue). Panels Aa-Ab and Ba-Bb show magnifications of boxed areas in panels A and B for α-actinin (red) and sarcomeric myosin (green).

6.3.3 Remarkable differences in gene expression between MPC and C2C12 mBAMs; MHC isoform conversion upon electrical stimulation in MPC mBAMs

RNA expression levels of genes encoding for muscle differentiation and maturation markers were quantified using qPCR. As can be seen in Figure 6.4 and 6.5, the most striking difference between C2C12 and MPC mBAMs was the mere divergence in expression levels between the two. Myogenin and actin were expressed at higher levels in the C2C12 mBAMs, whereas the expression of α-actinin was not significantly different between the two cell sources. Expression levels of all other genes were higher in MPC mBAMs compared to C2C12 mBAMs.

The myogenic regulatory factors (MRFs) are important transcription factors in the differentiation from myoblasts to myofibers. MyoD and myogenin play a role in early differentiation, whereas Myogenic Regulatory Factor 4 (MRF 4) and Muscle Lim Protein (MLP) are essential for myotube maturation. A decrease over time was observed for MyoD and myogenin in C2C12 mBAMs as well as in MPC mBAMs (Figure 6.4, panels A and B).

One of the late MRFs, MRF4, showed an increase over time, with a decrease in expression caused by electrical stimulation in both C2C12 and MPC mBAMs when started on differentiation day 4 (ES2) (Figure 6.4, panel C). This decrease persisted

during the 48-hour recovery period (ES2 rec). Start of stimulation on differentiation day 2 (ES1) also caused a decrease in MRF4 expression in both C2C12 and MPC mBAMs, which did not persist after recovery (ES1 rec) in the C2C12 mBAMs. MLP expression remained constant over time in MPC mBAMs, whereas in C2C12 mBAMs, an increase was found over time (Figure 6.4, panel D). MLP expression was markedly increased by electrical stimulation started on day 2 (ES1) of differentiation in the MPC mBAMs only. These results suggest that, as both MRF4 and MLP are late differentiation markers, electrical stimulation improves later stages of maturation in both C2C12 and MPCs mBAMs.

The expression of sarcomere proteins was also investigated to evaluate muscle maturation over time and as a result of electrical stimulation. In general, an increase in gene expression of sarcomeric proteins was observed over time in both the C2C12 and MPC mBAMs, except for α-actinin expression in the MPC mBAMs (Figure 6.5). Sarcomeric actin expression showed an increase caused by electrical stimulation in C2C12 mBAMs when stimulation was started on day 2 (ES1) (Figure 6.5, panel A). However, when started on day 4, a decrease in actin expression was found immediately after stimulation. Both effects did not persist after the recovery period. The effects of electrical stimulation on the expression of α-actinin were not consistent. Whereas stimulation started on day 2 triggered an increase in α-actinin expression in MPC mBAMs, a decrease was observed in C2C12 mBAMs (Figure 6.5, panel B).

The most striking differences between C2C12 and MPC mBAMs were found in the expression levels of myosin heavy chain (MHC) isoforms MHC-IId/x (MYH1), MHC-IIa (MYH2), MHC-IIb (MYH4), and perinatal MHC (MYH8). Overall, MHC expression was much higher in MPC mBAMs when compared to C2C12 mBAMs, and in both cell sources expression increased over time.

MHC-IId/x (MYH1) expression seemed to be unaffected by stimulation in both C2C12 and MPC mBAMs and only increased from day 2 until day 4, after which the expression remained stable (Figure 6.5, panel C). No remarkable effects of electrical stimulation were observed in MHC-IIa (MYH2) and MHC-IIb (MYH4) expression in C2C12 mBAMs (Figure 6.5, panels D and E). In contrast, in MPC mBAMs, electrical stimulation did strongly affect these two MHC isoforms. MHC-IIa (MYH2) was upregulated in all stimulated MPC mBAM samples and this upregulation even persisted after the recovery periods, whereas MHC-IIb (MYH4) expression was downregulated in the same samples. Expression of the perinatal form of MHC (MYH8) was also downregulated by electrical stimulation (Figure 6.5, panel F). However, this effect was visible in C2C12 mBAMs only immediately after stimulation started on day 2 (ES1), whereas in MPC mBAMs, this effect was observed at later time points; after the recovery period of ES1 (ES1 rec), immediately after stimulation started on day 4 (ES2), and after its recovery period (ES2 rec).

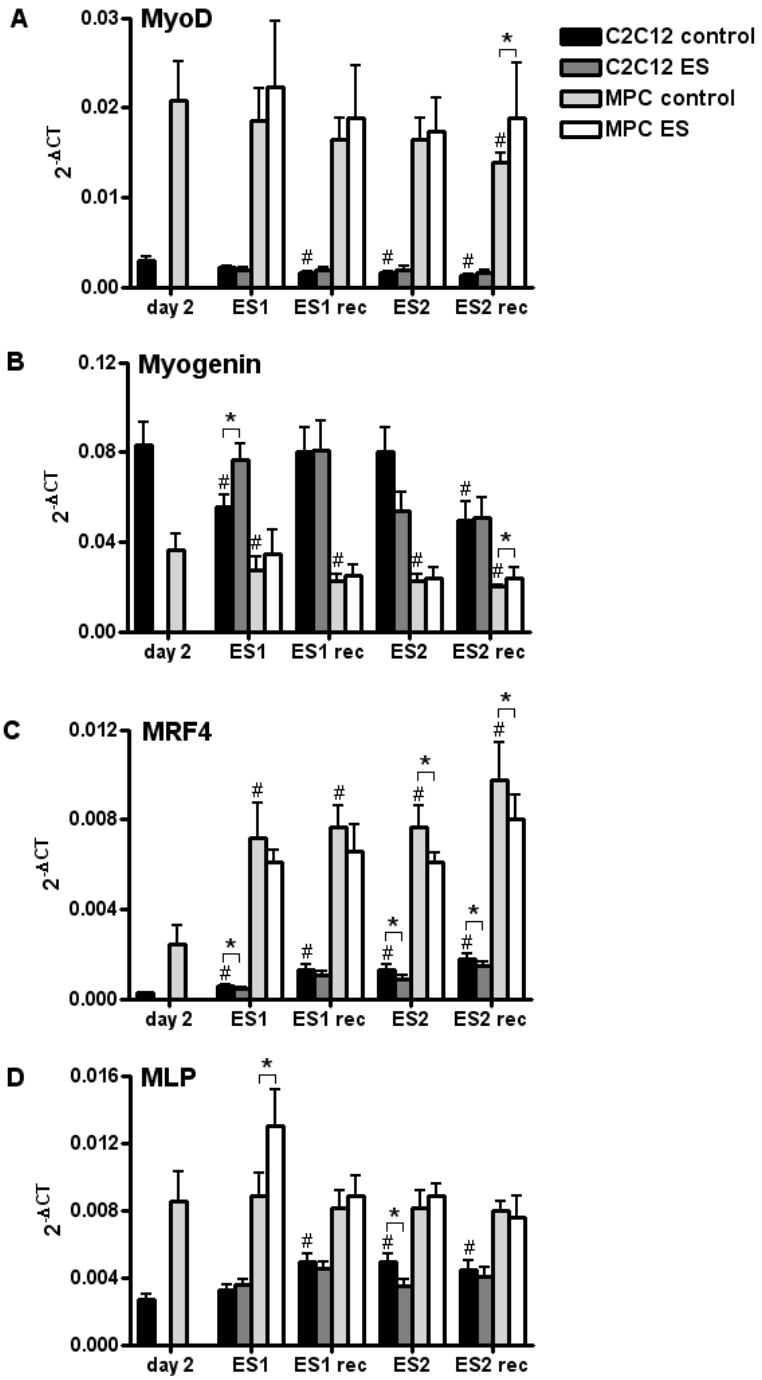

*Figure 6.4: Expression levels ($2^{-\Delta CT}$, y-axis) of myogenic regulatory factors MyoD (A), myogenin (B), MRF4 (C), and MLP (D) in time and with ES. BAMs of C2C12 and MPCs were subjected to ES protocols starting on day 2 (ES1) and 4 (ES2) after induction of differentiation. Recovery samples (ES1 rec and ES2 rec) were taken 48 hours after the 2-day pulsed ES protocols. * indicates a significant difference between ES and corresponding control (P<0.05), and # refers to a comparison of control constructs with respect to day 2 (P<0.05).*

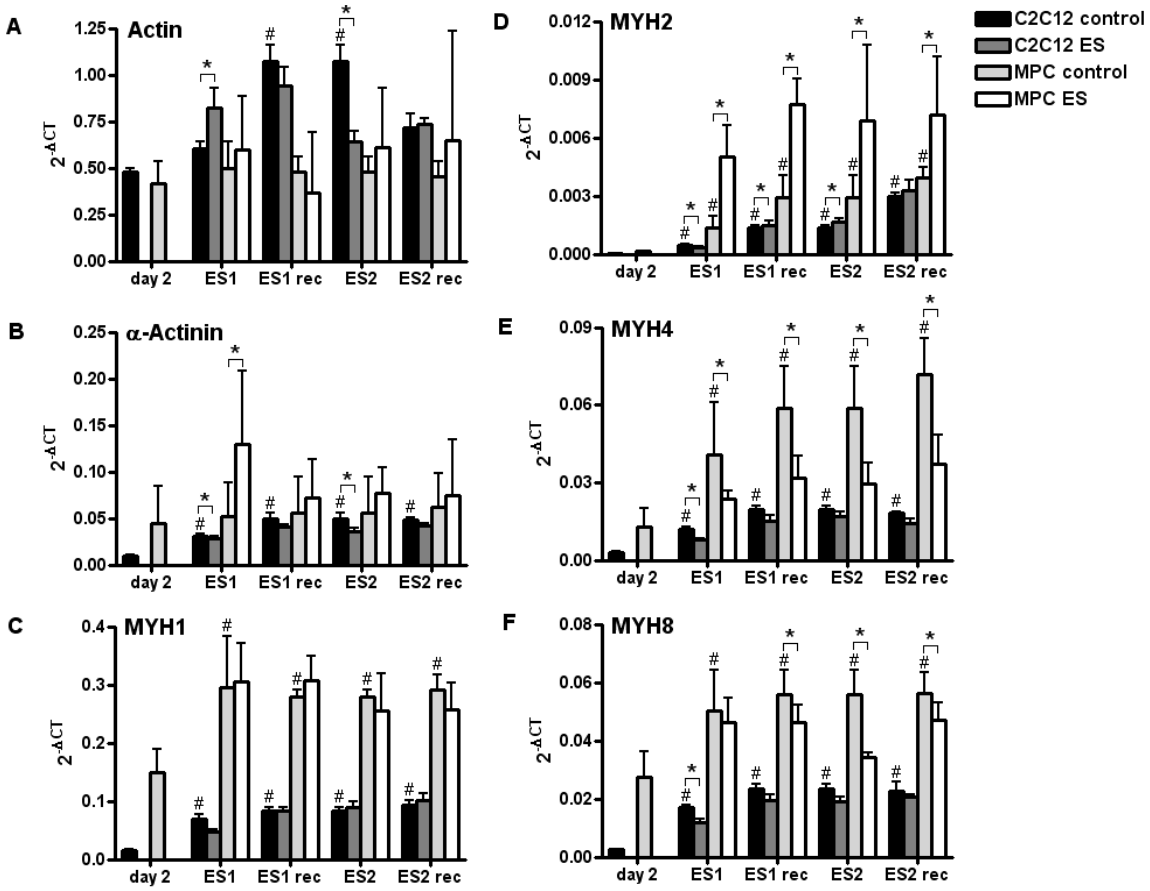

*Figure 6.5: Expression levels ($2^{-\Delta CT}$, y-axis) of sarcomere components actin (A), α-actinin (B), MYH1 (C), MYH2 (D), MYH4 (E), and MYH8 (F) in time and with ES. BAMs of C2C12 and MPCs were subjected to ES protocols starting on day 2 (ES1) and 4 (ES2) after induction of differentiation. Recovery samples (ES1 rec and ES2 rec) were taken 48 hours after the 2-day pulsed ES protocols. * indicates a significant difference between ES and corresponding control (P<0.05), and # refers to a comparison of control constructs with respect to day 2 (P<0.05).*

6.4 Discussion

Present-day skeletal muscle tissue engineering fails to result in the maturation level that is necessary for both regenerative medicine purposes as well as the *in vitro* production of meat. Our working hypothesis is that factors present in the *in vivo* niche are indispensable to achieve the desired maturation level (Boonen and Post 2008). First of all, a 3D environment possibly is superior to conventional monolayer cultures. This was demonstrated by the 3D gel system that was used in our study, a gel-mixture of collagen type I and Matrigel™, cast between anchoring points, in which mere culturing resulted in spontaneous alignment of myotubes and development of cross-striations over time. Another crucial observation in this study is the remarkable difference in response

between the established C2C12 myoblast cell line and primary myogenic progenitor cells (MPCs), both in control samples and upon electrical stimulation. The mBAMs that were created with MPCs were much more mature than the ones created from C2C12 myoblasts: MPC mBAMs had mature cross-striations of α-actinin and myosin, whereas C2C12 mBAMs only displayed α-actinin cross-striations. Moreover, contractions were only observed in MPC mBAMs. In addition, expression levels of myogenic regulatory factors and sarcomere proteins were generally higher in MPC mBAMs. Electrical stimulation exerted the most remarkable effect in the expression levels of MHC isoforms 1 and 4 in the MPC mBAMs. Stimulation caused a shift in the expression levels of these isoforms; MYH2 was upregulated, whereas MYH4 was downregulated upon electrical stimulation. In the C2C12 mBAMs, expression levels of MHC isoforms remained unchanged by the electrical stimulus.

The advantages of cell culture in 3D gels became apparent from histological analyses. The gel was attached to two anchoring points that were glued to the membrane of a 6-well Flexwell© culture dish, mimicking the function of tendons. In this set up, the cells created a tension in the constructs that was oriented in the direction of attachment. The myotubes therefore aligned in the direction of principal stress, as can be observed in the H&E staining of frozen sections of the samples, in accordance with other studies (Vandenburgh et al. 1996; Dennis et al. 2001; Gawlitta et al. 2008). The alignment of myotubes in one direction is important for the final architecture of tissue engineered muscle, as uniform alignment presumably allows for maximal force generation upon contraction. In addition, this alignment is pivotal for electrical conduction.

We (Langelaan et al. 2009) and others (Thelen et al. 1997; Bayol et al. 2005; Fujita et al. 2007) have reported synchronous contractions in 2D cultures of myotubes receiving an electrical stimulus. These observations were made in myotubes obtained from both the C2C12 cell line and the MPCs, whereas in the present study we only observed contractions in the MPC mBAMs. This effect could be a result of the higher number of myotubes present in the MPC mBAMs, compared to the C2C12 mBAMs, as was observed with immunohistochemistry. The fact that active contractions were observed in this 3D model system, verifies the effectiveness of the given electrical stimulus as was previously shown in 2D studies (Thelen et al. 1997).

Electrical stimulation results in an advanced sarcomere assembly in 2D cultures of C2C12 myoblasts as well as murine MPCs (Langelaan et al., 2009, Chapter 5). Sarcomere assembly was only observed after the application of electrical stimulation in C2C12 cultures, whereas in MPCs, sarcomere assembly always occurred and only the percentage of cross-striated tubes was slightly increased by electrical stimulation. The timeframe in which these sarcomeres form in the 3D model system was faster compared to monolayers of myotubes. For example, whereas no α-actinin cross-striations were observed in C2C12 control cultures in 2D after 5 days of differentiation, these were

invariably present in the 3D cultures after 4 days differentiation. A striking difference was found in the maturation level of the sarcomeres that had developed in the C2C12 versus the MPC mBAMs. The C2C12 mBAMs never displayed cross-striations in myosin, whereas the MPC mBAMs showed an increased assembly of cross-striations in myosin over time and upon electrical stimulation. In addition, α-actinin cross-striations were already present on day 2 of differentiation in the MPC mBAMs, while in C2C12 mBAMs they developed later and were advanced upon electrical stimulation. Others previously showed that C2C12 myotubes display α-actinin cross-striations (Fujita et al. 2007), but only form myosin cross-striations after long culture periods and under special circumstances (Engler et al. 2004b; Park et al. 2008). In contrast, but in accordance with our results, these myosin cross-striations are easily formed by MPCs (Boonen et al. 2009). Combining the observations on contractions and myosin cross-striations in MPC mBAMs, we conclude that the maturation level of mBAMs created with MPCs is evidently higher than those created with C2C12 myoblasts.

The maturation level of the mBAMs can also be monitored by expression analyses of MRFs and sarcomere proteins, as previously indicated by Langelaan and co-workers (Langelaan et al. 2009) in 2D cultures of C2C12 myotubes. We found that expression of early MRFs, such as MyoD and myogenin, was downregulated over time, whereas the late marker MRF4 was upregulated over time. No distinct additional effect of electrical stimulation was observed at this level. Most striking were the differences between the C2C12 and MPC mBAMs in the expression levels of these genes. The MPC mBAMs displayed a notably higher expression level of all MRFs, except for myogenin. This again confirms that maturation of myotubes in the MPC mBAMs is more advanced, compared to the C2C12 mBAMs. The high expression of MyoD in MPC mBAMs compared to C2C12 mBAMs can be explained by the difference in myosin heavy chain expression, as MyoD has been proven to be associated with myosin heavy chain isoform switches. In addition, MyoD has been shown to be highly sensitive for the absence or presence of electrical activity in muscle cells (Legerlotz and Smith 2008).

Most notably, an apparent difference in maturation level between C2C12 and MPC mBAMs was observed in the expression levels of MHC isoforms. Sarcomeric actin and α-actinin expressions were comparable in the mBAMs of both cell sources, but the MHC isoforms were expressed to a higher extent in the MPC mBAMs. Of the isoforms investigated, MYH1 was expressed at the highest level, followed by MYH4, MYH8, and MYH2 respectively. Other studies using primary myogenic cells also found this order of expression of MHC isoforms (Vandenburgh et al. 2008). In MPC mBAMs in culture, we found a switch to a fast isotype of MHCs instead of perinatal and slower isotypes. A switch from embryonic to more adult forms of MHC was to be expected and has been found more often as an effect of electrical stimulation (Pette and Vrbova 1999; Yan et al. 2007). Interestingly, electrical stimulation, either started on day 2 or 4 of differentiation, results in a remarkable switch of MHC isotype expression to a slower isotype, which

persisted over time. The faster type of MHC, MYH4, was downregulated by the electrical stimulation, whereas the slower type MYH2 was upregulated. *Ex vivo* studies on rat soleus muscle showed that a slow-to-fast switch is induced by high-frequency stimulation (150 Hz) (Hamalainen and Pette 1996). We used a low frequency stimulus of 1 Hz, resulting in a slower MHC isotype. Dependent on the final texture and muscle architecture that is required in the final tissue engineered muscle constructs, whether destined for regenerative medicine or meat production purposes, one might be able to tune the expression levels of the MHC isoforms by different frequencies of electrical stimulation.

MPCs are primary cells, designated to regenerate skeletal muscle, whereas C2C12 cells are a long standing cell line. Genetic make-up may account for better and faster maturation into muscle in MPCs. In addition, the cell harvest of MPCs was likely not completely pure. Although it contained mostly myoblasts, some co-isolated fibroblasts may have contaminated the culture. It was previously shown that such a heterogeneous mixture of cells is preferable for both differentiation and generation of peak force, as opposed to a homogeneous population of myogenic cells (Brady et al. 2008).

Finally, we conclude that primary myogenic progenitor cells (MPCs) are preferred over the established C2C12 cell line for the purpose of tissue engineered muscle, because of their excellent capabilities to develop into mature muscle tissue over time. Moreover, the MPCs reacted to the electrical stimulation by a switch in expression of MHC isotypes, while expression levels remained unchanged in the C2C12 mBAMs. The model system that we used for these studies, collagen/Matrigel^{TM} attached between Velcro anchoring points, was evidently useful for the creation of mature muscle tissue by mere culturing of the constructs and was predominant over the additional effect on muscle maturation caused by electrical stimulation. However, changing MHC isotype expression by electrical stimulation might be beneficial for the final texture of tissue engineered muscle.

6.5 Acknowledgements

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Chapter 7

Effects of a combined mechanical stimulation protocol: Value for skeletal muscle tissue engineering

This chapter is based on: Kristel J.M. Boonen, Marloes L.P. Langelaan, Roderick B. Polak Daisy W.J. van der Schaft, Frank P.T. Baaijens, Mark J. Post. *Effects of a combined mechanical stimulation protocol: Value for skeletal muscle tissue engineering*.

7.1 Introduction

Skeletal muscle is an appealing topic for tissue engineering because of its variety in applications for regenerative medicine (Levenberg et al. 2005; Boldrin et al. 2007), *in vitro* physiological model systems (Gawlitta et al. 2007; Vandenburgh et al. 2008) and meat production (Van Eelen et al. 1999). Since muscles are responsible for voluntary movement, posture, and balance, but are also involved in the protection of organs, loss of function due to traumatic injury or myopathies causes much morbidity. In addition to its physiological importance in most multicellular animals, muscle (meat) serves as a major source of dietary protein in western society.

Differentiation of muscle progenitor cells (MPCs) into skeletal muscle fibers is carefully regulated by the cellular environment *in vivo*. To improve *in vitro* differentiation, our working hypothesis is to recreate the *in vivo* "niche" of the MPC (Boonen et al. 2009). Niche factors that are proposed to be of importance for adequate differentiation include extracellular matrix proteins, neighboring cells, growth factors, matrix stiffness or stimulation by nerves. Another important factor present in the niche is mechanical stimulation (stretch). Not only is stretch involved in muscle development during embryogenesis, as a result of bone elongation and active movements of the embryo, in adults, stretch can also be a signal for muscle cells to hypertrophy (Vandenburgh 1987). Muscles are stretched, for example as part of physiological exercise by lengthening of muscles, either because of the action of antagonistic muscles or by eccentric contractions (Atherton et al. 2009). On top of that, of all tissue types exposed to mechanical stimuli *in vivo*, skeletal muscle shows the most obvious and rapid response to altered load, with striated muscle fiber hypertrophy after strength-training programmes, and atrophy in the absence of adequate mechanical stimulation (Benjamin and Hillen 2003).

Stretch has also been shown to cause muscle hypertrophy in *in vitro* cultures (Vandenburgh et al. 1989; Vandenburgh and Karlisch 1989) and to improve functionality of engineered muscle tissue (Moon du et al. 2008). Besides these effects, stretch can activate satellite cells (Tatsumi et al. 2001) and increase myoblast proliferation, while suppressing differentiation (Kook et al. 2008a; Kook et al. 2008b). Moreover, the introduction of a uniaxial ramp profile of continuously increasing stretch, mimicking bone elongation in embryogenesis, facilitates myotube alignment (Vandenburgh and Karlisch 1989).

Overall, reported results on mechanical stimulation *in vitro* seem somewhat contradictory, which might depend on the differentiation level of the cells at the start of the mechanical stimulation protocol and the environment these cells are cultured in. For instance, stretching a monolayer of cells that are attached to a membrane is undoubtedly completely different from stretching a 3D construct in which cells may have contact with neighboring cells and extracellular matrix on all sides. Therefore we

investigated the effects of mechanical stimulation on muscle development both in cells cultured in 2D monolayers and in 3D constructs. We kept stretch levels below 10%, since this level of stretch is thought to mimic *in vivo* mechanical stimulation, whereas stretches of 15% and above cause injury to the myotubes (Schultz and McCormick 1994).

To create a 3D environment, a gel-based system with cells incorporated is preferred, since it allows for dense packing of myofibers and, when constrained, allows for easy alignment of myofibers (Bian and Bursac 2008), both prerequisites for functional muscle tissue engineering. In contrast, when using a polymer based scaffold, fiber orientation is mostly random. Another aspect of the niche, the stiffness of the matrix, is important to consider in a 3D model system. Especially traditionally used scaffolds have a much higher stiffness than the stiffness of muscles and gel-based systems. We (Boonen et al. 2009) and others (Engler et al. 2004b; Boontheekul et al. 2007) have shown that substrate stiffness can be crucial for myotube maturation into cross-striated, functional muscle fibers.

We used fibrin gels to create a 3D environment with a stiffness in the lower kPa range. The collagen/Matrigel^{TM} gel used in previous experiments (Chapter 6) was not suitable for stretch experiments, because of limited elasticity. The use of fibrin is clinically approved and could therefore be used in transplantation studies for medical purposes. Furthermore, fibrin gels allow for evaluating the effects of stretch purely, without confusing results caused by additional extracellular matrix proteins present in other gel systems that are often used in skeletal muscle tissue engineering (Powell et al. 2002; Gawlitta et al. 2008; Vandenburgh et al. 2008). The work of Grossi and co-workers already showed that mechanical stimulation through different extracellular matrix receptors can result in variations in muscle differentiation (Grossi et al. 2007).

The use of a cell line for basic tissue engineering studies has proven to be useful (Okano and Matsuda 1998b; Matsumoto et al. 2007; Gawlitta et al. 2008). Cell lines such as the C2C12 murine myoblast cell line are easy to culture and readily fuse into myotubes upon serum deprivation. However, recent studies have indicated that differences do exist between primary cells and cell lines in their response to environmental cues (Maley et al. 1995; Boontheekul et al. 2007; Boonen et al. 2009). In this study, we investigated the effect of stretch in a 2D and 3D environment and compared the effects on muscle maturation in both C2C12 myoblasts and MPCs.

7.2 Materials & methods

7.2.1 Cell culture and construct engineering

7.2.1.1 Cell culture (2D)

MPCs were prepared from single fibers from intact mouse hindlimb muscles (EDL, TP, TA and soleus) with a method adapted from (Shefer et al. 2004; Collins et al. 2005) as described in (Boonen et al. 2009). Growth medium (GM) consisted of Dulbecco's modified Eagle medium (DMEM) Advanced (Invitrogen, Carlsbad, USA) containing 20% Fetal Bovine Serum (FBS; Greiner Bio-One, Frickenhausen, Germany), 10% Horse Serum (HS; Invitrogen), 1% chicken embryo extract (CEE, United States Biological, Massachusetts, USA) 100,000 IU/l penicillin and 100 mg/l streptomycin (Lonza, Walkersville, USA) and 4 mM L-Glutamine (Lonza). MPCs were passaged every 3 days at 30% confluency and grown in 1 mg/ml Matrigel^{TM}-coated (Sigma-Aldrich, St. Louis, USA) flasks.

C2C12 (ECACC, Porton Down, UK) murine myoblasts were cultured in GM, consisting of high glucose DMEM (Invitrogen), with L-glutamine, 15% FBS (Greiner Bio-One), 20mM HEPES buffer (Lonza), 0.1mM non-essential amino acids (Lonza) 100,000 IU/l media of penicillin and 100 mg/l streptomycin (Lonza). C2C12 cells were passaged every 2-3 days at 75% confluency. All cells were cultured in an incubator at 37°C and 5% CO_2.

A cell density of 25,000 cells/cm^2 was seeded in Uniflex® culture plates (Flexcell International, Hillsborough, USA) with two different coatings: laminin or collagen I. MPCs were induced to differentiate immediately after seeding by switching to differentiation medium (DM; growth medium without CEE). C2C12 cells were left to reach confluency for 3 days after which differentiation was initiated by a switching from GM to DM. C2C12 DM consisted of growth medium in which FBS was replaced by 2% HS (Invitrogen).

When small myotubes had formed (after 2 days in DM for MPCs and after 1 day in DM for C2C12 myoblasts in 2D monolayers), the mechanical stimulation protocol was started as described below (Figure 1).

7.2.1.2 Construct engineering (3D)

Fibrin constructs were created according to a protocol adapted from Matsumoto and co-workers (Matsumoto et al. 2007). Briefly, MPCs (passage 3) and C2C12 myoblasts (passage 20) were harvested and seeded into fibrin gel constructs at a density of $4 \cdot 10^6$ cells/ml. Cells were resuspended in a thrombin solution (2 IU/ml; Sigma-Aldrich) and mixed with a fibrinogen solution (3 mg/ml; Sigma-Aldrich) in a 1:1 ratio, containing 1.0 mg/ml ε-aminocaproic acid (ε-ACA) to prevent fibrinolysis.

Uncoated BioFlex culture plates (Flexcell International, Hillsborough, USA) were modified before addition of the cell-gel suspension. House-shaped pieces (approximately 5 x 7 mm) of Velcro with 'rooftops' facing each other, functioning as anchoring points of the muscle constructs, were glued (Silastic MDX4-4210, Dow Corning, USA) onto the silicone membrane of each of the wells of the 6-well plate. Well plates were sterilized by dipping in 70% ethanol and exposure to UV for about 30 minutes.

After preparation of the well plates, 375 µl of the cell/gel mixture was molded into shape between the anchoring points. One hour after molding the constructs into and between the anchoring points, 5 ml of growth medium, containing 1.0 mg/ml ε-ACA, was added to the culture. C2C12 constructs were kept in growth medium for 24 hours, after which medium was replaced by differentiation medium (DM). C2C12 DM consisted of growth medium provided with ε-ACA in which FBS was replaced by 0.4% Ultroser G (BioSepra, Cergy-Saint-Christophe, France). MPC constructs were cultured in DM directly after molding. MPC DM consisted of growth medium provided with ε-ACA, but without CEE.

One day after switching to DM, the mechanical stimulation protocol was started as described below. Medium was replaced every 48 hours, provided with fresh ε-ACA.

7.2.2 Mechanical stimulation

Uniaxial mechanical stimulation was applied using the BioFlex strain unit (Flexcell International). The protocol consisted of a 2-day ramp stretch of 0-2%, followed by an intermittent 3-hour on and 3-hour off intermittent dynamic stretch regime of 2-6% stretch (Figure 7.1).

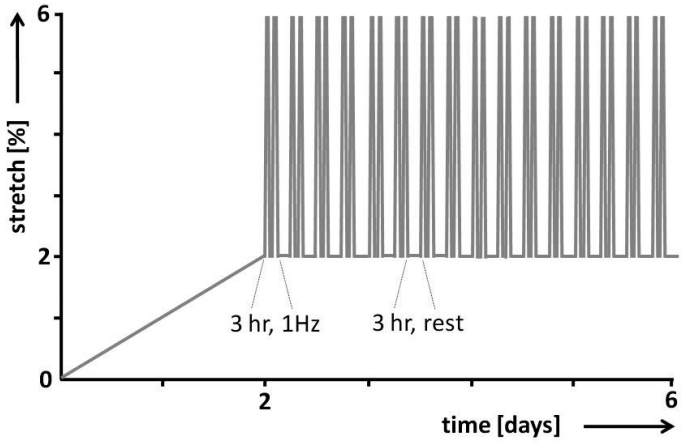

Figure 7.1: Mechanical stimulation protocol, consisting of a 2-day uniaxial ramp stretch (0-2%) period, and a 4-day intermittend uniaxial intermittent dynamic stretch (2-6%) period. Intermittent dynamic stretch was applied for 3 hours at a frequency of 1 Hz, followed by a resting period of 3 hours.

7.2.3 Fluorescent staining

7.2.3.1 Immunocytochemistry and immunohistochemistry

Cells were washed 2 times with PBS (Sigma-Aldrich) and fixed for 10 minutes in 10% formalin (Sigma-Aldrich). To prepare fibrin constructs for cryosectioning, they were washed 3 times with PBS and fixed for 1 hour in 10% formalin. Afterwards, constructs were washed with PBS and incubated overnight in 30% sucrose (Merck, Schiphol-Rijk, The Netherlands) in PBS. Subsequently, constructs were dissected from the anchoring points and placed in cryomolds (Sakura Finetek Europe BV., Zoeterwoude, The Netherlands), after which they were snap-frozen in Tissue-Tek OCT compound (Sakura Finetek Europe BV) using ice-cold 2-methylbutane (Sigma-Aldrich). The constructs were then sectioned into 10 µm slices, which were attached to poly-lysine coated glass slides.

Cells as well as sections were permeabilized with 1% Triton-X-100 (Merck) in PBS for 20 minutes. Subsequently, they were incubated twice for 10 minutes with 1% HS in PBS to block non-specific binding and washed 2 times for 10 minutes with NET-gel (50mM Tris pH 7.4, 150 mM NaCl, 5mM EDTA, 0.05% NP40, 0.25% gelatin). Afterwards, they were incubated for 2 hours with antibodies against myosin heavy chain (1:300) (MF20, developed by D.A. Fischman and obtained from the Developmental Studies Hybridoma Bank, maintained by The University of Iowa) and sarcomeric α-actinin (1:800) (clone EA-53, Sigma-Aldrich) in NET-gel with 10% HS. Next, cells and sections were washed 6 times for 5 minutes in NET-gel and incubated for one hour with Alexa Fluor 488-conjugated goat anti-mouse IgG2b (1:300) and Alexa Fluor 555-conjugated goat anti-mouse IgG1 (1:200) secondary antibodies (A21141, A21127; Invitrogen). Subsequently, they were washed 2 times for 5 minutes with NET-gel and stained for an additional 5 minutes with DAPI. Afterwards, cells and sections were washed 4 times 5 minutes in PBS and mounted on slides with mowiol (4-88; Calbiochem, San Diego, USA). Sections and cells were evaluated using fluorescence microscopy (Axiovert 200M, Zeiss, Göttingen, Germany).

7.2.3.2 Whole mount staining

Constructs were washed 3 times with PBS and fixed for 1 hour in 10% formalin (Sigma-Aldrich). Afterwards, they were washed once with PBS and permeabilized for 30 minutes in 1% Triton-X-100 (Merck) in PBS. Subsequently, they were incubated twice for 10 minutes with 1% HS in PBS to block non-specific binding and washed 2 times for 10 minutes with NET-gel. Next, constructs were stained with phalloidin-FITC (1:200; Sigma-Aldrich) for 40 minutes. Afterwards, they were washed 3 times for 10 minutes with PBS and mounted between slides with mowiol (Calbiochem). Constructs were evaluated using 2-photon confocal laser scanning microscopy (LSM 510 META, connected to an inverted Axiovert 200M, Zeiss).

7.2.4 Quantitative PCR

Total RNA was isolated according to the manufacturer's instructions (RNeasy^{TM}; Qiagen, Venlo, The Netherlands). Cells were washed 3 times with PBS and lysed in RLT buffer containing 1% β-mercapto-ethanol. Constructs were ground in RLT buffer containing 1% β-mercapto-ethanol using the Ultra-Turrax® T8 (IKA® Werke GmbH & Co. KG, Staufen, Germany). Synthesis of cDNA was carried out using 0.5 mM dNTPs (Invitrogen), 2 µg/ml random primers (Promega, Madison, USA), 10 mM DTT (Invitrogen), 4 IU/µl M-MLV (Invitrogen), M-MLV buffer (Invitrogen), and ddH_2O. Control reactions without M-MLV (-RT) were performed to screen for genomic DNA contaminations. The temperature profile of the cDNA synthesis protocol was as follows: 6 min at 72°C, 5 min at 37°C (subsequent addition of M-MLV), 60 min at 37°C, and 5 min at 95°C. Samples were stored at 4°C until use for quantitative PCR (qPCR). Stability of reference genes was investigated using a geNorm reference gene selection kit (PrimerDesign, Southampton, UK). The two most stable reference genes were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-2 microglobulin (B2M). Primer sets for MyoD, myogenin, MRF4, MLP, sarcomeric actin, α-actinin, MHC-IId/x, MHC-IIa, MHC-IIb (Sigma-Aldrich), and perinatal MHC (MHC-pn; PrimerDesign) were developed and validated for qPCR (Table 6.1). The qPCR reaction (MyiQ, Bio-Rad, Hercules, USA) was performed using SYBR Green Supermix (Bio-Rad), primers (0.5 mM), ddH_2O, and 1 µl cDNA. The temperature profile was: 3 min 95°C, 40x (20s 95°C, 20s 60°C, 30s 72°C), 1 min 95°C, 1 min 65°C, followed by a melt curve analysis. Target gene expression was evaluated using the ΔCT method. Results were normalized for reference genes and compared to corresponding control cultures. Values for ΔCT of target genes were subjected to statistical analyses comparing mechanically stimulated cultures with their corresponding controls and control cultures over time.

7.2.5 Statistical analysis

All data are presented as means and their standard deviations. Quantitative PCR analyses were n=3 for the 2D mechanical stimulation experiments, and n=6 for the 3D experiments. Fluorescent staining and histology were performed twice. A two-way ANOVA was performed to compare gene expression levels in the 2D experiments, investigating the effects of time. At the last time-point of the protocol in the 2D experiments another two-way ANOVA was performed to investigate the effect of the mechanical stimulation regime and coating. In the 3D experiments, a two-tailed, unpaired Student's t-test was used to compare gene expression levels between stimulated and non-stimulated control constructs. One-way ANOVA was performed to compare differences over time in the non-stimulated controls. Differences were considered significant for P values <0.05.

7.3 Results

The effects of a combined uniaxial mechanical stimulation regime, consisting of a 2-day ramp stretch to initiate myotube alignment, and a 4-day intermittent intermittent dynamic stretch protocol to promote myotube maturation, were investigated in a 2D and 3D model system for both the C2C12 cell line and MPCs. In 2D, cells were cultured on a laminin- or collagen I-coating. The effects of mechanical stimulation on myotube formation, presence of cross-striations, and expression levels of myogenic regulatory factors (MRFs) and sarcomere proteins were evaluated over time.

7.3.1 Stretch in 2D - downregulation of maturation markers

After the C2C12 myoblasts and MPCs had reached confluency, the differentiation process of the cells was initiated and one day later, the mechanical stimulation protocol (Figure 7.1) was started. Cells were evaluated directly after the ramp stretch or after the uniaxial intermittent dynamic stretch regime.

C2C12 myoblasts and MPCs readily formed equal amounts of myotubes on laminin-, and collagen I- coated membranes. Cross-striations of α-actinin in C2C12 myotubes were found only when cells were cultured on laminin-coated membranes (Figure 2, panels Aa-Ab), whereas myosin cross-striations were never observed in these cells (Figure 2, panel Ac). MPC myotubes displayed cross-striations in both myosin and α-actinin (Figure 2, panels Ba-Bc), which was independent of coating.

The gene expression analyses of MRFs and sarcomere components revealed that over time, muscle differentiation and maturation had taken place in both the C2C12 and MPC cultures, based on upregulated expression levels of these genes. The influence of coating and stretch on the expression levels of these genes was investigated after the 4-day stretch period. The results are summarized in Table 7.1. Expression of MYH4 was downregulated by stretch in both C2C12 and MPC myotubes. In MPC myotubes, stretch caused an additional downregulation of MRF4. Regarding the effect of different coatings, in C2C12 myotubes, only expression levels of MyoD, MLP and α-actinin were influenced, with higher expression levels in myotubes cultured on collagen type I compared to myotubes cultured on laminin. In MPC myotubes, coating largely influenced the expression of all genes, but myogenin, with higher expression in myotubes cultured on laminin-coating compared to collagen I-coating.

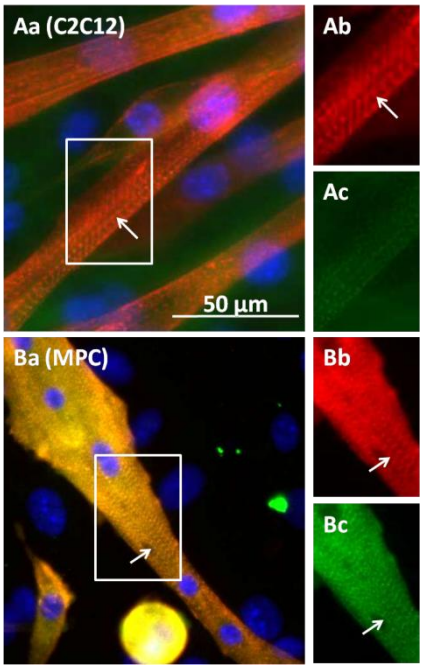

Figure 7.2: Cross-striations in C2C12 and MPC myotubes on laminin-coated membranes. Panels Aa and Ba show combined pictures of the myosin (green), α-actinin (red), and DAPI (blue) staining in C2C12 and MPCs respectively. Panels Ab and Bb show magnifications of boxed areas in panels Aa and Ba for α-actinin (red) and panels Ac and Bc for sarcomeric myosin (green).

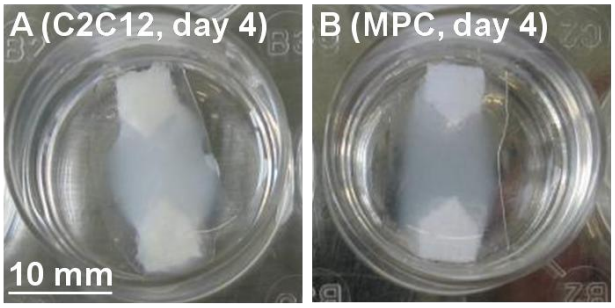

Figure 7.3: Control samples on day 4 of differentiation. Panels A and B show constructs of C2C12 and MPCs respectively.

		MyoD	MyoG	MRF4	MLP	actin	α-actinin	MYH1	MYH2	MYH4	MYH8
C2C12	stretch	-	-	-	-	-	-	-	-	↓ P<0.05	-
	coating	L<C P<0.05	-	-	L<C P<0.05	-	L<C P<0.05	-	-	-	-
MPC	stretch	-	-	↓ P<0.05	-	-	-	-	-	↓ P<0.05	-
	coating	L>C P<0.05	-	L>C P<0.01	L>C P<0.01	L>C P<0.01	L>C P<0.01	L>C P<0.01	L>C P<0.01	L>C P<0.01	L>C P<0.01

Table 7.1: Summary of qPCR results on day 8 for C2C12 and MPC 2D cultures. Values of ΔCT for all genes were subjected to a two-way ANOVA for each cell source, to investigate an effect of coating (L: laminin; C: Collagen I) and stretch. The effect of stretch is illustrated with an arrow (↓) for downregulation. P values are depicted for every significant difference.

7.3.2 Stretch in 3D – inhibition of differentiation and maturation

As a 3D model system, a fibrin gel was used to investigate the influence of stretch. The differentiation and mechanical stimulation protocols were similar to the 2D situation: after 1 day of culturing in differentiation medium the mechanical stimulation protocol, as depicted in Figure 7.1, was started. The constructs were analyzed on day 2 (before uniaxial ramp stretch), day 4 (after uniaxial ramp stretch), or day 8 (after uniaxial intermittent dynamic stretch) of the protocol. At these time points, we evaluated the distribution of myotubes throughout the sample by whole mount staining, examined the presence of cross-striations in frozen sections, and investigated the RNA expression levels of MRFs and sarcomere proteins, comparing stretched and control samples.

Macroscopic observations of the constructs revealed clear differences between C2C12 and MPC constructs (Figure 7.3). C2C12 constructs formed a dense, opaque structure, whereas MPC constructs remained lightly transparent and seemed less compact. Myotubes were observed in the whole mount staining of both types of constructs after 2 days of differentiation (Figure 7.4, panels Aa and Ba). Over time, myotube development progressed and was faster in the MPC constructs (Figure 7.4, panels Ab and Bb). The orientation of myotubes in the C2C12 constructs on day 8 was more pronounced in the direction of the orientation between the attachment points (Figure 7.4, panel Ac). This alignment appeared absent in the MPC constructs (Figure 7.4, panel Bc), presumably because of the lower myotube content.

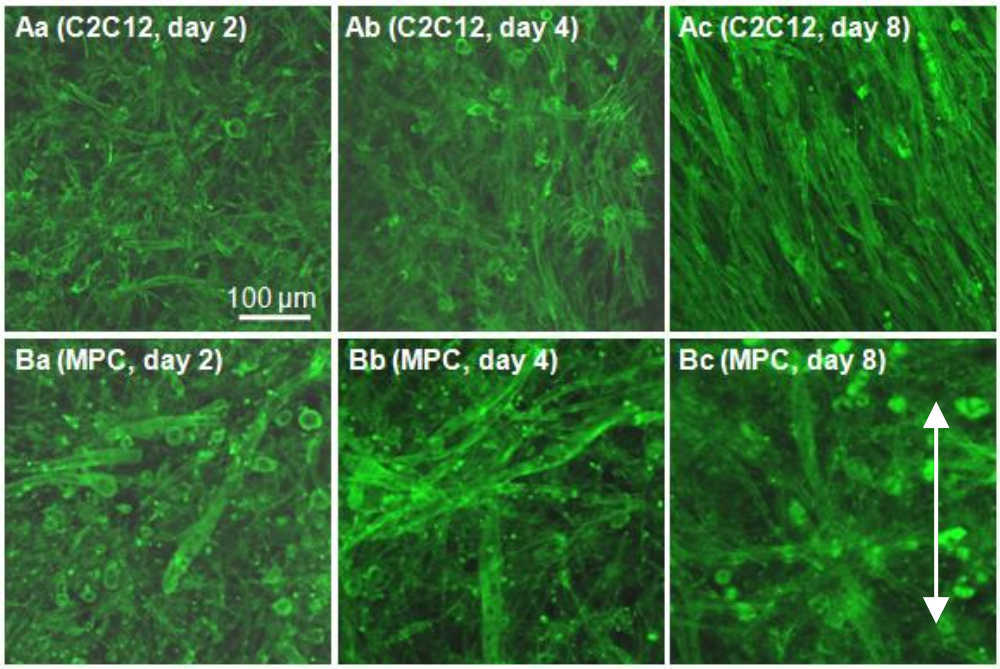

Figure 7.4: Whole mount cytoskeletal actin staining of C2C12 (Aa-Ac) and MPC (Ba-Bc) control constructs on day 2, 4 and 8 of differentiation. The direction of the samples, and the direction of attachment between the anchoring points, is from top to bottom, indicated by the arrow.

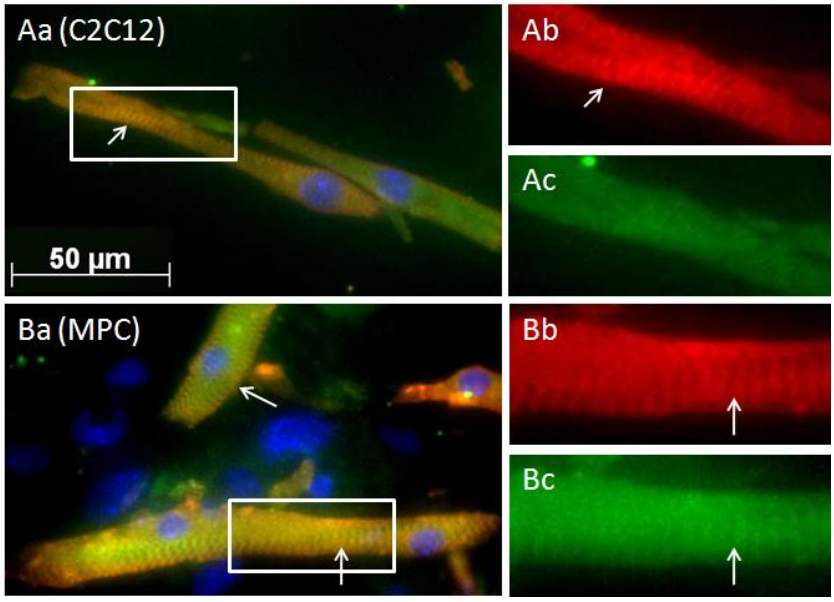

Figure 7.5: Cross-striations in C2C12 (Aa) and MPC (Ba) constructs. Frozen sections were stained for sarcomeric α-actinin (red), sarcomeric myosin (green), and nuclei (blue). Panels Ab-Ac and Bb-Bc show magnifications of boxed areas in panels Ab and Bb for α-actinin (red) and in panels Ac and Bc for sarcomeric myosin (green).

Sarcomere development differed remarkably between the C2C12 and MPC constructs. In C2C12, but not in MPC constructs, cross-striations were observed on day 4 of differentiation. By 8 days of differentiation, both constructs had mature cross-striations. Typical examples of the observed cross-striations are shown in Figure 7.5. Mechanical stimulation did not affect sarcomere development in C2C12 constructs, with similar levels of cross-striations in stretched and non-stretched constructs. By contrast, mechanical stimulation prevented the formation of cross-striated myotubes in MPC constructs, since none were observed after ramp and uniaxial intermittent dynamic stretch, whereas in the control constructs cross-striations were present after 8 days of differentiation (Figure 7.5).

The gene expression analysis of MRFs and sarcomere proteins showed that the expression levels of some genes (in particular MyoD, MYH1, MYH2, MYH8) substantially differed between C2C12 and MPC constructs (Figure 7.6 and 7.7). The MRFs are important in the differentiation pathway from myoblast to mature myofibers. Early MRFs, such as MyoD and myogenin, are mostly involved in the transition from myoblasts to myotubes, whereas late MRFs, such as MRF4 and muscle LIM protein (MLP), play a role in maturation of myotubes. In addition, MyoD is involved in myosin isotype switches. The expression levels of MyoD and the late MRFs increased over time in the C2C12 control constructs. On the other hand, in MPC control constructs, expression of myogenin and MLP decreased over time, suggesting more maturation in C2C12 constructs. The ramp protocol, evaluated on day 4, had very little effect on gene expression in both C2C12 and MPC constructs. Only for MRF4 in C2C12 constructs a small decrease was observed. Intermittent dynamic stretch, evaluated on day 8, caused a remarkable decrease in the expression of MyoD and myogenin in both C2C12 and MPC constructs. MRF4 was strongly reduced in C2C12 constructs, but was unaffected in MPC constructs (Figure 7.6). By contrast, in MPC constructs, a downregulation in MLP expression was found upon stretch, which was not observed in C2C12 constructs.

In addition to the expression analyses of MRFs, the expression of sarcomere proteins was investigated to explore the effects of stretch on myofiber maturation (Figure 7.7). Over time, the expression of MYH1, MYH2, and MYH4 was significantly increased in both C2C12 and MPC control constructs. The expression of actin, α-actinin and MYH8 increased in C2C12 constructs only, whereas the expression of actin slightly decreased in MPC constructs, again suggesting a higher maturation level in C2C12 constructs as compared to MPC constructs. After ramp stretch (day 4), only small changes were found. In C2C12 constructs, expression of actin and α-actinin was increased, and in MPC constructs, the expression of MYH4 was decreased. Intermittent dynamic stretch (day 8), however, induced significant decreases in the sarcomere proteins: downregulation of MYH 2-8 in both C2C12 and MPC constructs, and downregulated actin expression in MPC constructs (Figure 7.7).

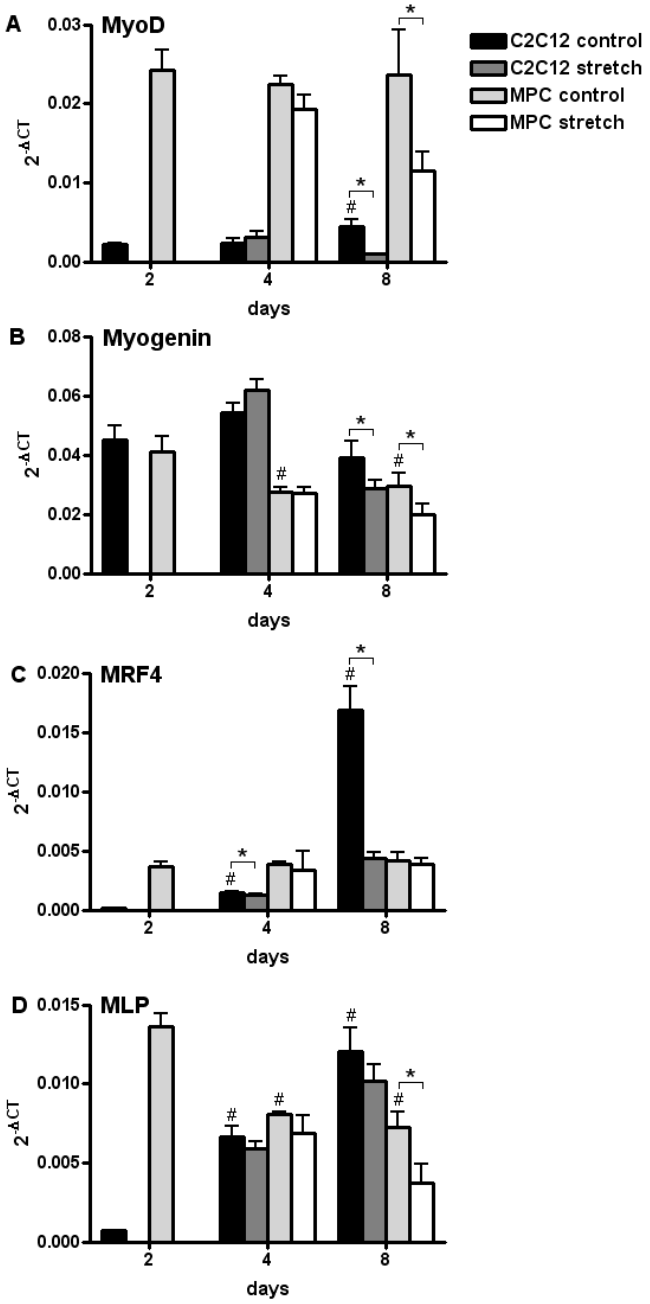

*Figure 7.6: Gene expression levels of myogenic regulatory factors MyoD (A), myogenin (B), MRF4 (C), and MLP (D) over time and upon mechanical stimulation (stretch). Constructs of C2C12 and MPCs were analyzed on day 2 (before uniaxial ramp stretch), day 4 (after uniaxial ramp stretch), and day 8 (after uniaxial intermittent dynamic stretch). * indicates a significant difference between stretched and corresponding control constructs (P<0.05), and # refers to a comparison of control constructs with respect to day 2 (P<0.05).*

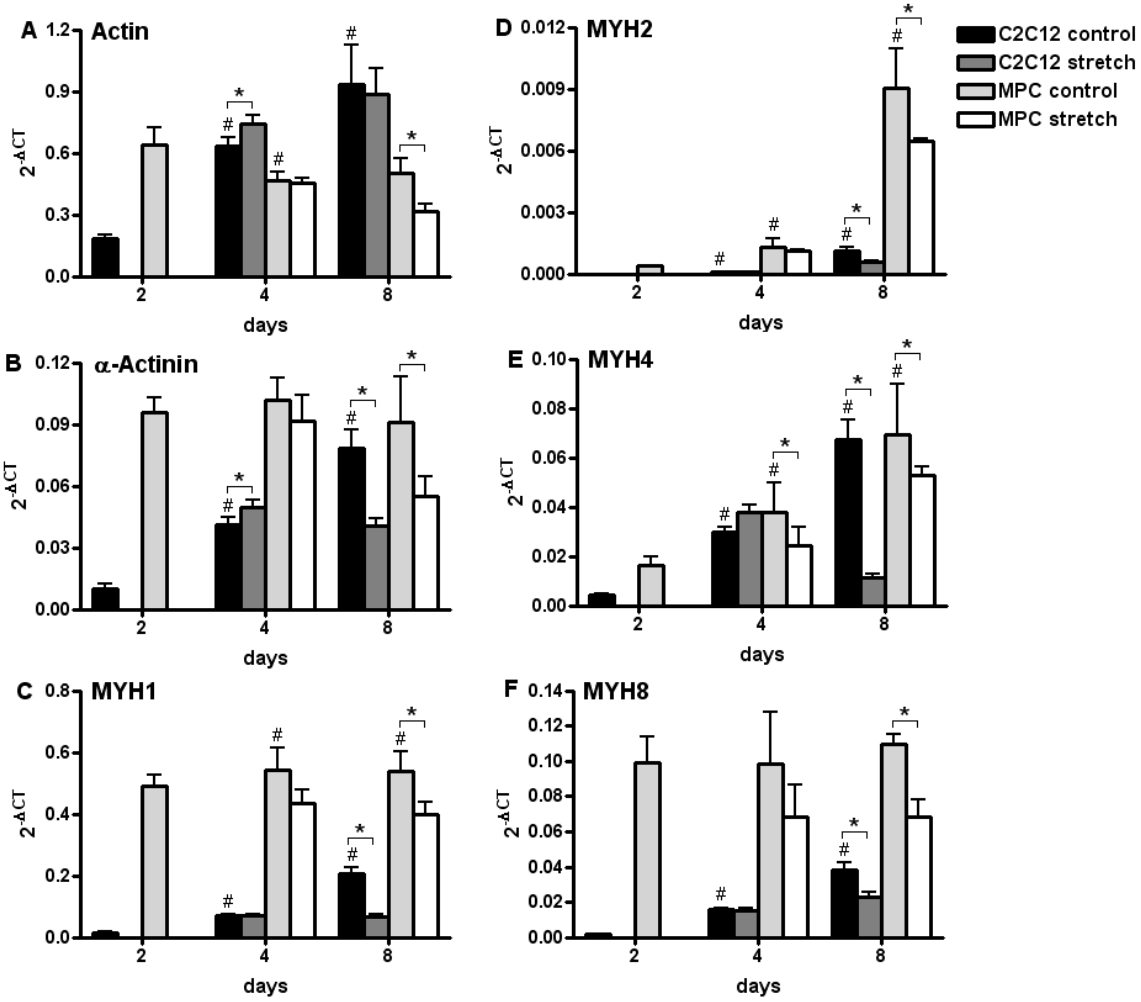

*Figure 7.7: Gene expression levels of sarcomere proteins actin (A), α-actinin (B), MYH1 (C), MYH2 (D), MYH4 (E), and MYH8 (F) over time and upon mechanical stimulation. Constructs of C2C12 and MPCs were analyzed on day 2 (before ramp stretch), day 4 (after ramp stretch), and day 8 (after uniaxial intermittent dynamic stretch). * indicates a significant difference between stretched and corresponding control constructs (P<0.05), and # refers to a comparison of control constructs with respect to day 2 (P<0.05).*

7.4 Discussion

Tissue engineered skeletal muscle can be useful for a number of purposes, mostly based on its application as a model system, employment in regenerative medicine, and more uncommonly for the *in vitro* production of meat. This study aimed at tackling some of the controversies that are associated with the use of stretch in tissue engineering strategies. While stretch is obviously an important niche parameter in the maintenance and adaptation of muscle tissue in vivo (Benjamin and Hillen 2003), its value for skeletal muscle tissue engineering purposes still needs to be proven. Certain mechanical

stimulation strategies show an increase in differentiation of muscle cells (Powell et al. 2002; Kurokawa et al. 2007; Moon du et al. 2008), but other protocols display an increase in proliferation, accompanied by a decrease in differentiation (Matsumoto et al. 2007; Kook et al. 2008a; Kook et al. 2008b). We set out to evaluate the differences in response to a standardized mechanical stimulation protocol in the physiological range in a 2D and 3D model system, and compared the C2C12 myoblast cell line and primary muscle progenitor cells (MPCs) in their response to these mechanical cues.

7.4.1 Stretch in a 2D model system

The combined mechanical stimulation protocol that we applied to both C2C12 myoblasts and MPCs cultured on protein-coated flexible membranes, consisted of a uniaxial ramp stretch to facilitate alignment in the direction of stretch (Vandenburgh 1988; Matsumoto et al. 2007), and intermittent dynamic uniaxial stretch to induce differentiation and maturation of the muscle cells (Vandenburgh et al. 1989; Moon du et al. 2008). Unfortunately, ramp stretch did not result in alignment of myotubes in the direction of stretch in our experiments; after 2 days, myotubes were still randomly oriented on the membrane. The maturation level of the myotubes of both cell sources was also not influenced by the uniaxial ramp stretch, since cross-striations and expression levels of MRFs and sarcomere proteins were not enhanced. These results suggest that the ramp stretch as used in our experiments might not be perceived as a stimulus by the cells.

Also uniaxial intermittent dynamic stretch did not provoke alignment of C2C12 and MPC myotubes in the direction of stretch, or influence the assembly of cross-striations. However, it mildly affected the expression levels of a few MRFs and sarcomere proteins in a negative manner, independent of coating, pointing to a decrease in maturation level of the cells. This decrease is in accordance with results from others using intermittent dynamic stretching protocols (Kook et al. 2008a; Kook et al. 2008b; Atherton et al. 2009), but at odds with observations made when using continuous dynamic stretching protocols (Vandenburgh and Karlisch 1989; Moon du et al. 2008). However, in the *in vivo* situation, training regimes are also intermittent and do lead to muscle hypertrophy (Benjamin and Hillen 2003). Therefore, it seems that the choice of protocol largely influences the outcome of mechanical stimulation studies and that the translation of the *in vivo* to the *in vitro* situation has not yet been achieved.

7.4.2 Stretch in a 3D model system

To relate the results found in the 2D model system to a somewhat more physiological situation in a 3D model system, we applied the same combined mechanical stimulation protocol to C2C12 myoblasts and MPCs in fibrin constructs. During the entire protocol,

constructs remained intact and no cellular damage could be observed. After uniaxial ramp stretch, orientation of myotubes in C2C12 constructs was more prominent in the direction of attachment between the anchoring points. However, because a similar alignment was found in control samples, this orientation is thought to be caused by tissue compaction and not by ramp stretch. The C2C12 myotubes caused a build-up of internal stress, because of the limitation of movement by attaching it to Velcro attachment points. Subsequently, because myotubes tend to align in the direction of principal stress, the direction of myotube alignment was longitudinal. However, this alignment was not observed in the MPC constructs, which is presumably caused by the lower myotube amount present in these constructs leading to lower internal stress. The uniaxial ramp stretch also did not affect maturation of C2C12 and MPC constructs much: Formation of cross-striations was not influenced and only small differences were observed at the transcriptional level, most prominently in the C2C12 constructs.

Uniaxial intermittent dynamic stretch also did not provoke alignment in the direction of stretch, for both C2C12 and MPC constructs. Intermittent dynamic stretch affected the formation of cross-striations only in MPC constructs, but rather surprisingly inhibited and not stimulated sarcomere assembly. C2C12 constructs displayed similar amounts of cross-striations of α-actinin in both mechanically stimulated and control constructs.

The most remarkable finding after intermittent dynamic stretch was the notable decrease in expression levels of all MRFs and sarcomere proteins in constructs of both cell sources, further supporting an impediment of maturation (Matsumoto et al. 2007). Inhibition of fast twitch MHCs has been observed previously as an effect of mechanical stimulation (Kumar et al. 2004; Kook et al. 2008b) and has also been interpreted as prevention of differentiation. In contrast, other studies report MHC isoform switches to more adult forms (Sakiyama et al. 2005; Kurokawa et al. 2007). However, in these cases, myotubes were continuously subjected to higher percentages of intermittent dynamic stretch, which could cause injury (Schultz and McCormick 1994), thus inducing pathophysiological rather than physiological adaptations.

In our hands therefore, 3D constructs reacted very similar to 2D cultures in response to stretch. In both experimental conditions, maturation was either unaffected or reduced. The observation that alignment was not achieved with any of the stretch regimens, particularly for MPCs, may offer an explanation for this lack of effect. C2C12s in 3D were aligned in the direction of stretch, but this was achieved apparently by internal forces generated during growth and tissue compaction. These forces may have stimulated maturation to an extent that could not be improved by exogenous application of intermittent dynamic stretch.

7.4.3 MPC versus C2C12

The two cell sources in this study reacted differently to stretch and coating. In 2D, the influence of substrate coating (laminin and collagen I) on the maturation level of the non-stimulated control cultures was considerable. Sarcomere assembly of C2C12 myotubes, visible in cross-striations of α-actinin, was only present on the laminin-coated membranes. By contrast, MPC sarcomere assembly occurred independently of the type of coating, and cross-striations of both α-actinin and myosin were present in all cultures. Expression levels of MRFs and sarcomere proteins were also influenced by substrate coating. Whereas in C2C12 myotubes, expression levels of genes affected by coating were higher in myotubes cultured on collagen I coated membranes, in MPC myotubes the effect was opposite; all genes affected by coating displayed higher expression levels in the MPCs cultured on laminin-coated membranes compared to collagen I coated membranes. In addition, more genes were affected by coating in MPC myotubes compared to C2C12 myotubes. Overall, and in accordance with previous results (Cimetta et al. 2009; Boonen et al. 2009), our results suggest that a laminin coating induces maturation best. As these cells are derived from satellite cells that are in close contact with the basal membrane that consists for 80% of laminin, they are presumably well equipped to interact with laminin (Girgenrath et al. 2005; Boppart et al. 2006).

In the non-stimulated 3D fibrin constructs, both C2C12 and MPC myoblasts differentiated into myotubes. Although MPC myotube formation was faster, the amount of myotubes was much higher in the C2C12 constructs. In addition, cross-striations were formed earlier in C2C12 constructs compared to MPC constructs. We believe that this is caused by the ECM protein-dependency of MPC proliferation and differentiation (Melo et al. 1996; Osses and Brandan 2002). MPCs are traditionally expanded in Matrigel^{TM}-coated flasks and lose their differentiation potential when cultured in conventional poly-lysine coated flasks (personal observations). Possibly, culturing in a “bare” fibrin environment has similar effects.

Also at the transcriptional level we observed increased maturation over time in the C2C12 control constructs, as evidenced by the augmentation of expression levels of MyoD and late MRFs (MLP and MRF4). On the other hand, in MPC control constructs, myogenin and MLP expression decreased over time. Probably, this means that C2C12 constructs are more mature compared to MPC constructs, also indicated by earlier presence of cross-striations.The effects of mechanical stimulation, both uniaxial ramp and intermittent dynamic stretch, resulted in similar trends in expression levels of the sarcomere proteins in constructs of both cell sources: no relevant changes after ramp stretch, and downregulation of sarcomere proteins after 4 days of intermittent dynamic stretch.

7.4.4 Conclusion

In conclusion, this research showed that dynamic mechanical stimulation in the physiological range restricts maturation into functional muscle fibers both in 2D monolayers of cells cultured on coated flexible membranes, and in fibrin constructs of both C2C12 and MPCs. We therefore conclude that the mechanical stimulation protocol we used is not recommended for tissue engineering purposes. The effect of mechanical stimulation showed a similar trend in constructs of both C2C12 and MPCs, which indicates that the C2C12 cell line is a relevant model system for research on mechanical stimulation regimes.

7.5 Acknowledgements

The authors want to thank Sarita Soekradj-Soechit for cryosectioning and histological analyses of the engineered muscle constructs. In addition, thanks to Leonie B.P. Niesen and Jo W. Habets for taking care of the animals. This work was financially supported by SenterNovem, grant ISO 42022. The MF20 antibody developed by Donald A. Fishman 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.

Chapter 8

General discussion

8.1 Introductory remarks

Tissue engineering of skeletal muscle in principle has three main goals. Most obviously, tissue engineered skeletal muscle can be used in regenerative medicine, for example in repair of defects caused by traumatic injury or for the treatment of muscular dystrophies (Levenberg et al. 2005; Boldrin et al. 2008; Serena et al. 2008). In addition, it could be useful as a model system for drug screening, pressure sore etiology and research on muscle physiology (Gawlitta et al. 2007; Vandenburgh et al. 2008). Last, a perhaps more exotic application of tissue engineered muscle is in food consumption. Muscle, in the form of meat, is a major source of dietary protein in the western world. Meat consumption has increased worldwide in the past decades, a phenomenon that is accompanied by an increase in pollution and land use and a decrease in animal welfare. By producing artificial meat industrially, these negative effects might be reduced.

The goal of the research presented in this thesis, is to improve the fabrication of tissue engineered skeletal muscle from primary cells. In the end, this knowledge can be used to produce tissue engineered skeletal muscle for meat production. To achieve this goal, some requirements need to be met (Chapter 2). Most of these requirements are also essential for the other goals of tissue engineered muscle. First of all, an appropriate cell source needs to be established. These cells should be able to undergo many rounds of population doublings, while preserving the capacity to differentiate into functional skeletal muscle cells. Second, these cells should be provided with appropriate signals and the appropriate environment to enable differentiation into functional skeletal muscle tissue. In the search for the best cell source, we decided to use myogenic precursor cells (MPCs) isolated from murine muscle (Chapter 1). To supply these cells with the appropriate environmental signals, we used the cells' natural *in vivo* environment (the niche) as an inspiration, as we hypothesized that these signals would be pivotal for appropriate cell functioning (Chapter 3). We then set out to systematically investigate the different environmental signals taken from the niche. In Chapter 4, we examined the influence of extracellular matrix protein coating and matrix stiffness on myogenic precursor cell (MPC) proliferation and differentiation in a 2D situation. We found that both protein coating and matrix stiffness considerably influenced both events. Proliferation was best on a substrate with an elasticity close to muscle *in vivo* elasticity, although classical culturing substrates (rigid glass coverslips) performed equally well. Furthermore, maturation into striated myofibers only occurred on elasticities higher than or equal to 10 kPa and was best on laminin-coated substrates. Next, we added electrical stimulation to this system (Chapter 5). Surprisingly, when electrically stimulated, Matrigel^{TM}-coated substrates now outperformed the laminin-coated substrates considering maturation. The electrical stimulation regime by itself only caused a slight advance in differentiation. Afterwards, the 2D system was expanded to a 3D system using a collagen I/Matrigel^{TM} gel-mix to produce murine bioartificial muscles (mBAMs) (Chapter 6). We then also compared the performance of the C2C12

cell line and muscle precursor cells (MPCs) and their response to electrical stimulation in this system. MPC mBAMs were found to be much more mature than their C2C12 counterparts. Moreover, mere culturing in this 3D system improved and accelerated differentiation and maturation to such an extent that the small, but positive effect electrical stimulation had on these phenomena seemed negligible in 3D. Last, we investigated the effects of stretch in a 2D and 3D situation and again compared performance of the C2C12 cell line to the MPCs (Chapter 7). In this 2D system, laminin was again the best protein coating for maturation, whereas stretch had only limited and this time negative effects. The 3D system that was used on this occasion (fibrin gel) had an adverse effect on MPC differentiation compared to the collagen I/Matrigel^{TM} gel-mix previously used, whereas C2C12 myoblast performance was excellent. Again, stretch was found to negatively influence maturation of both types of constructs. In conclusion, it has been shown in this thesis that the interaction between different types of niche factors, such as stiffness, extracellular matrix proteins and electrical and mechanical stimulation is complex and that the effect is also dependent on the cell type and dimensionality of the system. Laminin coating is best for maturation into functional muscle and substrate stiffness needs to be either close to physiological stiffness, or infinitely large. Moreover, the additional effect of electrical and mechanical stimulation on muscle maturation is small and therefore not considered to be essential for tissue engineering of skeletal muscle.

8.2 Ethical considerations

The market for meat is increasing dramatically because of growth of the world population and increase in the per capita income. Therefore, many animals live in bio-industry in tragic conditions. If it were possible to satisfy part of this market by producing meat in an artificial, industrial setting using stem cells, much animal suffering could be prevented. We expect that by doing fundamental research using murine cells, we will soon be able to extrapolate the results to animals that are being used for meat production, because the muscle regeneration process is similar. In addition, the knowledge used for the production of meat out of stem cells can be used for tissue engineering of muscle for regenerative medicine. Before being allowed to use mice for research projects, these projects need to be approved by the Dutch institutional animal care and usage committee, who test the necessity of animal use in the project. The animal discomfort in our experiments is relatively low, because the mice are euthanized before isolation of muscles.

8.3 Model systems

The choice for an *in vitro* model system for muscle tissue engineering is very important, because obviously, outcomes may differ depending on the model. Variables in this model system are the cell source, the (2D or 3D) environment, the matrix in which or on top of which cells are cultured and finally electrical or mechanical stimulation.

8.3.1 Cell source

Different cell sources can be used for skeletal muscle tissue engineering provided they undergo many rounds of population doublings and differentiate into skeletal muscle. Whereas in many tissue engineering strategies cell lines are being used (Okano and Matsuda 1997; Matsumoto et al. 2007; Gawlitta et al. 2008), eventually primary cells are preferred for consumption and regenerative medicine purposes.

8.3.1.1 MPCs vs. C2C12

The C2C12 murine myoblast cell line is probably the most widely used cell line in skeletal muscle research. The advantages are abundant: C2C12 myoblasts are easy to culture, population doublings are very rapid, differentiation into myofibers is easily controllable, results of experiments are extremely reproducible and no ethical questions are raised. However, it has been shown previously that differences do exist between primary cells and cell lines in their response to environmental cues (Maley et al. 1995; Boontheekul et al. 2007). The MPCs that we used are primary cells that are derived from murine satellite cells (SCs). Indeed, we also found differences in responses between the C2C12 myoblasts and MPCs, dependent on the environment they were cultured in. Whereas C2C12 myotubes formed α-actinin cross-striations only after approximately 4 days in murine bioartificial muscles (mBAMs) and myosin cross-striations only in special circumstances (Engler et al. 2004b), MPCs already show much α-actinin cross-striations at the first time point checked (after 2 days in mBAMs) and contain myosin cross-striations after 4 days in mBAMs (Chapter 5). In addition, MPC mBAMs contracted synchronously to the electrical stimulus, whereas no contractions were observed in C2C12 mBAMs. Overall, MPC mBAMs were considered much more mature compared to C2C12 mBAMs (Chapter 6). However, in the fibrin gel that was used for the stretch experiments, C2C12 myoblasts were able to produce much more myotubes in the constructs compared to MPCs. They also formed α-actinin cross-striations faster than MPCs, although they were still unable to form myosin cross-striations (Chapter 7). Because the extracellular matrix protein content of the two gel systems differs considerably, it would therefore seem that the protein dependency differs between the two cell sources, possibly related to a difference in integrin repertoire.

Interestingly, RNA expression levels of different myogenic regulatory factors (MRFs) and sarcomeric proteins differ tremendously between C2C12 and MPC constructs. Part of this difference can be explained by a divergence in reference gene expression level, but large differences remain after correction for this. We currently do not have an explanation for this phenomenon. However, effects of culturing and stimulation trigger similar effects in changes of expression levels in both cell types.

Both for electrical stimulation and stretch, overall effects on maturation were similar in C2C12 myotubes and MPCs, making C2C12 myoblasts a useful model system keeping in mind that results should be verified using MPCs.

8.3.1.2 Alternative cell sources

For the production of tissue engineered muscle, other cell sources should not be ignored. In the case of meat consumption purposes, embryonic stem cells are of interest. Theoretically, these cells can be expanded indefinitely and would therefore significantly reduce the number of animals used for meat production. Unfortunately, embryonic stem cells from farm animals have not yet been established (Keefer et al. 2007; Talbot and Blomberg 2008). However, satellite cell derived myoblasts have been isolated from pig and show promising results in *in vitro* culture and differentiation (Wilschut et al., 2008). In addition, bone marrow derived stem cells have shown some degree of plasticity and might be able to give rise to muscle fibers (Gussoni et al. 1999; Gang et al. 2004). Obviously, muscle derived stem cells (MDSCs) should also not be disregarded. Although we did not succeed in isolation of these cells (Chapter 1), their use remains promising (Peng and Huard 2004).

8.3.2 Culture environment

MPCs have been shown to lose proliferation and differentiation capacity after mere culturing and passaging (Machida et al. 2004; Collins et al. 2005; Montarras et al. 2005). To investigate the effect of different niche factors on the proliferation and differentiation potential of the MPCs, we therefore used these primary cells without prior expansion (Chapter 4 and 5). However, for tissue engineering strategies, much higher numbers of cells are required and therefore cells needed to be expanded. Fortunately, when cultured on Matrigel™ until passage 3, and when kept subconfluent, proliferation and differentiation potential of MPCs remained good. Occasional pre-plating for 30 minutes in uncoated culture flasks was necessary to separate non-myogenic cells, but overall culture quality was excellent (Chapter 6 and 7). For the 3D setting, we chose to use a gel system instead of a scaffold, since gels allow for dense cell seeding and alignment because of compaction. Two different gel systems were used in this thesis: A collagen I/Matrigel™ gel-mix and a fibrin gel. Performance of the MPCs in

the different systems was strikingly different: Whereas almost only cross-striated fibers were formed in the collagen I/Matrigel™ gel-mix and a mature, structural muscle tissue was formed (Chapter 6), much fewer myotubes were formed in the fibrin gels and these myotubes also matured slower compared to the myotubes in the collagen I/Matrigel™ gel-mix (Chapter 7). When comparing mere culturing in a 2D environment with a 3D environment, it was clear that the 3D environment was much more stimulative of myotube differentiation and maturation compared to the 2D environment.

8.4 Niche factors

Since we were inspired by the *in vivo* niche in our quest to engineer functional, mature muscle tissue, several niche factors were investigated separately or combined for their effects on MPC proliferation, differentiation and maturation. The interplay turned out to be complex. A schematic overview of the different niche factors that were investigated and their effects on myoblast proliferation and differentiation can be found in figure 8.1.

8.4.1 Matrix stiffness

First of all, the effect of matrix stiffness was investigated. Previously, it was already shown that proliferation of murine primary myoblasts was affected by substrate stiffness (Boontheekul et al. 2007). We showed that proliferation of MPCs was best on physiologically elastic substrates (Collinsworth et al. 2002) (20 kPa) (Chapter 4). For differentiation into cross-striated myotubes, it had been shown that C2C12 myotubes only developed characteristic myosin cross-striations when cultured on substrates with elasticities close to physiological stiffness (Engler et al. 2004b). However, in MPCs, we observed development of myosin cross-striations on all substrates with an elasticity higher than or equal to 10 kPa when using MPCs, underlining the potential differences between primary cells and a cell line (Chapter 4). In the electrically stimulated samples, matrix stiffness was also found to be of influence: Effects of electrical stimulation were visible at an earlier time point on 21 kPa cells than on coverslips (Chapter 5).

Remarkably, stiffness of the gels in the 3D experiments is possibly much lower than the threshold that was found in the 2D setting for formation of cross-striations. Cox and co-workers previously measured stiffness of C2C12 mBAMs and found it to be 0.38 ± 12 kPa (Cox et al. 2008), and stiffness of the fibrin gels we used was found to be around 0.25 kPa (Georges et al. 2006). However, cross-striations were formed in both gel systems, suggesting that stiffness might be perceived differently by the cells in a 3D environment. It cannot be ruled out though that mBAMs created from a gel with physiological stiffness result in even better maturation.

8.4.2 Extracellular matrix proteins

In a 2D culture environment, extracellular matrix protein coating was found to influence maturation only. The development of cross-striated, functional muscle fibers was best on laminin and poly-lysine coated substrates for regular culture conditions as well as on softer substrates (Chapter 4), although Matrigel™-coating was generally reported to be superior for differentiation (Maley et al. 1995; Langen et al. 2003). However, when myotubes cultured on laminin-coated substrates were stimulated, whether electrically (Chapter 5) or mechanically (Chapter 7), the stimulation had no or even a negative effect on maturation. Possibly this effect is due to less mechanical integrity in the connection between laminin and integrin α7β1 (Boppart et al. 2006; Liu et al. 2008) compared to the connection between other extracellular matrix components and their integrins.

In general, it has been shown that the presence of endogenous extracellular matrix proteins (fibronectin or basement membrane (BM) proteins) is indispensable for differentiation (Melo et al. 1996; Osses and Brandan 2002). Indeed, we found that in a fibrin gel system without BM proteins present (Chapter 7), differentiation was inferior compared to myotubes cultured in a collagen I/Matrigel™ gel-mix (Chapter 6).

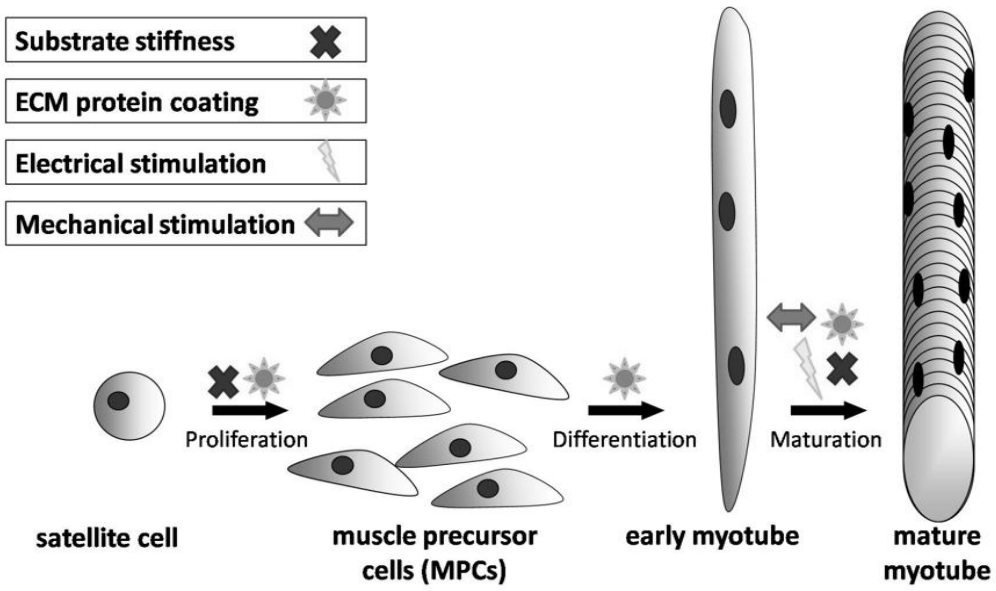

Figure 8.1: Effects of different niche factors on proliferation, differentiation and maturation of MPCs as described in this thesis.

8.4.3 Electrical stimulation

Electrical stimulation was investigated in 2D and 3D settings. In both cases, when using our specific stimulation protocol, only a small advance in maturation was found when comparing presence of cross-striations and contractions that was comparable to other studies (Kawahara et al. 2006; Park et al. 2008). In 3D, the most important finding was a switch in myosin isotype caused by electrical stimulation. Our protocol was probably perceived as a low-frequency stimulus and therefore evoked a switch to a slower myosin type (Chapter 6), a finding frequently seen after stimulation (Wehrle et al. 1994; Bayol et al. 2005). In 2D stimulated cultures a decrease in MHC8 was found that was similar to the decrease in the 3D culture system, pointing to a comparable isotype switch. We believe that, although control over myosin isotype might be useful, the electrical stimulation regime as we used it is not essential for myofiber maturation. However, its usage as a tool to measure strength of contractions and thus monitor maturation of the muscle tissue seems promising.

8.4.4 Mechanical stimulation

We investigated the effects of a mechanical stimulation protocol that consisted of a ramp stretch, followed by intermittent dynamic dynamic stretch. The ramp stretch was investigated with the purpose of facilitating myotube alignment, but failed to do so. In the 2D setting, no effect was found of the ramp stretch on gene expression levels of MRFs and sarcomeric proteins and the formation of cross-striations. In the 3D setting, just small effects were seen in the C2C12 constructs only. Intermittent dynamic mechanical stimulation was found to evoke a decrease in myotube maturation both in 2D and in 3D situation. Expression levels of all investigated MRFs and sarcomeric proteins decreased, similar to other studies (Kumar et al. 2004; Kook et al. 2008b). In MPC constructs, a delay in the formation of cross-striations was observed. Apparently, the mechanical stimulation protocol that we used was not beneficial for myotube maturation and is therefore in its current form not useful for tissue engineering purposes.

8.5 Future perspectives and recommendations

8.5.1 Limitations of culture systems

Several niche factors have been investigated in this thesis, together with the effect they have (separately or combined) on muscle development. Although some effects and their interactions have been clarified, the picture remains incomplete.

Substrate stiffness was shown to be important for maturation in a 2D setting in the sense that a threshold stiffness had to be overcome before cross-striations were

observed (chapter 4, (Engler et al. 2004b)). However, probably the stiffness of the gels that we used in the 3D setting is an order of magnitude smaller (Georges et al. 2006; Cox et al. 2008). Still, cross-striations were formed in both gel systems used. It therefore remains to be determined whether this stiffness threshold actually exists or that it is merely a 2D artifact. Similarly, whereas laminin-coating was best for maturation in simple monolayer culture (Chapters 4 and 7, (Cimetta et al. 2009)), laminin did not support higher levels of maturation in response to stimulation, whether electrical or mechanical. Instead, when collagen- or Matrigel^{TM}-coating (containing collagen IV) was used, attachment of the cells and thereby propagation of signal seemed better (Chapters 5 and 7). The choice for the right protein coating might therefore be dependent on the environment cells are put into. For electrical stimulation investigations, one single stimulation protocol was used (Chapters 5 and 6). Although timing of the start of the electrical stimulation protocol was thoroughly investigated by Langelaan and co-workers (Langelaan et al. 2009), other variables can be changed indefinitely, thereby also changing the effect of the stimulus. The most important limitation of our studies is probably the duration of the stimulus. Whereas the effect of stimulation seemed to be only temporal, this may not be the case when the stimulus is provided for a longer period of time. Correspondingly, in the mechanical stimulation experiments, one single stimulation protocol was chosen, based on pilot experiments (Chapter 7). Stimulation protocols found in literature differ enormously and observed results therefore range from increasing proliferation with inhibition of differentiation (Matsumoto et al. 2007; Kook et al. 2008a; Kook et al. 2008b) to enhancement of differentiation (Powell et al. 2002; Kurokawa et al. 2007; Moon du et al. 2008). We also observed a decrease in maturation, but one should be aware that effects may largely depend on the protocol used.

8.5.2 Applicability of tissue engineered skeletal muscle

In order to use tissue engineered skeletal muscle in practice for meat production or regenerative medicine, a number of criteria need to be met, which are remarkably similar for the two purposes.

First of all, the debate for the right stem cell source is still undecided. Whereas the MPCs that we used are indeed capable of producing mature, functional muscle tissue, there is still room for improvement in the proliferative capacity of these cells. However, MPCs might be useful for regenerative medicine, because their use allows for the usage of patient specific, autologous cells. For meat production, embryonic stem cells would be most useful, because of their unlimited proliferative capacity. However, differentiating these cells into skeletal muscle remains problematic (Zheng et al. 2006). Moreover, skeletal muscle tissue contains additional cell types other than muscle cells, such as fat cells and fibroblasts that are essential for tissue structure and taste. To recreate realistic

skeletal muscle tissue, these cells should be considered for co-culturing techniques (Edelman et al. 2005; Brady et al. 2008). Functional vascularization is pivotal for implantation to be effective, but is also a prerequisite for large scale production of skeletal muscle for *in vitro* meat production, because of diffusion limitations in static 3D culture systems. In order to produce reasonably sized constructs, a tubular system that can provide the cells in these engineered constructs with nutrients and a way for disposal of waste products inevitably needs to be present (Jain et al. 2005; Levenberg et al. 2005). In addition, muscle fibers need to be densely packed and aligned in engineered muscle constructs for both regenerative medicine or consumption purposes (Bian and Bursac 2008). As sarcomere organization into cross-striations and the ability of muscle constructs to contract are inevitably required for regenerative medicine, we think that it might also be of importance for meat texture and "bite". This architecture of muscle cells provides the tissue with a structure that can either resist strength and mere implantation techniques, or offers the desired texture for meat consumption purposes. To create these desired properties, the choice for a culture system is of importance. Gel systems, like the ones used in this thesis seem to be promising, since they allow for the recreation of *in vivo* muscle structure by permitting high density cell seeding and consecutive cell alignment and compaction. By co-culturing with endothelial cells, creation of a vasculature also seems possible (Levenberg et al. 2005). We cannot conclude yet on the importance of electrical and mechanical stimulation protocols. Electrical stimulation can obviously be used as a tool to measure maturation level of a construct, but the fact that it advances maturation might also be of importance, since time can be an important factor in regenerative medicine as well as in the meat industry. In addition, mechanical stimulation deserves much more investigation, because of its known effects on hypertrophy *in vivo* (Vandenburgh 1987). When the roles of the different types of stimulation are elucidated, they can be used in the development of a bioreactor, which is the first step towards upscaling tissue engineering of skeletal muscle. Another important step in this upscaling process is the exclusion of any animal components from the culturing process. Currently, we use bovine and equine serum as well as chick embryo extract in MPC culture medium. These components should be replaced, possibly by a cocktail of growth factors that could be produced in yeast cells or bacteria (Halasz and Lasztity 1990). We believe that, eventually, tissue engineering of skeletal muscle for regenerative medicine as well as for meat production is feasible. Although we do not expect many ethical objections for the purpose of regenerative medicine, consumption of artificial meat could meet some resistance. Therefore, product marketing will be of significant value in the future. Given its potential societal impact, further endeavors to create artificial meat from animal stem cells need to be promoted.

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Samenvatting

Het tissue-engineeren van skeletspierweefsel is op verschillende manieren interessant voor onderzoekers. Voor de hand liggend is het gebruik van engineered spierweefsel in de regeneratieve geneeskunde, bijvoorbeeld in de behandeling van spierziekten of om defecten te herstellen die het gevolg zijn van een trauma. Bovendien kunnen dit soort spieren gebruikt worden als modelsysteem voor bijvoorbeeld onderzoek naar geneesmiddelen of het ontstaan van drukwonden. Iets exotischer is het onderzoek met als doel de productie van consumptievlees. De huidige vorm van bio-industrie heeft een grote invloed op het milieu voornamelijk vanwege het hoge verbruik van water en graan, de uitstoot van broeikasgassen en de verspreiding van besmettelijke ziekten. Bovendien gaat het gepaard met dierlijk lijden. Door spierweefsel op grote, industriële schaal te tissue-engineeren zouden deze nadelige bijkomstigheden kunnen verminderen.

Om dit soort vleesproductie te verwezenlijken moet aan een aantal voorwaarden worden voldaan: Ten eerste moet er gebruik kunnen worden gemaakt van een celbron die lang kan prolifereren zodat er veel cellen ontstaan, maar die nog steeds de mogelijkheid hebben om te differentiëren naar spiercellen. Ten tweede moeten de juiste omstandigheden gecreëerd worden om deze cellen geschikte signalen aan te bieden om volwassen spierweefsel te vormen in een drie dimensionale (3D) omgeving. Aangezien spiercellen zelf niet gebruikt kunnen worden, omdat deze bestaan uit vele gefuseerde cellen en zelf geen delingscapaciteit meer bezitten, maken we gebruik van satellietcellen. Deze cellen zijn normaal gesproken in het lichaam verantwoordelijk voor de verrassende regeneratieve capaciteit van de spier en zijn daardoor uitermate geschikt als stamcelbron. Het enige probleem is dat het lijkt of deze cellen hun stamcel-capaciteiten verliezen op het moment dat ze in kweek worden genomen. Onze hypothese is dat dit veroorzaakt wordt doordat de cellen weggehaald worden uit de specifieke omgeving waar ze zich normaal bevinden: de niche.

Verschillende niche-factoren kunnen een rol spelen in het functioneren van een satellietcel: bijvoorbeeld groeifactoren, extracellulaire matrix (ECM) eiwitten, andere nabije cellen, elektrische signalen van zenuwen, rek door beweging en groei en de elasticiteit van zijn omgeving. In dit proefschrift hebben we de invloed van verschillende van deze factoren op zichzelf en met elkaar gecombineerd op het gedrag van satellietcellen uit muizen onderzocht.

We hebben aangetoond dat het gebruik van verschillende ECM eiwitten cruciaal is voor alle facetten van satellietcel gedrag (proliferatie, differentiatie en maturatie). De beste maturatie (gekenmerkt door de aanwezigheid van karakteristieke dwarsstreping en de

aanwezigheid van spontane contracties) is waargenomen op laminine coating. Dit is niet verwonderlijk aangezien het laminine-netwerk in het basaal membraan zich *in vivo* direct aan de zijde van de satellietcel bevindt. Wat betreft elasticiteit van de omgeving is in dit proefschrift bewezen dat de waarde van de elasticiteit hoger dan een bepaalde grenswaarde dient te zijn om maturatie tot dwarsgestreepte spiervezels te bereiken. Bovendien is de elasticiteit van de omgeving van invloed op de proliferatie van myoblasten met de hoogste proliferatie op een ondergrond met een elasticiteit die vergelijkbaar is met die van spierweefsel maar ook op klassieke kweekbodems. Van elektrische stimulatie hebben we aangetoond dat het de maturatie tot volwassen spiervezels versnelt, gedemonstreerd door de eerdere aanwezigheid van dwarsstreping en de opregulatie van maturatie markers. Daarnaast lijkt elektrische stimulatie bepalend voor welk type myosine er wordt aangemaakt (d.w.z. of het snelle of langzame spiervezels worden). De vorm van mechanische stimulatie die door ons is gebruikt had een negatief effect op maturatie, uitgedrukt in een vertraging in de aanwezigheid van dwarsstreping en de downregulatie van maturatie markers. Door het gebruik van verschillende modelsystemen is duidelijk geworden dat differentiatie in een 3D omgeving vele malen gunstiger is in vergelijking met een 2D omgeving.

Concluderend kan gesteld worden dat we hebben aangetoond dat bepaalde niche-factoren belangrijk zijn voor het functioneren van satellietcellen. Met dit proefschrift is een belangrijke stap gezet in de richting van het ontwikkelen van een kweeksysteem voor tissue-engineered vlees.

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Kristel, september 2009

Curriculum vitae

Kristel Boonen werd op 10 april 1982 geboren te Eindhoven. In 2000 behaalde zij haar gymnasium diploma aan scholengemeenschap St. Ursula te Horn. Na een multidisciplinaire, internationale bachelor opleiding aan het University College te Utrecht volgde zij aan de Universiteit Utrecht een master-opleiding in de richting “Developmental Biology and Biomedical Genetics”. In het kader van deze opleiding koos ze voor een major bij Medische Fysiologie in Utrecht, waarna een minor aan de Technische Universiteit Eindhoven bij de faculteit biomedische technologie volgde. Deze omgeving beviel, zodat ze in 2005 begon aan een promotieonderzoek bij deze zelfde vakgroep, resulterend in dit proefschrift. Sinds november 2009 is zij in als klinisch chemicus in opleiding verbonden aan het Catharina ziekenhuis te Eindhoven en het St. Anna ziekenhuis te Geldrop.