Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Hans Alan Whitburn Haugen

Expansion of bovine skeletal muscle cells in a lab-bench bioreactor using edible microcarriers made of eggshell membrane

Master's thesis in Biotechnology
Supervisor: Eirin Marie Skjøndal Bar
Co-supervisor: Sissel Beate Rønning and Mona E. Pedersen
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Abstract

Cultured meat is an emerging biotechnology where meat is produced in a bioreactor out of animal stem cells. Aimed at addressing issues with slaughtered meat, the technology could save animals from industrial farming, prevent future pandemics, and help partially spare the environment. This experiment focused on the expansion of bovine skeletal muscle satellite cells from sirloin cow steak on edible eggshell membrane based microcarriers.

Skeletal muscle satellite cells attach to flasks coated with Entactin-collagen-laminin and microcarriers. After attachment, they proliferate and expand in numbers before differentiating and becoming meat. Expanding large amounts of cells is a major hurdle which complicates cultured meat mass production.

The cells were fed with a proliferation medium with chemical components mimicking blood. The cells were added to a BioFlo 120® lab-bench bioreactor from Eppendorf, together with eggshell membrane microcarriers and proliferation media. Every couple of days, a sample was extracted from the bioreactor and the amount of cells were calculated. The cells in the sample were also observed under a microscope after hoechst staining. The results were compared to an experiment using a general-purpose microcarrier called Cytodex® 1 from Cytiva.

PicoGreen™ dsDNA quantification, as well as glucose and lactate concentrations were measured. qPCR was attempted on samples from the bioreactor. Studies of Muscle Satellite Cells proliferation and differentiation, by microscopy, Western blots (MHC, Myostenin, Myogenin, MLC, Tubulin, Desmin, Actin) and qPCR (MyoD, Myogenin, Desmin), were also conducted.

The microcarriers of eggshell membrane did not function well with the bioreactor used in the experiment as the bioreactor impeller did not allow the cells to remain suspended. Further research will need to be conducted to find a way to properly prepare the eggshell based microcarriers, or alternatively use a different reactor with a different impeller design.

Sammendrag

Kultivert kjøtt er en moderne bioteknologi der kjøtt blir produsert i en bioreaktor ut av stamceller fra dyr. Formålet er å erstatte slaktet kjøtt. Teknologien kan redde dyr fra industriell kjøttproduksjon, forhindre fremtidige pandemier, og delvis føre til en mindre påkjenning på planetens økosystemer. Dette forsøket fokuserte på å oppformere bovine skjelettmuskel satellittceller fra mørbradbiff på spisbare eggeskallmembrankarriere.

Skjelettmuskel satellittceller fester seg til Entactin-collagen-laminin belagte labflasker og mikrokarriere. Etter at de fester seg så proliferer de og øker i antall før de differensierer og blir til kjøtt. Å oppformere større antall skjelettmuskelceller har vært et stort hinder som kompliserer kultivert kjøtt masseproduksjon.

Cellene ble matet med et prolifereringsmedium med kjemiske egenskaper likt blod. Cellene ble inokulert i en BioFlo 120® bioreaktor fra Eppendorf, sammen med eggeskallmembrankarriere og prolifereringsmedium. En prøve ble tatt ut av reaktoreren annenhver dag og antall celler ble beregnet. Cellene i prøven ble også studert under et mikroskop etter hoechst staining. Resultatene ble sammenlignet med et eksperiment der generelle mikrokarriere (Cytodex® 1) fra Cytiva.

PicoGreen^{TM} dsDNA kvantifikasjon, samt glukose- og laktatkonsentrasjonmålinger ble tatt. Et qPCR forsøk ble gjort på prøvene fra reaktoren. Muskelsatellittcelle proliferasjon- og differensieringsevne ble også studert ved mikroskopi, Western blot (MHC, Myostenin, Myogenin, MLC, Tubulin, Desmin, Actin) og qPCR (MyoD, Myogenin, Desmin).

Mikrokarrierene av eggeskallmembran fungerte dårlig med bioreaktoren som ble brukt i forsøket. Impelleren gjorde at cellene ikke klarte å holde seg suspendert i løsningen. Videre forskning kan vise hvordan en kan inokulere bioreaktoren slik at eggeskallmembranene holder seg suspendert og homogenisert, alternativt så kan en annen impeller eller reaktor anvendes.

Thanks

I would like to thank my advisor Eirin Marie Skjøndal Bar at NTNU. Eirin helped me during my bachelor as well, and I am very happy she was willing to help me again for my master thesis. I would not have completed my bachelors nor my master thesis without her help.

I would also like to thank my advisor at Nofima, Sissel Beate Rønning. I contacted Sissel in 2020 and asked if I could join her group at Nofima to learn more about cultured meat. I am very proud she let me join Nofima Ås for my master thesis. Sissel has been incredibly knowledgable and patient. I could not have wished for a better advisor.

I would also like to thank my second advisor at Nofima, Mona E. Pedersen. Mona has helped me a lot in the lab at Nofima and has been a great and encouraging advisor.

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I would like to thank Nofima's Dimitrios Tzimorotas. Dimitrios is an expert on biotechnology and bioreactors. I would not have finished my master without his help. I have enjoyed the discussions we have had in the lab and over lunch in the cantine.

I must also thank my brothers, Torbjørn and Erik Haugen, for supporting me. Finally my parents for moral support and encouragement.

Hans Alan Whitburn Haugen

Ås, Norway 28th August 2022

Contents

Figures

Tables

Acronyms

csv Comma Seperated Values plain-text digital file. 18

Diff Differentiation. xii, 23, 37

DMEM Dulbecco's modified eagle medium. ix, 9–11, 30

ECL Entactin-collagen-laminin. iii, v, ix, x, 10–12, 14

ECM Extracellular Matrix. 3, 4, 8, 53, 55, 56

ESM Eggshell membrane. 55, 56

FBS Fetal bovine serum. 5

FCS Fetal calf serum. 53

MCs Microcarriers. viii, xi, xvii, 2, 7, 8, 18, 19, 23–26, 28, 31, 54, 56

MuSC Muscle Satellite Cells. iii, vii–xii, xiv, xv, xvii, 2, 4, 6–15, 17, 20, 22–25, 30, 31, 33, 34, 47, 52–57, 59

PBS Dulbecco's phosphate buffered saline. ix, 11, 15, 19, 20, 24–26, 28

Prolif Proliferation. 23

RPM Rotations per minute. xi, 13, 16, 26, 31, 54

Glossary

4-(2-aminoethyl) benzene-sulfonyl fluoride (AEBSF) phosphate protease inhibitor prevents a samples enzymes from destroying the sample. 21

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favorable artificial environment. See cellular agriculture, proliferate, differentiate and seeding. 2, 4

Cellular agriculture is to make animal-sourced food from cell culture. See cell culture. 2

centrifugation or spinning down is a lab technique where a centrifuge is used to separate substances in falcon tubes with centrifugal forces by rotating the falcon tubes at high speeds. See pellet. 13

confluent or cell confluency is when cells growing on an adhesive surface completely cover the flask, and there is no room for cells to grow as a monolayer. In this experiment, no more than 70 % of the flask would be covered with cells before splitting to prevent differentiation during proliferation. Differentiated sattelite muscle cells can not proliferate. x, 13–15, 33

differentiate or cell differentiation is when a cell type changes and becomes a new specialized cell type. This is especially typical of stem cells. 6, 7, 20, 33

Dulbecco's Phosphate Buffered Saline (PBS) is an inert solution commonly used in cell biology research. 11, 12, 17, 20

Extracellular matrix (ECM) is a network of proteins and other molecules that surround, support, and give structure to cells and tissues in the body. 8

Fetal Calf Serum (FCS) is a common component of animal cell culture media. It is harvested from bovine fetuses taken from pregnant cows during slaughter. The product is also marketed as fetal bovine serum (FBS). There exists an expensive alternative called Ultroser G which is not sourced from animals. 5, 10, 30, 53

in-vitro is when cells perfrom outside their normal biological context in a lab. 10

LAF bench a laminar flow bench provides a sterile working environment for working with samples and cells to reduce the risk of contamination. ix–xi, 9–11, 13–15, 17, 25, 26

Lysis is when a cell's membrane is destroyed and you end up with a lysate which includes the cells proteins, DNA etc.. 21

Myogenic means originating in muscle tissue. 6

pellet is a small round clump of cells which accumulate at the bottom or lower side of a falcon tube after centrifugation. It is then possible to remove the media the cells were suspended in without removing the cells themselves. x, xi, 13, 14, 17, 26

proliferate is a term used to describe cell growth and devision, when cells first expand and then devide. In other words, proliferation is when cells grow and increase in number. x, 6, 14, 15, 33

quiescent Quiescence is the reversible state of a cell in which it does not divide but retains the ability to re-enter cell proliferation. 6, 7

Seeding is to spread cells from a sample to a culture vessel, such as a flask or bioreactor. This is done to perfrom cell culture activities. See cellular agriculture and cell culture. vii, 13

Splitting is to move cells grown in 2D from a set of flasks into a new greater set of flasks. This is often done to prevent cell differentiation. viii, 15

supernatant is the liquid lying above a pellet after centrifugation. See pellet and centrifugation. 16, 26

transcriptional activator is a protein (transcription factor) that increases transcription of a gene or set of genes. 6, 7

Chapter 1

Introduction

90 % of the biomass of all mammals in the world comes from domestic animals (60 % of total), plus the biomass from humans (30 % of total). Most animals are not roaming in the wild, meat production is astonishingly huge worldwide. The majority of people asked by the late statistician Hans Rosling's independent educational non-profit Gapminder[1] completely underestimate the massive amount of animals that are raised to be eaten or used for other agricultural purposes. If people were more aware of how small a share the world's wild biomass is, perhaps they would be more concerned with protecting nature (Rosling, 2022d). Increases in global demand for animal products have already led to intensification of industrial livestock farming, and this trend is expected to magnify as millions of households are lifted out of poverty in developing countries (Rorheim et al., 2016, p. 2).

Roughly 80 % of agricultural land is used for animals. Most farmland is used for animals to eat grass or to grow crops to feed them. When you stand in a supermarket, the aisles with meat and dairy take up only a small part of the store. It's difficult to realise that these products use 80 % or more of all agricultural land in the world. The land use is one way that meat production contributes to climate change. A lot of that land could have been forest (Rosling, 2022b).

Today, only 9 % of people live in low-income countries. For most companies in most industries, global competition intensified during the past 30 years as the majority of the world's population moved from living in low-income countries to middle-income countries (Rosling, 2022a). The "developing and developed" dogma of old has become a myth[2]. The world has become much wealthier and people all around

[1] https://gapminder.org

[2] See the 200 years that changed the world by Hans Rosling: https://www.gapminder.org/videos/200-years-that-changed-the-world/

the world are having fewer children (Rosling et al., 2018, p. 26).

Around 11 % of the world's population don't have enough food. This is less than what most people expect. In 2019, around 9 % of the world population (690 million people) didn't have enough food. In 2020 it is estimated to have increased to 11 %, because of increased poverty caused by the Corona crisis (Rosling, 2022c). World hunger is a solvable problem, and as people become wealthier they will eat more meat (Rorheim et al., 2016, p. 2).

The current meat industry is the main source of the pandemic diseases, keeping livestock pose a significant disease risk to human-beings (Rorheim et al., 2016, p. 1). Around 60 % of all known human diseases and 75 % of the most damaging emerging diseases are zoonotic (animaltransmitted) in origin. Most pathogens in recent years, such as bovine spongiform encephalopathy (BSE), and all forms of influenza (swine, avian, etc.) are transmitted through livestock (Rorheim et al., 2016, p. 2).

Cellular agriculture has been suggested to partially address issues associated with the environmental impact, animal welfare and sustainability challenges of conventional animal farming (M. J. Post et al., 2020). To upscale production, microcarriers offer a convenient method for growing adherent cells in bioreactors. Cell culture flasks are not feasible for industrial scale production when thousand fold increases in production volume are needed. Microcarriers (MCs) serve as a scaffold that adherent cells can attach to, allowing them to proliferate while a bioreactor keeps the cell-microcarrier complex freely suspended in the media (ChemoMetec, 2017). Processed eggshell membrane powder combined with collagen has shown promise as a biomaterial for tissue engineering (Rønning et al., 2020). Expanding Muscle Satellite Cells (MuSC) on edible eggshell membrane MCs in stirring bioreactors has been previously attempted. By using edible MCs, the process of upscaling production becomes much cheaper as it skips the process of removing the MCs from the final product (Hagen, 2019).

To further the research on eggshell membrane microcarriers, a method to expand MuSC on eggshell membrane MCs in a BioFlo® 120 lab-bench bioreactor has been developed and several experiments has been conducted to see if eggshell membrane can be used to reliably and cheaply upscale cultured meat production.

Theory on cultured meat and skeletal muscle satellite cells is presented in chapter 2. The methods and materials used for expanding bovine skeletal muscle cells in a lab-bench bioreactor using edible microcarriers made of eggshell membrane are described in chapter 3. The results of the experiment are presented in chapter 4. A discussion of the experiment's findings and what they implicate is prestented in chapter 5 and finally the conclusions are presented in chapter 6.

Chapter 2

Theory

For the last several years, research has been done on cultured meat by both the European Union (EU) and the United States (Edelman et al., 2005). Already in 2002 a meat experiment was conducted by NASA for the purpose of feeding astronauts on long space missions (Chiles, 2013). In 2007, the *in vitro* meat consortium was founded by the EU (Goodwin & Shoulders, 2013). In 2008, the first *in vitro* symposium was held in Norway (Midgley, 2008). In 2013, the first hamburger was cooked and showed off by Dutch researchers at a livestream event in London. Dr. Mark Post led the research and said the product would be in supermarkets within 10 to 20 years. The hamburger cost more than $330 000 US dollars to produce at the time (Bhat et al., 2015).

Meatable, a Dutch biotechnology company led by some of the same people who made the first cultured meat hamburger, recently showed off inaugural pork sausages for the first time. Cultivated meat is transitioning from research projects to something more akin to actual products (Sawers, 2022).

Skeletal muscle becomes meat after an animal dies (post-mortem). Meat consists of 90 % myofibers and 10 % connective tissue and fat cells (Listrat et al., 2016). Satellite cells are found in skeletal muscle. They are placed beneath the basal lamina. Some of the satellite cells found beneath the basal lamina are myogenic while others are non-myogenic cells (Sinha et al., 2017). During exercise or injury, the cells activate to repair muscle tissue (Meiliana et al., 2015). Post-natal muscle consists of multinucleated muscle fibres and ECM. Muscles are highly adaptive and can fill many different functional requirements. During repair the muscles will first grow. This is known as hypertrophy; the muscle cell size increases. Next, during hyperplasia, cell quantity increases (Stern & Mozdziak, 2019). Hyperplasia occurs during early embryonic development, producing the amount of muscle cells an animal will have throughout its life. Post-natal growth is due to hypertrophy of existing

cells (Wigmore & Stickland, 1983).

Mammals produce hormones and growth factors to enable hyperplasia. Cell culture needs the same hormones and growth factors in the culture medium to sustain cell proliferation and differentiation. Producing such compounds on an industrial scale has yet to be solved, research on the effects of such compounds on human health does not yet exist (Chriki & Hocquette, 2020).

A satellite cell will undergo self-renewal when a daughter cell starts proliferating or becomes quiescent. Some satellite cells differentiate with either stochastic or asymmetric cell distribution (Zammit et al., 2006). MyoD, myf-5, myogenin and myf-6/MRF4/herculin are the basic helix-loop-helix transcription factors which play a key role in controlling the development of skeletal muscle (Bach et al., 2004). See figure 2.2.

Muscle ECM consists of two layers. The first layer is linked directly to the plasma membrane of myofibers. There is a second external layer in the reticular lamina (Sanes, 2003). ECM provides muscle tissue with elastic properties, structural support, helps the cells with cell signalling and muscle cell transmission to the tendon which attaches the muscles to bone (Grzelkowska-Kowalczyk, 2016).

Lactic acid is produced almost continuously in all muscle fibres in living animals. The tricarboxylic acid cycle (TCA) changes lactic acid to pyruvic acid oxidatively. If the cell lacks oxygen, lactic acid is transported into the muscle fibre. Skeletal muscle tissue will use glucose as an energy source, the sugar is transformed into chemical energy (ATP) by a process known as glycolysis (Puolanne et al., 2002). There are fast and slow muscles. Fast muscles depend on anaerobic metabolism (anaerobic glycolysis) and can quickly contract. Slow muscles use aerobic metabolism and are more resistant to fatigue (Argiles et al., 2016).

To prevent lactic acid acidification, it is important to carefully monitor metabolism and provide sufficient oxygen to muscle cells during cultured meat production. Harmful waste products must also be removed during production of cultured meat. This can be done by periodically changing the media every few days during the production process (Datar & Betti, 2010).

Cultured meat begins with harvesting Muscle Satellite Cells from an animal by biopsy or a small incision. To remove some of the fibroblasts in the sample, which can easily dominate a cell culture due to their proliferation potential, one can place the cells on a uncoated flask. The fibroblasts will attach to the plate first and the satellite cells can be removed without fibroblasts (M. Post & Hocquette, 2017). See figure 2.1.

After satellite cell acquisition one can begin muscle cell proliferation by providing the cells with an appropriate culture medium in culture flasks (Alexander et al., 2017).

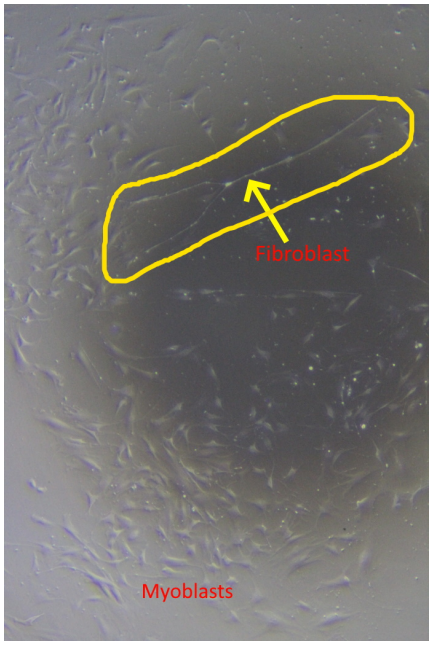

Figure 2.1: An example of a fibroblast muscle cell surrounded by myoblast muscle cells.

The media typically includes Fetal Calf Serum (FCS), also known as Fetal Bovine Serum (FBS), sourced from calves. A synthetic alternative called Ultroser G is also available (Gstraunthaler, 2003). Satellite cells will not proliferate forever. Human muscle cells from satellite cells have a maximum capacity of 45 cell divisions; they can only double in number 45 times (Hughes et al., 2016).

After proliferation and continuous splitting in culture flasks (to prevent premature differentiation) the cells can be moved to a stirring bioreactor to upscale production. Since muscle cells are anchorage dependant, microcarriers can be used which will provide cells with a larger surface area for growth compared to culture flasks (Moritz et al., 2015).

A bioreactor with sterile media provides the most cost-effective method to expand muscle cells. Once the desired amount of cells has been reached, a binding protein is added and stirring is stopped. Harvesting the cells and pressing them to a hamburger or sausage is the final step of the production of cultured meat (van der Weele & Tramper, 2014).

Resource efficiency an cost-efficiency is important in food production. Before scaling up to a larger bioreactor it is common to experiment with smaller lab-bench bioreactors and spinner flasks (Verbruggen et al., 2018). A bioreactor with microcarriers provide a larger surface for cell proliferation and aeration (Nienow, 2006). Furthermore, it becomes easier to transfer the correct nutrients to the cells, it decreases costs and supports longer term cellular agriculture expansion (Gupta et al., 2014). Bioreactors are available in all sizes, some vessels hold only a few millilitres of media, while others can hold volumes up to thousands of litres (WuXi-Biologics, 2018).

Bioprocess monitoring becomes easier with a bioreactor and process monitoring is an important part of a bioreactor experiment (Landgrebe et al., 2010). Bioprocess metrics include metabolic indicators such as glucose, glutamine, glutamate, lactate and ammonium measurements, as well as osmolarity, cell viability, viable cell density and total cell density measurements (López-Meza et al., 2015). Glucose concentration is the most important metric to monitor as this is where the cells receive their energy and main nutrients from.

Glucose and glutamine are the main nutrients consumed by proliferating cells (Hosios et al., 2016).

There are three main categories of microcarriers. The first category is nonporous and microporous microcarriers. The second category is microcarriers made of a matrix of dextran or polystyrene. Lastly, the third category of microcarriers are made of cellulose or gelatin. Cytodex® 1 is a microporous microcarrier (Badenes et al., 2016). Cytodex® 1 has high performance for cell proliferation of primary cell lines compared to other microcarriers (Cristina et al., 2005). Eggshell membrane carriers are also microporous (Andreassen et al., 2022).

Myogenic differentiation plays an important role in muscle development and is regulated by a number of transcription factors (Jang & Baik, 2013, p. 1). See descriptions below and figure 2.2.

MyoD (Myoblast determination protein 1) acts as a transcriptional activator that promotes transcription of muscle-specific target genes and plays a role in muscle differentiation. MyoD induces fibroblasts to differentiate into myoblasts (UniProt, 2022c).

Myogenin acts as a transcriptional activator that promotes transcription of muscle-specific target genes and plays a role in muscle differentiation. During terminal myoblast differentiation, plays a role as a strong activator of transcription at loci with an open chromatin structure previously initiated by MyoD. Plays also a role in preventing reversal of muscle cell differentiation. Myogenin induces fibroblasts to differentiate into myoblasts (UniProt, 2022d).

Desmin is a muscle-specific filament essential for proper muscular structure and function. In adult striated muscle they form a fibrous network connecting myofibrils to each other and to the plasma membrane from the periphery of the Z-line structures (UniProt, 2022b).

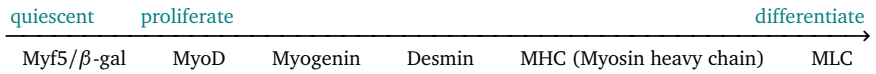

Figure 2.2: How gene expression changes over a Muscle Satellite Cells lifetime as it goes from a satellite cell and differentiates into a myoblast and then proliferates before differentiating and fusing with other myoblasts into a multinucleated myotube (Jang & Baik, 2013, p. 2).

Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells (UniProt, 2022a).

MHC (Myosin heavy chain) acts as a transcriptional activator that promotes transcription of muscle-specific target genes and plays a role in muscle differentiation. MYOD induces fibroblasts to differentiate into myoblasts. (UniProt, 2022c).

MLC (Myosin regulatory light chain) plays a role in cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion (UniProt, 2022e).

Myogenin Acts as a transcriptional activator that promotes transcription of muscle-specific target genes and plays a role in muscle differentiation.Essential for the development of functional embryonic skeletal fiber muscle differentiation (UniProt, 2022d).

Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain (UniProt, 2022g).

Myostatin acts as a negative regulator of skeletal muscle growth (UniProt, 2022f).

Myogenic differentiation is a highly orchestrated sequential program to generate mature skeletal muscle. This event is initiated from muscle precursors called myoblasts, which arise from the somatic mesoderm. Myoblasts differentiate into mononucleated myocytes in the early differentiation stage, and they fuse into multinucleated myotubes in the late stage of differentiation (Jang & Baik, 2013, p. 1). See figure 2.3.

Muscle Satellite Cells repair injured muscles and have a tremendous proliferative potential. MuSC have stem cell characteristics, as they are capable of self-renewal and differentiation along a specified molecular pathway (Hawke & Garry, 2001, p. 536). They lay dormant in a quiescent state until activated. Activated MuSC differentiate into myoblasts. Myoblasts are capable of self-renewal. Myoblasts will at a later stage differentiate into myocytes. Myocytes are able to fuse to each other into myotubes, or to newly formed myofibers in order to restore damaged muscle (Massenet et al., 2021, p. 3). MuSC cells can be used to produce cultured meat (Chal & Pourquié, 2017, p. 1).

Like most mammalian cells, MuSC are anchorage dependent. They attach to surfaces of MCs and coated culture flasks. Cell attachment is a crucial parameter, low attachment efficiency will lead to a low expansion yield. Cell attachment involves interaction between several cell adhesion molecules (CAMs) and substrates on the surface of the microcarrier. In order to enhance cell attachment and proliferation, one can modify the MCs (Bodiou et al., 2020, p. 4).

Eggshell membrane is available as a by-product of the chicken egg processing (breaking) industry. The structure of the carrier is fiber-like. Eggshell membrane carriers stimulates different cell types (such as fibroblasts, epithelial, and endothelial cells) to both grow and differentiate (Rønning et al., 2020, p. 2).

There are four strategies for MCs modification. Changing the chemical composition is possible by changing hydrophilicity and charge. Coating the MCs is possible with ECM proteins, recombinant proteins and synthetic chemicals. It is possible to change MCs stiffness, elasticity, size, shape and topography. Finally, one can also change seeding conditions, such as pre-incubation protocols, amount of cells added to reactor, seeding density, the MCs density, medium composition, agitation protocol and bioreactor impeller design (Bodiou et al., 2020, p. 4).

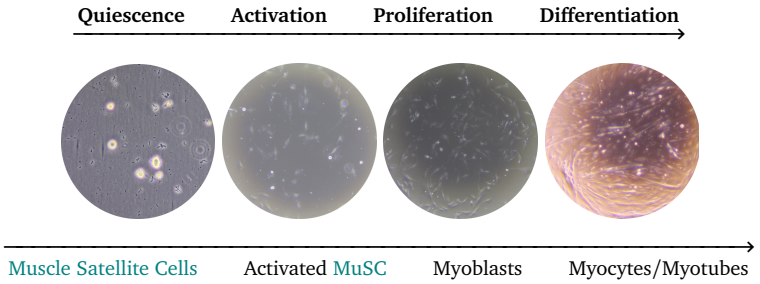

Figure 2.3: Muscle Satellite Cells become activated and differentiate into myoblasts. Myoblasts proliferate and become myocytes and myotubes (Massenet et al., 2021, p. 3).

The Extracellular matrix (ECM) surrounding muscle, consisting of proteins and other molecules (polysaccharides, RNA, etc.), support and give structure to muscle cells and tissue. ECM plays an important role in maintaining homeostasis and regulating the development of skeletal muscle. Collagen is the most abundant component in skeletal muscle tissue ECM. Collagen can be divided into many different subgroups. Collagen fiber type I, III, V, and XI form the fiber in the skeletal muscle, and have a good biomechanical performance (Zhang et al., 2021, p. 4). Eggshell membrane carriers, with its fibrous structure and collagen components, enhances fibroblast and keratinocyte proliferation and myofibroblast differentiation (Vuong et al., 2018). Eggshell membrane carriers have proven to be an excellent biomaterial for MuSC cells with high cell attachment, high cell proliferation and metabolic activity combined with low cytotoxicity (Andreassen et al., 2022). Thus further investigation is still needed and could be a vital step in making cultured meat production economically feasible.

Chapter 3

Materials and methods

3.1 Proliferation and differentiation of Muscle Satellite Cells

Muscle Satellite Cells proliferation and differentiation was studied by microscopy (see subsection 3.1.7), Western blots (see subsection 3.1.8) and qPCR (see subsection 3.1.9). Proliferation media and differentiation media (see subsection 3.1.1), 6-Well Plates (see subsection 3.1.3) and flasks (see subsection 3.1.2) were prepared for the experiment.

3.1.1 Preparation of media for MuSC expansion experiment

The ingredients for the proliferation media were taken out of storage. See table 3.1. The ingredients were defrosted and brought into a LAF bench in a sterile cell lab. The various ingredients were mixed directly into a 500 ml DMEM flask with an electric motorized lab pipette. The flask was marked *proliferation media* with a water-proof marker. The solution was stored in a fridge for later use. See figure 3.1.

The same was done for the differentiation media. Note that the ingredients are slightly different, see table 3.2. The flask was marked as *differentiation media*.

Figure 3.1: Proliferation media was prepared in a LAF bench. The ingredients were stored in falcon tubes in a freezer and were defrosted in a shaking water bath. The correct amount of each ingredient to add to a 500 ml DMEM flask had already been prepared by the cell lab leader.

Table 3.1: Proliferation media mixture

Proliferation media ingredients
500 ml DMEM with Glutamax-I from Thermo Fisher
10 ml Fetal Calf Serum (FCS), 2 % serum
10 ml Ultroser® G Serum Substitute for Animal Cell Culture from Life Sciences
2,5 ml Pen/Strep 10.000 units per ml
2,5 ml Fungizone (250 μg per ml Amphotericin)

Table 3.2: Differentiation media mixture

Differentiation media ingredients
500 ml DMEM with Glutamax-I from Thermo Fisher
10 ml Fetal Calf Serum (FCS), 2 % serum from Thermo Fisher
2,5 ml Pen/Strep 10.000 units per ml
2,5 ml Fungizone (250 μg per ml Amphotericin)
25 pmol insulin

3.1.2 Preparation of 6-Well Plates for proliferation and differentiation of Muscle Satellite Cells experiment

For MuSC to grow in-vitro, vessel surfaces are prepared with Entactin-collagen-laminin (ECL) to make the cells attach to the surface-treated culture vessel. See table 3.4 for the materials used.

Coating is done by first preparing a coating medium. See table 3.3. 2 ml coating media was added each well of each plate. In total, four plates were prepared, two parallels for proliferation and two for differentiation.

The flasks were incubated for 2 hours in a LAF bench before the coating media was removed and each well was rinsed with Dulbecco's Phosphate Buffered Saline (PBS). PBS is not harmful to cells and was added to each well, and then sucked out with a Integra Vacusafe lab Aspiration System to remove excess coating media. The coated plates were kept in a fridge until use. See figure 3.2.

Table 3.3: Coating medium for 6-Well Plates

Coating media ingredients for coating four 6-Well Plates
750 μl Coating medium ECL Cell Attachment matrix from MilliporeSigma 50 ml DMEM with Glutamax-I from Thermo Fisher (2 ml per well)

Figure 3.2: ECL based coating medium was added to each well in the 6-Well Plate, the treatment makes the surface of the culture vessel more adhesive for the MuSC cells. 2 ml coating medium was added per well to treat the vessel, and then removed and washed with PBS after setting for 2 hours.

3.1.3 Preparation of T175 lab flasks for MuSC expansion experiment

Coating is done with a cell scraper to cover the entire flask. See 3.4 for the materials used.

The coating medium, simply 175 μl ECL, was added to the flask with an automatic pipette, forming a line along the bottom of the flask, and then spread thoroughly with a cell scraper to make the ECL properly cover the entire bottom surface of the T175 flask. See figure 3.3

The flask was incubated for 2 hours in a LAF bench. Alternatively, flasks can be kept overnight in a fridge to let the ECL coating settle to the now adhesive cell culture flask.

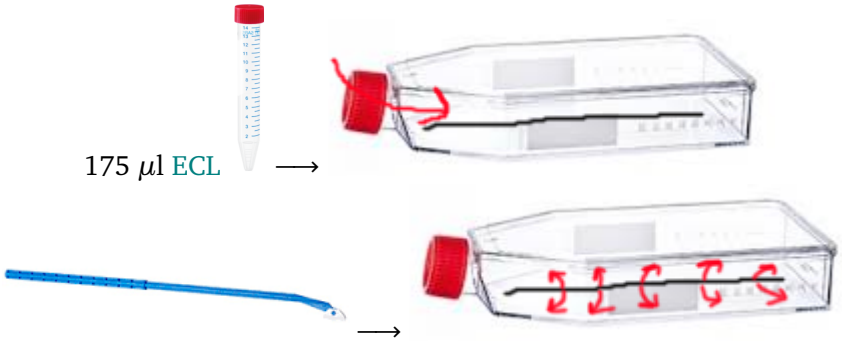

Figure 3.3: ECL was added to the long flat surface at the bottom of a T175 flask like the one on the top right. The coating was spread using a cell scraper as shown bottom right in the figure above. Cells will later be added and attach to the bottom surface of the flask.

Table 3.4: Materials used for preparing flasks and 6-Well Plates for MuSC

Materials used to coat flasks and 6-Well Plates with ECL
Coating medium ECL Cell Attachment matrix from MilliporeSigma
Four 6-Well Plates
Large T175 flask
Sterile Dulbecco's Phosphate Buffered Saline (PBS) 1X from Thermo Fisher
Fortuna ScanLaf clean bench from LaboGene
Laboratory automatic pipette 200 μl
Cell scraper
Fridge

3.1.4 Seeding and growing Muscle Satellite Cells

Isolated Muscle Satellite Cells frozen in cryogenic media were retrieved from a liquid nitrogen storage container[1]. See figure 3.4. The cells were brought into a sterile cell lab where they were brought up to 37°C in a shaking water bath. Proliferation media was also heated to 37°C in advance. See figure 3.5. See table 3.6 for an overview of all the equipment and materials.

Figure 3.4: A liquid nitrogen storage container (left) used to freeze and store cells for biotechnological experiments. The MuSC were kept in such a container, protected from icicles with freezing media inside vials (right).

10 ml heated media was added to a 15 ml falcon tube with an electric pipette inside a LAF bench. The 1 ml contents of the vial were then also added to the falcon tube with an automatic pipette. See figure 3.6.

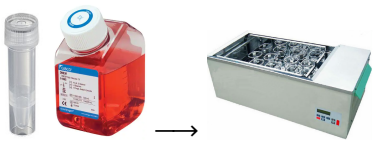

Figure 3.5: A shaking water bath (right) can quickly heat samples and media up to a desired temperature. Humans have a physiological temperature of 37°C which is the temperature used in the experiment during incubation and cell work.

A centrifuge programmed at 550 RPM and set to spin for 5 minutes was used to perform centrifugation on the falcon tube. The cells were separated from the media and were collected into a pellet at the bottom of the falcon tube. See figure 3.7.

The cryogenic media was sucked out of the falcon tube with an Integra Vacusafe Aspiration System, leaving the cells inside the falcon tube. See figure 3.8. 10 ml warm proliferation media was added to the falcon tube and the mixture was transferred to a T175 flask. Another 15 ml proliferation media was added to the flask to give the cells plenty of warm media to grow in. See figure 3.9.

Figure 3.6: After thawing, the cryogenic media must be quickly removed from the bovine muscle satellite cells to prevent damaging the cells. The cells were moved into a LAF bench together with the preheated proliferation media. A 15 ml falcon tube with 10 ml proliferation media was prepared and the bovine muscle cells were added to the falcon tube.

The flask was then incubated at 37°C until 70 % confluent. See figure 3.10.

[1] See a video of actor Shakeel Mowlaboccus from The University of Western Australia retrieving a vial from liquid nitrogen storage here: https://www.youtube.com/watch?v=eQuqJxHNOt0

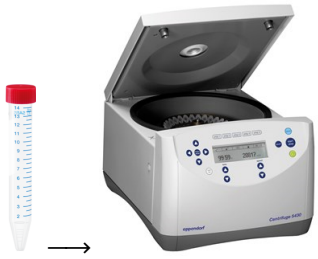

Figure 3.7: To remove the cryogenic media from the cells, a centrifuge was used. A centrifuge uses centrifugal force to separate compounds, leaving the heavier cells in the bottom of a centrifuge tube, also known as a falcon tube. This enables easy removal of the undesired media in the tube.

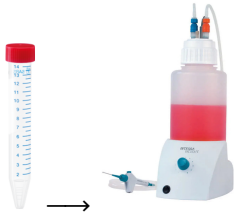

Figure 3.8: Cryogenic media can be sucked out of a centrifuged falcon tube, leaving the cells in a pellet at the bottom side of the tube. The falcon tube was brought back inside a LAF bench and a Integra Vacusafe lab Aspiration System was used to suck out the cryogenic media.

Figure 3.9: The cells in the falcon tube received proliferation media and were moved to a T175 flask (right) coated with ECL.

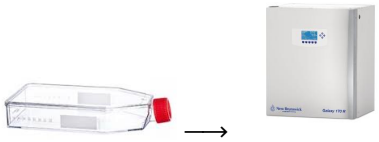

Figure 3.10: The cells were moved to a incubation cupboard and proliferated until they became confluent.

Table 3.5: Materials for the MuSC proliferation experiment

Equipment and materials used to proliferate MuSC
Shaking water bath
Floating foam tube rack for water bath
Proliferation medium
Bovine MuSC in cryogenic media from liquid nitrogen storage
15 ml falcon tubes
Eppendorf 5430 Centrifuge
T175 lab flasks coated with ECL (see subsection 3.1.3)
Fortuna ScanLaf clean bench from LaboGene
Laboratory automatic pipette 1000 μl
Electric automatic motorized pipette
Integra Vacusafe lab Aspiration System
Galaxy 170 R incubation cupboard from Eppendorf

3.1.5 Splitting cells

Muscle Satellite Cells should be between 70 % and 90 % confluent when they are split to keep them proliferating. See figure 3.11.

Before beginning the experiment, trypsin, PBS and the cell proliferation media was heated to 37°C in a shaking water bath. See figure 3.13.

The T175 flask with cells were brought out of the incubation cupboard and placed in a LAF bench.

All the media inside the T175 flask was sucked out with an an aspiration system and the flask was then washed with 10 ml PBS to remove remnants of the growth media left in the flask. The MuSC cells will still remain attached to the flask wall. See figure 3.14.

10 ml trypsin was added to the flask and the flask was placed back to incubate for 5 to 8 minutes at 37°C. Trypsin is toxic to the cells, so it is important to keep track of time. See figure 3.15 and 3.16.

The flask was then taken out of the cupboard and observed under a microscope. They should look like they did on day 0. See figure 3.12.

The majority of the cells were free and suspended in the trypsin solution. They were then transferred into a 50 ml falcon tube with an electric motorized pipette. 10 ml proliferation media was added to the falcon tube. See figure 3.17.

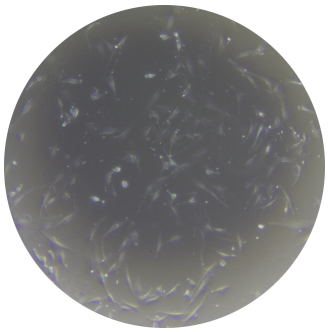

Figure 3.11: MuSC seen under a microscope at 4x magnification. They become confluent over time as they proliferate. To prevent differentiation it is important to split the cells into new flasks.

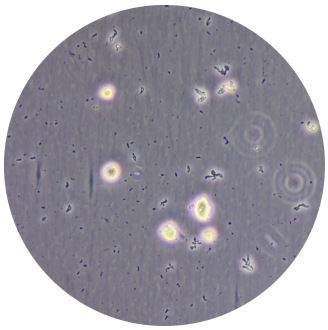

Figure 3.12: MuSC seen under a microscope at 10x magnification. This is what the cells look like at day 0 of the experiment, before they attach to the flask. Trypsin will make them return to this state. After trypsination, cells loosen from the flask surface; they become detached from the flask wall and look like little shiny beads again.

The falcon tube was inserted into a centrifuge and span for 5 minutes at 550 RPM. See figure 3.18.

The supernatant was then sucked out. The pellet was left inside the falcon tube. See figure 3.19.

The cells were resuspended in 10 ml proliferation media. The cells were counted with a NucleoCounter®. See subsection 3.1.6.

The cells were put inside two coated flasks, doubling the amount of flasks one started with. 15 ml proliferation media was added to each flask and the flasks were put into an incubation cupboard at 37°C. See figure 3.20.

Figure 3.13: Trypsin and PBS were pre-heated in a shaking water bath at 37°C in preparation to the splitting experiment.

Figure 3.17: With the cells freed, 10 ml proliferation media and trypsinated cells were added to a falcon tube.

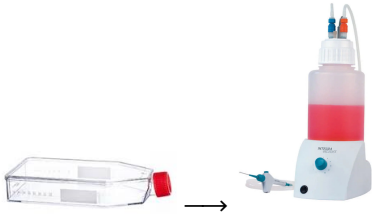

Figure 3.14: The proliferation media used to feed the cells during incubation was removed from the flasks.

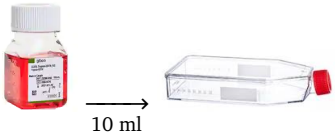

Figure 3.15: 10 ml trypsin was added to the flask with an electronic motorized pipette.

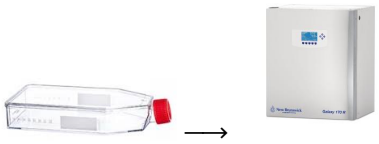

Figure 3.16: The cells were moved to an incubation cupboard at 37°C as the trypsin worked on the cells, detaching them from the surface of the flask and freeing them into the trypsin solution. It is important to not allow the cells stay too long in the trypsin solution, a timer was set for 8 minutes.

Figure 3.18: The falcon tube was centrifuged for 5 minutes at 550 RPM.

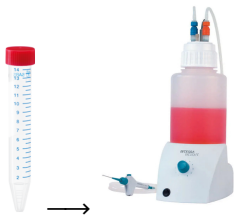

Figure 3.19: Trypsin was sucked out of a centrifuged falcon tube, leaving the cells in a pellet at the bottom side of the tube. The falcon tube was brought back inside a LAF bench and a Integra Vacusafe lab Aspiration System was used to suck out the trypsin.

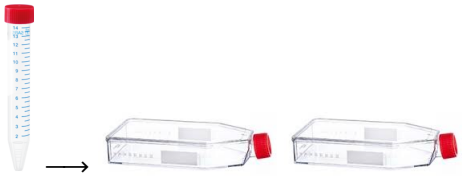

Figure 3.20: The cells were split into two T175 flasks, ready to continue proliferating. 25 ml proliferation media was added each flask. The flasks were then incubated at 37°C.

Table 3.6: Materials for the MuSC splitting experiment

Equipment and materials used to split MuSC
Shaking water bath
0,05 % Trypsin-EDTA (1X) from Thermo Fisher
Sterile Dulbecco's Phosphate Buffered Saline (PBS) 1X from Thermo Fisher
Proliferation medium
Coated T175 flasks with confluent Muscle Satellite Cells
50 ml falcon tubes
Eppendorf 5430 Centrifuge
Laica DM IL LE inverted laboratory microscope
Fortuna ScanLaf clean bench from LaboGene
Laboratory automatic pipette 1000 μl
Electric automatic motorized pipette
Integra Vacusafe lab Aspiration System
Galaxy 170 R incubation cupboard from Eppendorf

3.1.6 Cell counting with NucleoCounter®

A NucleoCounter® NC-202™ from ChemoMetec was used to count the cells during proliferation. See subsection 3.1.5 and figure 3.21. The Via2Cassette™ contains two fluorescent dyes, AO (Acridine Orange) and DAPI (4',6-diamidino-2-phenylindole), deposited in the first part of the flow system (ChemoMetec, 2020, p. 6).

A suspended cell culture can be measured by taking the cassette sample intake tip into a solution with cells and fully pushing down on the piston for sample loading on top of the cassette. This will extract a part of the sample into the counting chamber which can be inserted with the cassette into the NucleoCounter® machine.

The measurement will be cells per ml solution, with quantities of the total amount of cells, and how many live and dead cells there are in the sample. The results can be stored via a computer and accompanying computer software as a pdf or a csv file.

Figure 3.21: NucleoCounter® NC-202™ (top) from ChemoMetec with an accompanying Via2-Cassette™ (middle). Samples can be taken with the cassette and then be used to measure cell quantity with the NucleoCounter® machine and ChemoMetec's PC software (bottom).

Counting cells attached to Cyotodex® 1 Microcarriers

The NucleoCounter® can also count cells attached to Cyotodex® 1 Microcarriers. This is done with a lysis buffer (ChemoMetec, 2021). For this experiment, Lysis 1 Acidic Lysis Buffer from ChemoMetec was used.

Microcarriers offer a convenient method for growing adherent cells in bioreactors. Cell culture flasks are not feasible for industrial scale production when thousand fold increases in production volume are needed. Microcarriers serve as a scaffold that adherent cells can attach to, allowing them to proliferate while a bioreactor keeps the cell-microcarrier complex freely suspended in the media (ChemoMetec, 2017).

Treatment of cell samples with Lysis 1 facilitates detachment from microcarriers, disaggregation, and cell lysis resulting in a suspension of released single nuclei (ChemoMetec, 2021, p. 1). See table 3.7 for materials required.

Two 250 μl samples, diluted with PBS, was taken from a stirring bioreactor (a homogenous solution) into two separate 1,5 ml micro-centrifuge tubes. See 3.2.2.

250 μl lysis buffer was added to one of the micro-centrifuge tubes. The lysed tube was vortexed and a sample from this tube was extracted into a Via2-Cassette™.

The ChemoMetec PC software was configured to take a Microcarriers measurement by changing the protocol in the protocol browser. Refer to the manual (ChemoMetec, 2021, p. 8).

The cassette was inserted into the NucleoCounter® and the sample was ran through the machine.

A second cassette Via2-Cassette™ cassette was filled with the contents of the untreated micro-centrifuge sample tube. When prompted, this sample was inserted into the NucleoCounter®. The cell quantity measurement was presented and recorded.

Table 3.7: Materials for counting cells on Cyotodex® 1 Microcarriers with NucleoCounter® NC-202™

Equipment and materials to count cells from a bioreactor with NucleoCounter® NC-202™
Homogenous Microcarriers cell sample in suspension
1,5 ml micro-centrifuge tube
Lysis 1 Acidic Lysis Buffer from ChemoMetec
Lab bench vortexer
NucleoCounter® NC-202™ from ChemoMetec
Via2-Cassette™ from ChemoMetec

3.1.7 6-Well Plates incubation

To study proliferation and differentiation, cells were moved to 6-Well Plates when there were enough cells for 50 000 cells per well. There were in total four plates, so 1,2 million cells had to be grown in flasks. Once this was achieved the cells were trypsinated, spun down and the trypsin sucked out akin to subsection 3.1.5. The cells were moved to the 6-Well Plate's wells, mixed with 4 ml proliferation media for each well. The plates were incubated in an incubation cupboard at 37°C.

A Laica DM IL LE laboratory microscope was used to take pictures of the cells on

every day of the experiment. See 3.8 for materials used for this experiment and chapter 4 for results.

Two plates were going to be used to study how MuSC cells differentiate. On day 4, two plates were taken to a freezer at -80°C after the proliferation media had been sucked out of the wells and each well rinsed with PBS. The two remaining plates were kept for longer, the proliferation media in each well was replaced with differentiation media. These plates were taken to the freezer after 8 days in total.

Two wells on each board were used for Western blot. The remaining wells were used for the qPCR experiment. See figure 3.22.

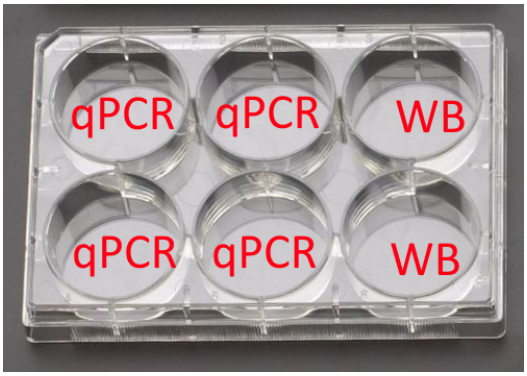

Figure 3.22: Four of the wells of each plate were used for the qPCR experiment. The remaining wells were used for the Western blot experiment.

Table 3.8: Materials for 6-Well Plates incubation experiment

Materials for 6-Well Plates proliferation and differentiation experiment
Laica DM IL LE inverted laboratory microscope
Four coated 6-Well Plates
0,05 % Trypsin-EDTA (1X) from Thermo Fisher
1,7 million MuSC cells in T175 flasks
Sterile Dulbecco's Phosphate Buffered Saline (PBS) 1X from Thermo Fisher
Proliferation medium
Differentiation medium
Eppendorf 5430 Centrifuge
Fortuna ScanLaf clean bench from LaboGene
Laboratory automatic pipette 1000 μl
Integra Vacusafe lab Aspiration System
Galaxy 170 R incubation cupboard from Eppendorf

3.1.8 Western blot

Western blots were made with Desmin, MHC, MLC, Myogenin, MyoD, Actin and α-tubulin primary antibodies. See table 3.9 for materials used. The secondary antibodies were ECL plex CY3 from goat and and ECL plex CY5 from rabbit.

First, the cell plates with non-differentiated and differentiated cells were taken out the freezer (-80°C) and put on ice.

100 μl RIPA buffer + 10 μ phosphate cocktail + 5 μl 4-(2-aminoethyl) benzene-sulfonyl fluoride (AEBSF) phosphate protease inhibitor per ml was first added the the cell plates. Lysis, the process of freeing the cell contents, including the proteins of the cells, was performed over 30 minutes on ice. A cell scraper was used to transfer the lysis to cold eppendorf tubes. The tubes were spun down at 13,300 rpm (max) on a bench-top centrifuge for 15 min at +4°C. The supernatant was transferred to a new cold tube. 2x5μl of the lysis was used to measure the amount of protein with a DC protein assay from BioRad in a 96 wells board. The absorption at 750 nm was measured after 15 minutes. After this was done, 20 μl sample was prepared for each gel well. See table 3.10. 4 μl DTT was added to each sample.

5 μl sample buffer (NuPAGE Sample reducing agent) and 2 μ reducing agent (NuPAGE LDS Sample Buffer) were added to the tubes. The tubes were incubated in a heated water bath at 70°C for 10 minutes.

The samples were then ready to be run on a gel. Three gels were prepared, two parallels of each sample were run on each gel. One NuPage 3-8% Tris-Acteate gel to be run in Tris-Acetate SDS running buffer and two NuPage 4-12% Bis-tris to be run in MOPS SDS running buffer.

200 ml running buffer with 500 μl antioxidant were mixed together and added to the inner gel chamber, 200 ml running buffer was also added to the outer chamber. 1,5 μl ECL plex rainbow marker was added with 10 μl sample per well and ran at 200 Volts for 1 hour.

After running the gels, they were blotted on paper with the iBlot machine. Thereafter, 2 % ECL was added to TBS-Tween (2 grams per 100 ml) and the gel membrane was shaken on a lab shaker for 1 hour, and then incubated with the primary antibodies of interest overnight at 4°C. The had at this point the antibodies added to it from rabbit and mouse to detect the various proteins of interest. The paper was washed with TBS-Tween three times in 10 minute increments. A secondary antibodies were added to the paper and washed with TBS-Tween three times more, 10 minutes for each wash. The paper was dried and pictures of the bands were taken with the G-Box machine.

Table 3.9: Materials for Western blot experiment

Materials to perform Western blot
desmin (cat #Ab8592 (#2) from rabbit, 53 kDa, 1:2000 dilution)
MHC-2x (cat #6H1 DSHB (#81) from mouse, 220 kDa, 1:200 dilution)
MLC (cat # T14.s DSHB (#11) from mouse, 20 kDa, 1:1000 dilution)
MyoD (cat #Sc-304 (#11) from mouse, 20 kDa, 1:200 dilution)
myogenin (cat #Sc-12732 (#53) from mouse, 34 kDa, 1:200 dilution)
actin (cat #Ab1801 (#17) from rabbit, 42 kDa, 1:500 dilution)
α-tubulin (cat #T5168 Sigma (#1) from mouse, 50 kDa, 1:10.000 dilution)
ECL plex goat C-mouse IgG, CY3 (cat #PA43009V)
ECL plex goat a-rabbit IgG, CY5 (cat #PA45011V)
Ice
iBlot system from Thermo Fisher
Syngene G-box with a Syngene UV Transilluminator
NuPAGE Sample reducing agent (Invitrogen, NP0004)
NuPAGE LDS Sample Buffer (4x) (Invitrogen NP0007)
Phosphate coctail
DTT (dithiothreitol) - Thermo Fisher Scientific
RIPA buffer
Lab shaker
TBS-Tween tablets from Medicago AB (lot 243502)
Eppendorf tubes
Bench-top centrifuge
ECL plex rainbow marker
Antioxidant
NuPage 3-8 % Tris-Acteate gel ran in Tris-Acetate SDS running buffer
NuPage 4-12 % Bis-tris ran in MOPS SDS running buffer
Well combs
Casting tray
Electrodes
Voltage source
Gel box

3.1.9 Real-time qPCR of 6-Well Plate experiment and a Cytodex® MuSC bioreactor experiment

Real-time qPCR of the 6-Well plate experiment proliferation and differentiation experiment, and one of the bioreactor experiments (bovine MuSC attached to Cytodex® 1) were conducted. All cell work on mRNA was done with all tubes placed on ice to slow down enzymatic activity, particularly RNases in the environment. Isolating mRNA was done slightly differently for the two ex-

Table 3.10: Western blot samples extraction quantities to achieve desired protein concentration

Sample	Amount to extract for 4 gels	distilled H_2O for 4 gels
Prolif plate 1 well 1	54,5 μl	1,5 μl
Prolif plate 1 well 2	46,5 μl	9,5 μl
Prolif plate 2 well 1	37,0 μl	19,0 μl
Prolif plate 2 well 2	35,5 μl	20,5 μl
Diff plate 1 well 1	33,5 μl	22,5 μl
Diff plate 1 well 2	39,6 μl	16,4 μl
Diff plate 2 well 1	29,4 μl	26,6 μl
Diff plate 2 well 2	36,1 μl	19,9 μl

periments, depending on whether the cells were attached to a 6-Well plate or Microcarriers.

Gene expression of bovine Muscle Satellite Cells during expansion in a bioreactor was analysed by taking 1 ml MC/-cell suspension into a microtube. The mRNA was isolated from the MuSC suspended in proliferation medium by centrifuging the microtube at 300g for 5 minutes. The media was carefully removed and the sample washed with 1x PBS and then centrifuged again. 350 μl RLT buffer was added with DTT. The sample was vortexed and the protocol from RNeasy mini kit from Qiagen was then used to isolate mRNA.

Gene expression of bovine Muscle Satellite Cells during proliferation and differentiation was analysed by lysing the 6-Well Plates with 350 μl RLT lysis buffer and transferring the lysis to a microtube. An RNeasy mini kit was used to isolate mRNA.

1,5 μl sample was measured with Nanodrop to find the mRNA quality. After isolating mRNA, it was turned into a more stable form called cDNA. This was done with TaqMan®Reverse Superscript Transcription kit from Applied Biosystems. For the 6-Well proliferation experiment and bioreactor experiment, cDNA was generated from about 1,0 to 2,5 ng mRNA. The differential plate had between 34,0 to 100,0 ng mRNA. The samples were inserted into a GeneAmp PCR system to create cDNA. qPCR was then carried out using a TaqMan Gene expression Master Mix and QuantStudio5 PCR System. The amplification protocol was initiated with 2 minutes at 50°C, followed by denaturation for 10 minutes at 95°C, then 45 cycles of denaturation for 15 seconds at 95°C, annealing of TaqMan probes and amplification at 60°C for 1 minute.

For the bioreactor experiment, pPCR was performed on 1 biological replicate, with 3 technical replicates. Fold change relative to the Day 0 (control) for each gene was analysed. For the 6-Well experiment, proliferating gene expression was compared to differentiation. See results in chapter 4.

Table 3.11: Materials for qPCR experiment

Materials to perform qPCR measurements
Samples of MuSC on 6-Well Boards
Samples of MuSC on Cytodex® 1 MCs suspended in proliferation media
Micro Star 17 Centrifuge from VWR
Dulbecco's phosphate buffered saline
RNeasy MiniKit (Qiagen, cat #74104)
TaqMan Gene expression Master Mix (Life Technologies)
GeneAmp PCR system (9700)
TaqMan®Reverse Superscript ViLO (Invitrogen, Carlsbad, CA, USA)
QuantStudio5 PCR System (Applied Biosystems, Foster City, CA, USA) PCR System

3.2 Expanding cells in lab-bench bioreactor

Three bioreactor experiments with Cytodex® 1 MCs were conducted. Another three attempts were made with microcarriers of eggshell membrane. 800 ml proliferation media without antibiotics was prepared and 8 million cells were grown in culture flasks to inoculate the bioreactor for each experiment. See table 3.13 for the proliferation media recipe, and table 3.12 for the materials used for these experiments.

3.2.1 Eggshell membrane MCs preparation

For the first eggshell membrane bioreactor experiment, 400 mg eggshell membrane Microcarriers were measured on a lab scale and transferred to a 50 ml falcon tube. For the final two experiments, 900 mg eggshell membrane was prepared. 35 ml 70 % ethanol was added to the falcon tube to remove microbial contaminants. The Microcarriers were mixed with the alcohol by turning the tube a few times and was then allowed to settle. The alcohol was removed with an Integra Vacusafe Aspiration System. 50 ml sterile PBS was added to microtube and then removed again, three times, to wash the carriers before being added to the reactor.

3.2.2 Cytodex® MCs preparation

According to the manufacturer, there should be 114 mg Cytodex® 1 Microcarriers per 100 ml media. Since we were using 800 ml media, 912 mg Cytodex® 1 was measured on a lab scale in a 250 ml schott flask. The solution

was mixed with 100 ml PBS and left to swell in PBS for 3 hours, before being washed twice with PBS. The MCs were then autoclaved at 121°C for 20 minutes. The PBS was removed before the carriers were added to the bioreactor with the MuSC.

Expansion of bovine Muscle Satellite Cells in a bench-top bioreactor

Figure 3.23: A BioFlo® 120 lab-bench bioreactor from Eppendorf running with bovine Muscle Satellite Cells cells attached to Microcarriers.

The bioreactor was configured according to the settings in table 3.15. The bioreactor was autoclaved in a Getinge autoclave before the experiment started, and 8 million cells, 800 ml bioreactor proliferation media (see table 3.13) and microcarriers were added to the reactor. The reactor was allowed to stand 1 hour without stirring to allow the cells to attach to the microcarriers. The reactor experiments where conducted according to the plan in table 3.14, where new glucose in the form of replacing media and additional microcarriers were added on day 7 and 15. The agitation was temporarily paused and the reactor taken into a LAF bench during this process. The differences in each experiment are explained in table 3.16.

At each sampling day, 10 ml media was removed from the middle of the vessel and another 8 ml was extracted and split for subsequent analysis as described in the subsection below (3.2.2). Day 0 sampling was performed 20 minutes after inoculation.

Sampling

On each sample day 8 ml media was extracted from the middle of the bioreactor vessel to retrieve a sample of media and microcarriers for further analysis. Before taking out a sample, 10 ml was removed and discarded to clear the instruments of old media from the previous sample retrieval.

The sample was transferred to a falcon tube inside a LAF bench and then spun down at 550 RPM for 5 minutes. 900 μl of the supernatant was transferred to an eppendorf tube for lactate measurements. Another 900 μl of the supernatant was transferred to another eppendorf tube for glucose measurements. About 5 ml of the supernatant was taken from the sample falcon tube to a new empty falcon tube for use in a a spectroscopy experiment.

The pellet was then resolved with 2 ml PBS. The remaining sample was divided into six marked eppendorf tubes; 250 μl to a tube marked *NucleoCounter untreated*, 250 μl to a tube marked *NucleoCounter lysis*, 100 μl to a tube marked *Hoechst staining*, 100 μl to a tube marked *IF*, 850 μl to a tube marked *RNA* and 850 μl to tube marked *Protein*.

The tubes were taken out of the cell lab and into a freezer (-80°C).

3.2.3 Myogenic potential after expansion

A 20 ml sample was extracted from the reactor and spun down in a centrifuge. Two wells of a 6-Well Plate were prepared. For one well, the sample was first trypsinated and flushed through cell strainer (100 μl Nylon Mesh), filtering out the cells from the MCs. 10 ml trypsin was used. The cells were counted with NucleoCounter®. The other well was trypsinated and filled with 4 ml sample, unfiltered, and had the microcarriers sucked away with an aspiration system. A repeat of the proliferation and differentiation experiment was conducted. See 3.1.7. The goal was to use 50 000 cells per well, but since there were so few cells only two wells were filled.

3.2.4 Hoechst staining and observation under Zen microscope

The hoechst sample tube had 400 μl PBS added to it. Hoechst stain (1 drop NucBlue) was added to 100 μl suspension sample, followed by 20 min of incubation in the dark, wrapped in aluminium foil. 150 μl was transferred to two microscopy cover glasses which were then protected with a cover slip. Pictures of the cells attached to Microcarriers were taken with a fluorescence microscope from Zeiss (Axio Observer Z1 microscope).

3.2.5 Glucose and lactate measurements

A Merck Reflectometer RQflex® 20 was used to measure glucose and lactate concentration quantities in the bioreactor vessel for each day of the bioreactor experiments. See figure 3.24 and table 3.17 for the materials used. See chapter 4 for results.

Figure 3.24: The Merck Reflectometer RQflex® 20 (top) was used to make glucose and lactate measurements from the bioreactor samples. The RQflex® 20 can be programmed with little strips to make optical measurements of both glucose and lactate from a liquid sample. The glucose and lactate test strips arrive in little vials (bottom).

Samples from the bioreactor were taken out of the freezer (-80°C) and thawed on the lab bench.

The Merck Reflectometer RQflex® 20 was first recalibrated and then programmed to make glucose measurements. A glucose test kit from Supelco included a barcode strip for programming the reflectometer to make glucose measurements. The glucose test procedure was transferred to the instrument via the barcode strip by selecting *Add a new method* on the user interface of the reflectometer and then inserting the program strip into the bar code scanner (Merck, 2017, p. 9). See figure 3.25.

The glucose samples were diluted 10 fold, the test measuring range for the kit was 1 - 100 mg/l glucose. 150 μl sample and 1350 μl distilled water was transferred into a 2 ml microtube for each sample.

Once programmed, the procedure was chosen and confirmed by pressing the *START measurement* button (the red button).

The test strip with both reaction zones was immersed into the sample (as described in the package from Merck) and at the same time the *START measurement* button was pressed to start a 15 seconds timer. It is very important that immersing the sample and pushing the *START measurement* is done at the same time.

The measurement takes in total 60 seconds. 15 seconds into the test, the test strip was removed from the sample and the excess liquid was carefully allowed to run off the sample strip via the long edge of the strip onto an absorbent paper towel.

An acoustic signal starts before the end of the reaction time and the strip was inserted all the way into the strip adapter.

The measurement was taken automatically after the end of the reaction time. The measurement result was displayed on the screen automatically stored on the device.

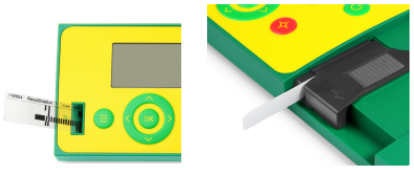

Figure 3.25: The reflectometer is calibrated and programmed to make various measurements with a bar code strip, inserted into the bar code scanner on the front face of the machine (left). Once programmed, a sample test strip which has been immersed into a sample (or calibration strip during recalibration) can be inserted into the strip adapter (right). Easy to follow instructions on how to operate the machine are presented the screen of the reflectometer and in the user manual (Merck, 2017).

For lactate, a lactate test kit from Merck was used. The measuring range was 3,0 - 60,0 mg/l lactic acid, as described in the packaging. The samples were diluted 5 times. 300 μl sample and 1200 μl distilled water was transferred to a 2 ml microtube for each sample.

The procedure was done the same way as the glucose measurement. The lactic acid test takes in total 300 seconds. The device was programmed with the lactic acid procedure with a bar code and measurements were taken after pushing the *START measurement* button and immersing the test strip in the sample for only 2 seconds. Excess liquid was allowed to drip of the strip and when instructed the strip was inserted all the way into the adapter port. The measurement was written down.

3.2.6 PicoGreen DNA concentration quantification

The DNA concentration was measured with the Picogreen method for the final bioreactor experiment with eggshell membrane. A Quant-IT Picogreen ds DNA Assay was used to detect ds DNA.

1 ml of reactor sample (originally planned for an RNA experiment) was washed with PBS and then dissolved in an RLT lysis buffer. The solution was then incubated at 55°C for 15 minutes. The sample was then vortexed. MCs were allowed to settle in the sample tube and 1 μl of the supernatant was then added to 99 μl 1xTE into 96-well plate and mixed 4 times with a multipipette.

A standard curve was prepared by adding 6 μl DNA standard (100ng/μl)

to 594 μl 1xTE and serial dilutions. 100 μl of each standard in duplicates was added in a 96-well black fluorometer plate. 100 μl 1:200 dilution of PicoGreen was added to each well and mixed 4 times with a multipipette. A 5 minutes incubation period followed, this step was done in the dark.

The fluorescence signal was measured using a Synergy H1 hybrid multimode reader. Average DNA concentration of parallels were calculated using the standard curve. See results in chapter 4.

Table 3.12: Materials for bioreactor experiment

Materials for performing the bioreactor experiments
800 ml proliferation media for bioreactors (see table 3.13)
8 million bovine MuSC cells in culture flasks ($8 \cdot 10^6$ cells)
0,9 grams Cytodex® 1 from Cytiva Life Sciences (5 cm^2 per ml, 4400 cm^2/g)
400 mg Microcarriers of eggshell membrane (50 mg per 100 ml proliferation media: 50·8)
70 % Ethanol Alcohol
Fisherbrand sterile cell strainer 100 μl Nylon Mesh cat no. 22363549
BioFlo® 120 Bench-top Bioreactor from Eppendorf
Pitch blade bioreactor impeller from Eppendorf
Lauda Microcool MC 600 Chiller
Lab scale
Integra Vacusafe Aspiration System
HS Lab Steam Sterilizer Autoclave from Getinge
250 ml schott flask
Microcentrifuge tubes
NucBlue™ Live ReadyProbes™ Reagent (Hoechst 33342) from Thermo Fisher
Aluminium foil
ZEISS Axio Observer Z1 microscope
Microscopy cover glass and a cover slip
Quant-IT Picogreen ds DNA Assay from Invitrogen
RLT lysis buffer from Qiagen GmbH
Synergy H1 hybrid multimode reader from BioTek Instruments Inc.

Table 3.13: Proliferation media mixture for bioreactor experiments

Proliferation media ingredients without antibiotics used for all bioreactor experiments
800 ml DMEM with Glutamax-I from Thermo Fisher
16 ml Fetal Calf Serum (FCS), 2 % serum from Thermo Fisher
16 ml Ultroser® G Serum Substitute for Animal Cell Culture from Life Sciences

Table 3.14: Plan for bioreactor experiments

Procedure protocol planned for bioreactor experiments
Day 0: Start experiment. No stirring. Sample after 1 hour
Day 1: Sample
Day 3: Sample
Day 5: Sample
Day 7: Sample, added new MCs and media
Day 9: Sample
Day 11: Sample
Day 13: Sample
Day 15: Sample, added new MCs and media
Day 17: Sample
Day 19: Sample
Day 21: Sample

Table 3.15: Settings for bioreactor for bovine MuSC expansion experiment

Bioreactor settings for bovine MuSC expansion experiment
pH set to 7,3
No sparging, accomplished by setting reactor to 5 % CO_2, 95 % Air
Temperature set to 37°C
Stirring on set to 50 RPM

Table 3.16: Parameter changes across the bioreactor experiments

Experiment	**Cells**	**Media**	**Weight microcarriers**	**Duration**	**RPM**
1st Cytodex®	9 million	800 ml	912 mg	11 days	40
2nd Cytodex®	9 million	800 ml	912 mg	19 days	40
3rd Cytodex®	8 million	800 ml	912 mg	27 days	40
1st Eggshell	8 million	800 ml	400 mg	11 days	40
2nd Eggshell	11 million	800 ml	900 mg	4 days	100
3rd Eggshell	5 million	600 ml	900 mg	22 days	100

Table 3.17: Materials for glucose and lactate measurements

Materials to perform glucose and lactate measurements
Reflectoquant® RQflex® 20 Reflectometer from Merck Millipore
Strips from Glucose Test (kit includes protocol) from Merck Millipore
Strips from Lactic Acid Test kit (kit includes protocol) from Merck Millipore
Samples in microcentrifuge tubes from bioreactor
2 ml microtubes
Paper towel

Chapter 4

Results

The proliferation and differentiation experiment results are presented first. The micrographs taken of the Muscle Satellite Cells in one of the wells of a 6-Well plate can be found in figure 4.1, 4.2, 4.3 and 4.4. One can clearly see the cells first proliferate and fill the well as the cells become more confluent, and then differentiate.

Next, results form the Western blot experiment is shown, bands of desmin and actin are in figure 4.5, tubulin and MLC in figure 4.6, interesting non-specific bands in figure 4.7 and finally myostatin in figure 4.8.

Results from the qPCR experiment in figure 4.9 and figure 4.10.

NucleoCounter results for all bioreactor experiments are found in figure 4.11 and 4.12.

Glucose measurement results for all bioreactor experiments are shown in figure 4.13 and lactate results in figure 4.14.

PicoGreen results for the final (3^{rd}) bioreactor experiment on eggshell membrane are in figure 4.15.

Zeiss micrographs of microcarriers and MuSC cells are in figure 4.16, 4.18, 4.17, and 4.19.

Finally, the results from the myogenic potential experiment are presented in figure 4.20, 4.21 and 4.22.

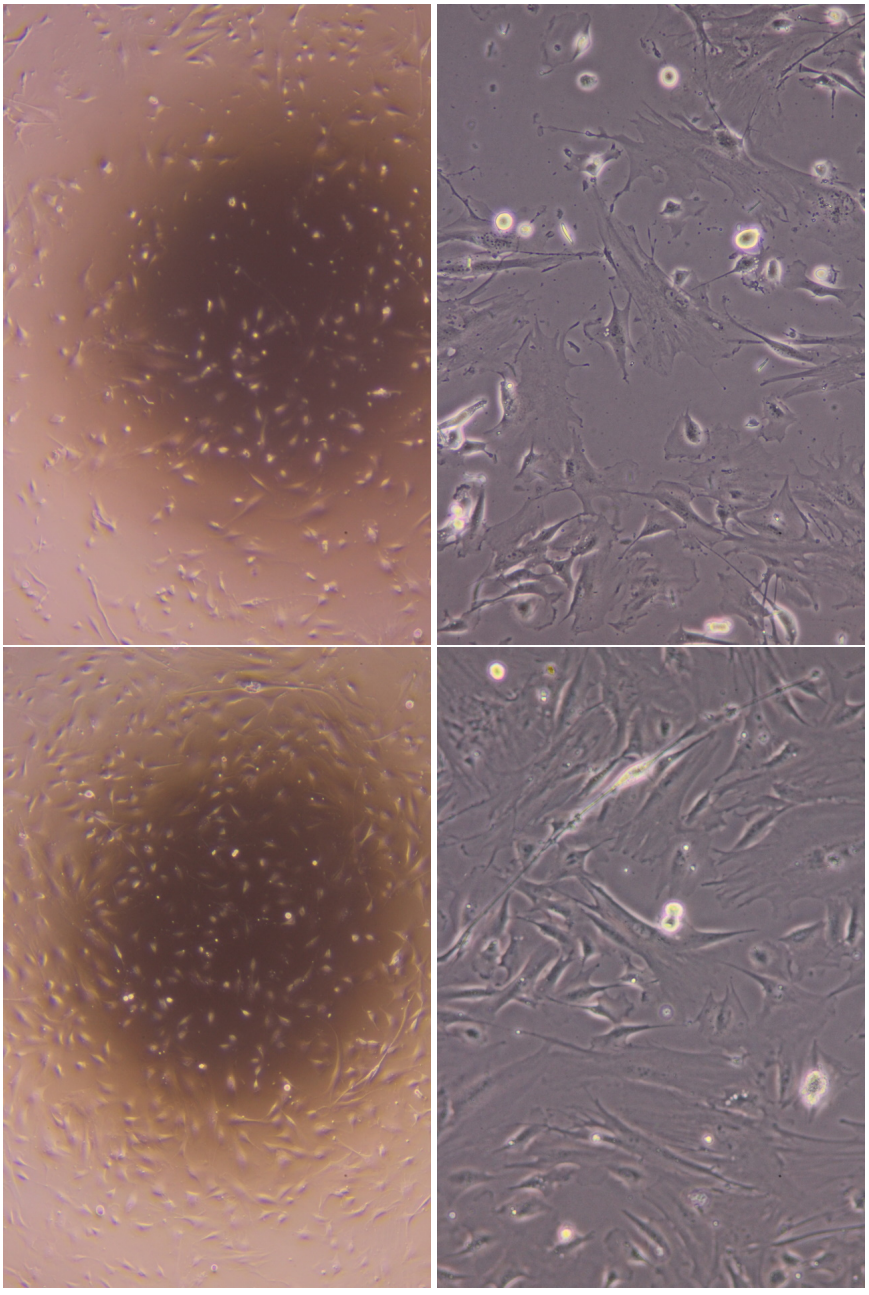

Figure 4.1: Day 1 (top) and day 2 (bottom) from a 6-Well plate well in proliferation media. 4x magnification (left) and 10x magnification (right). Muscle Satellite Cells start out as proliferating cells and will increase in quantity. The well becomes more confluent over time.

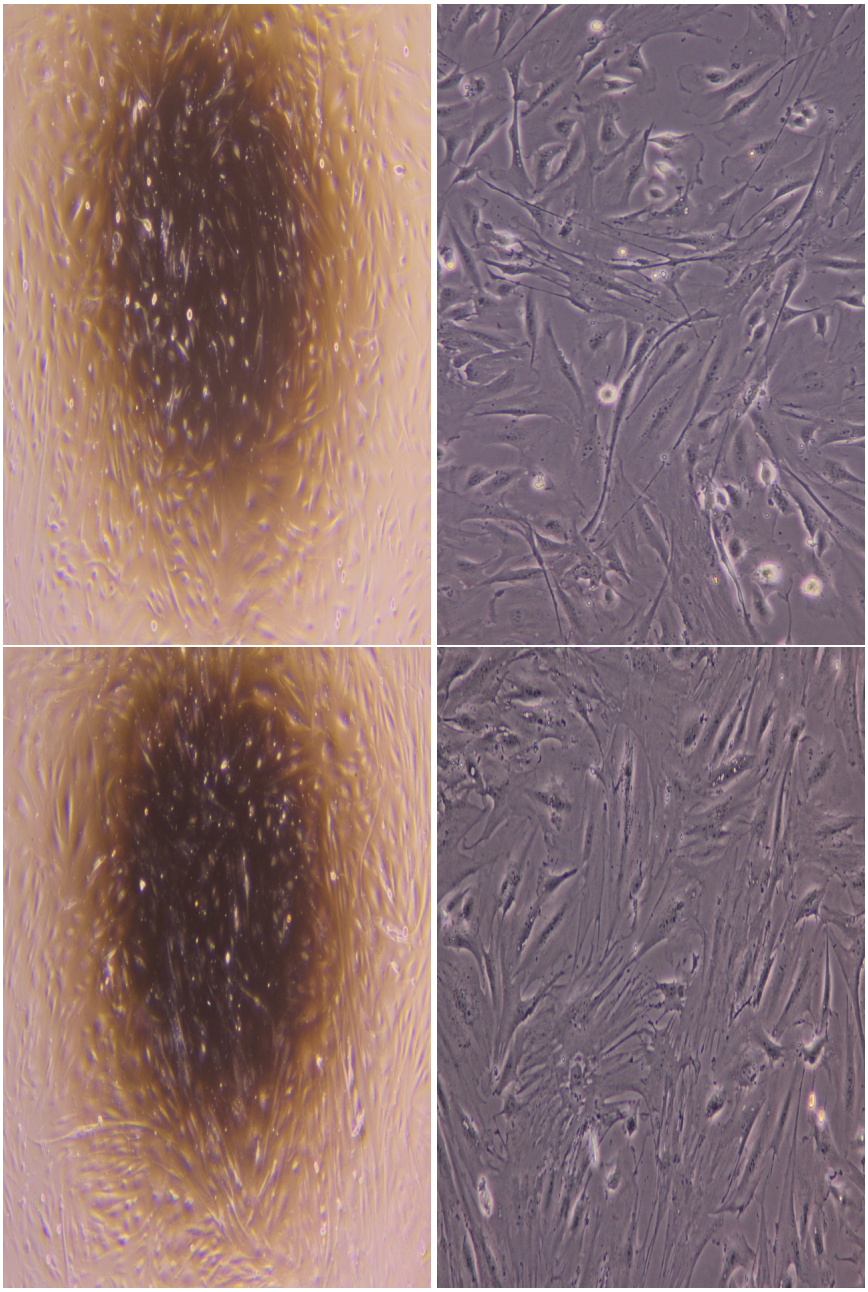

Figure 4.2: Day 3 and day 4 in proliferation media. 4x magnification (left) and 10x magnification (right). The wells have become more confluent and some of the cells have started to fuse and become myofibers.

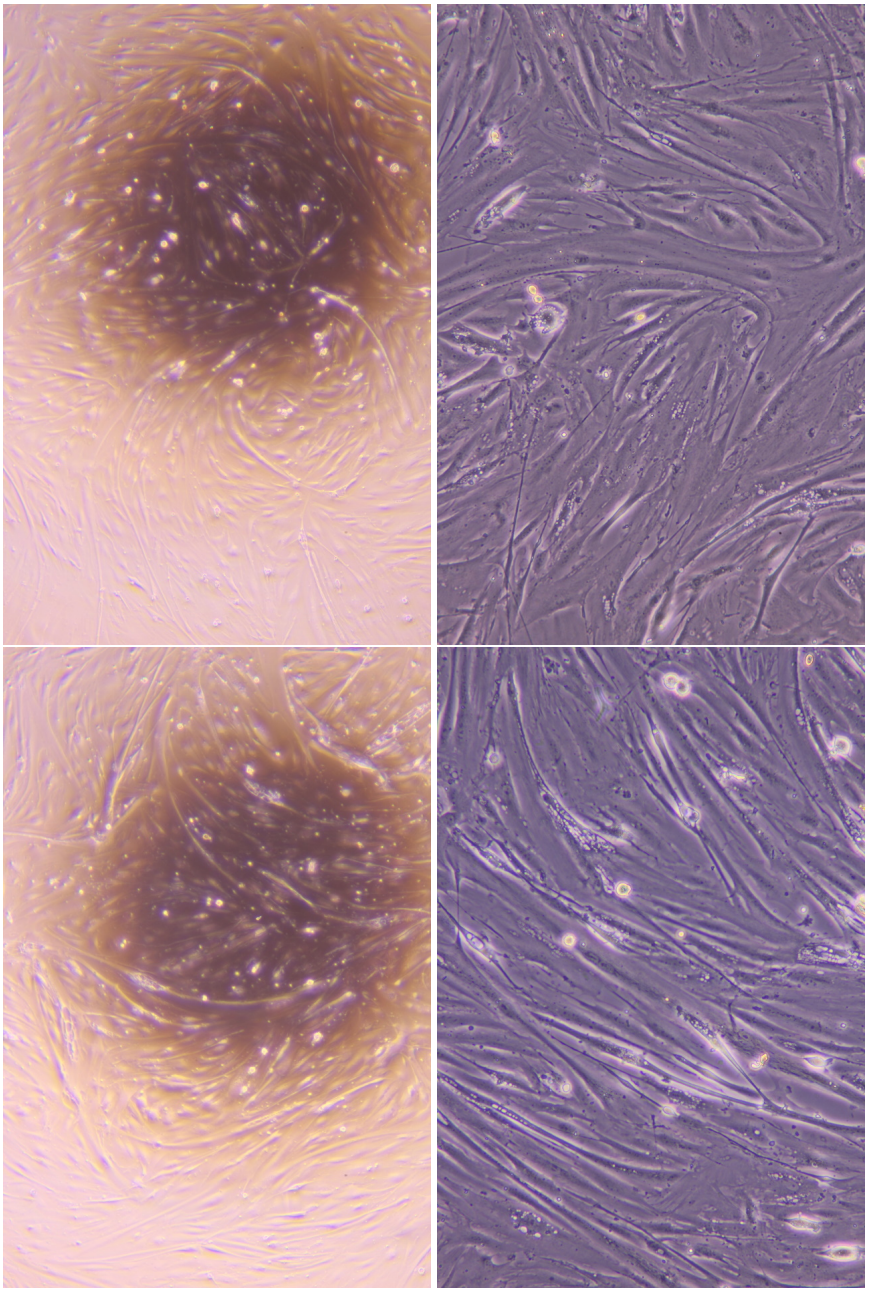

Figure 4.3: Day 5 and day 6, media has been changed to differentiation media (differentiation media was applied on day 4). 4x magnification (left) and 10x magnification (right). Myofibers are starting to become more apparent. The cells are aligning to form muscle.

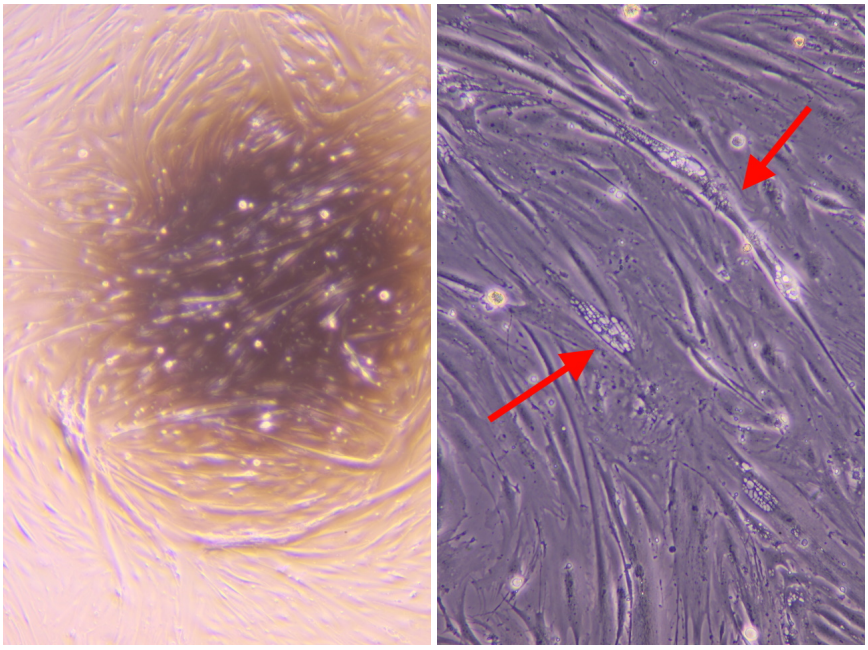

Figure 4.4: Differentiation of bovine cells in coated flasks after seven days in total. For the final three days, the cells were in Diff media. The cells start as out myoblasts, but over time they merge together and form myofibers (shown with red arrows). The images are taken at 10x magnification (left) and 4x magnification (right). The cells were in 6-Well Plates and grown for both proliferation and differentiation. The plates were later harvested and used in a to identify proteins typical of bovine cell proliferation and differentiation.

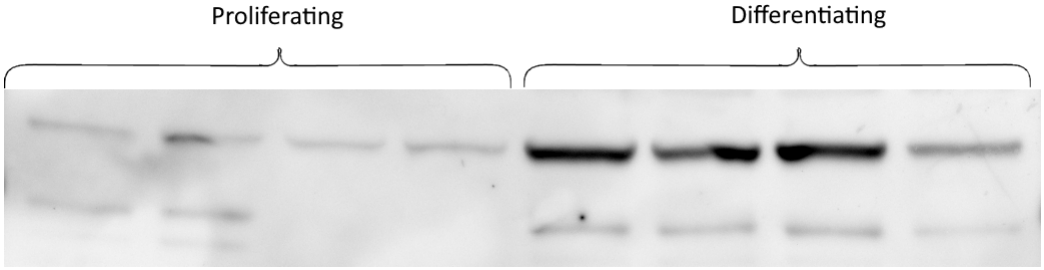

Figure 4.5: 4-12 % gel A under CY5 light showing Desmin (53 kDa) clearly visible (top band). Actin (42 kDa) partially visible (bottom band).

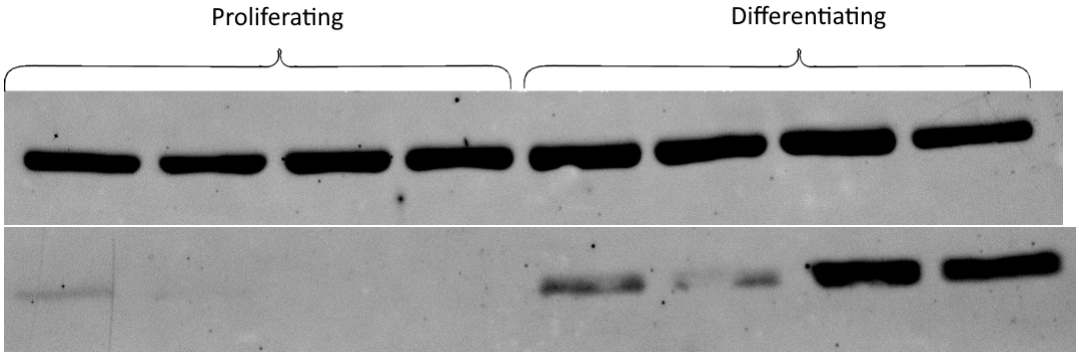

Figure 4.6: 4-12 % gel A under CY3 light clearly showing tubulin (top band) at 53 kDa. MLC (myosin light chain) shown bottom (20 kDa).

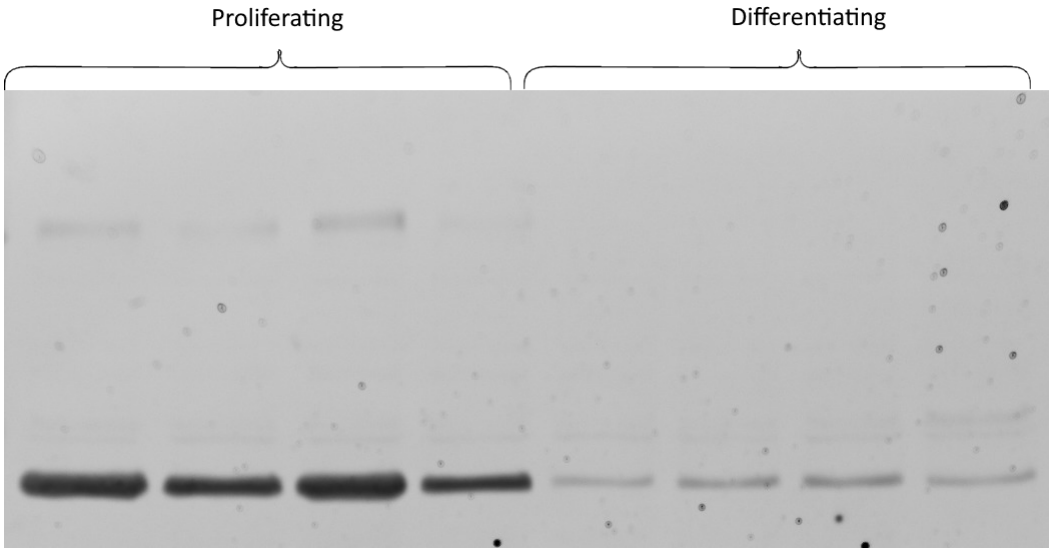

Figure 4.7: 4-12 % gel A under CY3 light showing non specific bands at 65 kDa, although it could be MHC (220 kDa).

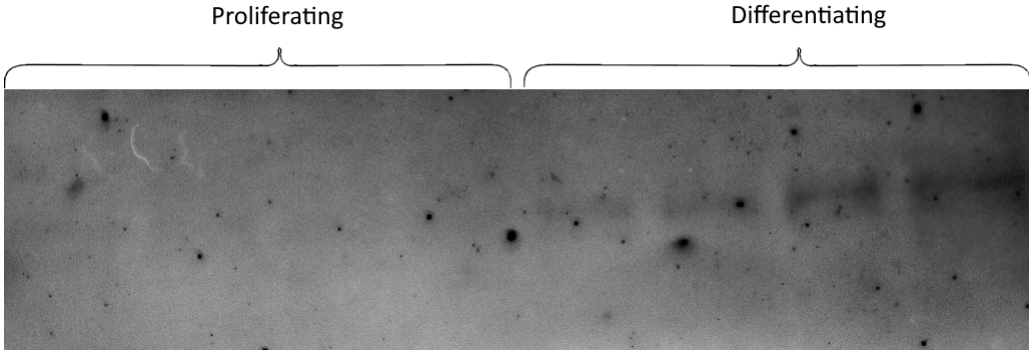

Figure 4.8: 4-12 % gel B under CY3 light. Myostatin (35 kDa) might be visible here.

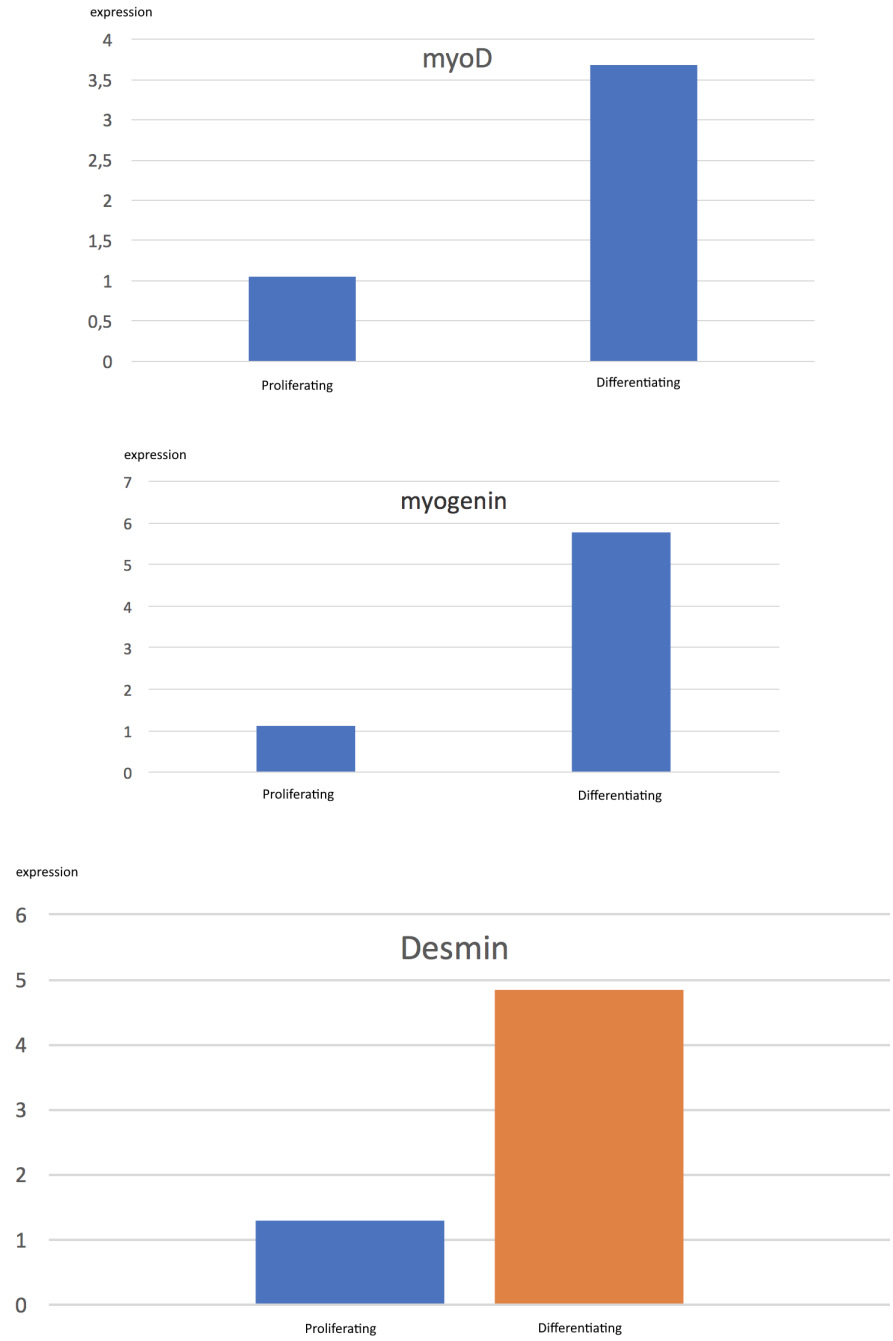

Figure 4.9: rPCR signals showing expression of MyoD, Myogenin and Desmin in proliferating cells (left column) compared to differentiating cells (right column). The samples were from the 6-Well Plates experiment seen in figure 4.4.

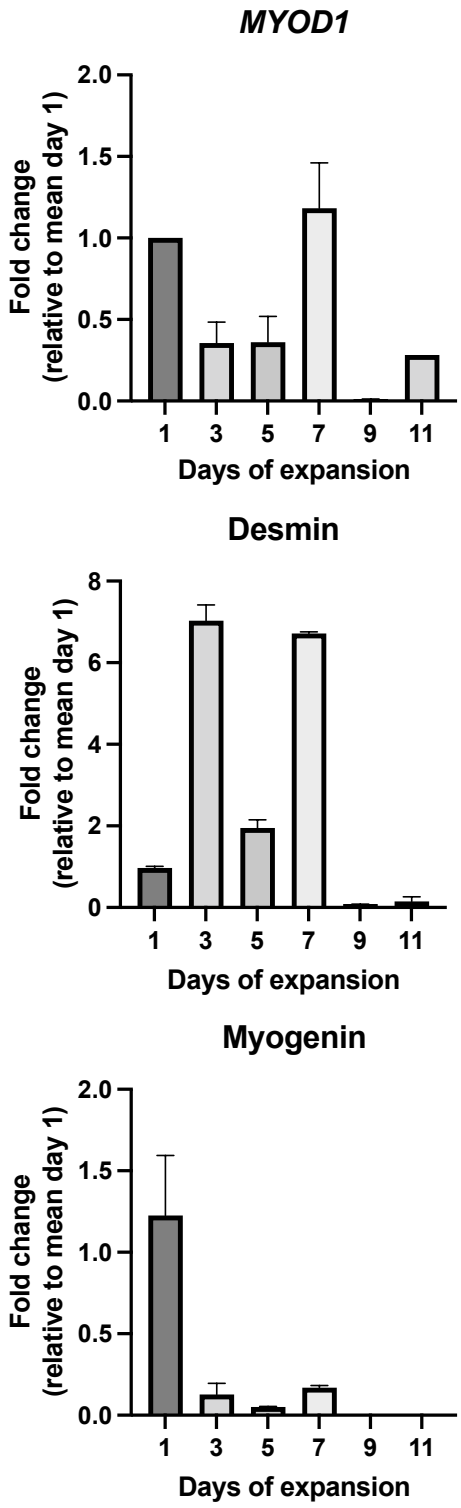

Figure 4.10: MyoD, desmin and myogenin rPCR expression from the first Cytodex® bioreactor experiment. Only 11 days were completed before this experiment was stopped.

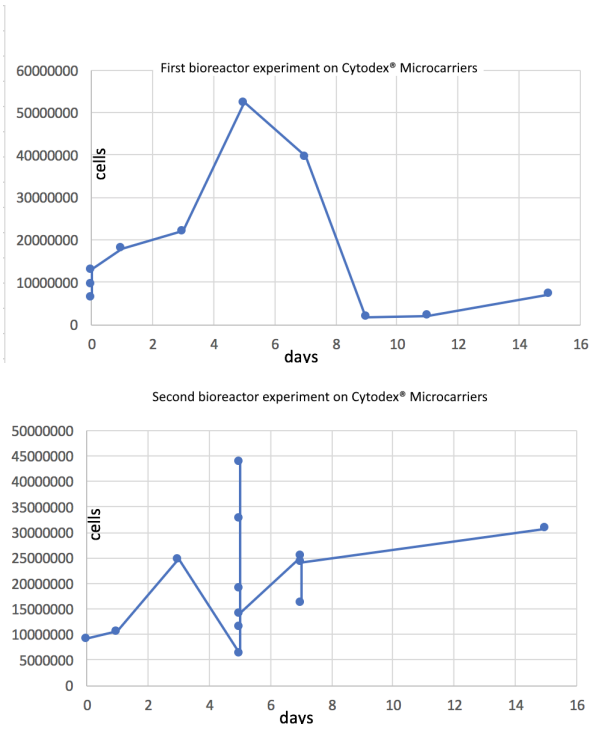

Figure 4.11: NucleoCounter cell count plot for the two first bioreactor experiments, going from day 0 through to day 15. The bioreactor was inoculated with 9 million cells (day 0) for the first experiment, and 9 million cells for the second experiment. Microcarriers from Cytodex® was used (0,912 grams were prepared for 800 ml medium). The first experiment was stopped early as cells died on 7 as CO2 was accidentally set to 0,0. The experiment was redone. The second attempt was stopped early as cells were thought to have differentiated as they were added to reactor, resulting in poor growth. The reactor was refilled with media (containing glucose and more carriers) on day 7.

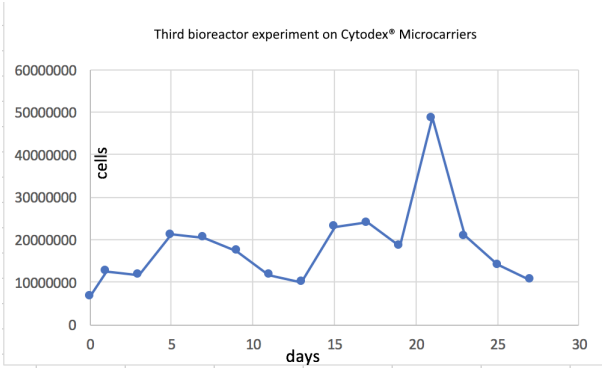

Figure 4.12: Final Bioreactor experiment with Cytodex® microcarriers cell count plot day 0 through 27. The bioreactor was inoculated with 8 million cells. Microcarriers from Cytodex® was used (0,912 grams were prepared for 800 ml medium). This experiment was considered a success. The reactor was refilled with media (containing glucose and more carriers) on day 7 and day 15.

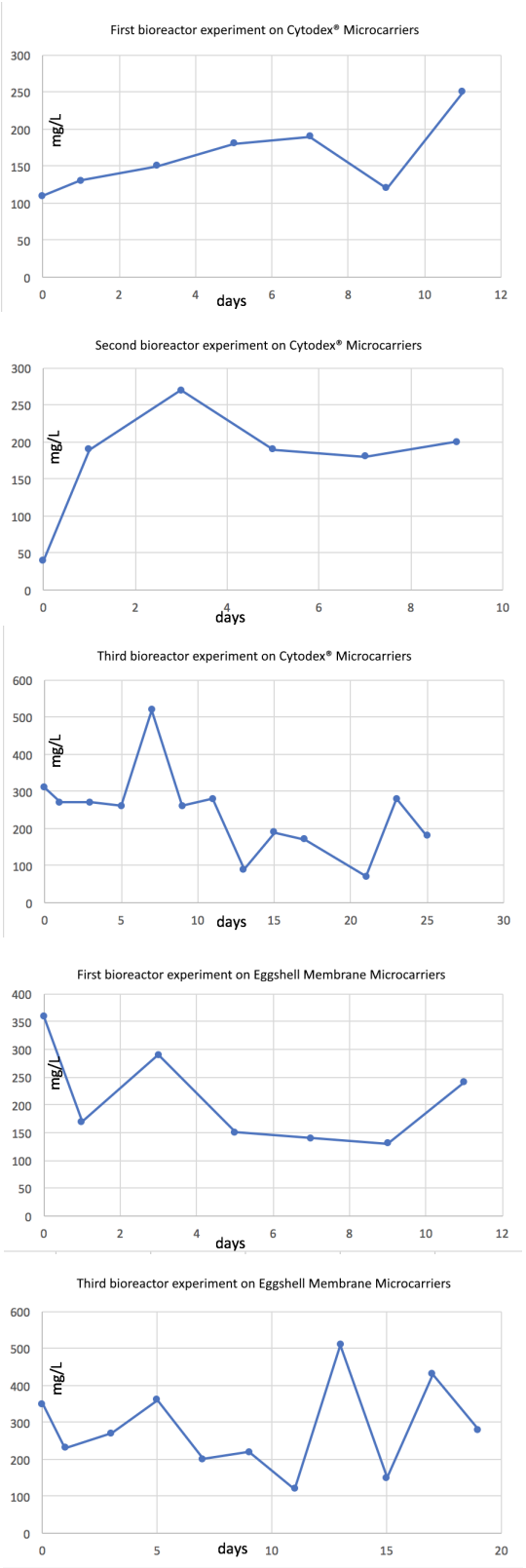

Figure 4.13: Glucose measurements for all the bioreactor experiments. There are three bioreactor experiments where Cytodex® microcarriers was used and three with egg shell membrane carriers. *Since the second eggshell membrane experiment ended after only four days, it is not included here.*

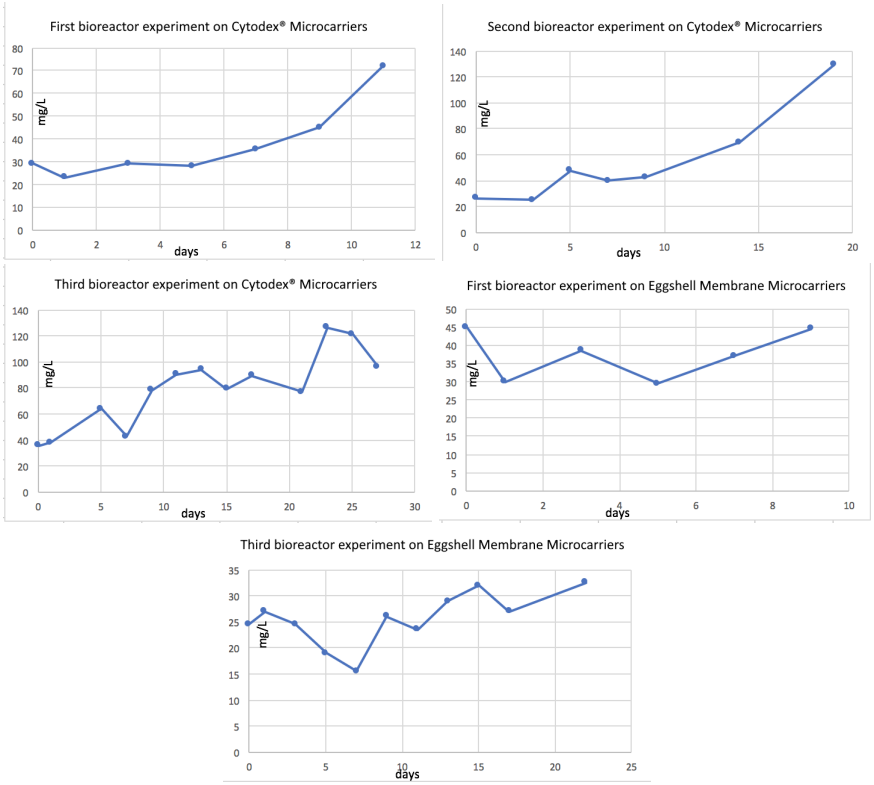

Figure 4.14: Lactate measurements for all the bioreactor experiments. There are three bioreactor experiments where Cytodex® microcarriers was used and three with egg shell membrane carriers. The reactor was refilled with media (containing glucose and more carriers) on day 7 and day 15 for each experiment. *Since F8 was ended after four days, it is not included here.*

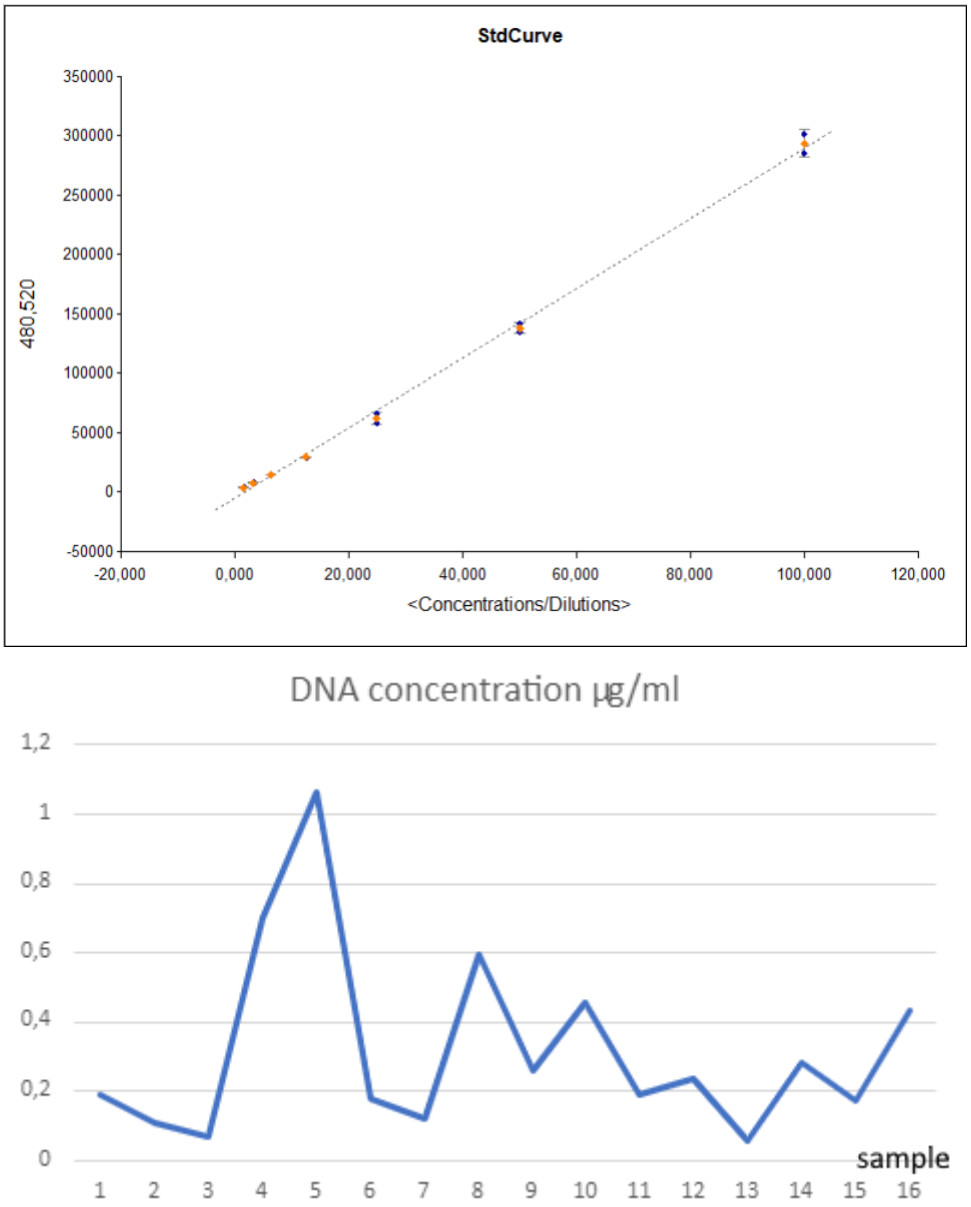

Figure 4.15: Standard curve from PicoGreen™ experiment (top) with y = 2873,6x and $R^2 = 0{,}9972$. DNA concentration in μg/ml from the last bioreactor experiment on eggshell membrane (bottom) reckoned out by taking the blanked data from the PicoGreen measurements and dividing by y (2873,6).

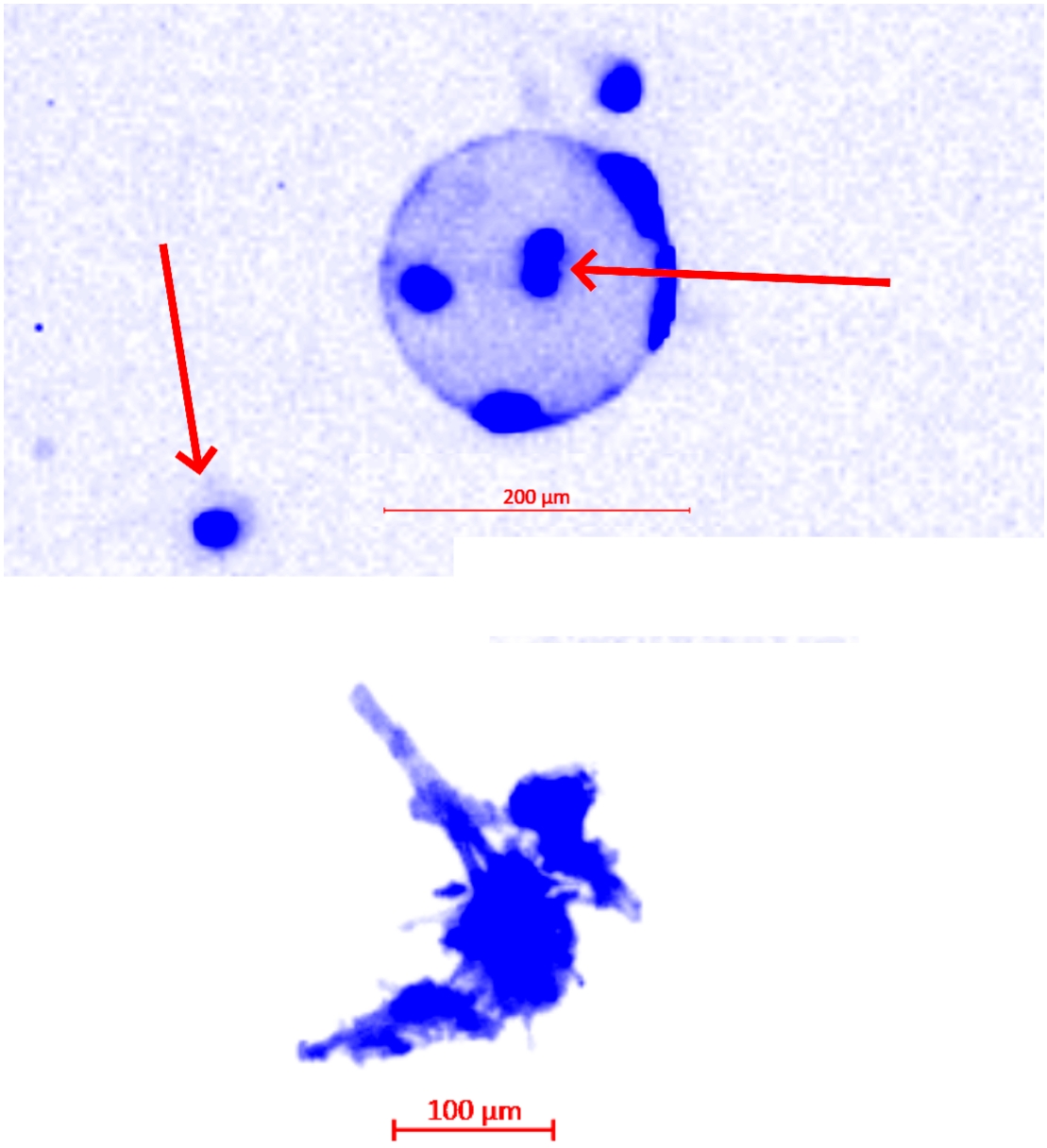

Figure 4.16: A single Cytodex® microcarrier (top) with numerous cells attached to it. Some of the cells are free and not attached to microcarriers. Red arrows show some of the cells. A micorcarrier made of collagen and eggshell membrane is also displayed (bottom). Micrographs of the hoechst stained microcarriers were taken with a ZEISS Axio Observer Z1 microscope with a 49 DAPI Reflector.

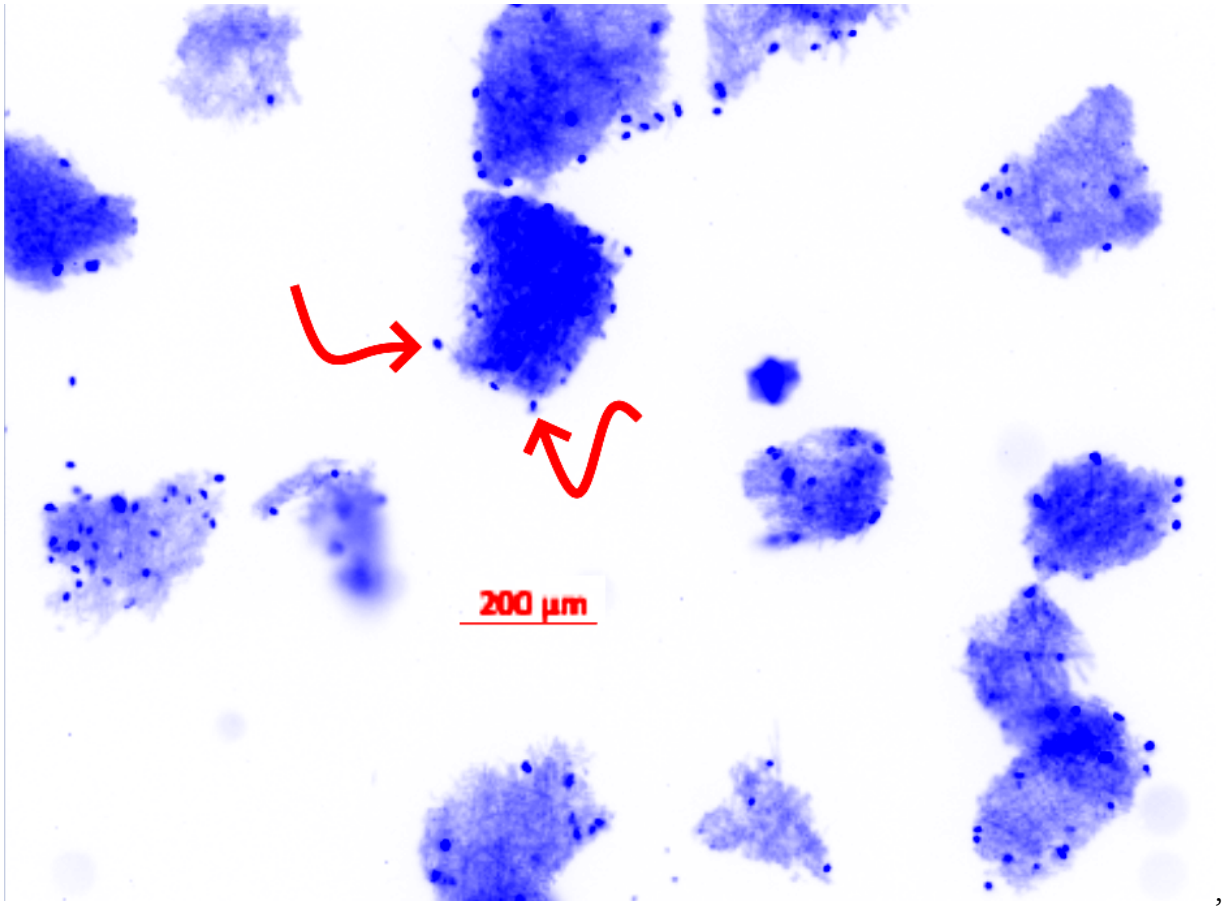

Figure 4.17: Micrograph (taken with a ZEISS Axio Observer Z1 microscope) from the first eggshell membrane microcarrier bioreactor experiment. One can clearly see cells growing on the carriers. The cells have been hoechst stained. Red arrows show some of the MuSC cells.

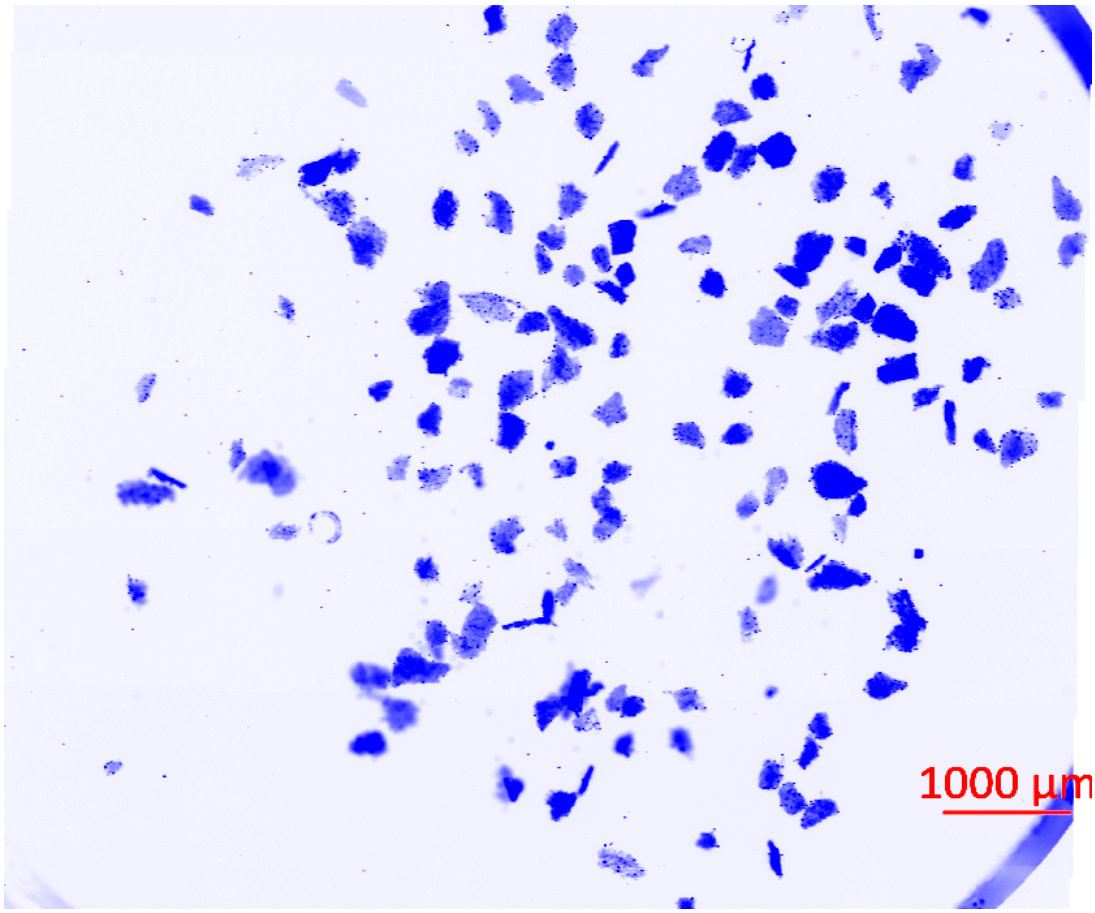

Figure 4.18: Another micrograph from the first eggshell membrane bioreactor experiment. The cells have been stained with hoechst staining, they can be seen attached to the eggshell carriers. Micrograph taken with a ZEISS Axio Observer Z1 microscope.

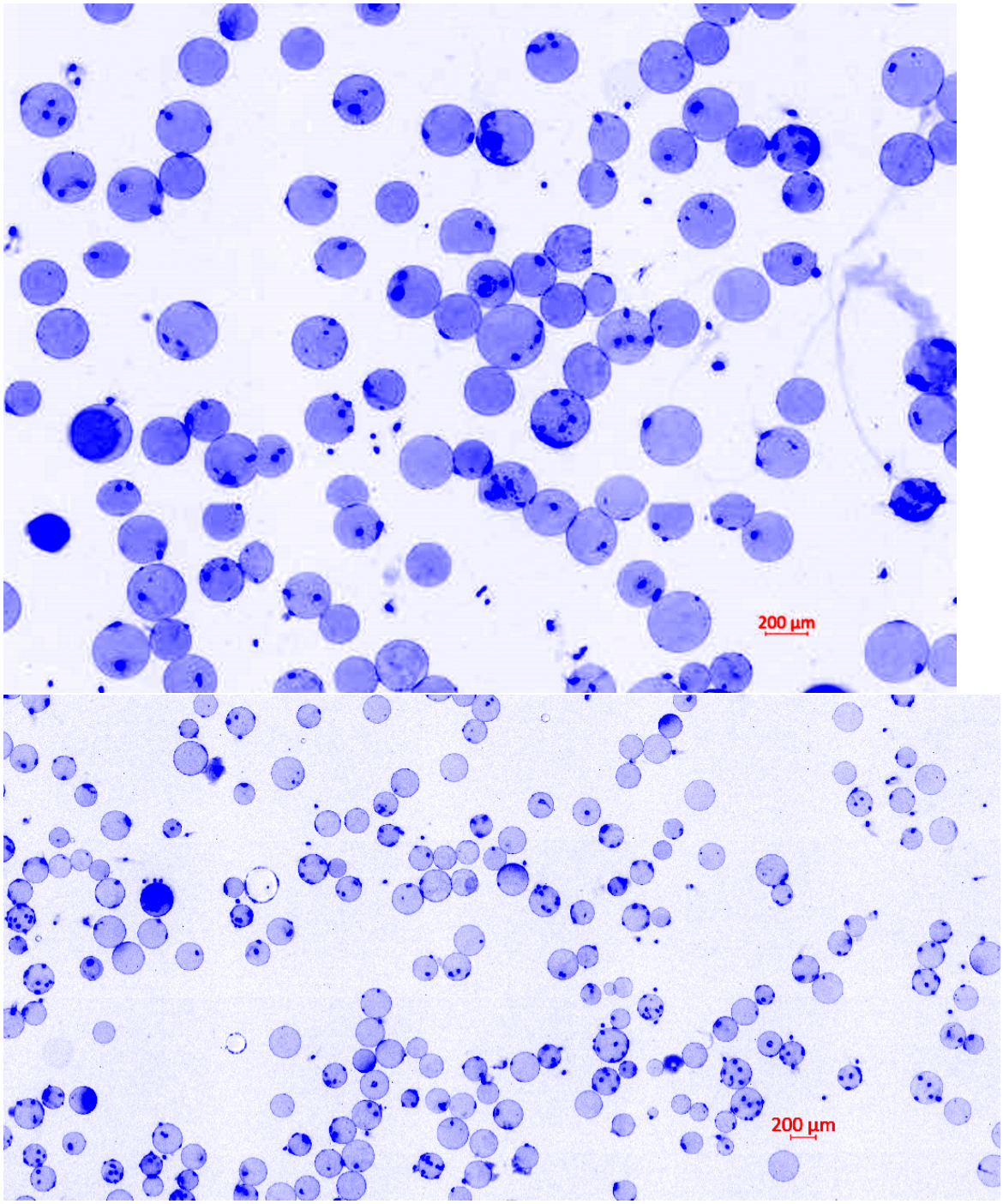

Figure 4.19: Day 0 (top) and day 5 (bottom) of the first Cytodex® 1 experiment, seen under a ZEISS Axio Observer Z1 microscope after hoechst staining. As the cells proliferate, the carriers fill with cells.

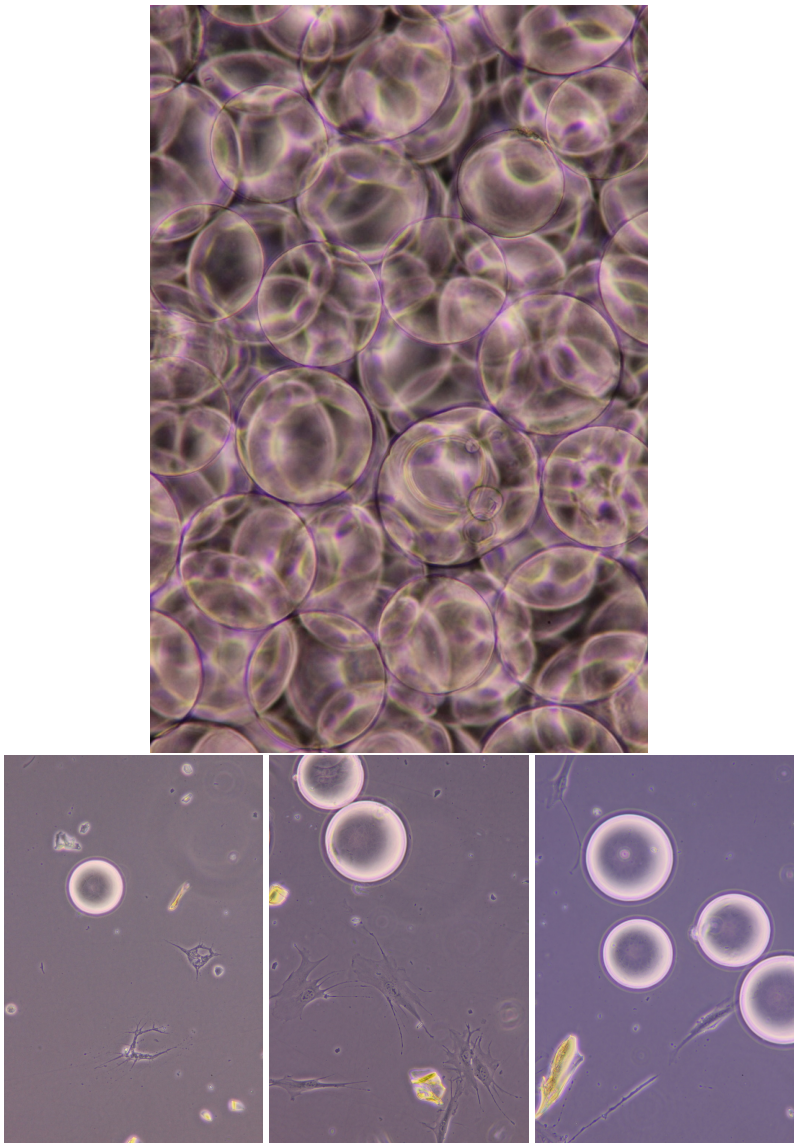

Figure 4.20: Myogenic potential experiment non-filtered sample from the second Cytodex® bioreactor experiment. The well is filled with microcarriers (top micrograph, 10x magnification) which were sucked away to expose cells (bottom row, 10x magnification). Results from day 1 (left), day 3 (middle) and day 7 (right).

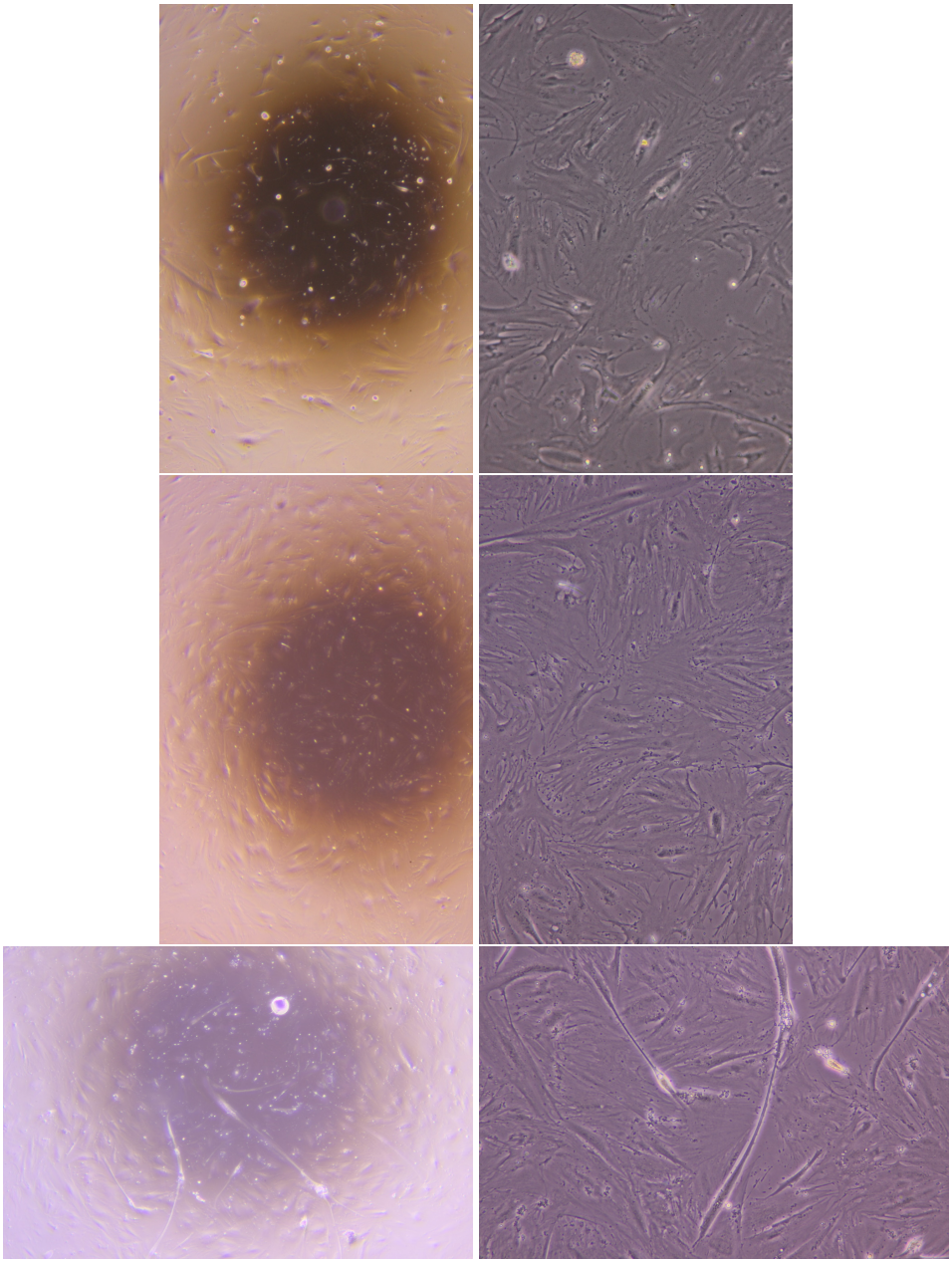

Figure 4.21: Myogenic potential experiment micrograph from a filtered sample from the second Cytodex® bioreactor experiment. 4x magnification is shown on the left column, and 10x magnification on the right column. Day 1 (top), day 3 (middle) and day 7 (bottom) are shown. Day 1 (top) and day 3 (middle) show cell proliferation, no myofibers were seen in the samples. The last image shows some cell differentiation (day 7).

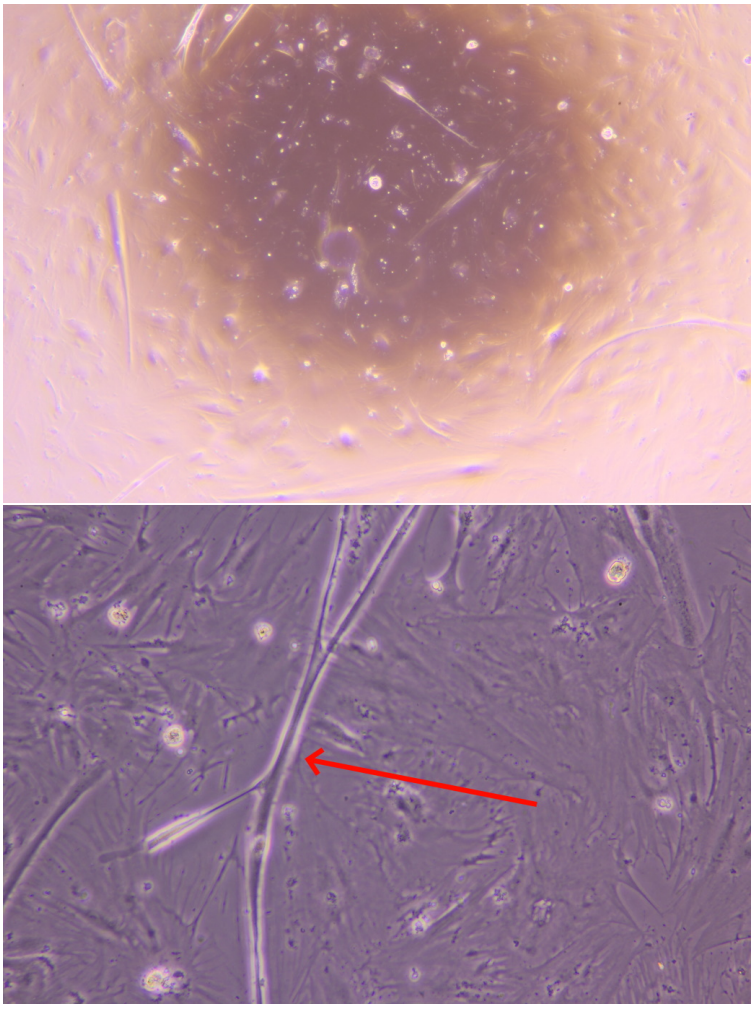

Figure 4.22: Myogenic potential experiment filtered sample on day 11. Myofibers have started to form, some MuSC cell differentiation has occurred. The red arrow shows early myofiber formation. Top micrograph is at 4x magnification, while bottom micrograph is at 10x magnification.

Chapter 5

Discussion

The Muscle Satellite Cells were from a primary cell line. Since the cells have not been modified, they were close to their *in vivo* state and exhibited normal physiology. Primary cells will eventually stop proliferating. It is difficult to make secondary MuSC cell lines (cells which proliferate forever) so it was important to establish optimised culture conditions (Merck, 2022).

As MuSC age, they lose their muscle fibres to atrophy. It is thought this originates from the proteolytic pathway. The loss of muscle fibres might be due to apoptosis (planned cell death), which is typical of mammalian cells (Dirks & Leeuwenburgh, 2002). Apoptosis can be triggered by internal factors, such as cell stress, or external factors from foreign cell signals in the ECM (Rønning et al., 2017).

The 6-Well plate experiment showing Muscle Satellite Cells proliferation and differentiation went as expected. The first four days of the experiment, shown in figure 4.1 and figure 4.2, show cells increasing in quantity and density. The well becomes more and more confluent over time. The myoblasts begin to differentiate already on day 3, but it isn't before day 5 and 6 they really start to fuse and become myofibers. This is seen in figure 4.3. By day 7, seen in figure 4.4, the cells have started aligning to form muscle fibres. Similar results can be found other experiments on Muscle Satellite Cells (Lambrechts et al., 2016). In other MuSC differentiation experiments, it is typical to use differentiation media from the beginning of the experiment, instead of adding it at day four (Mattick et al., 2015). The differentiation medium used had Fetal Calf Serum (FCS) in it, its constituents varies greatly from batch to batch which has in other experiments caused spontaneous differentiation when new media is added to the wells (Hagen, 2019, p. 58). Various hormones which can appear in FCS can cause spontaneous contractility in muscle cells due to a shift in calcium action (Aswad et al., 2016). Both the differentiation and proliferation medium contained the antibiotic

PenStrep which is known to enhance transcription factors relating to proliferation and differentiation (Ryu et al., 2017). Antibiotics can't exist in food for consumption (Gstraunthaler, 2003). The experiments which included media with antibiotics and Fungizone (Amphotericin B) were done for research only.

The Western blot experiment was partially successful. Western blots of proliferating and differentiating Muscle Satellite Cells cells were attempted for MHC, Myostenin, Myogenin, MLC, Tubulin and Desmin. MHC and Myogenin were a bit tricky, but the rest were successfully shown on blots. Desmin in figure 4.5 is mainly present in differentiating Muscle Satellite Cells as expected. Desmin is found in adult striated muscle and helps connect myofibrils to each other and to the plasma membrane (UniProt, 2022b). MHC and Myogenin could have been redone on new blots.

Tubulin is found in all eukaryotic cells and was used as a marker (UniProt, 2022g). MLC (myosin light chain) in figure 4.6 shows how this protein mainly appears later in the differentiation stage of the MuSC life-cycle. This is expected (Jang & Baik, 2013, p. 1).

Interesting non-specific bands were found on the blot for MHC, shown in figure 4.7. They appear at 65 kDa.

Myostatin is seen faintly in figure 4.8. It appears to be mainly present in the differentiation side of the blot. Myostatin is a negative regulator of skeletal muscle growth (UniProt, 2022f).

Eggshell membrane carriers is a by-product biomaterial. With minimal processing, it can provide food-grade, low-cost, microcarriers which is biocompatible with MuSC. Further development can be a vital step in making cultured meat production economically feasible (Andreassen et al., 2022). Using animal sourced microcarriers will not remove the world's reliance on feedstock. However, humanity might decide to keep chickens and continue the large scale chicken egg processing industry which the carriers rely on. It could also be a good temporary compromise before finding other sources. Cultured meat would in any case potentially greatly reduce the amount of cattle (Melzener et al., 2020, p. 1). New media without any antibiotics and a serum not sourced from calves should also be devised before commercialization of cultured meat (Gstraunthaler, 2003).

Bioreactors make it easy to monitor pH, oxygen, temperature, aggregation and measuring metabolites such as lactate, glucose, glutamine and ammonia (Nilsson, 1988). The microcarriers of eggshell membrane did not function well with the bioreactor used in the experiment as the bioreactor impeller did not allow the cells to remain suspended. Pitch blade bioreactor impeller from Eppendorf Bioflow® 120 spins in the centre of the vessel. Maybe if the impeller was at the bottom of the reactor, it would force the microcarriers into suspension. In an attempt to make the eggshell MCs become suspended, various reactor speeds were tested. The problem of having really fast RPM in the reactor is that the stress destroys the

cells. A better solution should be considered. Changing the milling size and thus the size of the microcarriers could solve the problem.

When counting cells on eggshell membrane, using PicoGreen™ might be the best option as Lysis buffer 1 from ChemoMetec does not dissolve eggshell based microcarriers resulting in readings in the NucleoCounter® being inaccurate.

The NucleoCounter® worked well during splitting of cells, but for the bioreactor experiment the samples had too few cells to be accurately counted on the machine. As seen in figure 4.11, when taking a measurement six times in a row on day 5 one receives very different results. A better sampling method might be devised where the samples for the NucleoCounter® are less diluted, alternatively PicoGreen™ cell counting method could be used when counting a smaller amount of cells.

Expression of MyoD, Myogenin and Desmin was higher in the differentiating cells compared to the proliferating cells in the rt-qPCR experiment. This is expected as these genes play a role in promoting cell differentation. MuSC differentiation, protein denaturation, lactic acid accumulation and anaerobic glycolysis metabolic reactions are responsible for producing the textural quality, appearance and taste of meat (Datar & Betti, 2010).

The bioreactor experiments using Cytodex® 1 microcarriers looked good after hoechst staining under a Zeiss Z1 microscope. Cytodex® 1 microcarriers are designed for primary cells (Cytiva, 2020). The results from the NucleCounter® were not as favourable, NucleCounter® is designed to count cell concentrations within the limits of $5 \cdot 10^4$ cells/mL to $2 \cdot 10^7$ cells/mL on microcarriers (ChemoMetec, 2021, p. 2). The measurements were below the minimum requirement. Low cell concentrations will negatively affect the counting precision (Chemometec, 2020).

Cytodex® microcarriers are typically not used as a part of the final comestible product (Marga et al., 2017). One great benefit of eggshell based microcarriers is that the carriers are digestible and can be completely incorporated into the final product, circumventing the need to remove the carriers (Hagen, 2019, p. 59).

Eggshell membrane (ESM) powder consists of collagens, glyocoproteins, cysteine-rich eggshell membrane proteins (CEMPS), carbohydrate and antibacterial protein (Marga et al., 2009). ESM has been used in traditional Asian cultures to treat wounds (Vuong et al., 2017). Muscle cells are surrounded by collagen *in vivo*, it is a major structural protein in muscle Extracellular Matrix (ECM) (Gillies & Lieber, 2011). In a spinner flask experiment with Cytodex® 1, conducted by Ferrari et al., large microcarrier-cell-aggregates would develop on the carriers, making it hard for the cells to get access to nutrients which lead to cell death (Ferrari et al., 2012). With its fibre-like structure, eggshell membrane carriers could potentially outperform Cytodex® 1 by preventing microcarrier-cell-aggregates from appearing. Andreassen et. al. found eggshell membrane carri-

ers support excellent MuSC attachment with cells covering the entire MCs surface while demonstrating high cell proliferation and metabolic activity combined with low cytotoxicity (Andreassen et al., 2022). Excellent cell attachment of MuSC on eggshell membrane can be confirmed here, see figure 4.17.

ECM is important for structure alone. It also contributes to cell signalling, regulation of cell activation and maintenance of cell identity (Thomas et al., 2015). Recently, edible scaffolds of fish gelatin and alginate were developed for myoblast growth with excellent performance (Acevedo et al., 2018). The irregular surface found on ESM could affect its biological function as it will randomly distribute the cells attached to it, furthermore the pores and roughness of the material will affect cell development (Li et al., 2014). Skeletal muscle tissue requires a uniform and reproducible architecture to produce dense muscle tissue (Langelaan et al., 2010).

Another raw material which has been suggested for use as microcarriers is residual raw materials of turkey (Jurgilevich et al., 2016). Unlike eggshell membrane based carriers, these carriers have a tendency to float, making cell adhesion difficult as the material will be at the top of the reactor as the cells float to the bottom. When the cells attached, they would sink to the bottom of the bioreactor. Eggshell membrane carriers suspend nicely when cells are not added to the reactor. It is possible that when MuSC cells attach to eggshell membrane they become heavy and sink just like they do on turkey based carriers.

For qPCR bioreactor experiment, MyoD and Desmin are at about the same levels throughout the experiment. Myogenin however drops significantly after the first day of the experiment. This might be because the cells mainly try to proliferate as they grow in the bioreactor.

Glucose generally trended downward as the bioreactor experiments were conducted (with the exception of the first bioreactor experiment which was stopped after 9 days), as expected. Glucose from the proliferation medium becomes ATP through glycolysis, it is converted to chemical-energy by mitochondria in the MuSC cells (Moritz et al., 2015). New media was added to the reactor on day 7 and 15 of the experiments, one can see an increase in glucose on those days in the third bioreactor experiment with Cytodex® and the third bioreactor experiment with eggshell membrane carriers. Refreshing the medium when cultivating muscle cells is necessary, it will help regulate glucose, lactate, glutamine and the concentration of ammonia (Schop et al., 2008). Lactate measurements generally increase as the cells leave lactate as an immune response in the media (Pösö & Puolanne, 2005). This is also as expected. High pH is a burden for the cells, reducing lactate has been shown to improve cell productivity (Xu et al., 2018). There is a desire to better improve bioreactors and reactor techniques. For stirred culture vessels, fed-batch mode can improve culture performance (Hambor, 2012). Lactic acid increases in hypoxic (low oxygen) systems (Chakravarthy et al., 2001). Regulating glutamine concentration could also affect lactate levels as degrada-

tion of lactate occurs when glutamate is lowered (Zagari et al., 2013).

DNA concentration was measured for the last eggshell membrane bioreactor experiment with frozen samples. Since the DNA concentrations were low, there can be inconsistencies in the quantification of DNA, as seen in other DNA quantification methods using DNA-binding fluorescent dyes (Nakayama et al., 2016). Small amounts of DNA in a sample for dsDNA analysis can give uncertainty during dsDNA quantification, most dsDNA quantification methods are highly dependent on the determination size by the bioanalysis (Robin et al., 2016). Since most of the cells were lost at the bottom of the bioreactor, the PicoGreen^{TM} measurement for the final eggshell membrane experiment shows very little cDNA per mL media. Solving the suspension problem will likely improve the results.

The Muscle Satellite Cells cells seem to have myogenic potential after a bioreactor experiment as the cells started to differentiate into myotubes after seven days, where the first five were spent in proliferation media and the final days were in differentiation media. Some cells may have been lost on the cell straining filter during sowing.

The BioCommand® software for the Eppendorf BioFlow® bioreactor was not set correctly after refilling microcarriers and media on day 7 of the first bioreactor experiment. CO_2 was accidentally left off, turning the proliferation media purple, increasing the pH in the solution and killing off most of the cells. DMEM goes purple at high pH. According to the reactor the pH had reached over 9 when it was supposed to be 7,3. To prevent these problems in the next experiments, BioCommand®'s automatic alarm feature put to use (Scientific, 2011). Automatic emails were sent from the BioCommand® software when parameters went out of range on the next experiments.

Further research can include ways of properly preparing eggshell based microcarriers so they remain suspended in the BioFlo® bioreactor. Alternatively, a different impeller design or a different bioreactor could be tested. One could even design an impeller. The microcarriers of eggshell membrane did not function well with the bioreactor used in the experiment as the bioreactor impeller did not allow the cells to remain suspended. It might be possible to treat the cells before putting them into the reactor by for example vortexing to make them stay suspended in the reactor.

Chapter 6

Conclusion

Since most of the cells were lost at the bottom of the bioreactor, the PicoGreen™ measurement for the final eggshell membrane experiment shows very little cDNA per mL media. Solving the suspension problem will likely improve the results.

The NucleoCounter® worked well during splitting of cells, but for the bioreactor experiments the samples had too few cells to be accurately counted on the machine. A better sampling method might be devised where the samples for the NucleoCounter® are less diluted, alternatively PicoGreen™ cell counting method could be used when counting a smaller amount of cells.

The Cytodex® bioreactor experiments looked good after hoechst staining under a Zeiss Z1 microscope even though results from the NucleCounter® suggested otherwise.

MHC and Myogenin Western blots could have been redone on new blots.

Glucose generally trended downward as the bioreactor experiments were conducted (with the exception of the first bioreactor experiment which was stopped after 9 days), as expected. New media was added to the reactor on day 7 and 15 of the experiments, one can see an increase in glocuse on those days in the third bioreactor experiment with Cytodex® and the third bioreactor experiment with eggshell membrane carriers, as expected. Lactate measurements generally increase as the cells leave lactate as an immune response in the media. This is also as expected.

The Muscle Satellite Cells cells seem to have myogenic potential after a bioreactor experiment as the cells started to differentiate into myotubes after seven days, where the first five were spent in proliferation media and the final days were in differentiation media.

The microcarriers of eggshell membrane did not function well with the bioreactor used in the experiment as the bioreactor impeller did not allow the

cells to remain suspended. Further research will need to be conducted to find a way to properly prepare the eggshell based microcarriers, or alternatively use a different reactor with a different impeller design. It might be possible to treat the cells before putting them into the reactor by for example vortexing or trying eggshell membrane microcarriers of different sizes and thus make them stay suspended in the reactor.

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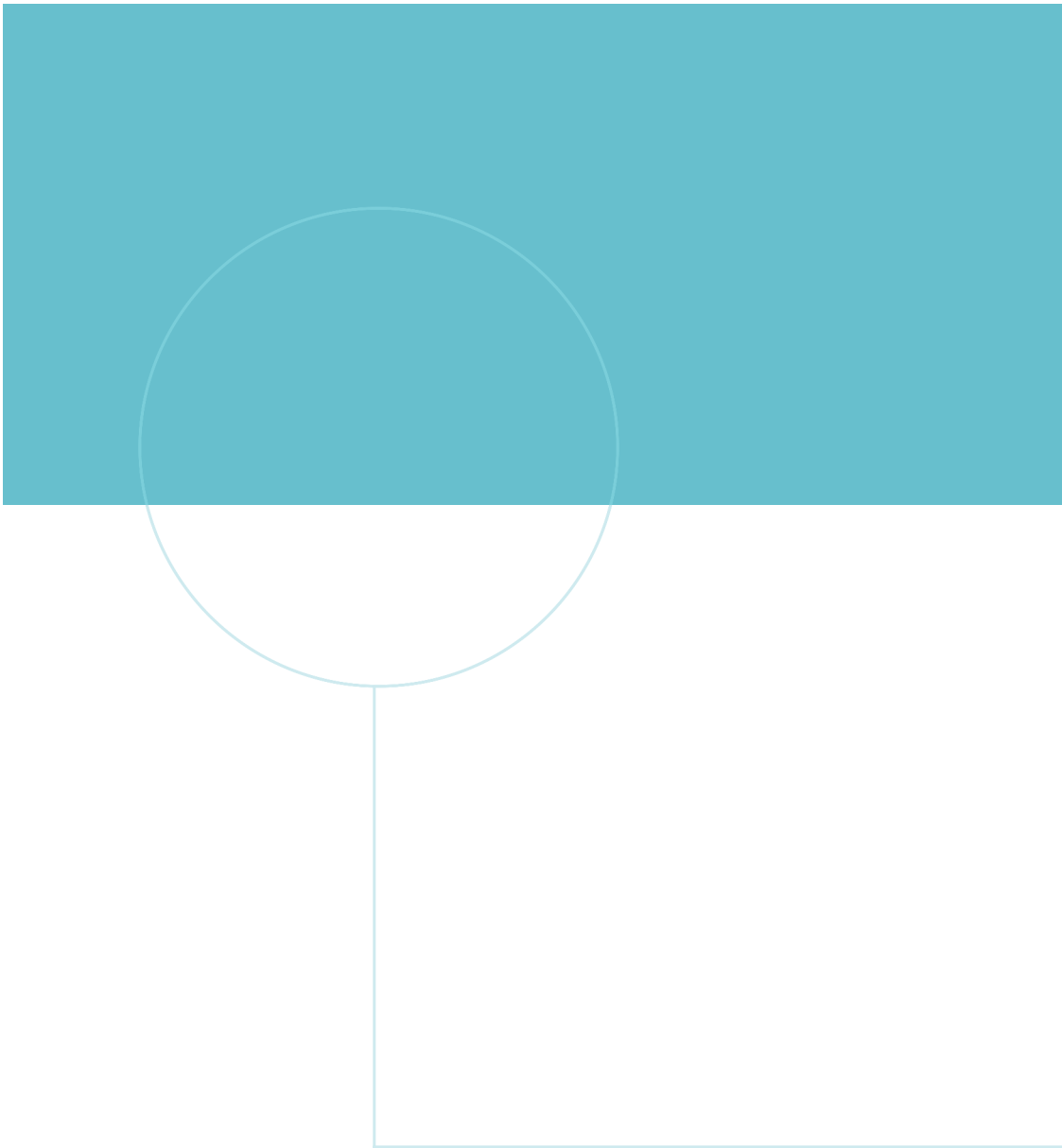

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