Isolation and characterisation of bovine muscle stem cell progenitors

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FACULTY OF SCIENCE
Department of Chemistry

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ISOLATION AND CHARACTERIZATION OF BOVINE MUSCLE STEM CELL PROGENITORS

Diploma Thesis

submitted to the Department of Chemistry,
Faculty of Science, University of Zagreb
for the academic degree of Master in Chemistry

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This Diploma Thesis was performed at Division of Biochemistry, Department of Chemistry, Faculty of Science, University of Zagreb under the mentorship of [titula, ime i prezime mentora] and under assistant mentorship [titula, ime i prezime neposrednog voditelja].

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University of Zagreb
Faculty of Science
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Diploma Thesis

ABSTRACT

ISOLATION AND CHARACTERIZATION OF BOVINE MUSCLE STEM CELL PROGENITORS

Josip Čačković

This thesis investigates the technical feasibility of bovine muscle progenitor stem cells as a source for *in vitro* meat cultivation. The cells were isolated using enzymatic digestion. Bovine muscle progenitor stem cells were identified using the paired box protein, Pax7 using immunocytochemical assays and fluorescence microscopy. The cells were treated with SB203580, a common inhibitor for the p38 mitogen activated protein kinase pathway to examine its influence on the cell's proliferation and differentiation capabilities. However, it didn't yield the expected outcome. Collagen type 1, gelatine and laminin were used as coatings and compared to see their influence on the selectivity of the target cell type, of which laminin was the most promising. Lastly, cells were sorted via fluorescence activated cell sorting using CD29, CD56 as positive, and CD31 and CD45 as negative cell surface markers, which proved to be an auspicious method for the purification of the cell culture.

(67 pages, 25 figures, 7 tables, 146 references, original in English)
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Keywords: cultured meat, characterization, fluorescent activated cell sorting, p38 MAPK, isolation, satellite cells, surface coating

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Diplomski rad

SAŽETAK

IZOLACIJA I KARAKTERIZACIJA MATIČNIH STANICA PREKURSORA MIŠIĆNIH STANICA IZ GOVEDA

Josip Čačković

Ova disertacija istražuje tehničku izvedivost korištenja matičnih stanica prekursora mišićnih stanica iz goveda za uzgoj mesa *in vitro*. Stanice su izolirane primjenom enzimatske digestije. Matične stanice prekursori mišićnih stanica iz goveda identificirane su pomoću transkripcijskog faktora Pax7, uporabom imunocitokemijskih testova i fluorescentne mikroskopije. Stanice su tretirane SB203580, inhibitorom metaboličkog puta p38 mitogen-aktivirane proteinske kinaze, kako bi se ispitao njezin utjecaj na sposobnosti proliferacije i diferencijacije stanica. Međutim, korištenje istog nije dalo očekivani rezultat. Stanično posuđe tretirano je raznovrsnim premazima: kolagen tip 1, želatina i laminin, kako bi se promatrao njihov utjecaj na proliferaciju i selektivnost prema stanicama od interesa. Laminin se pokazao kao najobećavajući. Konačno, stanice su sortirane putem fluorescentno aktiviranog sortiranja stanica primjenom CD29, CD56 kao pozitivnih i CD31 i CD45 kao negativnih površinskih staničnih markera, što se pokazalo izvrsnom metodom za pročišćavanje kulture stanica.

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Ključne riječi: cultured meat, karakterizacija, fluorescentno aktivirano sortiranje stanica, p38 MAPK, izolacija, satelitske stanice, premazi staničnog posuđa

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PROŠIRENI SAŽETAK

Svjetska populacija raste iz dana u dan, prema nedavnim modelima UN-a do 2050. trebalo bi biti čak 9 milijardi ljudi na svijetu. Iako je to porast populacije od 30 % u odnosu na brojeve iz 2010-ih godina, pretpostavlja se da će prateće poljoprivredne potrebe porasti značajno više. Određeni podaci govore kako bi konzumacija mesa i mesnih prerađevina mogla porasti čak 50 % u istom periodu. Takav razvoj mesne industrije mogao bi uzrokovati druge neželjene ishode poput širenja površina za ljudsku uporabu, bilo urbanizacijom ili krčenjem preostale divljine u neposrednoj blizini ljudi, kao i povećanu emisiju stakleničkih plinova.[1] Znanstvena zajednica pristupa rješavanju problema na više načina, od kojih je uzgoj mesa *in vitro*, u laboratoriju,[2] opcija vrijedna spomena.

Ovaj rad se bavi inicijalnim tehničkim aspektima uzgoja mesa *in vitro*, poglavito razvojem staničnih kultura koje bi se mogle koristiti u tu svrhu. Većina istraživanja usmjerena je na govedo jer se govedarstvo smatra jednim od najvećih uzročnika porasta količine stakleničkih plinova i zauzima najviše prostora u poljoprivrednom sektoru.[3] Naše istraživanje usmjereno je na pročišćavanje svježe izoliranih primarnih mišićnih matičnih stanica goveda i njihovu karakterizaciju s namjerom razvoja stanične linije u kasnijim istraživanjima. Stanice su izolirane jednom tjedno iz svježih biopsija goveda dobivenih od lokalne bio-mesnice. Rezultirajuća stanična kultura heterogena je populacija stanica. Najzastupljenije stanice u takvoj kulturi su govеđe mišićne progenitorske stanice, u nastavku govеđe mišićne satelitske stanice (engl. Bovine muscle progenitror stem cells ili bMuCs), koje se imunocitokemijskim testovima i fluorescentnom mikroskopijom identificiraju kao stanice koje eksprimiraju protein jezgre Pax7. Kako bi pokušali povećati selekvinost prema bMuCs-ima uspoređeni su sljedeći premazi posuđa za staničnu kulturu: neobložene, kolagen tipa 1, želatina i laminin. Uz selektivnost prema željenim bMuCs-ima, uspoređujemo i njihov utjecaj na sposobnost diferencijacije stanica inicirane uskraćivanjem seruma. Laminin i laminin pentapeptide pokazuju se kao najutjecajniji premazi za stanično posuđe. Laminin poboljšava uspješnost izolacije za 50 % relativno naspram prethodno korištenog premaza kolagena tipa 1. Također je znatno uspješniji u održavanju udjela bMuCSa u staničnoj kulturi tijekom većih pasaža. Pročišćavanju primarne stanične kulture pristupili smo pomoću fluorescentno aktiviranog sortiranja stanica (FACS, engl. *fluorescent activated cell sorting*) koristeći sljedeće površinske

markere: CD29, CD31, CD45 i CD56.[4] FACS omogućuje povećanje udjela bMuCSa u kulturi na veće razine, no što su bile domah nakon izolacije te su ostvareni udjeli veći od 55 % bMuCSa u kulturi. U konačnici, istražujemo uporabu uobičajenog inhibitora SB203580 za inhibiciju p38 MAPK puta, što je jedan od načina na koji se transkripcijski faktor MyoD fosforilira uslijed čega dolazi do početka diferencijacije. Ideja je spriječiti neželjenu diferencijaciju bMuCs-a inhibiranjem tog signalnog puta i povećati proliferativne sposobnosti izoliranih bMuCs kultura. Nažalost korištenje SB203580 inhibitora nije urodilo značajnim poboljšanjem u sprečavanju diferencijacije bMuCSa u kulturi ili očuvanju njihovih karakteristika matičnih stanica.

Cilj ovog rada uspostavljanje je bMuCS stanične kulture koja ima sljedeće karakteristike: preživljavanje bez inhibitora smanjenje udjela ne-bMuCS stanica u kulturi, selektivnu diferencijaciju u mišićno tkivo (miotube) i poboljšane proliferativne sposobnosti. Korištenjem određenih metoda kao što su FACS i premazi staničnog posuđa lamininom ili lamininom pentapeptidom optimizirali smo proces izolacije matičnih stanica prekursora mišićnih stanica iz goveda i njihovog uzgoja *in vitro*. Rezultati i metode korištene u ovom radu mogu poslužiti kao dobar temelj za daljnjja istraživanja koja će ići u smjeru uspostavljanja dugoročnih kultura bMuCS i njihovog masovnog kultiviranja u svrhu uzgoja mesa *in vitro*.

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§ 1. INTRODUCTION

The population of the world is still in its growth phase, with the latest UN models predicting around 9 billion people by the year 2050. And although it is an estimated 30% increase in population since the 2010s, the accompanying agricultural needs are expected to rise even faster, with the consumption of meat growing by 50% in comparison to 2010 numbers. Such development could cause unwanted outcomes; the expansion of the areas under human influence, be it through urbanization or by taming the remaining wilderness in the immediate human vicinity; as well as increased greenhouse gas emissions.[1] The research community is tackling the problem with a variety of possible solutions, of which the cultivation of *in vitro* meat is a notable option.[2]

This thesis will deal with initial technical aspects of *in vitro* meat cultivation, especially the development of cell cultures that could be used for that purpose. Most of the research is focused on bovine meat, as the cattle (beef) industry is considered one of the biggest contributors to greenhouse gas emissions and land detractors in the agricultural sector.[3] Our research is based on freshly isolated primary bovine muscle progenitor cells and their purification, with the goal to establish a cell line for *in vitro* meat cultivation in further research. Cells were isolated once a week from fresh bovine biopsies secured from a local bio-butcher. The resulting cell culture is a heterogenous cell mixture. Bovine muscle progenitor cells (or bMuCS for short) are the most prevalent in such a cell culture, which are identified as cells which express the paired-box protein 7 (Pax7) and determined using immunocytochemical assays and fluorescence microscopy. In an attempt to increase the selectivity of dishes for bMuCS, after isolation, we compared the following coatings for cell culture dishes: uncoated, collagen type 4, gelatine, and laminin. In addition to the selectivity toward the desired bMuCs, we also compared their influence on the differentiation ability of bMuCs initiated by "starvation" induced by serum withdrawal. We decided to try to purify the primary cell culture mixture by using fluorescent activated cell sorting (FACS), using the following surface markers: CD29, CD31, CD45 and CD56.[4] Lastly, we investigated the use of the common drug SB203580 for the inhibition of the p38 MAPK pathway, which is one of the ways how the transcription factor MyoD is phosphorylated, and differentiation is initiated. The idea is to prevent unwanted differentiation of bMuCs by inhibiting this metabolic pathway and to increase the proliferative abilities of our

culture. Our prime goal is establishing a bMuCS cell culture that has the following characteristics: survival without antibiotics, depreciation of non bMuCS cells in the culture, and selective differentiation into muscle tissue (myotubes).

§ 2. LITERATURE REVIEW

2.1. Meat industry today and the prospects of *in vitro* meat cultivation

The number of people living, breathing, and eating on this planet is growing every day and with it our craving for meat. According to the latest UN projections, the world's population is going to increase by approximately 30% of today's numbers to 9 billion in 2050 followed by a 50% increase in meat consumption.[1] The latter has more relevance because it cannot be explained only by population growth, but also through societal development. And while some argue that higher meat consumption is a sign of the "westernization" of the developing world, others share the opinion that higher meat consumption is only a nutritional transition phase throughout the development of a society from a developing nation to a developed nation.[5,6] This brings additional concerns to the table if we also consider the climate effects of the agricultural sector, especially the meat industry, its greenhouse gas emissions and deforestation effects. 13.2% of all global greenhouse gasses were emitted by the livestock industry in 2018,[7] in comparison with only 8% by non-animal foods. Figure 2.1. shows that meat nutritional staples contribute most of the greenhouse gas emissions in the agricultural sector, compared with a couple of other selected food items.

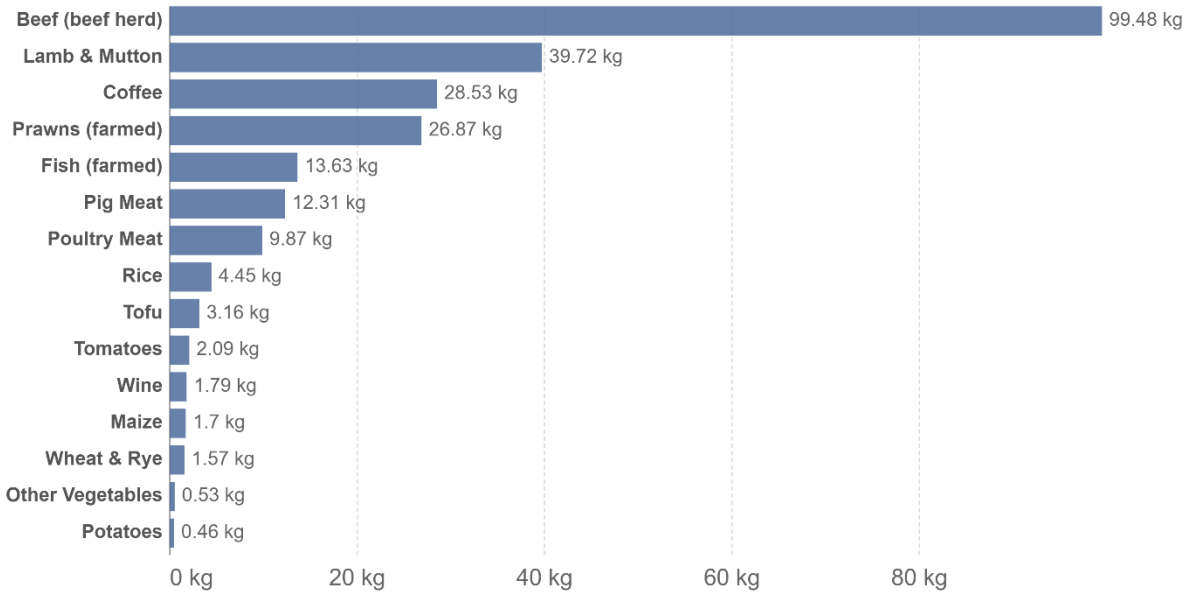

Figure 2.1. Greenhouse gas emissions by food staples, measured as an equivalent of carbon dioxide mass throughout 100 years per a kilogram of the food staple.[7]

Land use is another ill effect exacerbated by the growing meat industry, because, as mentioned above, it is crucial for further development of the human society, but also for the preservation of the existing biodiversity by safeguarding our existing flora and fauna.[8] Unfortunately, land is limited, especially land that is viable for human habitation and agricultural practices. Of the ~150 000 000 km^2 available land on Earth, around 30 000 000 km^2 is used for meat production, accounting for the farmhouses, grazing and growing of animal feed.[9] Grazing fields are mostly created by deforestation which inhibits the natural sequestration of carbon dioxide and depletes biodiversity in the surrounding area. Figure 2.2. shows that meat staples again have significant impact on the land use of the agricultural sector.

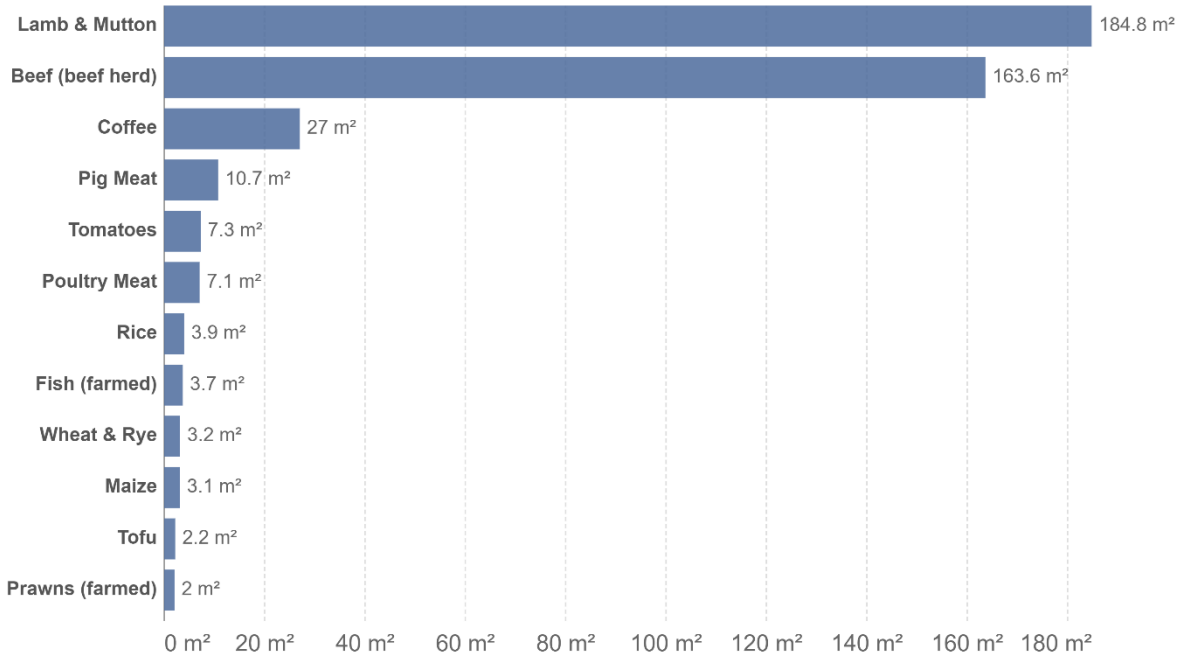

Figure 2.2 Necessary land surface (measured in square meters, m2) for generating 100g of protein per food staple.[9]

Bovine and ovine staples are outliers in both previously mentioned environmental categories. One reason for it could be the average time needed to raise cattle or a lamb ready for slaughter. It takes from 10-30 and 6-10 months for bovine and ovine, in comparison to 4 months for hogs.[10,11,12]

With land use in mind, the feed to edible protein ratio should be taken into consideration as well. The amount of protein fed to and converted to edible protein in the final product is 8.5% (ovine), 6.7% (grass-fed bovine), 12.1% (bovine), 23.3% (porcine) and 33.3% (pullum).[13] The feed conversion rate is the biggest driver of land use in the meat industry, especially when we consider the time and food necessary for breeding an animal.

2.1.1. Cell sources for in vitro meat

We begin with the starting cell population on which there is no consensus which species nor their breeds should have priority. Research is currently focused on bovine, porcine and pullum with some rare instances of ovine and crustaceans.[14,15,16] The end goal is a complex product, because as we know, meat isn't formed only by muscle tissue. In addition to muscle fibres, meat also contains some fat from adipose cells and connective tissue, so it is theoretically

possible to start from a heterogenous cell culture, but it would certainly weigh on the proliferation step later. The methodology of cell sourcing also isn't standardized, some groups are experimenting with genetically modified somatic muscle cells,[17] where they use CRISPR to knock out the CDKN2[18] by causing frameshift mutations which stop the expression of p15 and p16 which both inhibit CDK4 whose overexpression leads the immortalization of the myogenic cell line.[19] Others are investigating the potential reprogramming of fibroblasts into induced pluripotent stem cell with the abilities to differentiate into muscle fibres by overexpressing of the MyoD transcription factor using a *doxycycline inducible Tet-on dual plasmid system*.[20] Contrary to that, we have used a method without genetic modifications for this thesis by isolating fresh satellite cell cultures and have also experimented with different coatings for higher selectivity and using FACS for sorting our satellite cells to cultivate a reliable bMuCS culture that could be used for *in vitro* meat cultivation further on. Each one of the forementioned approaches has its own hardships. First, let's consider the option of genetically modifying bovine cells for the purpose of *in vitro* meat cultivation, there isn't sufficient genome sequencing data available for all bovine breeds, which can have a significant impact on the later efficacy and standardisations of cell cultures/lines which can put a burden on the R&D and fiscal side, although less than for the non-genetically modified options. Additionally, genetically modified products wouldn't be suitable for regulatory approval in all markets and regions, and consumer acceptance still proves to be a hurdle, especially in a region like the EU where genetically modified food faces low public and consumer acceptance.[21]

Besides, the non-genetically modified option of creating cell cultures or cell lines has problems of its own. For example, the satellite cells that we have used for this thesis are the *bona fide* cells responsible for muscle regeneration after.[22] Their main disadvantage is maintaining their proliferative capabilities because they tend to differentiate into myotubes and myofibrils (even in low confluency) and stop replicating. However, data suggest that there is a smaller subpopulation of satellite cells in the main satellite cell population, which has far greater regenerative and proliferative capabilities and efforts are under way to develop selection protocols for extracting those cells from the primary culture.[23]

2.1.2. Cell culture and scale up

Large scale mammalian cell culture has been available since the later part of the 20th century, due mostly to unintended innovations in cell medium production, incubators and serum productions and the general awareness of necessary aseptic conditions. Although large-scale cell culture is available today and has room for improvisation, *in vitro* meat is in a league of its own.

Culture medium is the primary challenge when trying to grow *in vitro* meat, as it is necessary to provide an equilibrium of numerous parameters (i.e., sugars, ammino acid share, growth hormones, pH, etc) to promote cell growth, it should be economically feasible for cultured meat to even be competitive and, above all, safe for people. Foetal bovine serum (FBS) is another hard to replace component.[24] It is collected from the blood of unborn calves in the last two thirds of gestation, they're mostly accidentally discovered when slaughtering pregnant cows.[25] It is a vital supplement in human and mammalian cell culture as it contains hormones, minerals, vitamins, transport proteins and growth factors.[26] FBS has its own set of problems, it has large variations in composition from "lot to lot", they can also have diverging or unintended interactions with test subjects (i.e., the results can never be fully reproducible with to different lots).[27,28] An extra factor here to keep in mind is that FBS is a by-product of the meat industry, ergo its availability is determined by the industry's market performance. As *in vitro* meat is set on being a substitution for the contemporary meat industry it cannot be feasible procuring such a vital component from a source that is supposed to shrink. Today, there are FBS-free chemically defined culture media available on the market from multiple vendors but there are also published protocols for those media for specific cells.[29] Both solutions are quite weary both in terms of time and fiscal contributions. Transcriptomics are pushing the boundaries by providing information on cell surface proteomics, which can further help to develop a cell culture medium for specific breeds or animals with relative connections.[30,31] The cells, if properly cultured, should at least be able to go through 20 and preferably over 30 doubling cycles during the proliferative stage of production. For the desired higher doubling rate, more attention should be focused on mechanisms that determine differentiation with the end goal of having the ability to stop it or initiate it by the composition of cell culture medium.[32] One of such potential biochemical mechanisms that was explored is the p38 MAP kinase pathway

which has the potential for preserving the proliferation and stemness of the satellite cell culture.[33]

The whole culturing process should at one point be automated with minimal human interactions for mitigating the potential contamination of the cultures.[34] The establishment of cultured meat "factories" has been criticized as more of an art project rather than a scientific undertaking by some.[35] It is true that the initial capital cost can't compete with the conventional meat industry, but there is room for improvement. Lowering the cost of the culture medium, a wave of innovations in the bioreactor manufacturing, tissue engineering advances or other unforeseeable advancements are all factors that could drive down the final consumer price for cultured meat and make it a viable option on the market.

2.2. Satellite cells – biochemical background and their characterisation

2.2.1. C2C12 cell line

Cells from the C2C12 cell line are immortalized mouse myoblast cells that have the ability to initiate myogenesis in low serum conditions („serum starvation“).[36] During differentiation, they express the myosin heavy chain protein and form multinucleated myotubes similar to those found in wild-type cells.[37] They are a vital research tool in myogenesis investigation.[38,39,40] The C2C12 cell line was used in this research as a training tool for cell culture technique and for comparing the morphology and functions of this established cell line with our isolated bovine muscle progenitor stem cell cultures.

2.2.2. Skeletal muscle and satellite cells

Skeletal muscle is formed by postmitotic multinucleated muscle fibres (the contractile units of muscle), and they are formed by fusing large numbers of mononucleate myoblasts.[41] Later on, it was discovered that there were quiescent cells lying on the myofiber surface, but beneath its basement membrane, giving it the name “satellite cell” (from here on ”SC”).[42] It was proven that SC give rise to myoblasts by isolating viable myofibers, using enzymatic digestion, with cohorting the SC and culturing them to establish a proliferating SC culture.[43] Their main objective is the regeneration of muscle tissue during an injury by activating them and differentiating into myoblasts.[44] For that, they first must be activated by a signal from the myofibers, caused usually by exercise, injury, or disease.[45]

When SC are activated, they proliferate and yield myoblasts. Those myoblasts additionally proliferate and later commit to differentiation when they are called myocytes. Myocytes are mononucleated cells that have the ability to fuse together forming myotubes or fuse themselves with already existing myotubes.[46,47] Myotubes are multinucleate, elongated cells consisting of at least three myocytes and in the vicinity of a myofibril. Myofibrils are long, tubular organelles of the myotube, which form the contractile apparatus. During maturation, myotubes develop into myofibers. Myofibers cytoplasm is filled with myofibrils and their nuclei are located peripherally.[48] Figure 2.3. depicts the forementioned myofiber formation.

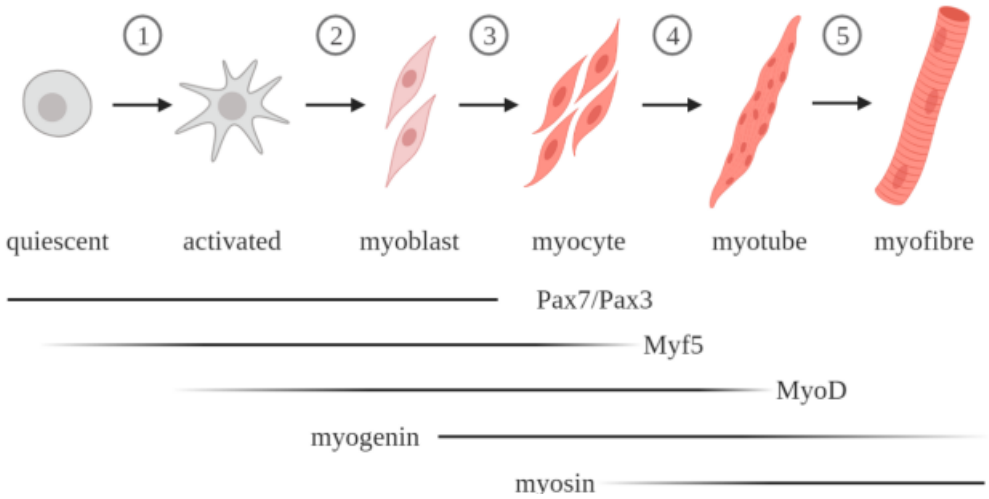

Figure 2.3. Formation of a myofiber with the necessary transcription factor and the time frame of their expression. Transition steps: (1) activation, (2) proliferation, (3) differentiation, (4) fusion and (5) maturation. Figure based on and edited.[47]

2.2.3. Molecular markers of satellite cells

Figure 2.3. also reveals additional information about the crucial myogenic transcription factors during distinct stages of myofiber formation. The most prominent SC molecular markers are as follows: Pax3/ Pax7, MyoD, myogenin and isomers of myosin heavy chain proteins.[49]

Pax7, also known as paired box protein 7, is a transcription factor that is involved in the proliferation of satellite cells, myogenesis and muscle regeneration.[50] Pax7 is present in the nuclei of SCs that are quiescent, and it is the most notable marker for their identification.[51] It can bind to DNA as a heterodimer with Pax3.[52] Pax7 and Pax3 control the activation signals that activate the quiescent SCs and are therefore suitable biomarkers for the identification of SCs.[53]

In addition to Pax7, activated SCs also upregulate and co-express the transcription factor MyoD (a.k.a. myoblast determination protein 1).[54] MyoD and Pax7 must maintain an important balance during SC determination and differentiation. If SCs return to their quiescent state, they downregulate their MyoD expression and if they fully mature and initiate terminal differentiation, then Pax7 is downregulated and MyoD expression is maintained.[55] Although MyoD is considered as the main regulator of myogenesis, there is research that suggests that Myf5 (a.k.a., Myogenic factor 5) could functionally substitute MyoD, as the two are more homologous to each other than to other myogenic regulatory factors.[54] The lack of both

however, is a major obstacle for myogenic programming as their deficiency in SCs results in mild differentiation and growth defects during development.[56] It is suggested that both have complementary functions during myofiber development rather than being able to substitute one another and that Myf5 upregulation precedes that of MyoD.[57] The details of their functions or the intercorrelated effects of one on the other are yet still to be determined as there are multiple suggestions how the myogenic progression could work. Some argue that it's possible that only MyoD or Myf5 are separately upregulated upon SC activations before initiating the co-expression of the other; others propose that Myf5 directs myoblast proliferation while MyoD is responsible for preparing myoblasts for differentiation.[58,59] When everything is considered and despite the unknown regarding the exact functions of MyoD and Myf5 one thing is sure, and that is the reliability of MyoD as a biomarker for myoblasts that tend to differentiate.[60]

Myogenin (a.k.a. Myf4 or myogenic factor 4) is also a crucial transcription factor during myogenesis, where it is required for the early differentiation of committed muscle progenitor cells into myofibers. Its expression directly initiates myoblast differentiation and their fusion into myofibers and is vital for the regulation of myofiber size.[61,62,63] It has been shown that genetically modified mice with mutated Myogenin in large amount do not form multi nucleated myofibers, which shows that myogenin is a crucial biomarker for terminal myoblast differentiation.[63]

Biomarker analysis of SCs wouldn't be complete without MyoHC (a.k.a. myosin heavy chain) which is a part of the larger myosin family.[64] The myosin family makes up around half of the total protein in skeletal muscle.[65] It is vital in generating contraction, as it is a mechanoenzyme that binds ATP in its catalytic head domain. The nucleotide binding, hydrolysis and product release are associated with the induction of conformational change or contraction in more simpler terms .[66] The presence of MyoHC in myotubes indicate full maturation the muscle progenitor cells and their functionality making them a crucial endpoint marker for myogenesis.[67]

In short, Pax3/ Pax7, MyoD, MyoG and MyoHC are all viable suiters for the confirmation of the different stages during the life of satellite cells. We used exclusively Pax7 for the conformation of SCs after isolation or sorting and for assessing the purity of the bMuCs culture over time. For differentiation experiments, we used MyoHC as the main confirmation for the cell's full development. The most crucial difference between the two is that Pax7 is a transcription factor located in the nuclei and MyoHc is a molecular motor protein located in the

cytoplasm of multinucleated myotubes. One of the drawbacks of these markers is that they can only be identified with immunocytochemical assays when the cells are fixated and thus the results will always be gathered retrospectively, which isn't ideal if such a method is to be used in quality assurance labs for a scaled up *in vitro* meat project. But for research purposes, as in this thesis, it didn't prove to be an obstacle.

2.2.4. P38 MAPK pathway

Mitogen-activated protein kinases (MAPK from here on) are serine/threonine-specific protein kinases that are heavily involved cell cycle functions, such as proliferation, gene expression, differentiation, mitosis and apoptosis. They all share similarities on a base level as they're activated by phosphorylation from another protein kinase, termed MAPK kinase (MAPKK), which is also phosphorylated and activated by a protein kinase, termed MAPK kinase kinase (MAPKKK). MAPKKK can be activated through a variety of cytokines, growth factors, cellular stresses or through interaction with the Ras protein family, a subtype of small GTP-ase proteins.[68]

For our research, the most notable member of the MAPK family is the p38 MAPK, of which four isomers have been identified in mammals, p38-α (MAPK14), p38-β (MAPK11), p38-γ (MAPK12) and p38-δ (MAPK12).[69] They are all expressed widely in the organism with varying expression patterns in different tissues. P38-α is ubiquitously expressed in all cell types and tissues, while p38-β is highly expressed in the brain, thymus, spleen; p38-γ is almost exclusively found in skeletal muscle and p38-δ is found in the pancreas, intestine, adrenal gland and kidneys.[70,71,72] They all catalyse the reversible phosphorylation of proteins when they are in their active state (dually phosphorylated). The most important phosphorylation, regarding myogenesis, that is undertaken via the p38 MAPKs is the phosphorylation of MyoD in the nuclei. MyoD isn't the only transcription factor that is being phosphorylated via this metabolic route, nor is p38 the only way myogenesis can be initiated. A brief graphical summary of the p38 MAPK pathway is given in Figure 2.4

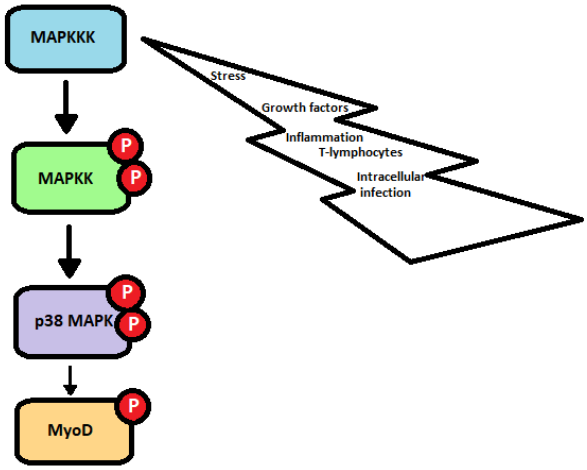

Figure 2.4. A simplistic schematic description of the p38 MAPK pathway with the most common activators on the right in the "bolt" and a simple phosphorylation cascade on the left. Edited image.[73]

The phosphorylated MyoD is transcriptionally active and thus myogenesis is initiated.[74] We were hoping to score on that note and investigated potential p38 MAPK inhibitors. We chose the SB203580 molecule that is described as an inhibitor for the whole p38 family, whose structure is shown in Figure 2.5. SB203580 is a pyridinyl imidazole compound that competes with ATP for the catalytic site of the p38 MAPK, thus hampering the phosphorylation of it and preventing the activation p38 MAPK.[75]

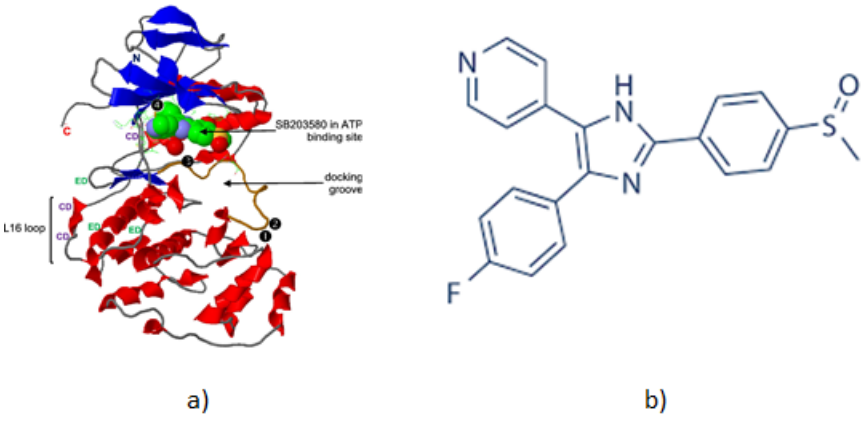

Figure 2.5. a) Crystal structure of p38 with SB203580 occupying the ATP binding site. SB203580 is shown in green. The activation loop is shown in orange. b) Chemical structure of pan-p38 inhibitor molecule SB203580.[76,77]

Unfortunately, most of the research on the inhibitor has been performed in other mammals, such as rats, mice, and rabbits. We wanted to investigate the use of this inhibitor molecule to examine its influence on the p38 MAPK pathway within the satellite cells of the isolated bMuCS cultures. The idea was that by inhibiting this pathway in our culture, the phosphorylation of MyoD would also be inhibited, thus leaving our cells unable to initiate myogenesis, and enabling them to proliferate for longer.[78,79,80,81,82,83]

2.2.5. Satellite Cell niche and the basal lamina

Stem cells generally tend to "live" in a specific microenvironment within their native tissue, which is commonly referred to as a "niche". This niche provides the quiescent stem cells with a protective environment against depletion, and it transfers signals for their activation, proliferation and differentiation. Changes to the niche components can result with loss of stemness from the stem cells throughout the body.[84] The niche also offers structural integrity to the cells, physically separating the stem cell pool from the rest of the tissue. Extracellular matrix components have a major contribution in the niche as they represent the structural and signal transferring components of the niche.[85] Currently, it isn't possible to replicate the multifaceted three-dimensional niche in its *in vivo* presence, but researchers have undertaken steps towards recreating the niche *in vitro* and investigating the influence of different ECM components on the proliferation, migration and differentiation of satellite cells.[86,87,88]

In addition to potential *in vitro* meat production, the satellite cell niche is also of great interest because of it's the regenerative capabilities that it provides to the muscle tissue. SCs provide muscle tissue with the capacity to recover from a variety of injuries (i.e., myotoxin injection, mechanical crush, prolonged freeze injury and *ex vivo* mincing and replacement).[89] Providing SCs with an environment that more closely resembles their *in vivo* niche could prove to be of significance in their *in vitro* cultivation.[90] SCs reside within the basal lamina adjacent to the plasma membrane of the muscle tissue as it is depicted in Figure 2.6.

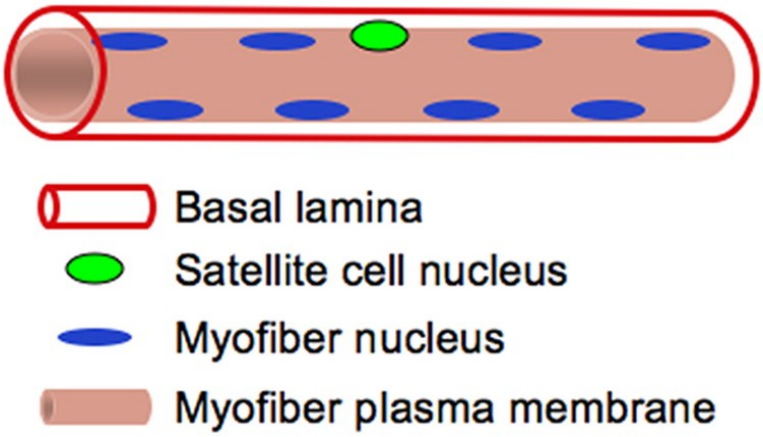

Figure 2.6. A schematic representation of the satellite cell's location, figure is credited to Yablonka-Reuveni Z. et al. and edited.[91]

The SCs are surrounded by collagens, laminins, fibronectins and glycosaminoglycans that form proteoglycans together with proteins, they all comprise the SCs ECM *in vivo*. Yet not all off the components are part of the basal lamina, some are in the reticular lamina.[92] As seen from Figure 2.7., the biggest constituents of the basal lamina where SCs reside are collagen type 1 and laminin, which both assemble two cross-linked networks, connected by the glycoprotein nidogen.[92]

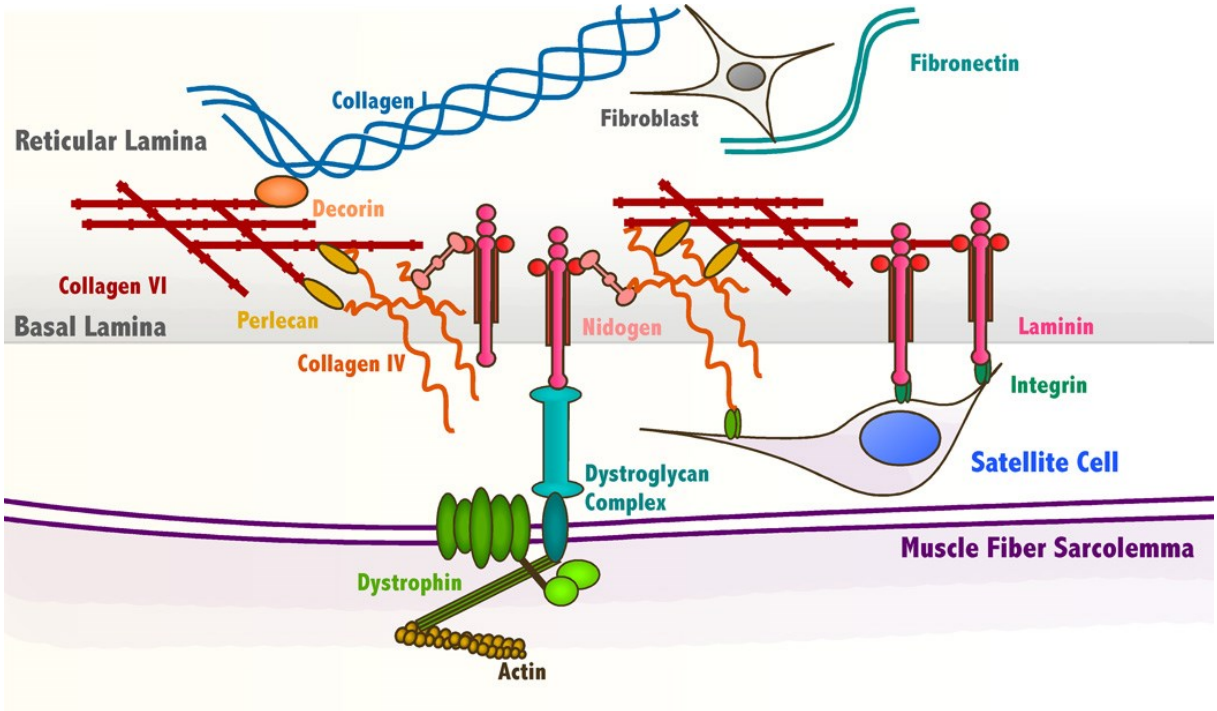

Figure 2.7 A detailed schematic diagram of the SC niche and its components, based on and edited (https://doi.org/10.3109%2F03008207.2014.947369).[89]

In our research on the coating and scaffolding effects of certain ECM components, we investigated three common ECM components used in stem cell research; collagen type 1, Laminin and Gelatine. There is research that suggest that replicating the satellite cells *in vivo* surroundings could prove vital for maintaining their stem cell capacity.[93]

Collagen type 1 is the most common component of the basal lamina that form network-like structures in the ECM and as such provides a scaffold for the SCs. It is highly conserved in vertebrates and regulates cell adhesion and migration. It has six α chains and can be found in at least three hetero-trimeric triple helical form which can later generate dimers, tetramers, and lateral connections in a supramolecular assembly fashion. Its structural representation is how in Figure 2.8.[94,95]

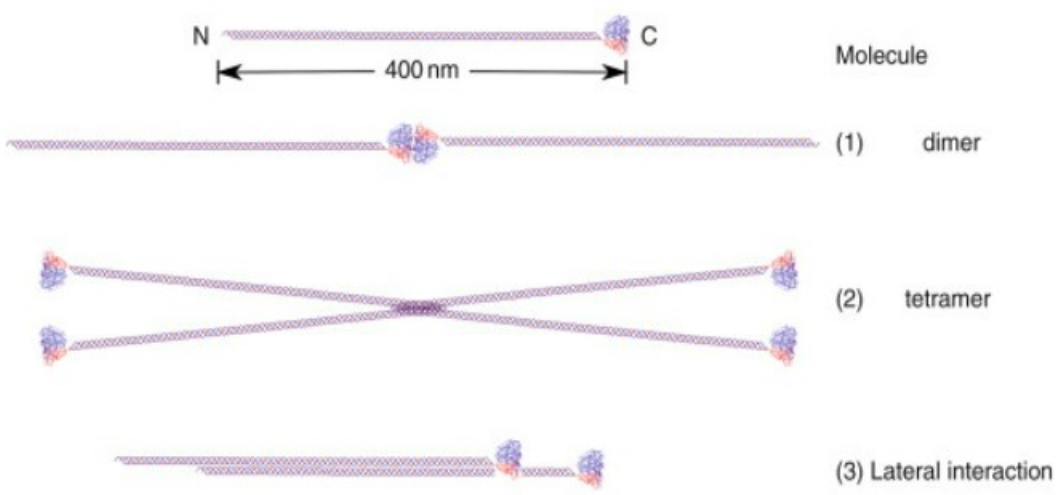

Figure 2.8. Graphical depiction of various collagen type 1 supramolecular assembly's, edited image.[95]

Laminins are the major non-collagenous components of the basal lamina separating the epithelia from the connective tissue. There are essential parts of the basal membrane as most of their null mutations prove to lethal.[96] They are heterotrimeric glycoproteins comprised of three separate chains α, β, and γ which look almost as a "cross", with one longer and three shorter arms.[97] Laminins have the ability to bind with other laminins, proteins such as collagen type 1, and to cells via the integrin receptors expressed on their surface.[98] The main structure of laminin is depicted in Figure 2.9.

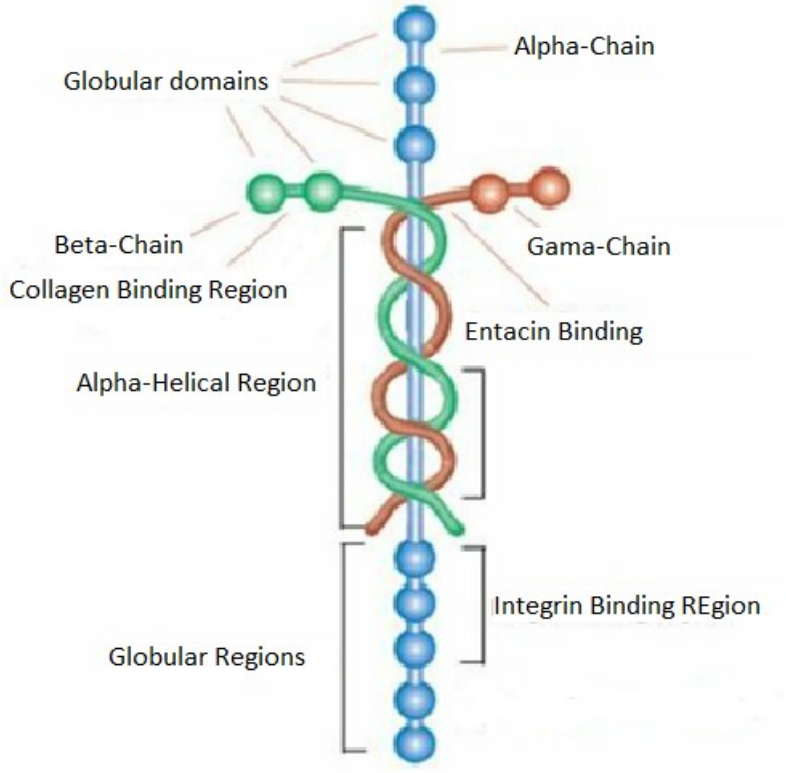

Figure 2.9. Schematic depiction a single laminin heterotrimer with its most notable binding sites, edited image.[99]

Collagen type 1 and laminin are fairly expensive to be used in mature cultivation process but are viable options for when it comes to proof-of-concept research. As a more economically friendly variation for culture dish coating, we have also investigated gelatine. Gelatine is an industrial biopolymer produced from animal skin and bones using diluted acids, which thermally denaturalise the collagen within them. It is used in various industries, the food industry as a solvent (i.e., candy, marshmallow) or for preservation (i.e., frozen dairy foods), pharmaceutical industry as a drug carrying agent or matrix for implants. One of gelatine's most useful properties is its gel formation at lower temperatures due to a collagen fold conformation which enables it to form hydrogen bonds.[100,101]

2.2.6. Satellite cell identification using FACS

Fluorescent activated cell sorting (FACS) with the right parameters has proven itself as a reliable method for the purification of heterogenous cell populations. The biggest advantage of FACS is that even live cells can be stained and sorted with only minor damage. It uses lasers

and conjugated antibodies for single cell detection. Each cell is analysed for visible light scatter, forward and side scatter, and fluorescent parameters. The forward scatter analysis produces information about cell size and the side scatter analysis provides information about the inner complexity of the cell, while the fluorescently conjugated antibody analysis provides detailed information on the protein expression in/on certain cells or just identification of a stained cell.[102] A simplified depiction of the general workings of FACS is shown in Figure 2.10.

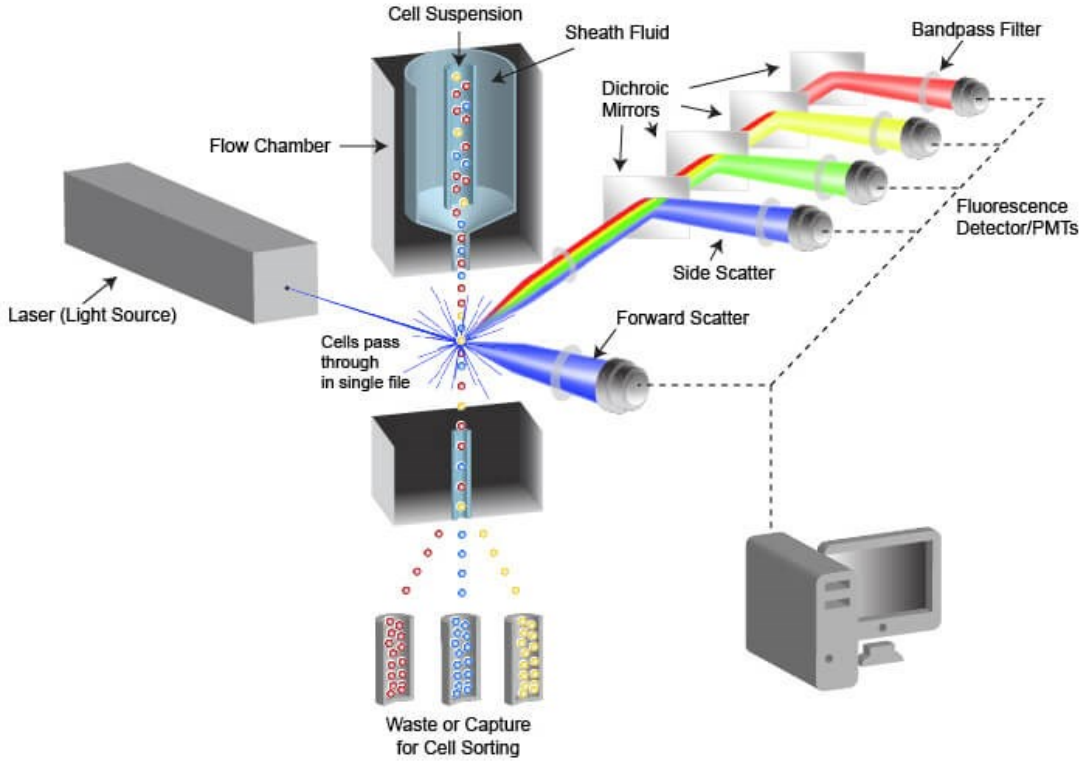

Figure 2.10. Schematic depiction of a FACS system and its crucial components.[103]

The cell suspension is transferred to the cell sorter in a specialised "FACS tube", it is put in the sorter which creates a uniform single cell stream by sucking the cells through a nozzle and mixing them with sheath fluid inside the flow chamber. After exiting the nozzle, the single cells are illuminated by a light source and both the scattered light and fluorescent emission are detected by multiple detectors (if a multicolour panel is being used). FACS detectors use light filters rather than CCDs (charge coupled device). When a signal from a cell is in predetermined amplitude limits for the detector, a charging pulse is generated and transferred to the electrically conducting fluid, at the lower end of the nozzle, which in combination with the desired cell creates a charged droplet. The timing of this charge transfer is precise because of the known

length of path and the speed with which the cells pass through the nozzle. Depending on the number of parameters that are being analysed, cells can be charged with multiple positive or negative charges that later enable their separation. After the cells have been charged, they pass through an electrostatic field where charged droplets deflect in their separate containers and non-charged droplets pass through in theirs.[104]

For our research, we used direct conjugated fluorescent antibody labelling in contrast to secondary antibody labelling, as we wanted to further expand our sorted cell culture. We targeted our satellite cell population with four different labels, two positive and two negative. CD29 and CD56 were positive labels that and CD31 and CD45 our negative labels. We chose these four based on previous experiences drawn from S. Ding et al.[33] They've shown that CD56 is a suitable marker for SC identification, as well as others have previously.[105,106,107]

CD29 is a dual marker that is specific for SCs and fibroblasts and thus we'll use their dual presence an affirmation of SCs.[107] CD31 is a marker of endothelial and CD45 is a marker of hematopoietic cells, they'll serve for the negative selection of cells that are present alongside SCs in our isolated primary cell culture. [108,109]

§ 3. MATERIALS AND METHODS

3.1. Bovine muscle progenitor stem cell culture

All activities surrounding cell culture techniques that are mentioned in this section were performed under a laminar flow hood. That and *good laboratory practice* provide a minimal hazard environment regarding cell culture contaminations. In addition, before passaging the cells, it is necessary to warm up/incubate at 37°C all solutions that are going to be used (i.e., trypsin, growth medium, PBS). The solution temperature can be crucial for the activity of certain components within it (i.e., trypsin's cleavage activity which is used for separating adhering cells from cell culture surfaces). Table 3.1. also provides useful information on the volumes of solutions used during cell culture for varying culture dish sizes.

Table 3.1. Volumes of often used solutions for varying culture dish formats in ml.

Flask size	PBS±	Trypsin	Growth medium	Different coating solutions
T12,5	2.5	1.5	2.5	1.5
T25	5	2	5	2
T75	10	4	10	4
T175	20	8	20	8
6-well	2.5	1.5	2.5	1.5

Table 3.2. provides a useful guide on all the mediums used in this research.

Table 3.2. Names and compositions of the most frequently used media in this thesis

	Ingredients and composition
Isolation medium 1	DMEM HG*, 20% FBS**, $2 \cdot 10^{-4}$ mol/dm^3 L-Gln***, 10 mg/dm^3 Primocin****
Isolation medium 2	DMEM HG, 20% FBS, $2 \cdot 10^{-4}$ mol/dm^3 L-Gln, 10 µg cm^{-3} SB203580, 10 mg/dm^3 Primocin
Growth Medium 1	DMEM HG, 20% FBS, $2 \cdot 10^{-4}$ mol/dm^3 L-Gln
Growth Medium 2	DMEM HG, 20% FBS, $2 \cdot 10^{-4}$ mol/dm^3 L-Gln, 10 µg cm^{-3} SB203580
Growth Mouse	RPMI *****, 10 % FBS
Differentiation medium	DMEM HG, 2% Horse Serum, $2 \cdot 10^{-4}$ mol/dm^3 L-Gln (for bMuCS)

* DMEM HG (BioWest, Nuaillé, France, #MS01BO)

** Primocin (InvivoGen, San Diego, CA, USA, ant-pm-2)

*** FBS (PAN Biotech, Aidenbach, Germany, #200802)

**** L-Gln (PAN Biotech, Aidenbach, Germany, #5290321)

***** RPMI 1640 (BioWest, Nuaillé, France, L0498-500)

****** Horse Serum (ThermoFisherScientific, Kandel, Germany, 302200BNZ)

3.1.1. Cell isolation

The SC isolation protocol that was regularly used was established "*in house" by* ms. sc. Jannis Wollschlaeger and performed under sterile conditions using a laminar flow hood. The bovine satellite cells were always isolated out of a fresh (within 30 minutes of the isolation procedure) *post-mortem* biopsy obtained from a local "bio-butcher" (Organic butcher Griesshaber), in addition all samples were the same age, 2 years. This isolation protocol used the enzymatic digestion approach for tissue dissociation and cell harvesting. The first step in the isolation protocol was to prepare the protease solution for the enzymatic digestion. The protease powder from *Streptomyces griseus* (Sigma Aldrich, Darmstadt, Germany, #SLCB5965) was weighed for a final mass concentration of 3 mg ml^{-1}, diluted in DMEM HG and Primocin was added for a final concentration of 10 mg dm^{-3} Primocin, then the prepared solution is sterile filtered through a 0.2 µm mesh and incubated at 37°C. It is necessary to filter every solution, that was made *in house*, through a 0.2 µm mesh to minimize the contamination risk.

The muscle tissue is then placed on a sterile glass plate and mechanically cut and separated from the dermis, epidermis, connecting and fat tissue. The size should be 2-5 mm, so that enough muscle tissue surface area is available for the protease treatment. The final amount should be between 10-20 g of muscle tissue and placed in a 50 ml tube. Then it is necessary to add the previously prepared 3 mg ml^{-1} protease solution in a 1:1 volume to weight ratio to the minced muscle tissue. The 50 ml tubes should then be placed horizontally on a shaker (150 rpm) and incubated at 37°C for three hours.

After the three-hour digestion, the isolation protocol proceeds with further downsizing of the sample by pushing the homogenic mixture through a 500 µm cell strainer into a urine cup and then transferring it in the same 50 cm^3 tubes. The mixture is then centrifuged at 1000 xg for 10 minutes. In the next step, the supernatant is used by decanting it into a fresh 50 cm^3 tube and mixed with 10 cm^3 of erythrocyte lysis buffer (155 mmol dm^{-3} NH_4Cl, 10 mmol dm^{-3}

$KHCO_3$, 0,1 mmol dm^{-3} EDTA, pH 7.3, in distilled H_2O) for 5 minutes; after which 10 cm^3 of basal medium DMEM HG is added. The mixture is then again centrifuged at 1000 xg for 10 min. This time, the cell containing pellet is used and the supernatant is discarted. The pellet is resuspended in 20 ml of basal medium and pushed through a 40 μm cell strainer into a fresh 50 ml tube and centrifuged at 1000 xg for 10 min. The supernatant is again discarted and the cell pellet is resuspended in isolation medium 1 or 2 and transferred in an uncoated culture flask for three hours for separating the fast-attaching fibroblasts.

After three hours of preplating, the bovine satellite cell solution is transferred into a collagen type 1 coated culture flask and put into an incubator at 37°C and 5% CO_2 for further growth. The coating of cell culture dishes is explained in section *3.1.7.*

* For certain experiments, the growth medium was altered to either include the p38 MAPK inhibitor, SB203580 (Biozol, Eichen, Germany, SEL-S1076). It is vital to note that the cells are cultivated with Primocin until they reach confluency for the first time and need to be passaged. During the first passaging procedure, the cells are deprived of the Primocin and continue to be cultivated without the use of any antibiotics.

3.1.2. Medium exchange

The first medium exchange is due two days after the isolation. The old growth medium is aspirated, (this is necessary to get rid of unwanted cell types or worse (other organisms), which might be floating in the isolation medium). 5 ml of PBS+ (containing Mg^{2+} and Ca^{2+} [Corning, Corning, NY, USA, #0001023471]) is added to the primary cell culture, the flask is gently rinsed and the PBS+ is aspirated, in the end 2.5 ml of fresh growth medium with Primocin is added, and the cells are put back into the incubator at 37°C and 5% CO_2. This protocol can also be used for media exchange of growth medium if the cells need more time to reach confluency. But this protocol should never be used for cross medium exchanges (i.e., exchange of growth medium 1 for isolation medium 1).

3.1.3. (First) Passaging of cells

The old growth medium is aspirated from the culture dish, add the proper amount of PBS+ and gently shake it so that the whole culture dish is washed with PBS+.The PBS+ is then aspirated,

the Trypsin solution (0,05% Trypsin in EDTA) is added , and gently spread over the whole surface of the culture dish and put in the incubator (5% CO2 and 37°C) for 4 minutes. The trypsin is used for dissolving the extracellular proteins that attach to the culture dish surface, enabling the separation of attached cells from the culture dish. After 4 minutes, the cells are taken out of the incubator, examined under a brightfield microscope to confirm that they have detached from the surface, and put under the laminar flow hood. Growth medium is added to the Trypsin solution in a 1:1 volume ratio, the cells are gently resuspended with the medium, this is necessary for a maximum cell content extraction from the old culture flask. The suspension culture is transferred into a 50 cm^3 tube and the cells are centrifuged for 2 min at 500 xg.

3.1.3.1 Cell counting

After the centrifugation, the excess growth medium/trypsin suspension, which doesn't contain cells, and floats above the cell containing pellet is aspirated. Caution is advised, as the cell pellet should be left unscathed from this. The "dry" cell pellet is then resuspended with 2ml of fresh growth medium. An aliquot of 10 µl is taken and transferred to a 96-well plate for cell counting. 10 µl of trypan blue is added to the aliquot and resuspended with a pipette. A 10 µl aliquot is taken from the 96-well plate and placed in the "Neubauer counting chamber". The total number of cells in the tube is the calculated via the given formula:

$$\frac{n(numberof\ cells\ in\ all\ quadrants)}{4} \times 10^4 \times 2 \times 1$$

Where *n* represents the cell count in each quadrant, 10^4 is a multiplication factor for converting the cell concentration in the "Neubaeur counting chamber" from 0,1 mm^{-3} to cm^{-3}, 2 is the dilution factor, and 1 represents the initial volume in cm^3. After counting the cells, the necessary cell number that is going to be seeded for the next passage is calculated (i.e., for a T75 0.4*10^6 cells are enough for the bMuCS to grow to confluency in 3 days), also it is needed to seed at least 3-5 wells of a 48-well plate that are going to be fixated after 24h afterwards for further analysis and cell identification. Furthermore, the proper amount of growth medium (DMEM HG, 20%FBS, $2 \cdot 10^{-4}$ mol/dm^3 L-glutamine) or (DMEM HG, 20% FBS, $2 \cdot 10^{-4}$ mol/dm^3 L-

glutamine, 10 μg cm^{-3} p38 inhibitor), is added to the 50 ml tube, the cells are resuspended and transferred into a newly prepared culture dish. *

*If it is the first passaging of a primary culture after isolation, this is the point where the usage of Primocin is stopped, and the culture is freed from antibiotics.

3.1.4. Freezing of cells

The procedure for freezing the cells follows the same procedure as in *3.1.3.* and *3.1.3.1*, but after the cell counting, the cells are again centrifuged for 2 mins at 500 xg. The biggest difference regarding the passaging protocol is the use of and preparation of the freezing medium instead of the growth medium, because it is necessary to freeze the cells with DMSO, for it prevents the formation of intra- and extracellular crystals from forming during the freezing process. Otherwise, the forming crystals could damage or even kill the cells during their freezing process and even further downgrade their viability potential after thawing.
As forementioned, the same procedure protocol is followed as in *3.1.3.* and *3.1.3.1*. The freezing medium is prepared with respects to the cells being frozen. Usually, it's similar to the growth medium (i.e., DMEM HG, 20% FBS, 10% DMSO, $2 \cdot 10^{-4}$ mol/dm^3 L-Gln). For freezing the cells properly, it is necessary to freeze $0.5 \cdot 10^6 – 1.5 \cdot 10^6$ in 1ml of freezing medium. After counting, the rest of the growth medium is aspirated from the 50 ml tube, the cells are resuspended with the freezing medium and distributed in aliquots of 1ml per each cryotube. Before the cells are stored at -196°C in the liquid nitrogen tank it is necessary to pre-freeze them at -80°C first for 24h in a special cryo box that gradually decreases the temperature by 1°C per minute.

3.1.5. Thawing of cells

A 50 ml tube with 9 ml of warm growth medium is prepared. The cells are slowly thawed in the water bath (37°C) until there is only a small ice chunk left in the cryotube. The cells are transferred into the prepared warm culture medium in the 50 ml tube and the cell solution is centrifuged for 5 minutes at 200 xg. Afterwards, the excess medium is aspirated, the cell pellet

is resuspended in fresh growth medium (i.e., DMEM HG, 20%FBS, $2 \cdot 10^{-4}$ mol/dm^3 L-glutamine) and transferred to the newly prepared and culture dish.

3.1.6. Fixation of cells

Cells that are being fixated have usually been seeded beforehand into a 48-well plate.
Aspirate medium. Wash cells with 500 µl PBS+ three times. Aspirate PBS+, add 200 µl of Histofix (Carl Roth, Karlsruhe, Germany, Roticlear A538.1) and let it sink in for 15 minutes at room temperature with the lid closed. Wash cells twice with 500 µl of PBS+, after the second wash don't aspirate the PBS+ from the wells so that the fixated cells don't dry up before immunocytochemistry staining. Seal the well-plate with parafilm and store the cells in the fridge at 4°C.*

*don't store the cells for more than 2 weeks after fixating them.
**it is not necessary to fixate the cells under a laminar flow hood.

3.1.7. Differentiation of cells (serum starvation)

For bMuCS cell differentiation the cells must reach a confluency of at least 90%. Differentiation is performed in 48-well plates, approximately $4 \cdot 10^4$ cells per well are needed for staining of Pax7, MyoHc, control(s), MyoD and MyoG. The cells are passaged and seeded following the "passaging of cells" protocol from section *3.1.3.,* with the difference being that after cell counting, the cells are seeded in a 48-well plate with addition of 500 µL of growth medium. The cells go through a medium exchange after they have firmly adhered to the culture dish surface and reached the desired confluency (90 %). During the medium exchange, the old growth medium is exchanged for a differentiation medium, for reference see Table 3.2. Therefore, the reduction in serum availability leads to cell stress and the cells begin to differentiate. The experiment is designed so that the cells were fixated at certain time points, usually day 0 (when the medium exchange to differentiation medium occurs), day 2 and day 7, when the cells are expected to fully differentiate and form adult muscle fibres.

3.1.8. Culture dish coatings

To examine the influence of coating solutions on the characteristics of the isolated bMuCS, the cell culture dishes had to be coated first. Table 3.2. contains the used coating solutions and their concentration ranges. For the comparison of coating concentrations, each standard concentration was made from the stock solution, or weighed, to minimize dilution errors later. The standard gelatine solutions were prepared by weighing precalculated amounts of gelatine powder under a laminar flow hood, while sterile water (Mili Q) was incubated to 60°C in parallel. The warmed-up water was used to dissolve the gelatine powder, and to decrease contamination risks, the solutions were filtered through a 0.2 µm sterile filter mesh. The standard collagen type 1 solutions from Table 3.3. were prepared by dissolving a calculated and aliquoted amount of the stock solution in 0.1 % acetic acid (CH3COOH), so that the two amount to 50 cm^3. The standard laminin solutions were prepared by dissolving a calculated amount of the stock solution in sterile basal medium (i.e., DMEM HG).

Table 3.3. Used coating solutions and their concentration ranges used in this thesis.

Coating	Manufacturer	γ / mg cm^{-3}	γ / mg cm^{-3}	γ / mg cm^{-3}	γ / mg cm^{-3}	γ / mg cm^{-3}
Gelatine	GELITA® Limed Bovine Bone Gelatine; 641919; GELITA Deutschland GmbH, Eberbach, Germany	0.1	0.5	1.0	2.0	5.0
Collagen type 1, rat tail	Merck, Darmstadt, Germany, #3785919	0.1	0.25	0.5	1.0	2.0
Laminin, Engelbreth-Holm-Swarm mouse	Corning, Corning, NY, USA, #3290891	0.001	0.005	0.01	0.03	0.05
Laminin pentapeptide (929-933)	ThermoFisherScientific, Kandel, Germany, #N13F023	0.01				

All coatings were performed in a similar manner. Volumes were added depending on the culture dish format as per Table 3.1. and the concentrations from Table 3.2., the solution should be spread evenly with gentle shakes if necessary. The dish is then closed off and put in an incubator at 37°C for one hour, so that the coating solution can adhere to the culture dishes. After adhesion during the incubation period, the excess solution is aspirated, and the dishes are sealed off with

parafilm. The collagen type 1 and gelatine coated dishes are ready for use as such or can be stored (at 4°C), whereas laminin coated dishes need an additional layer (enough to cover the whole surface area of the dish) of basal medium if they are going to be stored (at 4°C). Additionally, the laminin coated dishes need to be washed with sterile PBS+ as per Table 3.1. before seeding cells on to them.

The excess collagen solution can also be saved for further coatings, but the same solution shouldn't be used for more than three separate coating treatments. It is not recommended to reuse the diluted laminin coating solution more than once because of large concentration variations following reuse. The collagen type 1 and gelatine coated culture dishes can be stored at 4°C for up to two weeks, whereas it is not recommended to store the laminin coated culture dishes for periods longer than two weeks.

3.1.9. bMuCS doubling rate

The doubling rate was calculated using the following formulas:

$$DR = \frac{\log(\frac{N(t)}{N(0)})}{\mathrm{t} * \log 2}$$

Where *DR* is the doubling rate expressed in d^{-1} (or how much the population will grow in 1 day), *N(t)* is the cell count at a given time point, whereas *N (0)* is the cell count at the starting point and *t* is the time measured in days. The other formula is used to calculate the time necessary for the doubling of a cell population expressed in hours:

$$DT = 24/DR$$

Where *DT* is the time necessary for an observed cell population to double, expressed in hours, *24* is a numerical value that has h d^{-1} as a unit of measurement attached to it, and *DR* is the forementioned doubling rate expressed in d^{-1}.

3.2. Cell identification

3.2.1. Immunocytochemical biomarker staining

For us to be able to visualise our biomarkers of interest, immunocytochemical staining with antibodies was performed to visualise the endogenous markers within the bMuCS. The previously fixated cells were stained within 7 days from their fixation. It isn't necessary to perform this procedure under the laminar flow hood as the cells are already dead. It is still advised to perform it with caution as careless action can result in the dislodging of the cells from the well surface. The procedure described here is intended for a 48-well plate and the volumes can be adjusted for other well sizes.

The cells are firstly permeabilized with 200 µl of 0.1% of Triton-X dissolved in PBS+ for 5 minutes. The cells are once washed with PBS+. 200 µl of blocking solution is added (1% BSA in PBS+) for 30 minutes at room temperature. Primary antibody solutions diluted in blocking solution are added (150 µl per well) as per Table 3.4. are added to the cells and incubated over night at 4°C. After incubation, cells should be washed three times with washing solution (0.1% Tween (VWR International GmbH, Darmstadt, Germany, SAFSP9416) diluted in PBS+) and put on a shaker for 5 minutes at 60 rpm. The next step is to incubate the cells with the secondary antibody (150 µl), as described in the Table 1, for 45 minutes. Again, wash cells with washing solution three times and put them for 10 minutes at 60 rpm on a shaker after each washing cycle. Incubate the cells for 10 minutes with DAPI (1000x diluted stock solution) for nuclear staining. Wash cells once with 500 µl of PBS+ and store cells in 500 µl of PBS+ at 4°C in the dark . The cells were later imaged using a Axio Observer microscope, (Carl Zeiss AG, Jena, Germany) and the images were analysed as described in the section *3.2.2.*

Table 3.4. Antibodies used in this thesis for immunocytochemical analysis.

Protein of interest	**Primary antibody**	**1° Antibody Manufacturer**	**Secondary antibody**	**Dilution in blocking buffer**
Pax7	Monoclonal mouse Pax7 (γ = 34 µg/ml)	University of Iowa, Iowa City, IA, USA	Goat IgG anti-mouse IgG (H+L)-Cy3 (Dianova, Germany) 1:200	1:4

MyoD	MyoD Mouse sc-377186 (γ = 200 µg/ml)	Santa Cruz Biotechnology, Dallas, TX, USA	Goat IgG anti-mouse IgG (H+L)-Cy3 (Dianova, Germany) 1:200	1:100
MyoG	Monoclonal mouse Myogenin McAb (γ = 150 µg/ml)	Proteintech, Rosemont, IL, USA	Goat IgG anti-mouse IgG (H+L)-Cy3 (Dianova, Germany) 1:200	1:400
MyoHC	MyoH ½ mouse sc-53088 (γ = 200 µg/ml)	Santa Cruz Biotechnology, Dallas, TX, USA	Goat IgG anti-mouse IgG (H+L)-Cy3 (Dianova, Germany) 1:200	1:40
IgG1	Normal mouse IgG1 sc-3877 non-conjugated (γ = 200 µg/ml)	Santa Cruz Biotechnology, Dallas, TX, USA	Goat IgG anti-mouse IgG (H+L)-Cy3 (Dianova, Germany) 1:200	1:40
IgG2a	Normal mouse IgG2a sc-3878 non-conjugated (γ = 200 µg/ml)	Santa Cruz Biotechnology, Dallas, TX, USA	Goat IgG anti-mouse IgG (H+L)-Cy3 (Dianova, Germany) 1:200	1:20

*Plates shouldn't be stored for more than two weeks before imaging.

3.2.2. Imaging by using fluorescence microscopy

The cell images of the fixated cells were captured on a Axio Observer microscope (Carl Zeiss AG, Jena, Germany), and analysed via ImageJ (National Institute of Health, Bethesda, MD, USA), a freely available program for processing of images. The cell images were taken under the conditions described in Table 3.5. and captured in at least two channels, blue for DAPI stained nuclei and the red channel for our protein of interest. All images that were used for the data in this thesis are representative images, meaning there were at least three images taken from each well that were used for establishing an average value for the well. Additionally, for

Pax7 and other target staining's (i.e., MyoHC, MyoG...), the average percentage of three separate wells was taken for the final value. For counting our cells, split channel images are imported first into ImageJ to get a simple RGB representation as in Figure 3.1. a). The image is then transformed into an 8-bit image for further processing using; **Image → *Type* → *8-Bit oder 16-Bit*** and get an image like Figure 3.1.b)

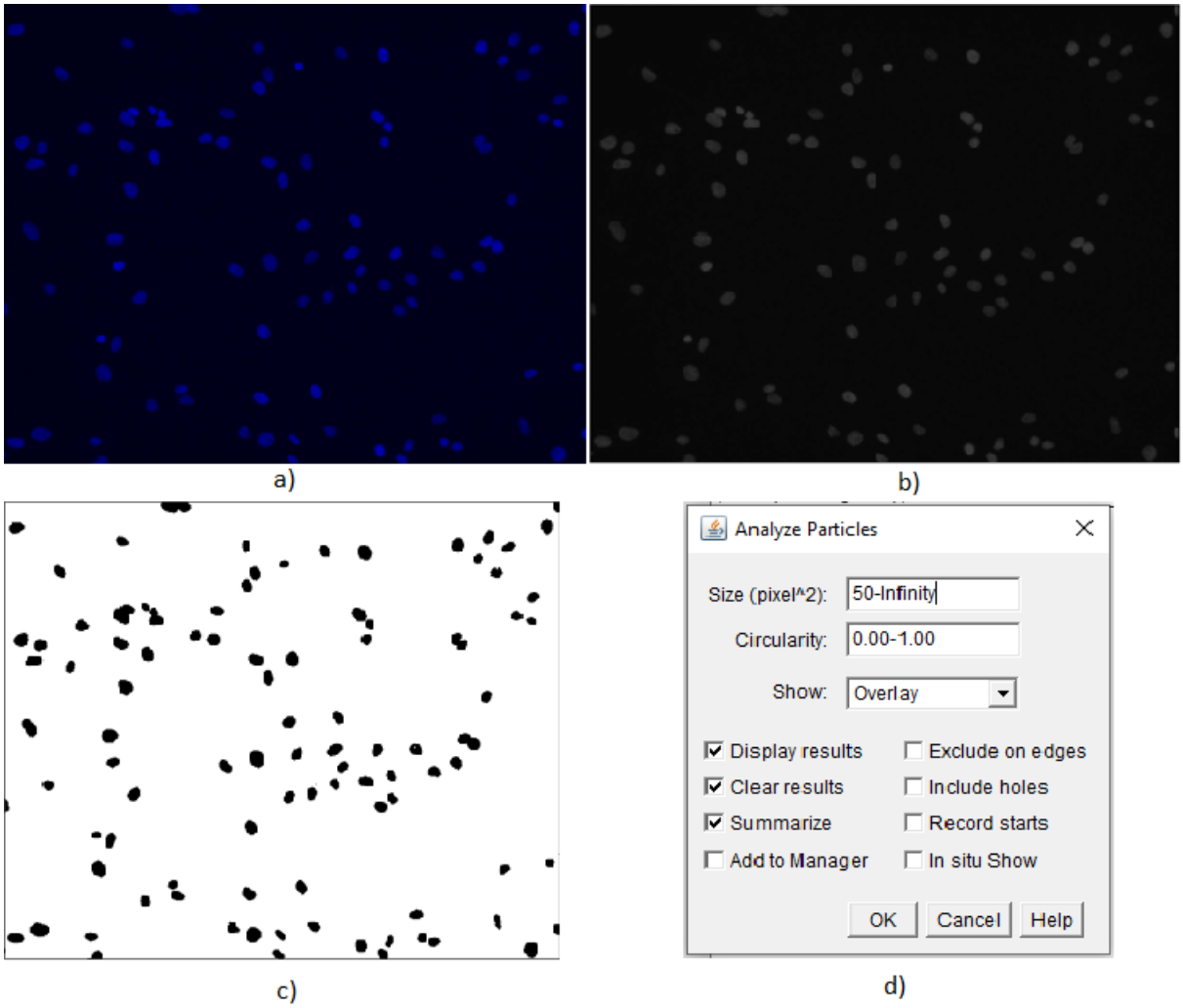

Figure 3.1. Brief depiction of the *image analysis* process. a) representative image of a DAPI stained nuclei shown via the blue channel, captured on a fluorescent microscope. b) an 8-bit version of image in a). c) processed image b) with only nuclei, threshold set to exclude background noise. d) a screenshot of the "analyse particles" window in *ImageJ* with the necessary parameters for automatic cell counting.

Further on, the threshold is adjusted to get a realistic representation of the stained cells by using ***Image → Adjust → Threshold → Apply*** to get something as depicted on Figure 3.1.c). This step is more important with the cells that are expressing the proteins of interest rather than the DAPI labelled cells because not all cells express the protein with the same intensity, depending on their origin or their maturity stage. The next step is to separate cells that seem to form one surface using ***Process→ Binary→ Watershed.*** The final step is to initiate the counting of the cells themselves by using ***Analyse → Analyse Particles*** and parameters as depicted on Figure 3.1.d). After the total number of cells per image is counted via the blue channel, the counting proceeds with counting the cell of interest, in this case the Pax7 positive cells (red channel). The result is the fraction of PAX7 positive cells in the primary cell culture. In more mature experiments, the differentiation potential of the bMuCS (muscle satellite cells) is observed via MyoHC staining. The procedure is the same regarding the total cell count, but it differs in the way the differentiated cells are confirmed. The major difference with MyoHC staining being only the cells covered by the MyoHC expressing area are counted as positives.

Table 3.5. Biomarkers and their fluorescence microscopy parameters.

Marker	DAPI	IgG1	IgG2a	Pax7	MyoD	MyoG	MyoHC	2° Ab ctrl
Channel	Blue	Red	Red	Red	Red	Red	Red	Red
Exposure time (ms)	100	400	400	1500	1400	250	1100	400

3.3. FACS antibody staining

Our cells were alive while being stained and sorted, thus extraordinary caution was applied regarding the speed and quality of the procedures. The uttermost importance was given to preserving the viability of the cells and their wellbeing.

Before the staining procedure itself, 40 ml of a "FACS-buffer" solution was prepared by mixing FBS with PBS- for a FBS end concentration of 20% (8ml FBS in 32ml PBS-). After preparing the "FACS-buffer" solution, it is necessary to sterilely filter it through a 0.2 μm mesh, for contamination risk reduction, and keep it at 4°C until further use.

This protocol follows the passaging cells protocol until afterwards the cells counting, with the distinction that the cells are washed with PBS- and that the cells are diluted through a 40 μm cell strainer, as well as the culture flask being additionally washed with growth medium to get as much of the cells out. It is important to mention that from this point onward, the cells should be kept at 4°C at every step where it is possible to keep the temperature constant. Table 3.6 contains all Fluorescently conjugated antibodies that are used for this thesis.

Table 3.6. FACS conjugated antibodies used in this thesis

Fluorescent labelled antibody	Dilution	Manufacturer
Mouse anti-sheep CD45: RPE (γ=200 μg/ml)	1:50	Bio-rad, CA, USA
Mouse IgG negative ctrl: RPE (γ=200 μg/ml)	1:50	Bio-rad, CA, USA
CD56 anti-human, FITC (γ=200 μg/ml)	1:50	Miltenyi Biotec, NRW, Germany
REA ctrl human IgG1: FITC (γ=200 μg/ml)	1:50	Miltenyi Biotec, NRW, Germany
CD29 anti-human: Vio Bright V423 (γ=200 μg/ml)	1:50	Miltenyi Biotec, NRW, Germany
REA ctrl human IgG1: Vio Bright V423 (γ=200 μg/ml)	1:50	Miltenyi Biotec, NRW, Germany
Rabbit anti-human CD31: Alexa Fluor 750 (γ=200 μg/ml)	1:10	Bioss Antibodies, MA, USA
Human IgG ctrl: Alexa Fluor 750 (γ=200 μg/ml)	1:10	Bioss Antibodies, MA, USA

After the counting, it is necessary to calculate the correct proportions of fluorescently labelled IgG antibody controls, fluorescently labelled CD antibodies for FMO (fluorescent minus one) controls and for the bulk of the sample that is going to be sorted according to Table 3.7.

Additionally, it is necessary to split up the cell in accordance with Table 3.7., each control in its own 15 ml tube sample and the sorting sample into a 50 ml tube if the volume is too much.

Table 3.7. Composition and division of FACS tubes for multicolour analysis.

Antibodies	Unstained (negative control)	IgG controls
Composition	100 µL "FACS-buffer"	84 µL "FACS-buffer" 2 µLCD29 ctrl 2 µLCD45 ctrl 2 µL CD56 ctrl 10 µL CD31 ctrl

	Gating controls				
Antibodies	CD29 CD31 CD45	CD29 CD31 CD56	CD31 CD45 CD56	CD29 CD45 CD56	Sample cells
Composition	86 µL"FACS-buffer" 2µL CD29 2µL CD45 10µLCD31	86 µL"FACS-buffer" 2µL CD29 2µL CD56 10µLCD31	86 µL"FACS-buffer" 2µL CD45 2µL CD56 10µLCD31	94 µL"FACS-buffer" 2µLCD29 ctrl 2µLCD45 ctrl 2µL CD56 ctrl	84 µL"FACS-buffer" 2µLCD29 2µLCD45 2µL CD56 10µL CD31

Later, the cells are centrifuged for 10 minutes at 300 xg, the supernatant is carefully discarded using a pipette. Each cell pellet is re-suspended in the "FACS-buffer" and the proper number of antibodies is added as in the Table 3.7. The content should be properly mixed and incubated at 4°C for 10 minutes in the dark. Each designated tube is washed with an addition of 2 ml of "FACS-buffer", then centrifuged again for 10 minutes at 300 xg. The supernatant is discarded again carefully, and the cell pellets is resuspended with "FACS-buffer" ($1.5 \cdot 10^6$ cell per 150 µL and 150 µL for less than that). The samples are then transferred into special FACS tubes and put in a box filled with ice, for slowing the cells metabolism down and mimimizing stressful conditions, in which they will be transported to the FACS facility. Cells were sorted at The

University of Tubingen using a Multiapplication cell-sorter MA900 (Sony, San Jose, CA, USA).

*Before the sorting itself, it is necessary to add 100x diluted 7-AAD (BD Biosciences, NJ, USA) in the unstained control for live dead staining.

§ 4. RESULTS AND DISCUSSION

The aim of the research presented in this thesis is to determine if it is technically possible to establish a bovine muscle progenitor stem cell (bMuCS) cell culture that could be used as a cell source for *in vitro* meat cultivation. This chapter will summarize the performed experiments. The optimization of the cell isolation process and bovine muscle progenitor stem cells was performed in tandem. Supply chain issues during *the 2020.-2022. COVID-19 Pandemic* hindered the possibility of a logical chronological progression in the research where we would first optimize the isolation process, and then go further into the cell culture characterization and purification process.

4.1. bMuCS cell culture isolation

The isolation process was performed as previously described in the section 3.1.1. Cell isolation. Although the cell isolations were performed on a weekly basis, not all cultures were later used, as contaminations occurred since there were isolations with insufficient Pax7 positive cells, and as some isolations had a suboptimal number of total cells for further characterization assays. The average fraction of satellite cells (Pax7-positive cells) in a bMuCS culture after the first passage out of all cell isolations is *28.51±14.25 %*, from which only isolations with a fraction higher than 25% were considered for characterization assays. This value is dissatisfying when compared to other contemporary published cultured meat research, where groups are claiming over 90% efficiency when sorting cells via FACS is included.[33,110] It is somewhat consistent with older publications[111] and when comparing research that only focuses on enzymatic digestion and preplating as techniques used during isolation.[112,113] A representative bMuCS cell culture after isolation alongside a representative C2C12 cell culture is shown in Figures 4.1.

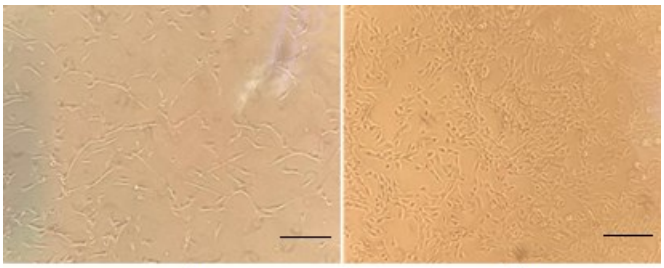

Figure 4.1. a) bMuCS cell culture (left) after the first passage and b) C2C12 mouse myoblast cell culture (right) captured via brightfield microscopy at 20x zoom with the scale bars set at 100 μm.

The C2C12 cell culture used in this thesis proliferated much faster in comparison to all the isolated bMuCS cultures. The C2C12 culture doubled every 17.4 h, from passage 33-36. This value is in conjunction with other data using the same cell line.[114,115] The isolated bovine satellite cells have an *in vitro* reported doubling time range of 30-70h,[110,116] while the bMuCS cultures isolated for this thesis had a doubling time range of 26- 40 hours. The doubling rate was calculated using the following formulas from *3.1.9.* The data collected in this thesis observes the doubling rate only from passage to passage without generating a growth curve. As mentioned in other publications, one of the reasons for such diverse doubling rates, from culture to culture, could be the heterogeneity of such isolated bovine cultures, as they are not pure satellite cell cultures.[117,118]

4.2. bMuCS identification

As forementioned in section 3.1.2., cells were identified using selected cell markers for certain development stages. Figure 4.2. shows the C2C12 cell stained for DAPI and for Pax7 co-stained with Cy3 as the secondary fluorescent antibody.

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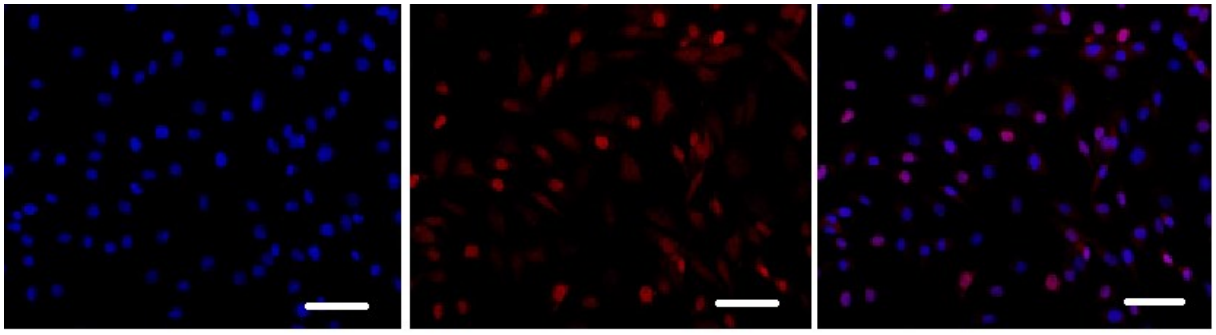

a) b) c)

Figure 4.2. Fluorescence microscopy images taken of bMuCs alphaG (passage1) with a) showing the blue channel (DAPI), which shows all nuclei present in the well, b) showing the red channel (Pax7-Cy3), which shows all the cells positive for the Pax7 protein and c) showing a merged channel image where all cells (blue) are overlayed with the Pax7 positive cells. White marker on each figure is 100 μm.

In the left picture, we can see all the cells in culture stained DAPI in blue. DAPI binds to AT rich regions in the DNA and enables us to count the overall number of cells in culture.[119] The middle picture presents cells that are stained with Pax7-Cy3, our cells of interest. Satellite cells are represented as purple dots and "only blue" dots are other cell types that don't express Pax7. Pax7 proved to be quite a troublesome marker for satellite cell identification as its concentration varied depending on the stage at which the satellite cells found themselves at the moment of fixation. Furthermore, it showed quite a weak signal and cells that were imaged for Pax7 were visualized by longer exposure, as well as the attached secondary antibody, to fluorescent emitting light.. All of this meant that cell counting was only possible manually.

MyoG and MyoHC staining on the other hand, proved to be quite simple. Additionally, MyoG staining wasn't used for quantitative analysis, but rather as a monitoring marker during differentiation.[63] Figure 4.3. shows the results of MyoG staining.

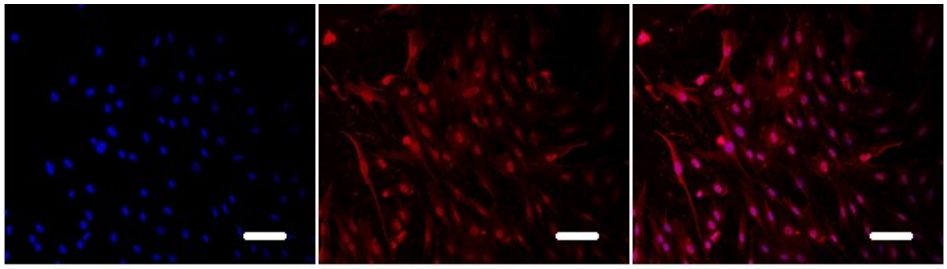

Figure 4.3. Fluorescence microscopy images taken of bMuCS alphaL (passage 3, day 2 of differentiation) with a) showing the blue channel (DAPI), which shows all nuclei present in the well, b) showing the red channel (MyoG-Cy3), which shows all the cells positive for the myogenin and c) showing a merged channel image where all cells (blue) are overlayed with the myogenin positive cells. White marker on each figure is 100 µm.

MyoHC was used for quantitative analysis during differentiation assay to measure the fusion index in our cell culture so that we can estimate how many cells in our culture can mature into muscle fibres.[66]

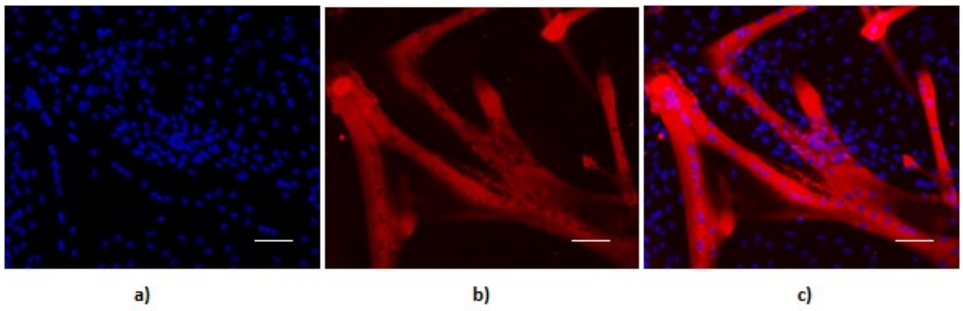

Figure 4.4. Fluorescence microscopy images taken of bMuCS alphaL (passage 3, day 7 of differentiation) with a) showing the blue channel (DAPI), which shows all nuclei present in the well, b) showing the red channel (MyoHC-Cy3), which shows all the cells positive for the MyoHC and c) showing a merged channel image where all cells (blue) are overlayed with the MyoHC positive cells. White marker on each figure is 100 µm.

MyoHC is shown in figure 4.4, as it is a motor protein expressed in mature muscle fibres that follows that fibres morphology. The forementioned fusion index is a metric that is calculated by counting all the nuclei within a fibre (here the blue dots within the red MyoHC) and dividing them by the total amount of cells in culture. To make a confident estimate, it was necessary to

take at least three representative images (regarding cell distribution and myofiber formation), or otherwise known as technical replicates.[67]

4.3. Cell culture dish coatings

4.3.1. Comparison of gelatine, laminin and collagen type 1 coating for bMuCS cultivation

In section 2.2.4., we discussed the use of three different coatings for our cell culture flasks so that we could compare them and their effect on our bMuCS culture. The extracellular matrix composition can have profound impact on the behaviour of satellite cells, be it by preserving their stemness, their proliferation rate or myogenesis initiation.[84] All coating experiments were performed with three technical replicates and two biological replicates (αG and Φ2). The cells were thawed, seeded onto a 6-well well plate and cultivated for three passages. Each coating was tested for a range of 6 concentrations that were found in the research literature. The first coating that was tested was gelatine, as it is one of the cheapest, most widely available and used coatings in mammalian cell culture.[120] Figure 4.9.

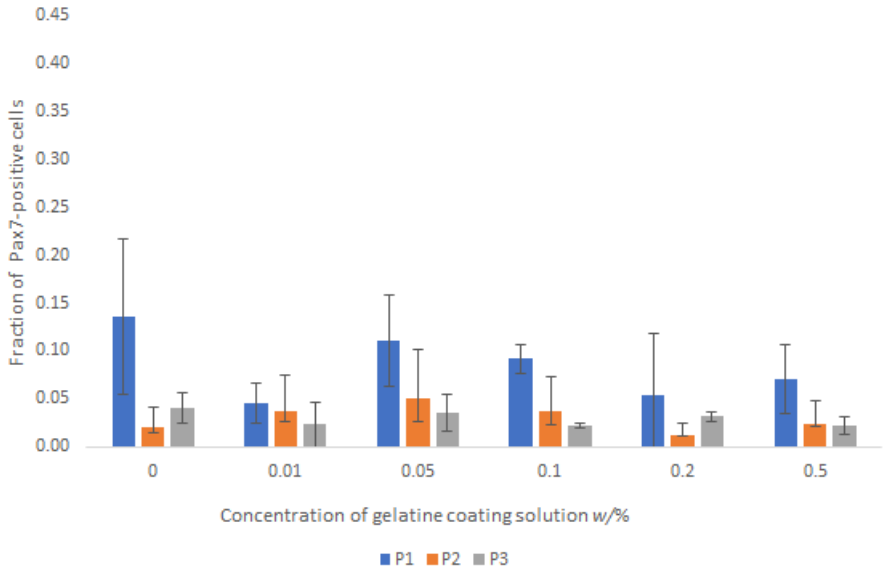

Figure 4.9. Graphical plot of Pax7 positive cells within the bMuCS Φ2 (Passages 1-3). Cells were cultured for three consecutive passages on culture dishes coated with gelatine solutions, with a gelatine concentration range: w = 0.01%, 0.05%, 0.1%, 0.2% and 0.5% (*P<0.05).

The gelatine coatings didn't present a significant advantage when they were compared with non-coated culture dish surfaces. The highest fraction of Pax7 positive cells was seen in the first passage of the non-coated dish (0.14±0.08) % and the third passage as well (0.04±0.02)%. They didn't influence the bMuCS culture cell proliferation in any way, nor did they show any

selectivity towards the satellite cells within the culture itself. Although gelatine is a readily available and cheap coating, unfortunately it can't provide any benefit in the *in vitro* meat cultivation. These results are in line with other publications regarding gelatine.[121]

The next investigated coating was collagen type 1. This type of collagen has been mentioned in other research regarding satellite cells.[122,123] The results of the collagen type 1 coating can be seen in the Figure 4.10.

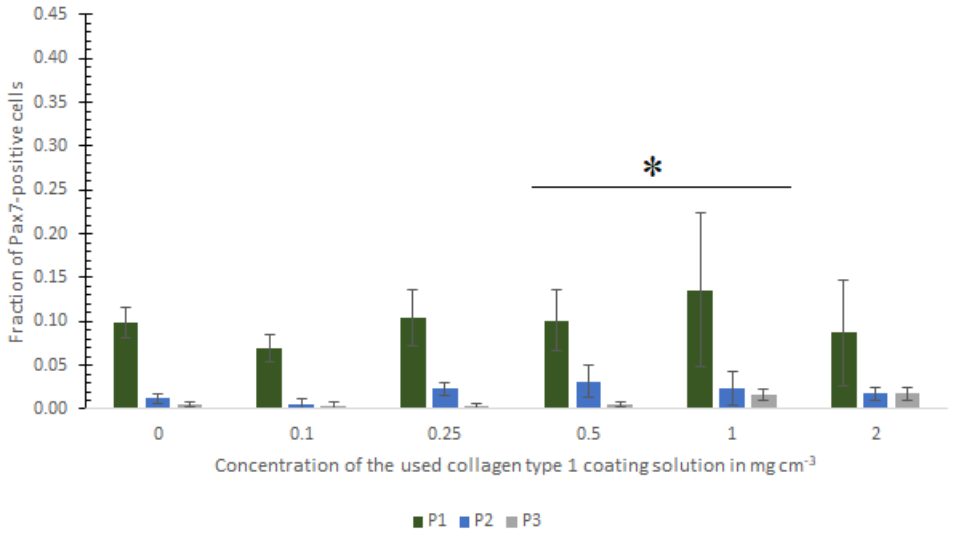

Figure 4.10. Graphical plot of Pax7 positive cells within the bMuCS alpha G (Passages 1-3). Cells were culture for three consecutive passages on culture dishes coated with collagen type1 solutions, with a collagen type 1 concentration range: 0.1 mg cm^{-3}, 0.25 mg cm^{-3}, 0.5 mg cm^{-3}, 1.0 mg cm^{-3} and 2.0 mg cm^{-3}. (*P<0.05)

Similarly, to gelatine, collagen type 1 also didn't present any significant advantage when compared to non-coated culture dish surfaces. Though in contrast to gelatine, the highest fraction of Pax7 positive cells for the first and third passage were detected at 1,0 mg cm^{-3} (0.14±0.09) % and (0.02±0.01) % respectively. This suggests that collagen type 1 does show a minimum influence on the satellite cell culture, although its effects aren't significant. The concentrations from 0,1-0,5 mg cm^{-3} didn't have any influence on the proliferation of the observed bMuCS culture nor on the selectivity towards the satellite cells within. The higher concentrations of collagen type 1 influenced the observed bMuCS cultures in a manner that hindered their proliferation, which could be an indication of minimal cytotoxicity. These results can be compared to similar research, as most of the publications present contradicting results

in the use of collagen type 1 as a coating for satellite cells. Some argue that this divergence in results may be attributed to species.[124]

The last coating that was investigated was laminin with a concentration range from 1-50 µg cm^{-3}, as those were the most common concentrations found in other published research.[125,126] Results for the laminin coating experiment are shown in Figure 4.11.

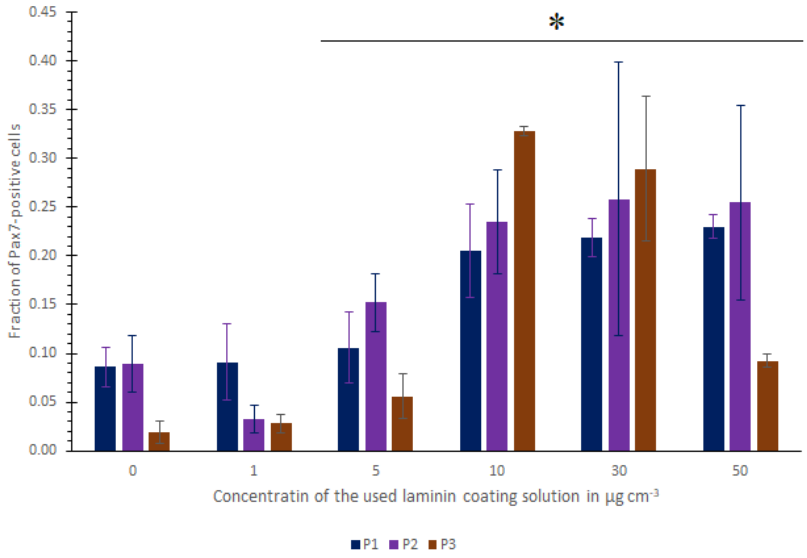

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Figure 4.11. Graphical plot of Pax7 positive cells within the bMuCS alpha G (Passages 1-3). Cells were culture for three consecutive passages on culture dishes coated with laminin solutions, with a laminin concentration range: 1 µg cm^{-3}, 5 µg cm^{-3}, 10 µg cm^{-3}, 30 µg cm^{-3} and 50 µg cm^{-3}. (*P<0.05)

In Figure 4.11., it is clearly shown that a 1 µg cm^{-3} laminin coating solution won't yield any advantages when compared to a non-coated culture surface, as its Pax7 positive cell fraction decreases through the three passages. The other laminin concentrations don't show a significant advantage when compared to the non-coated culture dish surface, but they do show a robust trend by which higher laminin concentrations favour higher fractions of Pax7 positive cells in the culture for longer periods of time and higher passage numbers. When comparing the values through the passages, the concentrations of 10 µg cm^{-3} and 30 µg cm^{-3} produced the highest values for the fraction of Pax7 positive cells in the culture, where in the third passage it was (0.33±0.01) % at 10 and (0.29±0.07) % at 30 µg cm^{-3} .This is in conjunction with previous data available on the use of laminin as a coating in *in vitro* meat cultivation.[31,127,128] One

disadvantage of higher laminin concentrations that we have observed is that cells don't proliferate at concentrations of 30 µg cm^{-3} of laminin and higher, although this wasn't investigated later. In regards with the results from Figure 4.10., laminin coating at a concentration of 10 µg cm^{-3} is our choice regarding further bMuCs cultivation optimizations.

4.3.2. Collagen type 1 vs. Laminin preplating comparison

The next point of interest in the optimization of bMuCS cultivation was the substitution of collagen type 1 0.5 mg cm^{-3} for preplating using laminin coating at a 10 µg cm^{-3} concentration. As such, cells were isolated as described in section *3.1.1.*, but the cell mixture was split in two at the end of the isolation and one null-passage was cultivated in a 10 µg cm^{-3} laminin coated and the other in a 0,5 mg cm^{-3} collagen type 1 coated culture flask. The preplating is regarded as an uncomplicated technique used for harvesting satellite cells with a higher yield during isolation.[121,129,130] Pre-plating is mostly used to get rid of the fast-adhering fibroblasts right after isolation, usually by leaving the isolated cell mixture from 20 minutes to 5 days (4 hours in our case), in an initial culture flask and then transferring the cell mixture to a new coated culture flask afterwards. It can also be achieved by shaking the cell mixture on a shaker so that the fibroblasts attach, and the satellite cells stay suspended in the culture medium.[131] The goal of this experiment in the thesis was to investigate whether the coating solution used in the culture flask after the preplating can have an influence on the efficiency of the satellite cell isolation procedure. This experiment was performed with three biological replicates (αN, αO and αP) to subdue any donor variations. The result is shown in Figure 4.12.

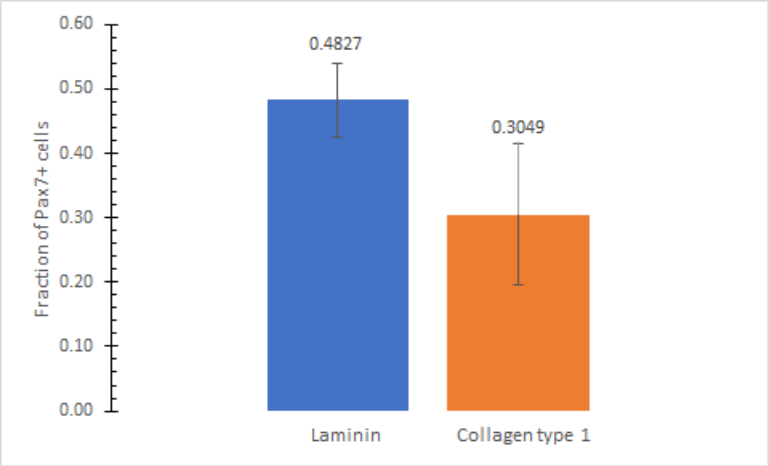

Figure 4.12. Graphical depiction of the preplating experiment using collagen type 1 0.5 mg cm^{-3} and laminin 10 μg cm^{-3} as coating for culture flasks. Cells were divided right after the isolation and cultivated. *($P<0.05$; n=3)

Although the data from Figure 4.12. doesn't show a significant advantage for the use of laminin instead of collagen, it does depict a clear trend for the use of laminin. The average yield after isolation rose to 48.27±5.73 % when using laminin as a coating, in comparison to the 30.49±10.99 % harvest of satellite cells when using collagen type 1 as a coating. These results have helped to further optimize the satellite cells isolation protocol used in this thesis. Unfortunately, these results can't be compared to other research as there is none available that specifically discuss the use of laminin coating for preplating during satellite cell isolations.

4.3.3. Myotube formation potential of different coating solutions (gelatine, laminin, and collagen type 1)

After investigating the influence of different culture dish coating on the optimization of bMuCS cell culture cultivation, we investigated the influence of those coating during differentiation. One would be forgiven to presume that different coating materials can also influence the maturation process of satellite cells, when it has been shown that those coatings have an influence on their proliferation capabilities. There has been some research on coating and satellite cell differentiation.[111,113,132] This experiment followed the procedure described in section *3.1.7.*, using three technical replicates of the αP bMuCS culture. The medium exchange occurred at Day 0, one set of cells was further fixated on day 2, and lastly, the fixated cells from day 7 were used for the cell's differentiation potential using the fusion index, the results of which can be observed on Figure 4.13.

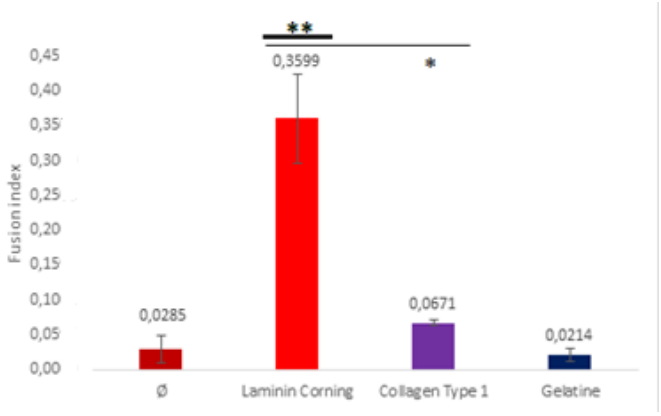

Figure 4.13. Graphical depiction of the varying fusion indexes for each coating solution at their optimal concentration (10 μg cm^{-3} for laminin, 0.5 mg cm^{-3} for collagen type 1 and 0.05% for gelatine). The αP (passage 3) donor was used following the differentiation protocol (*3.1.7.*). The cells were stained for MyoHC and their fusion index was analysed via fluorescent microscopy. (*P<0.05; **P<0.005)

Figure 4.13. shows that laminin coating is again the best coating option even for differentiation, when as much as 35.99±6.33 % of cells showed the ability of myotube formation, while the others formed myotubes in the following percentages 2.85±2.01% for non-coated, 6.71±0.42 % for collagen type 1 and 2.14±0.92 % for gelatine coated culture dishes. The myotube formation (fusion index) analysis has been carried out via fluorescent microscopy and images of the different coatings' influence on the myotube formation can be seen in Figure 4.14.

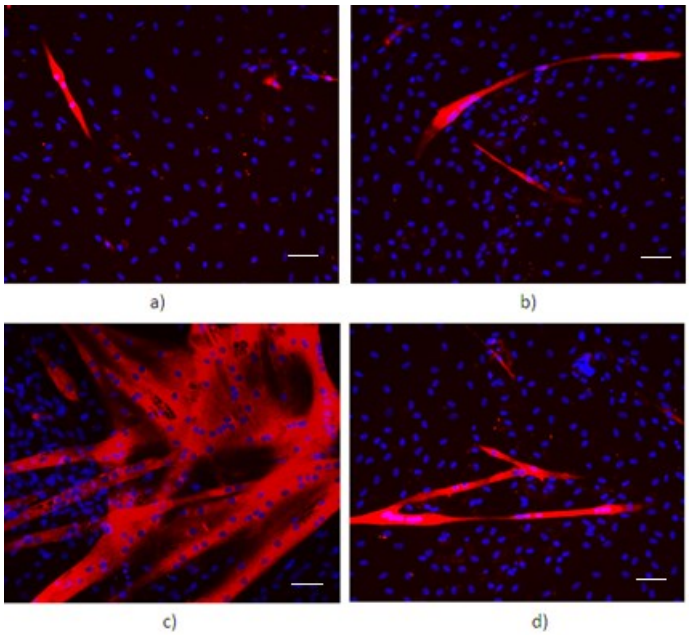

Figure 4.14. Representative fluorescent microscopy merged channel images of bMuCS αP (P3; day 7 of differentiation) stained for MyoHC with Cy3 (red) as secondary antibody and DAPI (blue). With a) depicting myotube formation on non-coated, b) gelatine, c) laminin and d) collagen type 1 coated culture dishes.

It is necessary to note that the same αP passage, when stained for Pax7 showed only 25 % positive cells. This suggests that there are other cells in the bMuCS culture that are capable of myogenesis, and it is in line with contemporary cultured meat research.[133,134,135] Additionally, cells grown and differentiated on laminin seem to show a much more complex nature of their fusion, where it seems as if multiple fibres are interconnected (Figure 4.14.c), whereas the other coating don't seem to produce such complex structures during muscle fibre formation. All of this is in accordance with other bovine cultured meat research that confirms the use of laminin coatings during the culture differentiation.[31,113,136]

4.3.4. Whole laminin vs Laminin pentapeptide

In this thesis, the whole protein laminin was used during the coating material comparison. Upon further search, we were able to find a cheaper laminin version, which was a pentapeptide, corresponding to the sequence 929-933 of the B1 chain.[113] This specific part of the B1 laminin chain is also a cell attachment domain.[137] These findings motivated us to investigate if the economical laminin pentapeptide would suffice as a viable substitution for the whole laminin protein when used for coating purposes in cultured meat research. For this comparison, we used the αG bMuCS culture again for three passages with three technical replicates. The results of the comparison are visible in Figure 4.15

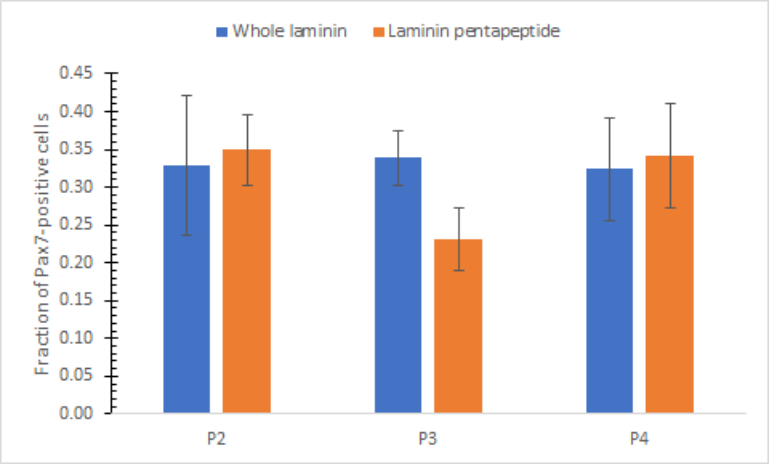

Figure 4.15. Comparison of the whole laminin protein and a laminin pentapeptide as coating for culture dish surfaces via the fraction of Pax7+ cells within bMuCS αG (P1-3). For both coatings a 10 μg cm^{-3} solution was used. (n=3; *P<0.05)

The results of the laminin comparison from Figure 4.15. show that there isn't any significant deviation from the collected values. At the beginning of the experiment, there were (0.33±0.9) % of Pax7 positive cells in the whole laminin coated culture dishes and (0.35±0.05) % in the laminin pentapeptide coated culture dishes, whereas after three passages the whole laminin coated culture dishes had a (0.32±0.07) % of Pax 7 positive cells in comparison to the (0.34± 0.07) % of the laminin pentapeptide coated ones. These results show again that laminin can sustain the satellite cell population within the bMuCS culture for multiple passages. It is safe to say that the pentapeptide version can be used as a viable whole laminin substitute, without any drawbacks. Unfortunately, these findings can't be compared with other research, as there aren't any available on the use of laminin pentapeptide 929-933 for bovine *in vitro* meat cultivation.

Furthermore, all the above-mentioned results position laminin as the most favourable coating material for *in vitro* meat cultivation. It showed remarkable advantage during differentiation, where it enabled the best and most complex structure formation during myotube formation. It also showed amazing selectivity towards satellite cells for preplating purposes, but also during further cultivation.

This thesis gathered a lot of positive data for the use of laminin as a coating for bovine *in vitro* meat cultivation, but it also only investigated the use of simple materials. This research could further expand on the possibilities of different coating solutions that are comprised of a

multitude of materials (i.e., *Matrigel*). More complex coating matrices that resemble the *in vivo* bovine satellite cell niche could prove to be even more beneficial in their cultivation.

4.4. P38 MAPK inhibitor SB203580

In the section 2.2.3., the reasoning was established to investigate the use of SB203580 as a promising drug in the p38 MAPK pathway that could lead to higher proliferation as well prove to be a regulator for myogenic differentiation of satellite cells.

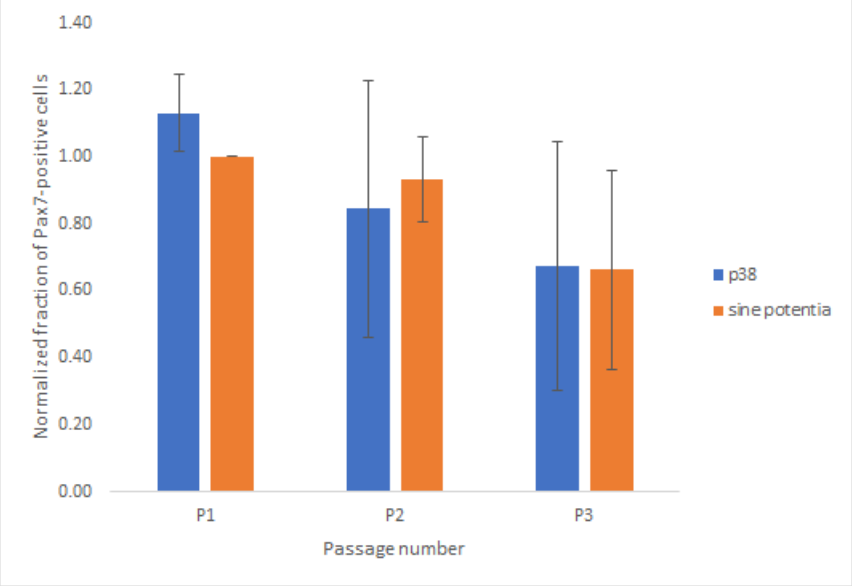

Figure 4.8. Graphical depiction of the influence of SB203580 on the bMuCS cell culture in comparison to non-treated bMuCS. The cells were cultured with growth medium 1, as a control and growth medium 2 as the observed culture. The results are presented as averages and standardized at the beginning to the value of the average of the control bMuCS cultures. (n=3)

Three separate biological replicates were used for this experiment, with each having at least three technical replicates. All three cultures used contained 30 – 40 % of Pax7 positive cells within them, and all were cultivated for three passages continuously. The data was collected using immunocytochemical staining of cells with Pax7 primary antibody and Cy3 as the secondary fluorescent antibody via fluorescence microscopy. All targeted bMuCS cultures for this experiment were cultured with the same growth medium (DMEM HG, 20%FBS, $2 \cdot 10^{-4}$ mol/dm^3 L-Gln and 10 µg ml^{-1} of SB203580), whereas the control bMuCS cultures were cultured in the same growth medium without the addition of SB203580. The cells were followed from the isolation and as such, the control/target conditions in their growth medium

were established right at the beginning, to minimize any delays of the p38 inhibitors activity, that could be shown when using frozen cells. The 10 μg cm^{-3} was used as other research, such as Ding et al.,[31] noting that lower concentrations didn't have any influence on the cells whereas concentrations of 20 μg cm^{-3} tended to have a negative influence on the culture's viability. The values for Figure 4.8. have all been normalized to the average Pax7 positive cell fraction of the control cultures. The results from Figure 4.8. show that although there is a slightly higher fraction of Pax7 positive cells at the beginning (1.13±0.11) %, there isn't a significant distinction between the target and the control culture. Moreover, that difference isn't noticeable anymore after three consecutive passages where the cultures containing the p38 inhibitor have a (0.67±0.37) % fraction of Pax7 positive cells, and the not treated cells have (0.66±0.30) % Pax7 positive cells when compared to the normalized value of the non-treated cultures at the beginning. During the three passages, the average fraction of Pax7 positive falls for approximately 10% after each passage. From all of this it can be concluded that using the p38 MAPK inhibitor doesn't provide any significant benefits in comparison to using the growth medium without SB203580. There are a few possibilities why SB203580 didn't show any benefits regarding preservation of the stemness of the bMuCS cultures. One reason could be that, although p38 MAPK activity is necessary in the satellite cell myogenesis initiation,[138] it isn't the only regulation site in satellite cell. Other possible routes for initiating satellite cell proliferation and differentiation include the use of the Fibroblast growth factor (FGF),[139] transforming growth factor beta (TGF-β),[140] and the insulin-like growth factor family (IGF-1 and IGF-2)[141] or via the extracellular signal-regulated kinases (ERK1 and ERK2).[142] Additionally, the γ-p38 isoform is presumed to be responsible in the signal transfer that initiates myogenesis, as it is the most prevalent p38 isoform found in muscle tissue,[74] and as the SB203580 molecule isn't described as a specific γ-p38 inhibitor. It is also possible that SB203580 won't produce a significant inhibition in the muscle tissue, or in other words the phosphorylation of MyoD will be able to continue as before. Lastly, as mentioned in section *2.2.2.,* MyoD expression is coregulated with myf5, and thus lower activation of MyoD (via the SB203580 inhibition of the p38 MAPK pathway) could lead to an upregulation of myf5 as a substitute.[55] As they are homologues, myf5 could then take over the role of the myogenesis initiator in the satellite cells with suppressed MyoD activation via the p38 MAPK pathway.[54] When considering the above mentioned, it could be argued that the inhibition or temporary

downregulation of both MyoD and myf5 could lead to a higher proliferation rate of *in vitro* cultured satellite cells.[143]

4.5. FACS analysis

As mentioned in section *2.2.6*, it is expected that bMuCS can be isolated from a heterogenous cell culture by a multicolour FACS assay, using select cell surface markers (clusters of differentiation), CD29 and CD56 as positive markers and CD31 and CD45 as negative markers.[33] Figure 4.5. shows the results after sorting of bMuCS alphaK using all 4 forementioned cell surface markers.

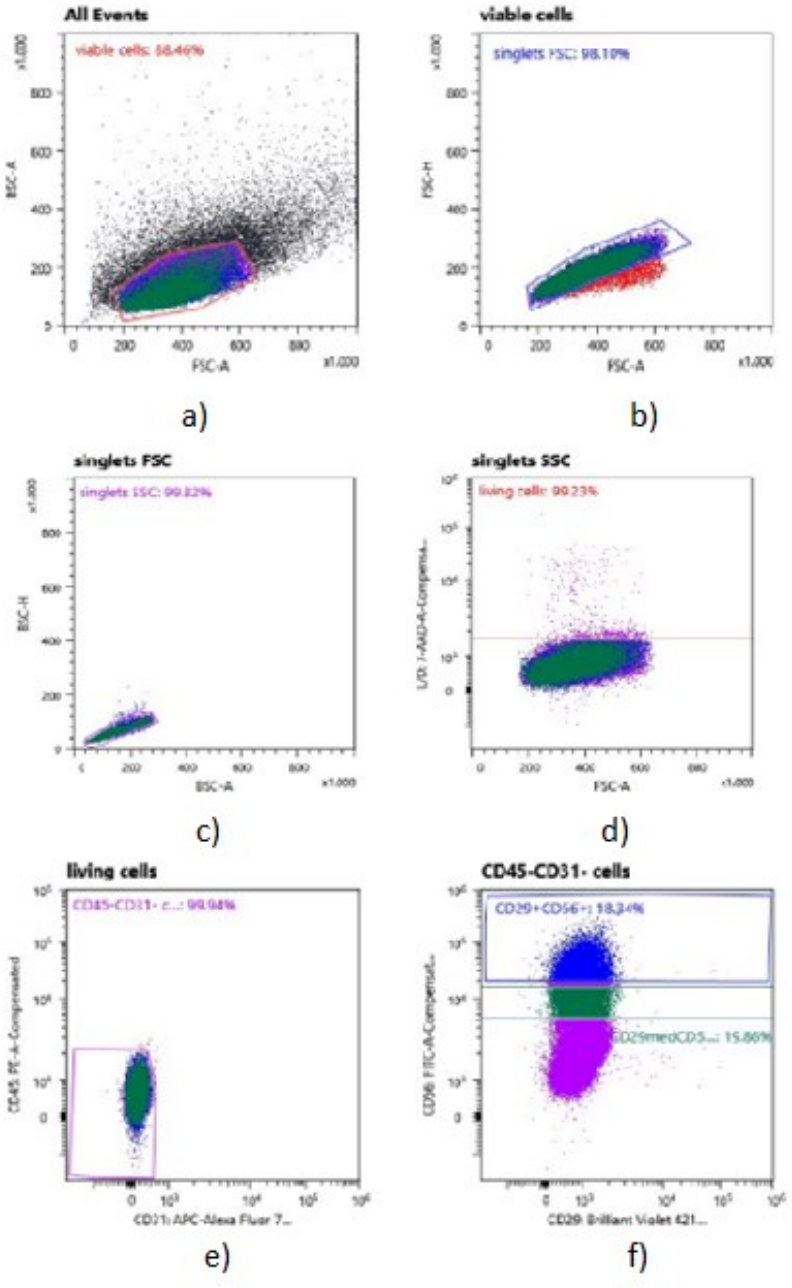

Figure 4.5. FACS plots of isolated bMuCS alphaK. Graphs are further explained in the text.

The first "all events" graph depicts a plot of all cells in the culture. The area of interest is selected, to eliminate debris from the analysis, and analysed in the "viable cells" graph to select the single cells and remove double cells out of the analysis. In the "FSC" graph, the living cells are selected via their approximate size and the cells positive for 7-AAD are removed (dead cells), as the stain can only penetrate the cells membrane if it is not intact. The "SSC" graph plots the cells in culture depending on their complexity. Also, we have the "CD31- and CD45-" plot (third from the left below) where we arbitrary set an intensity threshold below which we can confidently say that the selected cells don't express our negative markers, depicted in green, and those cells won't be considered further on. Lastly, we have the "CD29+ and CD56+" plot (furthest to the right below) where we also see a certain intensity threshold above which we expect to find only double positive cells that express our positive selection markers, depicted here in blue. The cells depicted in blue were sorted and further cultivated to examine their cultivation properties. The purified cell culture was further on analysed using Pax7 immunocytochemical staining, which is shown alongside their previous analysis in figure 4.6.

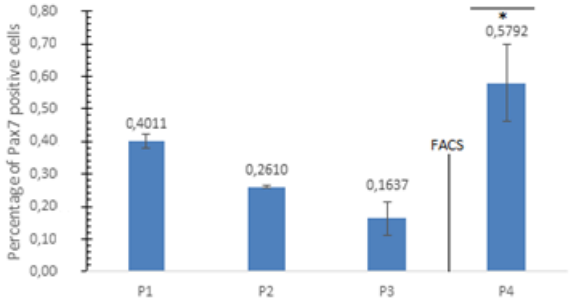

Figure 4.6. Fraction of Pax7 positive bMuCS αK cells before and after FACS (*P<0.05). The cells were cultivated for three passages after isolation with growth medium and then sorted, the fourth passage shows the fraction of Pax7 positive cells after sorting.

As mentioned previously, a large quantity of cells that was required for FACS analysis, thus the bMuCS had to be cultivated longer and for more passages. Figure 4.6 clearly shows the influence of FAC-ing on the bMuCS culture purification as there were only (0.16±0.02) % Pax7 positive cells before in comparison to (0.58±0.12) % of Pax7 positive cells after the sorting procedure. This trend is also visible with the αG culture whose Pax7 changes are shown in

Figure 4.7. Whereas the FACS gating parameters for αG can be found in Figure A in the Appendix.

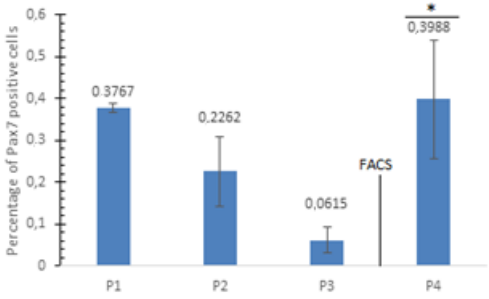

Figure 4.7. Fraction of Pax7 positive bMuCS αK cells before and after FACS (*P<0.05). The cells were cultivated for three passages after isolation with growth medium and then sorted, the fourth passage shows the fraction of Pax7 positive cells after sorting.

Here it can be again observed how the Pax7 fraction changes from (0.06±0.03) % before to a staggering (0.40±0.14) % of positive Pax7 cells in the culture after the sorting. The most surprising thing to see is that in both instances, the fraction of Pax7 positive cells after the sorting exceeds the initial Pax7 fraction obtained right after the isolation, which is (0.40±0.02) % for the αK culture and (0.38±0.11) % for the αG culture. This shows that FACS can be a promising technique for further bMuCS purifications *i*f necessary. Unfortunately, however, these results are subpar when compared to data published by other groups, where most claim that they have achieved a purity of well above 90 % Pax7 positive cells for isolated bovine satellite cell cultures.[33,144]

One downside to the use of FACS as a purification technique for cells intended to be used in *in vitro* meat cultivation is that the fluorescent antibodies used for cell sorting stay attached to the cells, although its concentration would drop through higher passages. The end goal of *in vitro* meat cultivation is that it can be edible and brought to a consumer market, and it should withstand all the regulatory health standards of ordinary meat products. There hasn't been any research on the influence on human health of such antibodies present in meat. One possibility is that the conjugated antibodies won't cause a reaction within the satellite cell culture during cultivation or further processing as the culture don't have an immune system of their own that could react to such stimulus.[145] Additionally, the fluorescent molecules found in the conjugate must be tested for their toxicity as well.[146] It could prove to be an obstacle to

getting regulatory approval for a product that uses this technique. Another downside to the attached FACS antibodies is that we cannot say how they further on influence the binding of the primary Pax7 antibodies used for satellite cell identification. Unfortunately, this wasn't further investigated due to time restrictions and forementioned cell count demand.

§ 5. CONCLUSION

This thesis investigated the technical feasibility of *in vitro* meat cultivation using satellite cells to establish a viable long term cell culture. The cells were identified by visualising the Pax7 transcription factor using fluorescence microscopy. This enabled us to follow the number of satellite cells within our bMuCS culture and further examine how we can selectively optimize their culture conditions.

We investigated if we could purify our bMuCS culture and sort only satellite cells from it. This proved to be the right way forward, as the sorted cells have a higher fraction of satellite cells in culture afterwards. FACS has its own downside, as it is economically restrictive and time consuming if no *on-site* sorter is available. Additionally, the influence of the attached antibodies must be investigated in long-term culture as well for human health to provide a framework towards regulatory approval of such a product. Laminin culture dish coating proved to a positive and easily implementable technique to nourish the satellite cells in culture. Laminin coating enhanced the selectivity of the culture dishes towards satellite cells in comparison to other cell types. It also proved to enhance the myofiber fusion potential of satellite cells during differentiation. Lastly, the p38 MAPK and its inhibitor SB203580 were investigated as a hope to develop a simple controlling mechanism with which the cells could be cultivated long-term and myogenesis initiation regulated. Unfortunately, SB203580 didn't prove itself as such and its use in the research was abandoned.

This thesis showed that *in vitro* meat cultivation has a long way to go, research wise, before it reaches the consumer market. It points to the right steps that should be taken from here on to establish a bMuCS culture for in *vitro* meat cultivation, but it doesn't solve other problems. One of the ways how *in vitro* meat cultivation can further be optimized is finding a cost-saving alternative growth medium.

§ 6. LIST OF ABBREVIATIONS AND SYMBOLS (prema potrebi)

7-AAD	7-Aminoactinomycin D
ATP	Adenosine triphosphate
CDKN2	cyclin dependent kinase inhibitor 2
DMEM HG	Dulbecco's modified Eagle medium high glucose
CRISPR	clustered regularly interspaced short palindromic repeats
DAPI	4′,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
FACS	Fluorescent activated cell sorting
FBS	Foetal bovine serum
GHG	Greenhouse gas
GMO	Genetically modified organism
iPSC	induced pluripotent stem cells
MyoD	myoblast determination protein 1
MyoG	Myogenin
MyoHC	Myosin heavy chain
NCAM	Neural cell adhesion molecule
PBS	Phosphate buffered saline
UN	United Nations

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§ 8. APPENDIX

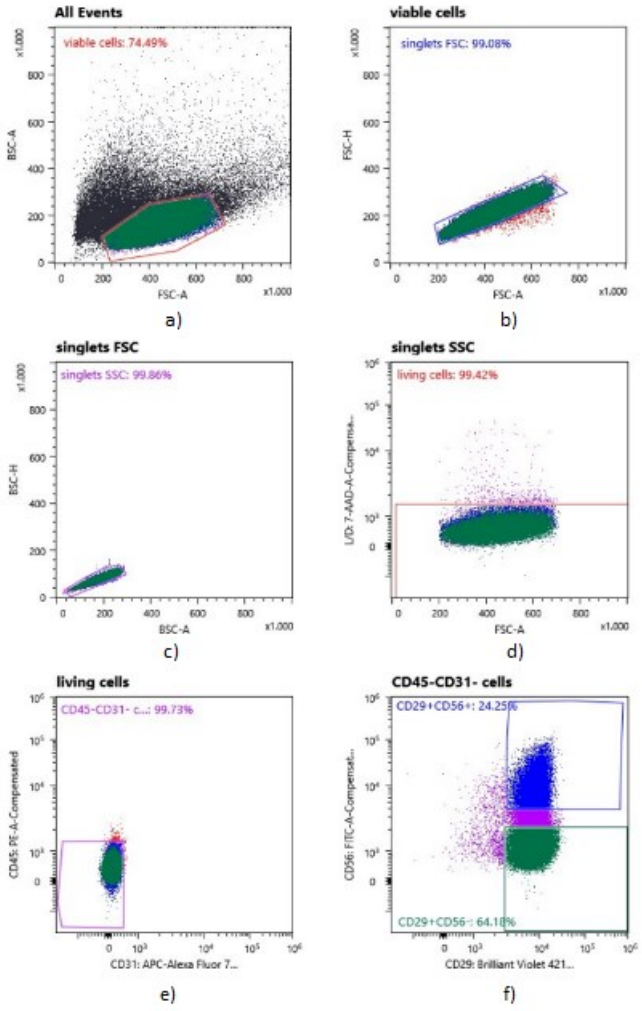

Figure A. Figure 4.5. FACS gating plots of isolated bMuCS alphaK. Graphs are further explained in the text.

The first “all events” graph depicts a plot of all cells in the culture. The area of interest is selected, to eliminate debris from the analysis, and analysed in the “viable cells” graph to select the single cells and remove double cells out of the analysis. In the “FSC” graph the living cells are selected via their approximate size and the cells positive for 7-AAD are removed (dead cells), as the stain can only penetrate the cells membrane if it is not intact. The “SSC” graph plots the cells in culture depending on their complexity. Further on we have the “CD31- and CD45-” plot (third from the left below) where we arbitrary set an intensity threshold below

which we can confidently say that the selected cells don't express our negative markers, depicted in green, and those cells won't be considered further on. Lastly, we have the "CD29+ and CD56+" plot (furthest to the right below) where we also see a certain intensity threshold above which we expect to find only double positive cells that express our positive selection markers, depicted here in blue. The cells depicted in blue were sorted and further cultivated to examine their cultivation properties. The purified cell culture was further on analysed using Pax7 immunocytochemical staining seen in Figure 4.7.

§ 9. CURRICULUM VITAE

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2013–2017	High School Gimnazija Tituša Brezovačkog, Zagreb
2017–2020	Bachelor of science; Chemistry, Faculty of Science, University of Zagreb
2021-2022	Erasmus+ international student exchange, internship at the University Reutlingen in the AG Kluger, Reutlingen, Federal Republic of Germany

Honours and Awards

2021	Rector's award, *On-line Znanstvene čarolije*

Activities in Popularization of Science

2018-2023 Otvoreni dan Kemijskog odsjeka, Znanstvene čarolije, BASF Kid's Lab