MUSCLE PROGENITOR CELLS FOR CULTIVATED MEAT

OPTIMIZATION OF MUSCLE PROGENITOR CELL ISOLATION TECHNIQUES FOR PRODUCTION OF CULTIVATED MEAT

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LAY ABSTRACT

Traditional meat production is associated with numerous challenges including animal welfare concerns, human health concerns, and harmful environmental consequences. The global population is predicted to reach 9.7 billion by 2050, emphasizing the importance of alternative food sources to meet the increased food demand. Cultivated meat is a promising new protein source, with the intended purpose of providing a sustainable food source with reduced ethical concerns compared to conventional meat. While there are several challenges to overcome throughout the production process, a major consideration in the early stages of cultivated meat production is cell sourcing. Muscle cells harvested from a tissue biopsy are one proposed starting cell source which has the potential to make up most of the tissue in cultivated meat products. This thesis aimed to improve upon previously published protocols used for muscle cell isolation and provide an optimized cell population for use in cultivated meat production. The cell sorting protocol described in this thesis provides a highly efficient technique for muscle cell purification and long-term growth. The resulting cell population has many characteristics that are pertinent to cultivated meat and may advance the early stages of production.

ABSTRACT

Traditional meat production has major sustainability and ethical concerns. Cultivated meat helps to address these concerns by reducing the need for mass animal farming. Muscle progenitor cells (MPCs) harvested from skeletal muscle are a promising cell source for cultivated meat. While various protocols have been developed for MPC isolation, which protocol is best suited for the cultivated meat industry requires further investigation. Therefore, the purpose of this thesis was to optimize the MPC isolation technique to produce a pure myogenic cell population and provide the cultivated meat industry with standardized procedures for production. For these proof-of-concept experiments, skeletal muscles harvested from the hindlimb muscles of mice were used. Cells were isolated from the harvested muscle then subjected to one of three protocols for MPC enrichment: pre-plating, ice-cold treatment (ICT), or fluorescence activated cell sorting (FACS). The pre-plating and ICT protocols resulted in impure cell populations with few MPCs after one week in culture. Therefore, FACS using two cell-surface markers, NCAM and CD34, was employed as a more specific method for MPC sorting. CD34+NCAM1- cells grew quickly, however, unwanted cell types remained following FACS. In contrast, CD34+NCAM1+ cells had a consistent small, rounded shape and slow proliferation rate. These cells remained viable in culture for several months and had high Pax7 expression, indicating they were a pure population of myogenic cells. CD34+NCAM1+ cells maintained their capacity to differentiate after culturing for an extended period, demonstrating their potential use for cultivated meat production. The results of this study provide a better understanding of the differences between previously published MPC isolation techniques. Future studies will investigate the potential for CD34+NCAM1+ cells to be grown on a larger scale. These

experiments provide insight into MPC populations that may exist in livestock species and will help to streamline the early stages of cultivated meat production.

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LIST OF ABBREVIATIONS AND SYMBOLS

bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CAM	Cell Adhesion Molecule
CD	Cluster of Differentiation
CMV	Cytomegalovirus
CO_2	Carbon Dioxide
CXCR4	Chemokine Receptor type 4
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's Phosphate Buffered Saline
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
eMyHC	Embryonic Myosin Heavy Chain
ESC	Embryonic Stem Cell
FACS	Fluorescence Activated Cell Sorting
FAO	Food and Agriculture Organization
FAP	Fibroadipogenic Progenitor
FBS	Fetal Bovine Serum
HGF	Hepatocyte Growth Factor
ICAM	Intercellular Cell Adhesion Molecule
ICT	Ice-cold Treatment
IGF	Insulin-like Growth Factor
iPSC	Induced Pluripotent Stem Cell
kDa	Kilodalton
MACS	Magnetic Activated Cell Sorting
MAPK	Mitogen Activated Protein Kinase
MCAM	Melanoma Cell Adhesion Molecule
MCK	Muscle Creatine Kinase
MHC	Myosin Heavy Chain

MPC	Muscle Progenitor Cell
MRF	Myogenic Regulatory Factor
MSC	Mesenchymal Stem Cell
Myf5	Myogenic Factor 5
MyoD	Myogenic Differentiation Protein 1
NCAM	Neural Cell Adhesion Molecule
PDGF	Platelet Derived Growth Factor
PECAM	Platelet Endothelial Cell Adhesion Molecule
SEM	Standard Error of the Mean
SP	Side Population
TGFβ	Transforming Growth Factor Beta
VCAM	Vascular Cell Adhesion Molecule

DECLARATION OF ACADEMIC ACHIEVEMENT

Alexandra Steele was the primary author and responsible for majority of the data collection and analysis. Mekayla Truong assisted with data collection. Zoya Tabunshchyk assisted with flow cytometry training and cell sorting. Dr. Thomas Hawke was responsible for conceptualization and design of the study and assisted with experimental planning.

LITERATURE REVIEW

Chapter 1: Cultivated Meat

1.1 Introduction to Cultivated Meat

The global population is increasing, leading to challenges including an increased food demand and environmental decline. Currently, we are heavily dependent on animal sources for food which are responsible for 25% of global protein consumption[1]. The Food and Agriculture Organization (FAO) reported that 18% of human induced greenhouse gas emissions come from livestock animals and have predicted that global meat consumption will increase 73% by 2050 compared to 2010 measurements[2,3]. On top of this, an overwhelming proportion of the world's water supply and habitable land are involved in meat production, particularly from raising and producing feed for livestock[1,4]. As a result, there has been an uprising in the development of alternative protein sources that have potential to feed a larger population. Despite their increasing supply in supermarkets and restaurants, plant and fungal protein sources are avoided by many people for several reasons, one being that they simply prefer meat[5]. Additionally, most plant products lack all the essential amino acids that are required for a healthy diet[6]. Therefore, cultivated meat has been proposed as another alternative protein source that may more closely mimic traditional meat with regards to taste, texture, and composition.

Cultivated meat, also referred to as lab grown, cell-based, or in vitro meat, is meat created using cell culture and tissue engineering techniques. The goal is to create a meat product that is the same as traditional meat in all aspects, with a largely reduced impact on the environment and improved animal welfare. Production begins with selection of specific cell types which will be sorted into the various progenitor cell types required (Figure 1). Several

different cell types need to be grown to make up all the tissues present in meat, including skeletal muscle, adipose tissue, and connective tissue. These cells will be quickly expanded in large bioreactors before being collected and seeded into scaffolding[7]. The progenitor cells will be differentiated into mature tissue to create a complete, meat-like product[7]. With cultivated meat, there is an opportunity to supplement the finished product with additional nutrients and fine tune the overall taste and texture, which is not possible with traditional meat[8].

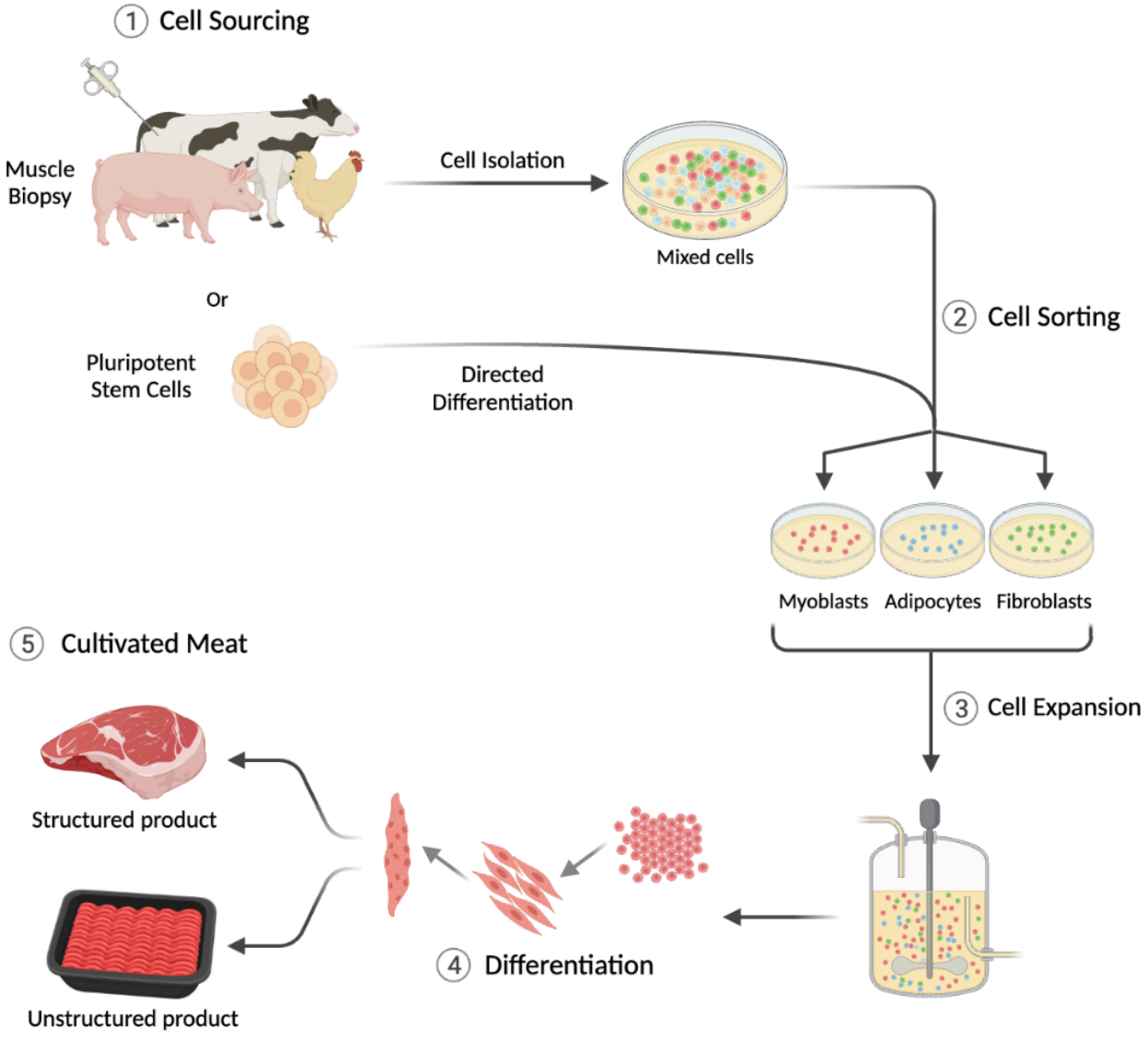

Figure 1. General process of cultivated meat production.

1.2 Cultivated Meat as a Sustainable Alternative

To continue to feed the global population on a planet with finite resources, cultivated meat may be a necessary alternative to traditional meat to cut back on land, water, and energy use. One life cycle assessment showed cultivated meat would have a significant reduction in greenhouse gas emissions, land use, and water use compared to conventional meat from beef, pork, sheep, and poultry[9]. Additionally, the estimated energy use of cultivated meat was lower when compared to conventionally produced meat from all animals assessed other than poultry[9]. Although these assessments are based upon smaller scale models and a full analysis of the large scale production process has yet to be conducted, the general consensus is that the switch from conventional meat to cultivated meat will lessen the overall impact on the environment.[9–11] There will ultimately be a reduction in deforestation and use of valuable resources with cultivated meat, and by making use of processes such as media recycling and using renewable energy sources, sustainability could be improved even further[9,11].

Another advantage to cultivated meat over conventional meat is the reduction in antimicrobial use that will be required for production. Resistance to antibiotics poses a threat to global health which is worsened by the vast use of antibiotics in livestock animal housing facilities[12]. Animals such as pigs are confined to small spaces where hundreds of animals live in close contact, making antibiotic use mandatory to maintain health and hygiene among the animals[12]. While antibiotics will likely be required during the early cell sourcing stages of cultivated meat production, the remaining stages are not likely to involve antibiotic use[12]. As a result, the amount of antibiotics used during cultivated meat production will be negligible when compared to the thousands of tonnes of antibiotics used for traditional meat production each year[12,13].

Meat consumption is currently responsible for approximately 22% of all foodborne illness cases each year[14]. Bacteria and parasites such as Salmonella and E. coli are often introduced in conventional meat slaughterhouses and pose a health risk to consumers if meat is improperly handled[8]. As cultivated meat will be produced in facilities where sterility is prioritized and routine testing will be conducted, a reduction in foodborne illness from meat consumption can be expected[8]. This reduction in exposure to pathogens may provide the additional bonus of a longer shelf life and reduced food waste with cultivated meat compared to traditional meat[15]. Finally, the transmission of zoonotic diseases often originates from livestock animals and accounts for millions of deaths each year[16]. Pandemics or endemics caused by viruses such as COVID-19, H1N1, and H5N1 are thought to have arisen from a wild or livestock animal which was eventually transmitted to humans through animal farming and meat consumption[17]. As cultivated meat would eliminate the need for mass animal farming and drastically reduce human exposure to animals, it is expected that there would be a great reduction in transmission of zoonotic diseases[17].

1.3 Current Status of the Cultivated Meat Industry

Hypotheses about the potential to grow meat using cell culture techniques have been documented as far back as the 1930s, however, the first real experiments investigating the possibility of cultivated meat weren't conducted until the late 1990s[7,18]. The first cultivated meat product to be publicly consumed was created in 2013 at the University of Maastricht in Holland, and since then the industry has grown at a rapid pace[7,18]. Compared to just two companies in 2014, by the end of 2022, there were over 150 public companies dedicated towards cultivated meat production, on six different continents[19]. At the end of 2020, the Singapore Food Agency approved the first ever sale of a cultivated meat product which was sold in a Singapore restaurant

shortly after[20]. Since then, the food and drug administration has approved the sale of cultivated meat in the U.S. and approval in Canada will likely be not far behind[20]. Techno-economic assessments suggest that production could be advanced enough by 2030 to have cultivated meat produced at price parity with traditional meat on the market[21].

While the efforts and progress made toward establishing the cultivated meat industry have been tremendous, there are still numerous production, economical, and regulatory hurdles which must be overcome before there will be widespread production and consumption of cultivated meat. In the early stages of production, a major challenge is choosing the best cell source for large scale growth and differentiation. The ideal cell source will be able to grow in a bioreactor and will likely need a microcarrier to support growth for adherent cell types. Other production obstacles include eliminating the use of animal products in the growth media, designing suitable bioreactors, microcarriers, and scaffolding to support the mature tissue, and reducing the cost of production. Aside from production obstacles, there are challenges associated with consumer acceptance and regulatory approval which also must be considered.

Chapter 2: Cell Sources and Requirements

2.1 Potential Cell Sources for Cultivated Meat

There are several cell sources with the potential to produce a cultivated meat product that mimics real skeletal muscle. One method may be to use pluripotent cell sources including embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs)[7]. These cells can be transformed into virtually any cell type, including those which make up the various tissues present in meat: skeletal muscle, adipose tissue, and connective tissue[7]. While ESCs raise ethical concerns due to their origin from an embryo, these concerns can be avoided through the creation

of iPSCs from adult somatic cells. Pluripotency can be induced through the expression of four transcription factors: c-Myc, Klf4, Oct3/4, and Sox2[22,23]. Creation of iPSCs has traditionally been done in mouse or human cells, using the transcription factor sequences originating from that respective species. However, the species conservation of these four transcription factors between human/mouse and livestock animals is remarkable[24]. Specifically, cattle, goat, horse, pig, sheep, and rabbit have a minimum of 84% sequence similarity to mouse or human, demonstrating the possibility of iPSC line development in these species[24]. Several studies have demonstrated the successful development of porcine, bovine, ovine, and chicken iPSC lines with comparable efficiency to mouse and human iPSCs[25–30]. However, most iPSCs developed from livestock animals to date have inadequate transgene silencing, resulting in a lack of complete pluripotency[25]. This ultimately affects the progression of these iPSCs down the desired cell lineage[25]. While the use of pluripotent stem cells has promise, reprogramming strategies can be complex and costly, and additional research needs to be conducted into the development of pluripotent cell lines from livestock species[7].

Another cell source which can be used for production of cultivated meat is primary cells harvested directly from an animal. A skeletal muscle biopsy can be taken from a live or post-mortem animal, eliminating the need to kill the animal for meat production. Adult stem cells, including satellite cells, mesenchymal stem cells (MSCs), or fibroadipogenic progenitor cells (FAPs) may then be isolated from the skeletal muscle[7]. In combination, these three cell types can make up every tissue present in meat[7]. MSCs can be isolated from bone or skeletal muscle and differentiated into myoblasts, adipocytes, fibroblasts, and endothelial cells[7,31]. FAPs may be isolated directly from the interstitial space of skeletal muscle or created by directed differentiation of MSCs[7,31]. FAPs may then be used to make up the connective tissue or lipid

content of meat by differentiation into fibroblasts or adipocytes, respectively[7]. Finally, satellite cells are myogenic stem cells that can differentiate into mature myofibers, making up the bulk of a meat product.

2.2 Muscle Progenitor Cells

Satellite cells reside underneath the basal lamina of skeletal muscle fibers[32]. While most of the time they remain mitotically quiescent in adult muscle, satellite cells can be activated upon stimulation such as injury or to induce muscle hypertrophy[33]. Once activated, satellite cells can undergo asymmetric division in which one daughter cell self-renews to replenish the satellite cell pool, and the other becomes an actively proliferating myoblast (Figure 2). For the purpose of this research, satellite cells and myoblasts will be collectively referred to as myogenic progenitor cells (MPCs). MPCs are identified by expression of the canonical paired box transcription factor Pax7[34,35]. When in close proximity to each other, MPCs have the ability to fuse together into one elongated myotube, containing several nuclei. These differentiated myotubes form bundles of mature muscle fibers, composing the macroscopic structure of skeletal muscle. While muscle fibers are abundant in adult skeletal muscle, these cells do not have the ability to proliferate and produce progeny. Thus, actively proliferating MPCs are of interest for use in cultivated meat production. MPCs represent about 30% of the nuclei in young skeletal muscle that is still developing, however, only about 2-7% of the nuclei in adult skeletal muscle are MPCs[36,37]. Therefore, for their use in cultivated meat production, isolation of MPCs must be precise and efficient in order to maximize the cell yield.

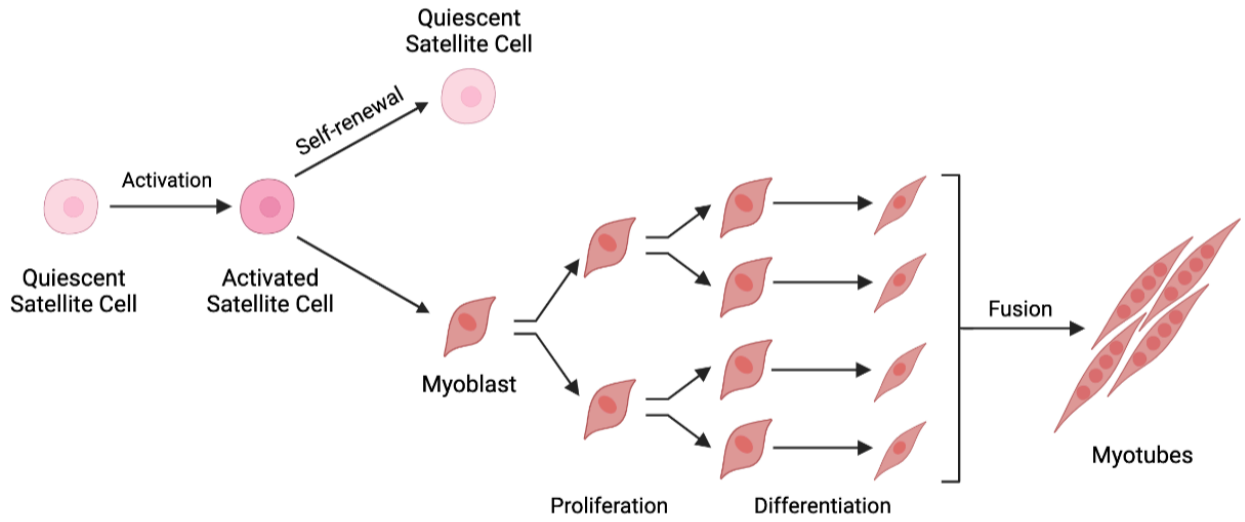

Figure 2. Muscle progenitor cell lifecycle.

MPCs can be isolated directly from a skeletal muscle biopsy of an animal. Factors such as species, breed, age, and sex of the donor animal may impact the behaviour of the isolated cells. Satellite cell number has been shown to decline drastically from birth to adult age and therefore a lower cell yield is expected when harvesting muscle from an adult animal compared to animals still in development[38,39]. Females tend to have fewer satellite cells per fiber and therefore may also result in a reduced cell yield compared to male donors[38]. Additionally, myoblast fusion into myotubes has been shown to be reduced in MPCs isolated from adult cattle and pigs compared to young cattle and pigs.[40] In contrast, myoblast fusion was increased in adult sheep compared to young sheep, pointing out species differences that may occur with differing donor animals[40]. Within a single species, the breed of animal chosen may also have impacts on MPC growth and differentiation. One study comparing MPCs from several cattle breeds found the speed of MPC differentiation to be faster in Belgian Blue cattle compared to the other four breeds assessed[41]. In addition, MPCs from Belgian Blue cattle demonstrated a greater cell longevity shown by a higher fusion index at high passage numbers compared to the other

breeds[41]. This suggests MPCs from Belgian Blue cattle may be the optimal choice for bovine cultivated meat production due to their longer lifespan and greater capacity for differentiation. These intra-species differences suggest the breed chosen for cell sourcing may have profound impacts even in the later stages of cultivated meat production.

2.3 Basic Cell Culture Requirements

Standard mammalian cell culture requires nutrient supplementation delivered to the growing cells through a growth medium. The most common supplement to cell culture media is an animal derived serum, usually fetal bovine serum (FBS)[42]. FBS has become the gold standard in cell culture due to its nutrient rich composition that is essential for cell maintenance[43]. However, there are numerous concerns about the use of FBS for production of cultured meat. First, ethical concerns are raised due to the high number of fetal cows that are sacrificed for collection of FBS, and the use of FBS during production of cultured meat introduces an additional, unwanted animal product[44,45]. Additionally, the increasing cost of FBS makes the use in large scale production unattainable[46]. Lastly, the exact composition of FBS is undefined and has considerable batch-to-batch variability due to seasonal and geographical variation[47]. Overall, an expensive, animal derived product with a poorly defined composition is less than optimal for the purposes of cultivated meat.

As an alternative, serum free media have been developed to eliminate the need for animal product supplementation. However, these serum-free alternatives can still be very expensive due to the addition of recombinant growth factors[48]. Growth factors such as basic fibroblast growth factor (bFGF), insulin like growth factor (IGF), and transforming growth factor beta (TGFβ) are essential for basic cellular processes but account for majority of the cost of these alternative

media[48,49]. To reduce the cost associated with production of recombinant growth factors, products derived from plant or fungal sources may be used. For example, molasses and yeast extract could supply many of the essential amino acids, vitamins, and carbohydrates needed for cell survival and could be used in combination with other plant or fungal sources to support cell growth[49]. These products may act as partial replacements to the expensive growth factors required but must be investigated further[49]. One other potential alternative to animal serum may be to use part of or whole chicken eggs as a nutrient supplement to the growth media. Chicken eggs are inexpensive and contain all the factors necessary to support growth of a chick embryo, making their use in cell culture plausible. Egg yolk supplementation has been shown to support growth of neuronal, endothelial, and smooth muscle cells, but growth of MPCs using egg derived nutrients has not yet been investigated[50,51].

Adherent cell types such as MPCs often require a surface coating of extracellular matrix proteins to facilitate cell attachment to the culture dish and ultimately improve cell proliferation and differentiation. Matrigel and Geltrex are two frequently used surface coatings, originally derived from a mouse tumor which produced high levels of extracellular matrix proteins[52,53]. While these coatings have been shown to improve MPC growth compared to uncoated surfaces, their compositions are not fully defined and subject to batch-to-batch variability[52,53]. As an alternative, synthetic extracellular matrix proteins such as collagen, laminin, or fibronectin may be used independently or in combination to provide a defined attachment site for adherent cells[54]. These synthetic proteins eliminate the need for an additional animal derived product in culture, but optimization may be required to facilitate cell attachment to the same degree as Matrigel and Geltrex[55].

Chapter 3: Muscle Progenitor Cell Isolation Techniques

3.1 Techniques for Muscle Progenitor Cell Purification

Isolation of muscle progenitor cells (MPCs) from skeletal muscle involves incubation of the muscle tissue in digestive enzymes to free the cells from surrounding tissue. There are many different digestive enzymes which may be used, including collagenases, dispase, pronase and/or trypsin[56]. Protocols for MPC isolation are variable and differ in the type of enzymes used and incubation time which may result in differing populations of cells isolated[56].

While each protocol varies, standard techniques for the isolation of cells from skeletal muscle consistently result in not only the isolation of MPCs, but a mixture of several other cell types, including fibroblasts and endothelial cells[55]. As a result, various protocols have been developed to eliminate the unwanted cells within MPC culture. A simple technique which is used frequently is pre-plating[57]. Pre-plating takes advantage of the innate property of fibroblasts and other non-myogenic cells to adhere to surfaces faster than MPCs, allowing them to be selectively removed from culture[57]. A more recent technique developed to improve the purity of MPC culture is the use of ice-cold treatment (ICT)[58]. ICT uses a cold stimulus to promote detachment of MPCs from the culture surface so they can be separated from unwanted cell types[58]. Both pre-plating and ICT allow for a quick separation of cell types based upon differing adhesion characteristics.

Another method to separate MPCs from other cell types is to use a Percoll density gradient[59,60]. Percoll contains small silica particles which can be diluted to various densities to create a density gradient[59]. When added to the gradient, the isolated cells will settle to their

corresponding density following centrifugation. The cells of interest can then be collected from the appropriate density phase and cultured separately from the other cell types.

Two more involved techniques for the purification of MPCs are fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS). FACS and MACS use antibodies bound to fluorophores or magnetic beads, respectively, to sort out the cells of interest based upon their cell surface receptors. FACS uses flow cytometry to analyze each cell individually and sort based on cell size, granularity, and surface marker expression[61]. On the other hand, MACS uses a magnetic column which is simpler and less expensive than FACS equipment but results in less specific sorting[61]. The markers chosen to sort live cells by FACS or MACS must be localized to the surface of the cell to allow for antibody binding. Expression of numerous cell surface markers have been identified on MPCs, resulting in many possible FACS and MACS sorting strategies. Cell surface markers present on unwanted cells such as fibroblasts may also be identified and excluded from the collected cell population, allowing for highly specific cell selection. MPC surface markers and sorting strategies are discussed further in the next section.

3.2 Muscle Progenitor Cell Markers

MPCs can be identified by the presence of various myogenic proteins that are expressed at different phases throughout the myogenic lifecycle. As mentioned previously, the transcription factor Pax7 is highly expressed in quiescent satellite cells. Satellite cell expression of Pax7 is conserved among rodents, humans, and many livestock species including pigs, chickens, cows, and sheep[62–64]. Once satellite cells are activated and become proliferative myoblasts, they will maintain expression of Pax7, but at reduced levels[56]. These $Pax7^{low}$ cells are committed to the

myogenic lifecycle and can no longer replenish the satellite cell pool[56]. Other transcription factors known as myogenic regulatory factors (MRFs) control the progression of satellite cells throughout the myogenic lineage[65]. There are four MRFs, known as myogenic factor 5 (Myf5), myogenic differentiation protein 1 (MyoD), myogenin, and MRF4[65]. Myf5 is first expressed in satellite cells which will commit to the myogenic lineage[62,66]. MyoD is then expressed in activated satellite cells and proliferating myoblasts, along with Myf5[66]. Increased expression of MyoD, along with the other two MRFs, myogenin and MRF4, initiates the differentiation of myoblasts into myocytes[66,67]. In the early stages of differentiation, muscle creatine kinase expression is induced and can be observed in the cytoplasm of the cell[68]. Finally, in later stages of myoblast fusion and myotube formation, expression of various myosin heavy chain (MHC) isoforms can be observed, forming part of the contractile unit of mature muscle fibers[69].

While MPCs are usually defined by their expression of Pax7, the nuclear localization of Pax7 deems it unusable as a marker for live cell sorting. Therefore, previous researchers have identified numerous cell surface markers expressed by MPCs. Most sorting strategies use cluster of differentiation (CD) markers or cell adhesion molecules (CAMs). Table 1 lists many of the known surface markers expressed on MPCs that have been used for MPC sorting. While these markers have been identified on MPCs, it is important to note that most of them are also expressed on other cell types, making their use alone limited. Successful separation of MPCs from the other cell types present in skeletal muscle requires the use of multiple cell surface markers in combination. To eliminate many of the unwanted cell types further, cell surface markers expressed on the unwanted cells but not on MPCs may also be used as negative markers and excluded from the cell collection. Commonly used negative cell surface markers for MPC sorting are also listed in table 1. Other markers which have been identified on MPCs but not used

for sorting include the calcitonin receptor, caveolin-1, c-Met, epidermal growth factor receptor (EGFR), ICAM-1 (CD54), jagged-1(CD339), M-cadherin, nestin, and syndecan 3[70–78].

Table 1. Cell surface markers used for MPC sorting.

Positive Cell Selection Markers	**Cell Type**	**Species**
Beta 1 integrin (CD29)	Satellite Cells	Mouse[79,80], Cattle[63,81], Pig[82–84], Human[85]
CD34*	Satellite Cells	Mouse[79,86–88]
NCAM-1 (CD56)	Satellite Cells	Mouse[89], Cattle[63], Pig[82,83], Human[85,90–93]
KAI-1 (CD82)	Satellite Cells	Human[94,95]
VCAM-1 (CD106)	Satellite Cells	Mouse[79,96]
MCAM (CD146)	Muscle Interstitial Progenitor Cells	Mouse[97], Human[95,98]
CXCR4 (CD184)	Satellite Cells	Mouse[79,80], Human[92]
CD318	Satellite Cells	Human[94]
Alpha 7 integrin	Satellite Cells	Mouse[79,87,88,99,100], Human[101]
SM/C-2.6	Satellite Cells	Mouse[102,103]
Syndecan 4	Satellite-SP Cells	Mouse[77]
Negative Cell Selection Markers	**Cell Type**	**Species**
Integrin alpha M (CD11b)	Macrophages and Neutrophils	Mouse[87,88,100], Human[92]
PECAM-1 (CD31)	Endothelial Cells	Mouse[87,88,96,99,100,102], Cattle[63], Pig[82,83], Human[71,85,92]
CD34*	Hematopoietic Cells, Endothelial Cells	Human[71,92]
CD45	Hematopoietic Cells	Mouse[80,86–88,96,99,100,102,103], Cattle[63], Pig[82,83], Human[71,85,92]
Mac1	Macrophages and Neutrophils	Mouse[80]
Sca1	Hematopoietic Cells	Mouse[80,86–88,96,99,100,102]
Ter119	Erythrocytes	Mouse[86]

*In contrast to mouse MPCs, CD34 is not expressed in human MPCs and is therefore sometimes used as a negative sorting marker in human tissue.

Chapter 4: Scale-Up Considerations

Standard mammalian cell culture typically involves small cell culture dishes or flasks with a relatively low number of cells and small volumes of growth media, in the range of millilitres. Cell growth for production of cultivated meat, however, will involve an enormous scale up from standard practice, involving trillions of cells grown in bioreactors which may exceed 20,000 litres in volume[104]. As a result, there are numerous considerations which must be taken into account when scaling up cell growth for production of cultivated meat.

4.1 Microcarriers

In order to survive and proliferate, many cell types require paracrine signaling from other cells in their proximity. Therefore, cells must be seeded at a suitable starting density within the bioreactor[105]. This will require sequential upscaling from smaller to larger vessels, known as seed-train scaling, to maintain exponential cell growth[105]. Additionally, cell growth in a bioreactor typically involves suspension culture, unlike standard conditions for MPCs which are dependent on a surface to adhere to. To be grown in a bioreactor, anchorage dependent cell types will require one of the following three processes; growth in a bioreactor with anchoring points such as a fixed bed bioreactor, growth in aggregates, or a surface to adhere to in the form of microcarriers will need to be provided[106]. Aggregate growth involves 3D clusters of cells which use other cells as attachment points. Various cell types have been grown in aggregates, however, poor control over aggregate size often results in inadequate nutrient diffusion to the centre of the aggregate[106,107]. Instead, microcarriers are a more likely solution for MPC suspension culture.

Various types of microcarriers exist, differing based upon their size, surface charge, and surface coating[106]. Bovine myoblasts have been successfully grown on microcarriers in a small

spinner flask[108]. Positively charged, uncoated microcarriers were the quickest to initiate exponential cell growth for the bovine myoblasts, but other microcarriers were deemed more suitable for different cell types, indicating the best microcarrier is cell type specific[108]. Mouse myoblast cell lines, as well as primary rabbit, pig, and chicken MPCs have also been successfully grown on microcarriers, demonstrating the potential for microcarrier supported MPC growth from various species[109–111].

The material used to design the microcarriers will determine how the cells are processed once they have grown to a sufficient density. The simplest option may be to use microcarriers made of edible material so that the cell collection stage does not require their removal. Potential materials for edible microcarriers could include polysaccharides, peptides, and lipids which are already used in the food industry for various reasons[106]. These microcarriers will impact the overall sensory characteristics of the final product and may still be partially removed to reduce the total content[106]. Another option is to use microcarriers made of biodegradable material. MPCs have been grown previously on biodegradable materials such as alginate and collagen[112,113]. Microcarrier degradation may involve thermal, chemical, or mechanical processes, but care must be taken to avoid cell damage[106]. The final option is to use non-edible, non-biodegradable microcarriers which are completely removed in the final growth stage, just prior to cell collection[106]. Microcarrier removal may involve enzymatic dissociation, mechanical detachment, or dissociation using temperature variation[106]. Viable bovine myoblasts have been successfully grown and detached from non-edible microcarriers while maintaining the ability to differentiate, however, dissociation can be difficult and greatly reduce the cell yield[108]. When using non-edible microcarriers, sufficient degradation or removal must be performed to align with regulatory standards of the food industry.

4.2 Bioreactor Design

For cultivated meat production to be economically feasible, the bioreactor design must optimize cell growth and minimize inefficiencies. There are four standardized bioreactor processes termed batch, fed-batch, continuous, and perfusion[114]. Batch bioreactors grow cells to their maximum density with no additions or waste removal, while the fed-batch process involves supplementation with fresh growth media to improve the growth rate[114]. Continuous culture involves constant supplementation with fresh growth media at various rates, while waste is collected in a separate vessel[114]. Finally, perfusion culture is similar to continuous culture but with a cell collection method to harvest the growing cells and recycle the used media[114]. Each of these processes may be used at different stages of production to maximize cell collection while minimizing cost.

There are many different bioreactor designs, each using different technologies to optimize cell growth. A stirred tank bioreactor is most often used for mammalian cell culture[115]. The constant mechanical mixing maintains the cells in suspension and can achieve high cell densities at a manageable cost[115]. While these bioreactors have been successfully used with mammalian suspension cells, they may result in higher cell death of MPCs which are less resistant to the shear stress associated with mechanical mixing[104]. Other bioreactors such as packed bed or hollow fiber bioreactors may be used as an alternative to stirred tank bioreactors, with reduced shear stress[115]. These bioreactors make use of microfibers to create a large surface area for growth of adherent cells and allow nutrient supplementation through the fiber lumen, rather than by mechanical mixing. However, these bioreactors are often single use resulting in more waste production, and the complex design results in an increased cost[115].

The chosen bioreactor process must allow for sufficient nutrient delivery to all cells within the bioreactor, as well as waste removal. Adequate oxygen levels may be established using spargers which will maintain the dissolved oxygen content[116]. This method of oxygen delivery by aeration results in bubbling of the growth media which is not optimal for cell viability[104]. As an alternative, oxygen may be supplied upstream to ensure sufficient oxygen saturation before the growth media is provided to the bioreactor[104]. Carbon dioxide sensors will also likely be employed to ensure the pH is kept in range to maintain optimal conditions[104,116]. Finally, waste products must be efficiently removed to prevent growth inhibition. Ammonia and lactate are two by products of normal cellular processes which inhibit cell growth[117]. Careful monitoring of these metabolites will be required to achieve maximum cell densities, and waste removal through perfusion processes may be required[117].

4.3 Scaffolding

Once the cells are harvested from the bioreactor, they will need to be seeded into a scaffolding material which will provide structural integrity to the final product. The scaffold will allow adherence and differentiation of the seeded cells to create tissue that is representative of traditional meat. Scaffolds have been developed for human organ generation in which replicating the structure is extremely important for the creation of functional tissue[118]. These tissue engineering techniques can be used in cultivated meat production with much less precision, as the final structure needs to mimic the texture of normal meat, but not function[118]. Like the microcarriers used for cell expansion, the scaffolding material will likely be made of inexpensive and edible or biodegradable materials[119]. Scaffolds made of proteins present in the extracellular matrix such as collagen and gelatin have been proposed, however, derivation of these proteins from animals is not ideal and the use of recombinant proteins will greatly increase cost[119].

Instead, plant derived protein isolates from plants such as soy, wheat, or oats may provide a suitable scaffolding material at a much lower cost[119]. These plant proteins may need further modifications to improve cell adhesion to the same degree as the extracellular matrix proteins[119]. Other suitable and inexpensive scaffolding materials are plant-derived polysaccharides such as starch or cellulose[119]. Whether the scaffold is made of proteins or polysaccharides such as cellulose, it will contribute to the overall protein or fiber content of the final product and can be used to improve the nutritional content[120].

Synthetic scaffolds may be created in various structures, including porous scaffolds, fibrous scaffolds, or in the form of hydrogels[121]. Porous scaffolds are a sponge like structure with many empty spaces which allow for cell attachment and nutrient diffusion throughout[121]. The structure and texture of these scaffolds would be designed to mimic the perimysium of skeletal muscle[121]. On the other hand, fibrous scaffolds would be made of elongated fibers, with empty spaces between the fibers allowing for cell growth and nutrient circulation[121]. For the creation of structured meat products, fibrous scaffolds may be better suited than porous scaffolds for the formation of aligned myofibers which are present in structured meat[121]. Hydrogels, which are polymer matrices, would provide another scaffolding option that would very closely mimic the extracellular matrix[121]. The hydrogel should be flexible, allowing for the cells to remodel the hydrogel shape as they migrate throughout it[121]. Parts of the hydrogel may eventually be replaced by the extracellular matrix of the seeded cells to create a texture very similar to traditional meat[121].

Decellularization of plants or fungi may also be useful to provide structural support to the growing cells[119]. Unlike decellularized animal tissue which leaves behind just the extracellular

matrix, plant and fungus decellularization leaves intact cell walls in which animal cells could then be seeded into[119]. These scaffolds may need additional processing to support adherence and growth of animal cells, and the decellularization process must be performed with non-toxic chemicals[119]. Lastly, a scaffold free, cell sheet approach may be used to avoid the need for additional scaffolding materials[121]. This approach entails growing 2D cell sheets which are stacked together into a 3D structure and relies on the cells to secrete their own extracellular matrix[121]. This technique has been less studied than the other scaffolding approaches and may require a substantial amount of space to grow the 2D structures[121].

PURPOSE

Conventional meat production raises sustainability and ethical concerns and may not sustain the growing population. Cultivated meat may address these concerns, however, which cell source(s) represent the most ideal population for the cultivated meat industry still requires much study. Muscle progenitor cells (MPCs) harvested from skeletal muscle are a promising cell source to make up the bulk of a cultivated meat product. While numerous protocols have been developed for the isolation of MPCs, standard protocols for the isolation and culture of MPCs are not well defined and often result in impure cell populations. Therefore, the purpose of this research is to optimize the isolation of MPCs to produce a pure population of myogenic cells and provide the industry with standardized procedures for production.

METHODS

Primary Cell Isolation

Muscle Collection

Wild type C57BL/6 mice were euthanized via cervical dislocation. Tibialis anterior, extensor digitorum longus, gastrocnemius, plantaris, soleus, and quadriceps muscles were collected, weighed, and placed into a cell culture dish containing a small volume of 1X Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific, Gibco™, catalog #14190144).

Cell Isolation

Following collection, mouse hindlimb muscles were immediately brought into a sterile environment for cell isolation. A mixture of digestive enzymes (see below) dissolved in Ham's F10 Nutrient Mix (Thermo Fisher Scientific, Gibco™, #11550043) was prepared in a 50 mL conical tube. The muscles were shredded using forceps and transferred into the conical tube for enzymatic digestion. The muscle slurry was incubated for a total of 90 minutes in a shaking incubator at 37°C. The muscle slurry was then filtered through a 100 µm cell strainer followed by a 40 µm strainer to remove large pieces of tissue. The strainer was flushed with 1X DPBS to collect any remaining cells. The cell suspension was centrifuged for 4 minutes at 400 g and the supernatant was discarded. The pellet was resuspended in growth media made of Ham's F10 Nutrient Mix containing 20% fetal bovine serum (FBS; Thermo Fisher Scientific, Gibco™, catalog #10437028), 4 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, catalog #AFL3718) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Gibco™, catalog #15140122). The cells were plated and put into an incubator at 5% CO_2 and 37°C.

Methods for Enzymatic Digestion

To obtain the optimal cell population, three methods of enzymatic digestion were compared. Method 1 invovled incubation of the harvested muscles in Ham's F10 media containing 4 U/mL dispase II (Thermo Fisher Scientific, Gibco™, catalog #17105041) and 112.5 U/mL of collagenase IV (Thermo Fisher Scientific, Gibco™, catalog #17104019) for 45 minutes. The muscle slurry was centrifuged at 400 g for 4 minutes and the supernatant was discarded. The pellet was then resuspended in 0.05% tryspin-ethylenediaminetetraacetic acid (EDTA; Thermo Fisher Scientific, Gibco™, catalog #25200056) and incubated for another 45 minutes. Method 2 involved incubation in Ham's F10 media containing 4 U/mL dispase II and 0.5 U/mL collagenase B (Sigma-Aldrich, catalog #COLLB-RO) for 90 minutes. Method 3 involved incubation in Ham's F10 media containing 4 U/mL dispase II, 112.5 U/mL collagenase IV, and 112.5 U/mL collagenase I (Thermo Fisher Scientific, Gibco™, catalog #17018029) for 90 minutes.

Cell Culture

C2C12 Cells

The C2C12 mouse myoblast cell line (ATCC, Manassas, VA) was used as a positive control. C2C12 cells were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Gibco™, catalog #11995065) supplemented with 10% FBS. Growth media was changed every two to three days and cells were passaged when they reached a confluence of approximately 80%. To induce differentiation, the growth media was replaced with differentiation media made of high glucose DMEM containing 2% horse serum (Thermo Fisher Scientific, Gibco™, catalog #26050088).

HEK293 Cells

The human embryonic kidney (HEK293) cell line (ATCC, Manassas, VA) was used as a negative control. HEK293 cells were grown in high glucose DMEM supplemented with 10% FBS. Growth media was changed every two to three days and cells were passaged when they reached a confluence of approximately 80%.

Mouse Primary Cells

Mouse primary cells were grown on collagen coated plates in media made of Ham's F10 Nutrient Mix containing 20% FBS, 4ng/mL bFGF and 1% penicillin-streptomycin. The plates were coated with 5 $\mu g/cm^2$ of rat tail collagen (Thermo Fisher Scientific, Gibco™, catalog #A1048301) diluted in 20 mM acetic acid. The first media change was performed two to three days following cell isolation and every two days after that. The cells were passaged when they reached a confluence of approximately 80%. To induce differentiation, the growth media was replaced with differentiation media made of DMEM containing 2% horse serum.

Pre-plating Protocol

To enhance the proportion of myogenic cells, several pre-plates were performed. Cells were removed from the plate using 0.25% trypsin-EDTA and replated in growth media on a new dish. The cells were incubated at 37°C and 5% CO_2 for a specific length of time ranging from 1 to 3 hours. The growth media along with any non-adherent cells were removed and plated in a new dish. Any cells that remained on the dish following the 1-to-3-hour incubation were assumed to be contaminating cell types, thus they were discarded.

Ice-Cold Treatment

The ice-cold treatment (ICT) protocol described by Benedetti et al. (2021) was used to enhance myogenic purity of the isolated primary cells[58]. Briefly, the dish containing the heterogenous cell population was washed three times with 1X DPBS at room temperature. Ice-cold DPBS was then added to the dish and placed on ice for 30 minutes to allow for selective detachment of the MPCs. The detached cells were collected, centrifuged, and resuspended in growth media before plating into a new dish.

Proliferation Assays

The proliferation rates of various cell types were measured. An equal number of cells were plated in three wells of a 12 well plate then incubated at 37 °C and 5% CO_2. Cell counts were done in triplicate 24- and 48-hours following plating. The Bio-Rad TC20 Automated Cell Counter was used for counting and trypan blue staining was used to measure cell viability.

Adenoviral Infection

To test the myogenicity of the isolated primary cells, an adenovirus with a muscle creatine kinase (MCK) promoter and enhanced green fluorescent protein (eGFP) reporter was used (Ad(RGD)-tMCK-eGFP; Vector Biolabs). Cells were plated in a 24-well plate and infected with the adenovirus in a low volume of growth media. The cells were transduced with a multiplicity of infection of approximately 160. After six hours, the media was topped up to a normal volume. An adenovirus with a cytomegalovirus (CMV) promoter and GFP reporter was used as a control (Ad(RGD)-CMV-GFP; Vector Biolabs). The cells were imaged with a fluorescence microscope 24- and 48- hours following infection.

Immunocytochemical Analysis

As a measure of myogenic differentiation, embryonic myosin heavy chain (eMyHC) expression of the primary cells was observed. After culturing in differentiation media for at least five days, cells were washed with 1X DPBS and fixed in 4% paraformaldehyde ((Sigma-Aldrich, catalog #P6148) for a minimum of 15 minutes. After fixation, the cells were washed with 1X DPBS then incubated in blocking and permeabilizing solution made of 1% bovine serum albumin (BSA; BioShop, catalog #ALB003) and 0.2% Triton X-100 (Bio-Rad, catalog #1610407) for 1 hour at room temperature. The eMyHC primary antibody (F1.652, 1:10, Developmental Studies Hybridoma Bank (DSHB)) diluted in the blocking and permeabilizing solution was then added onto the cells and left overnight at 4°C. The next day, the cells were washed twice with 1X DPBS then incubated with the appropriate secondary antibody (Alexa Fluor™ 488 Goat anti-Mouse IgG1, 1:750, Thermo Fisher Scientific, Gibco™, catalog #A28175) diluted in the blocking and permeabilizing solution for 1 hour at room temperature. The secondary antibody was removed, and the cells were washed twice with 1X PBS. If needed, DAPI nuclear stain (BioShop catalog #DAP444) was added to the cells before imaging using a fluorescence microscope.

Microscopy

All images were taken on a Nikon Eclipse Ti microscope and analyzed on the NIS-Elements ND2 software (Nikon, Mississauga, ON).

Flow Cytometry and Fluorescence Activated Cell Sorting

Viability Staining

Following cell isolation as above, all primary cells isolated from the mouse skeletal muscle were plated on a collagen coated plate overnight. The following day, the plate was washed three times with 1X DPBS to remove debris. The cells were removed from the plate using 0.25% trypsin-EDTA, then filtered through a 40 μm strainer to minimize clumps. The cells were centrifuged at 300 g for 4 minutes and the supernatant was discarded. The cell pellet was resuspended in a fixable viability dye (LIVE/DEAD™ Fixable Lime (506) Viability Kit, Thermo Fisher Scientific, Invitrogen™, catalog #L34989) diluted in 1X PBS (1:1000) and incubated at room temperature for 15 minutes. The cells were centrifuged at 300 g for 4 minutes and washed once with 1X DPBS before further staining.

Flow Cytometry

Fixed cell flow cytometry was performed to analyze Pax7 expression of the various cell types. Methods were adapted from McKay et al. (2010)[93]. The collected cells were fixed dropwise in 70% ethanol at 4°C for a minimum of 15 minutes. Cells were washed in 1X DPBS then resuspended in a combined blocking and permeabilizing solution (2% BSA and 2% Triton X-100 in 1X DPBS) and incubated at room temperature for 1 hour. At this point, a small aliquot of the cells was kept separate to be used as a negative control. The cells were then pelleted and resuspended in 500 μL of Pax7 primary antibody supernatant (DSHB). Following incubation on ice for 45 minutes, the cells were pelleted and washed twice with the same blocking and permeabilizing solution. Both the main cell sample and negative control were resuspended in the appropriate secondary antibody (Alexa Fluor™ 488 Goat anti-Mouse IgG1, 1:750) diluted in the

blocking and permeabilizing solution. Following incubation at room temperature for 20 minutes, the cells were pelleted and washed twice with 1X DPBS. A Beckman Coulter Cytoflex LX flow cytometer was used to analyze the samples. Single stained C2C12 cells were used for compensation. Gating strategies were optimized to exclude small debris and large cell clumps, as well as dead cells indicated by the viability stain. The negative control sample was used to gate cells negative for Pax7.

Live cell flow cytometry was performed to analyze expression of several cell surface markers of the various cell types. Following viability staining, the cells were pelleted and resuspended in the appropriate primary conjugated antibodies (CD34: Alexa Fluor® 647 Rat anti-Mouse CD34, 1:100, BD Pharmingen™, catalog #560233; NCAM1: BV786 Rat anti-Mouse CD56, 1:500, BD OptiBuild™, catalog #748100; CD31: PE-Cy™7 Rat anti-Mouse CD31, 1:500, BD Pharmingen™, catalog # 561410; CD45: PE Rat Anti-Mouse CD45, 1:500, BD Pharmingen™, catalog #567111) diluted in FACS buffer (3% FBS and 5mM EDTA in 1X PBS). The cells were incubated in the dark on ice for 45 minutes before being pelleted and washed with 1X BSA. A Beckman Coulter Cytoflex LX flow cytometer was used to analyze the samples. Single stained C2C12 cells were used for compensation. Gating strategies were optimized to exclude small debris and large cell clumps, as well as dead cells indicated by the viability stain.

Fluorescent Activated Cell Sorting (FACS)

FACS was performed using methods adapted from Nederveen et al. (2019)[122]. Following staining with the viability dye, a small aliquot of cells was kept separate to be used as a negative control. The remaining cells were resuspended in NCAM1 (1:500) and CD34 (1:100) primary conjugated antibodies diluted in FACS buffer (3% FBS and 5mM EDTA in 1X PBS). The cells were incubated in the dark on ice for 45 minutes before being pelleted and washed with FACS

buffer. Prior to sorting, collection tubes were filled with growth media. The BD FACSAria II Cell Sorter was used for sorting the cells. Single stained C2C12 cells were used for compensation. Gating strategies were optimized to exclude small debris and large cell clumps, as well as dead cells indicated by the viability stain.

Statistical Analysis

All statistics were performed using GraphPad Prism version 9.4.1. For comparisons of two groups, a student's T test was conducted. For comparisons of three or more groups, an ordinary one-way ANOVA followed by Tukey's post-hoc test was performed. For analysis of two variables, a two-way ANOVA followed by Sidak's multiple comparisons test was performed.

RESULTS

Neither pre-plating nor ice-cold treatment (ICT) improved purity of myogenic cells harvested from mouse skeletal muscle.

Mouse hindlimb muscles were harvested and the cells were isolated using standard procedures. Following isolation, the cells were cultured for seven days before being switched to differentiation media. Few myotubes were present after culturing in differentiation media for five days, indicating the MPCs were either scarce in culture or were not capable of differentiating (Fig. 3B). To improve growth and survival of the MPCs, basic fibroblast growth factor (bFGF) was added to the primary cell media because it has been shown to stimulate MPC growth [123]. Additionally, plates were coated with collagen to increase adherence of the proliferating MPCs. The cells grown with bFGF supplementation were differentiated again on collagen coated plates. After five days of differentiation, some cells adopted an elongated shape, but were not multinucleated (Fig. 3C).

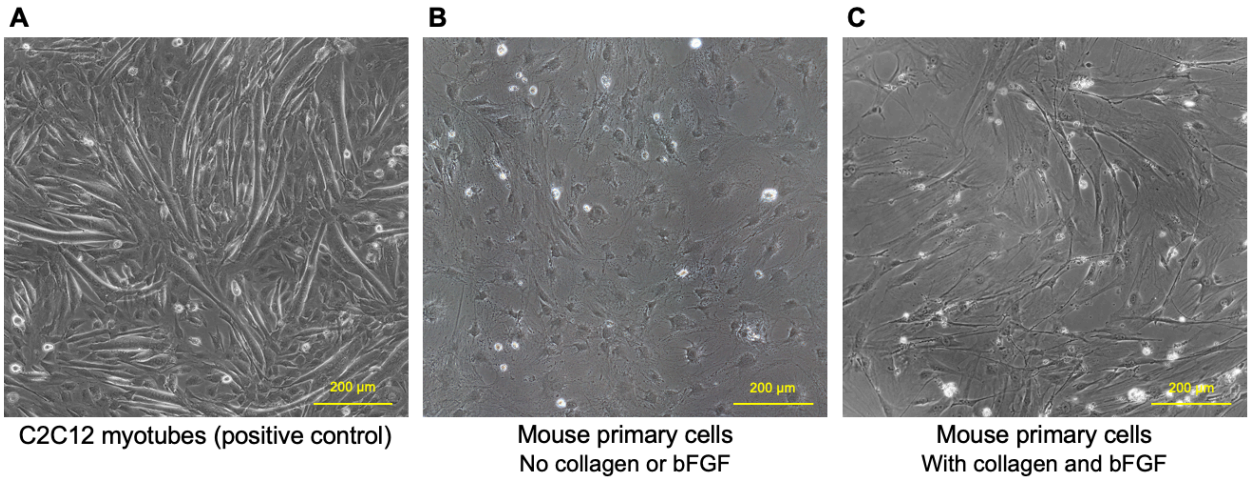

Figure 3. (A) Representative phase contrast image of C2C12 myotubes. This mouse myogenic cell line was used a positive control for comparison with primary cells. (B) Representative phase contrast image of mouse primary cells grown on an uncoated plate, without bFGF supplementation. (C) Representative phase contrast image of mouse primary cells grown on a collagen coated plate, with bFGF supplementation.

To purify the MPCs following isolation, the mouse primary cells were subjected to either the ice-cold treatment (ICT) protocol or a pre-plating protocol of 1, 2, or 3 hours. The ICT protocol resulted in a great reduction in cell yield, taking on average 10 days for the culture dish to reach confluence after the initial harvest compared to the pre-plating protocol, in which the cells took approximately four days to reach confluence. The cells cultured following a 1-hour pre-plate had a significantly lower proliferation rate compared to cells cultured following a 2- and 3-hour pre-plate (Fig. 4). Cells collected by ICT also had a significantly lower proliferation rate compared to cells cultured following a 2- and 3-hour pre-plate, and no difference from the 1-hour pre-plate (Fig. 4). The 2 and 3-hour pre-plates seemed to be too long and resulted in the loss of many cells. It was hypothesized that the slower proliferation rate of the cells cultured following the 1-hour pre-plate and ICT was due to a greater proportion of MPCs in culture, which have a slower proliferation rate than fibroblasts. All further experiments involving a pre-plate used a 1-hour pre-plate.

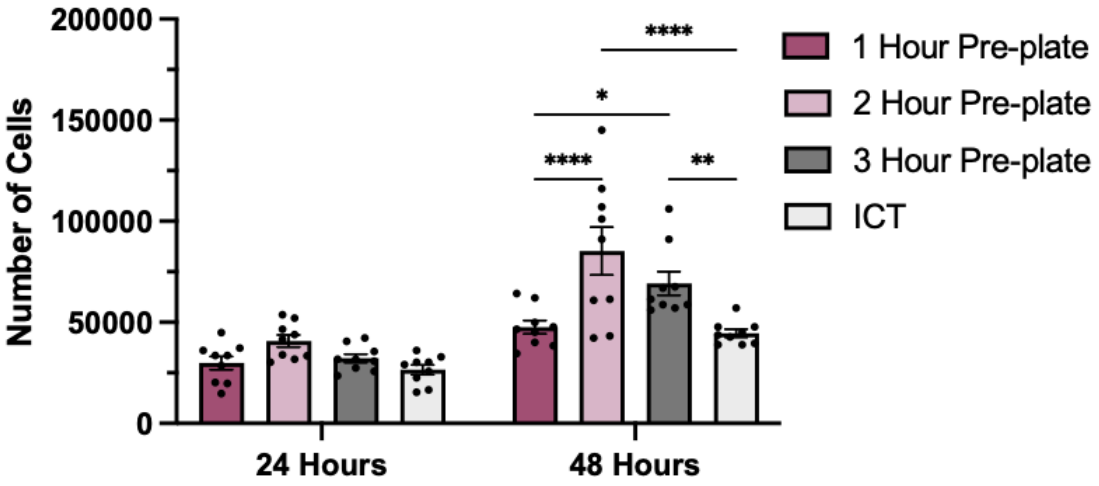

Figure 4. Mouse primary cell proliferation rates following pre-plating or ICT. Cells were plated at time zero and were counted at either 24 hours or 48 hours after plating (n=3). Data shown are mean ± SEM. A two-way ANOVA was performed, and asterisks denote statistical significance (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$). The significant differences due to the main effect of time are not reported here as the time variable was not of interest.

After culturing for ten days, the Pax7 expression of the cells cultured following a 1-hour pre-plate or ICT was analyzed by flow cytometry. An average of 6% or 16% of all live cells were Pax7 positive following the pre-plate or ICT protocols, respectively (Fig. 5A). The low percentage of Pax7 positive cells observed by flow cytometry would indicate many of the cells are non-myogenic, or, less likely, that they have progressed further down the myogenic lineage as to be Pax7 negative. To determine if these isolated cells were myogenic, they were cultured in differentiation media for one day before being infected with an adenovirus with a muscle creatine kinase (MCK) promoter and green fluorescent protein (GFP) reporter tag. No GFP expression was observed following adenoviral transduction into either the pre-plated or the ICT cells, indicating these cells are not myogenic (Fig. 6C, D). To further confirm the pre-plated and ICT cells were not myogenic, they were cultured in differentiation media for 5 days before being stained for embryonic myosin heavy chain (eMyHC). No eMyHC expression was observed in the pre-plated or ICT cells after five days of differentiation (Fig. 7B, C).

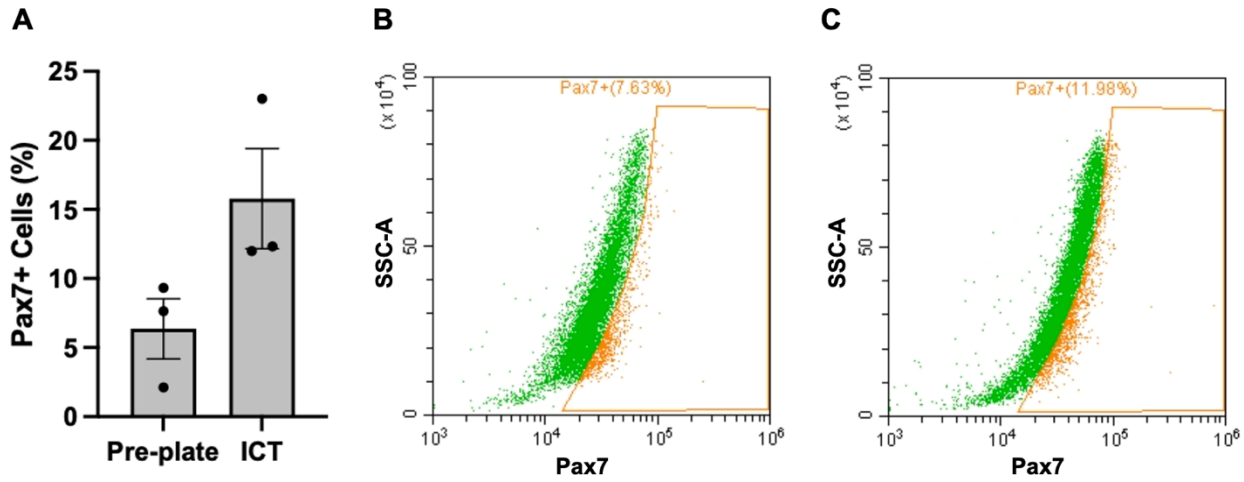

Figure 5. (A) Percentage of pre-plated and ICT cells that express Pax7, analyzed by flow cytometry (n=3). Data shown are mean ± SEM. (B) Representative flow cytometry profile of the Pax7 expression of pre-plated cells. (C) Representative flow cytometry profile of the Pax7 expression of ICT cells. Controls used for Pax7 gating are shown in the appendix.

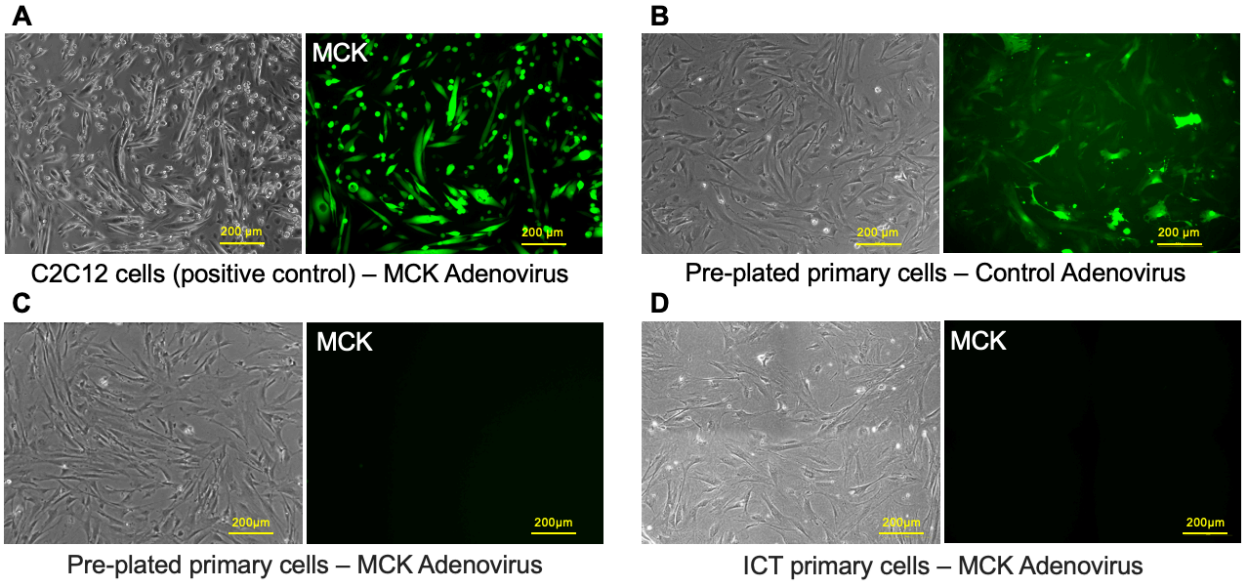

Figure 6. (A) Representative phase contrast (left panel) and immunofluorescent (right panel) images of GFP expression in C2C12 cells from the muscle specific adenovirus with MCK promoter. (B) Representative images of GFP expression in primary cells from the control adenovirus with CMV promoter to demonstrate the capacity of primary cells to be infected by the adenoviral vector. (C) Representative images of pre-plated primary cells infected with the muscle specific adenovirus with MCK promoter. (D) Representative images of ICT primary cells infected with the muscle specific adenovirus with MCK promoter (n=3).

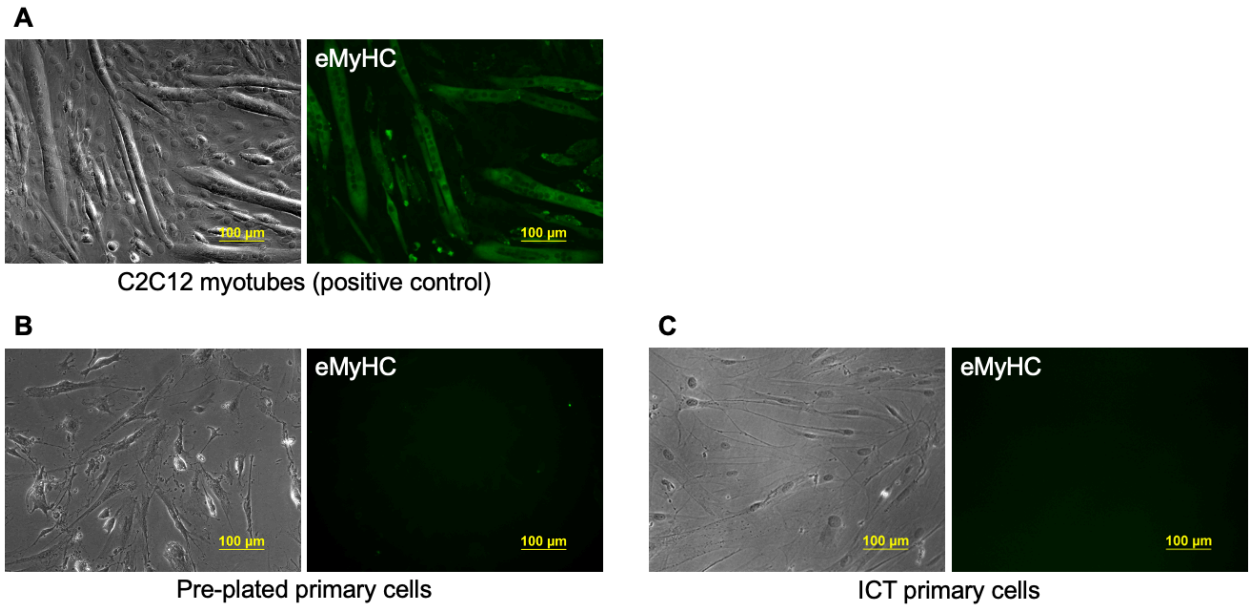

Figure 7. (A) Representative phase contrast (left panel) and immunofluorescent (right panel) images of C2C12 cells stained for eMyHC after five days in differentiation media (B) Representative images of pre-plated primary cells stained for eMyHC after culturing for five days in differentiation media. (C) Representative images of ICT primary cells stained for eMyHC after culturing for five days in differentiation media (n=3).

Optimized muscle digestion for MPC isolation.

To investigate the possibility that the enzymatic digestion of skeletal muscles harvested from mouse hindlimbs was less than optimal, three combinations of frequently used digestive enzymes were compared to determine which enzymes may be most efficient for MPC isolation. A combination of collagenase IV, dispase, and trypsin-EDTA (method 1) resulted in a significantly higher total cell number compared to the other two methods tested (Fig. 8B, C). Following isolation, the cells were fixed and the Pax7 expression was analyzed by flow cytometry. Method 1 resulted in a significantly greater number of Pax7+ cells compared to the other two methods tested (Fig. 8D). Therefore, the digestive enzymes used in method 1 were used for all further experiments.

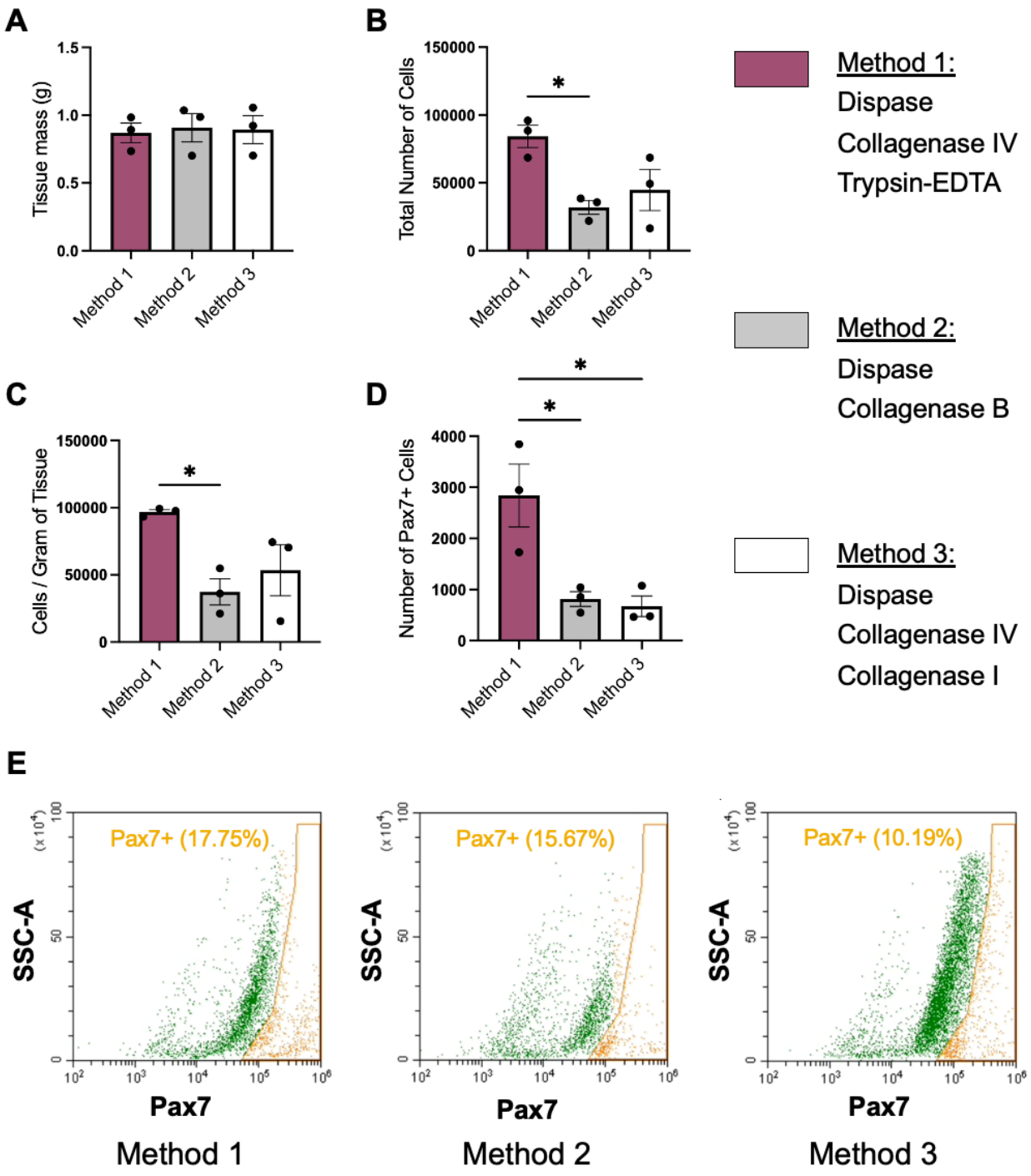

Figure 8. Comparison of digestive enzymes used for isolation of MPCs. (A) Tissue mass harvested to be used for cell isolation by each method. (B) Total number of cells isolated from each method. (C) Number of cells per gram of tissue isolated from each method. (D) Number of Pax7 expressing cells isolated by each method. Data shown are mean ± SEM (n=3). A one-way ANOVA was performed, and asterisks denote statistical significance ($p<0.05$). (E) Representative flow cytometry profiles of the Pax7 expression of cells isolated by each method.

FACS using NCAM and CD34 expression produces distinct subpopulations.

To improve the myogenic purity of the isolated primary cells, FACS was used to sort out the MPCs from other cell types. Expression of two cell surface markers, neural cell adhesion molecule 1 (NCAM1/CD56) and CD34 were observed on C2C12 cells, further supporting their use for sorting mouse primary cells (Fig. 9A). Once isolated, the mouse primary cells were plated overnight then collected and stained for CD34 and NCAM1 the following day. When sorting the primary cells, three cell populations were apparent: CD34-NCAM1-, $CD34^{Hi}$NCAM1-, and CD34+NCAM1+ (Fig. 9B). The CD34-NCAM1- population was not viable in culture and no further analysis was conducted. When cultured, the $CD34^{Hi}$NCAM1- population had a fibroblast like appearance, in contrast to the CD34+NCAM1+ population which appeared small and rounded; consistent with the morphology of quiescent muscle satellite cells[37] (Fig. 9C). When cultured for several days, a difference in proliferation rate between the two cell populations was very apparent. The $CD34^{Hi}$NCAM1- cells reached confluence and required passaging every few days while the CD34+NCAM1+ cells had very few cell divisions during the twelve-day period (Fig. 9D). This type of proliferation rate would be consistent with a population of cells that is more "stem cell- like", exhibiting a more a quiescent state[124]. Cell surface markers expressed on contaminating cell types are commonly used as negative sorting markers to exclude these unwanted cells from the final collection. When profiling the $CD34^{Hi}$NCAM1- sorted cells for CD31 and CD45, markers of endothelial cells and hematopoietic cells, respectively, a heterogenous mixture of cells are present (Fig. 9E)[63]. While majority of the $CD34^{Hi}$NCAM1- cells are negative for both CD31 and CD45, there are still many cells present which are positive for either of these markers (Fig. 9E). In contrast, when profiling the CD34+NCAM1+ cells,

98.5% are negative for both CD31 and CD45 (Fig. 9F). This would indicate the CD34+NCAM1+ cell population is homogenous, and likely myogenic.

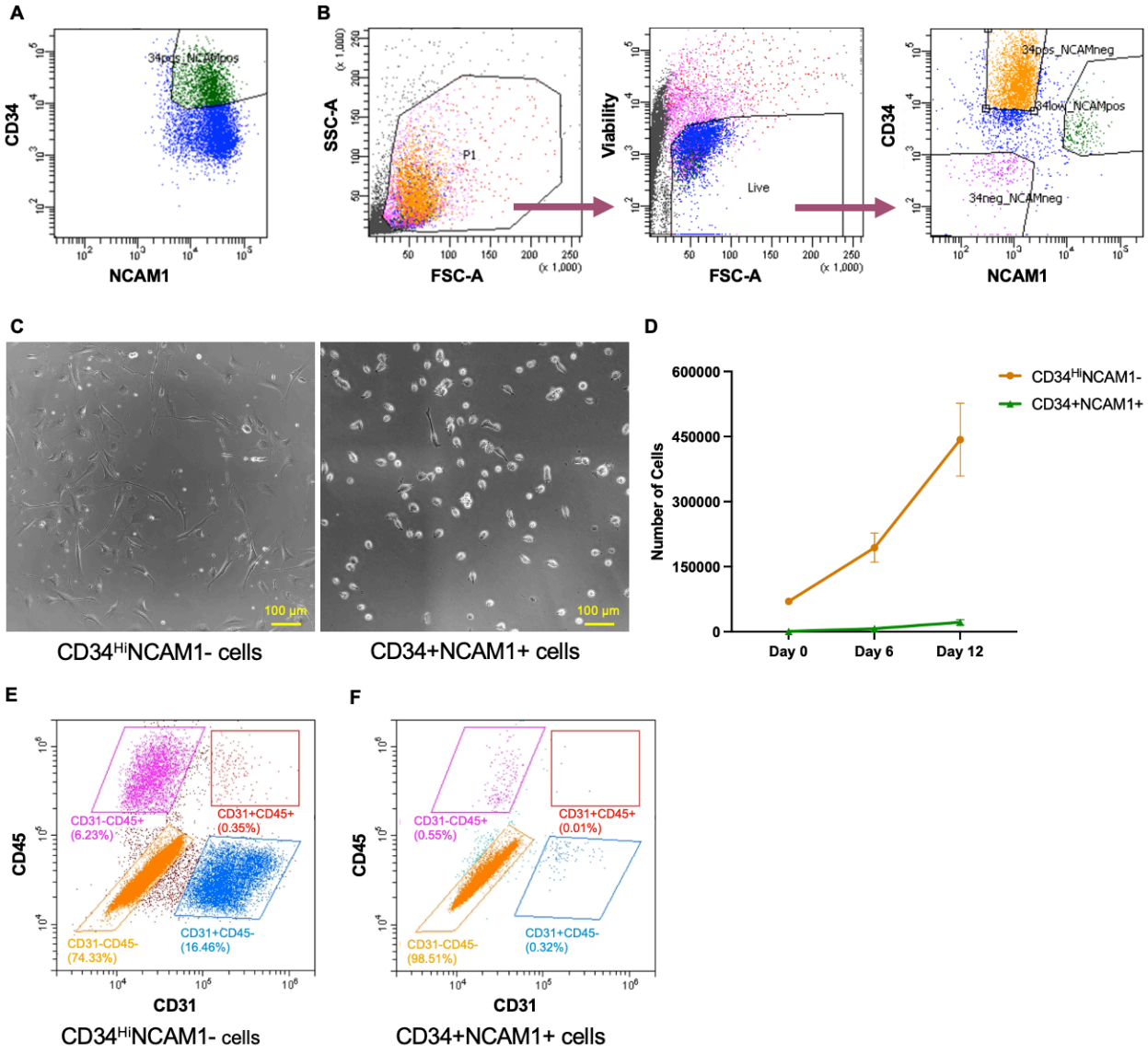

Figure 9. (A) Representative flow cytometry profile of CD34 and NCAM1 expression in C2C12 cells. (B) FACS gating strategy using CD34 and NCAM1. (C) Representative phase contrast images of the differing morphology of $CD34^{Hi}$NCAM1- cells and CD34+NCAM1+ cells. (D) Growth rates of $CD34^{Hi}$NCAM1- cells and CD34+NCAM1+ cells cultured for 12 days (n=3). Data shown are mean ± SEM. (E) Representative flow cytometry profile of CD31 and CD45 expression of $CD34^{Hi}$NCAM1- sorted cells. (F) Representative flow cytometry profile of CD31 and CD45 expression of CD34+NCAM1+ sorted cells.

CD34^{Hi}NCAM1- cells are not purely myogenic.

To assess the myogenic purity of the CD34^{Hi}NCAM1- sorted cells, the Pax7 expression was analyzed by flow cytometry. After culturing in proliferation media for twelve days, an average of 69% of the sorted cells were Pax7+ (Fig. 10A). Despite this significant degree of myogenicity by flow cytometric analysis, no myotube formation was observed following 5 days in differentiation media (Fig. 10C). Consistent with the lack of myotube formation, CD34^{Hi}NCAM1- sorted cells did not express eMyHC following culturing in differentiation media, further demonstrating a lack of myogenic differentiation (Fig. 10C).

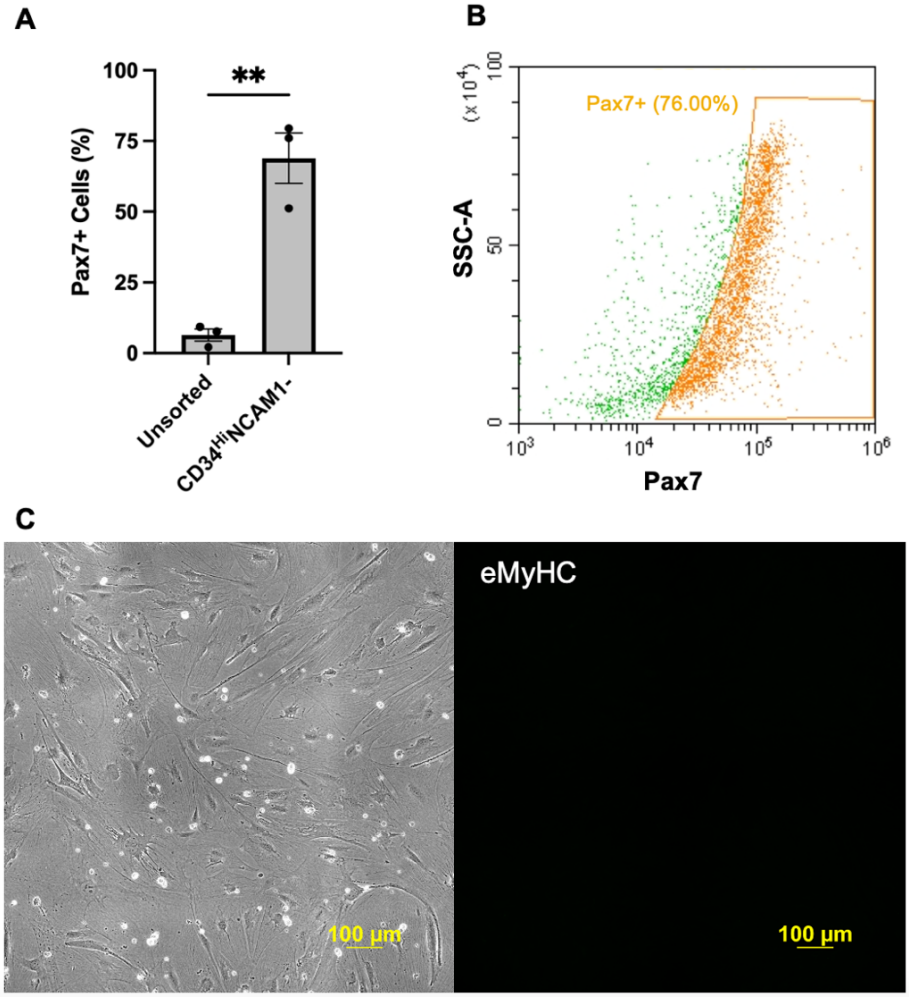

Figure 10. (A) Percentage of unsorted and CD34^{Hi}NCAM1- sorted cells that are Pax7+ (n=3). Data shown are mean ± SEM. An unpaired t-test was performed, and asterisks denote statistical

significance ($p<0.01$). (B) Representative flow cytometry profile of $CD34^{Hi}NCAM1-$ sorted cells. (C) Representative phase contrast (left panel) and immunofluorescent (right panel) images of $CD34^{Hi}NCAM1-$ sorted cells following 5 days in differentiation media, displaying a lack of eMyHC.

CD34+NCAM1+ cells are satellite cells which remain a homogenous population after culturing for several months.

CD34+NCAM1+ sorted cells maintained their slow proliferation rate and remained viable in culture for three months without any visible changes in cell morphology when compared to the first week of culturing (Fig. 11A, B). The expression of CD34 and NCAM1 was reanalyzed by flow cytometry and an estimated 99% of the CD34+NCAM+ sorted cells maintained expression of both these markers after culturing for three months (Fig. 11C). Finally, the Pax7 expression of the CD34+NCAM1+ cells was assessed by flow cytometry. After three months of culturing, 99% of the CD34+NCAM1+ sorted cells were Pax7+ (Fig. 11D). When compared to the C2C12 cell line, the CD34+NCAM1+ sorted cells had very high Pax7 expression demonstrated by the rightward shift in fluorescence on the flow cytometry profile (Fig. 11F, G). The mean Pax7 fluorescence intensity of the C2C12 cells was 81,407 a.u. while the mean Pax7 fluorescence intensity of the CD34+NCAM1+ cells was 369,639 a.u., demonstrating a 4.5-fold increase.

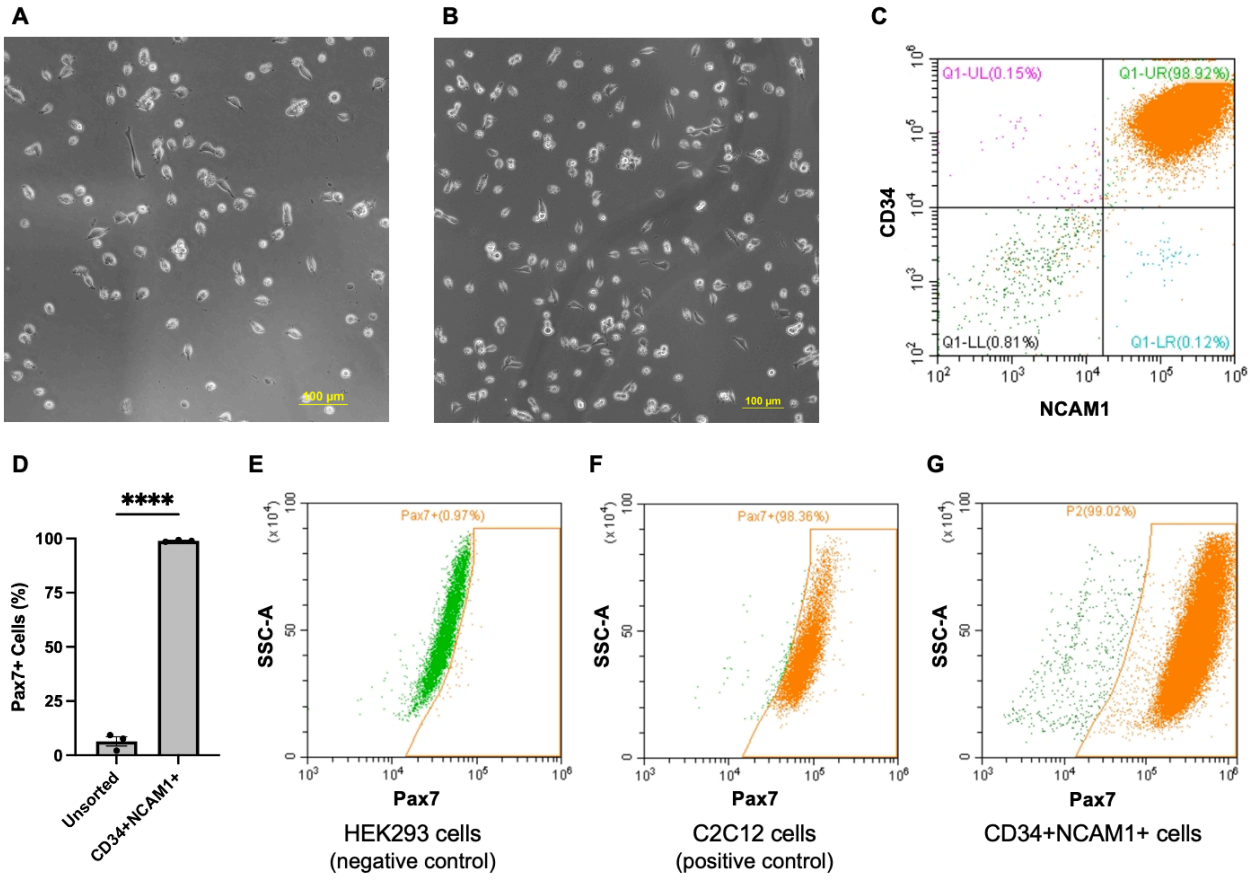

Figure 11. (A) Representative phase contrast image of the CD34+NCAM1+ sorted cells after one week in culture. (B) Representative phase contrast image of the CD34+NCAM1+ sorted cells after three months in culture. (C) Representative flow cytometry profile of the CD34 and NCAM1 expression of CD34+NCAM1+ sorted cells after three months in culture. (D) Percentage of unsorted and CD34+NCAM1+ sorted mouse primary cells that are Pax7+ (n=3). Data shown are mean ± SEM. An unpaired t-test was performed, and asterisks denote statistical significance ($p<0.0001$). (E) Negative control (HEK293 cells) used for gating Pax7 expression. (F) Positive control (C2C12 cells) used for gating Pax7 expression. (G) Representative flow cytometry profile of the Pax7 expression of CD34+NCAM1+ sorted cells.

<u>CD34+NCAM1+ cells maintain their differentiation capacity after culturing for several months.</u>

After culturing for three months, the CD34+NCAM1+ sorted cells were cultured in differentiation media to assess their capacity to form myotubes. The CD34+NCAM1+ cells began to elongate after a few days in differentiation media, and multinucleated myotubes were observed (Fig. 12A). However, CD34+NCAM1+ cell fusion and differentiation were to a lesser extent than the control C2C12 cell line in which larger myotubes with a higher myonuclear

number were observed (Fig. 12B). The differentiated CD34+NCAM1+ myotubes stained positive for eMyHC, further demonstrating their ability to differentiate (Fig. 12C).

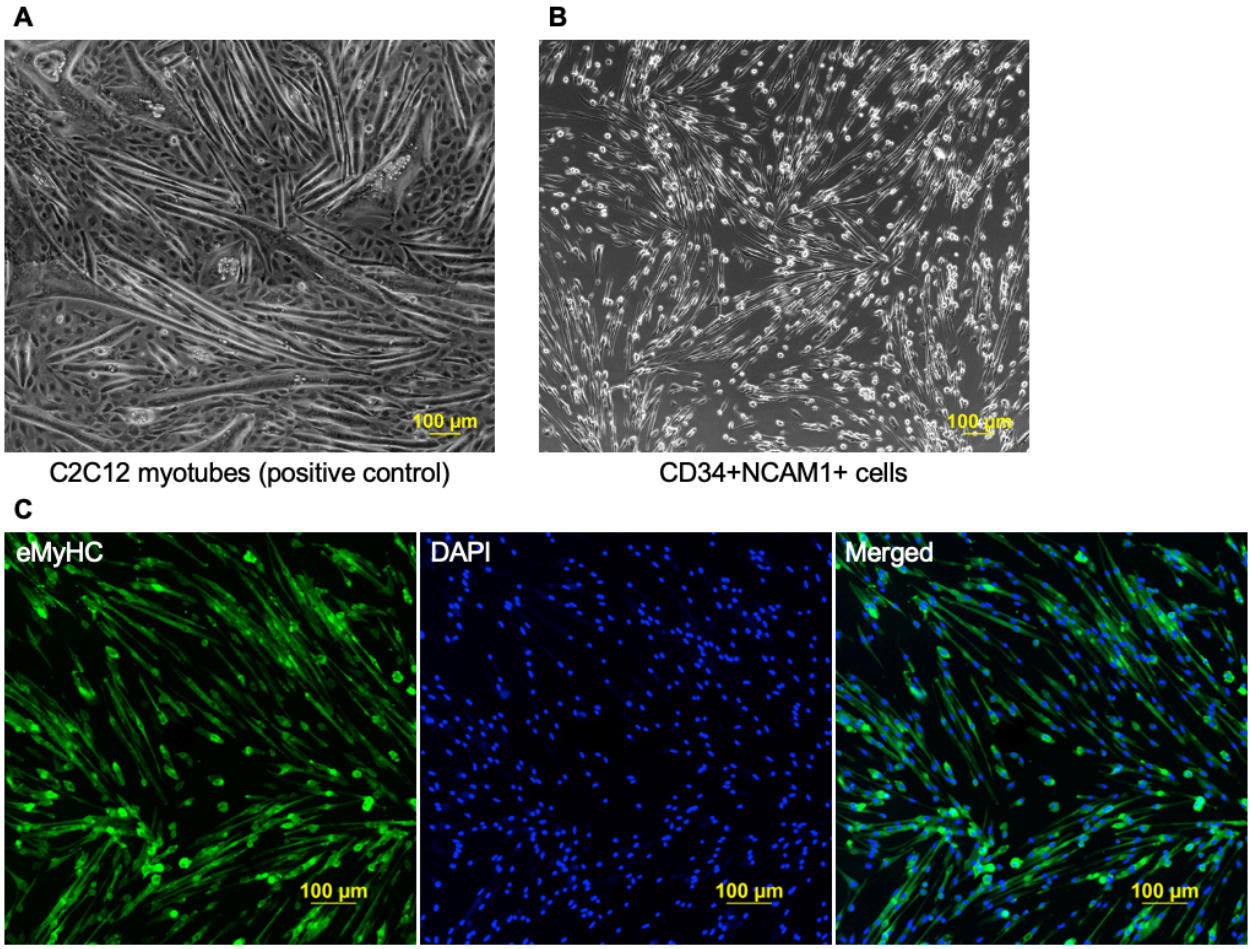

Figure 12. (A) Representative phase contrast image of C2C12 myotubes after culturing in differentiation media for seven days. (B) Representative phase contrast image of CD34+NCAM1+ sorted cells after culturing in differentiation media for seven days. (C) Representative immunofluorescent images of CD34+NCAM1+ sorted cells stained for eMyHC after culturing in differentiation media for seven days (n=3).

DISCUSSION

The global dependence on conventional meat as a significant contributor to our food intake is not sustainable, causing the production of alternative food sources such as cultivated meat to be essential. As the first step towards the creation of a high-quality meat product which meets regulatory standards, characterization of the initial cell source used is extremely important. Several cell sources have been proposed as a potential starting point for cultivated meat, however, a deeper understanding of the cell selection and growth requirements as they apply to the cultivated meat industry is needed. Muscle progenitor cells (MPCs) harvested directly from skeletal muscle have been chosen as the starting cell source for cultivated meat production due to their many advantageous properties over other proposed cell sources. The best methods for MPC isolation, purification, and culturing with respect to cultivated meat production are unclear, demonstrating a need for development of standardized procedures to be used in the cultivated meat industry, and defining the purpose of this research.

Currently used protocols for MPC growth are typically intended for basic in vitro studies and little research has been conducted regarding their application towards the cultivated meat industry[32]. For a product designed for the food industry, media containing as few additives as possible is optimal to reduce cost and to limit additional approval needed for non-food grade components. However, consistent with previous literature, we showed that MPC growth was not supported in basic growth media without supplementation with additional growth factors[123]. Previous studies have shown that media supplementation with basic fibroblast growth factor (bFGF) and a plate coating with type I collagen both increase the growth rate of primary myoblasts in culture[123]. However, our results showed that this alone could not support MPC growth, and further purification was required.

Pre-plating is one method that is very frequently used to improve the MPC fraction in culture. There are numerous different pre-plating protocols published, with large variations in the length of time used, ranging from 5 minutes to 24 hours[125–128]. Other factors such as the use of various plate coatings and growth media also vary between protocols[127,128]. For simplicity, we chose to compare pre-plating protocols of three different lengths of time, however, contrary to several of these published protocols, we observed consistently low myogenic purity. One notable difference between most previously published studies and this study was the number of days the cells were cultured for following the pre-plating protocol. Most studies involved culturing of the pre-plated cells for a very limited period, and few, if any passaging steps were performed before terminal differentiation was induced. In contrast, the cells grown in this study were passaged several times, because this type of expansion would be required when used for cultivated meat production. Additionally, complex plate coatings such as Matrigel were often employed in previous studies, however, these types of plate coatings were avoided in this experiment due to their undefined composition[127,128].

To improve upon the MPC enrichment that pre-plating provides, the ICT method was developed[58]. This technique was reported to produce a satellite cell population with 99% purity[58]. However, when the ICT protocol was performed on the isolated primary cells in this study, the time taken to reach a sufficient cell yield that could be analyzed was very long, and contaminating cell types were still present. The published protocol used a gelatin plate coating instead of collagen and grew the cells in media containing horse serum and chicken embryo extract rather than FBS[58]. Additionally, the skeletal muscle digestion involved different enzymes than the ones used in this study. Therefore, the low cell yield and myogenic purity observed in this study may be attributed to the differences in muscle digestion and culturing protocol used,

ultimately resulting in a cell population that was not susceptible to selective cell detachment by ICT. While changes to both the pre-plating and ICT protocols, such as the use of different growth factors or plate coatings, may have enhanced MPC purification further, these modifications did not align with the goals of this study. The requirements of a cell source to be used in the cultivated meat industry are different than those needed for short term in vitro experiments. Therefore, it was concluded that neither pre-plating nor ICT were suitable methods for the level of MPC purification that is required for the cultivated meat industry.

Fluorescence activated cell sorting (FACS) is frequently used as a method to sort cells with high specificity. There are numerous cell surface markers present on MPCs, and each could be used for cell sorting to produce different subpopulations. CD34 was originally identified as a marker for hematopoietic progenitor cells, while NCAM was identified on nerve cells[129,130]. Since then, expression of both cell surface markers on myogenic cells has been documented numerous times[131,132]. Previous studies have shown isolation of CD34+ cells from mouse skeletal muscle gave rise to both myogenic and non-myogenic cells[87,133,134]. This is consistent with our results in which using CD34 alone was shown to be insufficient for purification of MPCs, demonstrated by a lack of myogenic differentiation when the CD34+NCAM1- cells were cultured. CD34 has been shown to be associated with myoblasts which are committed to differentiation, with reduced levels of Pax7 and increased MyoD expression[135]. It is possible that these committed MPCs were lost when the CD34+NCAM1- cells were passaged numerous times over several days to weeks, explaining the reduced capacity to differentiate when compared to CD34+ cells assessed in previous studies[135]. As CD34 identifies both myogenic and non-myogenic cells, additional markers to be used in combination with CD34 are required for MPC purification. A marker which has been frequently used along with CD34 to purify MPCs from

mouse skeletal muscle is α7 integrin[87,134,136]. One previous study noted around 90% of cells sorted for CD34 and α7 integrin were Pax7 positive, however, using α7 integrin alone resulted in almost the same level of MPC purification as the combination of CD34 and α7 integrin[79]. This brings the use of CD34 for MPC purification into question and demonstrates that other skeletal muscle associated cell surface markers may be better suited.

NCAM has been used in combination with other markers for sorting MPCs from skeletal muscle of several species[83,89,90,137]. CD34 and NCAM have been used in combination to sort human MPC subpopulations, but we have not yet found a study which used this sorting strategy in cells derived from mice[138]. Here we showed that in mice, $CD34^{Hi}$NCAM1- cells had limited myogenic potential while CD34+NCAM1+ cells made up a homogenous MPC population. In humans, CD34+NCAM+ cells have been shown to be both myogenic and adipogenic precursors, dependent on whether they were grown in myogenic or adipogenic growth media[138]. These results are consistent with the mouse CD34+NCAM1+ cells isolated in this study which were shown to be myogenic precursors. Although not investigated, the mouse CD34+NCAM1+ cells isolated in this study could also have adipogenic potential when grown in suitable conditions. This demonstrates an area for future investigation into the potential development of adipocytes from the CD34+NCAM1+ precursors, which may provide another use for these cells in cultivated meat.

We demonstrated that the CD34+NCAM1+ cell population exhibited stem-cell like characteristics, consistent with quiescent satellite cells. However, a study by Capkovic et al. demonstrated that NCAM identified mouse MPCs which were committed to differentiation, contradictory to our results[89]. This study noted that there are multiple NCAM isoforms, and only the 120 kDa isoform was shown to increase myoblast fusion and differentiation[89]. This 120 kDA

NCAM isoform associated with myoblast fusion was the most abundant isoform in the NCAM+ cells isolated by Capkovic et al.[89]. Thus, it may be that a different isoform of NCAM was identified by the antibody used for sorting in our experiments, producing an MPC subpopulation that is more stem-cell like rather than committed to terminal differentiation.

The CD34+NCAM1+ cells were cultured for an extended period without changing phenotypes, demonstrated by a consistent cell morphology over the culturing period, and maintenance of expression of various myogenic markers. This consistency in cell phenotype, with no indication of cell senescence after being passaged several times is an optimal quality for the cell source to be used for cultivated meat. The cell expansion stage of cultivated meat production will require numerous cell doublings to achieve a sufficient yield, and a cell source which remains homogenous during this stage will be essential. The maintenance of Pax7 expression of the CD34+NCAM1+ cells after three months in culture was exceptional when compared to other MPC populations reported in the literature. A study by Ding et al. assessed bovine satellite cells isolated by a different FACS strategy and documented a reduction in Pax7 expression, from 92% of cells expressing Pax7 immediately after sorting, to approximately 45% of passage 5 cells[63]. In comparison, the CD34+NCAM1+ cells isolated in the current study were passaged a minimum of seven times without any reduction in Pax7 expression. Stout et al. created immortalized bovine satellite cells through genetic modification which could double over 120 times and demonstrated Pax7 expression in over 99% of cells following 35 passages[139]. While the CD34+NCAM1+ cells demonstrated consistent behaviour throughout the first seven passages, they should be cultured further to determine the maximum number of population doublings possible. The expression of myogenic markers like Pax7 should be observed again at

higher passages to determine if the myogenic potential is maintained when passaged to a similar extent to the immortalized bovine satellite cells.

Pax7 expression is used as the canonical marker of satellite cells due to its consistent expression and specificity to skeletal muscle cells[140]. A study by Rocheteau et al. demonstrated that Pax7 expression in MPCs exists on a continuum where $Pax7^{Hi}$ cells have low metabolic activity and delayed cell division, and $Pax7^{Low}$ cells are primed to commitment toward the myogenic lineage[141]. $Pax7^{Low}$ cells express markers associated with myogenic commitment and differentiation, including MyoD and myogenin, whereas $Pax7^{Hi}$ cells have little to no expression of these markers, and instead express markers of stemness[141]. In accordance with this is the high Pax7 expression of the CD34+NCAM1+ cells compared to the rapidly proliferating C2C12 myoblasts, suggesting the CD34+NCAM1+ sorted cells are a population of $Pax7^{Hi}$ satellite cells, in the quiescent state.

The final objective of this research was to assess the capacity of the CD34+NCAM+ cells to differentiate into myotubes. The CD34+NCAM+ cells maintained their ability to differentiate after several passages, demonstrated both morphologically and by the expression of eMyHC. Bovine satellite cells used by Ding et al. lost their capacity to differentiate after 10 passages, however, this lack of differentiation could be rescued when the growth media was supplemented with a p38 inhibitor[63]. P38 is a member of the MAPK pathway required for MyoD activation in satellite cells and subsequent progression down the myogenic lineage[142]. While a p38 inhibitor prevents early differentiation of satellite cells, stimulating the p38/MAPK pathway may improve differentiation when desired. Supplementing the differentiation media with additives which activate the p38/MAPK pathway may have the potential to improve CD34+NCAM1+ cell

differentiation to produce larger myotubes with an increased myonuclear number, however, this was not in the scope of this research.

In conclusion, this study provided evidence that standard techniques for MPC isolation and purification are not suitable for the purposes of cultivated meat production. We provided an efficient protocol for skeletal muscle digestion that improves upon MPC isolation over other published protocols. We then identified a FACS strategy that can be used to efficiently purify mouse MPCs which have the capacity to differentiate into skeletal muscle myotubes. The CD34+NCAM1+ population can be maintained in culture for extended periods without deficits in viability or the capacity to differentiate. Together, the described techniques contribute to the standardized protocols required for cell sourcing in cultivated meat production. These experiments provide insight into MPC populations that may exist in livestock species and will help to streamline the early stages of cultivated meat production.

FUTURE DIRECTIONS

While the CD34+NCAM1+ FACS strategy produced a highly pure population of MPCs, their growth rate in culture was remarkably slow. In order for the cell expansion stage to proceed in a timely manner, the cell source that is seeded into the bioreactor must have a doubling time that is drastically higher than the current doubling time of the CD34+NCAM1+ cells. Thus, methods to increase the proliferation rate of the CD34+NCAM1+ cells should be investigated. The growth media used to culture the CD34+NCAM1+ cells contained basic fibroblast growth factor (bFGF) because it is frequently used to promote MPC proliferation[123]. However, the use of other growth factors which have been previously implicated in satellite cell activation and proliferation, such as hepatocyte growth factor (HGF) and platelet derived growth factor (PDGF), may further promote cell proliferation[143]. Additionally, HGF, PDGF, and bFGF have all been reported to reversibly inhibit myogenic differentiation, which may be useful to prevent spontaneous early differentiation of the MPCs while they are grown to high densities in a bioreactor[143,144].

While methods to increase cell proliferation are assessed, future studies should also involve development of an animal product-free, chemically defined growth medium. One potential method to eliminate serum is to perform a slow adaptation protocol in which the use of FBS is reduced in a step wise manner until it is completely absent[145]. This process will require supplementation with additional recombinant growth factors to replace those present in FBS and maintain a suitable cell proliferation rate.

For cultivated meat to mimic traditional meat, the MPCs must be sufficiently differentiated into myotubes to create muscle fibers that are characteristic to the texture of a structured meat product. The CD34+NCAM1+ cells demonstrated some capacity to differentiate,

however, enhancing cell fusion and myotube hypertrophy may improve the sensory characteristics of the final product. Several additives including insulin like growth factor, dexamethasone, and growth hormone have been documented to promote myogenic differentiation in vitro through various mechanisms including activation of myogenic regulatory proteins and prevention of protein degradation in myotubes[143,144,146]. Further investigation into an improved differentiation media formulation should be conducted to maximize the myogenic differentiation of CD34+NCAM1+ cells.

The FACS strategy described was efficient for MPC purification from mouse skeletal muscle, but the use of this sorting strategy in tissue from other species was not assessed. The described cell isolation and sorting protocol should be repeated in tissue collected from livestock species to determine its use to produce cultivated meat from different species.

Finally, adaptation of the CD34+NCAM1+ cells from adherent to suspension culture should be performed. Analysis of various microcarriers and bioreactor designs should be assessed to achieve optimal cell growth required for the cell expansion stage of cultivated meat production.

REFERENCES

1. Mottet, A. *et al.* Livestock: On our plates or eating at our table? A new analysis of the feed/food debate. *Global Food Security* vol. 14 1–8 Preprint at https://doi.org/10.1016/j.gfs.2017.01.001 (2017).
2. *World Livestock 2011. Livestock in food security*. (FAO, 2011).
3. *Livestock's long shadow: Environmental issues and options*. (Food and Agriculture Organization (FAO), 2006).
4. Heinke, J. *et al.* Water Use in Global Livestock Production—Opportunities and Constraints for Increasing Water Productivity. *Water Resour Res* **56**, (2020).
5. Onwezen, M. C., Bouwman, E. P., Reinders, M. J. & Dagevos, H. A systematic review on consumer acceptance of alternative proteins: Pulses, algae, insects, plant-based meat alternatives, and cultured meat. *Appetite* vol. 159 Preprint at https://doi.org/10.1016/j.appet.2020.105058 (2021).
6. Grigg, D. & Sheffield, U. K. *The Pattern of World Protein Consumption*. *Pergamon Geoforum* vol. 26 (1995).
7. Reiss, J., Robertson, S. & Suzuki, M. Cell Sources for Cultivated Meat: Applications and Considerations throughout the Production Workflow. *Int J Mol Sci* **22**, (2021).
8. Chriki, S. & Hocquette, J.-F. The Myth of Cultured Meat: A Review. *Front Nutr* **7**, (2020).
9. Tuomisto, H. L. & de Mattos, M. J. T. Environmental impacts of cultured meat production. *Environ Sci Technol* **45**, 6117–23 (2011).
10. Sinke, P., Swartz, E., Sanctorum, H., van der Giesen, C. & Odegard, I. Ex-ante life cycle assessment of commercial-scale cultivated meat production in 2030. *Int J Life Cycle Assess* **28**, 234–254 (2023).
11. Hubalek, S., Post, M. J. & Moutsatsou, P. Towards resource-efficient and cost-efficient cultured meat. *Curr Opin Food Sci* **47**, (2022).
12. McNamara, E. & Bomkamp, C. Cultivated meat as a tool for fighting antimicrobial resistance. *Nat Food* **3**, 791–794 (2022).
13. Tiseo, K., Huber, L., Gilbert, M., Robinson, T. P. & Van Boeckel, T. P. Global Trends in Antimicrobial Use in Food Animals from 2017 to 2030. *Antibiotics (Basel)* **9**, (2020).
14. Bonny, S. P. F., Gardner, G. E., Pethick, D. W. & Hocquette, J.-F. What is artificial meat and what does it mean for the future of the meat industry? *J Integr Agric* **14**, 255–263 (2015).
15. Siddiqui, S. A. *et al.* Cultured meat: Processing, packaging, shelf life, and consumer acceptance. *LWT* **172**, 114192 (2022).
16. Karesh, W. B. *et al.* Ecology of zoonoses: natural and unnatural histories. *Lancet* **380**, 1936–45 (2012).
17. Espinosa, R., Tago, D. & Treich, N. Infectious Diseases and Meat Production. *Environ Resour Econ (Dordr)* **76**, 1019–1044 (2020).
18. Bhat, Z. F., Kumar, S. & Bhat, H. F. In vitro meat: A future animal-free harvest. *Crit Rev Food Sci Nutr* **57**, 782–789 (2017).
19. Bomkamp, C. *et al. 2022 Cultivated Meat State of the Industry Report*. (2022).
20. Chodkowska, K. A., Wódz, K. & Wojciechowski, J. Sustainable Future Protein Foods: The Challenges and the Future of Cultivated Meat. *Foods* **11**, (2022).

21. Vergeer, R., Sinke, P. & Odegard, I. *TEA of cultivated meat Future projections of different scenarios-corrigendum*. www.cedelft.eu (2021).
22. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–76 (2006).
23. Al Abbar, A., Ngai, S. C., Nograles, N., Alhaji, S. Y. & Abdullah, S. Induced Pluripotent Stem Cells: Reprogramming Platforms and Applications in Cell Replacement Therapy. *Biores Open Access* **9**, 121–136 (2020).
24. Ogorevc, J., Orehek, S. & Dovč, P. Cellular reprogramming in farm animals: an overview of iPSC generation in the mammalian farm animal species. *J Anim Sci Biotechnol* **7**, 10 (2016).
25. Su, Y., Zhu, J., Salman, S. & Tang, Y. Induced pluripotent stem cells from farm animals. *J Anim Sci* **98**, (2020).
26. Ezashi, T. *et al.* Derivation of induced pluripotent stem cells from pig somatic cells. *Proc Natl Acad Sci U S A* **106**, 10993–8 (2009).
27. Esteban, M. A. *et al.* Generation of induced pluripotent stem cell lines from Tibetan miniature pig. *J Biol Chem* **284**, 17634–40 (2009).
28. Cao, H. *et al.* Characterization of bovine induced pluripotent stem cells by lentiviral transduction of reprogramming factor fusion proteins. *Int J Biol Sci* **8**, 498–511 (2012).
29. Li, Y., Cang, M., Lee, A. S., Zhang, K. & Liu, D. Reprogramming of sheep fibroblasts into pluripotency under a drug-inducible expression of mouse-derived defined factors. *PLoS One* **6**, e15947 (2011).
30. Rosselló, R. A. *et al.* Mammalian genes induce partially reprogrammed pluripotent stem cells in non-mammalian vertebrate and invertebrate species. *Elife* **2**, e00036 (2013).
31. Ben-Arye, T. & Levenberg, S. Tissue Engineering for Clean Meat Production. *Frontiers in Sustainable Food Systems* vol. 3 Preprint at https://doi.org/10.3389/fsufs.2019.00046 (2019).
32. Mauro, A. Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol* **9**, 493–5 (1961).
33. Sousa-Victor, P., García-Prat, L. & Muñoz-Cánoves, P. Control of satellite cell function in muscle regeneration and its disruption in ageing. *Nature Reviews Molecular Cell Biology* vol. 23 204–226 Preprint at https://doi.org/10.1038/s41580-021-00421-2 (2022).
34. Seale, P. *et al.* Pax7 is required for the specification of myogenic satellite cells. *Cell* **102**, 777–86 (2000).
35. Lepper, C., Partridge, T. A. & Fan, C.-M. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development* **138**, 3639–46 (2011).
36. Pallafacchina, G., Blaauw, B. & Schiaffino, S. Role of satellite cells in muscle growth and maintenance of muscle mass. *Nutr Metab Cardiovasc Dis* **23 Suppl 1**, S12-8 (2013).
37. Danoviz, M. E. & Yablonka-Reuveni, Z. Skeletal muscle satellite cells: background and methods for isolation and analysis in a primary culture system. *Methods Mol Biol* **798**, 21–52 (2012).
38. Neal, A., Boldrin, L. & Morgan, J. E. The satellite cell in male and female, developing and adult mouse muscle: distinct stem cells for growth and regeneration. *PLoS One* **7**, e37950 (2012).

39. Snow, M. H. The effects of aging on satellite cells in skeletal muscles of mice and rats. *Cell Tissue Res* **185**, 399–408 (1977).
40. Gonzalez, M. L., Busse, N. I., Waits, C. M. & Johnson, S. E. Satellite cells and their regulation in livestock. *Journal of Animal Science* vol. 98 Preprint at https://doi.org/10.1093/jas/skaa081 (2020).
41. Melzener, L. *et al.* Comparative analysis of cattle breeds as satellite cell donors for cultured beef. *BioxRIV* (2022) doi:10.1101/2022.01.14.476358.
42. Subbiahanadar Chelladurai, K. *et al.* Alternative to FBS in animal cell culture - An overview and future perspective. *Heliyon* **7**, e07686 (2021).
43. Brunner, D. *et al.* Serum-free cell culture: the serum-free media interactive online database. *ALTEX* **27**, 53–62 (2010).
44. Jochems, C. E. A., van der Valk, J. B. F., Stafleu, F. R. & Baumans, V. The use of fetal bovine serum: ethical or scientific problem? *Altern Lab Anim* **30**, 219–27 (2002).
45. Brindley, D. A. *et al.* Peak serum: implications of serum supply for cell therapy manufacturing. *Regenerative Med* **7**, 7–13 (2012).
46. van der Valk, J. *et al.* The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. *Toxicol In Vitro* **18**, 1–12 (2004).
47. Gstraunthaler, G., Lindl, T. & van der Valk, J. A plea to reduce or replace fetal bovine serum in cell culture media. *Cytotechnology* **65**, 791–3 (2013).
48. Venkatesan, M. *et al.* Recombinant production of growth factors for application in cell culture. *iScience* **25**, 105054 (2022).
49. O'Neill, E. N., Cosenza, Z. A., Baar, K. & Block, D. E. Considerations for the development of cost-effective cell culture media for cultivated meat production. *Compr Rev Food Sci Food Saf* **20**, 686–709 (2021).
50. Sasse, M., Lengwinat, T., Henklein, P., Hlinak, A. & Schade, R. Replacement of fetal calf serum in cell cultures by an egg yolk factor with cholecystokinin/gastrin-like immunoreactivity. *Altern Lab Anim* **28**, 815–31 (2000).
51. Fujii, D. K. & Gospodarowicz, D. Chicken egg yolk-supplemented medium and the serum-free growth of normal mammalian cells. *In Vitro* **19**, 811–7 (1983).
52. Aisenbrey, E. A. & Murphy, W. L. Synthetic alternatives to Matrigel. *Nat Rev Mater* **5**, 539–551 (2020).
53. Gargotti, M., Lopez-Gonzalez, U., Byrne, H. J. & Casey, A. Comparative studies of cellular viability levels on 2D and 3D in vitro culture matrices. *Cytotechnology* **70**, 261–273 (2018).
54. Kühl, U., Ocalan, M., Timpl, R. & von der Mark, K. Role of laminin and fibronectin in selecting myogenic versus fibrogenic cells from skeletal muscle cells in vitro. *Dev Biol* **117**, 628–35 (1986).
55. Shahini, A. *et al.* Efficient and high yield isolation of myoblasts from skeletal muscle. *Stem Cell Res* **30**, 122–129 (2018).
56. Etienne, J., Liu, C., Skinner, C. M., Conboy, M. J. & Conboy, I. M. Skeletal muscle as an experimental model of choice to study tissue aging and rejuvenation. *Skelet Muscle* **10**, 4 (2020).
57. Richler, C. & Yaffe, D. The in vitro cultivation and differentiation capacities of myogenic cell lines. *Dev Biol* **23**, 1–22 (1970).

58. Benedetti, A. *et al.* A novel approach for the isolation and long-term expansion of pure satellite cells based on ice-cold treatment. *Skelet Muscle* **11**, (2021).
59. Matsuyoshi, Y., Akahoshi, M., Nakamura, M., Tatsumi, R. & Mizunoya, W. Isolation and Purification of Satellite Cells from Young Rats by Percoll Density Gradient Centrifugation. *Methods Mol Biol* **1889**, 81–93 (2019).
60. Che, X., Guo, J., Wang, B. & Bai, Y. Rapid isolation of muscle-derived stem cells by discontinuous Percoll density gradient centrifugation. *In Vitro Cell Dev Biol Anim* **47**, 454–8 (2011).
61. Choi, K. *et al.* Muscle stem cell isolation and in vitro culture for meat production: A methodological review. *Compr Rev Food Sci Food Saf* **20**, 429–457 (2021).
62. Yin, H., Price, F. & Rudnicki, M. A. Satellite cells and the muscle stem cell niche. *Physiol Rev* **93**, 23–67 (2013).
63. Ding, S. *et al.* Maintaining bovine satellite cells stemness through p38 pathway. *Sci Rep* **8**, (2018).
64. Wang, H. *et al.* Expression patterns and correlation analyses of muscle-specific genes in the process of sheep myoblast differentiation. *In Vitro Cell Dev Biol Anim* **58**, 798–809 (2022).
65. Hernández-Hernández, J. M., García-González, E. G., Brun, C. E. & Rudnicki, M. A. The myogenic regulatory factors, determinants of muscle development, cell identity and regeneration. *Semin Cell Dev Biol* **72**, 10–18 (2017).
66. Shirakawa, T. *et al.* Factors Regulating or Regulated by Myogenic Regulatory Factors in Skeletal Muscle Stem Cells. *Cells* **11**, (2022).
67. Hawke, T. J. & Garry, D. J. Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol (1985)* **91**, 534–51 (2001).
68. Tai, P. W. *et al.* Differentiation and fiber type-specific activity of a muscle creatine kinase intronic enhancer. *Skelet Muscle* **1**, 25 (2011).
69. Silberstein, L., Webster, S. G., Travis, M. & Blau, H. M. Developmental progression of myosin gene expression in cultured muscle cells. *Cell* **46**, 1075–81 (1986).
70. Gnocchi, V. F., White, R. B., Ono, Y., Ellis, J. A. & Zammit, P. S. Further characterisation of the molecular signature of quiescent and activated mouse muscle satellite cells. *PLoS One* **4**, e5205 (2009).
71. Charville, G. W. *et al.* Ex Vivo Expansion and In Vivo Self-Renewal of Human Muscle Stem Cells. *Stem Cell Reports* **5**, 621–32 (2015).
72. Yamaguchi, M. *et al.* Calcitonin Receptor Signaling Inhibits Muscle Stem Cells from Escaping the Quiescent State and the Niche. *Cell Rep* **13**, 302–14 (2015).
73. Barruet, E. *et al.* Functionally heterogeneous human satellite cells identified by single cell RNA sequencing. *Elife* **9**, (2020).
74. Cornelison, D. D. & Wold, B. J. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev Biol* **191**, 270–83 (1997).
75. Beauchamp, J. R. *et al.* Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *J Cell Biol* **151**, 1221–34 (2000).

76. Day, K., Shefer, G., Richardson, J. B., Enikolopov, G. & Yablonka-Reuveni, Z. Nestin-GFP reporter expression defines the quiescent state of skeletal muscle satellite cells. *Dev Biol* **304**, 246–59 (2007).
77. Tanaka, K. K. *et al.* Syndecan-4-expressing muscle progenitor cells in the SP engraft as satellite cells during muscle regeneration. *Cell Stem Cell* **4**, 217–25 (2009).
78. Martin, R. A. *et al.* Myogenic Cell Expression of Intercellular Adhesion Molecule-1 Contributes to Muscle Regeneration after Injury. *Am J Pathol* **190**, 2039–2055 (2020).
79. Maesner, C. C., Almada, A. E. & Wagers, A. J. Established cell surface markers efficiently isolate highly overlapping populations of skeletal muscle satellite cells by fluorescence-activated cell sorting. *Skelet Muscle* **6**, 35 (2016).
80. Sherwood, R. I. *et al.* Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafting skeletal muscle. *Cell* **119**, 543–54 (2004).
81. Naraoka, Y. *et al.* Isolation and Characterization of Tissue Resident CD29-Positive Progenitor Cells in Livestock to Generate a Three-Dimensional Meat Bud. *Cells* **10**, (2021).
82. Zhu, H. *et al.* Production of cultured meat from pig muscle stem cells. *Biomaterials* **287**, 121650 (2022).
83. Ding, S. *et al.* Characterization and isolation of highly purified porcine satellite cells. *Cell Death Discov* **3**, 17003 (2017).
84. Choi, K.-H. *et al.* Purification of Pig Muscle Stem Cells Using Magnetic-Activated Cell Sorting (MACS) Based on the Expression of Cluster of Differentiation 29 (CD29). *Food Sci Anim Resour* **40**, 852–859 (2020).
85. Xu, X. *et al.* Human Satellite Cell Transplantation and Regeneration from Diverse Skeletal Muscles. *Stem Cell Reports* **5**, 419–34 (2015).
86. Pannérec, A., Formicola, L., Besson, V., Marazzi, G. & Sassoon, D. A. Defining skeletal muscle resident progenitors and their cell fate potentials. *Development* **140**, 2879–91 (2013).
87. Fu, X. *et al.* Combination of inflammation-related cytokines promotes long-term muscle stem cell expansion. *Cell Res* **25**, 655–73 (2015).
88. Gromova, A., Tierney, M. T. & Sacco, A. FACS-based Satellite Cell Isolation From Mouse Hind Limb Muscles. *Bio Protoc* **5**, (2015).
89. Capkovic, K. L., Stevenson, S., Johnson, M. C., Thelen, J. J. & Cornelison, D. D. W. Neural cell adhesion molecule (NCAM) marks adult myogenic cells committed to differentiation. *Exp Cell Res* **314**, 1553–65 (2008).
90. Brady, M. A., Lewis, M. P. & Mudera, V. Synergy between myogenic and non-myogenic cells in a 3D tissue-engineered craniofacial skeletal muscle construct. *J Tissue Eng Regen Med* **2**, 408–17 (2008).
91. Martin, N. R. W. *et al.* Factors affecting the structure and maturation of human tissue engineered skeletal muscle. *Biomaterials* **34**, 5759–5765 (2013).
92. Bareja, A. *et al.* Human and mouse skeletal muscle stem cells: convergent and divergent mechanisms of myogenesis. *PLoS One* **9**, e90398 (2014).
93. McKay, B. R., Toth, K. G., Tarnopolsky, M. A. & Parise, G. Satellite cell number and cell cycle kinetics in response to acute myotrauma in humans: immunohistochemistry versus flow cytometry. *J Physiol* **588**, 3307–20 (2010).

94. Uezumi, A. *et al.* Cell-Surface Protein Profiling Identifies Distinctive Markers of Progenitor Cells in Human Skeletal Muscle. *Stem Cell Reports* **7**, 263–78 (2016).
95. Alexander, M. S. *et al.* CD82 Is a Marker for Prospective Isolation of Human Muscle Satellite Cells and Is Linked to Muscular Dystrophies. *Cell Stem Cell* **19**, 800–807 (2016).
96. Liu, L., Cheung, T. H., Charville, G. W. & Rando, T. A. Isolation of skeletal muscle stem cells by fluorescence-activated cell sorting. *Nat Protoc* **10**, 1612–24 (2015).
97. Mierzejewski, B. *et al.* Mouse CD146+ muscle interstitial progenitor cells differ from satellite cells and present myogenic potential. *Stem Cell Res Ther* **11**, 341 (2020).
98. Persichini, T., Funari, A., Colasanti, M. & Sacchetti, B. Clonogenic, myogenic progenitors expressing MCAM/CD146 are incorporated as adventitial reticular cells in the microvascular compartment of human post-natal skeletal muscle. *PLoS One* **12**, e0188844 (2017).
99. Motohashi, N., Asakura, Y. & Asakura, A. Isolation, culture, and transplantation of muscle satellite cells. *J Vis Exp* (2014) doi:10.3791/50846.
100. Sincennes, M. C., Wang, Y. X. & Rudnicki, M. A. Primary Mouse Myoblast Purification using Magnetic Cell Separation. *Methods Mol Biol* **1556**, 41–50 (2017).
101. Ozeki, N., Lim, M., Yao, C.-C., Tolar, M. & Kramer, R. H. alpha7 integrin expressing human fetal myogenic progenitors have stem cell-like properties and are capable of osteogenic differentiation. *Exp Cell Res* **312**, 4162–80 (2006).
102. Urbani, L., Piccoli, M., Franzin, C., Pozzobon, M. & De Coppi, P. Hypoxia increases mouse satellite cell clone proliferation maintaining both in vitro and in vivo heterogeneity and myogenic potential. *PLoS One* **7**, e49860 (2012).
103. Fukada, S. *et al.* Molecular Signature of Quiescent Satellite Cells in Adult Skeletal Muscle. *Stem Cells* **25**, 2448–2459 (2007).
104. Allan, S. J., De Bank, P. A. & Ellis, M. J. Bioprocess Design Considerations for Cultured Meat Production With a Focus on the Expansion Bioreactor. *Front Sustain Food Syst* **3**, (2019).
105. Frahm, B. Seed train optimization for cell culture. *Methods Mol Biol* **1104**, 355–67 (2014).
106. Bodiou, V., Moutsatsou, P. & Post, M. J. Microcarriers for Upscaling Cultured Meat Production. *Front Nutr* **7**, (2020).
107. Hookway, T. A., Butts, J. C., Lee, E., Tang, H. & McDevitt, T. C. Aggregate formation and suspension culture of human pluripotent stem cells and differentiated progeny. *Methods* **101**, 11–20 (2016).
108. Verbruggen, S., Luining, D., van Essen, A. & Post, M. J. Bovine myoblast cell production in a microcarriers-based system. *Cytotechnology* **70**, 503–512 (2018).
109. Bardouille, C., Lehmann, J., Heimann, P. & Jockusch, H. Growth and differentiation of permanent and secondary mouse myogenic cell lines on microcarriers. *Appl Microbiol Biotechnol* **55**, 556–62 (2001).
110. Kubis, H. P., Haller, E. A., Wetzel, P. & Gros, G. Adult fast myosin pattern and Ca2+-induced slow myosin pattern in primary skeletal muscle culture. *Proc Natl Acad Sci U S A* **94**, 4205–10 (1997).
111. Kong, Y., Ong, S., Liu, M. H., Yu, H. & Huang, D. Functional composite microbeads for cell-based meat culture: effect of animal gelatin coating on cell proliferation and differentiation. *J Phys D Appl Phys* **55**, 345401 (2022).

112. Rowley, J. A., Madlambayan, G. & Mooney, D. J. Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials* **20**, 45–53 (1999).
113. Sakar, M. S. *et al.* Formation and optogenetic control of engineered 3D skeletal muscle bioactuators. *Lab Chip* **12**, 4976 (2012).
114. Meyer, H.-P., Minas, W. & Schmidhalter, D. Industrial-Scale Fermentation. in *Industrial Biotechnology* 1–53 (Wiley-VCH Verlag GmbH & Co. KGaA, 2016). doi:10.1002/9783527807833.ch1.
115. Post, M. J. *et al.* Scientific, sustainability and regulatory challenges of cultured meat. *Nat Food* **1**, 403–415 (2020).
116. Santos, A. C. A. *et al.* Tissue Engineering Challenges for Cultivated Meat to Meet the Real Demand of a Global Market. *Int J Mol Sci* **24**, (2023).
117. Humbird, D. Scale-up economics for cultured meat. *Biotechnol Bioeng* **118**, 3239–3250 (2021).
118. Rybchyn, M. S., Biazik, J. M., Charlesworth, J. & le Coutre, J. Nanocellulose from *Nata de Coco* as a Bioscaffold for Cell-Based Meat. *ACS Omega* **6**, 33923–33931 (2021).
119. Levi, S., Yen, F.-C., Baruch, L. & Machluf, M. Scaffolding technologies for the engineering of cultured meat: Towards a safe, sustainable, and scalable production. *Trends Food Sci Technol* **126**, 13–25 (2022).
120. Ng, S. & Kurisawa, M. Integrating biomaterials and food biopolymers for cultured meat production. *Acta Biomater* **124**, 108–129 (2021).
121. Bomkamp, C. *et al.* Scaffolding Biomaterials for 3D Cultivated Meat: Prospects and Challenges. *Advanced Science* **9**, 2102908 (2022).
122. Nederveen, J. P. *et al.* Consistent expression pattern of myogenic regulatory factors in whole muscle and isolated human muscle satellite cells after eccentric contractions in humans. *J Appl Physiol* **127**, 1419–1426 (2019).
123. Rando, T. A. & Blau, H. M. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J Cell Biol* **125**, 1275–87 (1994).
124. Ono, Y. *et al.* Slow-dividing satellite cells retain long-term self-renewal ability in adult muscle. *J Cell Sci* **125**, 1309–17 (2012).
125. Gharaibeh, B. *et al.* Isolation of a slowly adhering cell fraction containing stem cells from murine skeletal muscle by the preplate technique. *Nat Protoc* **3**, 1501–9 (2008).
126. Xu, Z. *et al.* A modified preplate technique for efficient isolation and proliferation of mice muscle-derived stem cells. *Cytotechnology* **70**, 1671–1683 (2018).
127. Kim, K. H., Qiu, J. & Kuang, S. Isolation, Culture, and Differentiation of Primary Myoblasts Derived from Muscle Satellite Cells. *Bio Protoc* **10**, e3686 (2020).
128. Yoshioka, K. *et al.* A Modified Pre-plating Method for High-Yield and High-Purity Muscle Stem Cell Isolation From Human/Mouse Skeletal Muscle Tissues. *Front Cell Dev Biol* **8**, (2020).
129. Sidney, L. E., Branch, M. J., Dunphy, S. E., Dua, H. S. & Hopkinson, A. Concise review: evidence for CD34 as a common marker for diverse progenitors. *Stem Cells* **32**, 1380–9 (2014).
130. Hoffman, S. *et al.* Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. *J Biol Chem* **257**, 7720–9 (1982).

131. Petersen, Z. Q. *et al.* A population of satellite cells expressing CD34 represent a type of muscle stem cell in mice. *Exp Hematol* **28**, 114–115 (2000).
132. Covault, J. & Sanes, J. R. Distribution of N-CAM in synaptic and extrasynaptic portions of developing and adult skeletal muscle. *J Cell Biol* **102**, 716–30 (1986).
133. Montarras, D. *et al.* Direct isolation of satellite cells for skeletal muscle regeneration. *Science* **309**, 2064–7 (2005).
134. Joe, A. W. B. *et al.* Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. *Nat Cell Biol* **12**, 153–63 (2010).
135. Alfaro, L. A. S. *et al.* CD34 promotes satellite cell motility and entry into proliferation to facilitate efficient skeletal muscle regeneration. *Stem Cells* **29**, 2030–41 (2011).
136. Ieronimakis, N. *et al.* Absence of CD34 on murine skeletal muscle satellite cells marks a reversible state of activation during acute injury. *PLoS One* **5**, e10920 (2010).
137. Ding, S. Maintaining the stemness of satellite cells during long-term culture. (maastricht university, 2019). doi:10.26481/dis.20190327sd.
138. Pisani, D. F. *et al.* Hierarchization of myogenic and adipogenic progenitors within human skeletal muscle. *Stem Cells* **28**, 2182–94 (2010).
139. Stout, A. J. *et al.* Immortalized Bovine Satellite Cells for Cultured Meat Applications. *ACS Synth Biol* **12**, 1567–1573 (2023).
140. Al Tanoury, Z. *et al.* Differentiation of the human PAX7-positive myogenic precursors/satellite cell lineage in vitro. *Development* **147**, (2020).
141. Rocheteau, P., Gayraud-Morel, B., Siegl-Cachedenier, I., Blasco, M. A. & Tajbakhsh, S. A Subpopulation of Adult Skeletal Muscle Stem Cells Retains All Template DNA Strands after Cell Division. *Cell* **148**, 112–125 (2012).
142. Jones, N. C. *et al.* The p38alpha/beta MAPK functions as a molecular switch to activate the quiescent satellite cell. *J Cell Biol* **169**, 105–16 (2005).
143. Syverud, B. C., VanDusen, K. W. & Larkin, L. M. Growth Factors for Skeletal Muscle Tissue Engineering. *Cells Tissues Organs* **202**, 169–179 (2016).
144. Dayton, W. R. & Hathaway, M. R. Myogenic Cell Proliferation and Differentiation. *Poult Sci* **70**, 1815–1822 (1991).
145. Sinacore, M. S., Drapeau, D. & Adamson, S. R. Adaptation of mammalian cells to growth in serum-free media. *Mol Biotechnol* **15**, 249–57 (2000).
146. Sotiropoulos, A. *et al.* Growth hormone promotes skeletal muscle cell fusion independent of insulin-like growth factor 1 up-regulation. *Proceedings of the National Academy of Sciences* **103**, 7315–7320 (2006).

APPENDIX

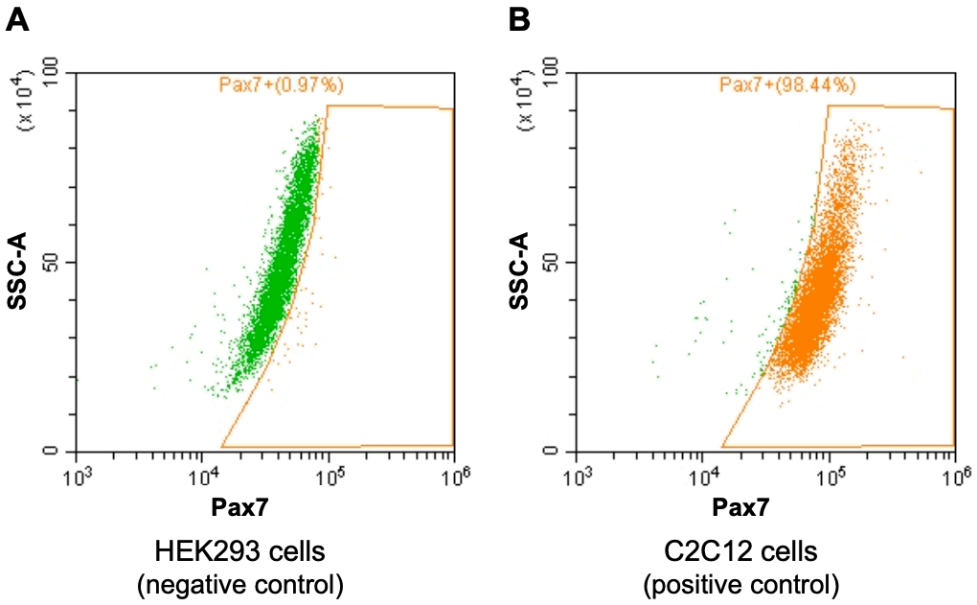

Figure 1. Controls used for Pax7 gating by flow cytometry. (A) HEK293 cells used as a negative control. (B) C2C12 cells used as a positive control.