Cultured meat from stem cells:
Challenges and prospects

Gonçalo Filipe Marques Fernando

Thesis to obtain the Master of Science Degree in

Biotechnology

Supervisor: Prof. Cláudia Alexandra Martins Lobato da Silva

Examination Committee:

Chairperson: Prof. Helena Maria Rodrigues Vasconcelos Pinheiro

Members of the Committee: Prof. Cláudia Alexandra Martins Lobato da Silva,

Dr. Ana Margarida Pires Fernandes-Platzgummer

December, 2020

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at Departamento de Bioengenharia (DBE) of Instituto Superior Técnico (Lisboa, Portugal) during the period April 2020-January 2021, under the supervision of Professor Cláudia Lobato da Silva.

Abstract

Cultured meat relates to meat products obtained by expanding muscle-resident cells in a culture medium and adequate scaffold, until an edible piece of tissue that resembles traditional meat is obtained. This technique, which is based upon advances of skeletal muscle tissue engineering, might not only bypass the need for animal exploitation through intensive agriculture systems, but also reduce the environmental and natural resource burden of conventional livestock production for meat, by reducing greenhouse gas emissions, land use and by controlling the surrounding production environment to avoid bacterial and viral infections that may pose detrimental health outcomes in consumers.

Such alternatives are needed to reduce resource expenses of a growing population. This review will highlight the starting points for cultured meat production, reference and describe a plurality of cell types that may be used in the process, including satellite cells and cells from the myogenic lineage. A short description of culture medium components and the challenges associated with media composition for cultured meat production will also be explained, as well as bioreactor and microcarrier choices for applications in the field. Overall, cellular agriculture remains a promising field which requires further investigation and funding for broad applicability in food production.

Keywords:

Cultured meat

Stem Cells

Tissue Engineering

Meat Substitutes

Satellite Cells

Skeletal Muscle

Resumo

A carne cultivada refere-se a carne obtida através da expansão de células de músculo num meio de cultura e estrutura adequada, até que um tecido semelhante a carne convencional seja obtido. Esta técnica, baseada em avanços na área de Engenharia de Tecidos, nomeadamente para músculo, tem potencial para ultrapassar a necessidade de sistemas de agricultura animal intensos, tal como reduzir os impactos ambientais que a produção de carne convencional acarreta, incluindo reduções em emissões de gases de estufa, uso de terreno, e controlando a produção de forma a evitar infecções com bactérias e vírus que podem prejudicar a saúde humana.

São necessárias alternativas como esta para reduzir o uso de recursos considerando a crescente população. Esta revisão pretende dar ênfase aos tipos de células para produção de carne cultivada, descrevendo vários tipos que poderão ser usados, nomeadamente células "satélite" e células da linhagem miogénica. Serão igualmente detalhados os componentes de meio de cultura utilizados e os desafios associados, tal como os vários tipos de bioreatores e "microcarriers"que poderão ser usados. Resumidamente, a agricultura celular aparenta ser uma área promissora que carece de mais investigação científica e investimento para aplicação em produção alimentar.

Palavras-chave:

Carne cultivada

Células estaminais

Engenharia de Tecidos

Substitutos de carne

Células Satélite

Musculo Esquelético

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
5-aza	5-aza-2'cytidine
ADIPOQ	Adiponectin
AT-MSCs	Adipose-tissue derived Mesenchymal Stem Cells
BAT	Brown Adipose Tissue
BC	Bacterial Cellulose
BR(s)	Bioreactor(s)
BSA	Bovine Serum Albumin
C2C12	Immortalized Murine Myoblast Cell-Line
C/EBPα/β/δ	CCAAT/Enhancer-Binding Protein Alpha/Beta/Delta
CA	Cellular Agriculture
DFAT	Dedifferentiated fat
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
ECs	Endothelial Cells
EGF	Epidermal Growth Factor
ESCs	Embryonic Stem Cells
FABP4	Fatty Acid-Binding Protein 4
FACS	Fluorescence Activated Cell Sorting
FAPs	Fibro/Adipogenic Progenitors
FBS	Fetal Bovine Serum
FGF-2	Basic Fibroblast Growth Factor
GF(s)	Growth Factor(s)
GHG	Greenhouse Gas
Hb	Hemoglobin
HGF	Hepatocyte Growth Factor
IBX	Methyl-isobutylxanthine
IGF-1	Insulin-Like Growth Factor 1
iPSCs	Induced Pluripotent Stem Cells
LEP	Leptin
LPL	Lipoprotein Lipase
Mb	Myoglobin
MC(s)	Microcarrier(s)
MRF4	Myogenic Regulatory Factor 4
MSCs	Mesenchymal Stem/Stromal Cells
Mstn	Myostatin
Myf5	Myogenic Factor 5
MyoD	Myoblast Determination Protein
MyoG	Myogenin
P38 MAPK	P38α/β Mitogen-Activated Protein Kinases
Pax3	Paired Box Protein 3
Pax7	Paired Box Protein 7

PBS	Phosphate Buffered Saline
PCL	Poly(ε-Caprolactone)
PDGRα	Platelet-Derived Growth Factor Receptor Alpha
PEO	Polyethylene Oxide
PLLA	Poly (L-lactic acid)
PLGA	Poly (lactic-co-glycolic acid)
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
RGD	Arginyl-Glycyl-Aspartic Acid
SCs	Satellite Cells
SDS	Sodium Dodecyl Sulphate
SFM	Serum-Free Medium
SkMCs	Skeletal Muscle Cells
Six1/4	Sineoculis Homeobox Protein ¼
SMAD 3	mothers against decapentaplegic homolog 3
SMCs	Smooth Muscle Cells
Sox7	SRY-related HMG box protein 7
TSP	Textured Soy Protein
TVPs	Textured Vegetable Protein(s)
UV	Ultraviolet Light
WAT	White Adipose Tissue
Wnt	Wingless-Related Integration Site
WISP	Wnt1-Inducible Signalling Pathway Protein
Zfp423	Zinc-Finger Protein 423

I. INTRODUCTION

Cultured meat is obtained by culturing few cells that can originate muscle from an animal source in an adequate medium and controlled environment, where cells can proliferate. Once an adequate number of cells are obtained, these can then be put into scaffolds and cultured until a muscle-like structure that resembles meat is obtained.

Since the first proof-of-concept of a cell-based beef hamburger (Post, 2014), and following recent developments in muscle tissue engineering, cultured meat has sparked the attention of both scientists and investors. This emerging technological field termed Cellular Agriculture (CA) has the potential to create animal products without animal slaughter or intensive resource exploitation. As of 2019, more than 55 CA companies have been established throughout the world (The Good Food Institute, 2019), exploring alternatives to conventional production of not only beef, pork, and poultry, but also fish and seafood.

The rationale behind the need of innovative agricultural techniques to provide products such as cultured meat is based on environmental, ethical and health grounds.

The rapid increase in human population, which will reach 9 billion people by 2050 (Godfray *et al.*, 2010) has elevated livestock production for meat consumption to unforeseen numbers, leading to an occupation of 70% of the total agricultural land for cattle raising and feed production (Steinfeld *et al.*, 2006) and simultaneously contributing to deforestation and terrestrial acidification (Gerber *et al.*, 2013; Poore and Nemecek, 2018). In contrast, initial predictions suggest that cultured meat production will potentially require reduced amounts of land (Tuomisto and de Mattos, 2011), since it does not rely on pasture grazing or intensive grain production for animal feed (Gerber *et al.*, 2013; Godfray *et al.*, 2010; Datar and Betti, 2010).

Additionally, meat production worldwide accounts for global greenhouse gas (GHG) emissions of 9% carbon dioxide, 39% methane and 65% nitric oxide (Steinfeld *et al.*, 2006). A study on the environmental impacts of food products has reported mean carbon emissions of 50 kg CO_2 equivalents for 100g of beef protein (Poore and Nemecek, 2018). Moreover, water usage is considerably high, considering 100 g of beef protein in intensive farming conditions may require up to $5x10^3$ L of water to be produced. Preliminary environmental assessments foresee that cultured meat will have a considerably lower impact in water expenditure and GHG emissions, particularly upon comparison with beef (Tuomisto and de Mattos, 2011), whereas the preliminary life-cycle analysis by Mattick and team have predicted a higher input of industrial energy required for cultured meat even when compared with beef, despite forecasting lower global warming potential (Mattick *et al.*, 2015). Other reports predict lower methane and nitric oxide emissions, but higher carbon dioxide (Lynch and Pierrehumbert, 2019).

There are also increasing health concerns associated with foodborne diseases. Intensive animal agriculture is pivotal in boosting zoonotic outbreaks, as well as antibiotic resistance (Anomaly, 2015). Cultured meat offers the potential to eliminate food-related pathogens, since it can be produced in sterile environments. In addition, there have been significant associations of red meat and processed meat products consumption with colorectal cancer (Carr *et al.*, 2016; Larsson and Wolk, 2006), ischemic heart disease (Tong *et al.*, 2019) and type 2 diabetes (Barnard *et al.*, 2014; Talaei *et al.*, 2017). While it is still unclear which factors are responsible for such associations, besides those attributed to cooking methods, cultured meat production will allow control and optimization of physical and chemical parameters that could reduce the concentration of target compounds,

therefore allowing the production of similar foods with reduced detrimental effects for consumers (Post, M., 2012; Ben-Arye and Levenberg, 2019).

Although there has been recent attraction into plant-based diets and the intent to reduce meat consumption from both health and environmental standpoints (Medawar *et al.*, 2019; Hemler and Hu, 2019; OECD/FAO, 2019), the few available polls indicate that humans following plant-based diets still represent a reduced portion of the global population (VRG, 2019). In addition, meat consumption is expected to increase, most significantly in Asian and South American countries (OECD/FAO, 2019). A multidimensional approach is therefore required to successfully transition to a more sustainable food system (Godfray *et al.*, 2010) and feed the growing population's appetite for meat, while simultaneously reducing its environmental and health impacts. Cultured meat can be an important part of such system, if successfully overcoming the ongoing challenges associated with its production and cost efficiency, which will be discussed herein.

II. PROCESS OVERVIEW

The cultured meat process can be briefly described in four stages (Figure 1). Firstly, a tissue biopsy is withdrawn from an animal donor, from which different cells that can give rise to muscle cells are extracted and isolated with different strategies (Ding *et al*., 2018; Post *et al.*, 2012).

Cells are put in an adequate culture medium that supports their growth, and can then be utilized to inoculate a bioreactor (BR), where adherent cells can grow in suspension attached to microcarriers (MCs). A medium recycling unit can be added to retain waste products that affect cell growth, whilst re-circulating mitogenic factors that improve cell proliferation and replenishing consumed nutrients (Allan *et al.* 2019; Specht *et al.*, 2018).

Afterwards, cells can be transferred to a BR that allows perfusion of medium through a porous scaffold, where cells can adhere. By switching to a myogenic differentiation medium, cells can fuse and differentiate into muscle cells, or myotubes and then mature into protein-rich muscle fibres under different stimuli (Langelaan *et al*., 2010).

These fibres can be retrieved from an inedible scaffold, or be maintained in a biodegradable or edible scaffold for further processing into meat products such as hamburgers. This can be achieved by adding adipose cells, flavourings and/or binders. In contrast, both adipose cells and supportive cells can be added to the embedded scaffold in the differentiation BR so that a muscle tissue construct akin to a steak can be obtained (Post *et al.*, 2020).

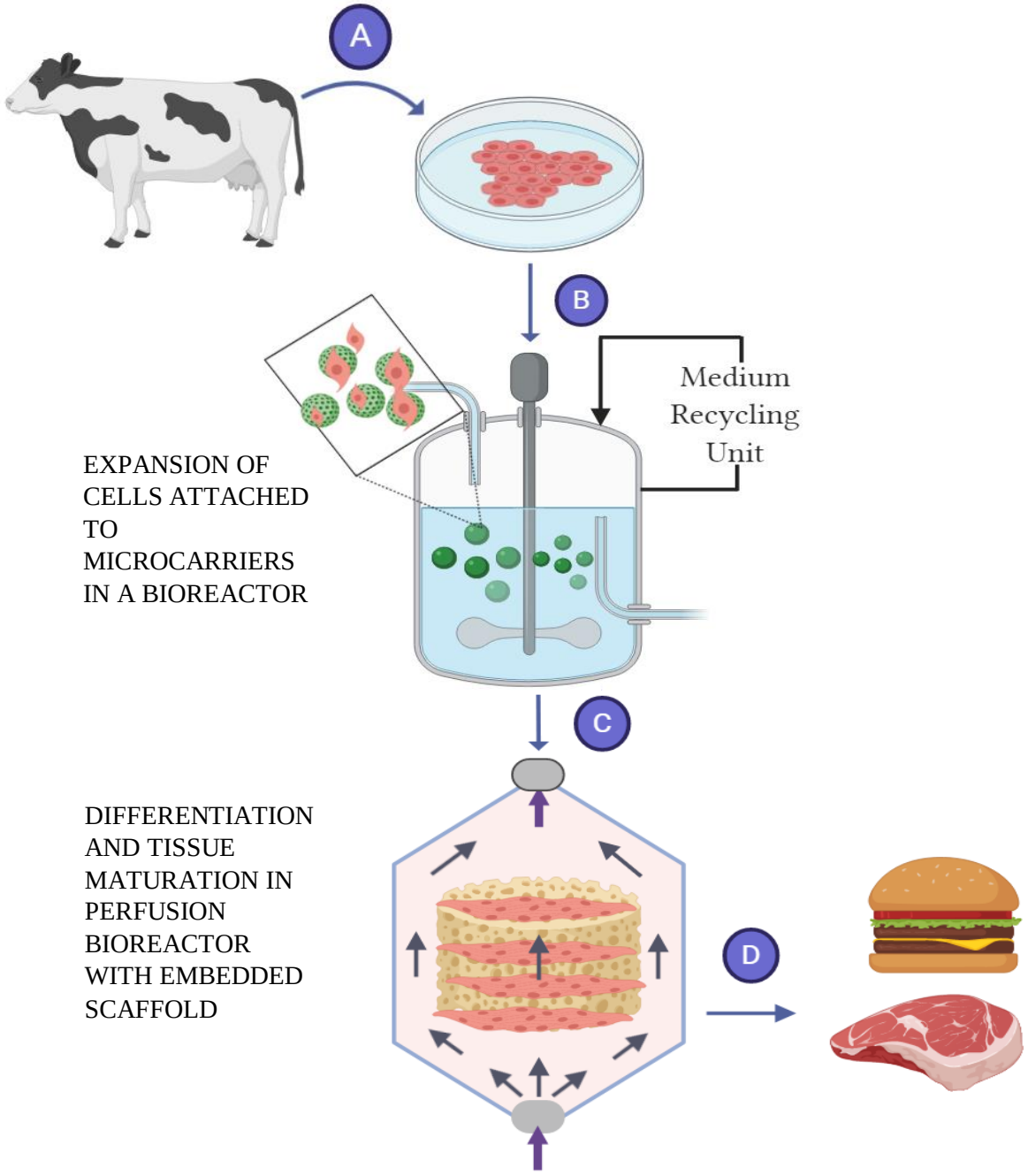

Figure 1- Schematic representation of the stages for cultured meat production. **(A)** A tissue sample is collected from an animal through a biopsy and cells are cultured in lab-scale equipment **(B)** Cells expansion occurs in a proliferation bioreactor (BR) where cells can adhere to small spheres, *i.e.*, microcarriers. **(C)** After expansion, cells can be used to inoculate a BR that ideally allows perfusion of culture medium through a scaffold where cells adhere. **(D)** After differentiation and maturation, mature muscle fibres can be used to produce hamburgers after further processing, or into more complex products such as steaks, by adding adipose cells and supportive cells into the scaffold before differentiation and maturation stages. Inspired in: Ben-Arye *et al.* (2019), Specht, L. (2018), Post, M. J. (2012). Created in BioRender.com

III. CELLS

III.1. STEM CELLS OVERVIEW

Stem cells are undifferentiated cells which can generate progenitor cells that ultimately give rise to a specific cell type (Fortier *et al.*, 2005; Nadig *et al.*, 2009). Stem cells have self-renewal capacity and can increase their population numbers with different proliferation rates. Some stem cells are totipotent, such as the zygote, which can differentiate into cells that form the endoderm, ectoderm and mesoderm layers, as well as extra embryonic bodies like the placenta, and the mesoderm layer originates muscle cells (Zakrzewski *et al.*, 2019). There are also populations of stem cells that display multipotency towards certain cell lineages, and more adult and committed stem cells to specific lineages.

Stem cells have been extensively studied as a potential therapeutic agent for a wide variety of illnesses or as a biological material for tissue engineering applications. Likewise, the process of producing cultured meat commences by choosing the adequate stem cell to isolate, proliferate and then differentiate into muscle fibres. There are several cell types as potential candidates for starting cultures of cultured meat, which will be discussed with more detail in the following sections.

III.1.1. EMBRYONIC STEM CELLS

Embryonic stem cells (ESCs) arise from the inner cell mass of the blastocyst, and are able to differentiate into all cell lineages and regenerate indefinitely (Ben-Arye and Levenberg, 2019;Wu *et al.*, 2016; Datar and Betti, 2010), making them a reliable cell source for cultured meat applications. Isolation of ESCs can be challenging (Fish *et al.*, 2020; Wu *et al.*, 2016), however ESCs allow the creation of cell banks which bypass the reliance on live animal donors for cell extraction (Ben-Arye and Levenberg, 2019).

ESCs can generate functional fat and muscle cells, although cultures can take upwards of 4 weeks to fully differentiate into the intended cell types (Cuaranta-Monroy *et al.*, 2014; Zheng *et al.*, 2006).A stable culture of bovine ESCs has been recently reported, by culturing these cells in a medium containing basic fibroblast growth factor (FGF-2) and a commercially available inhibitor of the Wnt-pathway (Bogliotti *et al.*, 2018).

III.1.2. INDUCED PLURIPOTENT STEM CELLS

Induced pluripotent stem cells (iPSCs) can be obtained from somatic cells (e.g., fibroblasts) through the expression of a combination of reprogramming factors, namely Oct 3/4, Sox2, C-Myc and Klf4 (Takahashi & Yamanaka *et al.*, 2006), which induces a pluripotent state in these cells. This feature makes iPSCs promising for application in cultured meat (Stanton *et al.*, 2019), since it reduces limitations with cell extraction from specific locations of the donor-animal. However, it will require comprehensive knowledge on the reprogramming and culture conditions of bovine cells, since the methods to generate iPSCs are usually described for rodents and humans, and these factors were recently found ineffective to sustain pluripotency of bovine-derived cells (Pillai

et al., 2019). In addition, the derivation process of iPSCs towards a specific lineage can be laborious, though there are synthetic biology techniques to accelerate the differentiation process using inducible transgenes, which allow these cells to differentiate into several cell types, including to the myogenic lineage (Pawlowski *et al.*, 2017).

Ultimately, utilizing genetically engineered cells might be overwhelmingly challenging for market acceptance, due to safety concerns with genetic modifications from a consumer standpoint. Even so, non-integrative strategies could have less impact on consumer perception. Alternatively, if pluripotency could be attained by adding food-grade molecules or peptides, the use of iPSCs would be largely advantageous over other cell sources for cultured meat production.

III.1.3. MESENCHYMAL STEM/STROMALCELLS

Mesenchymal stem/stromal cells (MSCs) are multipotent cells located in various organs and tissues, including bone marrow, adipose tissue, umbilical cord tissue, and placental tissues (Hill *et al.*, 2019).

One of the hallmark features of MSCs is their tri-lineage potential to differentiate into adipose cells, chondrocytes, and osteocytes. Furthermore, MSCs can commit to the fibrogenic or myogenic lineage under controlled conditions (Ben-Arye and Levenberg, 2019; Korovina, D., 2019; Okamura *et al.*, 2018; Witt *et al.*, 2017; Miao *et al.*, 2016), which makes them a promising starting cell type for cultured meat applications.

A recent report has shown that differentiation of bovine adipose tissue-derived MSCs (AT-MSCs) into mature muscle cells can be accomplished after 28 days with different culture medium strategies including growth factor (GF) cocktails and conditioned medium (Korovina, D., 2019). Other works have shown successful derivation of fetal bovine bone marrow-derived MSCs (BM-MSCs) into myogenic cells for 21 days, using commercially available myogenic induction medium, which resulted in high mRNA expression of late-myogenic markers (Okamura *et al.*, 2018).

Using murine MSCs, Witt and team have assessed their differentiation capacity to the myogenic lineage by adding different GFs to the medium and by co-culturing MSCs with myoblasts, which are myogenic-committed cells. The authors reported that MSCs were able to differentiate into muscle cells regardless of co-culture conditions and GF supplementation, expressing late myogenic markers (Witt *et al.*, 2017). Other studies with human cells have reported that BM-MSCs do not express myogenic markers after GF stimulation, while human AT-MSCs do (Kazprezycka *et al.*, 2019). Advantages of using MSCs include the fact that they have been widely studied in humans and mice for tissue engineering purposes and cell therapies. In addition, MSCs can be cultured in microcarrier systems or aggregates (Hanga *et al.*, 2020; Tsai *et al.*, 2017) and have been successfully expanded in different BRs (Timmins *et al.*, 2012). Moreover, BM-derived MSCs from fetal bovine have shown significant post-thaw viability, meaning that these cells might be cryopreserved (Okamura *et al.*, 2018). Thus far, and to the best of my knowledge, no cultured meat product has been reported using MSCs as a primary cell source.

III.2. SKELETAL MUSCLE CELLS

III.2.1. Satellite Cells (SCs)

SCs, or skeletal muscle stem cells, are small adult stem cells located between the basal lamina and the basement membrane of skeletal muscle fibres (Figure 2). SCs represent a population of cells that arises from a multipotent progenitor population (Bentzinger *et al.*, 2012), with a certain degree of heterogeneity, meaning that certain cells can originate both myogenic compromised cells and self-renew the SC population (Tierney and Sacco, 2016). These cells can maintain a quiescent state (*i.e.*, inactive) during adulthood, but can be activated *in vivo* in response to different stimuli such as injury or stress related with weight endurance (Fu *et al.*, 2015; Rocheteau *et al.*, 2014). When this occurs, SCs can migrate to the injury locus and differentiate into myoblasts or self-renewing daughter cells (Datar and Betti, 2010), allowing the regeneration of muscle fibres (Fu *et al.*, 2015; Tierney and Sacco, 2016).

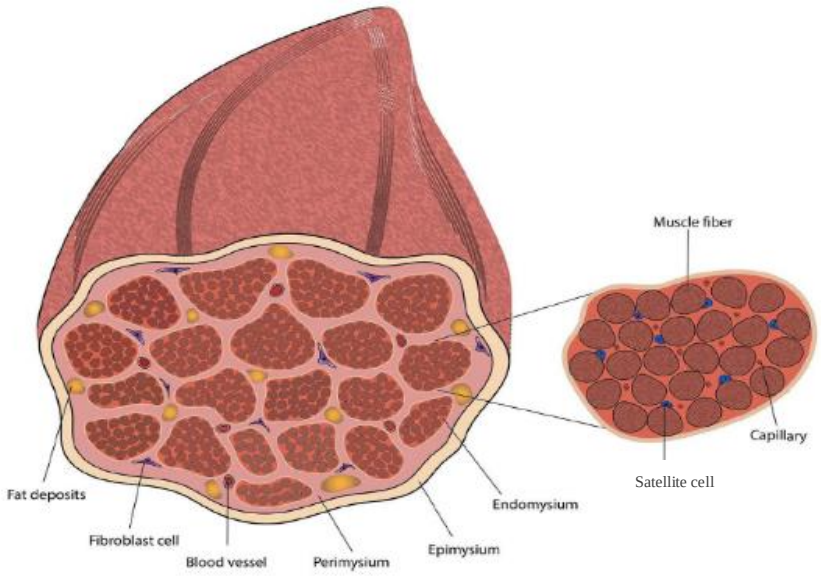

Figure 2- Schematic representation of dissected skeletal muscle tissue. Depicted in blue, satellite cells are adjoining muscle fibres in each fascicle. Fat deposits are depicted in yellow, and fibroblasts in purple, both located in the perimysium. From: Ben-Arye and Levenberg, 2019.

The isolation of SCs from muscle tissue can be achieved through various methods, including through fluorescence-activated cell sorting (FACS) with antigen markers CD56 and CD29 as positive markers, and CD31 and CD45 as negative markers (Ding *et al.*, 2018), after enzymatic breakdown of the tissue. Nonetheless, SC extraction is routinely performed via enzymatic digestion using collagenase, mechanical dissociation of sampled tissue, or a combination of both techniques in a more cost-effective fashion (Post, 2014; Tong *et al.*, 2015; Verbruggen *et al.*, 2018).

SCs can then be transferred to culture plates or spinner flasks, and medium is added for them to proliferate (culture medium considerations will be described later). Coating of culture plates with proteins such

as fibronectin, laminin and type I collagen might increase the proliferation capacity of SCs (Bentzinger *et al.*, 2012; Wilschut *et al.*, 2010). Subsequently, SCs can differentiate into myoblasts (Bentzinger *et al.*, 2012), which are myogenic progenitors. This process can be improved when cells are exposed to hypoxic conditions (Kook *et al.*, 2008). Substrate stiffness also influences myoblast differentiation capacity (Langelaan *et al.*, 2010). After a proliferation phase, these myoblasts start to merge and originate myotubes, which are elongated, multinucleated muscle cells (Bentzinger *et al.*, 2012; Datar and Betti, 2010; Post, M., 2014; Verbruggen *et al.*, 2018). Myotubes can then undergo maturation until myofibres are formed. For cultured meat purposes, myotube maturation was firstly achieved by culturing bovine myotubes around a circular surface made of agar (Post, M., 2014). A large number of these fibres was then used to assemble the first proof-of-concept of a cultured meat hamburger.

III.2.2. Stemness of Bovine SCs

Stemness refers to the ability of cells to maintain a stem-cell-like state. Usually, stemness decreases as myogenesis occurs (Bentzinger *et al.* 2012). In the asymmetric division of SCs, the progenitor cell differentiation to the myogenic lineage (i.e., myoblasts) is marked by the activation of the p38α/β mitogen-activated protein kinases (p38 MAPK; Tierney & Sacco, 2016). The p38 MAPK pathway regulates several factors in myogenesis of SCs, from their activation to proliferation as myoblasts, until differentiation into myotubes (Segalés *et al.*, 2016). Similarly, inhibition of the p38 pathway in bovine SCs allowed them to maintain their stemness potential, while still being able to differentiate in the myogenic lineage (Ding *et al.*, 2018). This was achieved using p38 inhibitor SB203580, a pyridinyl imidazole compound.

Previous research has shown that myostatin (Mstn), a growth differentiation factor, induces a quiescent state in satellite cells, and Mstn-/- rats have increased numbers of activated satellite cells (McCroskery *et al.*, 2003). Identifying factors that can maintain stemness of bovine SCs is imperative for the application of these cells in cultured meat processes. These factors will allow control of growth and differentiation of SCs. Ideally, food-grade factors from sustainable and low-carbon footprint sources should be preferred. For instance, a flavonoid from the leaves of *Apium graveolens* named apigenin was shown to decrease p38 MAPK pathway in rats with myocardial ischemia/reperfusion injury (Yang *et al.*, 2015). Currently, stemness capacity of SCs and other stem cells is usually reinforced by supplementing culture medium with different GF combinations (Ben-Arye *et al.*, 2019), but their production is costly and therefore there is still the need to unveil alternative ways to maintain the proliferative capacity of SCs, without inducing fusion and differentiation into myotubes.

III. 2.3. Myogenesis

Myogenesis is an elaborate process from which progenitor cells can give rise to mature muscle cells. To understand the mechanisms underlying cellular commitment to the myogenic lineage, it is essential to identify the major myogenic regulatory factors involved in the phenomenon.

Several transcription factors are associated with each stage of myogenic cell differentiation, as depicted in Figure 3. The *sineoculis* homeobox proteins 1 and 4 (Six1/4) are transcription factors required for adequate myogenic differentiation during embryonic myogenesis (Wu *et al.*, 2014), which is the earliest stage of differentiation into muscle lineages. These factors act alongside other myogenic regulators to determine the

myogenic fate of the progenitor cells (Liu *et al.*, 2010; Santolini *et al.*, 2016). In fact, Six1/4 expression decreases as specification increases (Figure 3). The paired box protein 7 (Pax7) is a transcription factor with roles in myogenesis, and previous studies in mice suggest that Pax7 is one of the drivers of SCs commitment to the myogenic lineage (Seale *et al.*, 2000), as it is highly expressed throughout the early stages of SC specification until differentiation into myoblasts (Bentzinger *et al.*, 2012; Ding *et al.*, 2018; Tierney & Sacco, 2016). Interestingly, overexpression of Pax7 prevents SCs from differentiating into myogenic cells (Olguin and Olwin, 2004).

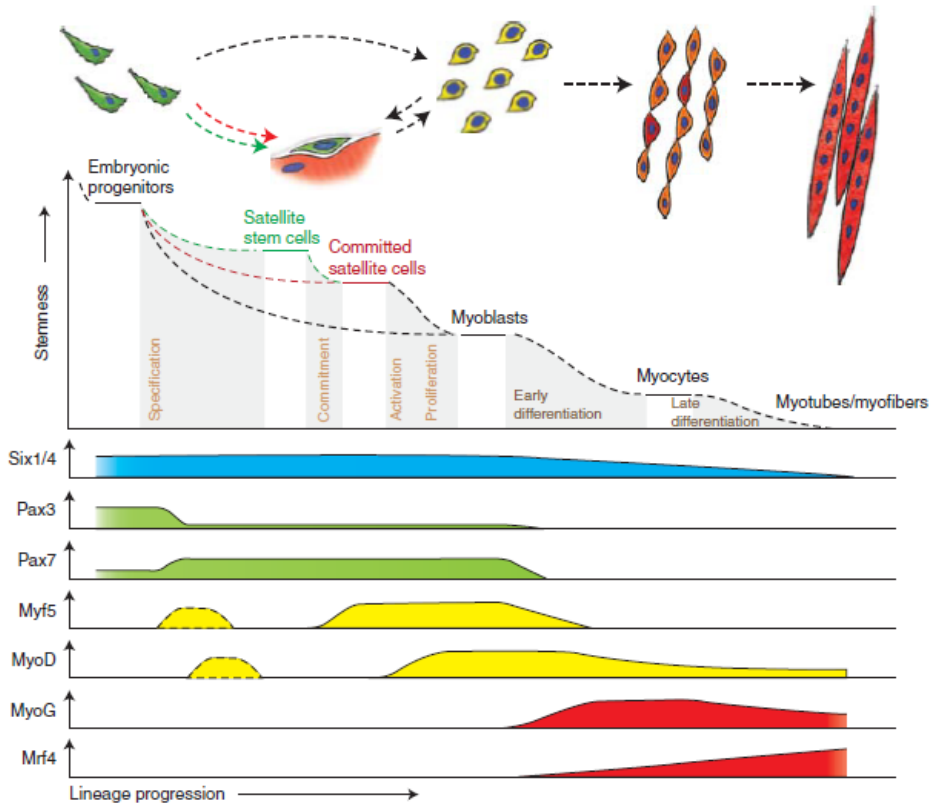

Figure 3- Expression of different regulation factors throughout myogenesis, from early progenitor cells through commitment of satellite cells to the myogenic lineage, until myotube formation. From: Bentzinger *et al.* (2012).

On the other hand, the expression of paired box protein 3 (Pax3) is observed in embryonic progenitor cells before specification into SCs (Bentzinger *et al.*, 2012). *In vivo* studies have shown that muscle-residing SC populations are decreased in mouse lacking the SRY related HMG-homeobox protein 7 (Sox7) gene (Rajgara *et al.*, 2017). Sox7 appears to be required for myoblast survival, and decreased expression in Sox7 leads to decreased Pax7 expression (Rajgara *et al.*, 2017). Both myoblast determination protein (MyoD) and myogenic factor 5 (Myf5) are usually required for the satellite cell differentiation into myoblasts (Seale *et al.*, 2000; Tierney & Sacco, 2016). In fact, MyoD can be used as an early marker for myoblast differentiation (Verbruggen *et al.*, 2018), although it also aids SC specification (Figure 3).

Myoblasts are usually MyoD+ (Tierney and Sacco, 2016). Myocytes however present a Myogenin (MyoG) positive phenotype (Bentzinger *et al.*, 2012; Seale *et al.*, 2000; Tierney & Sacco, 2016). Following alignment, myocytes start to merge and give rise to multinucleated myotubes, where expression of MyoG and

myogenic regulatory factor 4 (MRF4) is followed by the activation of muscle-specific genes and protein synthesis. As myotubes mature, the expression of both MyoD and MyoG decreases (Verbruggen *et al.*, 2018). In addition, myosin heavy chain (MHC), actin- 2, desmin and muscle lim protein (MLP) are late myogenic markers of myotubes and skeletal muscle fibres. Figure 4 resumes additional factors that allow specification of progenitor cells into mature myotubes. These include GF regulation, extracellular matrix (ECM) protein interaction, mechanical simulation, electrical stimulation, and substrate stiffness, which can influence each stage of differentiation, from a progenitor cell to a mature myotube (Langelaan *et al.*, 2010).

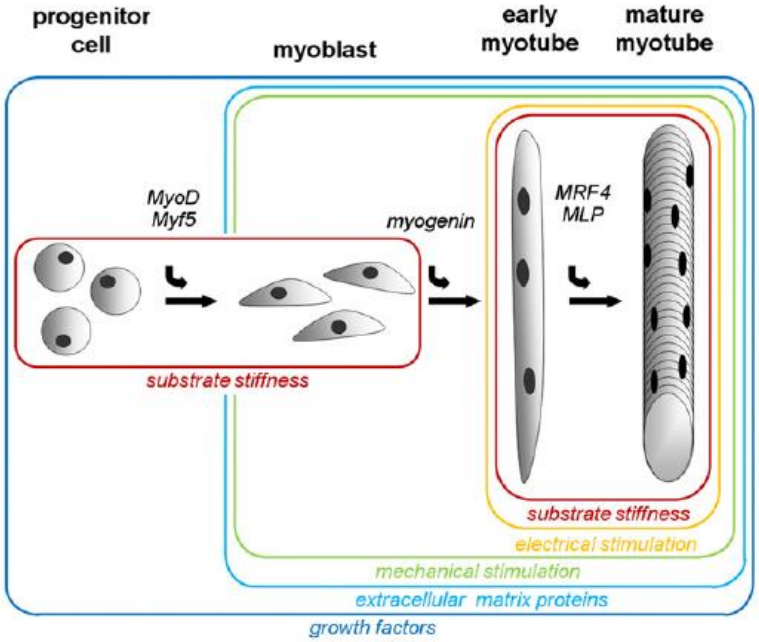

Figure 4. Mechanisms regulating myogenic differentiation of muscle progenitor cells. Substrate stiffness, growth factor exposure and regulation by myoblast determination protein (MyoD) and myogenic factor 5 (Myf5) are major mechanisms that drive progenitor cell commitment and induces their differentiation into myoblasts. Apart from substrate stiffness and growth factor regulation, myoblasts can generate early myotubes through additional interactions with extracellular proteins or by stimulating their mechanical properties, where myogenin becomes more expressed as myoblasts differentiate. Lastly, the formation of mature myotubes can is achieved through the above-mentioned mechanisms and by regulating the rigidity of the substrate or through electrical stimulation when myogenic regulatory factor 4 (MRF-4) and muscle lim protein (MLP) expression increases. From Langelaan *et al.* (2010).

Proliferation and maturation of muscle cells appears to be aided by electrical stimulation (Fujita *et al.*, 2007; Langelaan *et al.*, 2011), although other reports have witnessed only mild effects on myoblast maturation (Boonen *et al.*, 2011). Both electrical and mechanical stimulus can be used to induce myofiber alignment (Langelaan *et al.*, 2010; Gholobova *et al.*, 2015). Ultimately, cost-effectiveness of adding such procedures will have to be considered for large-scale production systems (Post, 2012).

III.2.4. ADIPOGENIC CELLS AND ADIPOSE TISSUE

Adipose tissue is a dynamic organ composed of a heterogeneous cell population which includes adipocytes, adipocyte progenitors, endothelial cells (ECs), blood and immune cells (De Sá *et al.*, 2017). There are two main forms of fat tissue with diverse physiological roles: white adipose tissue (WAT) and brown adipose tissue (BAT). During development, an adipogenic progenitor from mesodermal origin with low expression of Myf5 can commit into WAT lineage, whereas Myf5+ cells originate BAT (Zhou *et al.*, 2019).

In whole-meat cuts, different types of WAT can be found associated with muscle tissue, including subcutaneous fat deposits, intermuscular fat which can be found circumventing different muscles, and intramuscular fat (IMF) located within muscle tissues (Komolka *et al.*, 2014; Figure 5).

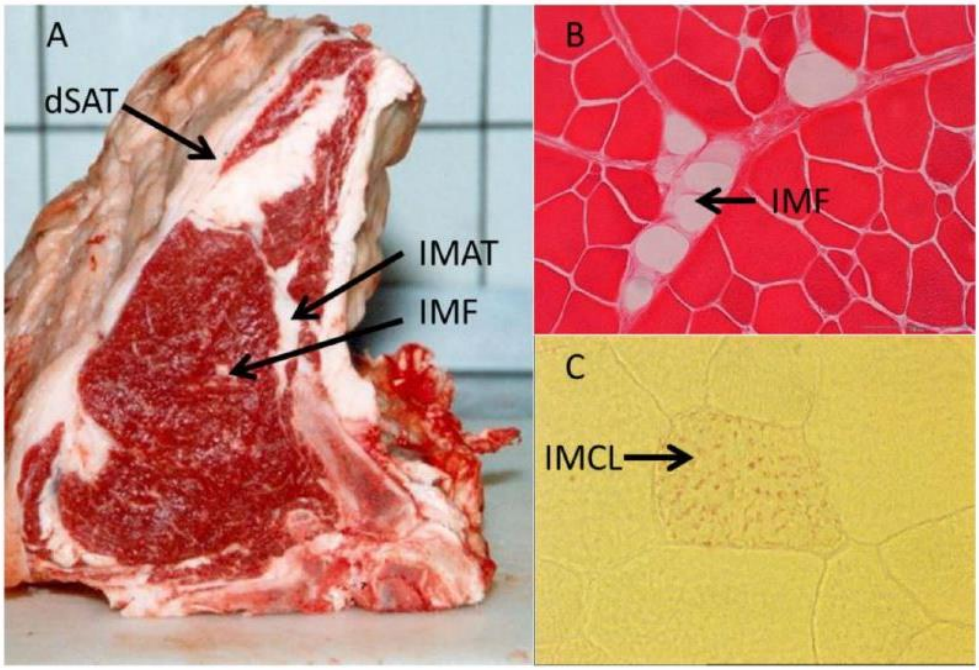

Figure 5-Depiction of muscle-associated adipose tissues in meat cuts **(A)** Location of deep subcutaneous adipose tissue (dSAT), intermuscular adipose tissue (IMAT) and intramuscular fat (IMF) in cattle muscle. **(B)** IMF location within *Musculus longissimus dorsi* of bovine, stained with Eosin. **(C)** Muscle cell from mouse *M. longissimus dorsi* and the location of intramyocellular lipids (IMCL) in red, after staining with Oil-red O. Adapted from Komolka *et al.* (2014).

For complex meat structures such as steaks, both intra- and intermuscular WAT could be of particular interest for texture mimicry, nutrition and optimal organoleptics, though IMF is mostly associated with meat quality (Komolka *et al.*, 2014). The presence of fat is essential for meat's distinguished taste, especially IMF content which benefits organoleptic properties of meat, such as moistness and texture (Motoyama *et al.*, 2016). During cooking, meat-associated lipids undergo lipolysis and generate unsaturated fatty acids, which are then oxidized into a plurality of compounds such as hydrocarbons, alcohols, and aldehydes (Flores, M., 2017). Lipid oxidation can favour meat taste, but also reduce it in a concentration-dependent manner.

The proportion of fat in meat products varies between species and the meat cut intended to replicate (Fish *et al.*, 2020). Despite the average fat percentage in bovine skeletal muscle revolving around 3%, muscle

from Wagyu cattle can have a network of IMF representing upwards of 30% of total weight. Therefore, achieving complex cultured meat products with such fat content, *i.e.*, marbled meat, will require considerable numbers of expanded adipose cells. A similar approach can be expected for applications in cultured minced meat products, where fat contents of as much as 35% can be found in conventional fast-food hamburgers (Barrado *et al.*, 2008).

Adipogenesis and Lipogenesis

The adipogenic phenomenon is a multifaceted process from which adipogenic-committed cells can generate mature adipocytes (Fish *et al.*, 2020; De Sá *et al.*, 2017), and it is based on hypertrophy and hyperplasia processes. Adipogenesis relates to hyperplasia, which is the increase in number of adipocytes, whereas lipogenesis is the process that generates sizable adipose cells through lipid accumulation. Adipocyte development is orchestrated by an array of regulatory processes including signalling pathways, transcription factors, hormones, GFs, and post-transcriptional mechanisms such as miRNA modulation (Figure 6).

Adipose-committed progenitors enter the adipogenic lineage via regulation of CCAAT/enhancer-binding protein alpha (C/EBPα) and peroxisome proliferator-activated receptor gamma (PPARγ), which are the most remarkable transcriptional co-regulators of adipogenesis. Firstly, C/EBPβ and C/EBPδ can activate C/EBPα and PPARγ to consequently induce adipogenesis through various mechanisms (Li & Tang, 2015; Lowe *et al.*,2011). In addition, zinc-finger protein 423 (Zfp423) can induce expression of PPARγ and drive adipogenic commitment (De Sá *et al.*, 2017), and knockdown of Zfp423 in bovine adipose cells from the stromal vascular fraction prevents their differentiation *in vitro* (Huang *et al.*, 2012).

Both insulin and insulin-like growth factor 1 (IGF-1) are important regulators of adipogenic commitment of WAT and BAT cells (Rosen & MacDougald, 2006), as demonstrated *in vivo* by reductions in adipose tissue formation in transgenic mice lacking insulin and/or IGF-1 receptors (Boucher *et al.*, 2016). Moreover, two isoforms of FGF (FGF-1 and FGF-2) have been reported to have pro-and anti-adipogenic effects in uncommitted MSCs (Zhou *et al.*, 2019). Regarding transcription growth factor beta (TGF-β), the signalling pathways of these cytokines are inhibited by Zfp423 in early commitment of adipogenesis, and TGF-β is highly expressed in cells with low adipogenic potential (Huang *et al.*, 2012). In addition, mothers against decapentaplegic homolog-3 (SMAD-3) phosphorylation by TGF-β receptor-associated ligands can lead to physical interactions of SMAD-3 with C/EBP isoforms, which ultimately suppress the expression of PPARγ (Li & Wu, 2020; de Sá *et al.*, 2017). Furthermore, Hedgehog and the canonical wingless-related integration site (Wnt)/β-catenin pathways are involved in the inhibition of adipogenesis in MSCs by targeting PPARγ, C/EBPα and C/EBPβ (Zhou *et al.*, 2019).

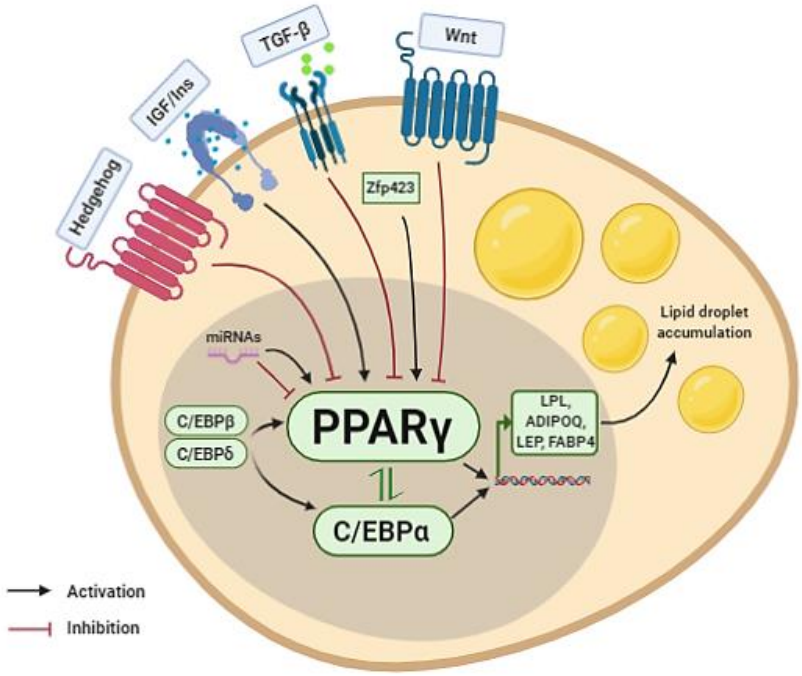

Figure 6-Simplified schematics of adipogenic regulation and some of the major drivers of adipogenesis. The main adipogenic regulators *C/EBPα* (CAAT/enhancer binding protein alpha) and *PPARγ* (peroxisome proliferator activated receptor gamma) can regulate each other following activation by *C/EBPβ* (CAAT/enhancer binding protein beta) and *C/EBPδ* (CAAT/enhancer binding protein delta), as well as *Zfp423* (zinc-finger protein 423). *IGF* (insulin-like growth factor) and *Ins* (insulin) can bind to their receptors and promote the activation of the central adipogenic regulatory network, whereas *Hedgehog*, *TGF-β* (transcription growth factor beta) and canonical *Wnt* (wingless related integration site) pathways can inhibit *PPARγ*and *C/EBPα*expression. Additional regulation is performed by different *miRNA* (micro-RNA) mechanisms, which can enhance or decrease the expression of the central regulators. Upon activation of *PPARγ*and *C/EBPα*, these factors can induce the expression of *LPL* (lipoprotein lipase), *ADIPOQ* (adiponectin), *LEP* (leptin) and *FABP4* (fatty acid-binding protein 4) genes, which can lead to the accumulation of lipids and maturation of adipocytes. Adapted from: Fish *et al.* (2020); De Sá *et al.* (2017). Recreated in Biorender.com.

After adipogenic commitment, C/EBPαand PPARγ can activate each other and subsequently induce the expression of adipogenic and lipogenic genes, including those that express leptin (LEP), adiponectin (ADIPOQ), fatty acid-binding protein 4 (FABP4) and lipoprotein lipase (LPL) (reviewed in Lowe *et al.*, 2011), which lead to the accumulation of tryacylglycerols in lipid droplets and consequently the differentiation and maturation of adipocytes. LEP and ADIPOQ are known adipokines, whereas the enzyme LPL and the transporter FABP4 are involved in the synthesis and transport of long chain fatty acids (Moisá *et al.*, 2013). A similar expression profile of these factors is documented in bovine adipose cells (Strieder-Barboza *et al.*, 2019; Arrighi *et al.*, 2015; Moisá *et al.*, 2013), where undifferentiated preadipocyte cultures have considerably lower expression of C/EBPα, C/EBPβ, PPARγ, LPL, LEP, ADIPOQ and FABP4, when compared to differentiated adipocytes (Strieder-Barboza *et al.*, 2019).

Overall, miRNA machinery appears to regulate several processes in adipogenesis, such as energy homeostasis and regulation of lipidic metabolic pathways (Romao *et al.*, 2014; Zhang *et al.*, 2017), by binding to target mRNAs and repressing gene expression post-transcriptionally.

The identification of adipose-specific miRNAs in livestock-derived cells has been previously assessed in bovine animals (Wang *et al.*, 2020; Romao *et al.*, 2014), where both pro-adipogenic (Ren *et al.*, 2020; Wang *et al.*, 2020; Wang *et al.*, 2018) and anti-adipogenic (Wang *et al.*, 2018) interactions have been elucidated. One study has identified miR-210 as a key post-transcriptional regulatory factor of intramuscular adipogenic differentiation, by inhibiting the expression of genes that originate Wnt1-inducible signalling pathway protein (WISP) (Ren *et al.*, 2020). WISP can attach to Zfp423 and prevent the activation of PPARγ, which is required for pre-adipocyte commitment.

ADIPOGENIC STARTING CELL TYPE

Extraction and isolation procedures for adipogenic progenitor cells from adipose tissue samples are relatively straightforward and have been previously described for bovine cells (Mehta *et al.*, 2019). Firstly, the freshly extracted sample can be digested using collagenase and, after a centrifugation step, the sample can be separated into three fractions: the upper fraction containing buoyant bodies (mature and burst adipocytes, as well as floating lipids), an aqueous supernatant composed of medium, FBS and collagenase residues, and the stromal vascular fraction underneath, from which adipogenic precursors can be isolated and cultured, including AT-MSCs and preadipocytes. In addition, other cell types could be used as sources of adipose tissue for cultured meat, which will be discussed below.

Fibro/Adipogenic Progenitor Cells (FAPs)

In recent years, a group of novel progenitor cells has been identified within the interstitial environment of muscle fibres. These cells have dual lineage commitment and can differentiate into fibroblasts and white adipocytes, and therefore named fibro/adipogenic progenitors (FAPs). This tissue resident subpopulation of MSCs has been identified in the stromal vascular fraction of muscle tissue samples and appears to be one of the major sources of intramuscular adipocytes (reviewed in Li *et al.*, 2020). Unlike skeletal muscle SCs, FAPs are platelet derived growth factor receptor α-positive (PDGFRα+), and human FAPs can be characterized by surface phenotype positive CD15+/PDGFRα+/CD56- (Arrighi *et al.*, 2015) or CD73+/CD45-/CD31- (Golovznina *et al.*, 2020).

IMF development starts at mid-conception while FAPs are actively proliferating. After birth, FAP cell populations start to commit to the adipogenic lineage and differentiate into preadipocytes, and later adipocytes (Li *et al.*, 2020). Indeed, expression levels of adipogenic-related genes such as C/EBPβ, PPARγ, LPL, ADIPOQ, LEP and FABP4 is similar between adipocytes differentiated from FAPs or from other adipose stem cells (Arrighi *et al.*, 2015).

Agricultural research has led to the identification of key-players in bovine IMF development (Park *et al.*, 2018), including differences between cattle breeds, heritability, and nutritional factors, while the latter can be modulated by different feeding and supplementation strategies, and provides insights into adipose cell cultures.

For instance, TGF-β, IGF-1 and FGFs are known factors that increase the proliferation capacity of FAPs (Li *et al.*, 2020), and the expression of TGF-β decreases as cells differentiate into the adipogenic lineage, both in FAPs and other MSCs populations (Arrighi *et al.*, 2015, Huang *et al.*, 2012; Ng *et al.*, 2008). On the other hand, FAPs differentiation into adipocytes and subsequent adipocyte hypertrophy in agricultural practices is usually achieved by restricting dietary supplementation of Vitamin A or adding PPARy agonists to feed. In addition, bovine IMF accumulation can be achieved by restricting Vitamin D3 intake, whereas the addition of Vitamin C appears to be beneficial for IMF development (reviewed in Park *et al.*, 2018).

Dedifferentiated fat (DFAT) cells

Unlike other cell types, mature adipocytes can undergo phenotypical changes and revert to a proliferative stage through a dedifferentiation process. Dedifferentiated fat (DFAT) cells are the progeny of these mature adipocytes. In addition to their proliferative features, DFAT cells can re-differentiate into lipid-accumulating adipocytes (Wei *et al.*, 2013). These cells are referenced as unipotent in recent reviews (Fish *et al.*, 2020), though other studies have reported transdifferentiation of DFAT cells into osteocytes and myocytes *in vitro* under controlled culture conditions and suitable medium (Kou *et al.*,2014; Kazama *et al.*, 2008).

A total of 10 days is required for redifferentiation of DFAT cells into adipocytes, where intracellular lipid droplet formation and accumulation can be observed through oil Red-O staining, coupled with phase-contrast microscopy (Wei *et al.*, 2013). In the same work, the highest observed differentiation rate was 30% when cells reached 100% confluence, which is not ideal for cost-effective production of cells for cultured meat. Hence, a thorough analysis into optimal conditions for differentiating DFAT cells will assure their application in CA.

IV. SUPPORTIVE CELLS

The properties of meat which are of consumers' interest, e.g., texture and flavour, are not exclusively attributed to muscle cells (Simsa *et al.*, 2019). Meat is comprised of muscle, adipose and connective tissue in different proportions according to tissue site (Ben-Arye and Levenberg, 2019; MacQueen *et al.*, 2019; Post and Hocquette, 2017).

Connective tissue is essential to the texture perception of meat (Ben-Arye and Levenberg, 2019; Post, M., 2012) and it is mostly made up of fibroblasts. In skeletal muscle, fibroblasts are located in the extracellular space of muscle fibres (Figure 2), and they can secrete ECM peptides that can be incorporated into muscle ECM (Chapman *et al.*, 2016).

In addition, both ECs and smooth muscle cells (SMCs) compose the vascular system and are promising cell types to use as feeder layers or in co-culture settings, which can affect the originating muscle constructs (Post *et al.*, 2020). Thus, both fibroblasts, ECs and SMCs should be investigated for their effects in muscle cell proliferation and creation of tissue-like constructs (Ben-Arye and Levenberg, 2019).

V. CULTURE MEDIUM

V.1. MYOGENICPROLIFERATION MEDIUM

Dulbecco's Modified Eagle's medium (DMEM) is a standard defined medium that can be used as the primary source of nutrients for myoblast proliferation (Verbruggen *et al.*, 2018). Basal medium like DMEM provides amino acids, vitamins, minerals and other essential components for cell survival and adequate growth. In addition, a combination of amphotericin, penicillin and streptomycin is usually added to bovine tissues before cell isolation to treat and prevent bacterial infections (Ding *et al.*, 2018; Xu *et al.*, 2018; Zhu *et al.*, 2017). Furthermore, supplementation of culture medium with hormones such as insulin ensures an adequate transport of glucose into cells (Gstraunthaler, G., 2003). Different medium formulations for bovine cell proliferation are resumed in Table 1.

Table 1- Proliferation medium for bovine cell lines.

		AT-MSCs	**Fetal BM-MSCs**	**Satellite Cells**	**Primary Myoblasts**	**Primary Myoblasts**
Proliferation Medium Components	**Basal Medium**	α-MEM (1g/L glucose)	DMEM	DMEM	DMEM/F12	α-DMEM
	Glutamine	2mM (Ultraglutamine)	-	-	-	4mM(L-glutamine)
	Antibiotic/ Antimycotic	-	P/S/A*	1% P/S/A	-	1% P/S/A
	FBS	10%	10%	10%	10%	20%
	HorseSerum	-	-	-	-	10%
	Insulin	-	-	-	1 uM	-
	bFGF	1 ng/mL	-	10 ng/mL	-	-
	IGF-1	-	-	100 ng/mL	-	-
	EGF	-	-	62 ng/mL	-	-
	$ZnCl_2$	-	-	50 µM	-	-
Reference		Hanga *et al.*, 2020	Okamura *et al.*, 2018	Ben-Arye *et al.*, 2020	Will *et al.*, 2015	Verbruggen *et al.*, 2018

Abbreviations: α-MEM- Alpha-minimal essential medium; A-DMEM- Advanced DMEM; AT-MSCs- Adipose tissue-derived mesenchymal/stromal stem cells; BM-MSCs- Bone marrow derived-mesenchymal stem/stromal cells; bFGF- Basic fibroblast growth factor; DMEM- Dulbecco's Modified Eagle's Medium; EGF- Epidermal growth factor; F12- Ham's F12 nutrient medium; FBS- Fetal bovine serum; IGF- Insulin-like growth factor 1; P/S/A- Penicillin/Streptomycin/Amphotericin; $ZnCl_2$- Zinc chloride.

*-100IU/mL penicillin, 100µg/mL streptomycin, 0.25 µg/mL amphotericin.

Fetal bovine serum (FBS) is also added to cell cultures (Table 1) and is composed of proteins such as albumin, GFs, minerals, and salts. It also contains fatty acids, protease inhibitors, namely α-antitrypsin or α2-macroglobulin, and transport proteins like transferrin and transcortin (Brunner *et al.*, 2010). Comprehensive lists of typical serum constituents have been previously described elsewhere (Freshney, R., 2015; Brunner *et al.*, 2010). FBS has become a staple in mammalian cell culture protocols, as it supports optimal cell growth and maintenance (Freshney, R., 2015). It is therefore usually added as a medium supplement to culture bovine skeletal muscle SCs and myoblasts, in concentrations ranging from 10-30% (Ding *et al.*, 2018; Verbruggen *et al.*, 2018; Zhou *et al.*, 2019; Zhu *et al.*, 2017). Interestingly, the bovine muscle fibres used to assemble the first cultured meat prototype were sustained in a medium containing 30% FBS (Post, M., 2014).

V.2. MYOGENIC DIFFERENTIATION MEDIUM

The process of *in vitro* differentiation of myoblasts into myotubes is often reported through chemical changes in medium composition (Table 2), namely by removing FBS in high concentrations and by adding horse serum in concentrations ranging 2-4%.

Table 2- Myogenic differentiation medium for bovine cell lines.

		Fetal BM-MSCs	**Satellite Cells**	**Primary Myoblasts**	**Primary Myoblasts**
Myogenic Differentiation Medium	**Basal Medium**	DMEM (high glucose)	DMEM	DMEM/F12	DMEM
	Glutamine	-	-	-	4mM (L-glutamine)
	Antibiotics/ Antimycotic	P/S/A*	1% P/S/A	-	1% P/S/A
	FBS	10%	10%	-	-
	Horse Serum	-	-	-	2%
	Insulin	-	-	0,1 µM	-
	IGF	-	100 ng/mL	-	-
	EGF	-	62 ng/mL	-	-
	Galectin	100 nM	-	-	-
	Dexamethasone	-	-	1 µM	-
	Linoleic Acid	-	-	1 µg/mL	-
Reference		Okamura *et al.*, 2018	Ben-Arye *et al.*, 2020	Will *et al.*, 2015	Verbruggen *et al.*, 2018

Abbreviations: BM-MSCs- Bone marrow derived-mesenchymal stem/stromal cells; DMEM- Dulbecco's Modified Eagle's Medium; EGF- Epidermal growth factor; F12- Ham's F12 nutrient medium; FBS- Fetal bovine serum; IGF- Insulin-like growth factor 1; P/S/A- Penicillin/Streptomycin/Amphotericin.
*-100IU/mL penicillin, 100µg/mL streptomycin, 0.25 µg/mL amphotericin.

An abrupt decrease in concentrations of FBS or GF can induce myotube fusion (Ben-Arye *et al.*, 2020; Post *et al.*, 2020; Manchinella *et al.*, 2017). Other strategies include the addition of myogenic-inducing

components to culture medium, including 5-aza-2'cytidine (5-aza), Galectin-1, dexamethasone, IGF-1 and epidermal growth factor (EGF; Ben-Arye *et al.*, 2020; Okamura *et al.*, 2018; Will *et al.*, 2015). Addition of selected fatty acids to a differentiation medium containing DMEM, 2% horse serum and antibiotics have also been reported to promote differentiation of SCs into mature myotubes (Xu *et al.*, 2018).

Myoblasts cultured in a differentiation medium composed of DMEM/F12, supplemented with dexamethasone, linoleic acid and insulin have reached fusion rates of 30.92% (Table 2; Will *et al.*, 2015). The same work has identified other suitable differentiation media, though not as efficient as the one mentioned above. These media formulations included the supplementation with other compounds such as FBS, transferrin, bovine serum albumin (BSA) and cytosine arabinoside.

If the starting cell type of choice has multi- or pluripotency, then additional differentiation steps must first ensure the differentiation of these cells into myoblast-like cells. This can be performed by adding small molecules to culture medium. For instance, both 5-aza and Galectin-1 have been shown to induce myogenic differentiation of bovine BM-MSCs (Okamura *et al.*, 2018).

V.3.SERUM-FREE MEDIUM (SFM)

Large-scale production of cultured meat will encounter considerable hurdles, including achieving an inexpensive animal-free culture medium without FBS. Ethical concerns related with the collection of blood from bovine foetuses are rather inescapable, since it relies on invasive procedures which may cause a certain degree of suffering to live foetuses (Brunner *et al.*, 2010). Another disadvantage of using FBS relies on the fact that the concentration of its components varies immensely from batch to batch, and its availability is limited. Furthermore, FBS might contain virus contaminants and prions (Brunner *et al.*, 2010; Hawkes, 2015; Lupi, 2003; Toohey-Kurth *et al.*, 2017), which could undermine the safety of cultured meat products and ultimately have damaging effects for consumers.

Notably, a recent study from Kolkmann and team has demonstrated that bovine myoblasts can be cultured under several commercially available serum-free media (SFM), including Fibroblast Basal Medium ™, StemPro™ MSC SFM, and mTeSR. Nonetheless, FBS-containing medium outperformed every commercial serum-free alternative tested (Kolkmann *et al.*, 2020).

To grow cells in a media free of FBS and other animal products, recombinant GFs such as FGF-2 and IGF-1 will most likely need to be added, since different GF combinations can stimulate SC proliferation and differentiation into the myogenic lineage (Ben-Arye and Levenberg, 2019; Syverud *et al.*, 2016).

Mitogenic molecules of plant origin are also promising substitutes of FBS in culture medium. An epithelial cell line of porcine origin has been previously cultured and successfully maintained when supplemented with plant-based reagent Prolifix, albeit slower than with FBS-containing media (Pazos *et al.*, 2004). Other strategies for successful establishment of serum-free cell cultures include gradual reduction of FBS content in medium, adaptation to SFM in increasingly higher concentrations while decreasing the concentration of FBS-containing medium, or cultivation of cells in conditioned medium (Van der Valk *et al.*, 2010).

V.4. ADIPOGENIC MEDIUM

Adipogenic medium has similar specifications to its myogenic counterpart, but require different supplementation depending on the initial cell type. For instance, in order to re-differentiate DFAT cells isolated from Wagyu steers, Wei and team applied a medium composed of DMEM/F12 supplemented with 10% FBS, troglitazone at 10µM/L, as well as methyl-isobutylxanthine (IBX), insulin and dexamethasone in concentrations of 27.8, 1.0 and 0.1 µg/mL, respectively (Wei *et al.*, 2013). At day 7, this medium was substituted by DMEM/F12 + 10% FBS and 1 µg/mL of insulin until day 10. Similar medium supplementation protocols for bovine adipocyte differentiation from adipogenic progenitors (AT-MSCs, FAPs and preadipocytes) have been described elsewhere (Jurek *et al.*, 2020; Mehta *et al.*, 2019; Choi *et al.*, 2013). However, optimal medium formulations need to be investigated, since the use of IBX, troglitazone and dexamethasone are not ideal for application in food for human consumption (Fish *et al.*, 2020).

A recent report elucidates the differentiation of bovine preadipocytes through supplementation with free fatty acids (Mehta *et al.*, 2019). In this work, a medium solution composed of monounsaturated ω-5, -7 and -9 fatty acids and two branched chain ones (Phytanic Acid and Pristanic Acid) in identical final concentrations (50µm), was applied to static 2-D cultures of preadipocytes, in which lipid accumulation could be observed after 2-3 days, as confirmed by Oil Red-O staining of lipid vesicles.

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VI. BIOREACTORS

Cells have been produced at scale for industrial purposes such as beer production that relies on fermentation by unicellular eukaryotes (yeast) and in pharmaceutical applications to produce recombinant proteins in mammalian cells (Allan *et al.*, 2019). Likewise, the up-scaling of cultured meat will most likely rely on the use of large-volume bioreactors (BRs) in lieu of culture flasks and culture plates (Bellani *et al.*, 2020; Swartz, E., 2019).

To successfully implement a cultured meat process which is both economically and technically feasible, it is necessary to take several points into consideration regarding BRs such as the mode of operation, nutrient supply, diminishing the shear stress applied to cells, and ultimately the type of bioreactor that satisfies these requirements (Allan *et al.*, 2019).

BRs should also allow monitoring of physical and chemical parameters such as pH, oxygen, carbon dioxide and waste products. In addition, they should be leak-proof, tolerate customization of flow rate, provide a easy configuration and be easy to assemble (Ahmed *et al.*, 2019; Mattick *et al.*, 2015). Cells from a smaller bioreactor can be utilized to sequentially inoculate larger bioreactors, in a methodology called seed-train proliferation, which can give rise to increasingly high cell densities, depending on which BRs are selected (Swartz, E., 2019).

The initial step is a proliferation stage, where the goal is to obtain large numbers of the starting cell type, for instance myogenic-committed SCs or MSCs, and it is expected to get as many cells per gram of expended glucose as possible, while reducing the concentration of waste products (Allan *et al.*, 2019) such as lactic acid and ammonia.

Afterwards, a differentiation stage where cells are able to fuse into myotubes in an adequate scaffold might need to occur in a bioreactor system that allows continuous perfusion (with cell retention) of culture medium (Specht *et al.*, 2018). A traditional perfusion bioreactor has a peristaltic pump that assures that medium is circulating through tubes and reaching the BR where cells are seeded in a scaffold, and being pumped out into a medium reservoir (Ahmed *et al.*, 2019). In addition, a plurality of sensors can be installed to recognise and control the concentration of target molecules such as toxins, waste products and nutrients such as glucose (reviewed in Ahmed *et al.*, 2019).

Culturing cells in perfusion allows cells to be kept in an exponential phase for longer, when compared to batch cultures (Kaisermayer and Yang, 2013). Static BR systems such as cell bags are a straightforward platform for expansion of ESCs (Li *et al.*, 2018), though dynamic bioreactor systems with perfusion appear to improve proliferation and viability of SCs and myoblasts (Cimetta *et al.*, 2007). Customization of flow rates in BRs set in perfusion is essential, since high-flow rates can disrupt cells, and minimal flow rates can generate nutrient and oxygen depletion (Ahmed *et al.*, 2019).

Various BR types can be chosen for cultured meat applications, including mechanically driven ones, such as stirred tanks or rocking motion BRs, and hydraulic ones such as hollow fibres and packed-/fixed bed BRs (Jossen *et al.*, 2018). These BR systems (Figure 7) have different configurations which will be discussed below, and different BRs can be used in proliferation and tissue maturation stages.

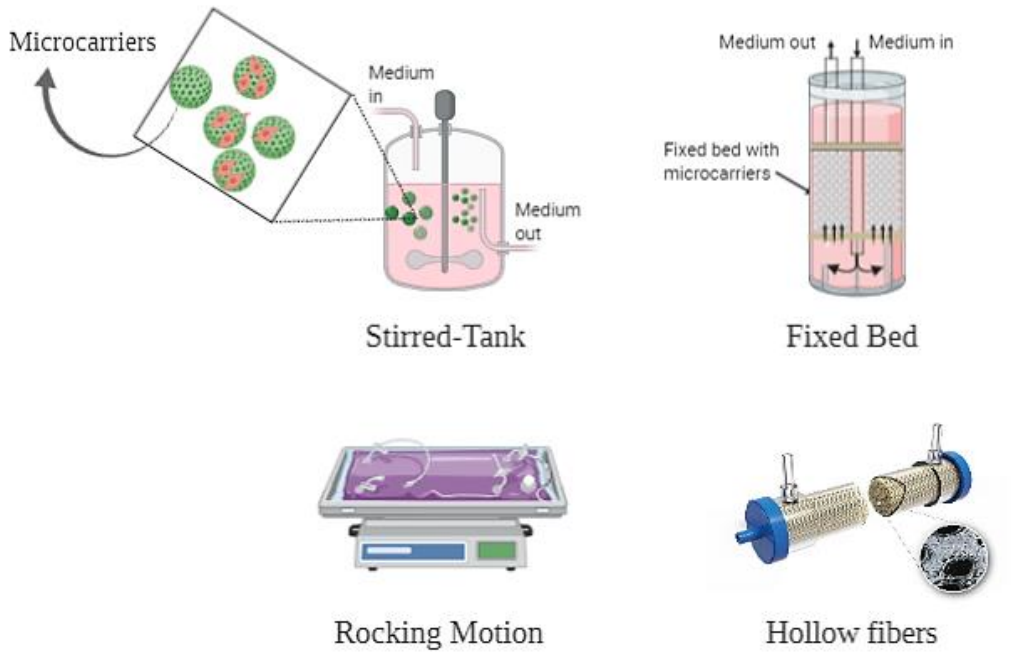

Figure 7- Possible bioreactor (BR) configurations for cell expansion, including mechanically driven (left) and perfusion (right) ones. Top left image represents a stirred-tank BR, where cells can grow in different feeding configurations and attached to microcarriers. Bottom left image represents rocking motion BRs, which are driven by a mechanical force which induces wave-like motion to the culture, and where cells can grow in suspension in disposable bags. Top right image is a fixed (or packed) bed bioreactor where medium is perfused through an immobilized bed of MCs where cells adhere and proliferate, while the bottom right picture shows the external look of a hollow fibre BR. Created in BioRender.com.

Stirred Tanks

Stirred tanks are impeller-driven BRs that allow culturing of cells in suspension, whether as single-cells, aggregates or attached to MCs (Stephenson and Grayson, 2018). There are various configurations for stirred tank BRs, and their working volumes range from millilitre scale (spinner flasks) to thousands of litres for industrial-scale BRs (Bellani *et al.*, 2020; Jonssen *et al.*, 2018; Siebliest *et al.*, 2016). Moreover, stirred tank cultures allow cell densities ranging $2x10^6$ cells/mL (Bellani *et al.*, 2020), and both bovine MSCs and SCs have been cultured in spinner flask systems (Hanga *et al.*, 2019; Verbruggen *et al.*, 2018).

Stirred tanks are advantageous because they can be scaled-up from bench-top settings to pilot and industrial scales in a straightforward way. In addition, stirred tank BR can be further automated and customized for processes of larger volumes (Bellani *et al.*, 2020), and can be used in seed train expansions, where small volume bioreactors inoculate others with larger volumes. On the other hand, stirred tanks require optimized cell-lines for adequate expansion and cells can be exposed high shear stress, due to agitation by the impeller that maintains the medium adequately mixed (Post *et al.*, 2020; Stephenson and Grayson, 2018).

Rocking Motion BRs

Rocking motion BRs allow gentle mixing of cells inside a disposable bag on a rocking platform. There are several commercially available rocking motion BRs, such as the WAVE (GE Healthcare), Finesse (Thermo Fisher) and Biostat (Sartorious; Stephenson & Grayson, 2018), and these BRs can be operated in batch, fed-batch and perfusion mode (Kaisermayer and Yang, 2013). BRs based on wave-like motion have been used to expand different cell types for biomedical applications, including MSCs (Timmins *et al.*, 2012). The size of the disposable bags ranges from 1 to 500 L (Stephenson & Grayson, 2018), however scale-up to volumes upwards of 100L can be demanding (Bellani *et al.*, 2020).

Rocking motion BRs are a powerful platform to grow cells intensively because they offer minimal shear stress to cells and allow cell densities averaging $2x\ 10^6$ cells/mL after cell expansion (Bellani *et al.*, 2020), although higher densities of $4.8\ x\ 10^7$ cells/mL have been obtained in WAVE BRs, when in perfusion mode (Kaisermayer & Yang, 2013).

Fixed-/packed- and Fluidized Bed BRs

Bed-based BRs refers to different BR settings comprising an immobilized surface, with MCs, scaffolds or porous fibres, inside a large vessel (Bellani *et al.*, 2020), through which medium is perfused. Fixed bed BRs have been previously utilized for biomedical purposes such as production of recombinant proteins for vaccine fabrication and viral vector production for gene and cell therapies (Cameau *et al.*, 2019; Rajendran *et al.*, 2014). Commercially available fixed bed BRs include iCELLis® bioreactors, which can have volumes up to 800L for cell manufacturing scales (Cameau *et al.*, 2019).

Fixed beds can achieve high cell densities on the range of $3\ x\ 10^6$ cells / mL due to their large available surface. In addition, packed bed BRs are advantageous because they can operate in different batch modes. In

contrast, these BRs allow the concentration of gradients which can be detrimental for cells and the packaging material used for the scaffold bed could make cell harvest more challenging (Bellani *et al.*, 2020).

Fluidized beds allow higher cell densities since fluidization is used for mixing, without requiring mechanical strength to mix, which is advantageous for cultured meat purposes. Conversely, there is a lack of data regarding the use of fluidized beds for volumes upwards of 100 L, and the cell yield at large scales is yet to be determined (Post *et al.*, 2020).

Hollow fibre BRs

Bioreactor systems based on hollow fibres allow cells to be seeded and proliferate in the internal and external parts of porous fibres, set in parallel within a cylindrical cartridge, through which medium can be perfused (Fish *et al.*, 2020). Cylindrical hollow fibre BRs are mostly used, though rectangular hollow fibre settings can also be manufactured (Eghbali *et al.*, 2016). These BRs have increased surface-to-volume ratio and can reach cell densities of $1x10^9$ cells/mL, since multiple cartridges can be set in parallel within the same device (Bellani *et al.*, 2020), which makes them appealing for large scale cell production.

Commercially available hollow fibres include the Quantum® cell expansion system, which has polysulfone fibres. This BR has been utilized for the expansion of myoblasts (Vang *et al.*, 2020), BM- and UC-MSCs (Mennan *et al.*, 2019), as well as T-cells (Coeshott *et al.*, 2019). Other configurations based on poly (L-lactic acid) (PLLA) hollow fibres have been already evaluated for expansion of C2C12 myoblasts, under both static and dynamic conditions (Bettahalli *et al.*, 2011).

For adequate cell expansion in hollow fibre membranes, cells can be first cultured on small-scale BRs such as t-flasks, until high confluence (70-80%) is achieved (Bettahalli *et al.*, 2011). Afterwards, cells can be detached from the surface of the flask using a trypsin solution, and subsequently be injected into the external part of the hollow fibres, to which they can attach to. However, having large scale applications in mind, cell harvesting from hollow fibres can be challenging (Bellani *et al.*, 2020). Therefore, adequate application of hollow-fibre BRs in cultured meat will rely on the choice of dissociation technique to retrieve cells from the fibres. Alternatively, using fibres from food-grade materials could bypass the need for cell dissociation steps (Fish *et al.*, 2020).

VII. MICROCARRIERS

Characteristics and applications

MCs are small particles that allow the expansion of adherent cells in bioreactors, because they provide a tuneable support matrix that can resemble natural tissue environment, and mammalian cells such as SC, myoblasts and MSCs are naturally adherent (Post *et al.*, 2020; Post and Hocquette, 2017). One of the key advantages of using a MC-based cell culture, in lieu of static 2D culture, is the high surface-to-volume ratio that MC systems provide, which in turn allows higher cell concentrations in culture (Verbruggen *et al.*, 2018; Park *et al.*, 2014) and can be ideal for large-scale applications in cultured meat.

Without growing attached to MCs, cells can still grow in aggregates (Figure 8). Nonetheless, drawbacks can arise from limited diffusion of nutrients, which can induce cell detachment from the aggregates (Swartz, E., 2019). MCs can be utilized in different BRs configurations, such as stirred tanks, packed and fluidized beds, as well as aerated ones, depending on MC features such as buoyancy and density (Bodiou *et al.*, 2020). In packed bed bioreactors, for instance, MCs are immobilized inside the vessel while medium is perfused (Figure 8).

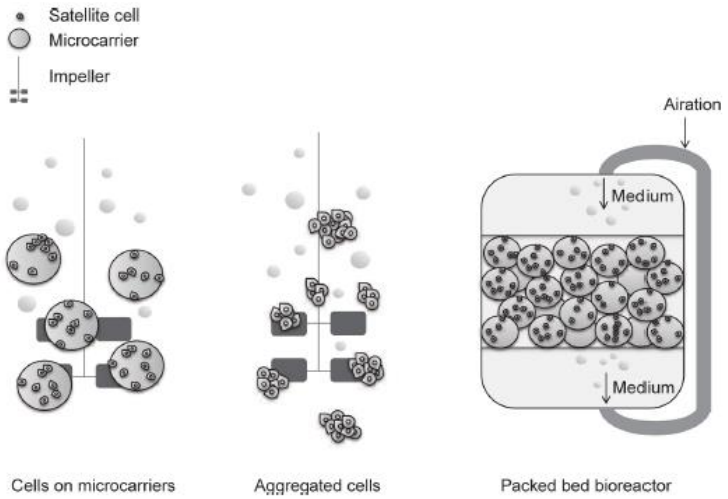

Figure 8- Expansion of satellite cells (SCs) in various configurations. SCs can proliferate in bioreactors attached to microcarriers (MCs), in cell aggregates, or in bioreactor settings such as packed-beds where MCs are immobilized and SCs can grow attached to MCs while medium is perfused through the packed-bed. Adapted from: Moritz *et al.* (2015).

MCs can be fabricated in several ways, including in-lab through water-in-oil emulsions (Kankala *et al.*, 2019) which is a straightforward method that allows the production of MCs in a cost-effective manner. It is based on the preparation of an aqueous phase containing the polymer of interest, which is then dispersed into an oil phase while stirring the container, and microscopic spheres are formed (Kankala *et al.*, 2019). This technique can be combined with freeze-drying to create highly porous particles of cellulose and poly (vinyl alcohol) (Zhang *et al.*, 2017), for instance.

Several considerations in MC choice might influence the attachment and successful proliferation of cells, such as adequate physical parameters such as stiffness and porosity, as well as surface charge and coating with cell-adhesion molecules (Bodiou *et al.*, 2020).

MCs can have different surface topography. Non-porous MCs allow cells to adhere to the surface while microporous MCs can have an increase in material transfer, but nonetheless have limited space for proliferation (Koh *et al.*, 2020; Figure 9). On the other hand, macroporous MCs have large pores that increase the surface area of MCs. For instance, Cytodex 1 MCs are non-porous, while Cytoline™ are macroporous with pore sizes ranging from 10 to 400 µm (Bodiou *et al.*, 2020).

The tensile strength of MCs can influence muscle cell adhesion, and a stiffness module ranging 2-12 kPa is expected to be advantageous to expand SCs (Bodiou *et al.*, 2020). Stiffness considerations with regards to scaffolds will be discussed in the next Section, which are also broadly applicable for MCs.

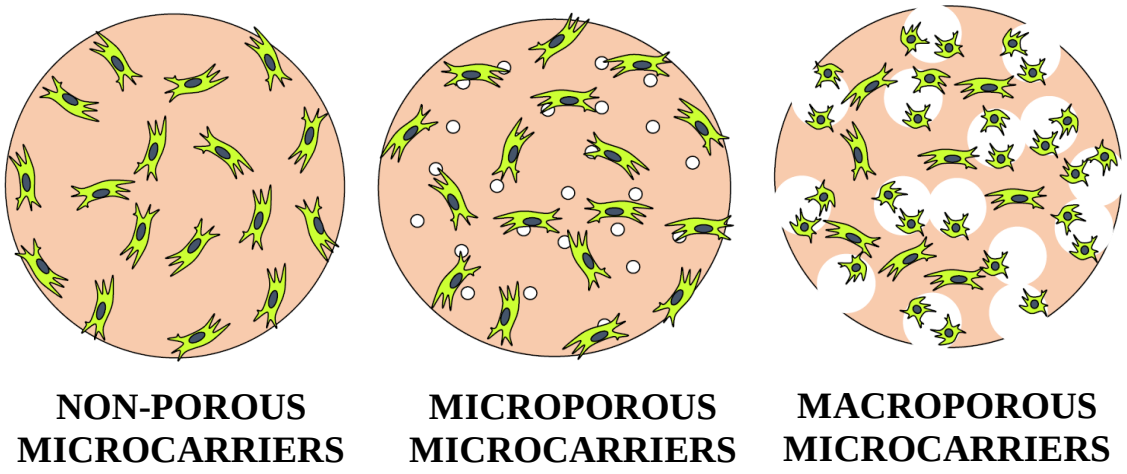

Figure 9- Representation of microcarrier systems with different porosity. The picture on the left shows nonporous MCs which allow cell adhesion in the exterior part of the structure. The picture in the centre depicts microporous MCs, while the picture on the right shows macroporous MCs where cells can grow through the pores and on the surface. Adapted from Koh *et al.* (2020).

MCs can have specific surface charge and chemistry. Commercial brands of MCs such as Cytodex 1 ® are positively charged and composed of crosslinked dextran, while CellBIND® MCs have a negative charge (Verbruggen *et al.*, 2018). Synthemax II® has no overall charge but is coated with arginyl-glycyl-aspartic acid (RGD) motifs (Weber *et al.*, 2010), and this amino acid sequence can be recognized by cell adhesion proteins such as integrins, allowing higher adherence capacity of mammalian cells (Rowley and Mooney, 2002). The three MC systems have been assessed for bovine myoblast expansion, and Cytodex 1® has shown to achieve the highest cell attachment rates (Verbruggen *et al.*, 2018). C2C12 myoblasts have also been cultured in Cytodex 1® (Figure 10; Pacak *et al.*, 2013) and chicken embryo myoblasts have been shown to adhere to Cytodex MCs and fuse into myotubes (Healthcare and Biosciences, 2005). Polystirene MCs from Pall have been recently used to culture bovine MSCs, where it was reported that cells could keep their multilineage potential after expansion (Hanga *et al.*, 2020). Cultispher ® is another common microcarrier used in tissue engineering applications based on gelatin (Timmins *et al.*, 2012), with pore diameters ranging 50 µm.

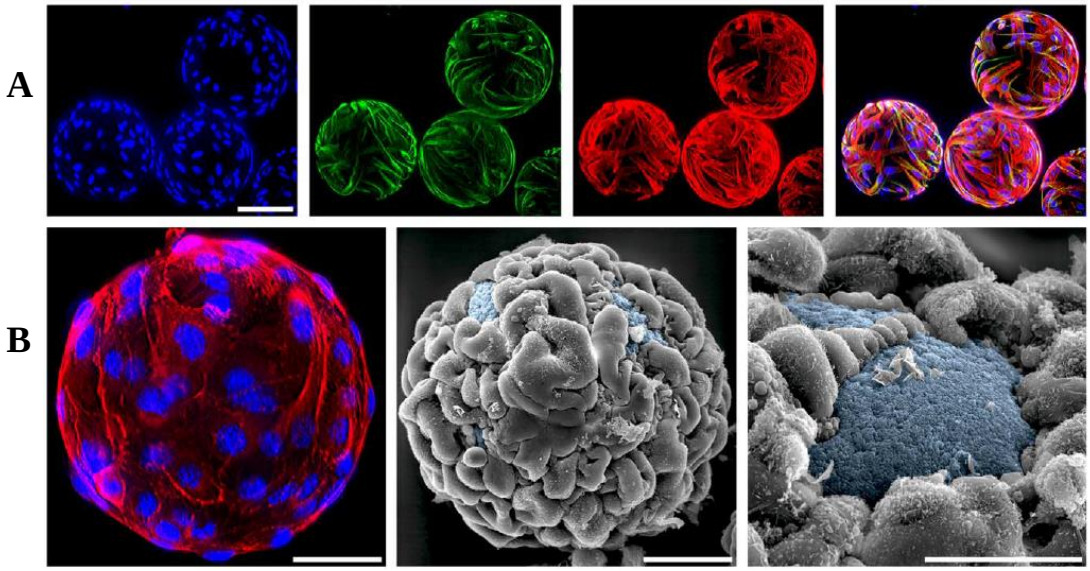

Figure 10-(**A)** Images depicting different staining procedures in C2C12 myoblasts attached to Cytodex 1 MCs Pictures show DNA stained with DAPI (in blue), antibody from actin detected with a conjugated antibody (in green), F-actin filaments with phalloidin staining (in red), and a merged picture. **(B)** Magnification of MCs with myoblasts stained for F-actin and DNA and two graphs obtained scanning electron microscopy where the matrix of Cytodex 1 MCs was coloured blue. Adapted from Pacak *et al.* (2013).

For adequate adhesion of cells, MCs can be coated with recombinant peptides like fibronectin and laminin, that bind to membrane-associated integrins of SCs (Bodiou *et al.*, 2020), or with other substrates which potentially increase cell adhesion, by enhancing the chemical and mechanical stability of microcarrier-cell complexes, with the purpose of mimicking natural tissue environment.

Commercially available coatings include Synthemax® II, which is composed of a synthetic protein that contains the RGD sequence of human vitronectin, an ECM peptide (Rodrigues *et al.*, 2018). Additionally, gelatin-derived type I collagen from different animals is commonly used to coat MCs, such as in the Cytodex 3 MCs (Koh *et al.*, 2020; Timmins *et al.*, 2012). Coating of MCs with recombinant proteins is less common in commercially available MCs (Bodiou *et al.*, 2020), but it could bypass the use of animal-derived peptides.

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Starting Parameters

During initial inoculation for cell expansion with MCs, BR design and stirring strategy must be considered, since continuous and intermittent stirring have different effects on cell adhesion and aggregation capacity of cells (Bodiou *et al.*, 2020). In addition, the density of MCs per cell needs to be assessed in order to find optimal concentrations of MCs for highest cell yields.

One study has reported that higher concentrations of ESC-derived hepatocytes in the beginning of the differentiation step in MC-based cell expansion, yield higher final cell concentrations than lower initial cell densities (Park *et al.*, 2014). Optimal seeding concentration of cells into MCs should also be determined and optimized, since it can influence overall cellular proliferation rate (Verbruggen *et al.*, 2018). If MCs are used in

agitated BRs with possible high shear stress, such as stirred tanks, the agitation can be reduced to minimum speed in order to favour cell adhesion to MCs. Some MC systems in diverse BR configurations are resumed in Table 3.

When cells like MSCs or myoblasts reach confluence, usually 72- 96h after seeding, new MCs can be added to culture which can promote bead-to-bead transfer, where cells can migrate and attach to neighbouring MCs (Verbruggen *et al.*,2018).

Edible MCs

Most commercially available MCs are tailored for biomedical applications and do not meet certain criteria for application in cultured meat, as they are often composed of polystyrene or other inedible polymers (Bodiou *et al.*, 2020), including those routinely used to expand mammalian cells of interest for CA applications (Table 3). Biodegradable polymers also hold potential for cultured meat (Kankala *et al.*, 2019), nonetheless the ideal MCs should comply with food production standards and therefore edible MCs remain very promising, since it would eliminate the need for a MC dissociation stage (Bodiou *et al.*, 2020).

A patent by the company Modern Meadows has highlighted the production of edible MCs composed of crosslinked pectin and cardosin (Marga *et al.*, 2017). Pectin is a polysaccharide mainly composed of galacturonic acid monomers and a major component of plant cell walls, and is commonly used as a gelling agent in the food industry, whereas cardosin is an RGD-containing aspartic protease found in artichokes and other species from the *Cynara* genus, which is used in food applications as a rennet substitute in cheese production. In this patent, the authors combine cystamine and pectin to produce pectin-thiopropionylamide composites, which can then be crosslinked with thiolized cardosin to form a hydrogel-like structure. Afterwards, microbeads with different diameters are produced from the hydrogel using a coaxial flow bead generator (Marga *et al.*, 2017).

In addition, beads composed of pectin crosslinked with edible chitosan can be produced using water-in-oil emulsions (Chacón-Cerdas *et al.*, 2020). Other edible polymers which could be used for cells to adhere include polysaccharides such as carrageenan, alginate and starch, peptide-based ones that are naturally functional, and lipid-based ones, which have been reviewed in Bodiou *et al.* (2020). Different polymers with applications in scaffold fabrication will be described more thoroughly in the next Section and could also be considered for fabrication of MCs.

Table 3- Distinct experimental settings for mammalian cell expansion, using bioreactor systems and microcarriers.

Donor Animal & Cell Type	Cell line and Manufacturer	Expansion setting; MC name and/or manufacturer	MC Size	MC Porosity	MC Material	MC Coating	Bioreactor Type	Cell Seeding Number	Days of Culture	Maximum Cell Density or Number	Ref
Human ESCs	H9	Cytodex 3® MCs	>175 µm	Micro porous	Dextran	Gelatin	Tissue Plates & Spinner Flasks	8x10^5 cells/mg bead	2 days	1 x 10^6 ESCs/mL, 5,9 x 10^5 differentiated hepatocytes/mL	Park et al 2014
Human ESCs	Q-CTS-hESC-2	Suspension; Cultilife™	n/a	n/a	n/a	n/a	1.5 L bag	2 x 10^5 cells/mL	5 days	6.7 x 10^5 cells/mL (1x10^9 cells per 1.5 L bag)	Li et al., 2018
Human iPSCs	Episomal iPSC line (Gibco), F002.1.12 line (TCLab)	Dissolvable MCs; Corning ®	200-300 µm	n/s	Crosslinked PGA-Ca^{2+}	Synthemax ® II (vitronectin) ; Matrigel; Human vitronectin	Spinner Flask	5.5 x 10^4 cells/mL	5-7 days	8,81 ± 1.45 x 10^5 cells /mL	Rodrigues et al., 2018
Human AD-MSCs	Freshly- isolated	MCs; Corning®	125-212 µm	Non-porous	Polystirene	CellBIND®	Spinner Flask	8,8x10^3 cells/mL	21 days	1x10^5 cells/mL	Tra et al., 2016
Human BM-MSCs Human UC-MSCs	Isolated from BM aspirates (Lonza) Isolated from human UCs	Cells seeded on HFs	n/a	n/a	n/a	n/a	PES-HFs (Quantum ® CES)	5-10 x 10^6 cells 5x10^6 cells	13+-1 days 7+- 2 days	1.31+- 0.84 x 10^8 cells 1.68+-0.52 x 10^8 cells	Mennan et al., 2019
Human Myoblasts	Clonetics human SM (Lonza)	Cells seeded on HFs	n/a	n/a	n/a	n/a	PES-HFs (Quantum ® CES)	1*10^6 cells	7-15 days	Total of 1 x 10^9 cells (with 10 days of culture)	Vang et al, 2020

Table 3- (continued)

Donor Animal & Cell Type	Cell line and Manufacturer	Expansion setting; MC name and/or manufacturer	MC Size	MC Porosity	MC Material	MC Coating	Bioreactor Type	Cell Seeding Number	Days of Culture	Maximum Cell Density or Number	Ref
Human T-Cells	Isolated from peripheral blood samples	Cells seeded on HFs	n/a	n/a	n/a	n/a	PES-HFs (Quantum ® CES)	85x10^6 cells	8 days	Total of 2,78x10^10 cells; 224 x 10^6 cells/mL	Coeshott et al., 2019
Murine Myoblasts	C2C12	MCs; in-house	280-370 µm	Macro-porous	PLGA	n/s	2D- 96-well plate	1x10^4 cells p/well	15 days	10 x 10^4 cells dynamic culture; 8 x 10^4 cells static culture	Kankala et al 2019
Bovine Primary Myoblasts	Freshly isolated	MCs; Cytodex® 1	190µm	Non-porous	Crosslinked Dextran	n/s	Spinner Flasks	1x10^6 cells/mL	6 days	3x10^6 cells/mL	Verbruggen et al., 2018
		MCs; Synthemax ® II	125-225 µm	Non-porous	Polystirene	ECM proteins					
Bovine AD-MSCs	Bovine ADSC (Cellider Biotech, Spain)	MCs; Pall	90-212 µm	Non-porous	Polystirene	n/s	Spinner flasks (100 mL), coated with Sigmacote	1500-6000 cells/cm^2 MC surface	9 days	28-fold increase in cell number with lowest seeding density (1500 cells/cm^2 MC surface)	Hanga et al., 2020
Primate Kidney Cells	Vero continuous cell line	Embbeded Microcarriers	n/s	n/s	Proprietary composition	n/s	Fixed-Bed (iCELLis Nano)	4x10^5 cells/mL	5 days	4x10^6 cells/mL	Rajendran et al., 2014

Abbreviations: 2D- Two-dimensional; AT-MSCs- dipose tissue-derived stem cells; BM- Bone marrow; BM-MSCs- Bone marrow-derived mesenchymal stem cells; C2C12- Immortalized murine myoblast cell line; CES- Cell Expansion System; ESCs- Embryonic stem cells; iPSCs- Induced pluripotent stem cells; MC(s)- Microcarrier(s); MHC- myosin heavy chain; N/A- Not applicable; N/S- Not specified; PES-HFs- Polyether sulfone hollow fibers; PLGA- Poly(lactic-co-glycolic acid); PGA- Polygalacturonic acid; Ref- Reference; SFM- serum-free medium; SM- Skeletal muscle; UC-MSCs- Umbilical cord-derived mesenchymal stem cells.

VIII. SCAFFOLDS

Cultivating tissue *in vitro* from livestock-derived cells will require the development of matrices that provide adequate conditions for optimal cell seeding, survival, migration, and differentiation. As mentioned in the previous sections (see Bioreactors/Microcarriers), both SCs, MSCs and myoblasts can adhere to MCs, which serve as micro-scaffolds for these cells, and proliferate in various bioreactor systems (Bodiou *et al.*, 2020; Hanga *et al.*, 2020; Verbruggen *et al.*, 2018). After a proliferation phase, myoblasts can then be transferred into an adequate structure, or scaffold, that offers mechanical support for muscle tissue development (Specht *et al.*, 2018).

Similarly to MCs, three-dimensional (3D) macro-scaffolds resemble natural tissue environment more apparently than two-dimensional (2D) ones, although modifications in 2D scaffolds can enhance their compatibility (Campuzano and Pelling, 2019). A schematic depiction of the differences between 2D and 3D scaffolds is represented in Figure 11.

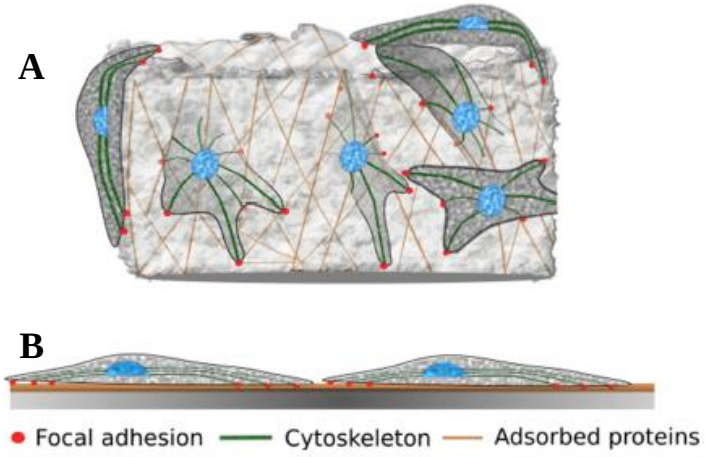

Figure 11-Structural differences between **(A)** three-dimensional (3D) and **(B)** two-dimensional (2D) scaffolds. 3D scaffolds allow cells to growth laterally and infiltrate the structure. From: Campuzano and Pelling (2019).

In 3D scaffolds with adequate porosity, cells can grow on the surface and migrate through the scaffold to adhere laterally and penetrate through the pores (Figure 11; Ben-Arye *et al.*, 2020; Bružauskaité *et al.,* 2016; Reilly and Engler, 2010).

Ideally, scaffolds should allow perfusion of nutrients to overcome diffusion limits typically associated with high cell densities, while simultaneously recreating the ECM environment. Furthermore, scaffold materials used for cultured meat production should be biocompatible, biodegradable, and ideally edible (Ben-Arye and Levenberg, 2019; Campuzano and Pelling, 2019).

An additional dissociation step to retrieve the cells from an inedible scaffold for further assembly into cultured meat products can be performed, nonetheless. If so, methodologies should be put in place to allow facilitated dissociation in downstream processing (Allan *et al.*, 2019). Moreover, the biopolymers used for cultured meat scaffolds should have minimal batch-to-batch variation and only represent a small portion of the cost of the final product (Post *et al.*, 2020; Allan *et al.*, 2019).

While the first prototype of a cultured meat hamburger was an assembly of muscle fibres previously seeded onto a hydrogel (Post, 2014), fibrous scaffolds are also valuable for muscle tissue engineering, as whole-muscle tissue and marbled cultured meat products will most likely require a combination of softer and stiffer scaffold materials to resemble the mechanical properties of different muscle layers, *i.e.,* perimysium, epimysium and endomysium (Ben-Arye and Levenberg, 2019; Figure 2).

Therefore, different scaffold properties and sources must be explored, including physical specifications (pore size distribution, pore diameter, pore directionality) and mechanical properties (tensile strength/stiffness, surface topography).

Porosity and pore size distribution of scaffolds can influence cell proliferation and differentiation of cells, and higher porosity appears to improve the growth of bovine SCs (Ben-Arye *et al.,* 2020). In addition, pore directionality improves muscle cell alignment and differentiation since it provides physical cues that drive myoblast growth (Basurto *et al.,* 2020). Likewise, alignment of fibrous scaffolds improves myoblast proliferation and facilitates the formation of myotubes with elongated morphology that express late myogenic markers (Cooper *et al.,* 2010).

The variations in tensile strength of biopolymers have been reported to influence the attachment and proliferation of different cell types (Figure 12). For instance, materials with elasticity modulus ranging 11-12 kPa can increase proliferation, adhesion, and self-renewal capacity of SCs *in vitro* and improve the engraftment percentage and regenerative capacity of muscle constructs *in vivo* (Gilbert *et al.,* 2010; Engler *et al.,* 2004). Similar results have been reported in MSCs, which acquired a muscle-like morphology and phenotype when cultured in microenvironments of elasticity ranging 11kPa (Reilly and Engler, 2010), whereas in more compliant surfaces (0.1 kPa) MSCs exhibited a nerve-like structure, and a bone-like morphology in stiff hydrogels (34kPa). Other studies report an optimal stiffness of 21 kPa for proliferation of murine myoblasts (Boonen *et al.*, 2009).

Interestingly, pre-adipocytes from mouse 3T3-L1 cell line proliferate and survive more significantly in moderate (7.9 kPa) and stiff (12.4 kPa) hydrogels (Figure 12-B), whereas adipocyte differentiation and lipid accumulation are more pronounced when these cells are cultured in more compliant matrices with a stiffness modulus ranging 3.3 kPa (Figure 12-C; Chandler *et al.,* 2011). Thus, the mechanical properties of scaffolds will need to be considered according to both the cell type of interest, and the phase of the cell cycle intended to replicate.

Moreover, surface topography of scaffolds has shown to influence the successful growth and differentiation of myoblasts, which adhere and differentiate more apparently in micropatterned scaffolds than in flat surfaces devoid of topographical cues (Orellana *et al.,* 2020).

A plurality of natural and synthetic materials have been previously used as scaffolds for skeletal muscle tissue formation, including whole decellularized plant tissues (Campuzano *et al.,* 2020), textured vegetable proteins (Ben-Arye *et al.,* 2020), silk protein fibres (Widhe *et al.,* 2010), gelatin, collagen or collagen-like proteins (MacQueen *et al.,* 2019; Yu *et al.,* 2014; Cimetta *et al.,* 2007), and poly- and oligosaccharides such as alginate (Rowley and Mooney, 2002; Yeo *et al.,* 2016), as well as chitosan (Cooper *et al.,* 2010). Each material possesses unique advantages and limitations for future applications in cultured meat, which will be briefly discussed below.

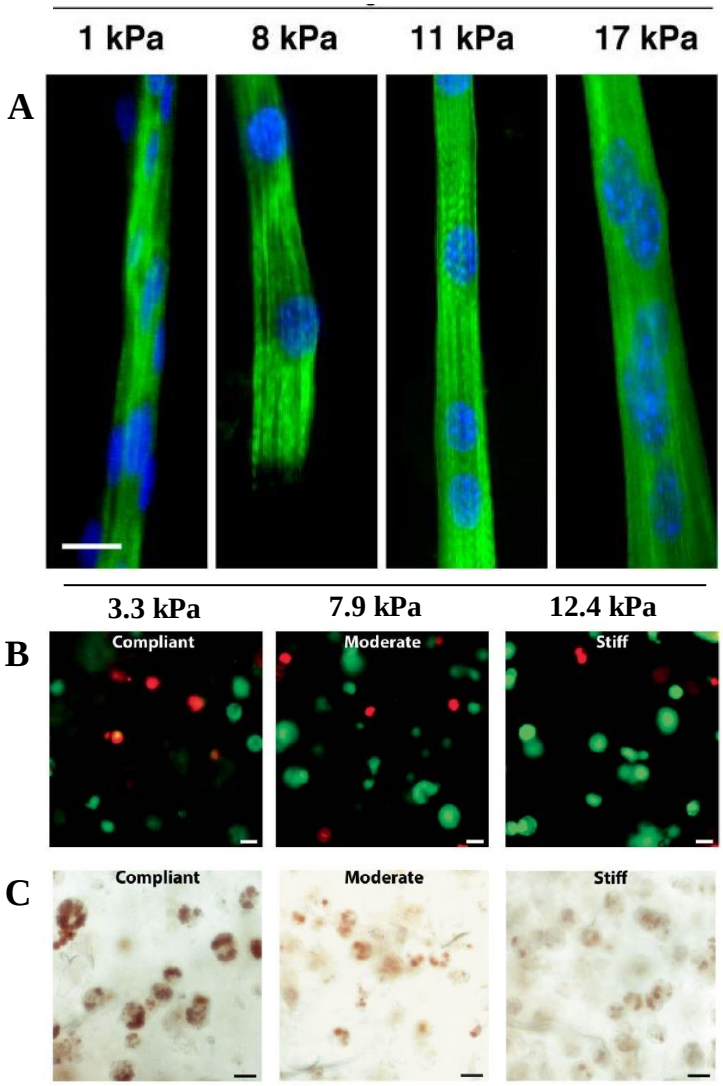

Figure 12- Effect of the elasticity of the microenvironment in muscle and adipose cell proliferation and differentiation. **(A)** Myotube development and alignment in collagen patterns with different stiffness, showing more pronounced alignment in scaffolds with substrate elasticity ranging 11 kPa. Nucleus can be seen in blue, and myosin fibres in green. Adapted from Engler *et al.* (2004). **(B)** Cultures of 3T3-L1 cells in hydrogels with different stiffness. Cell proliferation and viability is more pronounced in moderate and stiff hydrogels, when compared to compliant surfaces, as expressed through live-dead staining with live cells stained with calcein (in green) and dead cells with propidium iodide (in red). Scale bars= 20 µm. Adapted from: Chandler *et al*. (2011). **(C)** Differentiation of adipocytes from 3T3-L1 cell-line in hydrogels with different stiffness, showing higher lipid droplet accumulation in compliant surfaces, when compared to moderate and stiff ones, as shown by staining of lipid droplets with Oil Red O. Adapted from Chandler *et al*. (2011).

DECELLULLARIZED PLANT TISSUES

Plant tissues have promising applications in tissue engineering and cultured meat for their overall hydrophilicity and porosity (Fontana *et al.*, 2017). In addition, plant tissues are readily available at low costs and have a vasculature-like vein structure, as depicted in Figure 13, so they can be used as tissue perfusion scaffolds

(Gershalk *et al.*, 2017). Furthermore, plant tissues are composite materials comprising both soft and firm surfaces (Fontana *et al.*, 2017), which could allow the seeding of a diverse set of cell-types according to their mechanical and topographical requirements. For instance, the aligned topography of plant vascular bundle allows more evident myoblast alignment, when compared to the topographical cues of plant parenchyma (Campuzano *et al.*, 2020).

A plethora of plant tissues has been assessed for their potential as scaffolds for proliferation and differentiation of skeletal muscle cells. For example, decellularized tissues from apple and celery allow the proliferation of C2C12 myoblasts, where upwards of 4-fold increase in myoblast viability was reported after 12 weeks of culture (Modulevsky *et al.*, 2014). Furthermore, tissues from celery stalks allow the differentiation of C2C12 myoblasts into aligned myotubes with expression of late myogenic markers such as actin and MHC (Campuzano *et al.*, 2020). Additional cell types of interest in cultured meat research have been reported to grow in plant scaffolds, including ECs which were perfused into the veins of spinach leaves (Gershlak *et al.*, 2017), or fibroblasts sustained in apple-derived tissues (Modulevsky *et al.*, 2014).

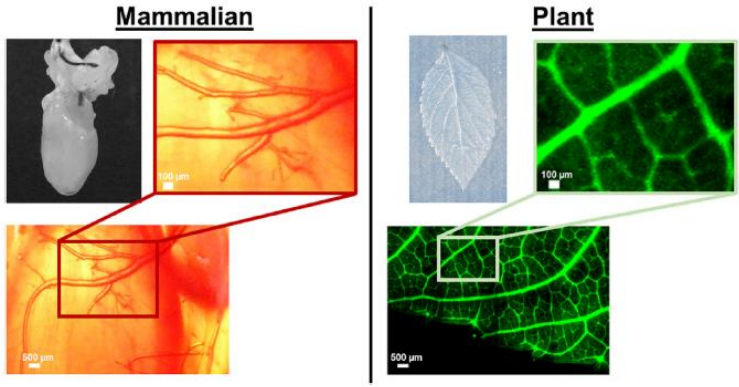

Figure 13-Similarities between mammalian and plant vasculatures. Vascular system of a decellularized mammalian heart from rat stained with Ponceau Red (left) and decellularized leaf and vein structure from summer lilac *Buddlejadavidii*, perfused with fluorescein-polyethylene glycol diacrylate to visualize veins in light green (right). From Gershalk *et al.* (2017).

TEXTURED VEGETABLE PROTEINS

Textured vegetable proteins (TVPs) show amounting potential as scaffold materials for cultured meat applications. TVPs are protein extracts from vegetable sources, usually legumes, which can be freeze-dried to obtain a porous, sponge-like structure TVPs do not require further modifications to improve its functionality (Ben-Arye *et al.*, 2020). These characteristics make TVPs an interesting scaffold material for cultured meat purposes.

Considering TVPs are produced from edible crops, applications thereof in cell-based meats could originate hybrid products with considerably lower costs for purchasers. In fact, a recent work has given a thorough characterization of the application of TVP scaffolds to grow and differentiate bovine cells (Ben-Arye *et al.*, 2020). Similarly, other works have demonstrated the efficacy of extrusion techniques to obtain fibres composed of pea and maize protein isolates (Krona *et al.*, 2017). These fibres allow successful proliferation of

C2C12 myoblasts and differentiation into myotubes, confirmed through visualization of actin filaments using confocal microscopy after staining.

CHITOSAN

Chitosan is a product of the deacetylation of chitin, an oligosaccharide naturally present in the exoskeleton of various crustaceans. In the food industry, edible films and coatings of chitosan can increase the shelf-life of fruits and vegetables (Jianglian and Shaoying, 2013), and the freezing capacity of fish products (Morachis-Valdez *et al.*, 2017). As a biopolymer, chitosan can form hydrogels with diverse mechanical properties (Drury and Mooney, 2003; Nie *et al.*, 2016) and complex microporous scaffolds (Li *et al.*, 2017), while being biocompatible and biodegradable. These attributes make chitosan an attractive scaffold material for cultured meat applications.

Chitosan is often utilized in combination with other materials, such as the biodegradable polymer Poly (ε-Caprolactone) (PCL; Cooper *et al.*, 2010), or bacterial cellulose (BC; Li *et al.*, 2017). Methods for chitosan fibre fabrication include applying a liquid nitrogen solution to BC, followed by freeze-drying, and ultimately layering it with chitosan (Li *et al.*, 2017), as depicted in Figure 14, which ultimately allows the formation of an aligned fibrous scaffold. In the same work, Li and team have described topographical and mechanical variations in BC-chitosan scaffolds according to their chitosan content, where 1% chitosan-BC scaffolds had the highest mean pore diameters (4±2 µm), overall porosity (upwards of 80%) and surface area (29.5 m^2/g scaffold), as well as the lowest crystallinity index (Li *et al.*, 2017).

Electrospinning can be used to crosslink PCL with chitosan and obtain 3D scaffold structures that allow the growth, proliferation, and differentiation of C2C12 myoblasts (Figure 16-C), characterized by the expression of MyoG and late myogenic proteins actin and MHC (Cooper *et al.*, 2010). Other works have shown that chitosan appears to promote adhesion and growth of myoblasts over the proliferation of fibroblasts (Iyer *et al.*, 2014).

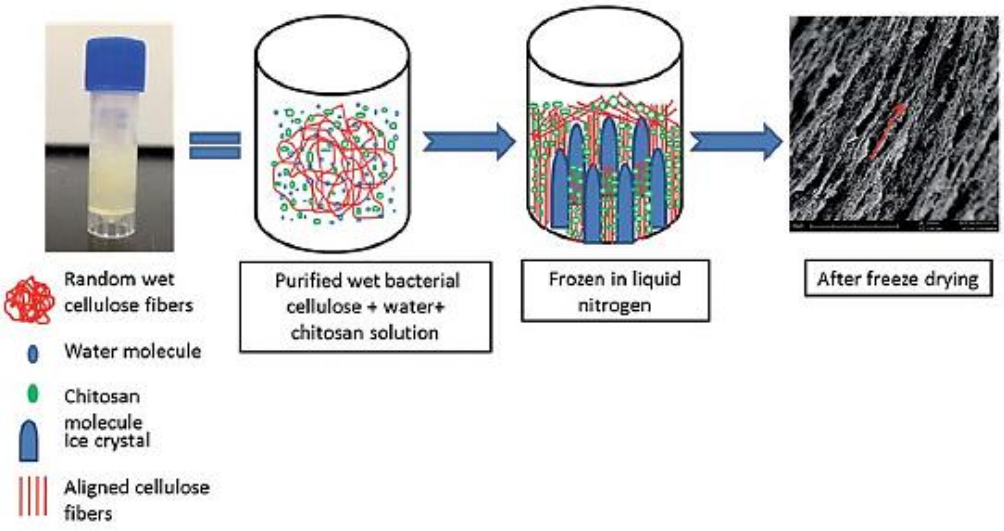

Figure 14- Fabrication of aligned scaffolds composed of chitosan and bacterial cellulose in aqueous solution, which are frozen in liquid nitrogen to generate vectorially aligned fibres, and then freeze-dried to obtain porous scaffolds. From Li *et al.* (2017).

ALGINATE

Alginate is a hydrophilic polysaccharide derived from brown seaweed (*Phaeophyceae*) such as those of *Laminaria* genus (Figure 15-A), and from bacteria of *Pseudomonas* and *Azotobacter* genera (Pereira and Cotas, 2020; Urtuvia *et al.*, 2017). This biopolymer can be used in the food industry to produce films and coatings for food preservation (McHugh, 2003). In addition, alginate has large applications in tissue engineering, since it can be arranged into hydrogels through ionic crosslinking with cations such as Ca^{2+} (Drury and Mooney, 2003; Sergeeva *et al.*, 2019) and into firmer macroporous structures after freeze-drying (Dar *et al.*, 2002; Enrione *et al.*, 2017), by using a combination of biomaterials and fabrication strategies (Enrione *et al.*, 2017; Bharatham *et al.*, 2014) or through chemical modifications to obtain highly-porous alginate sponges (Mohan and Nair, 2005).

Furthermore, alginate can form fibres through electrospinning using a carrier material, such as poly ethylene oxide(PEO) or dextran (Bonino *et al.*, 2011; Vigani *et al.*, 2018), or as part of a mixture of polymers (Apsite *et al.*, 2019). Production costs of alginate are also relatively low, ranging 6.5 to 11 $/kg for food-grade sodium alginate, and 13 to 15.5 $/kg for pharmaceutical-grade purity (Pereira and Cotas, 2020).

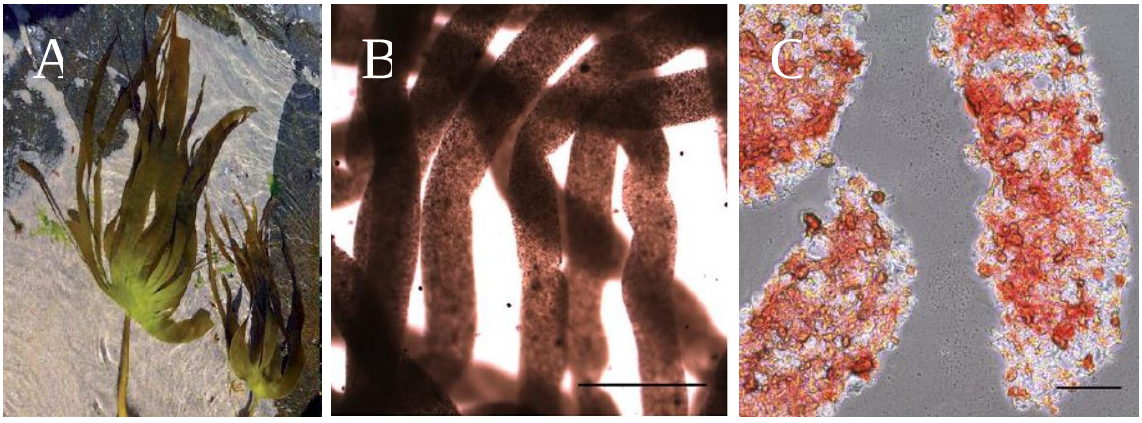

Figure 15- Alginate sources and capacity to sustain growth of bovine adipose cells. **(A)**Photograph of *Laminaria ochroleuca,* part of a genus commonly used for alginate extraction. Retrieved from Pereira and Cotas (2020). **(B)** Elongated alginate scaffolds seeded with bovine preadipocytes from the stromal vascular fraction of digested adipose tissue. Scale bar= 2000 µm. **(C)** Alginate scaffolds with preadipocytes after 72h in a medium with selected fatty acids, stained with Oil Red-O to observe and confirm lipid accumulation. Scale bar= 100 µm. Pictures (B) and (C) were adapted from Mehta *et al.* (2019).

Alginate is not inherently biocompatible, but its biocompatibility can be improved through chemical adjustments. For instance, the addition of RGD motifs to alginate has been shown to facilitate the adherence of myoblasts (Rowley and Mooney, 2002). In fact, a composite scaffold of low and high molecular weight alginate scaffolds with RGD peptides (Figure 16-A) has been shown to support the growth, survival, and migration of primary mouse myoblasts, as well as their differentiation into myotubes (Figure 16-B; Wang *et al.*, 2012). Previous research study from Chandler and team has described an increase in average cell number and total cell

area, when modifying alginate scaffolds with RGD motifs to culture mouse 3T3-L1 preadipocytes (Chandler *et al.*, 2011).

In addition, composites of sodium alginate, salmon gelatin, a gelling agent such as agar or agarose, and glycerol have been reported to support the adhesion and proliferation of C2C12 myoblasts (Enrione *et al.*, 2017). In this experiment, no additional peptide crosslinking was performed since collagen-based salmon gelatin naturally contains RGD sites, which serve as anchor points for cell adhesion. A bilayer of aligned PCL and methacrylated alginate fibres produced by electrospinning has also been deemed appropriate for the attachment and differentiation of myoblasts (Apsite *et al.*, 2019). The authors have found that the scaffolds were able to generate high percentages of aligned myotubes and a microtissue construct able to contract under electrical stimulation, confirming functionality akin to native skeletal muscle.

Alginate scaffolds have been shown to support adhesion and proliferation of lipid-accumulating preadipocytes from bovine (Figure 15-B and 15-C; Mehta *et al.*, 2019). In addition, other reports have described successful monolayer cultures of mouse L929 fibroblasts in porous alginate sponges, where the scaffold was reported to be biocompatible and nontoxic to cells (Mohan and Nair, 2005).

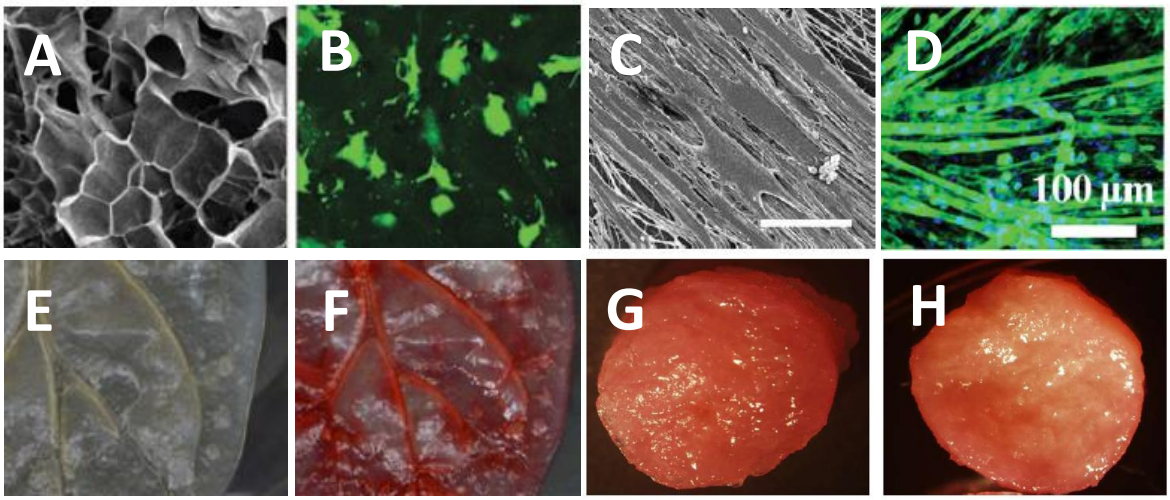

Figure 16: Overview of three-dimensional scaffolds for potential use in cultured meat applications. **A**- SEM imaging showing surface topography of lyophilized porous alginate scaffolds composed of low- and high-molecular weight alginate. Retrieved from Wang *et al.* (2012); **B**- Myotubes expressing green fluorescent protein in alginate scaffolds fabricated as in A. Retrieved from Wang *et al.* (2012); **C**- Muscle cell distribution on aligned chitosan-polycaprolactone nanofibrous scaffolds, using scanning electron microscopy. Scale bar 80 µm. Retrieved from Cooper *et al.* (2010); **D**- Muscle cell differentiation in silk protein mats, with fluorescently stained actin filaments (green) and nucleus (blue), 3 days after inducing differentiation into myotubes. Scale bar 100 µm. Retrieved from Manchineella *et al.* (2017); **E** and **F**- Imaging of decellularized spinach leaves, before and after staining with Ponceau red, respectively. From Gershlak *et al.* (2017); **G** and **H**- Cultured meat constructs (6 mm diameter sections) with bovine satellite cells (SCs) seeded on textured soy protein (TSP) scaffolds and a co-culture of bovine SCs and smooth muscle cells on TSP scaffolds, respectively. Retrieved from Ben-Arye *et al.* (2020).

GELATIN AND COLLAGEN-LIKE PEPTIDES

Gelatin has been extensively used as a scaffold material for tissue engineering, due to its biocompatibility and overall cost-effectiveness, since it is a by-product of livestock processing and naturally functional.

Fibrous gelatin scaffolds can be fabricated by electrospinning (Okutan *et al.*, 2014) and immersion rotary get spinning, and have been shown to sustain the proliferation of bovine aortic smooth muscle and rabbit skeletal muscle cells (MacQueen *et al.*, 2019). In addition, salmon-derived gelatin can be combined with alginate and a gelling agent (agar or agarose) to produce porous scaffolds suitable for C2C12 myoblast growth, exhibiting high cell viability and slow biodegradation, though with sub-optimal adhesion rates to the scaffold (Enrione *et al.*, 2017). A combination of both biomaterials into an edible film fabricated with micromolding techniques has been shown to allow the seeding, proliferation, and differentiation of C2C12 myoblasts as well as promoting significant expression of late myogenic genes such as MHC and desmin, when compared to flat surface films (Orellana *et al.*, 2020). In addition, gelatin-derived collagen sponges had been previously reported to allow adhesion and proliferation of C2C12 cells and SCs (Cimetta *et al.*, 2007)

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SILK

The potential application of silk fibres as scaffold materials in cultured meat is mostly attributable to silk's overall compatibility and physical properties. Firstly, silk-based scaffolds have been shown to allow growth and attachment of numerous cell types of interest for cultured meat, including fibroblasts (Widhe *et al.*, 2010), ECs (Liu *et al.*, 2011; Zhang *et al.*, 2015), muscle-progenitor SCs (Johansson *et al.*, 2016), SMCs (Liu *et al.*, 2011) and skeletal muscle myoblasts (Manchineella *et al.*, 2017).

Secondly, silk proteins can be arranged in various conformations, namely fibres, films, meshes and foams (Bauer *et al.*, 2013; Schacht *et al.*, 2016; Widhe *et al.*, 2010). In fact, different silk structures can adhere to different cell types with distinct degrees. For instance, fibroblasts seem to easily attach to both mesh, fibre, and film arrangements (Bauer *et al.*, 2013; Widhe *et al.*, 2010), whereas patterned films appear to particularly aid myoblast alignment (Bauer *et al.*, 2013).

Thirdly, silk scaffolds allow the fusion of myoblasts into myotubes with thick actin filaments (Manchineella *et al.*, 2017), as shown in Figure 16-D. Silk fibres can also be blended with a plurality of biomaterials. For example, combining recombinant silk with PCL and gelatin can form biocompatible fibres through electrospinning (Xiang *et al.*, 2018). Additionally, fibroin can also be isolated and utilized in combination with other polymers.

Much like gelatin, silk has naturally present bioactive sites that allow membrane proteins from cultured cells to adhere. Nonetheless, addition of RGD peptides still appears to increase myoblast adherence to silk scaffolds (Bauer *et al.*, 2013). Furthermore, recreating the ECM environment by adding proteins such as fibronectin and vitronectin in RGD-loaded silk scaffolds can increase the adhesion and proliferation of SCs (Johansson *et al.*, 2016).

IX. SCALE-UP CONSIDERATIONS

There are various parameters that should be considered for cost-efficient scaling of mammalian cells, in order to assure a sustainable bioprocess for cultured meat production that can reach price parity with conventional products. These include considerations with culture medium composition and cell expansion in different BRs.

Reducing overall costs of other medium constituents is imperative to achieve profitable, large-scale production of cultured meat. An article from the *Good Food Institute* has highlighted potential scenarios for effective cost reduction of culture medium production, using the Essential 8™ medium as a standard (Specht, L., 2020) and accounting for medium volumes of 20,000 L for the bioprocess, with costs ranging $337/L if ordered from the manufacturer. Essential 8 is a commercial SFM composed of DMEM, FGF-2, TGF-β, insulin, transferrin, sodium selenite, $NaHCO_3$ and ascorbic acid 2-phosphate. This analysis predicts that in order to get a significantly low-cost contribution of medium towards the final cultured meat product, in a way that the final price achieves price parity with conventional meat, medium costs should range $0.30/L (Specht, L., 2020). Although Essential 8 does not appear to be optimal for bovine muscle cell proliferation (Kolkmann *et al.*, 2020), similar cost reductions in optimized medium formulations for cells will guarantee cultured meat's feasibility, but requires thorough innovation. The highest cost inputs are mostly derived from commercially available GFs. For instance, FGF-2 costs upwards of $2 million/g, while pharmaceutical-grade TGF-β costs range $80 million/g (Specht, L., 2020). Even though small concentrations of GFs are required per litre of culture medium, they represent a large percentage of the cost when accounting for large scale medium requirements.

Alternative ways to reduce GF costs include reducing GF purity, generating less expensive equivalents, improving cost-efficacy of current production systems, or producing recombinant GFs in plants and bacteria (Kuo *et al.*, 2020; Post *et al.*, 2020; Ben-Arye and Levenberg, 2019; He *et al.*, 2016). In addition, conditioned medium from other cells and co-culture settings can reduce GF inputs (Post *et al.*, 2020; Korovina, D., 2019). Moreover, medium recycling units in BRs can improve overall medium costs (Post *et al.*, 2020; Allan *et al.*, 2019).

Glucose and amino acids are two essential medium components for mammalian cell proliferation, and microalgae might be a viable source of both nutrients (Okamoto *et al.*, 2020). These are usually obtained whether from grains or heterotrophic organisms. While glucose is inexpensive and readily available from different sources, amino acid supply must also support bulk purchases with minimum variability.

To select the ideal BR system for large-scale *in vitro* production of cells for cultured meat, the maximum cell density rates of the different apparatus must be considered. A recent review from Allan *et al.* (2019) estimates that 1 kg of meat composed solely of muscle cells requires 2.9×10^{11}cells to be produced. Bodiou and team suggest that 10kg of meat requires 10^{12} cells (Bodiou *et al.* 2020), which is within the same range of the aforementioned estimate.

Using previous estimates for average cell densities obtained with anchorage-dependent cells, Allan *et al.* (2019) calculated that the volume required to produce 1kg of meat in different BR settings is: 2,900 L in lab-scale culture flasks, 570 L for stirred-tank BRs, 110 L in packed-bed BRs, 48 L in fluidized-bed BRs, and 1.4 L in hollow-fibre BRs (Allan *et al.*, 2019). Therefore, there can be several pathways to achieve the intended number of cells for cultured meat products. After optimization in small-scale BRs and, in the case of adherent

cells, addition of MCs, cells can be expanded in bench-top BRs and then scaled up in BRs of increasing volumes, such as stirred tanks, or scaled-out by adding more BRs, like hollow fibres (Figure 17).

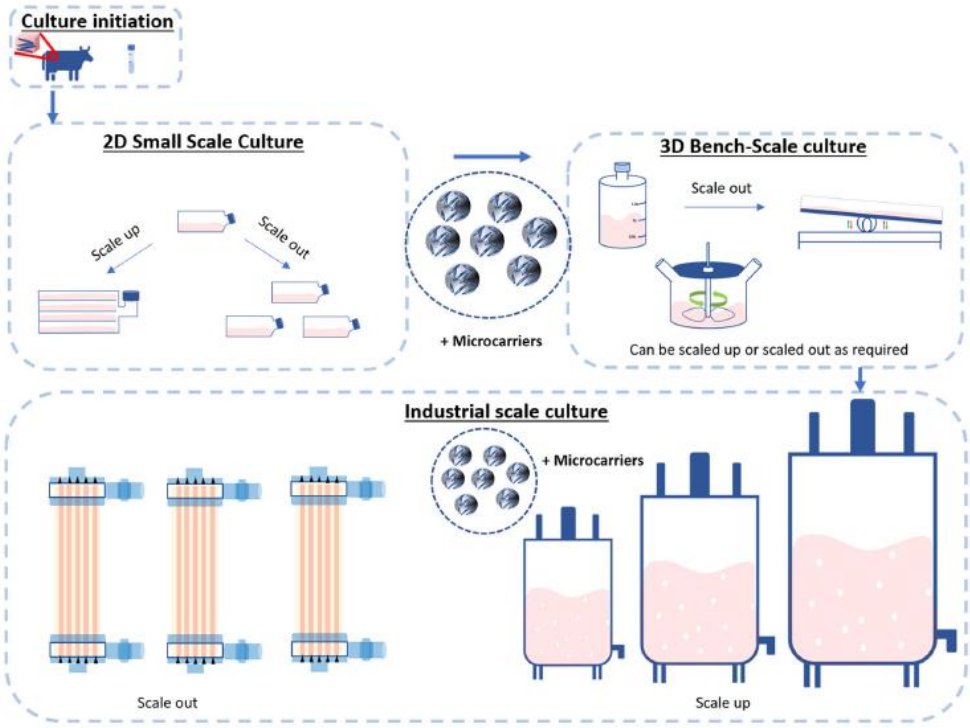

Figure 17- Pathway for large scale production of adherent animal cells. From a live biopsy or a cryopreserved vial, cells can proliferate in 2D cultures in hyper and t-flasks at small scale, and consequently be scaled up for larger devices in bench-scale cultures, after adding microcarriers. After reaching high densities, cells can be transferred the chosen devices for industrial scale production, whether scaling out by having several bioreactors, or scaling up the bioreactor chosen. From: Bellani *et al.* (2020).

Thus, large-scale production of cells for cultured meat application will be demanding and require significant optimization of chosen BR systems.

Optimized bioreactor conditions can be precisely predicted using microfluidics approaches and computational modelling of fluid dynamics (Swartz, E., 2019). Microfluidic devices can mimic the cellular environment at microscale and offer unparallel control of nutrient delivery and mechanical responses of cells to shear stress (Marques and Szita, 2017). Because these devices have working volumes downwards of 1 mL, microfluidic techniques can gather high-throughput data which can be used to optimize culture conditions before scale-up, in a cost-effective and timely fashion. Figure 18 shows the relevance of information that can be obtained from microfluidic devices and how it can provide important data for scaling up bioprocesses to larger volume cultures, in bench-top, pilot- and industrial-scale BRs.

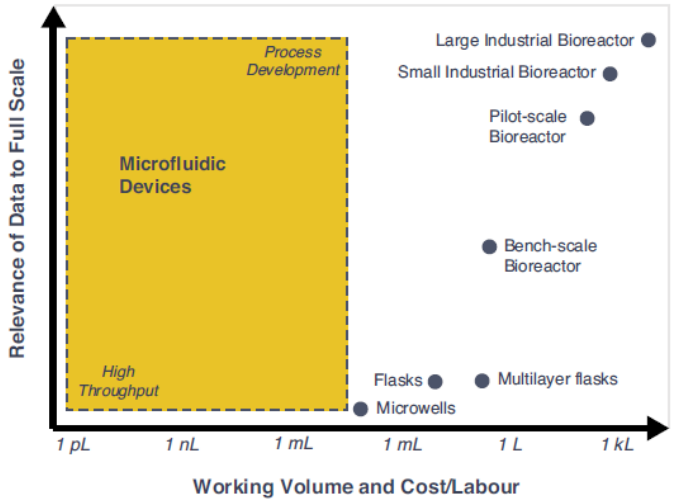

Figure 18- Relevance of data from microfluidic devices and BRs of different volumes, according to its cost and labour. While flasks and multilayer devices range mL to L scale, they offer limited data for scaling up a bioprocess. As volume increases, the data retrieved from the BR systems become more relevant for industrial scale endeavours. Microfluidics allow high-throughput screening on important parameters which are of interest for scaling operations. Adapted from: Marques and Szita (2017).

Due to its small scale, bioprocess variables such as oxygen levels and pH can be easily monitored with optical sensors, and variables can be adjusted to support the intended outcome (Marques and Szita, 2017).

In addition, computation fluid dynamics (CFD) models allow the prediction of fundamental physical parameters that can influence cell proliferation in BRs before scaling, using theoretical approximations that match experimental results (Villiger *et al.*, 2017). In this study, CFD predictions for oxygen transfer rates, mixing times and maximum shear stress were akin to the results from experiments in different BR volumes, from 15 mL to $15x10^3$ L. CFD can also be used to optimize bioreactor geometry and identify the add-ons required to achieve optimal mass transfer and energy dissipation rates for animal cell cultures (Li *et al.*, 2020). In the CFD modelling from Li and team, a screw-shaped air lift bioreactor with a volume of $300m^3$ and height of 13.75 m was designed, which was predicted to sustain a cell density upwards of $2x10^8$ cells/mL, by coupling the BR with microporous sparges and other improvements.

X. OBJECTIVES AND METHODOLOGY

Due to the pandemic restrictions put in place throughout the EU, my original thesis in Maastricht University (and Mosa Meat), which was intended to start in April 2020, was postponed in May and later cancelled in the midst of June. Hence, a state-of-the-art review of cultured meat production and considerations was put in place as an alternative thesis, after consideration and approval from the supervisor and the coordination of the course. In addition, five case studies will be analysed in the next chapter – "Results and Discussion" -, where results from other authors will be presented and discussed, with the intention of providing an assessment of recent advancements in the field of CA. Whenever necessary, an overview of the article will be provided, to give insights on the methods and aims of each study.

Keywords used during the literature search include: Bovine, Scaffolds, Edible, Microcarriers, Bioreactors, Cultured Meat, Cultured Meat, Cell-based Meat, Satellite Cells, Mesenchymal Stem/Stromal Cells, Embryonic Stem Cells, Co-cultures.

The set of criteria by which the peer-reviewed articles were evaluated for applicability as case-studies included, but were not limited to:

i) Novelty of the research
ii) Works should describe basic research with thorough experimental analysis, across a wide array of scientific disciplines within the realm of Biotechnology and Bioengineering
iii) Year of publishing should not be earlier than 2019
iv) Relevance of breakthroughs and direct implications in cultured meat research

A total of 35 research articles were primarily retrieved from PubMed and PMC databases[1], as well as Google[2]. From these, 12 papers were excluded since they did not comply with at least one criterion form the aforementioned. Consequently, 4 articles were excluded due to lack of broad applicability in the field, and lastly 5 articles were selected from the remaining 19, based on diligence of articles and avoiding an overlap in methodologies among selected articles.

XI. RESULTS AND DISCUSSION

XI. 1. Ben-Arye, T., Shandalov, Y., Ben-Shaul, S., Landau, S., Zagury, Y., Ianovici, I., ... & Levenberg, S. (2020). Textured soy protein scaffolds enable the generation of three-dimensional bovine skeletal muscle tissue for cell-based meat. *Nature Food*, *1*(4), 210-220.

Overview

This work from Ben-Arye and team intended to evaluate the capacity of soy TVP to serve as a scaffold for bovine skeletal muscle cell differentiation. Different co-culture techniques have been investigated, namely a monoculture of SCs, a co-culture of SCs and SMCs, one of SCs and ECs, and a tri-culture consisting of SCs, SMCs and ECs. The proteomic expression profile of co-cultures and monocultures was elucidated through comparative analytical methods, as well as through quantification of protein deposition in the scaffolds. In addition, the proliferation and differentiation medium used to culture bovine SCs was optimized through a full factorial statistical analysis before the experiments with TVP scaffolds, by seeding cells in PLLA-Poly(lactic-co-glycolic acid) (PLGA) scaffolds with different concentrations of medium components and assessing their morphology through immunostaining of actin filaments after differentiation. Ultimately, the mechanical attributes of bovine co-cultures were briefly assessed by measuring their elasticity modulus and ultimate tensile strength and comparing the results with native bovine muscle tissue. The sensorial attributes of the cultured meat constructs were then evaluated by a panel of volunteers.

Scaffold characterization

TSP scaffolds (one with 69% named TSP-1, and another with 53% protein content dubbed TSP-2) have shown to contain a wide range of pore diameters. TSP-1 had a wide range of pore sizes, with >400 µm diameter pores representing 32% of the total pore area. A heterogeneous distribution of pore sizes allows different cell types to adhere (Jahangirian *et al.*, 2019), which makes TSP an interesting scaffold for both cultured meat and biomedical applications. Moreover, microporous scaffolds allow communication between neighbouring cells and cell migration through the scaffolds (Bružauskaité *et al*., 2016). However, cell attachment is optimal in scaffolds with pore sizes of increasing diameters, though specific to cell type intended to use. For instance, large-size pores and fibre diameters are preferential for adhesion of SMCs and ECs in PCL scaffolds (Ju *et al*., 2010).

TSP-1 also contained the highest percentage of porosity, in the range of 56%. Highly porous scaffolds are advantageous for tissue engineering applications since they allow increasing nutrient flow to cells and adequate surroundings for ECM development (Jahangirian *et al.*, 2019).

Remarkably, both TSP scaffolds showed high pore interconnectivity and pore directionality. Pore directionality is essential for muscle cell differentiation, since the physical properties of the scaffold surface can influence myoblast elongation, and alignment is essential for cells to resemble native muscle tissue and remain contractile (Zidarič *et al.*, 2020). Primary cell seeding assays showed that TSP scaffolds can sustain fibroblast cultures for 3 weeks. A similar result was consequently observed with monocultures of bovine SCs, as well as with co-cultures, where authors reported that cells could effectively penetrate TSP scaffolds.

Cells and culture medium

The myogenic potential of bovine SCs cultured with optimized proliferation and differentiation medium (previously described in Table 1 and 2) was primarily assessed in PLLA/PLGA scaffolds before testing on TSP scaffolds. It was shown that the proliferation medium improved cell coverage and prevented the differentiation of SCs into myotubes in both PLLA/PGA and TSP scaffolds (Fig 19-A). Noticeably, statistical analysis following a factorial model of effects of medium compositions (control vs proliferation medium, IGF-1or EGF supplementation of differentiation medium) and interactions between them have shown that IGF had synergistic effects with the proliferation medium and that both EGF and IGF-1 addition to differentiation medium had beneficial effects in myotube formation.

IGF-1 is part of multiple proliferation processes in the development of different tissues, as it can regulate genes related with pluripotency and tissue-specific differentiation of MSCs (Youssef *et al*., 2017) and has important roles in FAP proliferation (Li *et al.*, 2020). In addition, IGF-1 has multiple roles in skeletal muscle tissue development, including the improvement of SC proliferation (Ahmad *et al*., 2020).

Previous studies had reported a positive effect of IGF-1 supplementation in early proliferation stages of human myoblasts in concentrations ranging 5-60 ng/mL (Witt *et al*., 2017). In this paper from Ben-Arye and team, the concentration of IGF-1 was 100 ng/ mL, in both proliferation and differentiation media (see Table 1 and 2). Addition of GFs in growth medium must be carefully examined if a commercial endeavour is to be put in place.

The costs of recombinant IGF-1 from rat, expressed in *E. coli* (Sigma-Aldrich), range 13.1 million € per gram of product, with ≥95% purity. Assuming previous estimates for the volume required to produce 1kg of meat in different BR settings (Allan *et al*., 2019), which were discussed in a previous Section (see Scale Up Considerations), a system like hollow fibres would require a volume of 1.4L to produce that amount of cultured meat. Under the assumptions that fresh medium is added every other day, the proliferation and differentiation process lasts 21 days, and no GF is produced by cells and re-circulated to the BR, a total of 14.7 L of medium containing 100 ng/mL of IGF-1 would be required for 1kg of meat. This would represent an overall amount of 0.001g of IGF-1, which would have a commercial cost in the range of 13,100€, solely for the addition of one medium component. While not considering cell density optimization and medium recycling, this oversimplified estimate only highlights the amounting cost-reduction required in culture medium to achieve competitive costs with conventional meat.

In addition to the cost-reduction methods for culture medium production highlighted by Specht, L. (2020), which included dropping GF purity and its concentration in medium overtime, and those discussed in previous sections (see Scale Up Considerations), production of recombinant GFs in-lab in *E. coli* has shown to reduce culture medium overall costs for iPSCs to 16$ per L, in which GFs represented a negligible portion of the cost after optimization (Kuo *et al*., 2020). In this study, 15 mg of an isoform of FGF-2 was produced after 2 days in a 1 L vessel containing engineered *E. coli*.

Additionally, EGF addition to differentiation medium had no significant effect on myotube coverage, but improved myotube area and shape complexity in a significant manner. EGF is part of muscle tissue microenvironment, where crosstalk between SCs and myotubes occurs upon muscle injury, and can be secreted by myotubes and other cells to activate senescent SCs (reviewed in Yin *et al*., 2013). Moreover, EGF substrates

have been shown to improve murine SC proliferation and activation, as well as an increase in expression of myogenic genes such as MyoG after differentiation (D'Andrea *et al*., 2019). A previous study with human myoblasts has reported that supplementation of 10 ng/mL of EGF to medium improved myoblast proliferation but had no effect in differentiation medium supplementation (Leroy *et al*., 2013). In the same work, the activity of EGF receptor was decreased following induction of differentiation, though with prominent expression during proliferation stages. However, these works have utilized FBS in the medium composition, which contains varying amounts of EGF, ranging 1-100 ng/ml (Freshney, R., 2015), meaning that determining optimal concentrations of EGF (and other GFs) for myogenic cell proliferation and differentiation is limited by other components in growth medium.

The optimized proliferation medium was shown to improve bovine SC coverage from 18–71% in TSP scaffolds (Fig. 19-B, left) and cell coverage on both scaffolds (TSP-1 and TSP-2) after growth in differentiation medium (Fig. 19-B, right).

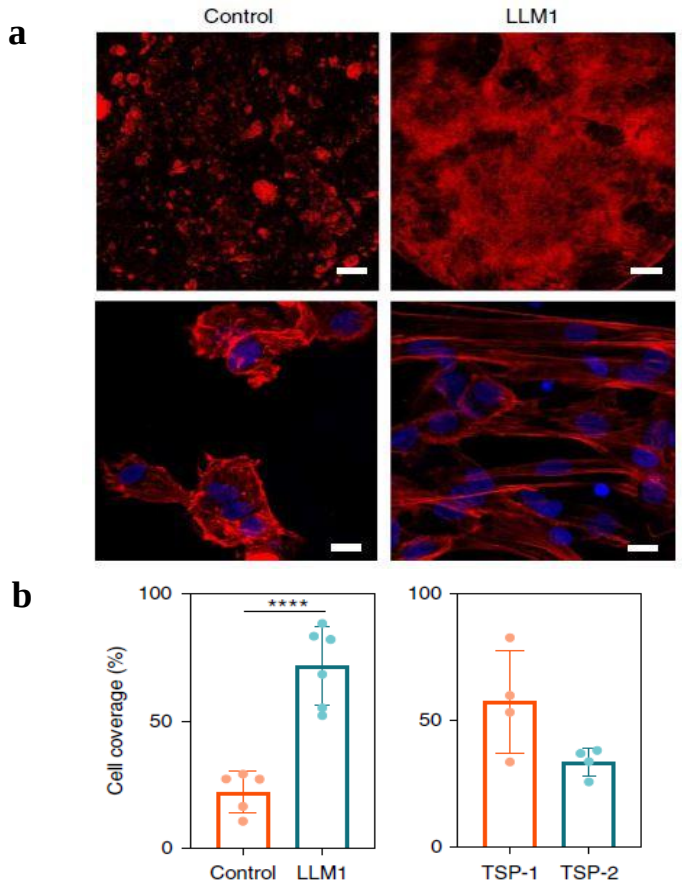

Figure 19- Effects of culture medium optimization on cell proliferation. (**a)** On top, bovine SCs with Dil staining, and bottom pictures depict the combination of phalloidin (red) and DAPI (blue), after proliferation stage in control medium (left) or the optimized proliferation medium LLM1 (right). Scale bar- 300 µm (top); 10 µm (bottom). **(b)** Cell coverage percentage after proliferation in control (n=5) or LLM1 (n=8) medium (left) and in different TSP scaffolds (n=4 for each scaffold type) after differentiation (right). ****P = 0.000061. Adapted from: Ben-Arye *et al.* (2020).

Myogenesis Co-cultures TSP scaffolds

Both SC/SMC co-culture and tricultures had high expression of the transcription factor MyoG than SC monocultures and co-cultures of SCs/ECs, as seen through immunofluorescence staining (Figure20-A). In addition, both SC/SMC co-cultures and tri-cultures had a stretched morphology (Figure 20-B). Western blot analysis of different co-culture experiments has shown higher MyoG expression in both SC/SMC co-cultures (3.6-fold) and SC/SMC/EC tri-cultures (4.6-fold), than in SC monocultures (Figure 20-C). Interestingly, MyoG gene expression in SC/SMC co-cultures was higher than in tri-cultures, as observed through quantitative PCR analysis. MyoG is a transcription factor expressed in differentiating SCs, myocytes and early myotubes (Yin *et al.*, 2013; Bentzinger *et al*., 2012), though this increase could be explained by overall outperformance of co-culture conditions in comparison with monocultures of SCs, which in turn enhance the flow of paracrine factors and cell-to-cell communication, that improve myotube formation in SC/SMC co-cultures and tri-cultures.

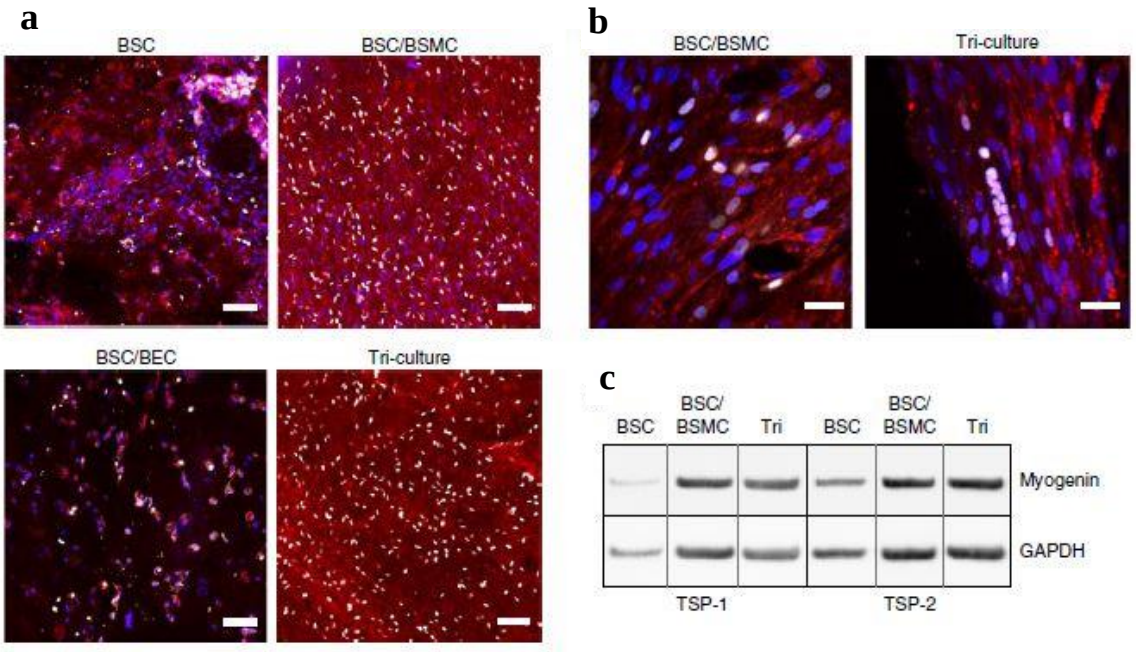

Figure 20-(a) Imaging of bovine monocultures of SCs, co-cultures of SCs with SMCs or ECs, and tri-cultures after immunofluorescence staining of SCs with Dil (red), nuclei with DAPI (blue) and myogenin-containing myotubes (white) following 7 days of culture in optimized differentiation medium. Scale bar- 300µm **(b)** Magnification of co-culture of SCs and SMCs (left) and tri-cultures of SCs, SMCs and ECs (right). Scale bar-30 µm. **(c)** Western blot analysis of different cell culture settings and expression patterns for late myogenic marker myogenin and housekeeping protein GAPDH. Abbreviations: BEC- bovine endothelial cells; BSC- bovine satellite cells; BSMC- bovine smooth muscle cells; GAPDH- glyceraldehyde 3-phosphate dehydrogenase; TSP- textured soy protein. From: Ben-Arye *et al.* (2020).

Restraints in growth capacity of skeletal muscle tissue *in vitro* is often observed due to the lack of an adequate vascularisation system (Ben-Arye and Levenberg, 2019; Gholobova *et al.*, 2015). This in turn limits nutrient and oxygen diffusion to some cells and can generate necrotic centres. Co-culture of myoblasts with ECs allows the formation of a pseudo-endothelial network (Gholobova *et al.*, 2015). Furthermore, vascular SMCs and

ECs are the main components of blood vessels and are known to interact with each other through direct and indirect pathways and promote atherogenesis (Li *et al*., 2018). Since blood vessels are part of functional skeletal muscle tissue (Ben-Arye and Levenberg, 2019), it is expected that recreating the microenvironment of native muscle tissue by co-culturing neighbour cell types of SCs and myotubes, can improve the overall functionality of the construct.

ECM deposition and mechanical assays

Trichrome staining has shown increased deposition of ECM proteins in both co-cultures of SCs/ECs, SCs/SMCs, and tri-cultures, without a significant effect from the type of TSP used. The highest increase in protein deposition and weight of the construct was observed in co-cultures of SCs/SMCs. 3.2-fold increase in collagen type I deposition was observed in SCs/SMCs constructs and a 3.8-fold increase in tri-cultures of SCs/SMCs/ECs, when compared to monocultures of SCs. Studies on protein deposition in scaffolds as an indicator of muscle construct improvement are limited.

ECM protein deposition in scaffolds is an important indicator of their adequacy for sustaining cells and allowing a microenvironment similar to native muscle tissue to be formed. Importantly, the amount of connective tissue can vary between different skeletal muscles (Burton *et al*., 2000), and the adequate ECM formation should be compared to the same animal and muscle intended to replicate.

The determination of Young's modulus and ultimate tensile strength of the muscle constructs, composed of different cell types, has shown that these cultured meat samples had mechanical attributes akin to those of native bovine skeletal muscle tissue. A panel of volunteers noticed that the addition of cells improved the sensorial attributes of the constructs and that these had organoleptic properties akin to conventional meat products.

XI. 2. Hanga, M. P., Ali, J., Moutsatsou, P., de la Raga, F. A., Hewitt, C. J., Nienow, A., & Wall, I. (2020). Bioprocess development for scalable production of cultivated meat. *Biotechnology and Bioengineering*.

Overview

This work aimed to determine a scalable cell expansion methodology for bovine AT-MSCs, by growing these cells attached to plastic MCs in spinner flasks and determining their physical and chemical attributes. Furthermore, the authors have assessed the multi-lineage potential of bovine AT-MSCs, namely adipogenic, chondrogenic and osteogenic differentiation capacity. Cell count parameters have been subsequently employed to determine different critical cell attributes, such as specific growth rate, population doubling time and cumulative population doublings. Additionally, various medium exchange strategies were examined, and their effects on glucose and lactate profiles were registered.

Preliminary expansion and initial characterization

The expansion of AT-MSCs in MCs was carried out in 100 mL spinner flasks (Figure 21-A). After 5 days of culture, AT-MSCs could be seen establishing bridges with MCs (Figure 21-B) and high cell viability was

further confirmed through live/dead staining, where live cells can be seen in green and a limited number of dead cells in red (Figure 21-C). Cell bridges are common in MC expansion of MSCs, and have been reported in fetal human MSCs cultured with different commercial MCs, where most of them formed large aggregates in culture (Goh *et al.*, 2013). In the work of Hanga and team, the authors reported that bovine the cells extracted express markers CD73, CD90 and CD105, and could be differentiated into osteogenic, chondrogenic and adipogenic lineages, which are defining characteristics of MSCs. These surface markers had been previously listed for the identification of bovine-derived MSCs in a review from Hill and team, where CD29, CD166, CD44 were also listed as positive markers, among others (Hill *et al*., 2019). Importantly, surface markers from different cells including MSCs can be species-specific, since human MSCs usually do not express CD29, CD166 and CD44 (Mohamed-Ahmed *et al*., 2018), and differences among equine, canine and bovine cell surface chemistry have been described (Hill *et al*., 2019). Further analysis is required to confirm adequate markers for cells from different animals, considering the heterogeneity of cell populations.

Osteogenic differentiation was confirmed through visualization of bone mineralisation and expression of alkaline phosphatase, whereas chondrogenic differentiation was visualized through staining macromasses in blue. The adipogenic potential of these MSCs was limited, as 12.1% ±2.8 of cells presented lipid droplets, out of 1982 cells. One study has reported higher adipogenic potential than chondrogenic or osteogenic in human AT-MSCs, and more prominently when compared to BM-MSCs (Mohamed-Ahmed *et al*., 2018), though the limitation in adipogenic potential could be also due to cell characteristics linked with donor animal features or physical conditions. Adipogenic commitment is regulated by multiple factors including Wnt/βcatenin pathway, and differentiation of porcine AT-MSCs has been reported to be inhibited *in vitro* by Wnt3a (Li *et al*., 2008), so Wnt3a inhibitors could help targeting adipogenesis of AT-MSCs.

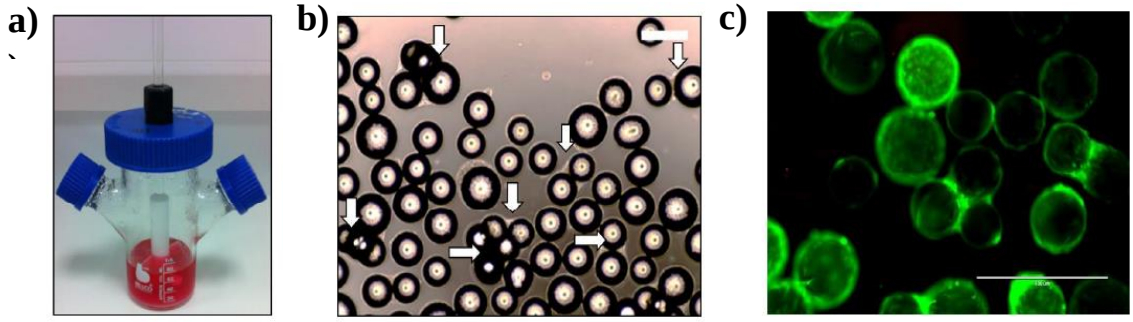

Figure 21- (a) Spinner flask used for cell expansion, volume= 100 mL; **(b)** Image of bovine adipose tissue-derived mesenchymal stem/stromal cells (AT-MSCs) in microcarrier cultures at day 5, using phase contrast. White arrows indicate cell-microcarrier bridges. Scale bar- 200 μm; **(c)** AT-MSCs in microcarriers at day 5 in culture after live (green)/dead (red) staining. Scale bar- 400μm. Adapted from Hanga *et al*. (2020).

This work by Hanga and team have not explored the capacity of bovine MSCs to differentiate into the myogenic lineage, which has been reported following stimulation with different medium supplements and culture strategies (Korovina, D., 2019). Such assessment would assure the feasibility of using MSCs as a cell

source for cultured meat, and failure to establish myogenic cells from MSCs would likely hinder their application in CA.

The first results following expansion of bovine AT-MSCs for 5 days in both monolayers and with MCs show that both culture conditions had similar fold increase in growth (Figure 22-A), specific growth rate (Figure 22-B) and cell population doubling time of 46 ± 1.34 hours in monolayer cultures, and 47.12 ± 8.7 hours in MC culture (Figure 22-C), where no significant differences were found ($p > 0.05$) among cell proliferation platforms.

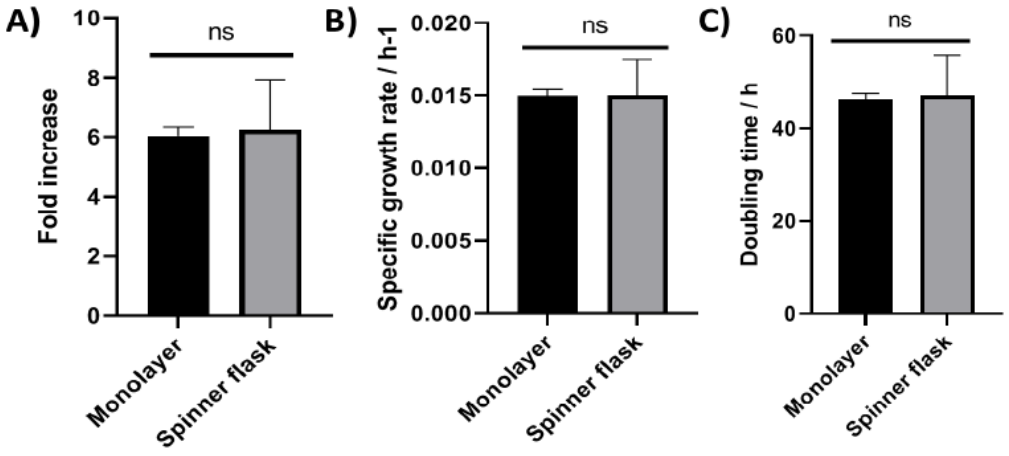

Figure 22- Cell quality attributes of bovine AT-MSCs at passage 3, following expansion in spinner flasks and in single layer cultures. Fold increase in growth **(A)**, specific growth rate **(B)** in hours^{-1}, and cell population doubling time **(C)** in hours. Data is presented as mean ± standard deviation. Experiments were performed three times (n=3). ns= not significant, through unpaired *t* tests analysis. Adapted from: Hanga *et al*. (2020)

Different MCs can impact population doubling times. Goh and team have shown human MSCs with doubling time ranging 48 h for Cytodex 3 cultures, which is within the same range as the results obtained in this study for bovine cells, although other MCs allowed doubling times for MSC ranging 33 h (Goh *et al*., 2013). In the same study, the fold increase of MSC populations was similar to those reported here, but the bench-top BR (1L) tested outperformed spinner flask cultures, which was not apparent in this study (Figure 22), though growth kinetics parameters were within the same range for both culture systems.

Bioprocess improvement of AT-MSC expansion

Different cell seeding densities (1500, 3000 and 6000 cells/cm^2) were analysed for their effect on growth kinetics, and the lowest seeding density (1500 cells/cm^2) generated the highest average fold increase of 28.8, whereas the cultures with 6000 cells/cm^2 had a 5.08-fold increase in growth. In addition, the highest growth rate and lowest doubling time was reported for cultures with 1500 cells/cm^2.In contrast, Verbruggen and colleagues had shown that initial cell densities impact bovine myoblast proliferation attached to MCs in spinner flasks, and higher cell concentrations ranging 5500 cells/cm^2 achieved more promising results (Verbruggen *et al*., 2018).

The impact of medium exchange in spinner flask cultures was assessed through glucose depletion and lactate accumulation profiles, where a 50% medium exchange generated the lowest fold increase in AT-MSCs cultures, but also a minimal increase in lactate concentration. In contrast, 80% medium exchanges yielded the highest fold increase in population and lowest doubling times, while lactate accumulation at the end of the culture was the highest, at 2.9 ± 0.17 mmol/L. Medium where cells grow can have increasing concentrations of paracrine factors, which are released by cells into the culture and can boost cell proliferation (Ben-Arye and Levenberg, 2019). Furthermore, conditioned medium is a promising strategy for cost reduction of medium components, and it is based on medium used by other cells to proliferate, which can then be used to grow the cell type of interest (Korovina, D., 2019).

A drop in glucose concentration at Day 3 was more accentuated when the density of 1500 cells/cm2 was employed, and consequently a steady decrease in glucose was reported until the end of the bioprocess. Interestingly, the most cost-effective medium exchange ratio was 80%, which generated significantly lower cost per number of cells obtained after the bioprocess, ranging 2£ in medium cost per million cells.

In this work, plastic-based MCs were used. Indeed, most commercially available MCs are based on inedible materials like polystyrene (Bodiou *et al.*, 2020), which deems them inadequate for incorporation in the final product. These hurdles can be overcome by adding a dissociation step where MCs can be separated from the cells (Allan *et al*., 2019), although it represents another bioprocessing step which carries associated costs. If the MC of choice is not edible, cell dissociation from MCs within the BR can be achieved using enzymes such as accutase with higher harvesting yields than filtration-based techniques (Rodrigues *et al.*, 2018). Therefore, MCs composed of edible materials are of augmented interest for cultured meat applications, and there is an increasing need for tailored MCs for CA applications.

XI. 3. Kolkmann, A. M., Post, M. J., Rutjens, M. A. M., van Essen, A. L. M., & Moutsatsou, P. (2020). Serum-free media for the growth of primary bovine myoblasts. *Cytotechnology*, *72*(1), 111-120.

Overview

This report intended to demonstrate the effect of different commercially available FBS-free culture media, namely FGM-CD SingleQuots Kit™ (FBM, Lonza, Germany),StemPro™ MSC SFM (StemPro™, ThermoFisher Scientific, The Netherlands), the Essential 8™Medium (Essential 8™, Life technologies, USA), theSTEMmacs™ HSC Expansion Media XF (STEMmacs™, Miltenyi Biotec, The Netherlands),mTeSR1TM (mTesR1TM, Stemcell Technologies,Canada), MesenCultTM ACF Culture Kit (Mesencult™, Stemcell Technologies, Canada) and TeSR™-E8™ (Stemcell Technologies, Canada), as an alternative to serum-containing medium (GM)in cultures of bovine primary myoblasts over time, namely at day 1, 4 and 6. In addition, the effect of culture medium supplementation with antibiotics, commercial serum-free additives, and GF mixtures was determined through cell viability assays. The effect of partial medium exchange (75%) in cell population growth was also evaluated.

SFM effects on cell proliferation

Commercial serum-free media FBM, TeSR-E8 and Essential 8 were reported to generate significantly higher cell numbers than regular DMEM cultures, as well as significant growth over time (Figure 23-A), though none of them had mitogenic properties akin to serum-containing GM, which exhibited the highest total cell numbers at day 4 and 6. STEMmacs medium failed to improve growth of bovine myoblasts (Figure 23-B). Similarly, GM had higher myoblast growth than different cocktails of serum-free media (Figure 23-C). Out of each combination tested, FBM-DMEM mixtures generated significantly higher number of cells than the negative control (DMEM). FBS is composed of numerous mitogenic molecules, including numerous GFs, insulin and transferrin (Brunner *et al.*, 2010), which makes it challenging to establish a supplementation strategy that affects cell behaviour as FBS. Nonetheless, these media were not tailored for myoblast proliferation, which could also substantiate the differences in efficacy amongst commercially-available media. Effective FBS-free and animal-free media have been reported to sustain various cell types such as iPSCs (Rodrigues *et al.*, 2018), ESCs (Kunova *et al.*, 2010), MSCs (Allen *et al.*, 2019) and mouse primary myoblasts (Cai *et al.*, 2019).

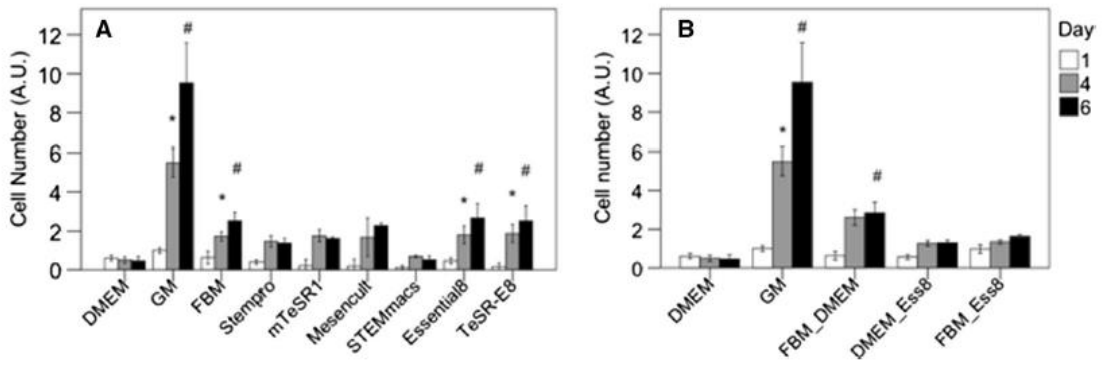

Figure 23- Bovine myoblast proliferation expressed in cell number with MTS in arbitrary absorption values (AU) for different serum-free media formulations (A) and mixtures of media (B) at 1, 4 and 6 days of culture. Every culture media had significant lower growth ($p<0.01$) than GM medium.

*- significant growth over time

#- significantly higher number of cells compared to DMEM cultures.

Retrieved from: Kolkmann *et al.* (2020)

Even so, effective SFM tailored for CA applications in bovine skeletal muscle cell development are lacking within commercial manufacturers. Achieving an effective SFM can be hypothesized through a top-down approach, where medium is fabricated by adapting previous formulations to the intended cell type, or a bottom-up approach where different medium supplements are tested for their applicability in a SFM (Jung *et al.*, 2012).

Effects of antibiotics, serum-free additives, and medium exchange rates

The authors have determined that antibiotic treatment led to reduced myoblast numbers when compared to cultures that did not contain antibiotics, after 4 and 6 days of culture, while no contamination was reported.

Such advancement is essential for the applicability of cultured meat in lieu of conventional animal agriculture (Specht *et al.*, 2018). Nevertheless, a more comprehensive analysis of contamination profiles of cells in culture without the use of antibiotics is required, which also investigates potential discrepancies related with cell and tissue source, as well as extraction procedures. Different SFM supplements, namely Xerum-Free™ (XFS) and Lipogro™ (Lipo) were shown to improve the growth of bovine myoblasts when cultured in DMEM-FBM media mixture, with significantly higher cell numbers with Lipo supplementation and a mixture of XFS and Lipo, when compared to the control medium of DMEM-FBM.

Further supplementation with a GF mixture composed of FGF-2, insulin, EGF and IGF was applied to myoblast cultures with Lipo, XFS and a control, where the GF mix generated higher cell numbers for every culture medium condition, both supplements generated increased cell numbers in combination with the GF mix after 4 days in culture, and Lipo was the only SFM supplement where a significant increase in cell number was witnessed (Kolkmann *et al.*, 2020).

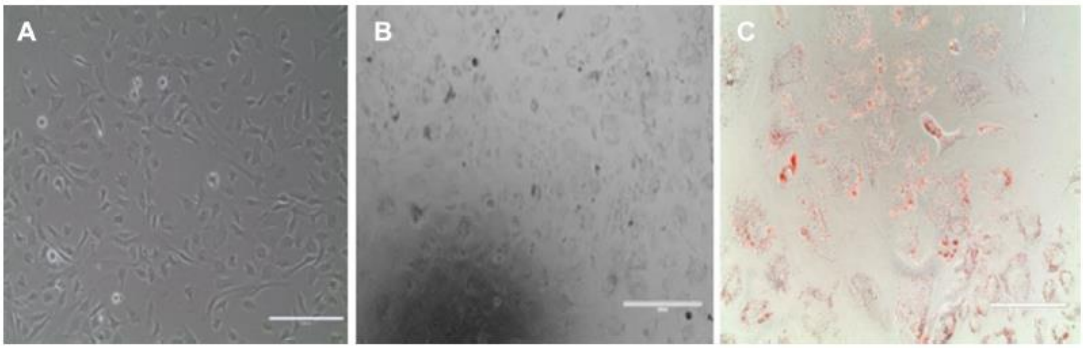

Figure 24- Myoblast photomicrographs in different culture medium settings. **(A)** DMEM **(B)** DMEM and Lipo **(C)** Oil-Red-O staining of culture with DMEM and Lipo showing lipid accumulation in myoblasts. Bars= 200 µm. Adapted from: Kolkmann *et al.* (2020).

GFs are known to impact proliferation and differentiation pathways of skeletal muscle cells (Ben-Arye & Levenberg, 2019). Interestingly, bovine myoblasts cultured with Lipo had a lipogenic phenotype, as expressed through Oil-Red-O staining of lipids inside myoblasts (Figure 24, A-C). Different strategies have been evaluated to promote transdifferentiation of muscle cells into adipose cells, although most were based in genetic engineering approaches, either through ectopic expression of adipogenic factors (Hu *et al.*, 1995), or more recently through abrogation of myogenic regulators such as MyoD (Chen *et al.*, 2019). Other strategies include co-culture systems, which have shown increase the expression of adipogenic genes in myogenic-committed bovine SCs, when cultured with preadipocytes (Choi *et al.*, 2013).

In this work, authors have assessed the effect of different feeding strategies, where a medium change of 75% was shown to improve cell proliferation with FBM, DMEM and the serum-containing medium GM and lead to growth inhibition in Stempro medium. An increase in myoblast number was reported for 75% medium exchange in cultures supplemented with Lipo, whilst cells grown on XFS-containing medium had higher cell numbers when 100% of culture medium was replaced. Medium exchange strategies had been investigated by Hanga and team with regards to bovine MSC culture, which have found that 80% medium exchange was more

cost-effective and allowed more pronounced cell proliferation (Hanga *et al.*, 2020), which is in accordance with the results presented in this work for bovine myoblast cultures.

XI. 4. MacQueen, L. A., Alver, C. G., Chantre, C. O., Ahn, S., Cera, L., Gonzalez, G. M., ... & Zimmerman, J. F. (2019). Muscle tissue engineering in fibrous gelatin: implications for meat analogs. *NPJ science of food*, *3*.

Scaffold fabrication

Using immersion rotary jet spinning, the authors obtained gelatin fibres after perfusion from a reservoir into an ethanol bath (Figure 25-A) with a rotating device that served as a collector, where gelatin fibres attached (Figure 25-B) and could then be manually extracted and peeled into smaller fibres (Figure 25-C). After freeze-drying, the gelatin scaffold exhibited a fibrous appearance, as confirmed through scanning electron microscopy (SEM) pictures (Figure 25-D).

The authors have then assessed the impact of different ethanol-water ratios in the bath vortex and reported significant morphological changes in the gelatin fibres, including fibre alignment and diameter. The correct alignment of scaffolds has shown to be required for adequate striation of skeletal muscle cells and myoblast alignment (Cooper *et al.*, 2011; Engler *et al*., 2004), and the topographical cues of the scaffold as well (Orelleana *et al*., 2020). It was noted that pure ethanol baths originated fibres with average diameters of 8.7 µm, whilst an average diameter of 2.9 µm was reported for gelatin scaffolds developed in 70% ethanol 30% water baths.

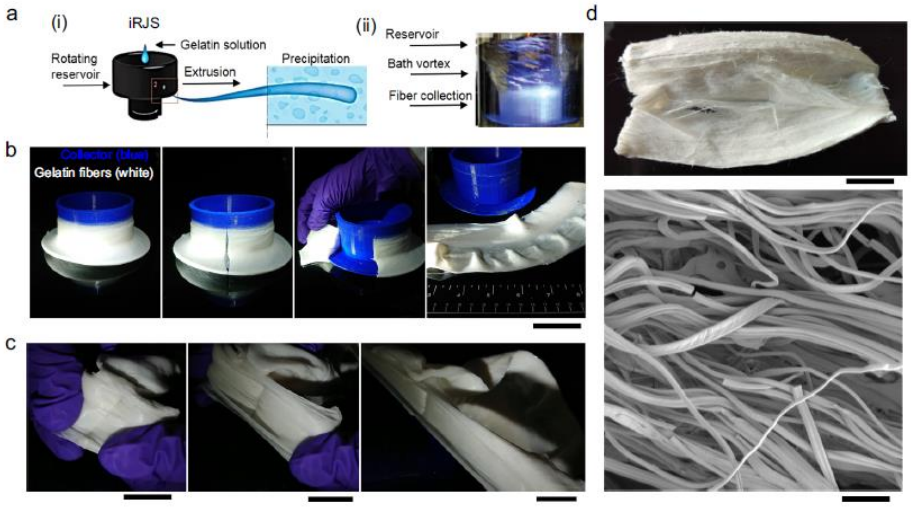

Figure 25- Fabrication of gelatin fibres using immersion rotary jet spinning techniques. **(a)** Gelatin solution permeates from a rotating reservoir into a bath vortex. **(b)** Fibres are removed from a cylindrical shaped collector (in blue) with rotating properties. Scale bar=10 cm. **(c)** Stripping gelatin scaffolds. Scale bar= 1 cm. **(d)** Photographs of fibrous gelatin scaffolds following freeze-drying. Scale bar= 1 cm. The bottom image depicts a scanning microscope image of the gelatin fibres. Scale bar= 50 µm. From: MacQueen *et al.* (2019).

Cell seeding in gelatin fibres and texture evaluation

In this report, the authors have evaluated the seeding efficiency of bovine aortic SMCs and rabbit SkMCs into spun gelatin fibres, where it was found that large diameter fibres supported tissue alignment, while short diameter fibres also promoted cell clumping. Adherence to gelatin scaffolds was reported to be more prominent than to plastic surfaces, for both cell cultures tested. Gelatin is a naturally functional scaffold known to comprise of peptide sequences such as RGD that are recognized by integrins on the cell surface, to where these adhere to (Davidenko *et al*., 2016).

In gelatin scaffolds crosslinked with microbial transglutaminase, it was reported that rabbit SkMCs were able to generate 3D tissue constructs with a cell density ranging 10^4 cells/mm^3.

Further analysis has been conducted on long-term culture of bovine aortic SMCs and rabbit SkMCs in gelatin fibres generated through chemical crosslinking, for 21 days (Figure 26). Microscopy observations indicate that the morphological features of the constructs were conserved, and highly dense tissues were reported. In addition, fibres seeded with rabbit SkMCs had more dispersed nuclei than bovine SMCs, and a similar result was reported for plastic 2D scaffolds.

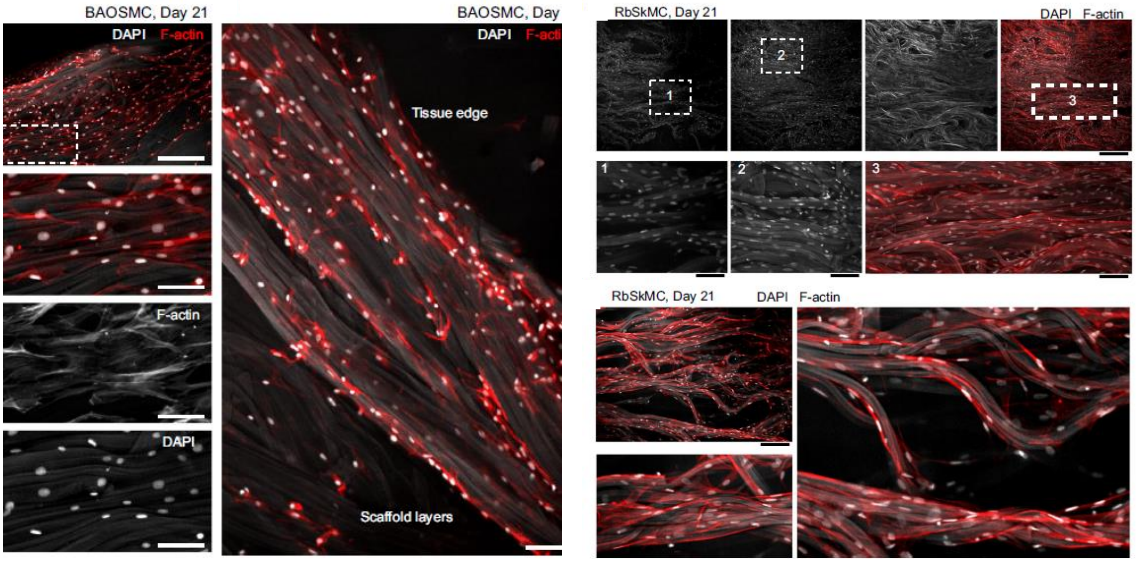

Figure 26- (A) Immunofluorescence staining of muscle constructs consisting of bovine aortic smooth muscle cells (BAOSMC) seeded in gelatin fibres after 21 days in culture, with nuclei stained by DAPI (in white) and actin filaments (red). Scale bar= 200 µm for top picture, 50 µm for bottom three pictures. **(B)** Magnification of muscle constructs from (A) where BAOSMC can be seen merging and spreading throughout the scaffold. Scale bar= 50µm. **(C)** Immunofluorescence staining of rabbit skeletal muscle cell (RbSkMC) nuclei with DAPI (in white) and actin filaments (F-actin) depicted in red. The top images (scale bar= 200 µm) show fibrous gelatin scaffolds in light grey, and the bottom images (scale bar=50 µm) show aligned nuclei throughout the scaffold layers. **(D)** Cultured RbSkMC in gelatin strands with different magnifications, and immunofluorescence staining as in (C). Scale bar= 200 µm (top left image), 20 µm (bottom left image), 20 µm (right image). Adapted from MacQueen *et al*. (2019).

The use of gelatin in scaffold production for cultured meat might pose several limitations, such as the presence of contaminants and zoonoses (An *et al.*, 2014). Gelatin is mostly composed of collagen and it is sourced from animals, therefore reducing its applicability in cultured meat. Several microorganisms possess different collagen-like proteins which resemble the triple helix structure of mammalian collagen. However, collagen contains hydroxyproline, which requires post-translational modifications to the amino acid proline, and bacteria do not naturally possess this molecular machinery (Yu *et al.*, 2014). Alternatively, recombinant collagen can be produced in bacteria, yeast, or plants (reviewed in Campuzano and Pelling, 2019), and both alternatives would circumvent the reliance on livestock-derived gelatin.

Using SEM and histology, the authors have compared the constructs with SkMCs from rabbit and SMCs from bovine to conventional meat products such as ground beef, rabbit muscle and bacon. The striation patterns and fibre alignment were observed through a combination of haematoxylin and eosin staining, showing a tissue distribution akin to processed meat products such as ground beef, with less pronounced striation and density. Additionally, the staining procedure displayed a similar pattern in collagen-like protein and collagen expression in both rabbit SkMCs and bovine SMCs, when compared to ground beef, bacon, and native rabbit skeletal muscle tissue.

A texture profile analysis indicated that both tissue constructs (bovine SMCs and rabbit SkMCs) had increased hardness after cooking, similar to ground beef, while rabbit skeletal muscle and beef tenderloin had a decrease in hardness after thermal exposure. Tensile elasticity analysis showed that uncooked beef tenderloin has a Young Modulus of 227.6 kPa, ground beef 207.4 kPa, Bacon 59.2 kPa, Prosciutto 82.2 kPa and Turkey 169.4 kPa. After 21 days of culture, fibrous gelatin constructs with rabbit skeletal muscle cells had a Young Modulus of 142.5 kPa, while the stiffness of bovine SMCs constructs at day 21 was roughly 389.4 kPa. Interestingly, these modules are considerably higher than the optimal stiffness of scaffolds for muscle cell proliferation (Engler *et al*., 2004; Boonen *et al.*, 2011), which highlights the need to consider the stiffness of whole tissue constructs as opposed to relying solely on mimicking native microenvironment of muscle cells. Indeed, perimysium endomysium and epimysium layers of skeletal muscle tissue have different physical and mechanical properties (Ben-Aerye & Levenberg, 2019) and therefore the substrate elasticity of scaffolds for cultured meat production is likely to be impactful in the texture perception of the product.

XI. 5. Simsa, R., Yuen, J., Stout, A., Rubio, N., Fogelstrand, P., & Kaplan, D. L. (2019). Extracellular Heme Proteins Influence Bovine Myosatellite Cell Proliferation and the Color of Cell-Based Meat. *Foods*, *8*(10), 521.

Characterizing bovine SCs, and the effect of heme proteins in cell proliferation

After initial isolation and expansion, immunofluorescence staining has shown that bovine SCs expressed the muscle SC marker Pax7. Following differentiation in tailored medium, SCs were shown to express the late myogenic marker Troponin-T, which verified the adequacy of the pre-plating method for isolation of cells and differentiation protocol. This method relies on separating SCs from other cells like fibroblasts by using different coatings, and a similar approach has been reported with SCs from mice (Yoshioka *et al.*, 2020).

The proliferation capacity of bovine SCs was reported to be increased at day 3 of culture if myoglobin (Mb) or haemoglobin (Hb) were added to the medium at a concentration of 3 mg/mL, when compared to a control consisting of SCs without the addition of heme proteins. The doubling time of SCs at day 7 was calculated to be lower for SCs with Mb (36.63 ± 0.74 hours) than for the control culture of SCs (41.67 ± 2.55 hours), and the highest doubling time was reported in cultures of bovine SCs with Hb (43.28 ± 0.66 hours). Previous reports have achieved a population doubling time of 32.3 h for human SCs between the first and second passage (Jarocha *et al.*, 2014), which is within the range obtained for Mb-supplemented cells, and both are slightly lower than the doubling time of bovine MSCs obtained previously in spinner-flask cultures (Hanga *et al*., 2020).

While comparing the effect of different Hb and Mb concentrations (1, 3 and 5 mg/mL) in cell proliferation for 7 days, the authors have reported an overall increase in bovine SC number when Mb was added, and higher Mb concentrations generated significantly higher proliferation efficacy at day 7. In contrast, the authors reported that supplementation with Hb resulted in linear reduction in cell numbers with increasing Hb concentrations, and overall had a null or marginally negative impact on bovine SCs proliferation. Indeed, concentrations of heme proteins in skeletal muscle differs, as roughly 1.5% of skeletal muscle protein fraction is Mb, and 0.5% is Hb (Pérez-Alvarez & Fernández-López, 2012).

Assessing the characteristics of bovine muscle constructs

After incubation of bovine SCs in a fibrin-based hydrogel, the generated muscle constructs were observed for 9 days to assess the effect of Mb and Hb on tissue colour, and images of day 1, 4, 7 and 9 were registered (Figure 27). A distinct red colour was reported in both Mb and Hb-containing tissue constructs, when compared to BSC cultures. Product mimicry is essential for societal acceptance of cultured meat products (Post and Hocquette, 2017), and therefore cultured meat products should provide a similar visual experience, which has been shown to be increased for Mb-containing constructs.

Mb is the most relevant molecule for muscle tissue colour (Fraeye *et al*., 2020) and therefore can enhance the sensorial experience of cultured meat. Other approaches to give similar colour attributes to cultured meat without Hb and Mb could rely on supplementation with heme analogs. For instance, leghemoglobin is derived from various legumes and can be produced in yeast (Fraser *et al*., 2018) to be used in plant-based meat alternatives. There are also multiple heme-like proteins from plants and algae that display similar functional properties as animal-derived ones (Becana *et al*., 2020), which could be cost-effective alternatives to recombinant globin production. In addition, muscle colour might be impacted by other heme-carrying compounds such as cytochromes (Pérez-Alvarez & Fernández-López, 2012).

Nonetheless, globins are not only important colour compounds, but also essential flavour molecules in meat (Fraeye *et al*., 2020), and their contribution to skeletal muscle tissue development discussed herein makes them an attractive supplement for growth medium formulations but require thorough research to define optimal concentrations for each molecule.

Overall, results here suggest that Mb is the most relevant globin during SC development *in vitro*. Thus, strategies should be put in place to evaluate which globins to incorporate in early stages of cultured meat production and others exclusively for flavour enhancement which can be done after cell culture.

The final weight of the different experimental groups was shown to be increased in both SCs with Hb (6.06 + 0.67 mg) and SCs with Mb (5.66 + 3.56 mg) than in SC muscle constructs (3.94 + 1.19 mg). The effect of using fibrinolysis inhibitors to prevent degradation of fibrin scaffolds was shown to increase the final weight of the constructs, which indicates the presence of the scaffold in the muscle constructs. Fibrinolysis inhibitors such as aprotinin and tranexamic acid have been previously utilized to maintain functionality and inhibit degradation of fibrin scaffolds, including studies with skeletal muscle tissue cells such as myoblasts and ECs (Gholobova *et al*., 2015).

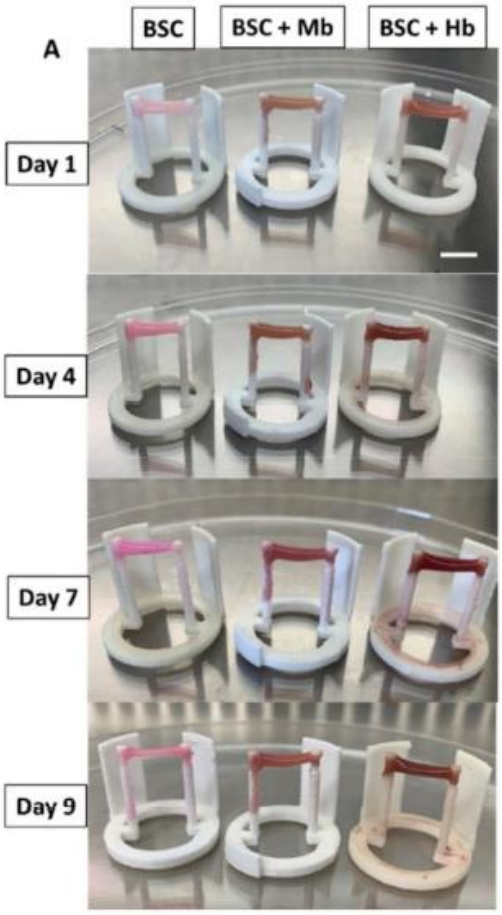

Figure 27- Pictures of muscle tissue constructs with BSC cultures, BSC+ Hb and BSC +Mb at day 1, 4, 7 and 9. A more accentuated red colour can be observed in muscle constructs with heme Hb and Mb, which increasingly noticeable as time progresses. Both Hb and Mb were added at a concentration of 3 mg/mL. Scale bar= 10 mm. Adapted from Simsa *et al*. (2019).

XII. CONCLUSION AND FUTURE PROSPECTS

Thus far, cultured meat production using tissue engineering techniques seems an innovative technological step in animal agriculture, as it might decrease the incidence of zoonotic outbreaks and antibiotic-resistant bacteria, whilst having minimal impact on animal welfare and potentially lower environmental impact.

However, a comprehensive and multifactorial approach towards implementation of such process in large scale is needed, including choices of primary cell sources, improvements in culture medium formulation towards xeno- and animal-free, chemically defined and cost-effective ones, tailored BRs and MCs for CA applications, as well as adequate scaffolds for growth and differentiation of cells into muscle fibres.

ESCs and iPSCs have great potential for establishing continuous cell-lines for products with multiple cell types, and generation of cell lines from both has been previously reported for various applications (Bogliotti *et al.*, 2018; Heo *et al.*, 2014). Using pluripotent cells in cultured meat production will rely on optimization of derivation protocols towards intended lineages, e.g. myogenic, adipogenic, fibrogenic, in order to establish a time-effective bioprocess.

On the other hand, MSCs can differentiate into multiple cell types including adipocytes with ease, though its myogenic differentiation has been also reported though under particular stimuli, such as culture medium and co-culture settings with myogenic cells. MSCs have enormous advantage due to their extraction from adipose tissue and many other sources, but will require further improvements in cell population doublings to have broad application in cultured meat. Their recent expansion in MCs in small-scale BRs makes them a promising cell source for application in the field (Hanga *et al.*, 2020). In addition, the application of bovine FAPs as adipogenic and fibrogenic sources remains to be investigated in large cultures but could be quintessential for applications in complex meat structures that involve marbling, such as steaks.

In contrast, the physiological attributes of SCs and the straightforward methods for their isolation makes them a promising cell type for cultured meat production, in addition to their specificity towards the muscle lineage (Langelaan *et al.*, 2010). However, their limited doubling capacity (Ben-Arye and Levenberg, 2019, Datar and Betti, 2010; Post, 2012) will need to be overcome, similarly to MSCs, to allow continuous processes without relying on weekly or monthly biopsies.

Furthermore, characterizing and evaluating side populations of certain cell lines and tissues for their expansion and differentiation properties (Pacak *et al.*, 2013) into desired cell lineages, could unveil novel cell populations with unmatched biological properties for CA applications. Ultimately, the cell types chosen for cultured meat products should allow consistent expansion and full description of optimal parameters, in order to establish a continuous process that relies on adequacy of biomass for scaling up between pilot and industrial facilities.

As described throughout this manuscript, achieving culture medium solutions for CA applications will be a stepping-stone towards broad applicability of the field to reach price parity with conventional animal-derived products. Efforts should focus on reducing GF cost burden, as well as achieving SFM that has similar mitogenic properties as FBS-containing media.

The choice of BRs will also largely impact the feasibility and cost-efficacy of cultured meat, and while further innovation will be required for process optimization, both stirred tanks and hollow fibres appear ideal for proliferation stages, while fixed-beds could be used in differentiation stages. As previously mentioned, it is still

uncertain if the process will be carried in one-step proliferation and differentiation or in two steps, but cultured meat products that do not require complex tissue architectures, like hamburgers, will probably be able to be developed using one BR that could accommodate growth of myogenic and adipogenic in edible MCs where upon medium changes, cells could differentiate into myotubes and then these small fibres could be processed and pressed, without requiring maturation and differentiation in a macro-scaffold.

The scaffold materials discussed have the potential to sustain the proliferation and differentiation of skeletal muscle cells for cultured meat applications, if adequate tuning of the scaffold is optimized for the cell type of choice. Composite scaffolds and 3D bioprinting offer direct applicability for the production of cultured meat products that have highly-complex tissue structures, such as steak-cuts. Other scaffold materials are being currently investigated and could represent significant cost reductions in fabrication and be sustainably-sourced. These include coconut- and mycelium-based scaffolds (reviewed in Post *et al.*, 2020).

Edible plants as scaffolds remain an interesting innovative field, mostly for cultured meat since the scaffold can remain in the final product if it enhances its organoleptic properties. For instance, fibres from jackfruit *Artocarpus heterophyllus*, commonly used as a meat substitute, have been used as a scaffold for turkey myoblast seeding and differentiation (Gibbons, M., 2018). In addition, scaffolds- based on protein isolates from plant sources that are already used as foods allow the creation of hybrid products with increased organoleptics. Likewise, hybrid products composed of plant-based products and cultured muscle and fat cells could improve cost margin towards price parity with conventional meat.

Overall, the chemical and mechanical properties of alginate, along with its cost-efficiency and accessibility of the raw material, make it a promising biomaterial for application in cultured meat. Considerations for application of alginate in cultured meat include the need for functionalizing the scaffold for higher cell adhesion, which requires an additional step in the bioprocess and consequently increases its production costs. In addition, alginate can have unpleasant odours (Gheorghita Puscaselu *et al.*, 2020) and could require further tuning for applications in cultured meat.

One of the hurdles towards the application of silk as a scaffold for cultured meat is its sourcing from animals, which undermines the concept of a truly animal-free animal product. Luckily, recombinant spider silk has been already synthesized in bacteria for different purposes (Kumari *et al.*, 2020). In addition, if the scaffold is to remain in the final product, the organoleptic properties and tensile strength of silk fibres in both raw and cooked forms of cultured meats must be thoroughly accessed for its similarity to conventional meat products.

Nonetheless, the scaffold material with most interesting properties and proven applicability with bovine cells is gelatin-based, such as the fibres obtained by MacQueen and team which were discussed in this manuscript (MacQueen *et al.*, 2019). However, recombinant production of collagen will most likely be required for application in cultured meat products and overcome the need for animal-derived products other than the starting cell types.

Composite scaffolds with different stiffness modules will be required for cultured meat applications if different cells types are expected to be utilized in co-cultures. Alternatively, muscle and fat cells can differentiate and mature in separate scaffolds and then be combined by layering the generated fat tissue onto muscle fibre constructs in proportions akin to natural muscle tissue (Zidarič *et al.*, 2020). For CA application, namely in cultured beef, cost-effectiveness of such processes should be examined.

Along with edibility, the material used in scaffolds should benefit the sensing experience of cultured meat (Ben-Arye and Levenberg, 2019), and similar stances are preferential for MCs as well. If the chosen material is neither biodegradable nor edible, dissociation methods can detach cells from the scaffold of choice, whether mechanically or enzymatically (reviewed in Allan *et al.*, 2019). However, such steps would require an increase in production costs and undermine the scalability of the process, which makes edible materials an attractive choice for cost-effective cultured meat manufacturing.

Recently, bioprinting has revolutionized the field of tissue engineering due to its precision and automation capacity, which not only allows the production of 3D scaffolds from a plethora of biomaterials, but also the injection of cells into scaffolds with unparalleled accuracy. Likewise, 3D bioprinting remains an interesting opportunity for significant improvement in spatial patterning of scaffolds, as well as tissue constructs for cultured meat. Furthermore, it represents another level of possible automation that could drive the efficiency of an industrial-scale production, if the devices are also able to scale-up, or otherwise scaled out.

If these challenges can be overcome, modifications implemented, along with thorough research into structural components of animal muscle tissue and their mimicry, cultured meat products may be commercially accepted in the years to succeed. Furthermore, it is tempting to envision a future food system which is not only more sustainable, but also that ensures the growing population's appetite for meat products, without relying on intensive animal exploitation.

It is important that a multidisciplinary effort is put to place by biotechnologists, cell biologists, biological, biomedical, chemical, and industrial engineers, as well as mathematicians, food and computer scientists, amongst many others, that allows such technological advancement to take place in an impactful way. Moreover, there is an immense gap in basic research which will require further support from public and private funding bodies, which assure that the industry can withstand the go-to-market challenges that will arise from scaling animal cell production for food products (Specht *et al*., 2018).

Due to the immaturity of the field, intentions of scaling up cultured meat processes are only taking place in 2020, as several companies approach scalability and prepare to open pilot facilities[3, 4]. Therefore, cost-efficiency and feasibility of these processes in large-scale are still unknown, and no comprehensive case-study can still be found when writing this manuscript.

Likewise, research and development in the field of mammalian cell production for food is very limited and in early-stages, so it is likely that cultured meat products will first be in the form of processed meats (such as hamburgers and meatballs) and blended products where plant-based ingredients are combined with animal cells. Further investigation into adequate co-culture settings, BRs, scaffolds, and overall advancements in tissue engineering, will be required for products such as steaks. Indeed, the first regulatory approval for cultured chicken muscle is a blended product in the form of nuggets, which has recently been approved in Singapore by regulatory bodies, and produced by the company Eat Just, which are already being sold in a selected restaurant in the city, under the brand Good Meat[5].

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