Full Title: Considerations for the development of cost-effective cell culture media for cultivated meat production

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Word Count: 13837

Running Title: Cultivated Meat Media…

Keywords: Cultured meat, serum-free media, muscle cells, media optimization, protein foods

Choice of Journal/Topic: *Comprehensive Reviews in Food Science and Food Safety*; Reviews and Trends

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ABSTRACT

Innovation in cultivated meat development has been rapidly accelerating in recent years because it holds the potential to help attenuate issues facing production of dietary protein for a growing world population. There are technical obstacles still hindering large-scale commercialization of cultivated meat, of which many are related to the media that is used to culture the muscle, fat, and connective tissue cells. While animal cell culture media has been used and refined for roughly a century, it has not been specifically designed with the requirements of cultivated meat in mind. Perhaps the most common industrial use of animal cell culture is currently the production of therapeutic monoclonal antibodies, which sell for orders of magnitude more than meat. Successful production of cultivated meat requires media that is food-grade with minimal cost, can regulate large scale cell proliferation and differentiation, has acceptable sensory qualities, and is animal ingredient-free. Much insight into strategies for achieving media formulations with these qualities can be obtained from knowledge of conventional culture media applications and from the metabolic pathways involved in myogenesis and protein synthesis. In addition, application of principles used to optimize media for large-scale microbial fermentation processes producing lower value commodity chemicals and food ingredients can also be instructive. As such, the present review shall provide an overview of the current understanding of cell culture media as it relates to cultivated meat.

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1. Introduction

Consumers are becoming increasingly aware of the broader impacts of their choices and behaviors. Common activities such as air travel, internet usage, and fashion textiles consumption have long been taken for granted, but their environmental, ethical, and socioeconomic implications are starting to become more widely understood (Higham, Cohen, Cavaliere, Reis, & Finkler, 2016; Morley, Widdicks, & Hazas, 2018; Niinimäki et al., 2020). Similarly, consciousness of the impacts of the global food production system and personal food consumption choices is reaching the mainstream. With the global population expanding rapidly and expected to reach almost 10 billion by 2050 (United Nations, 2019), innovation and expansion of the food system will need to occur to feed significantly more people over time.

The current food system has a sizable influence on the environment, with livestock production alone contributing a disproportionately large portion of global land use and greenhouse gas emissions (Henderson, Gerber, & Steinfeld, 2013; Lynch & Pierrehumbert, 2019; Rojas-Downing, Nejadhashemi, Harrigan, & Woznicki, 2017; Steinfeld et al., 2006). Besides the ethical animal welfare considerations regarding industrial animal agriculture, there are significant public health concerns associated with this practice as well. The large-scale rearing of animals in close quarters with commonplace prophylactic antibiotic administration provides prime breeding grounds for the emergence of transmittable antibiotic resistant bacteria or other disease-causing agents (Khanna, Friendship, Dewey, & Weese, 2008; Mathew, Cissell, & Liamthong, 2007). Moreover, risks of significant zoonotic disease epidemics, such as influenzas, HIV/AIDS, ebola, and COVID-19, can be associated with the use of animals for food (Andersen, Rambaut, Lipkin, Holmes, & Garry, 2020; Kalish et al., 2005; Li et al., 2014; Marí Saéz et al., 2015; M. I. Nelson et al., 2015; Taubenberger, 2006). Perhaps most importantly, traditional

animal agriculture is unlikely to meet the entire increased demand for meat and dietary protein as the population increases (Henchion, Hayes, Mullen, Fenelon, & Tiwari, 2017). Therefore, innovation in the field of cultivated meat (and cellular agriculture, in general) has accelerated over the past decade, as it has the potential to provide true meat products without these environmental, ethical, and technical issues.

Cultivated meat products will inevitably be composed of multiple types of cells. This adds to the complexity of the target product, as well as the technical challenges associated with its production. The cell types most likely to be important for industrial production include muscle satellite cells (or more generally, muscle precursor cells (MPC)), myoblasts, myocytes (also known as myotubes or myofibers), adipose-derived stem cells (ADSC), adipocytes, and fibroblasts. Satellite cells/MPCs, myoblasts, primary and secondary myocytes represent a range of muscle cells in order from the most stem-like to the most differentiated/mature. Adipocytes and their ADSC precursors are fat cells that could be useful for producing the fat component of cultivated meat products, while fibroblasts are connective tissue cells that could help provide texture and structure to the products. A major remaining technical hurdle for cultivated meat production is how to grow and manage all these cell types simultaneously. The term “muscle tissue cells” in the present review will be used in cases that refer to all these cell types collectively, while the more accurate and specific terms will be used when discussing a particular cell type.

There are several specific technical challenges still hindering low cost scale-up and commercialization of cultivated meat products; many of them are related to the design of the media that is used to culture the animal muscle tissue cells (Kadim, Mahgoub, Baqir, Faye, & Purchas, 2015; Stephens et al., 2018). In most other animal cell culture applications, expensive

and/or animal-derived ingredients play important roles in media formulations. Many of these components are commonly supplied by fetal bovine serum (FBS), which is the serum fraction of blood taken from a fetus within a slaughtered pregnant cow. Clearly, there are ethical concerns with this practice, but also FBS is inherently variable from batch to batch and is mostly undefined and expensive (Jochems, Van der Valk, Stafleu, & Baumans, 2002). We have previously demonstrated that these differences in FBS can directly lead to changes in muscle phenotype that could dramatically alter meat quality (Khodabukus & Baar, 2014). This leads to technical challenges when serum is used even in mainstream applications. As such, replacing serum or other animal-derived ingredients remains a primary challenge for cultivated meat media formulation, including for cell proliferation and differentiation. Other questions remain such as how media can be used to influence the organoleptic and nutritional qualities of cultivated meat products. In addition, with the large number of media components typically present in commercial media, sophisticated methods for experimental optimization of the components and their concentrations will likely be necessary to develop new media matched to the desired characteristics. Therefore, the present review seeks to provide insight into the myriad design considerations involved in cultivated meat media.

2. The Expected Modes of Cultivated Meat Production

In thinking about developing cell culture media for cultivated meat production, it is important to consider how media will influence product processing. Certainly, the main uses of media will be to grow stem cells and differentiate them into muscle, fat, and connective tissues. However, the way this is accomplished will depend on the desired product—either an unstructured product (for example, a burger or sausage product) or a structured product (such as

a chicken breast or beefsteak). Therefore, it is instructive to think about these two cases separately.

It is likely that the first products to market will be unstructured products. While various scenarios could be imagined for production of the necessary cells, perhaps the most likely would be to proliferate embryonic or induced pluripotent stem cells through all inoculum stages to the final growth and differentiation scale (Specht, 2020). If a cultivated meat cell culture scale were 25,000 L, as an example, which would be considered large-scale for mammalian-cell-based biotherapeutic production, approximately 10 successive cell inoculum stages would be needed (assuming a 20% inoculum at each stage, as would be common in biotherapeutics) (J. Zhang, 2014). With each one of these stages representing approximately three to four doublings, cells will need to survive in growth media, without differentiation, for 40-50 generations. In this scenario, it would only be in the last stage, largest scale bioreactor that the medium would be changed to promote differentiation to the required cell types (muscle, fat, and connective tissue cells), probably in separate bioreactors and then blended together to achieve the desired mix for the final product formulation (Hanga et al., 2020). While this would be easiest for true suspension culture, it is at least possible that growth of these typically adherent cells might need to be in conjunction with edible microcarriers, which could further complicate media optimization (Hanga et al., 2020; Verbruggen, Luining, van Essen, & Post, 2018).

An alternative approach to creating unstructured products would be to scale up growth of each constituent type of cell (muscle, fat, and connective tissue). This could be accomplished using more differentiated muscle tissue stem cells. Proliferation for a large enough number of generations to get to scale; however, could be problematic for these cell types. Their culture systems could be manipulated to enhance cell stemness and proliferation (Ding et al., 2018), but

much is still unknown about this possibility and it is outside the scope of the present review to discuss the complexities that would be implicated.

It is the ultimate goal of the cultivated meat industry to produce structured products as well. It is possible that all cells will be grown and differentiated on an edible scaffold, though supplying oxygen and nutrients throughout this structure will not be an easy task, nor will differentiation into more specialized tissue types *in situ* (Post, 2012). Another possibility would be to take the same approach as for unstructured products, except to take differentiating or differentiated cells from the last-stage production bioreactor as described above and print them onto an edible scaffold in the desired pattern. This could be followed by one to two generations of growth *in situ* to solidify the form of the tissue.

For either of these scenarios, using different sources of cells (e.g. beef, pork, chicken, turkey, fish, etc.) will likely necessitate re-optimizing unique formulations for both growth and differentiation media. While it is not yet clear just how different these optimized media will be for each desired production stage, scheme, and species, they will likely involve varying concentrations of the signaling substances that regulate proliferation and myogenesis. Regardless, all media will need to be food grade, animal product-free, and inexpensive in order to develop and sell a viable cultivated meat product.

The last point is that the media cost is critical to product viability. Therefore, it is important to think about the expected costs for media used for cell proliferation and differentiation at scale when considering feasibility of commercial processes for producing unstructured or structured products. Others have delved into the theoretical cost reduction potential of media for cultivated meat. It has been suggested that the media required to produce one kilogram of cultivated meat can feasibly achieve a cost as low as around 5.00 USD

(assuming around 23 L will be needed), which is below the average cost per kilogram of most conventional meat (Specht, 2020). This estimate is also far below current prices for commercial culture media, pointing to the critical importance of further development, even when accounting for economies of scale. These figures take into account that cultivated meat will be measured in terms of wet cell (product) weight, while most existing industrial cell culture products are measured in terms of dry cell weight.

3. Mainstream Media Formulations and Uses

Animal cell culture is a well-established technique used in several industries, such as biomedical research and biopharmaceutical production (J. Zhang, 2014). Unlike in the food industry, these industries operate with a very high final product value, which therefore justifies large production expenditures and minimal cost optimization effort. Nevertheless, cell culture's long history has yielded an understanding of the growth requirements of cells *in vitro* and an array of well-established media formulations (Eagle, 1955; Yao & Asayama, 2017). Animal cell culture media consists of everything cells need to survive, as well as any additional components needed to elicit desired cellular behaviors like proliferation, attachment, or hypertrophy. Fundamentally, basic media is composed of carbon and nitrogen sources such as glucose, glutamine, and other amino acids; vitamins and inorganic salts; signaling molecules like growth factors; and buffers (Yao & Asayama, 2017). There have been numerous published studies and reviews discussing general and particular aspects of conventional culture media (cited throughout the present review); therefore, the goal of this section is not to detail every basic aspect of culture media, but rather to describe how conventional media formulations can inform media development for cultivated meat applications.

In the biological and medical research fields, cell culture media is often taken for granted. To facilitate easier reproducibility and sound scientific control, researchers often use the same media formulations across a wide variety of studies with different cell types and culture protocols. A range of basal media are commercially available; they generally supply most, but not all, of the basic components needed for cell survival and growth, including glucose, amino acids, and vitamins. Traditionally, basal media formulations are supplemented with complex animal-derived components (namely serum) that supply many trace nutrients and signaling molecules (Yao & Asayama, 2017). Basal media formulations have evolved over time and have several permutations for various applications; their history and uses have been well-documented. Among them, Eagle's Minimal Essential Medium (MEM) (Eagle, 1959), Dulbecco's Modified Eagle Medium (DMEM) (Dulbecco & Freeman, 1959), and Ham's F-12 (Ham, 1965)—whose formulations are compared in Table 1—have emerged as the gold standards due to their versatility and widespread use, despite being originally developed more than 60 years ago for human HeLa or specific rodent cell lines. DMEM, noted by its excess of amino acids, is clearly not optimized for minimum cost, which is an example of the fundamental difference in approach to media formulation between the biomedical and cultivated meat industries. Furthermore, commercial media formulations like DMEM do not generally attempt to provide nutrient concentrations that are similar to the nutrient supplies that cells would be exposed to *in vivo*. This has been shown to alter the transcriptome and metabolic profile of cells grown *in vitro* compared to their counterparts *in vivo* (Voorde et al., 2019). Nevertheless, derivatives of DMEM are in widespread use in current laboratory-scale cultivated meat research due to their familiarity, availability, and convenience (Ding et al., 2018; Kolkmann, Post, Rutjens, van Essen, & Moutsatsou, 2020; Simsa et al., 2019; Verbruggen et al., 2018).

The prophylactic use of antibiotics is also common in cell culture for biomedical research, even with inherent undesirable side effects on the cultured cells (Kuhlmann, 1995; Relier et al., 2016). Despite an increased challenge in maintaining cell cultures free of bacterial contamination, the absence of antibiotics has been shown to allow for more facile serum-free media adaptation (Kolkmann et al., 2020). Moreover, the use of antibiotics in cultivated meat production could provide a problem for consumers sensitive to specific antibiotics and potentially contribute to the rise of antibiotic-resistant bacteria—both possible hinderances to consumer acceptance of the products. Standard use of antibiotics is therefore precluded from cultivated meat process design, as is typically the case in existing industrial-scale animal cell culture processes such as for monoclonal antibody production.

Balanced salt and buffer solutions form the basis of defined media formulations (Yao & Asayama, 2017). They are important in the regulation of pH and osmolarity and may secondarily be a source of some nutritional minerals. The pH and ion balances in culture media need to emulate physiological conditions (typically around pH 7.4) for optimal cell growth. Historically, phosphate buffered saline (PBS) has served as an important salt solution for cell culture (Dulbecco & Vogt, 1954). Bicarbonate buffer systems, in conjunction with ambient CO_2, are frequently employed in animal cell culture at bench scale, but this requires careful monitoring of the CO_2 concentration in the culture environment (Dulbecco & Freeman, 1959; Eagle, 1959). HEPES, one of the classical zwitterionic buffers described by Good *et al*. (Ferguson et al., 1980), is commonly used to supplement the bicarbonate system's buffering capacity; it displays quality buffering performance and relatively minimal cytotoxicity. However, it is an expensive ingredient and is often a primary cost driver of basal media formulations (Specht, 2020). As such, less expensive buffering agents, such as TES or complex ingredients (see below), could be

tested in cultivated meat media where buffering capacity is a concern. However, given that industrial culture processes allow for stricter control of CO_2 concentration compared to research settings, the less expensive bicarbonate buffer system alone is likely sufficient to control pH.

The stability and consumption rates of media components must also be considered. Whether due to light or heat exposure, mutual interactions, or variable consumption rates at different stages of the production process, the concentration and activity of media components will vary (Yao & Asayama, 2017; Zigler, Lepe-Zuniga, Vistica, & Gery, 1985). Many of the common media components can be adversely affected by excessive light and heat exposure (Neutsch et al., 2018). This means that conventional media for muscle tissue cell culture generally cannot be heat sterilized; instead, they are usually filtered or irradiated to ideally eliminate all microorganisms. Because heat sterilization is likely a more efficient process at industrial scale, formulation of cultivated meat media using more heat stable ingredients should be considered, though this idea has not yet been directly tested. Heat stable, defined serum-free media have been developed for other cell types and applications (Nagle & Brown, 1971), which lends credibility to the notion that this may be possible for cultivated meat.

It should be noted that the culture media is likely to influence the sensory qualities of harvested muscle/meat. Logically, residues of the media on or in the cells may impart flavor, texture, or color. For example, glutamic acid and asparagine are the well-known contributors to the umami flavor component of meat, and these amino acids are ingredients in some cell culture media (Kawai, Okiyama, & Ueda, 2002). As such, this introduces a new dimension to media formulation that must be considered. Some recent published studies with preliminary sensory tests indicate that laboratory-scale cultivated meat prototypes have acceptable organoleptic properties (Ben-Arye et al., 2020; Fraeye, Kratka, Vandenburgh, & Thorrez, 2020; Simsa et al.,

2019), and thus the sensory qualities of culture media do not appear to be of immediate concern. However, to date there have been no studies investigating sensory effects of media specifically, these sensory aspects of the media will become more important as the industry develops.

4. Nutrient Sources in Culture Media

Over the roughly 100-year history of cell culture, knowledge of the nutritional requirements of animal cells grown *in vitro* has developed (Eagle, 1955; Yao & Asayama, 2017). While different cell types within and between species understandably have different nutritional and culture requirements, there are several common requirements that general basal culture media formulations can easily satisfy. Muscle cells, like all cells, utilize extraneous substances as sources of energy and as building blocks for cellular structures. The important macro-scale ingredients in culture media that satisfy these basic cell requirements can broadly be classified into two groups: carbon sources and nitrogen sources. This concept is commonly applied in industrial microbial and animal cell culture. The rates and modes of carbon and nitrogen utilization are regulated by availability, extracellular signaling, and the proliferative status of the cells (Palm & Thompson, 2017). Necessary micro-scale ingredients for cultivated meat media meet the cells' vitamin and inorganic ion requirements, but these are less likely to significantly contribute to culture media costs at scale, so they require less discussion and investigation (Specht, 2020). Accordingly, the present section will discuss the function and cost reduction potential for carbon and nitrogen sources as they relate to cultivated meat.

Carbohydrates, amino acids, and lipids are the fundamental classes of carbon sources for metabolism of muscle tissue cells. While a variety of carbon-based molecules can be used as nutrition by microbes and by animals at the organismal level, most cultured animal cells lack the

biochemical ability at the fundamental level to efficiently utilize anything but glucose, glutamine, and a limited number of other amino acids and fatty acids (Leong et al., 2017; Palm & Thompson, 2017). Animal cells may make use of alternate sugar substrates, such as fructose, pyruvate, maltose, and sucrose, but generally these are metabolized significantly less efficiently than glucose and do not appear to be optimal for cell growth (Campbell, Onions, Kendall, Guo, & Scaramuzzi, 2010; Leong et al., 2017; Meyer, Vitavska, & Wieczorek, 2011). Likewise, while providing some fatty acids for cells in the culture medium may reduce the metabolic load involved in *de novo* fatty acid synthesis, most animal cells do not require extraneous fatty acids (Wu & Näär, 2019). The different cell types of potential importance in cultivated meat—myotubes, satellite cells/myoblasts, fibroblasts, adipocytes, and more stem-like precursors of these cells—have differing metabolic rates and needs, as do cells from the different species of interest (Agathocleous & Harris, 2013). This creates the potential need for media formulations specific for the cell type of interest in a particular meat product production line; however, the more subtle metabolic differences between the cell types require further elucidation.

Cells utilize a variety of carbon source uptake mechanisms, such as transporter-mediated uptake, receptor-mediated endocytosis, and nonselective macropinocytosis (Palm & Thompson, 2017). After uptake and phosphorylation, glucose is used as the substrate for several metabolic pathways to generate adenosine triphosphate (ATP) for energy and to synthesize biomolecules like nucleotides, fatty acids, and amino acids. The major catabolic glucose metabolism pathways include glycolysis (and subsequent fermentation or oxidative phosphorylation) and the pentose phosphate pathway (D. L. Nelson & Cox, 2017). Through glutaminolysis, glutamine functions as a carbon source by yielding α-ketoglutarate that is then used in a number of biosynthetic pathways (DeBerardinis et al., 2007; Yin, Qie, & Sang, 2012).

Oxygen is important as a final electron acceptor in oxidative phosphorylation of carbon sources, and it is very important that cultivated meat media be constantly supplied with an appropriate concentration of oxygen to ensure efficient carbon metabolism (Al-Ani et al., 2018). There are challenges associated with this oxygen requirement at industrial scale. While bioreactor process design is outside the scope of the present review, it is worth noting that efficient oxygen control techniques (such as cascade control format) have been established for existing industrial animal cell culture applications. When oxygen gas is added to bioreactors, the physical forces that the gas bubbles may create (especially as they reach the liquid/gas interface at the surface of the media working volume), and their effects on the cells, must be considered. Industrial cell culture media now commonly make use of synthetic block copolymer surfactant additives such as poloxamers (e.g. Pluronic®) to reduce cell damage caused by shear forces due to gas sparging and agitation (Jordan, Sucker, Einsele, Widmer, & Eppenberger, 1994; Tharmalingam, Ghebeh, Wuerz, & Butler, 2008). Poloxamer additives are likely usable for cultivated meat media since they have been approved by the US Food and Drug Administration for various food and drug applications. Moreover, protein components of cultivated meat media, such as plant hydrolysates or yeast extracts, could have surfactant properties that may reduce the need for synthetic additives like poloxamers.

Glutamine—and other amino acids—are perhaps most significant in their roles as nitrogen sources. Nitrogen is an integral component of functional and structural cell molecules, namely proteins and nucleotides. Heightened nitrogen anabolism is the driver of the increased nitrogen demand in proliferating cells (Meng, Chen, Lao, Liang, & Sang, 2010). Different animal species require trophic sources of different amino acids (essential amino acids), usually at least histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and

valine (Hou, Yin, & Wu, 2015). Some cell types have additionally been reported to be able to utilize the nitrogen in ammonia (Lie, Wang, Forbes, Proud, & Petersen, 2019; Stern, Dasarathy, & Mozdziak, 2017), but glutamine still carries great importance since it can be metabolized into many other amino acids used for protein assembly (Meng et al., 2010). Glutaminolysis is also a mode of NADPH regeneration—the reducing agent necessary for many anabolic pathways—further highlighting glutamine's importance in cell proliferation (D. L. Nelson & Cox, 2017). Because of problems with ammonia accumulation in the media due to glutaminolysis, animal cells in industrial cell culture are often genetically modified to produce their own glutamine from glutamate; however, genetic manipulation of cells for cultivated meat production may not be desirable or possible, but such a modification could be explored. Therefore, based on the present understanding of carbon and nitrogen source metabolism by animal cells, the availability of free glucose and glutamine in the media for proliferating cultivated muscle tissue cells seems essential, as well as a sufficient to supply the essential amino acids specific to the animal species in question.

Actively proliferating muscle cells (myoblasts), as are necessary in cultivated meat production, understandably have unique metabolic activities and needs. Much of the current understanding of myoblast metabolism is derived from studies on cancer cells, since cancer cells are likewise rapidly proliferating cells. The Warburg effect describes the preference of cancer cells to derive most of their ATP from glycolysis, and allow most pyruvate to be converted to lactate, even in the presence of sufficient oxygen (Heiden, Cantley, & Thompson, 2009). This effect is not limited to cancer cells; however, it is a hallmark of all rapidly proliferating cells. Glucose metabolism via the Warburg effect as opposed to oxidative phosphorylation yields several metabolites that are valuable to proliferating cells undergoing high rates of biosynthesis

and division (Heiden et al., 2009; Intlekofer & Finley, 2019; Kondoh et al., 2007). It would follow that media for cultivated meat production could potentially be formulated to stimulate and support the Warburg effect to promote muscle cell proliferation. The Warburg effect has successfully been induced *in vitro* by genetic manipulation and by other intrinsic means such as stimulation of hypoxia inducible factors (HIF) and inhibition of pyruvate kinase M2 isoform by tyrosine phosphorylation (Christofk et al., 2008; Hitosugi et al., 2009; Luo et al., 2011). These findings suggest additional methods of promoting *in vitro* muscle cell proliferation, such as modulating the oxygen concentration in culture media. However, many of the stimulatory mechanisms controlling the Warburg effect continue to be elusive, and its potential application to cultivated meat remains to be investigated.

Common animal cell lines used in biopharmaceutical production, such as Chinese hamster ovary (CHO) and murine myeloma lines (Sp2/0, NS0), have been selected or modified for highly efficient growth and metabolic behaviors that are also desirable in cultivated meat applications (Hunter, Yuan, Vavilala, & Fox, 2019; J. Zhang, 2014). Future research should investigate whether aspects of the culture media formulations used for these biotechnological cell lines can be translated to cultivated meat media, keeping in mind that the cells themselves (functional or not) are not the desired final product in existing biotechnological processes, whereas the cells are the end product in cultivated meat processes. Media formulations for existing cell lines are often proprietary, as are the media currently being developed in the commercial cultivated meat industry, meaning that translating knowledge between the two industries could prove difficult.

Vitamin requirements differ between *in vivo* and *in vitro* contexts, due to differential metabolic needs and synthetic capacities of various cell types. For example, some fat-soluble vitamins are important for specific cell types in certain contexts, and may in fact help promote

myogenesis (Lamarche, Lala-Tabbert, Gunanayagam, St-Louis, & Wiper-Bergeron, 2015), but in general they are likely not necessary in cultivated meat cell culture applications. The water-soluble (B and C) vitamins are considered to be the most important for inclusion in basal animal cell culture media, and they have been relatively well characterized in this application (Schnellbaecher, Binder, Bellmaine, & Zimmer, 2019). Importantly, vitamin C is essential for the production of collagen protein, by fibroblasts, that will be necessary for the structural integrity of cultivated meat. The water-soluble vitamins are not necessarily stable in final media solution for extended durations, so care must be taken to ensure a sufficient supply of fresh and active vitamins in cultivated meat media (Schnellbaecher et al., 2019). Production of vitamins via industrial microbe fermentation or chemical synthesis is relatively easy and inexpensive (Acevedo-Rocha, Gronenberg, Mack, Commichau, & Genee, 2019), making vitamin sourcing costs a less pressing issue in cultivated meat cost reduction. However, vitamin supply by minimally processed plant extracts has yet to be explored fully.

The requirements of inorganic ions (minerals) that help control cell growth, constitute cellular structures, and regulate media osmolarity, are similarly varied depending on cell type and context (D. L. Nelson & Cox, 2017; O'Neill, Awale, Daneshmandi, Umerah, & Lo, 2018). Minerals having a relatively high importance in general cell culture media include bicarbonate, calcium, iron, magnesium, phosphate, potassium, sodium, and sulfate. Minerals required in trace amounts that have roles in the molecular regulation of metabolism and cell function include chromium, cobalt, copper, iodine, manganese, molybdenum, selenium, and zinc (Eagle, 1955; D. L. Nelson & Cox, 2017). The major and trace minerals needed for muscle cell culture can generally be obtained relatively inexpensively (Specht, 2020). A few important trace minerals, such as selenium, are usually directly supplemented in commercial defined media (Yao &

Asayama, 2017), but other typical media components, as well as simple plant extract ingredients, should be studied regarding their ability to supply the required minerals. As an example, Primatone RL, a proprietary tryptic digest of meat, has been used as a complex low-cost supplement for industrial animal cell culture, satisfying several growth requirements (although it could be unsuitable for cultivated meat due to its animal origin) (Schlaeger, 1996). Ingredients serving multiple functions in cultivated meat media could help to control final media costs.

5. Regulation of Proliferation, Differentiation, and Protein Synthesis

As discussed above, at some point in the cultivated meat process, stem cells will need to be differentiated into muscle and other types of cells at the large scale. The media requirements for this step will almost certainly be distinct from those mainly supporting cell proliferation. The complex interplay of extracellular signaling molecules, intracellular signaling pathways, and transcription factor activity ultimately determines muscle cell behavior (Khodabukus & Baar, 2016). A thorough understanding of the signaling molecules and molecular pathways implicated in myogenesis benefits the cultivated meat researcher by providing insight into the functions that cell culture media need to recapitulate. By providing molecules that can activate the same complement of signaling pathways necessary for myogenesis or other types of differentiation, different cell culture media formulations can regulate the proliferation and differentiation of muscle cells. Because a primary goal of cultivated meat is to provide high-quality dietary protein, an understanding of the molecular pathways involved in cellular protein synthesis is important as well.

Myogenesis is the development of muscle tissue with the goal of repair, homeostasis, or *de novo* formation. *In vivo* it is a complex suite of events occurring in both embryonic

development and adult muscle regeneration, involving several stages with various molecular mechanisms and cell types. The proliferating cells are muscle precursor cells (MPCs; known as myoblasts in the embryo and satellite cells in the adult) that can differentiate and fuse to form *de novo* myotubes or join existing myofibers. The processes of proliferation, differentiation and fusion are associated with the activity of several known growth factors, transcription factors, and signaling pathways. These signaling factors have been studied in model systems from rodent to human, and for this review we assumed that the general molecular pathways are shared between all of these species for the sake of discussing our general understanding of what is necessary for cultivated meat production. Figure 1 provides a simplified graphical overview of the myogenic process.

In MPCs, the early myogenic regulatory factor (MRF) genes *Myf5* and *MyoD* drive proliferation (Cooper et al., 1999). These MRFs are essential for the proper formation of skeletal muscle, as their absence prevents the expansion of the myoblast pool (Cornelison, Olwin, Rudnicki, & Wold, 2000). During differentiation, a second group of MRFs, *MRF4* and *Myogenin* (Chargé & Rudnicki, 2004), are upregulated, driving differentiation and fusion, and helping to maintain the adult muscle structure. Beyond their biological functions, these genes serve as important markers for monitoring and optimizing media in cultivated meat applications.

Extracellular signaling molecules, which in most cases would need to be supplied by the media in cultivated meat, can regulate the activity of MPCs. A number of growth factors and cytokines have been determined to play a role in MPC activation and proliferation (Bentzinger, Von Maltzahn, & Rudnicki, 2010), such as: fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), insulin-like growth factors (IGFs), transforming growth factor-β (TGF-β), and other cytokines like tumor necrosis factor-α (TNFα) and leukemia inhibitory factor (LIF). FGFs,

especially FGF2, are known as potent activators of MPC proliferation and inhibitors of MPC differentiation (Wilkie, O'Neill, Butterwith, Duclos, & Goddard, 1995). HGF is predominantly important in the activation and chemotaxis of MPCs to promote muscle repair (Suzuki, Yamazaki, Guang, Kaziro, & Koide, 2000) and therefore is likely not important for cultivated meat applications. FGF is known to induce proliferation but block differentiation *in vitro* (Miller, Thaloor, Matteson, & Pavlath, 2000). IGFs promote muscle development through the stimulation of both MSC proliferation and differentiation (Engert, Berglund, & Rosenthal, 1996), as well as through stimulation of protein synthesis within developing myotubes (Musarò, McCullagh, Naya, Olson, & Rosenthal, 1999). TNFα may promote myoblast differentiation (S. E. Chen, Jin, & Li, 2007), while LIF promotes myoblast proliferation but not differentiation (Spangenburg & Booth, 2002). Conversely, TGF-β ligands such as TGF-β1 and myostatin are known to inhibit both the proliferation, differentiation, and growth of MPCs and myotubes (Carlson et al., 2009; S.-J. Lee, 2004). The presence of fibroblasts in coculture with MPCs has interestingly been shown to promote MPC proliferation, differentiation, and fusion (Mackey, Magnan, Chazaud, & Kjaer, 2017).

Though adipogenesis is a highly ordered process, the extracellular factors modulating adipogenesis are arguably less complex than those that control myogenesis. When desired in a cultivated meat application, differentiation of pluripotent, or adipose-derived, stem cells into adipocytes can be stimulated by insulin and IGFs, and can be monitored by measuring levels of the transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT enhancer-binding proteins (C/EBPs) (Rosen & MacDougald, 2006). Conversely, Wnt/β-catenin signaling can inhibit adipogenesis (Christodoulides, Lagathu, Sethi, & Vidal-Puig, 2009). The differentiation and proliferation of fibroblasts is not a pressing concern in cultivated meat, since

they are not the primary desired cell types in most cases, and they tend to be more robust to suboptimal conditions (Fernandes et al., 2016). The fibroblast's role in cultivated meat is not to provide substantial bulk, but rather to support myocyte and adipocyte growth and to manufacture enough extracellular matrix to produce a more realistic meat product. A specific concern related to fibroblasts, however, is that vitamin C will be important to supplement in the media for efficient fibroblast production of collagen. Overall, cultivated meat media will need to provide some of the above important growth factors, or substitutes thereof, to allow proper proliferation and differentiation of the desired cell types.

The above signaling molecules function to stimulate myogenesis through a multitude of molecular pathways. Like many growth factor receptors, the IGF-1 receptor is a primary activator of tyrosine kinase-linked signal transduction cascades such as the phosphatidylinositol-3 kinase (PI3K)-Akt, and extracellular signal-related kinase (MAPK/ERK) signaling pathways (Gredinger, Gerber, Tamir, Tapscott, & Bengal, 1998; Yu et al., 2015). These pathways can activate transcription factors that promote proliferation and increase protein synthesis via the mechanistic target of rapamycin complex-1 (mTORC1), helping drive muscle tissue development (Rion et al., 2019; Shavlakadze et al., 2010). Many cytokine receptors, such as the IL-6 receptor, invoke the JAK-STAT signaling pathway (Brooks, Dehkhoda, & Kragelund, 2017), while TGF-β receptors are serine/threonine kinase receptors and activate their own distinct signaling pathway involving SMAD protein phosphorylation (Carlson et al., 2009; Derynck & Zhang, 2003). The JNK/MAPK signaling pathway inhibits myogenesis, and it has been reported that several miRNAs function in muscle cells to repress this pathway while promoting the p38/MAPK pathway (Xie et al., 2018). Inhibition of the p38 pathway (by a small molecule therapeutic) has been implicated in the retention of stem-like proliferation capacity in

bovine primary satellite cells (Ding et al., 2018). Multiple Wnt protein signaling pathways also play a role in myogenesis through the Frizzled family of G-protein coupled receptors. The canonical β-catenin mediated Wnt3a pathway has been shown to have stimulatory effects on myogenic proliferation and differentiation by antagonizing Notch signaling (Jones et al., 2015; Otto et al., 2008), while Wnt7a can activate the noncanonical Wnt/planar cell polarity (PCP) pathway to induce MPC proliferation (Le Grand, Jones, Seale, Scimè, & Rudnicki, 2009). Taken together, cultivated meat media needs to facilitate coordinated stimulation of a variety of types of intracellular signaling pathways, and future research should investigate the most inexpensive and efficient ways of doing so (discussed below).

Protein synthesis is an energetically-expensive cell function (Lane & Martin, 2010), but myoblasts have the means to supply large amounts of energy for this purpose (Moyes, Mathieu-Costello, Tsuchiya, Filburn, & Hansford, 1997). Protein synthetic activity is regulated through mTOR-mediated signaling, and this has been reviewed extensively (Laplante & Sabatini, 2012; Wang & Proud, 2006). Amino acids can serve as regulators of protein metabolism, in addition to being the building blocks of proteins themselves. Leucine and its metabolites are known to play important roles in promoting protein synthesis in myocytes, through activation of mTORC1 (Anthony, Anthony, Kimball, & Jefferson, 2001; Bar-Peled & Sabatini, 2014; Wilkinson et al., 2013). The leucine metabolite, β-hydroxy-β-methylbutyrate (HMB), has been shown to enhance muscle protein synthesis and promote muscle tissue hypertrophy *in vivo* (Jackman et al., 2017; Wilkinson et al., 2013). Leucine and HMB, therefore, may be useful targets for enhancing *in vitro* muscle cell culture media; however, this has yet to be fully investigated.

Due to the cost associated with many of the purified growth factors and signaling molecules used for *in vitro* myogenesis and protein synthesis, less expensive alternative methods of stimulating the required cellular signaling pathways are likely necessary. One such method to achieve this could be to screen homology between amino acid sequences for cell signaling molecules and common plants or fungi. If significant sequence homology is identified in certain plants/fungi, those can then be tested for use in muscle cell culture media. One example of how plant extracts have been used as agonists for mammalian cell growth factor receptors is the use of cowpea (*Vigna unguiculata*) peptide extracts to stimulate insulin-associated cell signaling pathways (Barnes, Uruakpa, & Udenigwe, 2015; Venâncio et al., 2003). More generally, wheat and cotton peptones have shown promise in acting as substitutes for bovine serum albumin in the culture of bovine embryos (George, Kerschen, Van Nuffel, Rees, & Donnay, 2009).

6. Strategies for Serum-Free Media Formulation

Due to the cost, variability, contamination, and ethical issues mentioned earlier, the animal serum that is used in conventional biomedical cell culture media needs to be efficiently replaced if cultivated meat is going to meet the ethical and cost goals set by the market. The multifaceted problems associated with FBS, and other animal sera, are of significance to the biomedical realm as well, and have thus been reviewed extensively (Brindley et al., 2012; Even, Sandusky, & Barnard, 2006; Jochems et al., 2002; J. van der Valk et al., 2010; Jan van der Valk et al., 2018). The present section therefore focuses on the existing and possible strategies to replace animal sera in the context of cultivated meat.

Serum supplies not only the growth factors and hormones that stimulate proper cell growth *in vitro*, but also many nutrients (Yao & Asayama, 2017). Many of the major functional

constituents of serum have been revealed, but their concentrations are highly variable and there are still many unknown components (Freshney, 2010). For reference, Table 2 lists approximate concentrations of many of the known components of animal serum. For the above reasons, formulating serum-free media is not always as simple as supplementing basal media with a few growth factors. Like serum-containing basal media preparations, there is no single serum-free formulation strategy that works efficiently for every cell type in every situation; different cells have different requirements. As such, there are many serum-free formulations and supplementation protocols that have been described or made commercially available (Jan van der Valk et al., 2018). In almost all cases, however, these still contain animal- or human-derived ingredients, are not optimized for efficient muscle cell cultivation, and/or remain too expensive (Jan van der Valk et al., 2018). Commonly, serum-free media for biopharmaceutical production or medical research are supplemented with expensive growth factors (especially insulin, FGF2, and TGFβ) isolated from animal serum or produced recombinantly (Ejiri et al., 2015; Shiozuka & Kimura, 2000), since these industries are not encumbered by the same cost restraints as the food industry. Essential 8™ media is one such defined animal-free media formulation (given in Table 1) that is commonly used in research settings and is designed to promote stem cell growth, especially that of human pluripotent stem cells (hPSC) (G. Chen et al., 2011). As its name suggests, it consists of eight ingredients, one of which is normal DMEM/F12 basal medium. The remaining ingredients include insulin, FGF-2, and TGF-β as the primary recombinant growth factors, as well as additional vitamin, mineral, and buffer supplementation. Fibroblast Growth Medium™ is another similar commercially available serum-free media optimized for human primary fibroblasts. These two types of media were recently shown to have promise in cultivated meat applications, as they were able to support the proliferation of primary bovine myoblasts for

at least six days (Kolkmann et al., 2020). However, they achieved only roughly one doubling, compared to the roughly 3-fold proliferation achieved by 20% FBS and 10% horse serum medium. Further, the ability of these media to promote long-term and high-density proliferation of bovine myoblasts has not been assessed to date, nor has their ability to sustain myoblast markers or allow retention of differentiation capacity. These media would also be prohibitively expensive if used at production scale; nevertheless, they demonstrate the potential for animal myoblasts to be cultured in serum-free media and provide useful insight.

Clearly, much more innovation and optimization need to be performed to achieve efficient and low-cost serum-free media for cultivated meat. Further optimization of recombinant growth factor production holds promise as a solution for cultivated meat supplementation, potentially through genetic modification of the cultivated cells themselves, so that they endogenously express the proteins (Sunstrom, Baig, Cheng, Sugyiono, & Gray, 1998). However, much more research and optimization are required to achieve this at low cost. Additionally, this manipulation of the cultivated meat cells could ultimately deter potential consumers concerned about genetically modified foods. Another suggested method of replacing serum is the use of human blood platelet lysates, as a byproduct of conventional human blood donations (Burnouf, Strunk, Koh, & Schallmoser, 2016; Jan van der Valk et al., 2018). Platelet lysates contain many of the growth factors, cytokines, and attachment factors that are useful for cultivated meat production. However, there are several drawbacks to this potential approach, including cross-species compatibility, cost, and supply logistics (Hemeda, Giebel, & Wagner, 2014). Conditioned media systems have also been suggested as a solution to replace serum—the growth factors and other useful products that the cultivated meat cells produce themselves can be recycled and concentrated to support growth of other new cells in an interconnected system of bioreactors

(Pawitan, 2014). As will be discussed in more detail in a later section, there is the possibility that plant or fungal protein extracts can function as homologs for important growth factors and other regulatory proteins in cultivated meat media, but this remains to be investigated fully (Andreassen, Pedersen, Kristoffersen, & Beate Rønning, 2020; Venâncio et al., 2003).

In any case, cell lines must be adapted slowly over time to new media formulations, especially when introducing serum-free media (Ozturk, Kaseko, Mahaworasilpa, & Coster, 2003; Sinacore, Drapeau, & Adamson, 2000). Usually, an adaptation protocol will involve reducing the serum concentration by around 50 percent with each subsequent culture passage until less than one percent of the original concentration remains. In its place, the serum replacement formulation can be gradually introduced until the serum is no longer needed. While this general procedure has been performed with many animal cell types, it has not been well characterized for primary muscle cell lines. Achieving both a muscle cell line adapted to have a reduced requirement for growth factors and other serum components, as well as animal-free media that can inexpensively replace the function of serum, remains a critical goal of the cultivated meat industry.

7. Media Formulation for Large-Scale Food and Commodity Chemical Fermentations

Fermentation is used as a means of producing a large variety of products, from food to commodity chemicals to therapeutic recombinant proteins. While some animal cell lines, such as myeloma and CHO cells, are used at large scale to produce therapeutic proteins (J. Zhang, 2014), the price of these products (millions of dollars per kilogram) is such that process optimization and media cost reduction are not driving forces in commercialization. However, microbial food fermentations producing products such as beer and yogurt, and specialty chemical production

through fermentation, such as Vitamin C, citric acid, MSG, xanthan gum, and medicinal feed additives (antibiotics for livestock feed), necessarily need extensive media and process optimization to be competitive (Dahod, Greasham, & Kennedy, 2014). This is especially true of the latter category, in which media need to be optimized and inexpensive with multiple components supplying the required nutrients. In these cases, fermentations are generally large scale (>40,000 L) and product prices are low (generally less than $1-10/Kg), similar in scope to goal costs for cultivated meat.

In cases where media costs need to be kept especially low for profitability, the use of complex media ingredients is common. Generally, completely defined media are too expensive to make these processes viable. In many microbial fermentation systems, this means that complex ingredients (typically of agricultural origin) are substituted for defined sources of all key nutrients (Dahod et al., 2014). Table 3, adapted from (Kampen, 2014), compares the compositions of several of these complex agricultural byproducts. Many times, these complex ingredients supply not only one key nutrient, but several, allowing for fewer overall media ingredients. For example, instead of using purified sugars such as glucose as a carbon source, these microbial fermentations will use less refined sources of sugar, such as molasses, corn starch, or malt powder/maltose (Dahod et al., 2014). Of course, usability of these more complex sugars sources for cultivated meat production will depend on the ability of animal cell lines to utilize or at least tolerate sugars other than glucose, as they often contain fructose, sucrose, maltose, and other more complex carbohydrates. Similarly, these large-scale microbial fermentations often use more complex nitrogen sources that are by-products of agricultural processing of grains. Typical examples of these include corn steep solids, corn steep liquor, soy flour, or peptones/enzymatic hydrolysates of plant- or microbe-based material such as yeast or

soy extracts. The ability of animal cells to use these types of nutrient sources has not been extensively tested, and the potential cytotoxicity of the impurities found in them remains a concern. However, peptones of plants and fungi have been used as nutrient and metabolite sources for animal cells in specific applications using specialized cells such as CHO (Burteau et al., 2003; J. Y. Lee, 2009), but their usefulness in cultivated meat media has not been thoroughly investigated. Commercial grades of these latter materials can be quite inexpensive but may not be as defined or consistent as their laboratory analogs. When needed, fatty acids are often supplied by the addition of vegetable oils or semi-purified components such as oleic or linoleic acids, often direct precursors of products such as polyketide antibiotics in large scale production (Dahod et al., 2014).

Many of these complex ingredients supply more than just sugar or amino acids. Molasses and yeast extract, for example, are also rich in minerals (e.g. phosphorous or metals) and B-vitamins (Clarke, 2003). In Figure 2, the relative nutrient levels of two complex fermentation media are compared with those of Essential 8. The two media chosen are YPD, a common complex medium used for yeast growth, and an industrial medium (MFA) used to grow *Streptomyces* spp. for production of a medicinal feed additive (antibiotic for addition to animal feed). Media are compared for the broad categories of carbohydrates, free amino acids, minerals, and vitamins. While each production organism will likely have its own specific nutrient requirements within these broad categories, it can be seen from this figure that the complex ingredients that comprise the YPD and MFA media (the approximate nutrient profiles of which are provided in Table 3) can easily supply the general nutrition present in Essential 8 when supplied in some combination.

Because of the composition of these complex nutrients, they often preclude the need for specific buffers to be added to the media. They may also be able to substitute for specific proteins currently added to cell culture media if they contain analogs for the active domains of these proteins, though this has not been thoroughly studied to date. As discussed above, this latter point would be important to investigate, since the specific protein components of cultivated meat media (such as growth factors) are some of the most expensive components of current media (Specht, 2020). Finally, because of the complexity of these ingredients or limited solubility of their components, these ingredients can sometimes substitute for slow defined nutrient feeds that might otherwise be added in fed-batch fermentation processes (Dahod et al., 2014). This can simplify the processing but may lead to increased variability due to decreased control of nutrient additions.

Of course, there is a tradeoff between the use of complex and defined media ingredients. While complex ingredients can be considerably less expensive than their defined counterparts, they tend to introduce variability in processing (Dahod et al., 2014; Singh et al., 2017). This variability can be introduced because of seasonal differences in agricultural feedstocks or by processing differences between different suppliers of similar ingredients. Geographic differences of the agricultural feedstock origins can also contribute to variability if materials are sourced over a large area. Variability can also be a result of long-term storage of these materials at the production site. These agricultural materials will likely be stored at room temperature or outside temperatures in large quantities and may change over time. Some of them may be prone to chemical or microbiological degradation, or as in the case of molasses, may have solids that settle out over time causing variation in a non-stirred environment.

In addition to these potential media sources being nutrients for cell growth, they are all food grade and could potentially add desirable sensory qualities to the media, as mentioned above. Many of these ingredients are somewhat stable in high heat, allowing steam sterilization as the means for preparing for fermentation which is considerably less process intensive and expensive than filtration (typically used for cell culture media), especially as the fermentor tank itself still needs to be steam sterilized, even when filtration is used for the media. While some component degradation may happen at high temperatures, especially if nitrogen and sugar sources are mixed, it is at least possible that this could have positive sensory implications for the cultivated meat product downstream.

8. Media Optimization Methods

Once media components of interest have been determined through experimentation and/or theory, their concentrations need to be optimized. Due to the large number of components likely to be used in cultured meat processes (DMEM has 30-52, for example) this is a combinatorically difficult and time-consuming process, especially as critical interaction effects can occur (Ruotti et al., 2011). Additionally, due to physiological variability of cell lines, re-optimization may be needed as processes change and new components are identified, especially in an industry expected to work on a variety of different cell lines (M. Zhang et al., 2008). Therefore, efficient means of identifying and adjusting concentrations is necessary.

Traditionally, media design is initially conducted using a one-factor-at-a-time (OFAT) basis where a single component is considered for its effect on cell response. The drawback of this approach is that interaction effects can go unnoticed, leading to suboptimal media designs (Gupte & Kulkarni, 2003). This is illustrated in the Figure 3a, where the nonlinear interactions mask the

true optima if a simple OFAT method is used to follow the direction of steepest descent. *Design of Experiment* (DOE) techniques such as *Factorial*, *Plackett-Burman*, and *Central Composite* designs, where multiple nutrient concentrations are changed at once, allow for much faster optimization, and have successfully been used to characterize and optimize monoclonal antibody production and bacterial culture processes (Fisher, 1992; Horvath, Mun, & Laird, 2010; G. Zhang & Block, 2009; G. Zhang, Mills, & Block, 2009; G. Zhang, Olsen, & Block, 2007). These experiments are typically conducted at the 'lower' and 'upper' extrema of the media design space as seen in Figure 3b. This arrangement allows first order 'effects' for each media component to be estimated without being convoluted with other media component's effects via analysis of variance methods. These methods can be combined with response surface models (Figure 3c), such as a linear or polynomial model to map inputs X to outputs Y, to sequentially improve mixtures by predicting optimal media component concentrations (Chambers & Swalley, 2009). These methods can still require many experiments to be successful, and often become experimentally burdensome when optimizing more than 5 design variables, whereas typical industrial media may have 50-75 components (J. Zhang, 2014; M. Zhang et al., 2008).

Efficient media design has also been performed using stochastic optimization methods such as genetic algorithms, where media combinations are treated as chromosomes under natural selection pressures (Weuster-Botz, 2000). New media are computationally generated as stochastic combinations and mutations of previous media, with the goal of maximizing the fitness of the solution or objective function (maximizing biomass, for example) of each nutrient combination. More recently, stochastic methods have been augmented by mathematical surrogate models (neural networks, for example) that aid in prediction and store information about component effects and interactions through their parameters (G. Zhang et al., 2007), as shown in

Figure 3d. In this manner, as more experiments are conducted, the surrogate model enhanced stochastic optimizer can make better predictions about generating optimal media combinations. These methods can handle more nonlinear and interaction-heavy media design problems and have been successfully applied to designing microbial media with 19 components (G. Zhang & Block, 2009), resulting in more optimized media in half the experiments of traditional DOE. These efficient optimization methods continue to evolve and are increasing in robustness (Cosenza & Block, 2020), hopefully to the point where they can be utilized effectively by practitioners not expert in artificial intelligence and numerical optimization methodology.

While these latter methods have proven to be much more efficient than random or OFAT optimization, it is still important to accumulate knowledge about the organism of interest and its nutrient requirements and molecular physiology in parallel to the more black-box approach to optimization. Therefore, it is advantageous to conduct experiments with each cell line fully characterizing the use and effects of carbon and nitrogen sources, in addition to other key nutrients, as well as the production of key positive or negative metabolites over time in a basal medium. These data will facilitate understanding of processes that can be used to help design new experiments (for example, choosing concentration ranges of key nutrients or feed compositions and rates) and to troubleshoot process issues during scaleup and manufacturing.

9. Conclusion

Clearly, the design of culture media to achieve scalable, low-cost, and high-quality cultivated meat products remains a complex challenge. Success will likely require careful media formulation combined with innovative adaptation of cells and culture systems to these new media. Continuing research should be focused on developing the understanding of how the

molecular mechanisms controlling muscle cell growth and differentiation can be stimulated via more affordable and ethical means. This research can both inform and be informed by more mainstream biomedical research. However, media and processes are much more likely to resemble large scale microbial fermentation processes where commodity chemicals and food components are produced. Overall, the relatively rapid development and expansion in the cultivated meat space in recent years lends confidence to the notion that many of these remaining challenges can be overcome.

Acknowledgements

The authors would like to acknowledge New Harvest's generous financial support for E. O'Neill and Z. Cosenza, which facilitated preparation of this manuscript.

Author Contributions

E. O'Neill and D. Block conceptualized the scope and sections of the review. Z. Cosenza drafted the section and figure about media optimization. D. Block drafted the sections about the modes of cultivated meat production and media for large-scale fermentation and provided expertise on industrial fermentation. K. Baar provided Figure 1 and expertise regarding muscle tissue growth and differentiation. E. O'Neill performed the literature review, drafting and compiling the remainder of the manuscript. All authors reviewed and edited the manuscript.

Conflicts of Interest

The authors have no conflicts of interest relating to this manuscript.

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Table 1

Ingredient concentrations of common commercially available defined animal cell culture media used in biomedical research[a]

Component		Concentration (mg/L)				
		Minimal Essential Medium	**DMEM (high glucose, GlutaMAX , pyruvate)**	**Ham's F-12**	**DMEM/F -12**	**Essential 8**
Amino acids	Glycine		30	7.5	18.75	18.75
	L-Alanine			8.9	4.45	4.45
	L-Arginine hydrochloride	126	84	211	147.5	147.5
	L-Asparagine-H2O			15	7.5	7.5
	L-Aspartic acid			13.3	6.65	6.65
	L-Cysteine hydrochloride-H2O	31	63	35.12	17.56	17.56
	L-Cystine 2HCl				31.29	31.29
	L-Glutamic Acid			14.7	7.35	7.35
	L-Glutamine	292	862	146	365	365
	L-Histidine hydrochloride-H2O	42	42	21	31.48	31.48
	L-Isoleucine	52	105	4	54.47	54.47
	L-Leucine	52	105	13.1	59.05	59.05
	L-Lysine hydrochloride	73	146	36.5	91.25	91.25
	L-Methionine	15	30	4.5	17.24	17.24
	L-Phenylalanine	32	66	5	35.48	35.48
	L-Proline			34.5	17.25	17.25
	L-Serine		42	10.5	26.25	26.25
	L-Threonine	48	95	11.9	53.45	53.45
	L-Tryptophan	10	16	2.04	9.02	9.02
	L-Tyrosine	52	72	5.4	55.79	55.79

	L-Valine	46	94	11.7	52.85	52.85
Vitamins	Biotin			0.0073	0.0035	0.0035
	Choline chloride	1	4	14	8.98	8.98
	D-Calcium pantothenate	1	4	0.5	2.24	2.24
	Folic Acid	1	4	1.3	2.65	2.65
	Niacinamide	1	4	0.036	2.02	2.02
	Pyridoxine hydrochloride	1	4	0.06	2.013	2.013
	Riboflavin	0.1	0.4	0.037	0.219	0.219
	Thiamine hydrochloride	1	4	0.3	2.17	2.17
	Vitamin B12			1.4	0.68	0.68
	Ascorbic acid 2-phosphate					64
	Vitamin A					
	i-Inositol	2	7.2	18	12.6	12.6
Inorganic Salts	Calcium Chloride (CaCl2-2H2O)	200	264	44	116.6	116.6
	Cupric sulfate (CuSO4-5H2O)			0.0025	0.0013	0.0013
	Ferric Nitrate (Fe(NO3)3"9H2O)				0.05	0.05
	Ferric sulfate (FeSO4-7H2O)		0.1	0.834	0.417	0.417
	Magnesium Chloride (MgCl2-6H2O)	97.67	200	122	28.64	28.64
	Magnesium Sulfate (MgSO4) (anhyd.)				48.84	48.84
	Potassium Chloride (KCl)	400	400	223.6	311.8	311.8
	Sodium Bicarbonate (NaHCO3)	2200	3700	1176	2438	2438
	Sodium Chloride (NaCl)	6800	6400	7599	6995.5	6995.5
	Sodium Phosphate dibasic (Na2HPO4) anhydrous	140	141	142	71.02	71.02
	Sodium Phosphate monobasic (NaH2PO4-H2O)				62.5	62.5
	Sodium Selenite (Na2SeO3)					0.014
	Zinc sulfate (ZnSO4-7H2O)			0.863	0.432	0.432
Carbohydrates	D-Glucose (Dextrose)	1000	4500	1802	3151	3151
	Sodium Pyruvate		110	110	55	55
Lipids	Linoleic Acid			0.084	0.042	0.042

	Lipoic Acid			0.21	0.105	0.105
Growth Factors / Hormones	Insulin					19.4
	FGF-2					0.1
	TGF-β					0.002
	Transferrin					10.7
Other	Phenol Red	10	15	1.2	8.1	8.1
	Putrescine 2HCl			0.161	0.081	0.081
	Thymidine			0.7	0.365	0.365
	Hypoxanthine			4	2.39	2.39

[a] Formulations as supplied by Thermo Fisher Scientific, Waltham, MA, USA. Blank cells indicate the component is not included in the media formulation.

Table 2

Approximate nutrient and functional component concentrations in animal serum[a]

Proteins and Polypeptides	**40-80 g/L**
Albumin	20-50 g/L
Fetuin	10-20 g/L
Fibronectin	1-10 mg/L
Globulins	1-15 g/L
Protease inhibitors: α1-antitrypsin, α2-macroglobulin	0.5-2.5 g/L
Transferrin	2-4 g/L
Growth Factors	
EGF, PDGF, IGF-1 and 2, FGF, IL-1, IL-6	1-100 ug/L
Amino Acids	**0.01-1.0 uM**
Lipids	**2-10 g/L**
Cholesterol	3.867 mg/L
Linoleic acid	2.805-28.05 ug/L
Phospholipids	0.7-3.0 g/L
Carbohydrates	**1.0-2.0 g/L**
Glucose	0.6-1.0 g/L
Hexosamine	6-1.0 g/L
Lactic acid	0.5-2.0 g/L
Pyruvic acid	2-10 mg/L
Polyamines	
Putrescine, spermidine	8.815-88.15 ug/L
Urea	**170-300 mg/L**
Inorganic Ions	
Calcium	160.3-280.6mg/L
Chlorides	3.545 mg/L
Iron	0.559-2.793 mg/L
Potassium	195.5-586.5 mg/L
Phosphate	189.9-474.9 mg/L
Selenium	0.790 ug/L
Sodium	3.10-3.57 g/L
Zinc	6.538-65.38 ug/L
Hormones	
Hydrocortisone	3.625-72.49 ug/L
Insulin	5.778-577.8 mg/L
Triiodothyronine (T3)	13.02 ug/L
Thyroxine (T4)	77.69 ug/L
Vitamins	**10 ug - 10 mg/L**
Vitamin A	10-100 ug/L
Folate	5-20 ug/L

[a] Data are adapted from (Freshney, 2010). No specific species source is specified.

Table 3

Approximate average compositions of common agricultural byproducts used in industrial microbial fermentation[a]

Component		Beet Molasses[b]	Corn Steep Liquor[c]	Cottonseed Embryo	Bacto Peptone[d]	Yeast Extract
Amino Acids, %	Alanine	0.8			9.2	
	Arginine		3.3	2.9	5.8	0.78
	Aspartic Acid	1.5			5	5.1
	Cystine		1.9	1.52		
	Glutamic Acid	1.5			8.1	6.5
	Glycine	0.4	5.1	3.78	15.9	2.4
	Histidine		2.8	2.96	0.8	0.94
	Isoleucine		3.6	3.29	2.1	2.9
	Leucine	1.3	11.3	6.11	3.8	3.6
	Lysine		2.5	4.49	3.4	4
	Methionine		1.9	1.52	0.7	0.79
	Phenylalanine		4.4	5.92	2.8	2.2
	Proline				8.8	
	Serine				1.5	
	Threonine	0.6	4	3.31	1.1	3.4
	Tryptophan		0.2	0.95		0.88
	Tyrosine	0.7	5.8	3.42	0.6	0.6
	Valine	0.6	3.4	4.57	2.8	3.4
Vitamins, mg/100 g	Thiamine	0.01	0.5	0.399		0.32
	Riboflavin	1.1	0.1	0.482		1.9
	Nicotinic acid	8	1.6	8.33		
	Pantothenic acid	0.7	2.5	1.24		
	Folic acid	0.025	0.05			
	Pyridoxine-HCl		2	1.64		
	Biotin		0.01	0.152		0.14
Minerals, %	Potassium	6.4	4.5	1.72	0.2487	0.04
	Calcium	0.21		0.25		0.04
	Magnesium	0.12		0.74	0.0017	0.03
	Phosphorus	0.03		1.31		0.29
	Sodium	1.6	0.2		1.8127	0.32
	Iron	0.03	0.03		0.00078	
	SO3	0.74	0.25		0.32	
Sugars, %	Sucrose	48.9				
	Glucose	0.5	2.50		0.629	

[a] Most data adapted from (Kampen, 2014). Blank cells indicate no data available.

[b] Amino acid data additionally adapted from (Stark, 1961).

[c] Amino acid data additionally adapted from (Hull, Yang, Venzke, Kulhavy, & Montgomery, 1996).

[d] All data for Bacto peptone adapted from the supplier product sheet (Thermo Fisher Scientific, Waltham, MA).

Figures

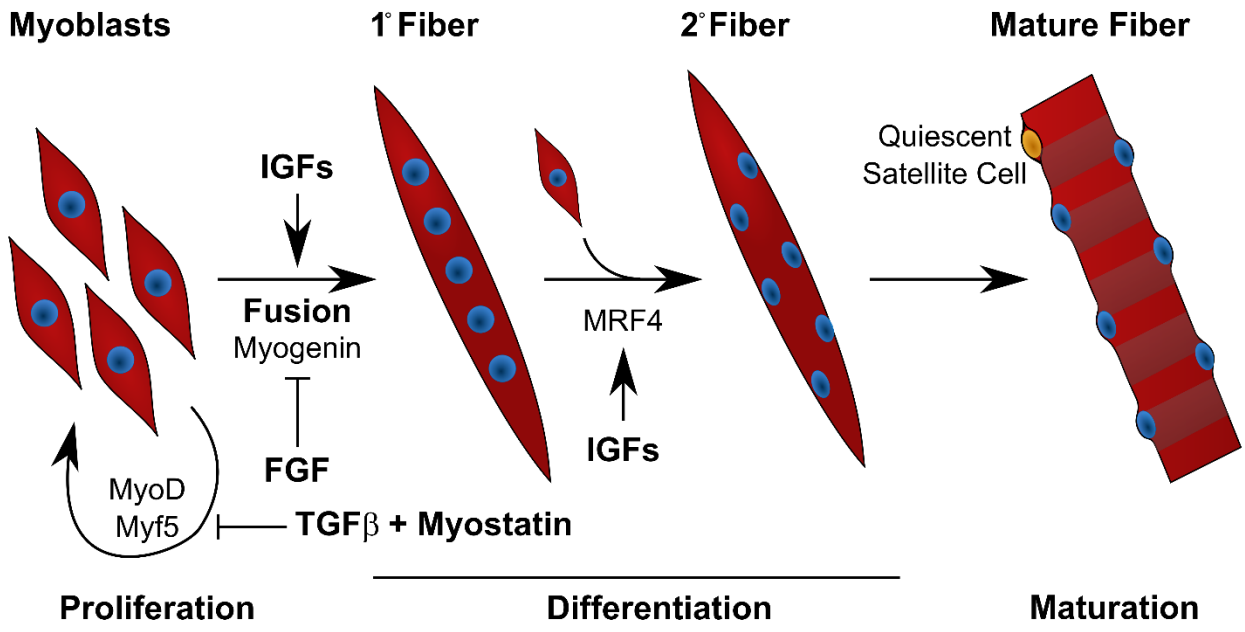

Figure 1: Overview of the myogenic regulation process relevant to cultivated meat media. | Upon activation of a quiescent satellite cell, actively proliferating muscle precursor cells are known as myoblasts. The myogenic regulatory factors (MRFs) *MyoD* and *Myf*5 drive this proliferation. Fibroblast growth factor (FGF) supports proliferation, while transforming growth factor β (TGF-β) and myostatin inhibit proliferation. Insulin-like growth factors (IGFs) support proliferation as well as differentiation. IGFs, along with the MRF myogenin, drive fusion of myoblasts into primary myofibers. Continued fusion and differentiation are supported by IGFs and MRF4, yielding secondary fibers, which ultimately develop into mature fibers (myotubes) complete with associated quiescent satellite cells.

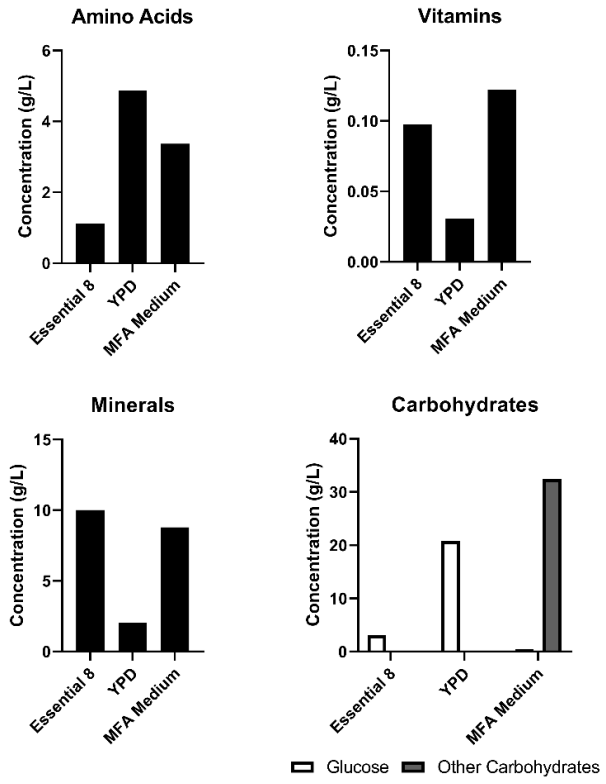

Figure 2: Nutrient profiles of example complex media

Approximate concentrations of four broad classes of nutrients between two examples of cell culture media containing complex and undefined ingredients. Yeast extract/peptone/dextrose (YPD) media is formulated for yeast cell culture and is composed of 1% yeast extract, 2% Bacto peptone, and 2% dextrose in water; the former two complex ingredients are described in more detail in Table 3. The example medicinal feed additive (MFA) medium to culture bacteria for the production of the antibiotic frenolicin is described in (US5593870A, 1994) and consists of corn oil, dextrin, corn steep solids, molasses, sodium formate, yeast extract, magnesium sulphate heptahydrate, monosodium phosphate heptahydrate, and calcium carbonate. The approximate nutrient profiles of the complex ingredients in MFA medium are given in Table 3. Essential 8 is a

defined medium for animal cell culture and is included here for comparison; its exact composition is given in Table 1.

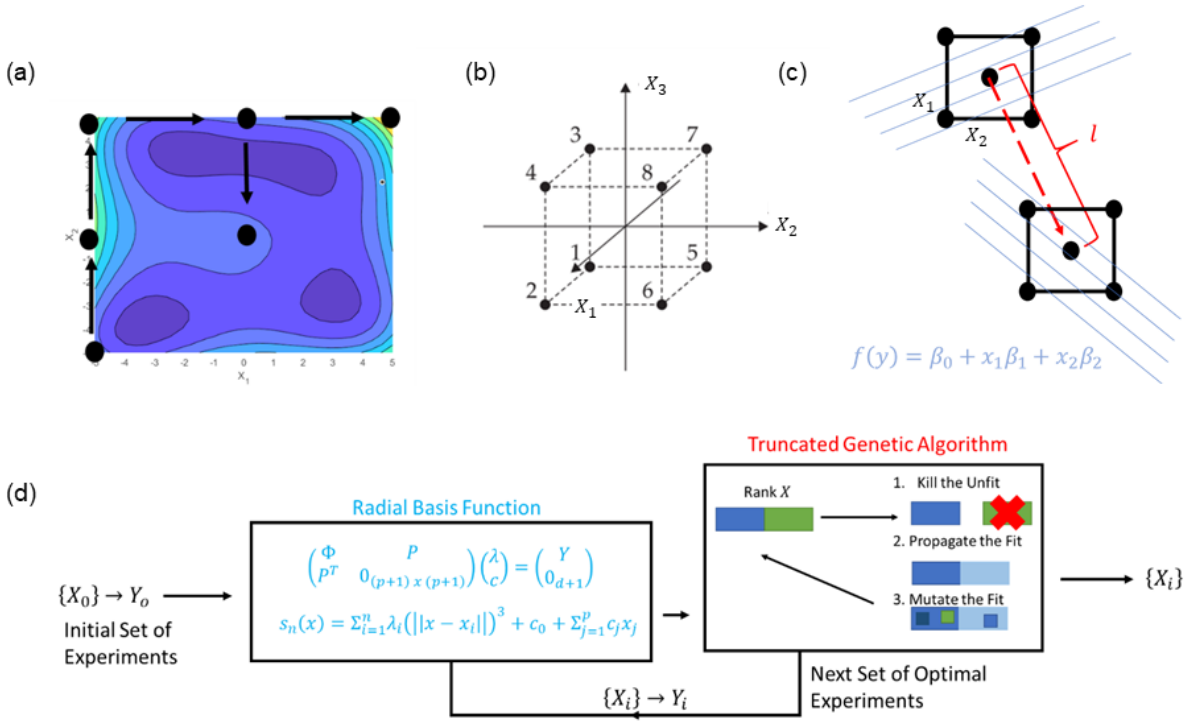

Figure 3: Experimental optimization methods. | (a) one-factor-at-a-time for local minimization problems where darker blue is more optimal response and X_1 and X_2 are media components, (b) full factorial design for three media components X_1, X_2, X_3 and two levels to estimate effects of each factor across each level, (c) response surface methodology with full factorial design for two factors and two levels (in black) combined with a linear model (in blue) to suggest an optimal direction of search (in red) for iterative component optimization, (d) a nonlinear radial basis transformation of inputs X models an approximation of the medium components effects on the response Y, this is used by a genetic algorithm with a truncation stopping criteria (stops when experiments get close to one another) to suggest new optimal media component concentrations based on the principle of natural selection.