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93923, Master's in Biotechnology

Cultured Meat from Stem Cells: Challenges and Prospects

EXTENDED ABSTRACT

Cultured meat is an innovative scientific endeavor to produce large-scale quantities of cells that can differentiate into muscle tissue and form common animal food products, without relying on slaughter, by using cell and tissue engineering techniques to grow cells in adequate medium and bioreactors.

This emerging field, broadly called Cellular Agriculture, intends to improve some of humanity's most pressing issues, including the need to feed 9 billion people by 2050 under sustainable development goals, without pressuring natural ecosystems and whilst reducing greenhouse gas emissions, land and water use.

Livestock industry is a major key-player in worldwide emissions of different pollutants, and has an enormous water footprint. In contrast, life-cycle analysis shows immense potential for cultured meat to surpass or diminish some of these challenges. In addition, intensive animal agriculture has a major role in boosting zoonotic diseases and antibiotic-resistant bacteria. Therefore, it is required a multidisciplinary approach to circumvent these effects, and cultured meat could allow a reduction and possible elimination of such events, by culturing cells in aseptic environments, where contamination is less likely.

The cultured meat process starts by extracting a piece of tissue from an animal donor, through a biopsy for instance, and then selecting an ideal cell population that has the potential to differentiate into muscle tissue, or fat tissue. These cells can be used to inoculate a bioreactor, where cells can grow in suspension in a growth medium with all the ingredients required for adequate cell expansion, until large cell densities are obtained. These cells can grow in microcarriers which mimic natural cell environment by allowing cells to grow attached to these spheres, which can have the required chemical cues for optimal cell adhesion. Afterwards, these cells can be used to inoculate a next bioreactor, in order to promote their differentiation and fusion into muscle cells, or myotubes. Thus, cells can be used to populate a tissue perfusion bioreactor with an immobilized scaffold, where cells can grow in a surface with physical cues similar to native muscle tissue, after changing the medium to a differentiation formulation, these cells can merge and form myotubes, and then mature into muscle fibers. Lastly, these fibers can be pressed, mixed with adipose cells or other components and then minced and shaped into processed meat products such as hamburgers. Alternatively, different cell types that compose the skeletal muscle niche can be put in co-cultures in the perfusion bioreactor to give rise to a complex tissue structure akin to a steak, for instance.

There are various types of cells that could be used as a starting cell type for cultured meat production. ESCs are derived from blastocysts and can give rise to most cell types, including muscle, fat and ECM cells. These cells have been previously extracted from bovine and because they can regenerate indefinitely, they are promising candidates for applications in cultured meat. In addition, iPSCs can be reprogrammed from different somatic cell types and originate pluripotent cells that can also originate most tissues of interest for this application. However, both cell types need thorough examination into their differentiation steps until muscle and adipose lineages, which can be challenging and require time-intensive methodologies.

In contrast, multipotent cells such as MSCs can differentiate into adipocytes and under certain stimulus into muscle cells and fibroblasts, and have been identified in bovine and shown to adhere and proliferate in microcarrier systems in suspension, which deems them an attractive source of cells for cultured meat. Additionally, SCs are muscle tissue-resident stem cells that commit solely to the myogenic lineage under normal conditions, and were used as a cell source in the first proof-of-concept of cultured meat.

The process through which SCs commit to the muscle lineage is called myogenesis and it is exemplary of the process that cells must go through *in vitro* settings for cultured meat applications. Among a plethora of transcription factors that regulate myogenic commitment of cells such as SCs, Pax7, Myf5 and MyoD appear to be major regulators of SC specification, which are expressed until SCs commit to myoblasts. Henceforth, the factors MyoG and Mrf4 are mostly expressed when myoblasts merge into multinucleated myotubes. This process is further regulated by numerous factors, including addition of different GFs, mechanical simulation and substrate stiffness. During the late myogenic process, both electrical simulation and substrate conditions are paramount for adequate myotube fusion and myofiber formation, which are essential in the differentiation step towards thin muscle fibers.

Besides the myogenic process, adipose tissue cells should be considered for application in cultured meat, since fat is an essential part of the experience of eating meat, as it enhances the organoleptics properties of muscle tissue, providing both flavor and texture. Similarly to myogenesis, adipogenesis is also a multifaceted process that comprises the specification of adipose progenitors into mature, lipid-accumulating adipocytes. This process is regulated by various factors, and both Zfp423 and IGF-1 are relevant inducers of adipocyte commitment, by regulating the major adipogenic factors, PPARγ and C/EBPα. These factors can generate a cascade of events that allow adipose-committed cells to express several factors, including LPL, LEP and ADIPOQ, which drive adipogenic differentiation and accumulation of lipid droplets.

It is clear that adipogenic progenitors can be extracted and isolated from the stromal vascular fraction of an adipose tissue sample, though other cell types also have adipogenic commitment, besides MSCs and pluripotent cells previously discussed, including FAPs which can be extracted from the stromal vascular fraction of muscle tissue and have dual-lineage commitment into adipocytes and fibroblasts, as well as DFAT cells which can originate proliferating adipose progenitors from somatic adipocytes that return to a proliferative, stem-like, state.

For complex meat structures, support cells such as fibroblasts, ECs, or SMCs appear to be essential for adequate structuring and functionality of muscle tissue, which will be required to produce muscle constructs akin to steaks and marbled meat structures. Fibroblasts are mostly relevant due to their ability to produce ECM proteins that offer cells native tissue-like surroundings, while ECs and SMCs are important components of

vascular systems and could allow pseudo-vascularization of muscle tissue, as well as improve the flow of autocrine and paracrine factors between cells in co-cultures.

In this manuscript, an overview of different culture medium formulations for expansion and differentiation of muscle cells and muscle progenitors is given, including the need to establish optimal concentrations of GFs such as bFGF and IGF-1 that appear to aid SC proliferations. Importantly, FBS has been routinely used for cultured meat applications, which will need to be substituted for further application in the field if the intended products are supposed to lack animal-derived components, other than starting cells. While proliferation of SCs and myoblasts is benefited from high FBS concentrations, the differentiation medium is usually composed of very low concentrations of FBS, also deemed starvation medium, where myoblasts fuse and originate myotubes. Importantly, culture medium components should have reduced batch variability and have consistent bulk production, if such processes are intended to be scaled into pilot or industrial facilities where the quantities of growth medium will be several orders of magnitude higher than those used in small-scale laboratories. On the other hand, adipogenic medium for cells to proliferate is akin to its myogenic counterpart, but several supplements are given to ensure differentiation into adipocytes, and some of these such as IBX are not ideal for food production, though recent efforts have established a differentiation method based on different fatty acid inputs, which could be a promising alternative for the aforementioned supplements.

An important part of the cultured meat process is the choice of adequate BRs for production of cells in large quantities, while providing an adequate environment for these to proliferate. Stirred tanks appear highly likely candidates for the proliferation phase since their scale up is relatively straightforward, though hollow fibers allow significantly higher cell densities to be obtained in smaller volume containers, though cell dissociation from the fibers can represent an additional effort in an industrial-scale process which can have associated costs. On the other hand, modifications in fixed and fluidized bed BR settings could allow the immobilization of a scaffold within the BR, thus allowing a differentiation process where myogenic cells could mature.

Under the assumption that adherent cell culture will be used in the proliferation stages of cultured meat process, MCs can allow cells to adhere and grow in suspension, whilst mimicking the native tissue environment. Various MCs are commercially available but nonetheless are not tailored for food applications, since they are mostly composed of inedible polymers. Edible MCs would allow the transfer of MC-cell complexes to an appropriate scaffold or, should the final product be a minced meat product, differentiation could occur on-site and MCs be incorporated in the final product, if the organoleptic properties of the meat constructs remains the same, or is enhanced. Importantly, different porosities and chemical modifications to MCs will assure adequate applicability in myogenic and adipogenic-committed cells, amongst a plethora of other factors, including substrate stiffness and topography.

These factors are also relevant when choosing the adequate scaffold, which functions as MCs to give cells an adequate surface to adhere to. Scaffold materials of interest include TVPs, alginate, silk and gelatin. Overall, scaffolds must be carefully tuned for the intended cell types, since different physical and mechanical cues can promote proliferation of muscle or fat cells in different manners. Thus, composite scaffolds and self-assembly of scaffolds with different cell types remains a challenge to be investigated. As alginate requires further functionalization with RGD peptides to adequately adhere mammalian cells, gelatin and silk-based scaffolds do not, though both protein solutions are extracted from animals and therefore could undermine the

original premise of cultured meat, which is to obtain meat products without animal-derived components other than cells.

In this manuscript, various case studies have been analyzed for their broad applicability in cultured meat research. Firstly, it was found that porous TVP scaffolds can be used for bovine SC proliferation and differentiation under co-culture settings with ECs and SMCs, as well as in monocultures (Ben-Arye *et al*., 2020). This study has identified IGF-1 and EGF as the most important factors in growth medium, IGF-1 having synergistic effects with the remaining proliferation medium, and both factors contributed to differentiation of SCs into myotubes. IGF-1 has important roles in skeletal muscle tissue development, as well as adipose tissue, however this article shows its relevance in bovine medium composition. Nonetheless, high concentrations (100 ng/ml) of GFs have high production costs, which need to be addressed for large-scale production of cultured meat, if achieving price-parity with conventional meat products is intended. Strategies to reduce GF costs include the use of conditioned medium, reduce GF purity or concentration in the medium, or produce GFs in alternative platforms that allow cost-effective scaling, such as in plants or bacteria.

The results from the expansion of bovine MSCs associated with MC systems in spinner flasks has shown that these cells could adhere and proliferate in the aforementioned conditions and maintain their three-lineage potential (Hanga *et al*., 2020). Despite the mounting application of this proof-of-concept, MSCs were cultured in plastic MCs which undermine the requirements for adequate application in cultured meat processes, since a dissociation step from MCs must take place. In addition, myogenic differentiation of MSCs has not been reported nor investigated, and therefore more research is required to validate the application of these multipotent cells in cultured meat production. Population doubling times of bovine MSCs were similar to those found in the literature.

Whilst investigating different SFM for application in bovine myoblasts, it was found that several commercially-available media could sustain bovine myoblast proliferation, though not as effectively as FBS-containing medium (Kolkmann *et al,* 2020). Among the various FBS-free medium tested, FBM and Essential 8 appeared the most promising, though not ideal. Interestingly, it was found that myoblast cultures benefited from partial medium exchanges, in lieu of complete medium replacement, and that myoblasts acquired an adipocyte-like phenotype when cultured with adipogenic supplements.

The fabrication of gelatin scaffolds using a novel technique comprising of immersion spinning under a rotary jet were analyzed and shown to produced gelatin fibers, where both bovine MSCs and rabbit SkMCs could adhere, proliferate and differentiate into striated muscle fibers, with a histological architecture akin to different native muscle tissues (MacQueen *et al*., 2019). In addition, rheological and texture parameters have shown that the fiber constructs had different stiffness as native muscle tissue, and similar to processed foods such as bacon.

When characterizing the impact of heme-proteins Hb and Mb addition to culture medium, it was found that both molecules impacted the color of bovine muscle constructs (Simsa *et al*., 2019). Nonetheless, Mb was the most relevant in bovine SC proliferation, where a concentration of 3 mg/mL allowed lower population doubling times (ranging 41.67 h) than Hb-containing medium at the same concentrations (roughly 43.28h), which is in the range of other SC doubling times reported in literature. It is therefore required to evaluate the need for addition of heme-proteins based on functionality, and Mb appears to be a regulator of SC proliferation, while Hb contributes more to color and taste of meat.

Concluding, this thesis has shown that cultured meat is a promising technique that could allow production of meat products without relying on intensive animal agriculture and resource exploitation. Nevertheless, a multidisciplinary approach needs to be carried out to guarantee the sustenance of the sector, and its application in large-scale production of meat products. Furthermore, these advancements will have tremendous impact in cost-efficiency of cell and tissue engineering since most knowledge is transferable for other mammalian cells, such as cost-effective culture medium and high cell density expansion. However, cellular agriculture remains an underfunded field which will require both public and private funding as well as a consortium of engineers, scientists and many other experts, to obtain a truly sustainable future food system without relying on intensive agricultural practices, to build animal-free animal products using biotechnology.

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