Cell Types for Cultured Meat Production: Current Challenges and Recent Advances

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Summary

Cultured meat, also known as cell-based or lab-grown meat, is made by placing cells that have the ability to self-renew and differentiate into muscle cells, in a nutrient-rich environment where they can grow and multiply, eventually forming muscle tissue that can be harvested and used for food. This process would allow meat to be produced without the need for intensive animal farming, which has the potential to help feed the world's growing population sustainably, efficiently, and safely. However, it's important to note that although early versions of cultured meat were shown to the public about a decade ago, the techniques used to make it cannot be scaled up to produce larger quantities. To successfully produce cultured meat on a large scale, an efficient production process is needed. This process depends on the optimal performance of cells involved in the production, including high proliferation rates, short cell doubling time, rapid and effective differentiation into muscle cells, and consistent behavior over multiple passages.

This review discusses the potential of four cell types - satellite cells (SCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) - in making cultured meat. The progress that has been made so far and the challenges that researchers are facing when trying to use these cells to create meat on a larger scale will be presented. Furthermore, this article suggests areas where more research and development is needed to make the process of cultured meat production more efficient and scalable. The information in the article is based on scientific studies and patents filed by companies that are working on developing cultured meat.

The review found that the most widely researched and popular type of cell for use in cultured meat production are SCs. However, one of the challenges with using SCs is retaining their proliferation and differentiation properties, but there are some advances in regulating cellular pathways that can help with this. Nevertheless, more research is needed to find cost-

effective ways of isolating highly purified SCs for successful commercialization of cultured meat. One shared challenge among the remaining cell types: MSCs, iPSCs, and ESCs, in cultured meat production is to differentiate them into muscle cells efficiently. Researchers have not yet developed protocols for forming myotubes using MSCs, and while iPSCs can generate myotubes, they are not fully developed. As for ESCs, there are no established protocols for making farm animal ESCs turn into muscle cells. To overcome this challenge, more research is needed to better understand the molecular mechanisms that control cell differentiation into muscle cells. While iPSCs and ESCs are still in the early stages of development, their potential is attracting more attention. However, there is limited information available on how to steer these cells to a myogenic fate due to a lack of public data, which can slow down the progress of this field. It is important for future research to carefully consider the pros and cons of each cell type, as well as any safety concerns related to their use.

Abstract

The production of cultured meat, also known as cultivated meat or clean meat, involves integrating biomaterials and cells with self-renewal and myogenic differentiation potential to engineer meat in vitro for consumption. It is hoped that this innovative approach would eliminate the need for intensive animal farming and slaughtering to produce animal proteins, while being more sustainable, efficient and safe. The successful large-scale production of cultured meat requires the development of an efficient production process, which partly depends on the optimal performance of cells involved in the process, including high proliferation rates, short cell doubling time, rapid and effective differentiation into muscle cells, and consistent behavior over multiple passages. This review evaluates the potential of satellite cells (SCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) in cultured meat production and discusses the current advancements and technical challenges related to their application. In addition, this review highlights areas for further research and development to improve the efficiency and scalability of the cultured meat production process through a review of scientific articles and relevant patents filed by cultured meat companies.

Keywords: cultured meat, meat substitute, cell sourcing, pluripotent stem cells, primary cells

1. Introduction

Cultured meat, a concept product made of cultivated animal cells as an alternative to conventional meat, has been gaining more attention in recent years due to the potential benefits it could provide. These claimed benefits include more efficient animal protein production, elimination of intensive farming practices, and reduced environmental impact through decreased fresh water and land use, and lower greenhouse gas emissions[1–3]. However, it is

important to note that cultured meat remains in the realm of research and development, and there is currently no established method for its large-scale, cost-effective production.

The initial step in the production of cultured meat is the acquisition of cells, which can be obtained from an animal tissue biopsy (i.e., primary cells) or from established cell lines, these cells must possess myogenic differentiation capacity to form myotubes, the building blocks of meat[4]. One of the main hurdles in the commercialization of cultured meat is the need to achieve large-scale and efficient production of cell biomass. This is crucial to ensure that the price of cultured meat is competitive and aligns with consumer expectations. To accomplish this, specific requirements must be met by the used cell types, such as accessibility, high proliferation rates, rapid and effective differentiation into muscle cells, and maintenance of genomic stability during the cell culture process. However, the four main types of cells used nowadays, namely satellite cells (SCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs), all have their limitations. For example, SCs have been the most commonly employed cell type for cultured meat research due to their robust myogenic differentiation, but many studies demonstrated that long-term in vitro culture of porcine, bovine and turkey SCs is limited to 20-30 population doublings and the expanded cells lose myogenic differentiation capacity[5–8]. On the other hand, iPSCs have the potential to overcome these limitations as they are capable of expanding indefinitely in theory, and there are various developed protocols to induce myogenesis[9–12]. However, the main disadvantage of iPSCs is that the differentiation process is often incomplete, resulting in difficulties in obtaining mature myotubes[9–12]. Additionally, the generation and cultivation of any of these four cell types are still associated with high costs, which pose a significant obstacle in terms of commercialization. Therefore, to date, none of the cell types is able to fulfill the requirements for cultured meat production.

The prototypes of cultured meat products were demonstrated to the public as long as 10 years ago, but the methods used to manufacture them cannot be applied at a bigger scale. For instance, the first cultured beef burger weighed 85 g and was comprised of 10,000 muscle strands derived from SCs[13] However, it is not known how many biopsies were performed to obtain starting cell population, how many doublings they went through, did they have a normal karyotype nor how well were they differentiated. Thus, the thorough characterization is required for understanding the given cell type's limitations before the upscaled process can be designed.

This work reviews scientific and patent literature available on characterization and application of SCs, MSCs, ESCs, and iPSCs in the field of cultured meat. The amount and depth of research published on these cells in cultured meat production vary. Therefore, this study evaluates the current advancements and limitations of each specific cell type and highlights areas for further research and development.

2. Cells

2.1 Satellite Cells

SCs are resident muscle stem cells that are responsible for muscle repair and regeneration[14]. In vivo they are located between the sarcolemma and the basal lamina of muscle fibers[14]. The utilization of SCs in cultured meat production is favored due to their ability to differentiate easily and exhibit muscle-specific properties. However, the challenges of limited proliferative abilities and the tendency to lose differentiation capacity during prolonged in vitro culture must be overcome in order to progress toward a scaled up process[5–8]. This chapter will highlight recent advancements in optimizing SCs performance, including the selection of appropriate donors, implementation of purification protocols, and preservation of their functional properties.

Given the prevalent use of primary SCs in cultured meat research, it is important to carefully select appropriate donors as SCs harvested from various animal species, breeds, ages, sexes, and muscle locations may have variations in their potential to be expanded and differentiated[15,16]. The impact of donor age on SC differentiation was discussed in a review, where the authors compared this effect in different species[17]. A decrease of myogenic differentiation capacity of SCs was observed in aging pigs and cows, but not in horses nor sheep. Melzener and colleagues highlighted the significance of breed-specific differences in bovine SCs through their comparative study. The results showed that Belgian blue and Limousin cattle exhibited significantly greater retention of differentiation capacity during prolonged passaging as compared to the other three breeds (Holstein Friesian, Galloway, and Simmental)[16]. These suggest that further research specific to species and breeds is required to examine the impact of SCs obtained from varying ages, sexes, and biopsy sites on their proliferation and myogenic differentiation.

In skeletal muscle, there are several cell types besides SCs, including fibro-adipogenic progenitors (FAPs), endothelial cells, mesenchymal cells, and others that can contaminate SC culture upon isolation[18]. Inadequate purification of bovine SCs can result in the overgrowth of FAPs replacing the myogenic cells, which was studied in detail in research by Messmer et al[18]. The research highlighted the importance of good purification of SC culture. The commonly used purification methods are magnetic-activated cell sorting (MACS), fluorescence-activated cell sorting (FACS), and pre-plating[6,7,19]. Messmer et al. showed that the use of FACS to sort bovine SCs based on the ITGA7+/ITGA5- strategy decreased contamination from FAPs, obtaining a more purified SCs populations compared to a sorting strategy based on CD29+/CD56+ used by Ding et al[6,7]. Building on the work of Ding et al., Choi et al. simplified and scaled up the process of purifying porcine SCs by using a single CD29 antibody in MACS and confirmed the efficiency for the enrichment of SCs[6,19]. However, FACS relies on expensive

equipment and antibodies, which can make cultured meat production process even more expensive. Li et al. claimed that the isolation method involving only pronase and dispase II digestion followed by 0.5 hour pre-plating has better expansion and differentiation efficacy than the method proposed by Ding et al, therefore avoiding the need for more expensive purification techniques[20]. While the search for optimized purification protocols for obtaining highly purified SCs continues, it's important that further research should also examine the relationship between the degree of purification and cell performance in order to determine the optimal level of purity necessary for cost-effective commercial-scale production. Additionally, conducting parallel studies comparing the efficiency and cost of various purification methods will provide valuable insights for selecting the most suitable method.

Prolonged cultivation of SCs is known to result in the loss of their proliferation and differentiation potential[5,6]. To overcome this challenge, researchers have attempted to manipulate various signaling pathways involved in cell growth, differentiation, survival, etc.[14,21]. In recent advances, specific compounds have been incorporated into the culture media to target signaling pathways such as YAP, MAPK, Wnt, and phosphatidylinositol-3-kinase (PI3K) for culturing farm animal-derived SCs[5,6,22–24]. For instance, research by Liu et al. found activating the YAP pathway with lysophosphatidic acid (LPA) could help maintain self-renewal and enhance the differentiation capacity under the high-density culture of porcine SCs[22]. Short-term inhibition of p38 MAPK signaling by SB203580 was shown to preserve expression of Pax7 in bovine and porcine SCs[5,7]. In addition, Park et al. observed that the proliferation of porcine SCs could be promoted while maintaining the expression of Pax7 by inhibiting the Wnt/β-catenin signaling pathway with CHIR99021[25]. Cytokines are signaling molecules and can regulate various physiological processes in vivo [26]. A study by Lei et al. identified an effective four-cytokine (long chain human IGF-1, platelet derived growth factor BB, basic fibroblast growth factor, and epidermal growth factor) combination for porcine SCs

expansion[26]. But further research is needed to assess the effectiveness of this strategy in long-term culture. To date, the compounds mentioned earlier are not yet approved for consumption, therefore, the use of food-grade compounds or food-grade alternatives would be preferred in development of food-safe culture media to meet regulatory requirements and promote consumer acceptance. As such, there are also studies on the effects of adding food-grade compounds in culture media. For example, supplementing the culture medium with Vitamin C or myoglobin showed improved proliferation and preserved myogenic properties in porcine and bovine SCs, respectively[27,28]. Natural flavonoids such as luteolin, naringenin, and quercetin were shown to promote porcine myoblast differentiation and maturation of myotubes in recent studies[24,29,30]. Advancements were made in maintaining stemness in SCs through various methods, but further investigation to evaluate the effects of certain compounds for long-term culture is necessary. Despite this, these compounds or their food-grade substitutes show potential for improving the performance of cell culture media or for developing new media.

Replacing fetal bovine serum (FBS) in culture media is another essential research goal in cultured meat development due to its animal origin, high cost and batch-to-batch variations. Two companies, Biftek and Joes Future Food, have developed FBS-free and chemically defined media for the expansion and differentiation of SCs respectively[31,32]. Biftek's patent describes the use of microbiota-derived postbiotics as an alternative to FBS in stimulating cell proliferation and growth, but the efficacy of the medium has not been reported[31]. Joes Future Food developed a chemically defined medium to induce myogenic differentiation of SCs by supplementing the basal medium DMEM/F12 with 19 compounds (Tween, oleic acid, linoleic acid, and vitamin E, etc.)[32]. This medium demonstrated thicker and longer muscle fibers compared to the general differentiation medium with 2% horse serum. Mosa Meat holds two patents in the development of culture media for SCs expansion and differentiation and has published two relevant academic papers[33–36]. Although bovine SCs expanded robustly in the

developed serum-free proliferation medium for 6 passages, their growth rates were lower than the medium supplemented with FBS[34,36]. Thus, the proliferation medium still needs to be optimized for efficiency. Moreover, while the developed differentiation media is effective at supporting myotube formation, the level of myotube formation that was observed was insufficient or not significant enough[33,35]. Therefore, further work is still needed to optimize serum-free media to improve cell performance and enable long-term culture. Integrating compounds mentioned in the previous paragraph (such as SB203580, CHIR99021, Vitamin C, luteolin, and naringenin) into existing serum-free media to inhibit or activate specific signaling pathways (such as p38/MAPK, Wnt/β-catenin) might be helpful. Supplementary Table 1 summarizes the patents filed by cultured meat companies that utilize cells with limited proliferative capacity. The table includes information on the types of cells used and/or patented, the focus areas of the patents, whether the cells were induced to differentiate, and any relevant academic publications, etc.

In summary, while there has been considerable research on enhancing the performance of livestock SCs, certain aspects such as the selection of optimal donors for cultured meat production remain an understudied area. Further investigation is required since the relationship between donor characteristics and in vitro cellular performance is essential for improving the overall efficiency of the production process. Advances were made in purification methods to obtain purified SCs, but it is yet to be determined at which degree of purification is most optimal for large-scale production. Some progress was made in development of culture media additives to improve SC performance. It is intriguing to further add them into developed serum-free media and investigate if they will show comparable effects. Additionally, it remains to be seen how the results will translate to large bioreactors since all the research is conducted at the laboratory scale.

2.2 Mesenchymal Stem Cells

MSCs are multipotent stem cells that have the ability to differentiate into at least three mesodermal cell types: osteoblasts, adipocytes, and chondroblasts[37]. These cells can be isolated from multiple tissue sources, including bone marrow, adipose tissue, skeletal muscle tissue, umbilical cord, and others[38–41]. MSCs from various species, including human, mouse, porcine, bovine and chicken, have been shown to express muscle-specific genes (MyoD, Myogenin, MyoG, Myf5, etc.) or proteins (Desmin, actin, etc.) under specific culture conditions[38–40,42,43]. This made them a promising candidate for use in cultured meat production. The following part will focus on the progress and advancements in using MSCs for myogenic differentiation.

The proliferation and differentiation potential of MSCs may differ depending on the tissue source from which they are derived due to differences in their microenvironment and distinct epigenetic modifications[38,39,44]. For example, studies showed that adipose tissue-derived MSCs (AD-MSCs) exhibited superior division rates in human and mouse models, while skeletal muscle derived MSCs (SM-MSCs) displayed enhanced myogenic differentiation potential in mice[38,39,44]. However, none of the three studies demonstrated the formation of myotubes, the ultimate proof of myogenic differentiation. Therefore, even if a particular source of MSCs exhibits higher expression of myoblast markers, it may not be useful for cultured meat production if it fails to form myotubes.

Some studies examine the muscle-generating capabilities of MSCs from farm animals. The treatment with 5-Aza-2′-deoxycytidine (5-Aza) had been shown to activate the myogenic differentiation potential and demonstrated muscle-specific genes expression (e.g. MyoG, MyoD, Myf5, etc.) in MSCs derived from porcine, chicken, and bovine bone marrow[40,43]. However, 5-Aza is a toxic compound known to cause cell death in concentrations > 100µM and therefore presents a hazard when applied in food manufacturing. When co-culturing porcine AD-MSCs with porcine SCs, Milner and colleagues demonstrated enhanced myogenic capacity, though the percentage of AD-MSCs contributing to myogenesis still remained as low

as 0.81%[45]. To date, there is no efficient protocol that would induce robust myogenic differentiation of MSCs.

Studies have explored culture conditions aimed at enhancing myogenic differentiation of SM-MSCs, with a primary focus on human or mouse models. Nevertheless, the results obtained from these studies have the potential to be extended to MSCs of farm animals. For example, Mikšiūnas et al. found that applying a heat stimulus to human SM-MSCs stimulated differentiating to the myogenic direction, especially for CD56 (+) subpopulations, expressed significantly more myogenic differentiation markers MyoD1 and MyoG and induced more efficient formation of myotubes[46]. Research by Testa et al. claimed that the addition of human-derived serum and platelet-rich plasma to human SM-MSCs had remarkable impacts on their proliferation and myogenic differentiation, leading to promoted myotube formation[47]. However, different purification methods are employed in these two studies, and their definitions for SM-MSCs lack precision. Testa et al. utilized a simple purification method by selecting the CD56+/CD90+/CD45- cell population. On the other hand, Mikšiūnas et al. identified MSC-typical cell surface biomarkers (e.g. CD105+/CD90+/CD73+/CD45-) and showed differentiation capacity toward osteogenic, adipogenic, and chondrogenic lineages, which is more consistent with MSC characteristics. Both of the derived SM-MSCs are heterogeneous populations and probably consist of satellite and non-satellite cells. But the ratios of these cell types may vary depending on the level of purification. As such, a standardized purification protocol for gaining homogeneous SM-MSCs is necessary to prevent confusion of these cells with SCs and increase the reliability of the results. Nevertheless, it's intriguing to explore the effects of heat stimulation on farm animal MSCs' or SCs' myogenic differentiation following the results of Mikšiūnas. The application of platelet-rich plasma, though, is not suitable for cultured meat production. An alternative approach could be to investigate the effects of myoglobin and hemoglobin on the myogenic differentiation of

livestock MSCs based on the results of adding them to culture SCs in a study by Simsa et al[48]. Another approach, a culture under hypoxic conditions may enhance cell myogenic differentiation efficiency, as reduced oxygen exposure can alter gene expression and affect cell behavior. One study reported that hypoxic conditions led to an increase in hybrid myotube formation when human or porcine bone-marrow MSCs were co-cultured with mouse primary myoblasts compared to co-cultures performed under normoxic conditions[49,50]. However, further research is needed to assess the long-term effects of hypoxia on MSC cultures, particularly in MSCs derived from farm animals.[49,50]

The application of MSCs in cultured meat production remains in its early stages. Currently, there is no established protocol for inducing efficient myogenic differentiation of MSCs, and myotube formation cannot be achieved solely through their use. There is limited research on the use of farm animal MSCs, and it is not widely utilized by companies. While Steakholder foods integrated MSCs into bio-inks for bioprinting in a patent, they did not induce myogenic differentiation[51]. Achieving the utilization of MSCs in cultured meat production through their sole use remains ambiguous, and co-culture with SCs may be a more viable option for utilizing this cell type.

2.3 Embryonic Stem Cells

ESCs are derived from the inner cell mass (ICM) of a blastocyst. They are pluripotent stem cells and have the potential to divide indefinitely and differentiate into any cell type, which are favored characteristics for use in cultured meat production[52]. However, the use of livestock ESCs for cultured meat production is still in its early stages of development. Unlike mouse models, there are more challenges of using livestock ESCs as there is limited knowledge on the key molecules and the appropriate gene expression levels required for maintaining the naïve state of farm-animal ESCs[53]. Despite this, ongoing research and various attempts are being made to address these difficulties.

For example, a stable bovine ESC line was efficiently established recently which allows for robust and prolonged propagation for more than 70 passages by supplementing the culture medium with fibroblast growth factor 2 (FGF2) and a small-molecule Wnt inhibitor (IWR1)[54]. In the case of porcine ESCs, Haraguchi et al. generated porcine ES-like cells using inhibitors CH99021 and PD184352 without gene modification from the ICM of porcine embryos. Telugu et al. derived ESCs from porcine blastocysts via KLF4 and POU5F1 upregulation using a lentiviral vector[55]. Although both cell lines demonstrated features of pluripotency, they were unable to sustain self-renewal over a prolonged period. A study led by Zhang and colleagues provided an alternative approach for generation of pluripotent porcine cells[53]. They established four naïve-like ESC cell lines morphologically similar to mouse ESCs which could be rapidly propagated without change in morphology for 130 passages. This was achieved through a novel method combining the reprogramming of embryonic fibroblasts by transfection of the 4 OSKM factors with a tetracycline-inducible vector and implementing somatic cell nuclear transfer into a mature oocyte. The resulting embryo was grown to the blastocyst stage and the inner cell mass was isolated for further culture and generation of naïve-like ESCs. Cells were cultured in a medium containing LIF and small molecular inhibitors CHIR99021, PD0325901, and SB431542 to improve efficiency[53]. The resulting porcine ESCs exhibited key features of ESCs, such as pluripotency markers, long-term self-renewal, and could spontaneously differentiate into cells expressing markers of the 3 somatic germ layers. This represents a significant improvement over previous studies and the established porcine ESC lines are promising for cultured meat production. Despite these advancements, producing authentic ESCs remains a challenge, and ongoing optimization of the culture conditions for farm animal-derived ESCs is still required. In addition, developing viral vector-free methods to obtain stable ESC lines is important to avoid potential risks associated with the use of viral vectors in food production.

The studies on inducing myogenic differentiation in ESCs have largely been focused on human and mouse cells for potential applications in regenerative medicine and drug screening, but the findings have implications for livestock ESCs as well. For example, BAF60C and JMJD3 genes are found to play a crucial role in the epigenetic regulation of human ESCs transition toward the myogenic lineage[56,57]. The expression of these genes can be further explored in ESCs from livestock species. Shelton et al. developed a method for differentiating mouse and human ESCs into highly enriched skeletal muscle lineage using GSK3 inhibitor CHIR99021, combined with the addition of FGF2 and N2 supplements[58]. This method is viral- and transgenic-free and holds promise for applying and validating the effects on farm animal ESCs. Research also has explored alternative methods such as the application of transient RNA-based protein expression in inducing myogenic differentiation. For instance, Bem et al. found that transient overexpression of miR145 and miR181 initiated myogenic differentiation and enhanced myotubes formation in human ESCs[59]. Another study conducted by Akiyama et al. observed an efficient and rapid myogenic differentiation of human ESCs (greater than 60% within 4 days) through the use of synthetic mRNAs encoding JMJD3c and MYOD1[57]. The same group later showed an even more efficient (>80%) method to induce myogenic differentiation of human ESCs by introducing mRNA encoding MYOD1 together with siRNA-mediated knockdown of OCT4[60]. The use of transient expression offers the advantage of reducing the risk of genomic integration and mutagenesis, making it a more suitable approach for the production of cultured meat. However, research in inducing the myogenic differentiation of farm animal ESCs is limited. Further research is needed to investigate the applicability of techniques used for human and mouse ESCs to farm animal ESCs and determine their effectiveness.

While ESCs hold great potential for the production of cultured meat, relatively few companies have utilized them in their processes. Super Meat holds a patent for using chicken

ESCs and testing muscle cell-related gene expression. However, they did not specify the differentiation methods used in their process[61]. On the other hand, Steakholder Foods holds patents for the harvest and propagation of bovine ESCs, but they have yet to reach the stage of differentiation[62,63]. Supplementary Table 2 summarizes the patents filed by cultured meat companies that employ pluripotent stem cells.

In summary, the utilization of ESCs for cultured meat production is not widespread at present. One of the key challenges is the lack of efficient protocols for inducing myogenic differentiation in ESCs derived from livestock. Further studies are required to validate existing protocols and to explore new methods for optimizing the use of ESCs in cultured meat production.

2.4 Induced Pluripotent Stem Cells

iPSCs, like ESCs, are also pluripotent stem cells, they can theoretically divide indefinitely and differentiate into various cell types, including muscle cells. The key difference is that iPSCs are derived from adult somatic cells, which are reprogrammed to an embryonic-like state by overexpressing the transcription factors OCT4, SOX2, KLF4, and c-MYC (referred to as OSKM)[55,64,65]. Despite the potential advantages of using iPSCs in cultured meat production, challenges persist in improving reprogramming efficiency, maintaining self-renewal and pluripotency, and inducing myogenic differentiation. The following will present recent advancements in addressing these challenges.

Recently, a lot of progress was made in the establishment of iPSCs from livestock. The successful generation of bovine iPSCs (biPSCs) has been reported using PiggyBac transposon systems or the sleeping beauty and piggyBac transposon systems[64,65]. Additionally, single-cell derived biPSCs with high cloning efficiency have been obtained[66]. As for porcine iPSCs (piPSCs), their successful generation has been made from porcine fibroblasts and MSCs using various protocols by utilizing viral vectors[55,67,68]. However, a persistent challenge is a low

reprogramming efficiency. Recently, sertoli cells have been proposed as a novel cell source for generating piPSCs through retroviral vector-mediated transfer of the OSKM transcription factors[69]. While a higher reprogramming efficiency was observed, it remains low at around 30%[68,69]. Further investigation is required to improve the reprogramming efficiency and also fully understand the underlying mechanism of the improvement, in order to facilitate the widespread application in the future. In addition, it's crucial to explore alternative methods for reprogramming that do not rely on the use of viral vectors to ensure consumer safety.

The challenge of maintaining the self-renewal and pluripotency of iPSCs is a crucial factor in their effective scale-up production. Recent studies have identified potential solutions to this challenge. For example, Zhang et al. found that the knockdown of AXIN2, the key component of WNT signaling, maintained the pluripotency and viability of piPSCs by enhancing the expression of cell cycle genes (such as CCND1) and reducing the expression of genes related to cell differentiation and apoptosis (such as BAX, CASP3, and CASP9) [70]. Wu et al. identified METTL3 as a key regulator of pluripotency and facilitated piPSCs self-renewal by using lentiviral vectors to express it in the cells[71,72]. Additionally, the study by Chakritbudsabong et al. showed that the addition of Lin28 to OSKM transcriptions factors helped maintain self-renewal and pluripotency of piPSCs until passage 40 with 100% efficacy. However, the reprogramming efficiency was 50% lower when compared to the OSKM transcription factor system[72]. It is necessary to investigate more simple and effective methods to improve reprogramming efficiency while maintaining the self-renewal and pluripotency of iPSCs, particularly for the production of cultured meat.

Inducing myogenic differentiation is the next step after generating iPSCs. Current methods include using small molecules, proteins, growth factors, etc., which vary depending on the source of iPSCs and the intended application[11]. Unfortunately, the majority of the studies still are focused on human or mouse models[11,12]. One study, however, performed myogenic

differentiation of piPSCs and demonstrated enhanced MYOG expression and sarcomere formation[9]. They applied a combination of GSK3B inhibitor CHIR99021, 5-aza-cytidine, and ectopical expression MYOD1[9]. However, the myotubes generated from piPSCs were in an early developmental stage and were considered immature. Further research is needed to determine the impacts of this inducing method on the pluripotency of piPSCs and to investigate its applicability to iPSCs of other species. There are no well-established myogenic differentiation protocols available for producing mature myotubes from farm animal iPSCs currently. Therefore, additional research is needed to either validate existing protocols that have been used for human and mouse models or develop new and efficient methods specifically for farm animal iPSCs.

With regards to the application of iPSCs in the cultured meat industry, there are at least three companies (Meatable, Roslin Technologies, and Steakholder Foods) that have claimed to be using or developing iPSCs, as stated on their website or in news[73,74]. In spite of this, there is a lack of publicly available patents or scientific literature that detail how these companies maintain self-renewal, and pluripotency, and induce myogenic differentiation of iPSCs.

In conclusion, iPSCs have great potential for use in cultured meat production. However, controlling this cell type is challenging and requires addressing technological difficulties. Furthermore, we should be careful of the possible negative effects of the lack of transparency in the technologies used by cultured meat companies to control and culture iPSCs as it affects the safety and quality considerations of the resulting cultured meat products. Additionally, without access to this information, it becomes difficult for the scientific community to evaluate and build upon these efforts, ultimately hindering progress in the field.

3. Conclusions

The use of these four cell types, SCs, MSCs, ESCs, and iPSCs in the context of cultured meat production is associated with a range of technical challenges. Among them, SCs are the

most extensively studied cell type in this area. One of the critical challenges for them is maintaining their stemness, which can be addressed by regulating the cellular pathways related to self-renewal and myogenic differentiation. However, a deeper understanding of these complex pathways is required to fully optimize their use. Additionally, the selection of appropriate donors and the development of cost-effective methods for isolating highly purified SCs are also crucial for the successful commercialization of cultured meat.

Although MSCs are not as muscle-specific as SCs, they have the advantage of being easy to obtain and culture, making them a valuable alternative for some applications in cultured meat production. ESCs and iPSCs are pluripotent cells and can differentiate into any cell type, which makes them a promising cell source for cultured meat. Successful generation of bovine and porcine iPSC and ESC lines has been achieved, but authentic ESC production remains a challenge, and reprogramming efficiency of iPSCs needs to be improved. Moreover, developing viral vector-free methods for iPSC reprogramming and maintaining self-renewal and pluripotency of iPSCs and ESCs to ensure consumer safety is crucial.

Another challenge faced by MSCs, iPSCs, and ESCs in cultured meat production is the lack of optimized and efficient myogenic differentiation protocols for farm animals. Specifically, there are currently no protocols available for myotube formation using MSCs, and while myotubes can be generated using iPSCs, they are often found to be immature. As for ESCs, myogenic differentiation protocols for farm animal ESCs have not been established. To address this challenge, further research is needed to understand the molecular mechanisms regulating the differentiation of these cells into muscle cells. Additionally, efficient and standardized protocols for their isolation and maintenance are necessary before they can be considered for commercial-scale production of cultured meat.

At present, SCs continue to be the most widely researched and popular cell type for use in cultured meat production. While the potential of MSCs in this area is being explored, the

results so far have not been highly promising for their standalone use. A more viable option might be co-culture with cell types that have better myogenic differentiation potential, such as SCs, until more efficient myogenic differentiation protocols are developed. Though iPSCs and ESCs are still in the early stages of development in the field, their utilization is attracting increasing attention. However, a lack of publicly available data or proprietary patents held by companies on controlling and culturing those cells can pose a risk to the rational and critical advancement of this field. Further research should take into account the advantages and disadvantages of each cell type, and their relevant efficiency and safety concerns.

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Supplementary Table 1

Summary of patents by cultured meat companies utilizing cells with limited proliferative capacity

Company Name	Patent Title	Patent Number	Patent status	Focus Area	Cell Types Used and/or Patented	Source Species	Differentiation Category	Scientific Evidence	Academic Publication	Cell Types Used in Academic Publication
Mosa Meat	Serum-Free Medium for Culturing a Bovine Progenitor Cell	WO 2021/158103 A1	Pending	Culture Medium	SCs	Bovine	NI	Proliferation Rates Analysis	37	Bovine SCs
					Stromal vascular fraction cells	Bovine	NI	Proliferation Rates Analysis		
	Hydrogels for cultured meat production	WO 2021/158105 A1	Pending	Scaffolding	SCs	Bovine	Myogenic Differentiation	1. Light Photomicrographs for Cellular Alignment; 2. IF Staining for Myosin, Filamentous-actin, and Desmin	N/A	N/A
	Serum-Free Medium for Differentiation of a Progenitor Cell	WO 2022/114955 A1	Pending	Culture Medium	SCs	Bovine	Myogenic Differentiation	1. RNAseq Analysis of LPAR1, LPAR3, OXTR, GCGR Genes; 2. Light Photomicrographs for Myocytes Morphology	34	Bovine SCs
Biftek	Microbiota-Derived Postbiotics: Alternative Supplement to Fetal Bovine Serum for Cultured Meat	US 2022/0098546 A1	Pending	Culture Medium	SCs	Bovine	Myogenic Differentiation	1. IF Staining for Pax 3/7 and MyoD; 2. Cell Cytotoxicity Assay for Cell Viability	N/A	N/A
Joes Future Food	Chemically Defined Medium for Differentiation of Muscle Stem Cells in Vitro	US 2022/0204923 A1	Pending	Culture Medium	SCs	Porcine	Myogenic differentiation	1. IF Staining for MyHC; 2. qPCR for MyoG, MyHC, and CAV-3 Expression; 3. Western Blotting for MyHC Protein; 4. Cell Differentiation Rates Calculation	N/A	N/A
Orbillion Bio	Using Organoids And/or Spheroids to Cultivate Meat	WO 2022/086926 A1	Pending	Bioprocess Design	SCs	Sheep	NI	1. Spheroids Diameters, Areas, and Roundness Measurement; 2. Spheroids Metabolic Activity Analysis; 3. Cells Viability Analysis	N/A	N/A
Steakholder Foods	Cultured Edible Meat Fabrication Using Bio-Printing	WO 2021/007359 A1	Pending	General Process	MSCs	Bovine	NI	1. Light Photomicrographs for Continued Cell Growth; 2. Live/Dead Fluorescent Staining for Cell Viability	N/A	N/A
					Myoblasts	Bovine	Unknown	Light Photomicrographs for Cell Distribution		
Aleph Farms	3d-Printable Protein-Enriched Scaffolds	WO 2022/162662 A1	Pending	Scaffolding	SCs	Bovine	Myogenic Differentiation	1. Live/Dead Assay for Cell Viability; 2. Cell Seeding Efficiency Analysis; 3. Alamar Blue Assay for Cell Metabolic Activity; 4. Dil Staining for Scaffold Coverage Assessment; 5. IF Staining for Desmin and Myogenin; 6.qPCR Analysis of MyoD and Myogenin	75	Bovine SCs

Supplementary Table 1 *Cont.*

Company Name	Patent Title	Patent Number	Patent status	Focus Area	Cell Types Used and/or Patented	Source Species	Differentiation Category	Scientific Evidence	Academic Publication	Cell Types Used in Academic Publication
Aleph Farms	Cultured Meat Compositions	WO 2019/016795 A1	Pending	Scaffolding	SCs	Bovine	Myogenic Differentiation	1. IF Staining for Myogenin; 2. Fusion Index Measurement	76	Bovine SCs
					Smooth Muscle Cells (SMC)	Bovine	Angiogenetic Differentiation	IF Staining for CD31		Bovine SMCs
					Endothelial Cells	Bovine	NI	IF Staining for Dil		Bovine ECs
					Fibroblasts	Bovine	NI	IF Staining for DiD		
	High Quality Cultured Meat, Compositions and Methods for Producing Same	WO 2020/100143 A1	Pending	Culture Medium, Nutrition	Embryonic Fibroblasts	Bovine	NI	1. Compounds (Beta-carotene, Iron, DHA, Elaidic Acid, Cvanocobalamin, Zinc, Lycopene, Canthaxanthin) Uptake from the Medium into Cells Analysis; 2. Accumulation of Cvanocobalamin Analysis	N/A	N/A
					Embryonic Myoblasts	Bovine	NI	Compounds (e.g. Zinc, Lycopene, and Canthaxanthin) Uptake from the Medium into Cells Analysis		
					Fibroblasts	Bovine	NI	Ferrous Bisglycinate Accumulation within Cells Analysis		
Upside Foods	Generation of Cell-Based Products for Consumption That Comprise Proteins From Exotic, Endangered, and Extinct Species	WO 2022/216742 A1	Pending	Genetic Engineering	SCs	Chicken	Myogenic Differentiation	Not Publicly Available	N/A	N/A
					SCs	American Alligator		Not Publicly Available		
					Unknown	Chicken	NI	1. qPCR for Collagen1A1 and Collagen1A2 Expression; 2. Staining for Collagen1A1 and Collagen1A2; 3. qPCR for MyH13 Expression; 4. Staining for MyH13		
	Apparatuses and Methods for Preparing a Comestible Meat Product	WO 2020/243324 A1	Pending	Bioprocess Design	Fibroblasts	Chicken	NI	Cell Retention Measurement	N/A	N/A
					Myoblasts	Chicken	NI			
	Compositions and Methods for Increasing the Efficiency of Cell Cultures Used for Food Production	US 2022/0411824 A1	Pending	Genetic Engineering	Fibroblasts	Duck	NI	Ammonia Levels Quantification	N/A	N/A
					Myoblasts	Duck				
	Comestible Cell-Based Meat Products Comprising Dry Cell Powder and Methods of Making Such Products	WO 2022/261647 A1	Pending	Bioprocess Design	Unknown	Chicken	Unknown	Hardness and Adhesiveness Measurement of Cell and Cell Powder Mixture	N/A	N/A

Supplementary Table 1 *Cont.*

Company Name	Patent Title	Patent Number	Patent status	Focus Area	Cell Types Used and/or Patented	Source Species	Differentiation Category	Scientific Evidence	Academic Publication	Cell Types Used in Academic Publication
Upside Foods	Systems and Methods for Cultivating Tissue on Porous Substrates	WO 2022/140185 A1	Pending	Bioprocess Design	Fibroblasts	Chicken	Unknown	Cell Retention Analysis	N/A	N/A
	Nutrient Media for the Production of Slaughter-Free Meat	WO 2021/248141 A1	Pending	Culture Medium	*Gallus* Cells	Chicken	NI	1. Cell Number Counting; 2.Cellular Viability Analysis	N/A	N/A
	Generation of Cell-Based Products for Human Consumption	WO 2022/232322 A1	Pending	Genetic Engineering	Embryonic fibroblasts	Chicken	Adipogenic Differentiation	1. Oil Red O Staining for Lipid Droplets Assessment; 2. Light Photomicrographs for Lipids Formation	N/A	N/A
					Embryonic Myoblastss	Chicken	Adipogenic Differentiation	Light Photomicrographs for Morphology and Lipids Formation		
					Embryonic fibroblasts	Chicken	Hepatic differentiation	1. qPCR for HNF4alpha, C/EBPalpha, and CYP3A4 Expression; 2. Light Photomicrographs for Cell Morphology; 3. Metabolites Analysis; 4. Fatty Acids Compositions Analysis		
Mission Barns	Scalable Bioreactor Systems and Related Methods of Use	WO 2021/207293 A1	Pending	Bioprocess Design	Fibroblasts	Duck	NI	Fluorescence Micrographs for Cell Distribution	N/A	N/A
					Adipocytes	Porcine	NI	Cell Density Measurment		
MyoWorks	A Scaffolding for Cultivated Meat and a Process for Making the Same	WO 2022/208525 A1	Pending	Scaffolding	Embryonic fibroblasts	Chicken	NI	MTT Assay for Cell Adhesion Efficiency Analysis	N/A	N/A
Integriculture Inc	Animal Cell Growth Promoter, Culture Medium for Animal Cell Culture, and Animal Cell Culture Apparatus	EP 3878941 A1	Pending	Culture Medium	Stomach, Muscle, Heart, Liver, Intestine, Brain, Bursa and Bone-derived cells	Chicken	NI	Cell Number Counting	N/A	N/A

NI - Not Induced

Supplementary Table 1 summarizes the patents filed by cultured meat companies that utilize cells with limited proliferative capacity. All of the patents included in the table are currently pending. The majority of these patents focus on culture medium, scaffolding, and bioprocess design. In most cases, the patents specify the cell types utilized in the process, with a preference for SCs and fibroblasts. While most of these patents provide scientific evidence to support their claims, the available evidence is not always comprehensive. Furthermore, there is a lack of relevant academic publications to support these patents.

Supplementary Table 2

Summary of patents by cultured meat companies utilizing pluripotent stem cells

Company Name	Patent Title	Patent Number	Patent status	Focus Area	Cell Types Used and/or Patented	Source Species	Differentiation Category	Scientific Evidence	Academic Publication	Cell Types Used in Academic Publication
Steakholder Foods	Harvesting Bovine Embryonic Inner Cell Mass Cells	WO 2022/154780 A1	Pending	Cell Harvesting	ESCs	Bovine	NI	Not Publicly Available	N/A	N/A
	Bovine Umbilical Cord Stem Cells as Feeder Layer for Embryonic Stem Cell Propagation	WO 2022/155377 A1	Pending	Cell Propagating	ESCs	Bovine	NI	Not Publicly Available	N/A	N/A
Super Meat	Pluripotent Stem Cell Aggregates and Microtissues Obtained Therefrom for the Cultured Meat Industry	WO 2022/149142 A2	Pending	Bioprocess Design	ESCs	Chicken	Myogenic Differentiation	1. Light Photomicrographs for Rhythmic Contractions; 2. MyHC, Pax6, and Phalloidin Staining	N/A	N/A
					ESCs	Chicken	Adipogenic Differentiation	IF Staining for Bodipy		
	Cultured Meat-Containing Hybrid Food	WO 2018/189738 A1	Pending	Nutrition	Hepatocytes	Not indicated specifically	NI	Cholesterol, Fat and Saturated Fat Content Measurement	N/A	N/A
					ESCs	Chicken	Adipogenic Differentiation			
					ESCs	Chicken	Erythroblasts Differentiation			
Upside Foods	Method for Scalable Skeletal Muscle Lineage Specification and Cultivation	WO 2015/066377 A1	Active	Genetic Engineering, Culture Medium	iPSCs	Porcine	Myogenic Differentiation	1. Western Blot of MyoD, Myf5, Myogenin and β-catenin; 2. IF Staining for Skeletal Myocyte Cell Surface Marker, NCAM	9	porcine iPSC
Aleph Farms	Cultivation Systems and Methods for Large-Scale Production of Cultured Food	WO 2020/222239 A1	Pending	Bioprocess Design	iPSCs, ESCs	Bovine	Unknown	RT-PCR Analysis of Pax 7 and Collagen type 1	N/A	N/A
	Edible Protein Products	WO 2022/097140 A1	Pending	Scaffolding	Pluripotent stem cells	Bovine	Myogenic and Fibroblastic Differentiation	qPCR Analysis of MyHC3 and COLIII	N/A	N/A
					Embryonic Fibroblasts	Bovine	Adipogenic Differentiation	Light Photomicrographs for Adipocyte Morphology		

NI-Not Induced

Supplementary Table 2 summarizes the patents filed by cultured meat companies that employ pluripotent stem cells. A small number of patents have utilized these types of cells, with ESCs being more commonly used than iPSCs. There is little to no information mentioned on how myogenic differentiation was performed in these patents. It suggests that ESCs have not yet been optimized for use in the cultured meat industry, further research and development are necessary for their successful implementation.