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MINI REVIEW

Sustainable Cell Sources for Cultivated Meat

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ABSTRACT

Cultivated meat (clean meat) is an emerging yet fast growing research field and industry with a great potential to overcome the limitations of traditional cattle meat production. Cultivated meat leverages the technologies of cell biology and tissue engineering, culturing multiple types of cells and assembling them into a tissue structure construct mimicking the muscle tissues of livestock animals. A sustainable cell source is the first and the utmost important component of cultivated meat technology. In this mini review, cell sources for the main cell types in cultivated meat (muscle cells and fat cells) are described. Stem cells with self-renewal and differentiation potential are the most prominent candidates. Progenitor stem cells from muscle tissues, mesenchymal stem cells isolated from many other tissues and induced Pluripotent Stem Cells (iPSCs) created from terminally differentiated cells have been used as cell sources for cultivated meat. To become a sustainable cell source, which can generate high quantity (extensive *in vitro* expansion) and high quality (stemness) cells for the making of cultivated meat, these cells still face the challenges and limitation intrinsically associated with *in vitro* culturing. The efforts and strategies to circumvent such limitations are also discussed.

Introduction

Cultivated meat is synonymous with cell-based meat, clean meat, textured meat or *in-vitro* meat, is one type of cellular agriculture. Due to the environmental and animal welfare concerns, along with continued demands for more protein based food by the increase of the global population, the need for the development of alternatives to the conventional livestock farming has become inevitable [1]. Cultivated meat employs the conventional cell culture techniques and tissue engineering methodologies to culture animal cells *in vitro* to form edible structures resembling conventional animal meat cellularly and organoleptically. In 2002, two different organizations made the first attempts at cultivated meat and showed the viability and sustainability of cultivated meat as a food source for the future [2]. One decade later, the first world's cultured burger, manufactured from cultured cells, was presented by Professor Mark J. Post. This was certainly one of the milestones in the cultivated meat field [3,4]. In recent years, cellular agriculture has attracted tremendous attentions from both academia and industry, the field of developing cultivated meat has been making progresses even leaps towards a unique research interest and a sustainable industry. Many cultivated meat companies all over the world have emerged since 2016. Moreover, the successful commercialization of lab grown chicken nuggets in Singapore in 2020 promised an even faster paced development of cultivated meat in the near future.

The technologies and biomaterials developed for tissue engineering are used to carry out the process of producing cultivated meat which is mainly composed of three primary technical foci, sustainable cell sources, scaffold biomaterials and bioprocessing. In this mini review, recent advancement, and challenges in developing sustainable cell sources will be reviewed.

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Meats for human consumption are mostly animal muscle tissues, which comprised of muscle fibers (average about 90%), fat, connective tissue and blood (10%) [5,6]. In the muscle tissues, skeletal myocytes, satellite cells, adipocytes, fibroblasts, endothelial cells and hematopoietic cells are the resident cells. Due to the predominance of muscle and fat tissue in meat, cultured muscle cells and fat cells become the building blocks for cultivated meat [7].

The first step of a common protocol to fabricate cultivated meat is to obtain starter cells from an animal by performing a biopsy [8]. Starter cells must be able to self-renew and differentiate into mature cell types that form the meat. Stem cells are of interest and promising candidates to be used as a starting cell source. Huge numbers of cells are needed to make meats in culture. Based on a rough calculation, ~ $2.9x10^{11}$ of muscle cells are needed for 1 kg of wet meat [9]. To achieve such a quantity, it is essential to have sustainable cell sources, which can proliferate/double significantly *in vitro* while preserving the functionality of the cells.

Cell sources for muscle cells

Cells from muscle tissues: Muscle fibers are made of mature muscle cells (myocytes) and myogenic progenitor stem cells (satellite cells). The formation of muscle fiber or myogenesis, starts with the fusion of activated satellite cells (myoblasts) either with one another to generate new multi-nucleated myofibers or with an existing myofiber to increase the pool of myonuclei and allow muscle growth [10]. Satellite cells are the cell source for initial muscle formation and muscle growth/regeneration [11]. Due to their self-renewal and differentiation ability, satellite cells are the most prominent cell type explored for cultivated meat [12]. Satellite cells are characterized by the expression of the paired type homeobox transcription factor 7 (Pax7) and the absence of the expression of Myoblast Determination Protein 1 (MyoD) ($Pax7^{+}MyoD^{-}$) [13]. The isolation of satellite cells from biopsies of muscle tissue can be achieved by enzymatic digestion and multiple selection steps including fluorescence-activated cell sorting [12,14]. Since the microenvironment "niche" plays a crucial role in maintenance of the stemness of satellite cells, the expansion of $Pax7^{+}MyoD^{-}$ satellite cells under the standard *in vitro* culture conditions has been challenging. For example, $Pax7^{+}$ satellite cells reduced from 100% to 30-50% in only three passages [15]. Currently, many approaches have been explored to overcome or reduce the loss of phenotype of satellite cells. It has been shown that P38-MAPK signaling pathway is involved in the loss of $Pax7^{+}$ satellite cells during in vitro culturing. With P38 inhibitors, bovine satellite cells showed enhanced proliferation and expression of Pax7+ [16]. Inhibitors against Methyltransferase Setd7 [17], JAK-STAT pathway [18] and activators of P53 pathway [19] have been shown to positively promote the proliferation and maintain $Pax7^{+}$ phenotype in murine muscle satellite cells. It is still unknown if these small molecules similarly regulate the growth of satellite cells isolated from larger livestock animals remain to be tested.

The Extra Cellular Matrix (ECM) components have been shown directly regulate the proliferation of bovine satellite cells [20,21]. The effects of ECM components such as collagen type I, fibronectin and gelatin, laminin and Matrigel were tested on the proliferation and stemness of porcine muscle satellite cells. Among these ECM components, coating of laminin or Matrigel sustained the proliferation and myogenic differentiation capacity of the porcine satellite cells [22].

Satellite cells isolated from muscle tissues have a high efficiency to differentiated into myoblasts and then form mature myocytes, therefore, muscle satellite cells remain to be the most explored cell sources for cultivated meats. To overcome the limitation of cell expansion, immortalization of these cells may be an important venue to explore. Cell lines developed via spontaneous or intentional (transfection of genes) immortalization have been considered and successfully used for cultivated meats [23]. Such advancement warrants opportunities for developing immortalized satellite cells or myoblasts.

Cells from non-muscle tissues: In addition to cells isolated from muscle tissues, cells from non-muscle tissues are also considered for making muscle fiber in cultivated meats. Bovine Mesenchymal Stem Cells (MSC) can be readily isolated from non-muscle tissues such as bone marrow [24,25], adipose tissues[26], and placental tissues[27-29]. These cells can self-renew and undergo multiple lineage specific differentiation [25,30,31]. While myogenic differentiation of MSC have been achieved, but the differentiation efficiency is low [27,32]. Adding growth factors seem to increase the myogenic differentiation of MSC cultured in ECM 3-dimensional constructs [33].

Even though MSCs have self-renewal ability, to reach the scale of cultivated meat, cells have to be expanded *in vitro* extensively. MSCs show reduced proliferation and stemness over long term culture [34,35]. Therefore, studies aiming to overcome this limitation are critical. The inclusion of growth factors such as Fibroblast Growth Factor (FGF), Hepatocyte Growth Factor (HGF) in culture showed differential effects on the growth and stemness of human MSCs. FGF promoted significant proliferation with compromised differentiation potential. But HGF promoted cell proliferation mildly but maintained the stemness of cells [36]. Extracellular matrix assembled by cultured cells have also shown promising effects on the proliferation and stemness of human MSCs [37,38]. It is expected that more efforts will be put into improving the quantity while maintaining the quality of bovine MSCs during *in vitro* expansion in the foreseeable future.

Besides using adult stem cells, re-programed cells are also considered as alternative cells sources. Re-programmed

cells such as induced Pluripotent Stem Cells (iPSCs) and trans-differentiated cells overcome the limitation on cell expansion. iPSCs can be induced from terminal different adult cells such as skin fibroblasts by overexpressing transcription factor genes Oct4, Klf4, c-Myc, and Sox2 [39]. Successfully differentiation of iPSCs to functional satellite like cells has been achieved with human iPSCs [40]. However, reports on using animal iPSCs as a source for myoblasts are limited [41]. Another type of re-programed cells that may be used as cell source for making muscle cells is transdifferentiated cells. Directly re-programing of fibroblasts into muscle progenitor cells has been achieved in small animals [42]. While the trans differentiation skipping creating iPSC cells is very attractive but its efficacy and safety for cultivated meat must be evaluated [43].

Cell sources for fat cells

Even though meat is predominantly composed of muscle fibers, fat occupies approximately 30% of the biomass, contributing to better flavor, taste, texture, appearance, and nutrition [44-46]. More positive organoleptic consideration has been declared by consumers for a meat consisting of the higher fat levels [47]. Lipids (fat molecules) and adipocytes (fat cells) are two components of food, having many differences in terms of biological and food properties. A large amount of lipids is stored inside of the adipocytes, representing more natural component of animal meat fat [48,49]. Adipocytes are the predominant component of White Adipose Tissue (WAT) dispersed in different deposits throughout the body, including subcutaneous and visceral fat. Progenitor or MSCs are the origin of the adipocytes, developing unilocular lipid droplets after the differentiation into mature adipocytes. To isolate cells from a biopsied tissue, collagenase type I is used in each species such as bovine, porcine, and other livestock species (duck, goose, turkey, goat, and sheep). Although subcutaneous WAT is the predominant source of cells, visceral WAT could be obtained mainly from chicken and fish for which collagenase type II is used to collect the cells. Adipogenic cells can also be harvested from muscle or intramuscular fat. Fibroadipogenic progenitor cells obtained from muscle can differentiate into mature adipocytes [50,51]. This adipogenic differentiation process may be coordinated by miRNA targeting Runx1 [52]. In the literature several studies have shown the results of adipogenic differentiation of myosatellite cells into fat cells [53-55]. Adipogenic differentiation of MSCs is induced by using a differentiation cocktail basically composed of insulin, dexamethasone, and Iso Butyl Methyl Xanthine (IBMX), and rosiglitazone. A wide variety of differentiation cocktails have been examined to promote adipogenesis [44,56]. Due to safety and cost-effectiveness concerns, optimization of the induction cocktails has become important. For example, using combinations of free fatty acids for bovine preadipocytes appears as a promising solution for differentiation and maturation of adipocytes [57].

The major cell types in WAT are mature adipocytes with different minor cell types known as Stromal Vascular Fraction (SVF) or Stromal Vascular Cells (SVCs) which consist of the mix of preadipocytes, mesenchymal stem cells (adipose-derived stem cells, ASCs), endothelial cells, immune cells, and smooth muscle cells [58,59]. Although ASCs in SVCs have ability to differentiate into other cell types such as osteoblasts, preadipocytes in SVCs the same are settled down to become mature adipocytes. Therefore, isolated cells may show differences in terms of cell types and differentiation pathways. While most of the cell types used were somatic cells from adult animals, some studies utilized immortalized embryonic sources [60]. Pluripotent stem cells such as embryonic stem cells or induced Pluripotent Stem Cells (iPSCs) are another promising cell type that could be applicable for scalable production of adipocytes in the wide range of animal species [61,62]. While iPSCs feature exceptional growth properties, they are formed by including reprogramming genes in somatic cells [63]. There are many reports showing the derivation of iPSCs from chicken, bovine, fish and porcine however, there has been no reports in the literature of successful differentiation of farm-animal derived iPSCs into mature adipocytes [46].

The expansion stem cell sources for adipocytes faces the same challenges as the expression of cell sources for myocytes does [64]. The proliferation and stemness decrease over a long period of *in vitro* culturing. The research efforts to overcome the limitation on expansion of stem cells for myogenesis as discussed in muscle cells may very well applicable to stem cells identified as cell sources for adipocytes.

Summary

The importance of developing cultivated meat has been widely recognized in recent years. Efforts from both academia and industry have contributed to the tremendous progresses in making cultivated meat. Currently, the most commonly used cell sources for making cultivated meat (Figure 1) are stem cells isolated from the tissues of livestock animals. This dependence on biopsies from live animals or carcass tissues undermines the mission of cultivated meat. On the other hand, birth tissues from livestock are often discarded. Therefore, the stem cells from these tissues warrant additional research and development efforts. The sustainability of stem cells as a cell source depends on the proliferative ability of stem cells and their differentiation ability (stemness) to become muscle or fat cells by demand. Both abilities are limited by extensive *in vitro* culturing. Efforts to overcome such limitation have shown encouraging results but more advancement in this aspect is called for. The advantages and disadvantages of different cell sources are highlighted (Table 1).

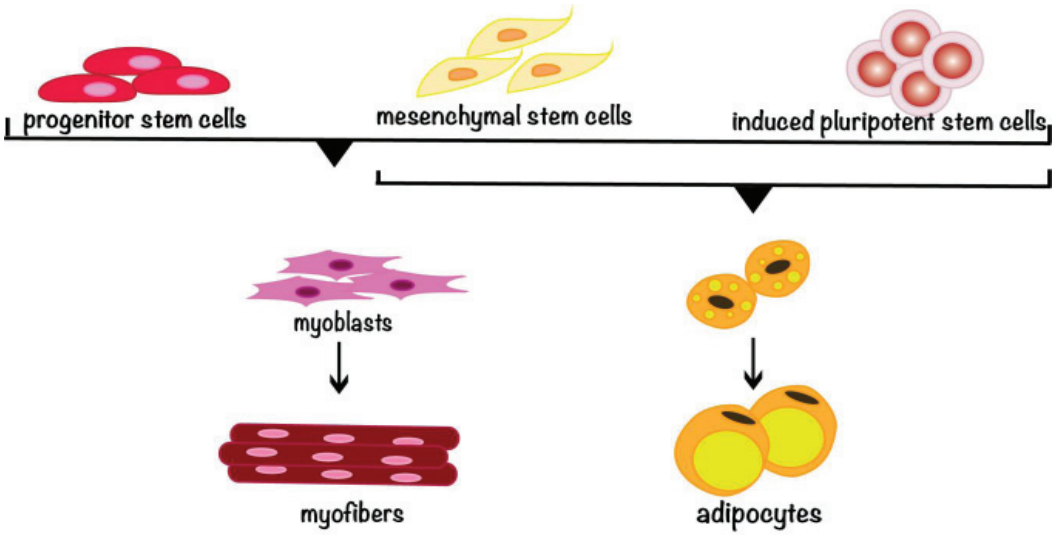

Figure 1 Summary of the cell sources to generate muscle cells and fat cells for cultivated meat.

Table 1: Comparison of different types of cells used as cell sources for cultivated meat.

Cell Types	Advantages	Disadvantages	Sources of cells
Progenitor stem cells (muscle satellite cells)	Easy to differentiate to the desired cell type	Limited proliferation potential; harvest cells from animal tissues	Biopsy of muscle tissue from live animal or carcass
Mesenchymal stem cells	Self-renewal (proliferation) Multiple tissue sources Differentiation to multiple lineages	The loss of proliferation ability and stemness during extensive in vitro expansion; require efficient and accurate lineage specific differentiation	Bone marrow, adipose tissues from live animal or carcass Placental tissue from birth
iPSC	Potential to be differentiated to muscle cells and fat cells from the same cell source	Creation of iPSC cells; rely on efficient differentiation, maturation	Multiple cell sources including terminally differentiated cells
Immortalized primary or stem cell lines	Unlimited cell expansion	Low efficiency of spontaneous immortalization; off-target effect of gene transfection; loss of the functionality or stemness of cells	Muscle satellite cells, mesenchymal stem cells

Perspectives

While only the cell sources are discussed here, the development and optimization of the scaffold materials for making the textured meats, the bioprocesses such as the vast expansion of cells by bioreactors, marbling the cultivated meat by additive manufacturing are all crucial for making the cultivated meat. A few comprehensive reviews on those topics are listed [9,12,65-67]. Even if cultivated meat is feasible and promising to evolve into a successful industry, it still has to overcome many challenges such as;

1. Developing effective and cost-efficient technologies.
2. Understanding and mimicking the textures and flavors of livestock meat.
3. Seeking governmental regulatory approvals.
4. Educating and improving customer acceptance of cultivated meat.

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