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Unlocking the potential of stem cells: Their crucial role in the production of cultivated meat

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ABSTRACT

Cellular agriculture is an emerging research field of agribiotechnology that aims to produce agricultural products using stem cells, without sacrificing animals or cultivating crops. Cultivated meat, as a representative cellular product of cellular agriculture, is being actively researched due to global food insecurity, environmental, and ethical concerns. This review focuses on the application of stem cells, which are the seeds of cellular agriculture, for the production of cultivated meat, with emphasis on deriving and culturing muscle and adipose stem cells for imitating fresh meat. Establishing standards and safety regulations for culturing stem cells is crucial for the market entry of cultured muscle tissue-based biomaterials. Understanding stem cells is a prerequisite for creating reliable cultivated meat and other cellular agricultural biomaterials. The techniques and regulations from the cultivated meat industry could pave the way for new cellular agriculture industries in the future.

1. Introduction

Currently, upcoming inevitable challenges are driving traditional agriculture to change. As the world population is estimated to reach approximately 10 billion by 2050, food production from the conventional agriculture industry would not be sufficient to nourish people globally because of the lack of arable land and the crisis of climate change (Eibl et al., 2021). To overcome the problems ahead, cellular agriculture is being considered as one of the potential solutions for food security. Cellular agriculture is an emerging research field of agribiotechnology that aims to produce agricultural products and by-products by culturing microorganisms, plant, and animal cells or tissues (Rischer et al., 2020). The resulting products of cellular agriculture are generally classified into acellular or cellular products. The former includes the by-products originating from the cells of microorganisms, plants, and animals, such as proteins, fat, food additives, pigments, flavours, and aroma components, while the latter includes *in vitro* cultivated cells or tissues themselves, such as meat and leather (Eibl et al., 2021; Rischer et al., 2020). Cellular agriculture is further classified by production methods based on tissue engineering or fermentation (Stephens et al., 2018). Although the term cellular agriculture was first coined in 2015, given its definition, practical studies on the field began with the discovery of plant cell totipotency in the early 20th century, followed by the development of *in vitro* culturing of animal cells and tissues, fermentation technology, and recombinant bacterial DNA production (Eibl et al., 2021; Haberlandt, 1902). Subsequently, various academic and industrial attempts have been conducted to create artificial agricultural products, such as secondary metabolite production by

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plant cells and tissues, ginsenosides, red dye shikonin, and vanillin (Eibl et al., 2021).

Cultivated meat, as the representative cellular product of cellular agriculture, is actively researched and its industrial scale is rapidly growing due to worldwide food insecurity, and environmental and ethical issues (Post et al., 2020). The concept of cultivated meat, which was originally proposed in Winston Churchill's book '50 Years Hence' nearly a century ago, became a reality in 2013 with the production of hamburger patties (Post, 2014; Reiss et al., 2021). The hamburger patty was composed of 10,000 individual muscle fibers differentiated from *in vitro* cultured bovine muscle stem cells using tissue engineering technology (Reiss et al., 2021). The manufacturing process of cultivated meat is generally composed of several steps, including isolation of muscle stem cells, primary and upscaled culture of muscle stem cells, and muscle differentiation and maturation (the details are well-reviewed in previous articles (Choi et al., 2021; Ong et al., 2021; Post et al., 2020)). Numerous endeavors have been carried out to optimize each material/process for producing cost-effective and consumer-satisfying cultivated meat. Along with the progress of technologies, support from legal and socio-cultural aspects, such as standards and safety regulations, have been simultaneously discussed. The fact that meat analogs could be obtained without raising domestic animals has explosively increased multidisciplinary investments and interest in cultivated meat. Eventually, in 2020, the cultivated chicken of Eat Just was approved as a novel food by the Singapore Food Agency (SFA) and has been sold in Singapore (Ng et al., 2021).

Four technologies including cells, culture media, scaffolds, and mass culture system are reportedly fundamental to producing cultivated meat. Of those, stem cells are considered the most important, as they are the fundamental and structural unit of cultivated meat and cellular agriculture, analog to seeds in conventional agriculture. Thus, a profound understanding of stem cell biology would aid in producing efficient and reliable *in vitro* cultured cell-based food materials. In this respect, this review will comprehensively discuss the basic stem cell biology and applications thereof in cultivated meat production. Then, the factors of production cost and safety considerations of cultivated meat will be analysed.

2. Stem cell biology

A one-cell embryo, known as a zygote, is formed by fertilizing oocyte and sperm, thereby creating an individual upon the gradual proliferation and transformation into specific tissues during fetal development following implantation (Rossant and Tam, 2017). In fetal development, the process in which ancestor cells such as zygotes and progenitors undergo changes into functionally specialized cells/tissues in molecular biological aspects is called 'cellular differentiation' (Gilbert and Barresi, 2017). Cellular differentiation occurs in a sequential manner over embryogenesis, and various functionally unspecified or undifferentiated cell populations appear in each stage of differentiation (O'Connor and Crystal, 2006). Additionally, in postnatal development, these undifferentiated cells enter cellular quiescence and, upon receiving activation cues such as injury, are responsible for tissue regeneration and growth through the vast expansion of the number of cells (Avgustinova and Benitah, 2016). Undifferentiated cells residing *in vivo*, or *in vitro* cultured cells thereof, are called 'stem cells'. The term 'stem cells' was first used by Valentin Haecker and Theodor Boveri to describe ancestor cells of organisms or germ cells in the late 19th century (Boveri, 1892; Haeckel, 1868). In the 1960s, Dr. Ernest McCulloch and James Till unveiled the existence and basic features of stem cells through sequential experiments using blood-forming stem cells, presently known as hematopoietic stem cells (Till et al., 1964). Unlike somatic cells, stem cells capable of self-renewal (the ability to create daughter cells identical to parent cells via mitosis) and cellular differentiation reflecting various features depending on their origins (Choi and Lee, 2019).

Stemness, including self-renewal and differentiation potential, is divided into several states based on their capacity (Tewary et al., 2018) (Fig. 1). The highest grade of differentiation potential acquired by cells is 'totipotency'. The totipotent cells themselves can develop into a whole individual and, to date, only zygote and early embryos reportedly represent totipotency. However, they are not considered stem cells because they are unable to self-renew and lose their potency as their development progresses (Condic, 2014). Totipotency remains challenging to be captured *in vitro* (Xu et al., 2022). Pluripotency is the ability to differentiate into every cell type in the body, except for extraembryonic tissues such as the placenta, while being unable to develop into a whole individual (Choi and Lee, 2019). A founder population called 'inner cell mass (or epiblasts)' in a blastocyst that came from a zygote gives rise to a fetus following implantation in the maternal uterus. Although these are not 'stem cells', as the temporary pluripotent cell population of embryogenesis does not have the ability of self-renewal, they can acquire self-renewal ability through *in vitro* culture under appropriate conditions, thereby turning into stem cells, which are embryonic stem cells (ESCs) (Evans and Kaufman, 1981). Additionally, another way to obtain pluripotent stem cells (PSCs) is through cellular reprogramming by genetic manipulation or nuclear transfer in somatic cells, which are known as induced pluripotent stem cells (iPSCs) and somatic cell nuclear transfer-derived (NT-) ESCs (Hochedlinger and Jaenisch, 2006; Takahashi and Yamanaka, 2006). Current technology could achieve somatic cell reprogramming with small molecules such as signalling activators and epigenetic modifiers without introducing ectopic expression of transcription factors (Ma et al., 2013). PSCs could differentiate into all types of cells in the body and infinitely proliferate for an extended period. Recently, early embryo-derived stem cells showing extended pluripotency capable of development into both embryonic and extraembryonic tissues have been established, but the information is still lacking (Gao et al., 2019). Unlike PSCs, multipotent stem cells, generally known as adult stem cells or tissue-specific stem cells, have less proliferative and committed differentiation potential into certain cell lineages. They could be isolated from various tissues of the fetus, juveniles, and adults and primarily differentiated into relevant or functionally related cells presented in their origin tissues. Various types of these cells, which are reportedly responsible for tissue regeneration, have been widely researched, such as hematopoietic stem cells from bone marrow, neural stem cells from the brain, and mesenchymal stem cells from several mesoderm tissues (Avgustinova and Benitah, 2016). However, stem cells gradually lose their ability to self-renew and differentiate as senescence is induced during an *in vitro* culture. Lastly, unipotent stem cells, which can give rise to a single type of cells, such as spermatogonial stem cells for spermatogenesis, have been reported (Liu et al., 2016).

Developmental biologist Conrad H. Waddington, known as the pioneer of epigenetics, addressed that the developmental competence of stem cells as described above is like a stone rolling downhill: stones at the top of a hill have difficulty maintaining a stable state and can move anywhere (Fig. 1). On the other hand, stones that reach the bottom of the hill are in a stable state and cannot move anywhere (Sieweke, 2015). Accordingly, because stem cells have distinct features from totipotency to unipotency based on a hierarchical order, cell types should be carefully chosen for producing cultivated meat through *in vitro* cell culture. Furthermore, mimicking an *in vivo* environment surrounding the cells, called a stem cell niche, is crucial for the cell maintenance *in vitro*. Generally, the physical space on which the cells reside and body fluid to supply nutrients are replaced by scaffold and culture media, respectively (Tewary et al., 2018). Because various types of stem cells originate from different tissues with distinct molecular biological profiles, their own tailored culture conditions are required to improve the culture efficiency and product quality. Furthermore, most of the research has been conducted mainly with humans and mice so far, which proved challenging to be transferred to livestock species. So, different prerequisites for culturing stem cells from different species are required, as they have different appearances and genomic backgrounds (Choi and Lee, 2019).

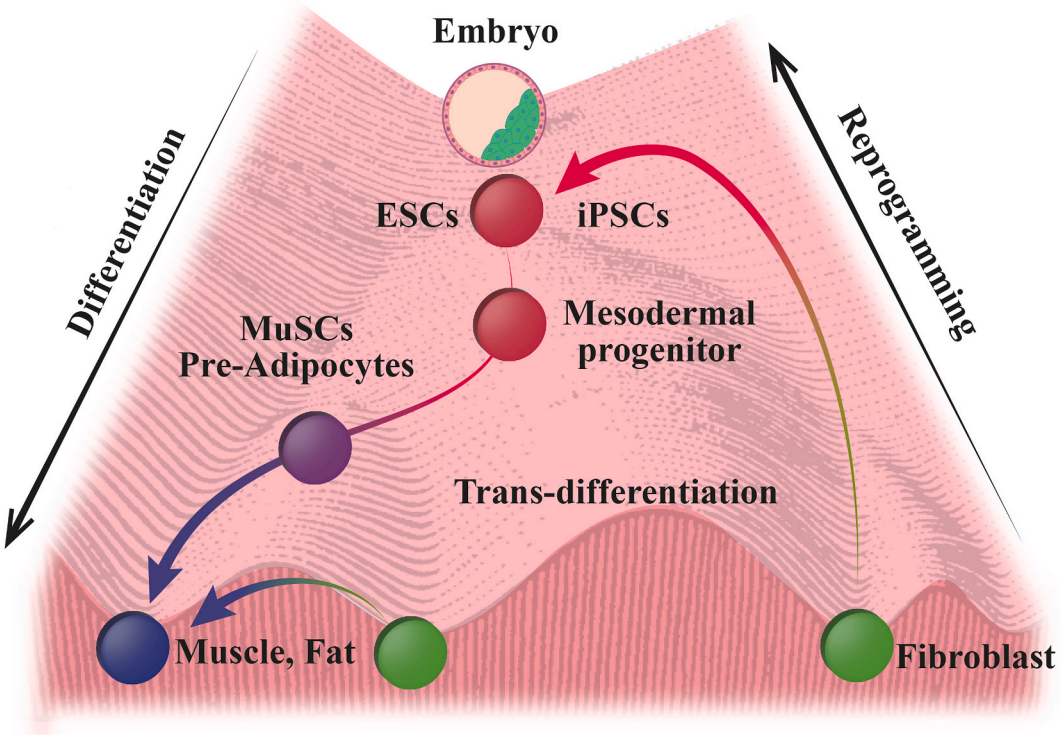

Fig. 1. Differentiation potential of stem cells. The transition from embryonic stem cells (ESCs) to muscle and fat can be explained by the differentiation potential capabilities of pluripotent stem cells. ESCs with pluripotent differentiation capacity are differentiated into muscle stem cells (MuSCs) and pre-adipocytes through mesodermal progenitors, which can differentiate into muscle and fat. Even the fibroblasts, which have been fully differentiated, can be transformed into muscles or induced pluripotent stem cells (IPSCs) via transfection of pluripotent genes.

3. Features of meat from domestic animals

Meat can be defined as "the flesh of an animal destined for consumption as food", including all edible parts of an animal, such as lean meat, fat, and intestines. Meat is one of the most important food resources for human nutrients including essential proteins, fats, trace minerals, and vitamins (Lee et al., 2020). Meat is not only valued for its nutrition but also for the pleasure of eating, sociocultural interactions, and much more. Meat is primarily composed of water (about 75%), proteins (around 20%), fat (ranging from 1% to 10%), and other components (Listrat et al., 2016). These compositions influence the quality of meat itself. Moreover, the tissue constituents of meat also affect its quality and composition. Meat exhibits diverse shapes and physiological functions, with approximately 90% consisting of muscle fibers and the remaining 10% comprising connective tissue, fat, vascular structures, and nerves (Listrat et al., 2016). Of these constituents, muscle fiber, connective tissue, and intramuscular fat play crucial roles in determining meat quality (Joo et al., 2013; Zhang et al., 2017). Especially, the molecular structure of the sarcomere, a basic functional unit of muscle, which is composed of contractile and cytoskeletal proteins such as myosin, actin, titin, nebulin, troponin-T, desmin, and filamin reportedly plays an important role in meat quality as well as muscle maturity (Lonergan et al., 2010). In fact, muscle fiber types affect meat quality itself. Adult muscle fiber can be classified as four types (type I: slow-oxidative; type IIA: fast oxido-glycolytic; type IIX and B: fast glycolytic) by metabolic properties (Joo et al., 2013; Zhang et al., 2017). Muscle with more type I muscle fibers tends to be red and has higher myoglobin contents, less toughness, higher juiciness, and more cooked meat flavor, while that with more type II muscle fibers is lighter, tougher, and has lower water holding capacity (Hwang et al., 2010; Joo et al., 2013; Kim et al., 2010; Renerre, 1990). Fat is known as a main component for meat flavor, texture, nutrition, and appearance, and these factors affect consumer's preference and willingness to pay (Fish et al., 2020; Lee et al., 2021). Based on these observations, a standard or grade for cultivated meat with mature ultrastructure is required, which might be evaluated by the similarity to meat from domestic animals and technologies to develop mature enough muscle structure by culture system. In addition, the composition of muscle and fat cells (80–90% in meat from domestic animals) and the ratio of scaffolds to the cells are very important for defining cultivated meat. Eventually, the final goal of manufacturing cultivated meat is to create meat *in vitro*, which must be fundamentally different from plant-based meat analogs.

However, for *in vitro* cultured muscle, reaching full maturity is proven challenging compared to that from domestic animals, so far muscle fibers mainly representing a fetal phenotype have been reported (Thorrez and Vandenburgh, 2019). Therefore, the eating quality and processing characteristics of cultivated meat might be different from conventional meat from domestic animals. Usually, research of *in vitro* cultured muscle cannot describe sarcomere structure in detail, and can only provide the contraction behaviour or myosin expression as evidence for muscle maturity (Fraeye et al., 2020; Ng and Kurisawa, 2021). Indeed, because these proteins are important for meat quality through the conversion of muscle to meat process post-mortem (Lonergan et al., 2010; Thorrez and Vandenburgh, 2019), the absence of mature muscle structure or post-mortem metabolism could result in somewhat different textural properties of meat. With an immature muscle structure and lack of connective tissue proteins, cultivated meat is usually suited for processed meat products, although some studies of *in vitro* cultures have reported meat with steak-form (Furuhashi et al., 2021; Kang et al., 2021). Also, information about the nutritional value of cultivated meat is currently very limited. Muscle stem cells are cultured in scaffolds for the production of cultivated meat; thus, the volume of the scaffolds might exceed that of muscle cells. Therefore, its nutritional value might be lower than that of meat from domestic animals in a given weight (Fraeye et al., 2020). Culturing fat and other tissues for the composition of cultivated meat is necessary to meet consumers' expectations and

achieve competitiveness in the market. Although there have been advances in meat analog production techniques, mimicking the flavors and mouthfeel of fat remains a challenge (Joshi & Kumar, 2015). Research flow for *in vitro* fat cultures might be similar to that of muscle tissue; cell sources, culture system, and tissue engineering should be considered (Fish et al., 2020) (Fig. 2). Co-culture or a combination with other tissue cultures could be a potential solution to improving flavor of cultivated meat.

4. Stem cells for muscle tissue

4.1. Muscle development

During embryogenesis, pluripotent inner cell mass (ICM; or epiblasts) sequentially develops toward myogenic progenitor via paraxial mesoderm, thereby generating muscle fibers and muscle tissues by cell-to-cell fusion (Chal and Pourquie, 2017). The myogenic progenitor cells settle down beneath the basal lamina of muscle fiber, and in turn, convert to quiescent myogenic satellite cells for the stem cell reservoir responsible for regenerating the muscle tissues for a lifetime during postnatal development. From the embryo to adulthood, various stem cell types including ESCs, muscle stem cells (MuSCs), and mesenchymal stem cells (MSCs), contribute to producing muscle, and ample research on muscle tissue regeneration has been conducted in the past to cure muscle dysfunction, and more recently to produce cultivated meat.

4.2. Pluripotent stem cells

Practically, PSCs represented by ESCs and iPSCs are considered ideal cell sources for producing cultivated meat without sacrificing animals due to their indefinite self-renewal capacity (Post et al., 2020). Authentic ESC lines from domestic animals including pigs and cows have recently been derived, while domestic animal iPSC lines independent of transgene expression have not been reported yet (Bogliotti et al., 2018; Choi et al., 2019). Although cellular reprogramming techniques are meant to be an alternative to create PSCs from somatic cells, genetically modified organism-driven issues related to food safety regulations and consumer acceptance remain unsolved (Sendhil et al., 2022). MuSCs and muscle fibers could be obtained from PSCs through *in vitro* recapitulation of embryonic myogenesis because PSCs resemble epiblasts of early

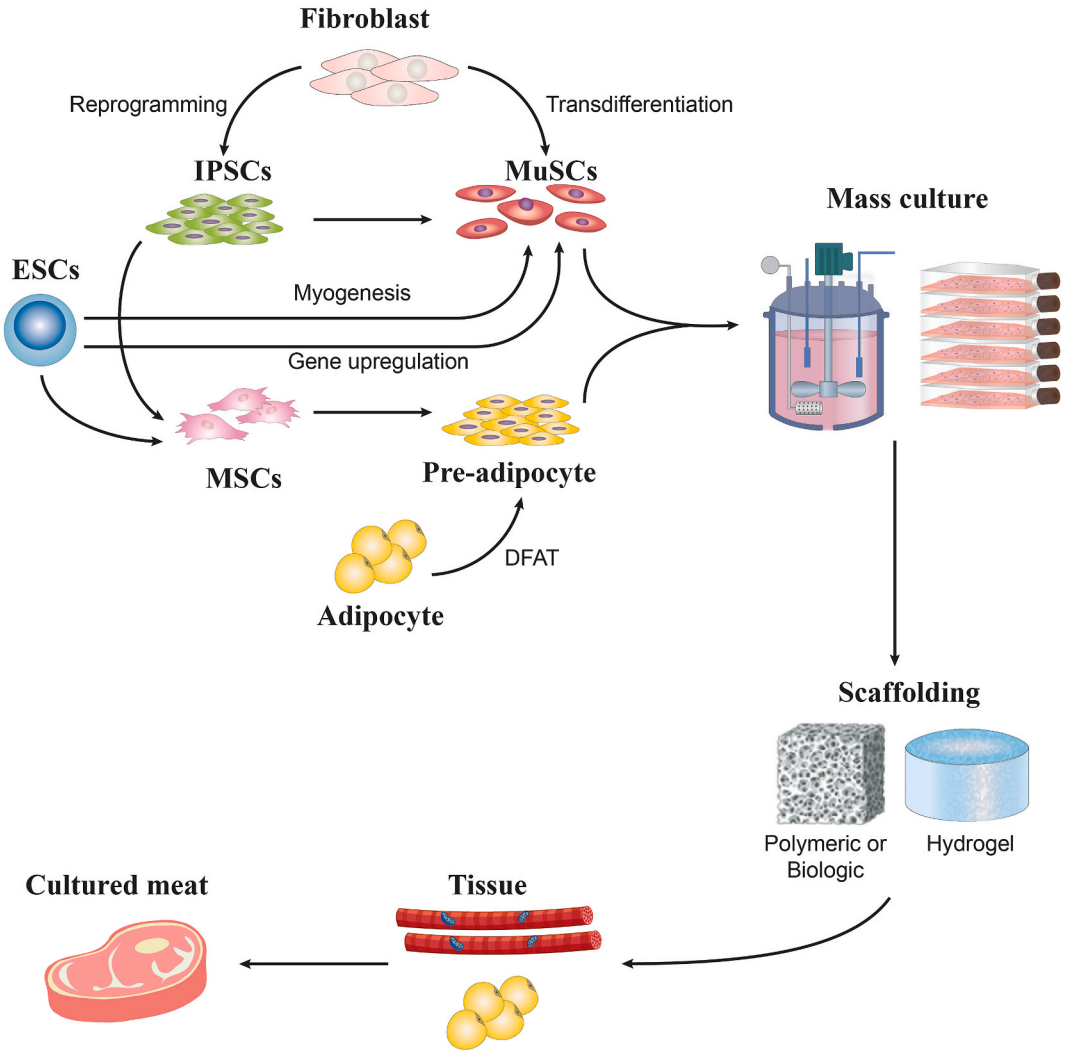

Fig. 2. General workflow for cultivated meat production using various stem cell types. Stepwise process of cultivated meat from embryonic stem cells (ESCs), induced pluripotent stem cells (IPSCs), mesenchymal stem cells (MSCs) and even terminally differentiated fibroblasts. Fully defined and appropriate culture condition of various cell types can be applied to the production of cultivated meat. Cells with different characteristics and origins are selected, followed by mass culture. The increased number of cells requires an environment in which the cells can attach, grow, and differentiate. Such an environment may be provided by the scaffold, and its requirements may be different for each cell type. Through this process, it is possible to produce muscle and fat that can be used for cultivated meat, and it is possible to produce several types of cultivated meat by controlling the ratio of these two tissues.

embryos in molecular biological aspects (Chal and Pourquie, 2017). *In vitro* myogenesis accomplished by various combinations of hormones and cytokines upregulates the myogenic determinants such as PAX7, MYOD1, and MYF5 in PSCs, leading to differentiation into MuSCs. According to recent research, myogenic differentiation rates range between 20 and 40% (Chal et al., 2018). A profound understanding of the *in vivo* environment involved in myogenic precursor specification would help in acquiring reliable resultants with high efficiency. However, maintaining stemness and regulating the differentiation path in stem cells having high potency require advanced cell culture techniques. Therefore, alternatives for producing cultivated meat have been investigated and are analysed herein.

4.3. Muscle stem cells

Various types of stem/progenitor cells resident in muscle tissues reportedly participate in skeletal muscle regeneration, such as myogenic satellite cells, MSCs, interstitial cells, fibroadipogenic progenitors, muscle side population cells, and pericytes (Klimczak et al., 2018). Of those, MuSCs including quiescent myogenic satellite cells and proliferating myoblasts are the main contributors to muscle fiber production (Kuang et al., 2007). Therefore, *in vitro* cultured MuSCs have the highest myogenic competency compared to other types of stem cells. MuSCs could be isolated from muscle tissues of fetuses, adults, and even after slaughtering. The yield and biological features of the isolated MuSCs are influenced by the conditions of donor animals such as age, breed, and sex (Choi et al., 2021). Additionally, the number of MuSCs varies depending on the location of muscle tissues.

Male animals have a greater number of MuSCs than female animals, especially proliferating satellite cells (Day et al., 2010). As muscle grows, the total number of stem cells increases while their density decreases, and in turn, satellite cell population declines losing its proliferative potential with aging (Campion et al., 1981). Stem cells from breeds having different genetic backgrounds have distinct myogenic ability features. For example, stem cells of the 'Belgian blue' breed harbouring GDF8 gene mutation show an enhanced proliferation capacity compared to those of other cattle breeds (Quinn et al., 1990). In fact, for *in vitro* culture, the stemness of MuSCs is also reduced with prolonged culture, despite coming from fetus or young animals, which indicates a constant supply of muscle tissues, such as from biopsies and slaughters, would be inevitable for the production of a continuous and sufficient supply of cultivated meat. Accordingly, along with research on the rejuvenation and extending the culture period of MuSCs, considering various conditions of animals would be a prerequisite for improving the isolation efficiency of MuSCs while minimizing the sacrifices of animals. Cellular immortalization can spontaneously occur by stress-induced gene regulatory network disruptions during long-term culture and could be used (Soice and Johnston, 2021). However, naturally immortalized cell lines are hard to obtain because of low yields and unpredictable physiological features resulting from genetic alteration.

4.4. Mesenchymal stem/stromal cells

Mesenchymal stem/stromal cells (MSCs) reportedly reside in various tissues such as bone marrow and adipose tissues in a quiescent state, and upon injury, they undergo activation to regenerate various tissues. To date, although their origin is still poorly understood, some research shows that pluripotent epiblasts differentiate into mesodermal progenitors via lateral-plate mesoderm formation by epithelial-mesenchymal transition (EMT), leading to their involvement in the formation of the body (Li et al., 2021). For *in vitro* culture, they could be isolated from several tissues including bone marrow, adipose tissue, and umbilical cord, or obtained by direct differentiation from PSCs. MSCs as multipotent stem cells could reportedly be differentiated into several mesodermal tissues including adipocytes, osteoblasts, chondrocytes, and myogenic progenitors (Joe et al., 2010). Indeed, the myogenic potential of MSCs has been controversial. *In vitro* cultured MSCs isolated from bone marrow highly expressed myogenic determinant genes by differentiation cues, although their biological functions were poorly characterized along with a low differentiation rate (Okamura et al., 2018). Furthermore, transplant studies using MSCs tagged by reporter genes have shown that MSCs are incorporated in muscle regeneration, thereby forming muscle fibers (Fukada et al., 2002). However, other studies have addressed that they have no myogenic potential after transplantation, as MSCs and MuSCs originate from different developmental lineages, lateral-plate, and paraxial mesoderm, respectively (Leinroth et al., 2022; Uezumi et al., 2010). MSCs secrete various cytokines to promote proliferation and differentiation of MuSCs and facilitate the infiltration of immune cells for inducing inflammatory responses, supporting the reconstruction of the muscle in indirect ways (Joe et al., 2010; Leinroth et al., 2022). In this respect, MSCs could be applicable for improving the growth of muscle tissues or for producing adipose tissue.

4.5. Genetic modification

Recently, genetically modified animals, including pigs and salmon, were approved as food by the US FDA (Dolgin, 2021; Waltz, 2017). Although both genetically-engineered animal- or cell-derived food materials have a long way to go for approval by the government around the world, the technology would have to be prepared for the potential upcoming food market. It is possible to transdifferentiate non-myogenic cells into MuSCs by ectopic expression of myogenic determinants such as *MYOD1* and *PAX3/7* applying the cellular reprogramming technique used to derive iPSCs (Chal and Pourquie, 2017). The transdifferentiation study using *MYOD1* first elucidated that cellular lineage could be altered by regulating a fate determinant (Davis et al., 1987). Fibroblasts, the most abundant cells in the human body, could be utilized for muscle production through the turn-on/off of genes at will. In addition, the upregulation of these genes allows PSCs to directly differentiate into muscle lineage by bypassing the sequential developmental process (Chal and Pourquie, 2017).

As somatic cells could acquire pluripotency by regulating gene expression, genetic manipulation allows for MuSCs to gain new traits, such as enhanced proliferation and differentiation capacities. Representatively, the immortalization of the cells has been widely applied for the establishment of stable cell lines by extending their life span, which enables maintenance of the stemness for an extended period without senescence (Soice and Johnston, 2021). As described above, MuSCs gradually lose their proliferation and differentiation abilities during *in vitro* culture. Naturally immortalized cell lines are hard to obtain and have generally been derived by engineering the genes that participate in cellular senescence and division. In particular, telomere synthesis enzyme, also called telomerase, and cell cycle activators such as *CDK4* and *BMI-1,* have been applied to accomplish the immortalization of MuSCs (Chua et al., 2019; Douillard-Guilloux et al., 2009). It has been proven that the shortening of telomeres, which are repetitive nucleotides at the end of chromosomes, is associated with the aging of cells (Soice and Johnston, 2021). To extend self-renewal using genetic engineering, careful approaches would be required, since constant proliferation impedes myogenic differentiation (Chua et al., 2019). Currently, CRISPR/Cas9, known as gene scissors, is highlighted as the next-generation genetic engineering technique. Their use promotes muscle growth through disruption of the myostatin gene without introducing ectopic genes, which indicates that among various genetic engineering techniques, gene ablations would be considered more amenable to meet the regulation (Wang et al., 2015).

5. Fat culture for cultivated meat production

The importance of fat has been emphasized recently, as it has a substantial impact on the juiciness and flavour of cultivated meat

(Hausman et al., 2014). Fat could be produced through adipogenesis, in which stem cells differentiate into adipocytes, and lipogenesis, in which triglycerides are accumulated in adipocytes. Adipogenesis begins with the commitment of MSCs or pre-adipocytes to an adipogenic lineage regulated by Zinc Finger Protein 423 (ZFP423), an upstream transcriptional regulator of pre-adipocyte-associated genes (Gupta et al., 2012). For further differentiation, preadipocytes are regulated by the transcription factor CCAAT/enhancer-binding protein (C/EBP) family induced by proliferator-activated receptor gamma (PPARγ) to generate mature adipocytes (Du et al., 2013; Hausman et al., 2014).

Although various cell types are capable of adipogenic differentiation *in vitro*, it is not yet clear which is the optimal source for producing fat for cultivated meat (Fig. 2). Characteristics of an ideal cell source include high proliferation and efficient differentiation capacity, low media requirements, homogeneity, stability, and adaptability to mass culture conditions (Fish et al., 2020). Except for mass culture adaptability, most of the features mentioned above have the potential to be resolved through the basic characterization of cells. Since there is a transition point between the regulation and differentiation of cells, efficient cell production is difficult without an accurate understanding of these properties, and additional negative aspects may upstart in the cultivated meat production process (Zhang et al., 2020).

5.1. Fat development

Adipogenesis proceeds as MSCs participate in the adipose lineage and differentiate into adipocytes. In this process, delicate regulation of the differentiation process is required. Because MSCs are multipotent adult stem cells and can differentiate into various cell types of mesodermal lineages, including adipocytes and osteoblasts, the interaction between cell cycle regulation and differentiation factors produces a series of events that ultimately lead to adipocyte production (Avgustinova and Benitah, 2016). The process of adipogenesis from multipotent stem cells has two stages that can be divided into expression patterns of specific genes. The first step, known as the determination process, involves the commitment of stem cells to pre-adipocytes. In the second phase, called terminal-differentiation, the pre-adipocytes gradually acquire physiological functions of mature adipocytes, including lipid transport and synthesis, insulin sensitivity, and adipocyte-specific protein secretion (Zhang et al., 2020). This is a complicated and delicate regulatory process in which gene expression is finely controlled (Zhang et al., 2020). In general, adipogenic differentiation is induced by treatment with 1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin (Fish et al., 2020; Tang et al., 2004). Various chemicals and methods are being studied in different species for a higher differentiation rate and long-term culture of adipocytes. Moreover, the differentiation of MSCs into a mesenchymal lineage is genetically manipulated by promoting specific transcription factors associated with a particular cell lineage (Fish et al., 2020; Zhang et al., 2020). To date, several transcription factors have been identified for the differentiation of MSCs into adipocytes. The adipogenic-specific peroxisome PPARγ is one of the representative transcription factors that regulates the expression of genes responsible for adipogenic differentiation.

5.2. Stromal vascular fraction (SVF)

The SVF of adipose tissue contains pluripotent cells that can differentiate into adipocytes, chondrocytes, and bone cells (Crossno et al., 2006). However, due to the short culture period and reduced differentiation potential by subculture, which requires continued animal sacrifice, the use of SVF-origin cells for fat culture is limited. To solve these problems, studying stem cells for stable and sustainable cell supply is necessary. Preadipocytes can be obtained using enzymes such as collagenase in SVF cells. SVF contains various cells such as endothelial, pericytes, T cells, and macrophages (Ramakrishnan and Boyd, 2018). Studies are underway to classify pre-adipocytes for the selection of cell lines that have the potential to differentiate into fat (Yu et al., 2020). The purely isolated preadipocytes can maximize the differentiation yield into adipocytes. Recently, multiple studies have been conducted on adipocyte culture for fat addition to enhance flavour, in addition to muscle culture. Dohmen et al. successfully sorted pre-adipocytes from muscles and applied them into alginate for fat tissue formation, but more research is needed to satisfy the cultivated meat industry (Dohmen et al., 2022).

5.3. Dedifferentiated FAT (DFAT)

As previously described, the adipogenic potential of SVF is drastically decreased during *in vitro* culture, which is considered a hurdle to producing cultivated fat. DFAT is supposed to be another candidate cell source for creating cultivated fat to overcome the disadvantages of SVF. Dedifferentiation of fully differentiated adipose can be accomplished through their ceiling culture. During the culture, lipid storage of adipose is reduced and, in turn, reverts to fibroblast-like shaped progenitor cells, so-called DFAT. DFAT reportedly has multiple differentiation capabilities into mesenchymal lineages such as adipocytes and osteoblasts (Matsumoto et al., 2008). Dedifferentiation of FAT has been studied in humans (Kishimoto et al., 2018), rats (Akita et al., 2016), mice (Yagi et al., 2004), and livestocks (Peng et al., 2015; Wei et al., 2013). Various research has shown that the enhanced adipogenic potential of DFAT is stably maintained with normal chromosomal karyotypes for an extended period (Peng et al., 2015). However, the major obstacle of DFAT to apply for producing cultivated meat would be their dependence on high serum concentrations (often 15–20%) to maintain a proliferative state *in vitro*, which is rarely encouraged for the creation of novel alternative protein food due to their price as well as animal welfare (Fish et al., 2020). Along with developing serum-free culture conditions for derivation and proliferation, the major features of DFAT remains to be solved for application to create cultivated fat.

6. Scaffold and tissue engineering

For *in vitro* culture, scaffolds as biomimetic materials recapitulate the *in vivo* stem cell niches by providing a 3-dimensional adhesive surface, which supports the *in vitro* organogenesis of stem cells and maturation of tissues, unlike 2-dimensional culture using a tissue culture plate (Ostrovidov et al., 2014). Hydrogel and porous scaffolds have been generally applied to organize artificial tissues from stem cells *in vitro* for a long time. Hydrogel is a hydrophilic polymer, which has been widely used to generate self-organizing artificial tissues from stem/progenitor cells, also called organoids. Mimicking the *in vivo* microenvironments allows the stem cells to recapitulate the developmental program, thereby fulfilling the *in vitro* organogenesis (Hofer and Lutolf, 2021). Stem cells differentiate into organs based on their developmental codes, indicating that the cells could be converted into more reliable tissues by organoid techniques compared to other current differentiation methods. Organoids resembling the brain were first generated in 2013; subsequently, muscle organoids have been reported using a hydrogel scaffold (Maffioletti et al., 2018). Although organoids have a high similarity to the actual organs in a biological aspect, the texture and shape of cultivated meat are hard to replicate. Porous scaffolds, as biomaterials with sponge-like structures, have more advantages to achieve the texture and shape of cultivated meat by providing the frames for cells to organize in the 3-dimensional structure compared with hydrogel scaffolds (Ben-Arye et al., 2020). However, the resulting tissues produced by porous scaffolds have a high portion of scaffolds and different histological structures unlike actual meat, which would cause a significant gap separating it from conventional meat in terms of nutrition and sensory characteristics.

Likewise, scaffold also plays a crucial role in creating cultivated fat in terms of differentiation efficiency and tissue maturity. Compared to 2D culture systems, 3D fat production improves efficiency and degree of

differentiation (Ma et al., 2018). While the 2D protocol generally produces only multi-layered lipid accumulation, the 3D system can produce large unilocular lipid droplets that are characteristic of mature adipocytes. Furthermore, 3D induction can induce a higher differentiation rate as compared to monolayer culture (Dohmen et al., 2022). In addition, scaffolding approaches have been developed to optimize the microenvironment of cultured fat. The physical properties of scaffolds play a pivotal role in determining the quality of adipocytes, crucial for various tissue engineering and regenerative medicine applications. It is evident that the mechanical stiffness of hydrogels profoundly influences adipocyte differentiation and function. Soft hydrogels resembling the natural elasticity of adipose tissue foster the formation of mature and functional adipocytes, whereas stiffer hydrogels may hinder their development (Ribeiro et al., 2016; Young et al., 2013). Additionally, the porosity and pore size of hydrogels are critical factors in ensuring optimal nutrient, oxygen, and waste product diffusion within the gel. An adequate pore size facilitates the exchange of essential molecules and promotes healthy adipocyte growth and metabolic activities (Habanjar et al., 2021; Loh and Choong, 2013). Furthermore, the swelling capacity of hydrogels, attributed to their water absorption and retention properties, significantly impacts the hydrated microenvironment required for adipocyte viability and function (Cao et al., 2021). Considering the degradation rate of the hydrogel, it is crucial for it to align with the various stages of adipocyte development and maturation, providing sustained support and allowing for extracellular matrix remodelling (Suh and Matthew, 2000). Lastly, the biochemical composition of the hydrogel, often functionalized with bioactive molecules, such as growth factors or extracellular matrix components, can regulate adipocyte behavior, promoting adipogenesis, enhancing adipocyte viability, and influencing metabolic activity (Li et al., 2018; Saldin et al., 2017). By thoroughly understanding and optimizing these physical properties, hydrogels can offer an ideal environment to support the growth, maturation, and maintenance of high-quality adipocytes.

In addition, to scaffold types, it is crucial to seek edible scaffold materials to produce cell-based food materials, including cultivated meat. To date, various edible materials, including natural extracellular matrix proteins, decellularized plants, and polysaccharides have been widely investigated to make artificial muscle tissues (Ben-Arye et al., 2020; Jones et al., 2021; MacQueen et al., 2019). Additionally, to achieve a reliable meat analogue to conventional meats, maturation of *in vitro*-produced muscles through mechanical stretching and electrical stimulations might be significant to obtain desirable texture and nutrition thereof (Langelaan et al., 2011; Powell et al., 2002). Accordingly, finding suitable scaffold biomaterials and developing tissue organization methods are pivotal to improving the quality of cultivated meat.

7. Production cost of cultivated meat

In 2013, Dr. Mark J. Post, a professor at Maastricht University in the Netherlands, and his colleagues proved that meat could be served by cultivating animal cells/tissues without slaughtering. They made beef patties using bovine muscle stem cells, which cost approximately 300,000 USD. Subsequently, emergent research teams have released other prototypes of cultivated meat and the cost thereof; however, different standards have been applied to calculate them, which can be confusing to potential consumers and relevant industries. The price of *in vitro*-produced tissues mostly depends on the production cost of cells, culture media, and scaffolds. For cells, this includes the price of donor animals, cell separation cost, and cell content in the final products, and for culture media and scaffolds, production cost and the amount used for culture would be considered for estimation of the inputs along with the culture technique and maintenance cost. However, for objective evaluation, the origin of cells, ingredients for culture, and composition and nutrients of the *in vitro* cultured cell-based foods should be disclosed along with the price. Additionally, delivery of objective information on cultivated meat considering the standard model based on conventional meats would aid in consumer's awareness and satisfaction, which might lead to the introduction of the cultivated meat grading system, thereby broadening the spectrum of an upcoming meat market.

The high cost of producing cultivated meat is a known factor that hinders the market entry of cultivated meat along with consumer acceptance and government regulations. Examination of potential consumers' willingness to pay (WTP) has shown that consumers have higher WTP for cultivated meat than traditional burgers by approximately two-fold if cultivated meat tastes equal to conventional meat and has environmental benefits (Kantor and Kantor, 2021). As mentioned above, although the production cost of cultivated meat has dropped rapidly through numerous research and developments, it still far exceeds that of conventional meat. To produce cost-effective cell culture products, the constant supply of cells using immortalized cell lines or embryonic stem cells without slaughtering is being considered as a key solution to reduce the cell supply price (Post et al., 2020). For culture media, fetal bovine serum (FBS) and growth factors are reportedly responsible for the high price. Numerous researchers in cell biology have long wrestled to replace FBS with inexpensive materials such as hydrolysates of by-products from animals and plant/microorganism extracts and produce growth factors from plant and insect cell culture systems (Andreassen et al., 2020; Cronin, 2020; Dohmen et al., 2022; Messmer et al., 2022; O'Neill et al., 2021). Furthermore, the price of the culture medium and scaffold can be reduced by replacing the ingredients with lower grade materials or by developing minimal culture conditions through the optimization of their concentration (Lyra-Leite et al., 2021; Ma and Suh, 2019). Automation of the culture process and cutting the cost of culture equipment should also be carried out along with the aforementioned factors (Bakmiwewa et al., 2015; Cronin, 2020; Lucendo-Villarin et al., 2020). Accordingly, in order to produce cost-effective cultivated meat, research on several different approaches should be simultaneously conducted (Table 1). Also, industrial preparation for upscaling the production facilities would be required to a tremendous cost reduction in comparison to the current R&D phase.

8. Safety of cell-based food materials

The technology development of cellular agriculture, particularly stem cell biotechnology, has led to the commercialization of cell-based food like cultivated meat in the market. However, several issues remain, including nomenclature (labelling), safety management, authenticity, and consumer perception (Post et al., 2020). Particularly, the safety management during and/or after production of cultivated meat should be secured because lab-grown meat technology, if not adequately managed, can pose direct threats to consumer health. The potential risks include microbial contamination from inadequate hygiene practices during production, allergenic reactions due to novel ingredients or additives, nutritional imbalances resulting from failure to replicate the optimal nutritional composition of traditional meat, unknown long-term effects that require continuous monitoring and research, and concerns related to antibiotic usage and the development of antibiotic-resistant bacteria, posing potential threats to public health. Proper regulation and stringent monitoring are vital to mitigate this concern.

In the case of food using new materials and technologies, it is necessary to establish safety evaluation processes suitable for the product. Currently, cultivated meat is not a natural sister product of existing meat, and it is difficult to have substantial equivalence with meat because, as stated above, completely different manufacturing processes are used to achieve mass production *in vitro* and to make it similar to real meat (Choi et al., 2021; Lee et al., 2020; Ramani et al., 2021). In this regard, cultivated meat is, in general, classified as a novel food, as it has no historical record. The EU, Canada, Australia, Singapore, Korea, and other countries have adapted the principle, while the US may use the term GRAS (generally recognized as safe) (Sergelidis, 2019). Recent regulatory approval for cultivated chicken and shrimp by

Table 1
Summary for various type of stem cells to produce cultivated meat.

	Embryonic stem cells	Induced pluripotent stem cells	Mesenchymal stem/stromal cells	Muscle stem cells	Cells derived by trans-differentiation technique	Immortalized cell lines	Genetically modified cells
Definition	Stem cells derived from pluripotent ICM or epiblast in early blastocyst.	Stem cells induced by the overexpression of pluripotency-related genes	Mesodermal precursor cells isolated from various tissues.	Stem cells for producing muscle tissues	Differentiation technique into desired cell type using reprogramming methods	Cell lines with spontaneously or artificially acquired extended longevity.	Cells with features enhanced or edited by genetic manipulations
Origins	Blastocysts	Somatic cells	Bone marrow, adipose tissues etc.	Muscle tissues	Somatic cells except for (excluding) myoblasts	Tissue-specific stem cells, somatic cells	Myoblasts, MSCs
Differentiation potentials	Differentiate into all types of cells in our body including muscle and fat tissues	Differentiate into all types of cells in our body including muscle and fat tissues	Differentiate into mesoderm cells such as adipocyte, chondrocyte, osteocyte, etc. not into myoblasts	Differentiate into only muscle fibre	Differentiate into muscle and fat tissues using different combinations of transgenes from different types of cells	Defects in myogenic differentiation due to continuous proliferation	Can be applied on both muscle and fat production
Proliferation ability	Infinite	Infinite	Restricted by aging	Restricted by aging	–	Infinite	Restricted by aging
Technical difficulty for differentiation	+++	+++	++	+	++	+	+
Cost-effectiveness	++	++	++	+	++	+++	++
Main advantage	No need to sacrifice the animals	No need to sacrifice the animals	Ample previous data available, huge potential applicable to various tissues	Easy to differentiate into muscle fibres		No need to sacrifice animals, cost-effective	
Present drawback	Hard to derive cell lines, Low differentiation rate to myoblasts	GMO issue, Low differentiation rate to myoblasts	Consistent sacrifice of animals for cell isolation	Consistent sacrifice of animals for cell isolation	GMO issue	GMO issue	GMO issue
Hazardous after ingestion	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
Entry barrier (regulation, industrialization)	Efficient differentiation method development	Regulations for GMO, Efficient differentiation method development	Efficient differentiation method development	Mass culture method development	Regulations for GMO	Regulations for GMO	Regulations for GMO

[a] Not expected to be an inhalation hazard after the regular cooking process of this material.

the Singapore Food Agency in 2020 adapted the novel food policy as well.

In terms of safety considerations, donor animals, origin and stage of the cells, cells before and after storage, manufacturing processes and ingredients, and cultured muscle after harvest should be listed and verified by an effective and reliable management scheme (Table 2: modified from a previous report (Lee et al., 2020)). Most materials (e.g., chemicals, nutrients, growth factors, scaffolds) for cultivated meat production are not approved yet for food use. In line with safety, the establishment of the cultivated meat standard and manufacturing process will help the industry to further develop other values (e.g., purity and density of muscle stem cells from their origin, cells-scaffolds ratio). In this regard, the establishment and adaption of harmonized regulation will secure the safety of cultivated meat after open discussions with various fields, regions, and people of interest. Recently, organizations in the United Nations, such as the World Health Organization and Food and Agriculture Organization have started to discuss the agenda with member countries. The US FDA's recent decision on safe-to-eat for human consumption of cultivated chicken meat may facilitate these actions.

Table 2
Potential considerations for safety management of cultivated meat.

Subject	Potential consideration	Safety management item
Donor animal	Biological source	- Common name - Breed - Nomenclature
	Carcass information	- Origin - Sex - Age - Registration number (if possible) - Disease infection - Pathogen - Chemical residue
Tissue	Biological source	- Common name (if not possible, muscle name instead)
Production process	Cell line establishment	- Detailed method - Equipment and used materials - Cryopreservation condition - Verification before/after cryopreservation - Risk assessment for materials (if using non-edible ingredients)
	Cultivated meat production	- Culture condition and materials - Equipment - Risk assessment for materials (if used non-edible ingredients) - Equipment and used materials - Method for basal media elimination (if necessary) - Storage condition
Product verification	Cell line	- Type and purity - Morphology - Stability (especially before/after cryopreservation) - Unintended chemical residue/ microorganisms
	Cultivated meat	- Appearance (shape, colour, added odour) - Proximate analysis - Amino acid content - Mineral content - Cell proportion
	Safety	- Human effect data - Allergenicity - Toxicological information - Digestibility - Recommended intake

9. Perspectives

We are standing before the door to the cellular agricultural era. Humanity has undergone a paradigm shift across society with remarkable advancements through sequential industrial revolutions. Civilization was built away from hunting and gathering through the 'Agricultural Revolution'. In addition, we have witnessed the establishment of a capitalist economy through the 'Industrial Revolution', the increase in agricultural production through the 'Green Revolution', and the advancement of information and communication technology through the recent '4th Industrial Revolution'. Accelerating the trend of research and development of innovative technologies, the world is headed toward significant changes through another technological revolution. Biotechnology is a field that utilizes biological systems and organisms to develop new products or techniques, which started with agriculture, including breeding. Food production has been dramatically increased by the Agricultural Revolution via the utilization of seeds. Likewise, numerous researchers are attempting to shift the paradigm on the production of foods using stem cells, which are known as the seeds of tissues in the body.

Stem cells, as progenitor cells that can differentiate into various cells in the body, have been highlighted as a promising source for curing human diseases and deciphering biological molecular mechanisms for a long time. Recently, through advanced stem cell technologies, animal muscle stem cells derived from various origins have been able to organize artificial tissues *in vitro* by supplementing nutrients, making developing food materials possible. Furthermore, those technologies have been expanded to new cellular agriculture products including leather, fur, and milk without raising domestic animals. In this respect, the paradigm shift to the production of sustainable agricultural products using stem cells could be called the 'Cell Revolution'. Just as human life has improved through the Agricultural Revolution and the Green Revolution, cultivated meat as the starting point of the 'Cell Revolution' can diversify our lives through expansion and consilience to various fields of industry. Accordingly, in order to pave the way for revitalizing cellular agriculture, it is crucial to further advance the basic technology derived from cultivated meat research and establish an industrial ecosystem and infrastructure. The next wave of stem cell research is coming along with the development of the cellular agricultural industry, which will open the ('Brave') new world.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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