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Advances and Challenges in Cell Biology for Cultured Meat

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Abstract

Cultured meat is an emerging biotechnology that aims to produce meat from animal cell culture, rather than from the raising and slaughtering of livestock, on environmental and animal welfare grounds. The detailed understanding and accurate manipulation of cell biology are critical to the design of cultured meat bioprocesses. Recent years have seen significant interest in this field, with numerous scientific and commercial breakthroughs. Nevertheless, these technologies remain at a nascent stage, and myriad challenges remain, spanning the entire bioprocess. From a cell biological perspective, these include the identification of suitable starting cell types, tuning of proliferation and differentiation conditions, and optimization of cell–biomaterial interactions to create nutritious, enticing foods. Here, we discuss the key advances and outstanding challenges in cultured meat, with a particular focus on cell biology, and argue that solving the remaining bottlenecks in a cost-effective,

scalable fashion will require coordinated, concerted scientific efforts. Success will also require solutions to nonscientific challenges, including regulatory approval, consumer acceptance, and market feasibility. However, if these can be overcome, cultured meat technologies can revolutionize our approach to food.

1. INTRODUCTION

Cultured meat (also referred to as cultivated meat) is a nascent biotechnology sector that aims to produce meat from cell culture rather than from the raising and slaughtering of livestock. This technology, along with other forms of cellular agriculture, has gained significant attention in recent years due to its potential to significantly reduce the negative externalities associated with the traditional meat and seafood industries, particularly with respect to environmental sustainability (such as greenhouse gas emissions and land and water usage), animal welfare, and food safety (1, 2). The first proof of concept, a burger produced from bovine primary stem cells, was unveiled a decade ago (3), and early products are now entering the market.

The process of cultured meat production involves the isolation and in vitro proliferation of animal cells (typically mammalian, avian, or fish) at a vast scale, usually prior to differentiation toward the tissue types found in meat (primarily skeletal muscle and fat). Many of these methods draw inspiration from the cell and gene therapy (CGT) industry, where cells have been harvested and manipulated outside of the body for medical applications for several decades (4), but price and scale considerations differ markedly. Various cell types can be used for this purpose, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), primary cells, and immortalized cell lines (of various flavors; **Figure 1**) (5). While the choice of starting cells can impact regulatory and marketing, as well as scientific, aspects of the technology, all strategies for cultured meat production involve the detailed understanding and accurate manipulation of stem cells throughout a complex, multistep process.

We review the most important recent advances in cultured meat science, with a particular focus on cell biological aspects of process development. We highlight the key challenges that remain to be addressed to bring cultured meat to market and explore promising avenues for future development.

Cellular agriculture: technologies that produce agricultural products, including meat and dairy, from cell cultures

Cell and gene therapy (CGT): medical treatments that repair or replace damaged tissues using living cells or genetic material

Embryonic stem cells (ESCs): totipotent stem cells derived from the inner cell mass of an embryo

Induced pluripotent stem cell (iPSC): pluripotent stem cell generated via the reprogramming of somatic cells to express embryonic transcription factors

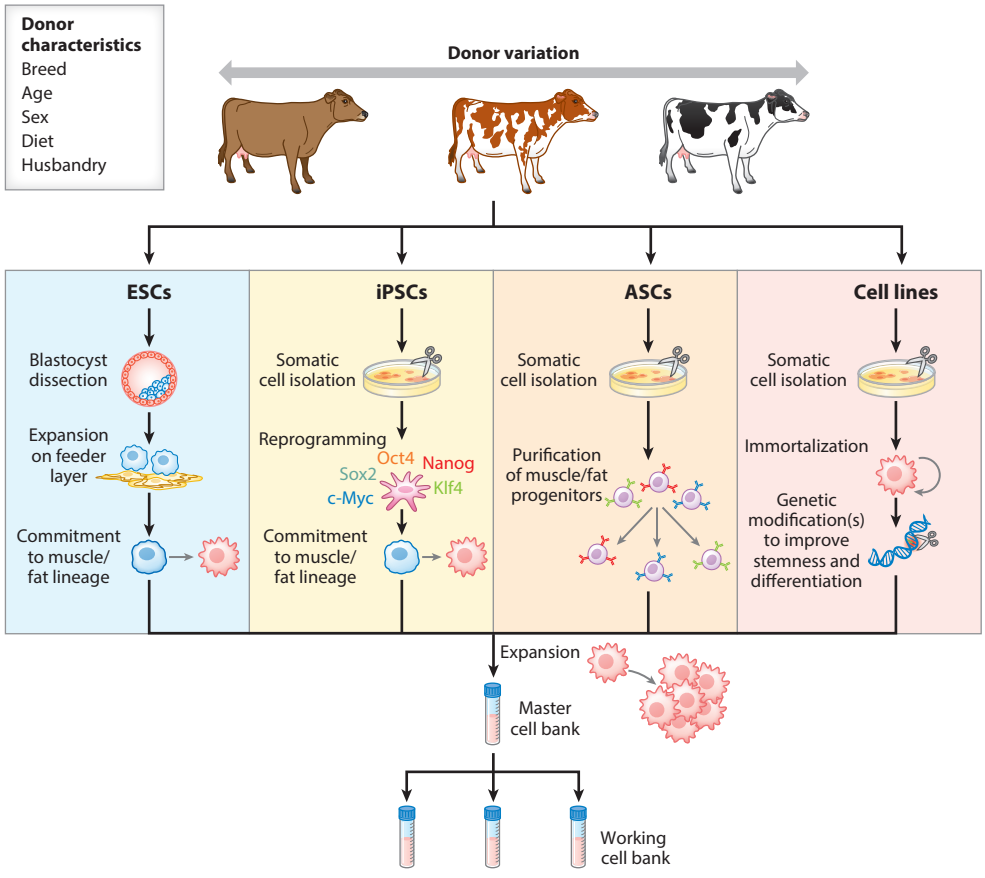
Primary cell: cell isolated directly from an organism and cultured without modification

Immortalized cell line: cell line that has spontaneously or otherwise acquired the ability to undergo indefinite cell division, bypassing normal cellular senescence checkpoints

2. ESTABLISHMENT OF CELL BANKS

Cultured meat production creates an unprecedented demand for highly pure and proliferative cells. While cell banking production and quality procedures are well established in the CGT industry, the vastly increased cellular requirements for food production necessitate large-scale culture methods and upscaled cryopreservation solutions, even for the most upstream steps of production. The exorbitant costs of current cell banking practices are also prohibitive for direct translation to the food industry. As for many aspects of cultured meat production, scale and cost thus represent significant challenges for cell banking.

The first decision required during cultured meat bioprocess design is selection of a starting cell type (**Figure 1**), which has numerous ramifications for the downstream process (5). Whereas ESCs, iPSCs, and immortalized cell lines offer clear advantages over primary cells in respect to their ability to proliferate indefinitely, they also come with regulatory, consumer acceptance, and, particularly for ESCs, ethical concerns (6). Ideal cells should be easy and efficient to isolate, highly proliferative in low-cost media, responsive to simple differentiation protocols, and robust to environmental changes. Even within primary cells, many options abound. For cultured fat production, for example, options include fat- or bone-derived mesenchymal stem cells (7, 8), dedifferentiated



	ESCs	iPSCs	ASCs	Cell lines
Advantages	• Unlimited proliferative capacity • Not genetically modified or reprogrammed	• Fairly easy to obtain • Unlimited proliferative capacity	• Easily obtained • Not genetically modified or reprogrammed • Established differentiation protocols available	• Unlimited proliferative capacity • Customizable to process needs
Disadvantages	• Ethical and consumer acceptance queries • Lack of well-defined protocols for differentiation in livestock species	• Low yield of genetic reprogramming • Lack of well-defined protocols for differentiation in livestock species	• Limited proliferation capacity • Need for frequent starting samples	• Difficult and expensive to derive • Consumer acceptance queries

Figure 1

Cell banking considerations for cultured meat production. Cell banks can be established from a variety of starting cell types and donor tissues. Although some approaches may be less susceptible to variability arising from donor animal differences, all rely on an initial tissue sample. Different strategies present different sets of challenges, and each has advantages and disadvantages that need consideration. Abbreviations: ASC, adult stem cell; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell.

fat cells (9), and even trans-differentiated muscle satellite cells (SCs) (10). However, most intramuscular fat in vivo derives from a population of mesenchymal cells found in skeletal muscle called fibro-adipogenic progenitor (FAP) cells (11), which mediate fatty and fibrotic tissue accumulation in muscle and hence might be more likely to recapitulate traditional intramuscular fat (12).

Satellite cell (SC): muscle-resident stem cell population with myogenic potential

Ultimately, the choice of cell type for cultured meat production depends on the specific needs of a particular production process and philosophy, making it hard to draw robust conclusions at this early stage of the field as to which strategies will ultimately be widely adopted.

Fibro-adipogenic progenitor (FAP) cells: muscle-resident stem cells with adipogenic potential

Regardless of the chosen strategy, cell banking requires the isolation of stem cells from a tissue sample, and donor animal selection is thus an important question. Cultured meat products have been developed from various species (13), but these cells may exhibit vast differences in growth behavior and medium requirements (14), posing potential hurdles for upscaled production. Age, sex, breed, and the sampling site itself may also affect stem cell yield and quality (15). For instance, proliferation rates can vary among cattle breeds (16). However, although numerous characteristics might be relevant, there is a dearth of literature comparing these traits with respect to cultured meat. Indeed, it is unclear to what extent physiological differences between donor animals are reflected in the yield and quality of derived stem cells at all. As the cultured meat field grows, producers may have greater control over other aspects of donor biology, both intrinsic (influencing genotype via selective breeding) and extrinsic (such as diet and husbandry). The extent to which manipulation of these factors can help to optimize cultured meat production processes remains unclear.

Primary cell-based bioprocesses in particular will require repeated sample collection (17), as well as isolation of stem cells on an unprecedented scale, hurdles that may prove challenging to resolve. Development of equipment for minimally invasive collection of donor samples in an aseptic and reproducible fashion will be required. The key challenge inherent to all cell isolation processes is efficient but gentle extraction, so as to obtain high yields of quality cells. Often achieved through physical mincing and then enzymatic dissociation, semi- and fully automated solutions for cell isolation will require tailoring to the tissue of interest and be able to accommodate differences arising from donor and sampling variability (18). The resulting cultures invariably consist of a mixture of cells and require further purification. This problem has been solved in various industries in several ways, though large-scale purification is likely to remain challenging from a cost perspective. For bovine stem cells, advances have been made in the characterization of the cellular heterogeneity of starting samples, which helped to identify reliable antibody panels for flow-based or magnetic cell sorting (12, 19, 20). Such strategies tend to result in significantly higher yields and purities than pre-plating or density-gradient centrifugation methods (21). Nevertheless, development of antibody panels for agriculturally relevant species is a challenge that should not be underestimated (in our experience, only approximately a quarter of human antibodies have been compatible with bovine). Cell-type-selective media formulations and attachment factors may help to enrich the desired cell type(s) in heterogeneous cultures, helping avoid technically and economically challenging cell purification procedures (12, 20). Similarly, whereas protocols for the generation, purification, and differentiation of ESCs and iPSC lines from model species are well-defined and thoroughly described in the literature, research on these cell types from livestock species is limited to a small number of recent studies (22, 23).

Cell banking for cultured meat will necessitate cell cryopreservation on hitherto unseen scales, regardless of cell type (24). Although existing solutions for large-scale bag-freezing of cells will initially be feasible, new solutions will be required as the industry scales. Large-scale automation of processes such as handling, filling, and labeling will be essential for standardization and optimal cell preservation. Cell banking is likely to be somewhat centralized, raising logistical and economic challenges related to the transport of large quantities of cryopreserved cells, should cultured meat scale to the point where it replaces a significant portion of the traditional meat industry. Chemically defined and animal component–free cryopreservation media are already available and will be the norm for regulatory reasons, although further cost reduction will be required.

A final major challenge with respect to cell banking is quality and safety assurance. Although some principles can be transferred from the CGT industry, diverging cell sources and downstream uses, along with the economics of the respective industries, will necessitate different approaches. Master and working cell banks must be subjected to rigorous testing to ensure freedom from adventitious agents (such as viruses and *Mycoplasma*); that cells are of high purity; and, crucially, that they are genetically stable (25). The scale of testing required will necessitate the development of new assays, with high accuracy and low cost, regardless of the cell types or strategies used. For example, detection and identification of cell culture contaminants by next-generation sequencing could be an approach with broad applicability (26). Overall, whilst cell banking certainly presents substantial challenges for cultured meat production, with further development and economies of scale, solutions likely will be found to overcome these hurdles.

Stemness: capacity of a stem cell to proliferate and differentiate

Population doubling (PD): number of times a culture of cells has doubled in number through mitotic division

Fetal bovine serum: blood-derived product obtained from fetal calves, commonly used as a supplement in cell culture media

Extracellular matrix (ECM): complex network of proteins, glycoproteins, and polysaccharides that surrounds and supports cells in tissues

3. PROLIFERATION AND STEMNESS

Cultured meat processes rely on the extensive proliferation of cells to generate sufficient biomass. Cell biological optimization of these promitotic conditions is critical to achieve proliferation phases that are robust, efficient, cost-effective, and animal-free. Choice of cell type dictates the challenges to an extent: Whereas primary stem cells invariably lose proliferation and differentiation capacity during long-term culture (a phenomenon described as loss of stemness), stable maintenance of pluripotent cells can require complex, expensive medium formulations (27, 28). Understanding how and why cell biology changes during proliferation will inform the design of interventions allowing longer expansion phases and differentiation at higher population doublings (PDs), increasing cultured meat yields and reducing process variability and costs.

The first challenge is achieving robust proliferation with medium formulations that are free of animal-derived components, particularly fetal bovine serum. In vivo, the stem cell niche [including neighboring cell types and the extracellular matrix (ECM)] provides intricate, context-dependent signals that direct cell behavior, which are hard to recreate in vitro. Nevertheless, serum-free medium formulations for proliferation of various cell types, including bovine SCs and FAPs, have now been developed, an important advance for the field (29, 30), whereas serum-free medium for iPSCs has been available for some time (31). These typically include albumin and cocktails of growth factors that activate important pathways related to proliferation and stemness (30, 32, 33), although formulations must be optimized for each particular cell type and species. These components are costly, and the switch to food-grade components and/or alternatives with similar performance is the next step in media development (34). Recombinant glycoprotein attachment factors also represent a significant cost in some processes (20, 35), and replacement of such factors with peptides containing adhesive motifs (such as RGD or YIGSR) is a promising area.

Maintenance of prolonged proliferation capacity in these media is a further cell biological challenge. During long-term culture, primary cells invariably enter a senescent state characterized by permanent cell-cycle exit, widespread gene expression changes, and striking cellular flattening and enlargement (36, 37) (**Figure 2**). Decreases in proliferation occur even prior to full senescence (reviewed in 38). In bovine SCs, proliferation rates drop to approximately 0.6–0.8 PDs per day until cells enter senescence at 20 to 30 PDs (19, 30), whereas the observed proliferative limit in FAPs is slightly higher (12). For industrial cultured meat production, proliferative capacity must be extended well beyond these limits (17). The causative relationship between cellular aging and decreased proliferation is key to understanding and addressing this challenge. Aging is characterized by an array of cellular changes, including altered signaling pathway activity, shifts in metabolism and morphology, and the accumulation of mutations at the genetic and epigenetic level (38). Various interventions are thus promising. Inhibition of p38 α/β MAPK signaling with

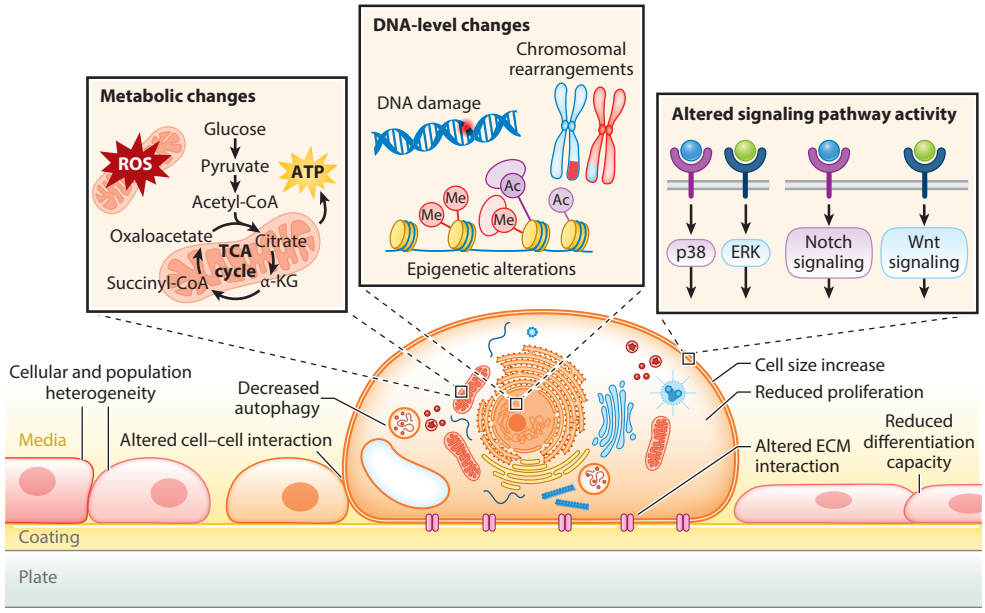


Figure 2

Hallmarks of cellular aging in vitro. Aging primary stem cells show a decreased proliferation and differentiation capacity, accompanied by an array of intracellular changes that affect cell size, morphology, and function, as well as interaction with the extracellular environment. Abbreviations: ECM, extracellular matrix; ROS, reactive oxygen species; TCA, tricarboxylic acid.

small molecules, such as SB203580, increases proliferation and differentiation capacity in bovine SCs (19), and high-throughput screening for compounds targeting other pathways may identify combinations that maintain stemness without resort to genetic engineering techniques (Section 7). Concentrations of NAD^+ decrease as cells approach senescence (39), and media compositions likely will need to be adjusted to account for (or avert) metabolic changes in glycolysis and oxidative phosphorylation. The extent to which DNA damage and/or epigenetic changes underlie cell aging phenotypes remains unclear. Telomere shortening eventually induces DNA damage response signaling (36, 40), and changes in DNA methylation at loci previously linked to senescence have been observed in various cell types (41). Limiting generation of reactive oxygen species, for example, by culturing cells in hypoxic conditions (42, 43) or by stimulating autophagy in aging cells (44), could be a promising approach to reduce oxidative stress and maintain proliferative capacity.

Primary cells also show a decline in differentiation potential with increasing PDs (12, 30, 33) prior to signs of senescence emerging, suggesting that loss of proliferation and differentiation capacity may be partly independent processes. Epigenetic changes may explain this reduced differentiation capacity; methylation of myogenic genes varies considerably in SCs from old and young muscle (45, 46). Several age-related signaling pathways have been identified that affect differentiation; SCs from aged donors show increased p38 α/β MAPK, impaired Notch, and alterations in Wnt signaling (47–49). Notch and Wnt are also linked to lower adipogenic potential in FAPs of aged animals (50). Whether these are also the key drivers in vitro remains to be elucidated, although targeting these pathways through media supplementation with growth factors (including fibroblast growth factor, hepatocyte growth factor, and insulin-like growth factor 1) has been shown to promote long-term culture of SCs (51). Other pathways that might

be important include JAK/STAT (Janus kinase/signal transducer and activator of transcription), PDGF (platelet-derived growth factor), and Hippo (52, 53). Compared with growth factors, small molecules that target distinct enzymes have the advantage of higher specificity but might also face different regulatory hurdles. Another important parameter in this respect could be substrate stiffness, which cells can sense in several ways. In vitro, cells encounter a stiffer and less complex extracellular environment, and increased ECM-related gene expression is a feature of cell adaptation and aging (54, 55). Ultimately, substrate stiffness for different cell types must be tested empirically but could offer routes for optimization; for instance, MYOD1 expression is highest in SCs cultured on 15-kPa substrates when compared to 5, 30, or 45 kPa (56).

A final challenge is cellular heterogeneity, which can occur through overgrowth by contaminating cell types or the emergence of different subpopulations or cellular states and can affect growth rates and variability of proliferation phases (20). Single-cell technologies have increased understanding in this respect (57), demonstrating that FAP cultures, for example, often contain both adipogenic and fibrogenic subpopulations (55). In SCs, subpopulations with differing self-renewal capacities may emerge upon serial proliferation (58). Less hierarchical models suggest that SCs in vitro may exist in parallel cell states that are dynamic and interchangeable during one culture passage, and which might change during cellular aging (20). Without tight controls, similar heterogeneities can also emerge within pluripotent cell cultures (27). Optimizing cultures to promote specific subpopulations or states is an important consideration for the design of proliferation phase conditions, which ultimately still represents one of the most challenging areas for cultured meat development.

4. DIFFERENTIATION (SIGNALING)

4.1. Muscle

Although some early cultured meat processes do not involve a differentiation step, consumer acceptance requires accurate mimicry of traditional meat, necessitating the creation of mature muscle fibers that recapitulate the full functionality, taste, and texture of skeletal muscle. Differentiation of SCs in vivo is well understood biologically (59, 60) and involves the induction of cell cycle arrest, sequential expression of master transcriptional regulators, and the fusion of myoblasts into multinucleated myofibers (**Figure 3**). However, recreating these steps in vitro, with additional constraints with respect to cost and use of animal-derived biomaterials, presents a major challenge.

Myogenic differentiation is an intricate process, involving interactions of multiple cell types and extracellular factors. In vitro, it is traditionally induced by serum starvation (an abrupt withdrawal of serum), which promotes a powerful cell cycle arrest, in turn initiating the differentiation cascade (61). Achieving a similar cell cycle arrest, and activating the same transcriptional programs in an animal-free fashion, while also compensating for the lack of stem cell niche, represents a tricky conundrum. Most culture methods replicate only a subset of the physiological inputs that impact SC differentiation during muscle regeneration in vivo, which include innervation, hormonal signaling, vasculature, and nutrition (62, 63). Generating serum-free formulations that drive robust differentiation across a range of donor animals (and even species) has proven to be challenging, but recent progress has been made using transcriptomic approaches to identify cell-surface receptors [including LPAR1 (lysophosphatidic acid receptor 1)] upregulated during the early phase of differentiation (33). Supplementing serum-free basal medium with ligands for these receptors resulted in differentiation efficiencies comparable to serum starvation. Further small molecules, including ERK (extracellular signal-related kinase) inhibitors, that drive robust differentiation and myoblast fusion in the absence of serum starvation have subsequently been identified (64). Nevertheless, major challenges remain with respect to understanding the complexity of these

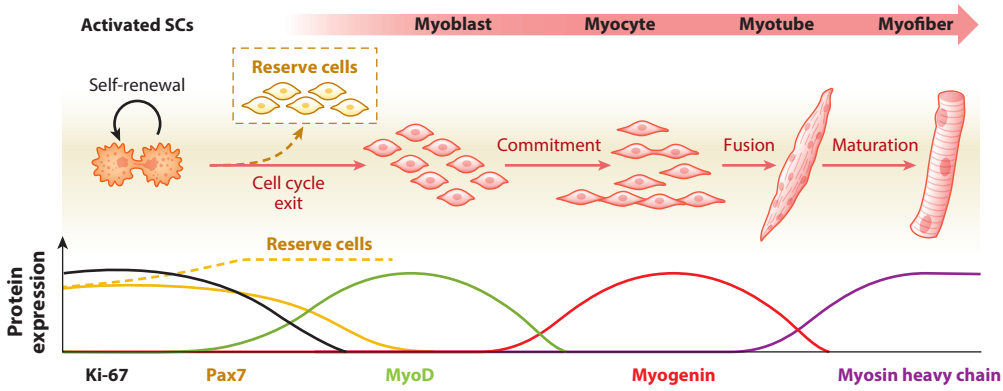


Figure 3

Overview of myogenic differentiation. Activated SCs differentiate into myoblasts or adopt a quiescent reserve cell phenotype that maintains the muscle stem cell pool. Myoblasts further differentiate into myocytes, which fuse to form multinucleated myotubes and subsequently mature into myofibers. This process is regulated by a hierarchy of transcription factors, including MyoD and myogenin, which drive the expression of muscle-specific genes. Abbreviations: MyoD, myoblast determination protein; Pax7, paired box protein 7; SCs, satellite cells. Figure adapted from Reference 60 (CC BY 4.0).

signaling pathways, and with the costs associated with stimulating them. For more pluripotent starting cell types, such as ESCs and iPSCs, robust induction of myogenic differentiation is likely to be even more challenging (65).

Cellular heterogeneity during myogenic differentiation in vitro poses an additional challenge (66). In vitro reserve cells (RCs, **Figure 3**), which are specified during differentiation but do not express myogenic transcription factors, reflect some properties of quiescent satellite cells in vivo where self-renewal ability is critical to the physiological repair of muscle. However, they are undesirable for cultured meat purposes because they reduce the proportion of differentiated cells (67). Although still poorly understood, advances have been made with respect to the signaling pathways that regulate RC formation. Notably, inhibiting Notch signaling can induce fusion of a subpopulation of RCs (68), and further adjustments could likely minimize the presence of RCs. Reaching the highest levels of maturity, with as many functional characteristics of in vivo muscle fibers as possible [including high concentrations of actomyosin, sarcomere organization (69), and myofiber contractility (70)], will nevertheless require further advances. One area of research in this respect is the development of culture medium formulations that support the maturation of fused cells into protein-rich myotubes. Several small molecules, including Notch and transforming growth factor-β1 inhibitors, have been identified as potential enhancers of maturation in iPSC-derived myotubes (71). Even with fully optimized media, however, 2D culture systems are fundamentally limited, highlighting the importance of tackling differentiation in the context of 3D muscle constructs (Section 5.1).

Reserve cells (RCs): quiescent, non-differentiating population of cells that emerge during myogenic differentiation

4.2. Fat

Although composed mainly of skeletal muscle tissue, fat is also essential for achieving the texture and flavor of meat (72). Adipogenesis is a complex, dynamic process during which cells undergo a significant shift in metabolism and accumulate large amounts of lipids (predominantly triglycerides) (73). This transformation drives the cells to become fully differentiated adipocytes: large spherical cells containing a unilocular lipid droplet (**Figure 4**). In living tissue, this process is affected by interaction with neighboring cells, surrounding ECM, and systemic physiological cues

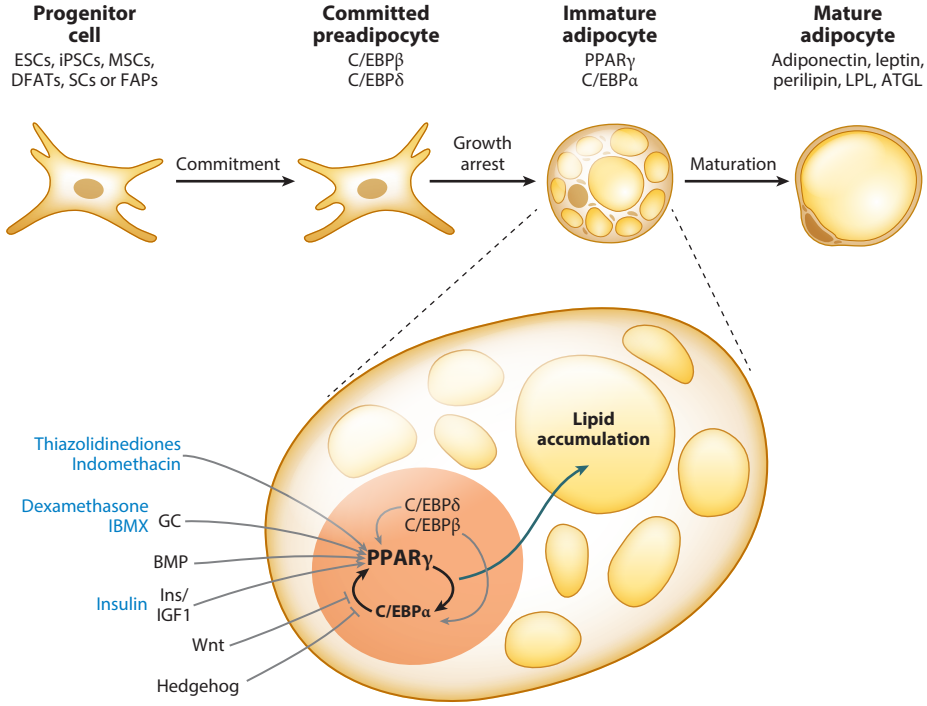


Figure 4

Regulation of adipogenesis in vitro. Adipogenic differentiation is regulated by waves of transcriptional activity. Inducers in the differentiation medium (*blue*) activate master regulators of adipogenesis, including PPARγ and C/EBPα. Signaling molecules like glucocorticoids, BMPs, and insulin can activate PPARγ and/or C/EBPα to induce preadipocyte differentiation, while other pathways suppress adipogenesis through direct inhibition of PPARγ–C/EBPα or other pro-adipogenic signaling cascades. Abbreviations: ATGL, adipose triglyceride lipase; BMPs, bone morphogenetic proteins; C/EBP, CAAT/enhancer binding protein; DFATs, dedifferentiated fat cells; ESCs, embryonic stem cells; FAPs, fibro-adipogenic progenitors; GC, glucocorticoid; Ins/IGF1, insulin/insulin-like growth factor 1; iPSCs, induced pluripotent stem cells; LPL, lipoprotein lipase; MSCs, mesenchymal stem cells; PPARγ, peroxisome proliferator-activated receptor gamma; SCs, satellite cells.

(74, 75). Replicating these interdependent signals in vitro is a difficult but critical challenge for cultured meat, which will lead to closer mimicry of animal fat tissue.

Adipogenic differentiation traditionally relies on the use of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine, combined with peroxisome proliferator-activated receptor (PPAR)γ activators such as thiazolidinediones or indomethacin (76) (**Figure 4**). These molecules function independently of serum; hence, animal-free differentiation of adipocytes has not proven as challenging to solve as for muscle. However, the food compatibility of such cocktails remains unclear (77). The differing nature of adipogenesis between model species (such as humans and mice) and ruminants presents a further challenge. For example, cattle use acetate rather than glucose as the principal precursor for lipogenesis (78). Mitić et al. (77) recently demonstrated that insulin and rosiglitazone are sufficient to induce fat differentiation in primary bovine, porcine, and ovine adipogenic precursor cells. Supplementation of cultures with lipid concentrates or single fatty acids is also known to induce lipid accumulation in fish and chicken (79, 80). However, whether this phenomenon is adipogenesis related, or simply passive lipid accumulation, is unclear. Furthermore, it can be costly and cytotoxic and may alter the lipid profile of the cells (81).

Hydrogel: 3D network of hydrophilic polymers that can absorb and retain water

Scaffold: 3D structure that provides mechanical support for tissue growth or differentiation

Aggregate: self-forming clump or cluster of cells that are physically connected by cell–cell interactions or via extracellular matrix

One of the main obstacles for cultured fat production is achieving a high level of differentiation in a robust and cost-effective fashion. Traditional 2D differentiation methods often lead to partially differentiated adipocytes with accumulation of lipids in small multilocular lipid droplets (76). Additionally, the current cost of reagents and high media:cell ratios, combined with lengthy differentiation times (often 4–6 weeks), make large-scale production of cultured fat unaffordable. One important advance will be the identification of more potent small molecules or bioactive compounds that directly activate master regulators of adipogenesis (including PPARγ and C/EBPα), increasing maturity and decreasing differentiation time. Development of 3D culture systems that better mimic the in vivo microenvironment and improve differentiation efficiency is also required to reduce the need for expensive and complex differentiation cocktails, and to achieve more consistent and efficient differentiation across different cell types and species (82).

Accurate imitation of the lipid composition of animal fat is another challenge. Understanding the metabolic pathways underlying fatty acid synthesis and tuning the media formulations to provide the ideal substrates are likely to be the main bottlenecks in this respect. There is also a need to develop more sensitive and high-throughput methods for detecting and quantifying lipid volumes and composition, particularly in small samples, for research and development purposes (83). Ultimately, addressing these challenges will be crucial for improving the nutritional and sensory quality of cultured meat. The challenge is to balance the cost and robustness of the differentiation process while still achieving a high level of differentiation efficiency and producing fat with desirable properties for use in food products. In the future, it may be possible to enhance the nutritional quality of cultured meat by modifying fatty acid composition, resulting in fat that contains lower levels of unhealthy saturated and trans-fats and higher levels of beneficial unsaturated fats, while still maintaining the authenticity of the final product.

5. DIFFERENTIATION (TISSUE FORMATION)

5.1. Muscle

Inducing myogenic differentiation in a robust fashion is necessary but not sufficient for the creation of edible skeletal muscle constructs. Various features of such systems are important for the formation of stable, scalable tissues, including cell physiology, medium composition, and mechanical and biochemical properties of biomaterials. Two-dimensional myogenic cultures are characterized by a low state of maturation, as cells adopt a random organization and detach from the substrate as they mature (84), but without careful engineering these issues can also occur in 3D tissue constructs.

Defining a low-cost, animal-free biomaterial for creation of hydrogels and/or scaffolds that support myoblast fusion and maturation remains a major challenge for cultured meat production and has been discussed at length elsewhere (85, 86). Cells must be able to adhere and interact, yet the material must also be degradable and provide mechanical resistance as contractility develops (**Figure 5**). Recent advances have included the use of soy and alginate as biomaterials for cultured muscle development, both of which are animal-free and food-grade materials with their own advantages and disadvantages. For textured soy protein scaffolds, additional functionalization is unnecessary for adhesion (87). Conversely, alginate can be used to create self-assembling, tunable hydrogels with beneficial mechanical properties and can degrade as cells migrate and secrete their own ECM (88, 89). For biomaterials that do not readily support cell attachment, functionalization with short peptide sequences is an attractive option. RGD-alginate is a well-studied system in tissue engineering (90, 91) and might offer a promising approach for cultured meat as well, although additional peptide sequences that more closely mimic native ECM might be beneficial for attachment, migration, and maturation. For cell types that proliferate readily in aggregates,

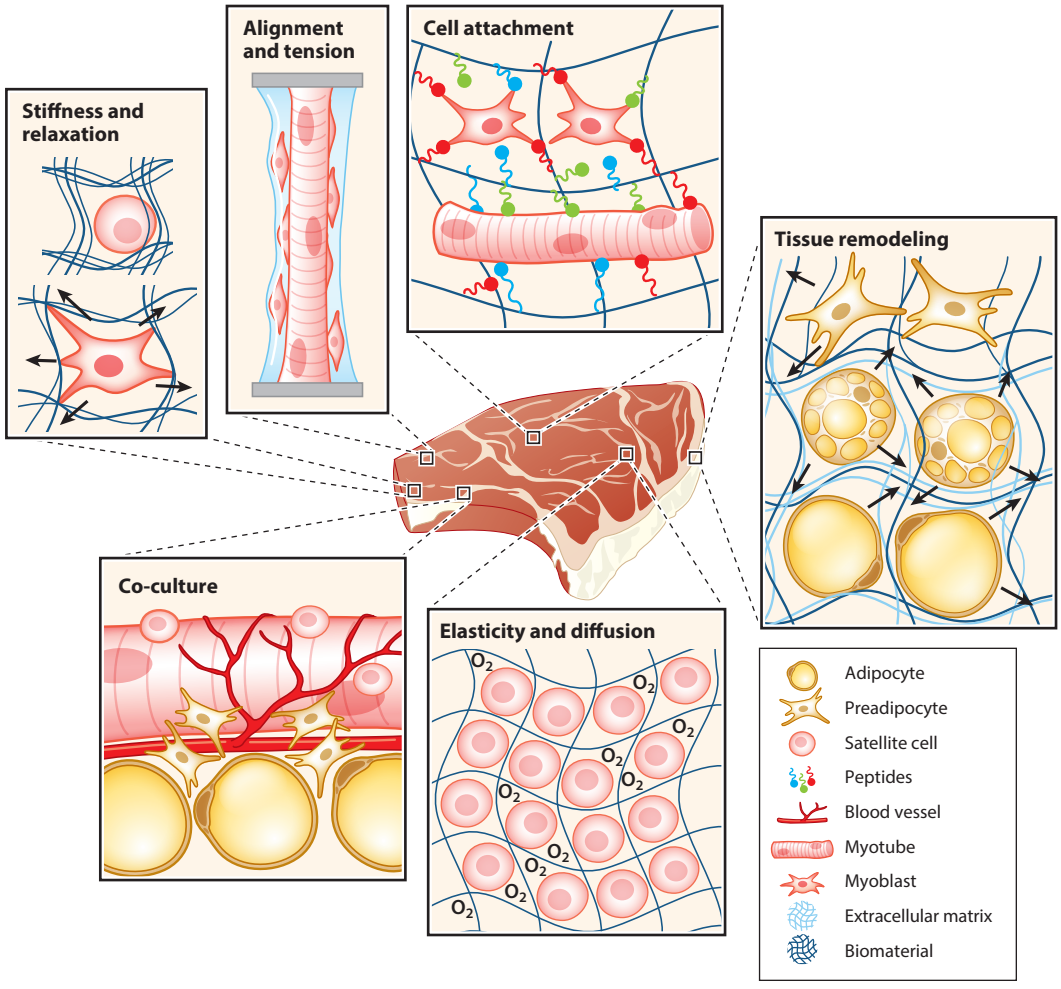


Figure 5

Biomaterial property considerations for optimal tissue engineering for cultured meat. A biomaterial must offer sufficient attachment, stability, and flexibility for the cell type being cultured, as well as the tissue being produced, such that sufficient differentiation and maturation can occur. Three-dimensional constructs will inevitably be limited in size without active transport of gases and nutrients.

differentiation in situ is also an attractive option from a bioprocess and economic perspective (because additional biomaterials and/or disaggregation steps are not required), although the level of differentiation obtained remains poorly understood in such systems (92, 93).

As mentioned (Section 4.1), achieving highly mature muscle is another major challenge that must be solved to develop products with sensory and nutritional characteristics matching those of traditional meat. Differentiation phases in cultured meat processes are typically on the order of 7–14 days, compared to months or years in vivo, although the speed with which muscle regeneration can occur in vivo suggests that achieving maturation on these timelines is not insurmountable. Once 3D culture systems are robust enough to allow fusion and early maturation, electrical pulse stimulation can be applied to stimulate contraction, promoting maturation and muscle protein accumulation (94). How electrical pulse stimulation might be deployed in large-scale bioreactors

Bioreactor: large vessel for producing biological products, such as cells

is unclear, however, and supplementation of acetylcholine (95) or other small molecules may represent a more scalable approach in this context.

5.2. Fat

Adipogenic tissue engineering poses related but distinct challenges to those discussed for muscle. Compared to highly structured skeletal muscle, fat tissue comprises more loosely organized connective tissue connecting large numbers of adipocytes (74), and lower degrees of cell motility and interaction are required during differentiation. Tissue constructs must nevertheless provide the flexibility to mature and sufficient cohesion to create a tissue-like structure (**Figure 5**). Several viable strategies for cultured fat production have been described in recent years and are reviewed extensively elsewhere (96, 97).

In the case of aggregate-based proliferation, an important decision is whether to use a hydrogel or scaffold for differentiation at all. Adipogenesis can occur in aggregates, as evidenced by adiponectin secretion and accumulation of lipid droplets (98), simplifying the process and avoiding costs and challenges related to biomaterial degradation and off flavors. However, a key advantage of some biomaterial-based systems, such as alginate hydrogels (12, 99), is that they can be extruded in various shapes, including microfibers, sheets (97), or beads, potentially allowing for larger and more complicated structures. Cell encapsulation also provides stability and cohesion, protecting against shear stress in bioreactor contexts (100). Regardless of the chosen strategy, the primary tissue engineering challenge remains ensuring proper gas and nutrient exchange, which limits the size of 3D constructs unless vascularization (or other channeling to the construct interior) can be achieved. Both solutions are possible on a laboratory scale but have cost, robustness, and scalability limitations that make them unsuitable for cultured meat applications without further development (96, 97).

Achieving full adipocyte maturation in short time frames is a further challenge in the design of cultured fat constructs. Unlike for muscle, it is unclear whether cell attachment to the substrate is required at all for adipogenesis, given observed results in alginate constructs devoid of functionalization, although contributions from cell–cell contact and attachment to self-deposited ECM cannot be discounted (12). Stiffness certainly has a major effect on differentiation, which tends to be better in hydrogels with lower Young's modulus (101). The cellular mechanisms underlying this are not fully understood, however; lower stiffness could contribute to increased induction of adipogenic pathways, improved flexibility within the biomaterial to allow lipid accumulation, or a combination of these and other factors. Hydrogels and scaffolds that do not require the use of animal-derived ECM or costly synthetic peptides are obviously preferred.

5.3. Co-Culture

The complexity of skeletal muscle arises from the function and interaction of multiple different cell types (102). To mimic this cellular heterogeneity in cultured meat products, co-culture approaches aim to differentiate at least two cell types in parallel, with several purported advantages (**Figure 5**). Paracrine or juxtacrine interactions between different cell types might improve the behavior of one or both. For instance, SCs cultured in the presence of endothelial and smooth muscle cells show increased alignment and higher expression of myogenic regulator myogenin on soy scaffolds, potentially through the deposition of ECM (87). Similar effects were observed when differentiating SCs in the presence of FAPs (103). Arguably the most tantalizing prospect of co-cultures is the introduction of vasculature that would allow increased thickness of tissues (104), and thus the production of cultured steaks.

However, although conceptually plausible, the technical difficulty of these approaches currently outweighs potential benefits. Vascularization by endothelial cells takes time, meaning cells

in the interior of thicker constructs are still deprived of nutrients. Furthermore, engineered vasculature in larger tissue constructs shows only limited functionality and stability. To differentiate multiple cell types in parallel, it is also necessary to create media that supports the co-differentiation of two or more cell types (105). Moreover, interactions between cell types may reduce, rather than increase, the extent of differentiation, as observed for the adipogenic differentiation of FAPs in the presence of SCs (103). Finally, bioprocess complexity increases considerably; multiple cell types must be proliferated either as co-cultures (introducing potential overgrowth effects) or as monocultures that are harvested and seeded simultaneously (introducing logistical challenges). Given the current technical challenges of co-culture and engineered vascularization, development of scalable cultured meat products that relies on parallel differentiation of multiple cell types is a tantalizing challenge that remains some way off. However, higher product complexity for improved texture and appearance might be achieved earlier through post-culture assembly of monocellular tissue constructs (106).

6. UPSCALING

Cultured meat requires the proliferation of cells at unprecedented scales. This expansion typically occurs in large bioreactors, which maximize cost efficiency through higher cell:medium ratios; reduced handling time; and active control of pH, dissolved oxygen, and temperature. The overriding challenge in these cultures is therefore to ensure sufficient nutrient delivery and waste removal at high cell densities. Understanding cellular responses to metabolic and mechanical stresses is thus critical for translating research-scale findings into robust and scalable bioprocesses (**Figure 6**). Overcoming biological limitations to process design, such as anchorage dependence (allowing for suspension growth of previously adherent cells), remains a challenge for future genetic engineering strategies (Section 7).

Cell density is a crucial operational target, as it directly determines both bioreactor yield and run cost. A major biological challenge is thus to leverage understanding of cellular metabolism to develop media formulations that maintain rapid growth, while minimizing production of growth-inhibitory metabolites such as ammonia (29). Understanding pathways involved in nutrient and waste metabolite sensing will inform design of interventions to metabolically reprogram cells for better bioprocess suitability (107). Although glucose is the primary carbon source fueling anabolic activity, excess can result in reduced proliferation (108). Glucose availability stimulates cellular anabolic responses through activation of the mammalian target of rapamycin complex 1 (mTORC1) (109). Amino acid supplementation also converges on mTOR signaling and is crucial to maintaining steady growth rates. Because mTOR controls several cellular responses, including cell size, proliferative rate, and glucose consumption rate, manipulating this signaling network offers intriguing possibilities for bioprocess optimization (110). Screening approaches to identify inhibitors have resulted in potential therapies to target aberrantly proliferating cells (111). For cultured meat, where cell proliferation is the ultimate goal, enhancing mTOR signaling via genetic or pharmacological strategies is underexplored and may prove a valuable direction for future research.

As a result of glucose and amino acid metabolism, waste metabolites, including lactate and ammonia, are released into the medium (112, 113). This accumulation can itself induce metabolic changes, resulting in altered growth profiles (114, 115) and protein production (116). Controlling accumulation of these metabolites is a key challenge for ensuring robust bioprocesses with maximal growth. In small-scale systems, regular medium changes are feasible. However, this solution is uneconomical at scale, with proliferation media typically accounting for 55–95% of the total cost of cultured meat (117). Other strategies include replacing glucose and glutamine with alternative sugars and amino acids (118, 119); for example, replacing glutamine with TCA (tricarboxylic

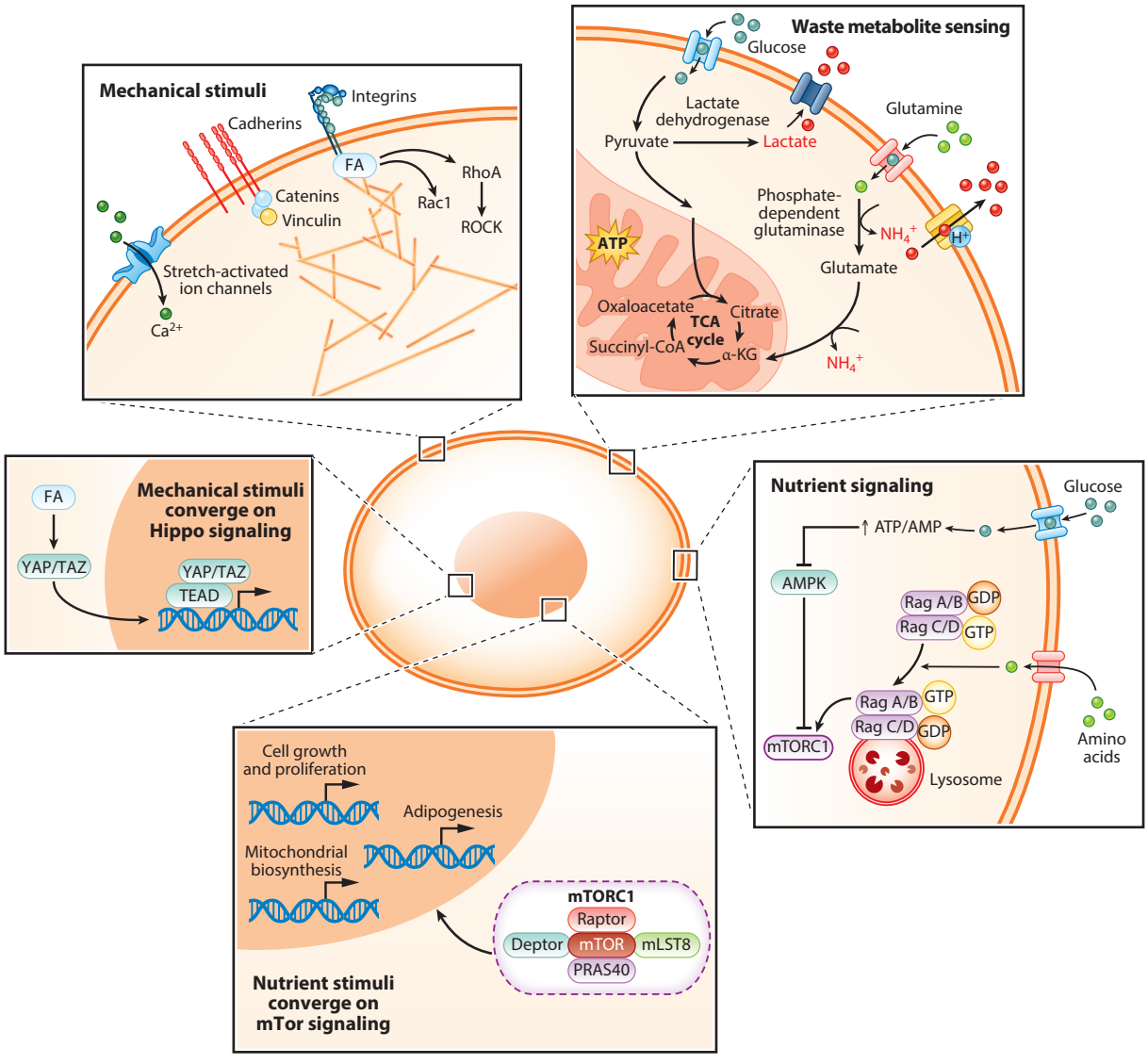


Figure 6

Cues and pathways involved in sensing of metabolic and mechanical stresses. Proliferating cells in upscaled systems, such as bioreactors, experience and sense a wide variety of stresses, the understanding and mitigation of which are crucial for achieving robust outcomes at scale. Similar considerations may apply for differentiation systems. Abbreviations: α-KG, alpha-ketoglutarate; AMPK, adenosine monophosphate-activated protein kinase; FA, focal adhesion; GDP, guanosine diphosphate; GTP, guanosine triphosphate; TCA, tricarboxylic acid. Figure adapted from images originally created in BioRender.

acid)–cycle intermediates such as α-ketoglutarate can reduce ammonia production (120). Nutrient concentrations required in the medium are largely set by the affinities of the cellular transport machinery, but because consumption rates are not uniform, medium recycling can also improve resource utilization. Partial recycling reduces the concentration of growth inhibitory metabolites, and given predictable rates of consumption, depleted nutrients can be topped up. Methods that have been explored to separate cells from medium include centrifugation and filtration (121), or

membrane-based filtration such as dialysis (122) and ultrafiltration/diafiltration (123). Challenges remain, however, with deploying these methods at scale, including contamination risk, nutrient loss, increased batch-to-batch variability, and potential cell death. Accurate in-line measurement of key metabolites is likely a prerequisite. From a cultured meat perspective, understanding the underlying biology that drives variation in nutrient consumption rate and designing interventions to minimize it are therefore central to a cost-effective scale-up.

Genetically modified organism (GMO): cell or organism with DNA altered using biotechnology to produce desired traits

A major challenge derived from the need for bioreactor cultures to be physically agitated is to ensure uniform nutrient and oxygen availability. The resulting turbulent flows generate shear stresses on cells (124), with higher rates of agitation in bioreactors largely correlating with reduced proliferation and increased cell death (125). Although the magnitude of these stresses can be approximated by metrics such as the Kolmogorov length scale or energy dissipation rate (126), the underlying cellular pathways that sense them remain to be elucidated. Shear stress can be partly alleviated by altering bioreactor design or through addition of protectants such as Pluronic F68 (125), but whether there exist endogenous mechanisms that can be exploited to protect cells from these stresses is an open question. Studies on fluid flows under controlled conditions show that low-shear regimes may actually trigger shear-protective signaling pathways; in endothelial cells, elevated bioavailable nitric oxide (NO) can act as a juxtacrine signal to prevent apoptosis (127). Even in myoblasts, hydrodynamic forces can induce NO production as a consequence of stretch-activated channel activity (128). Although not much is known about the efficacy of pharmacologically triggering NO signaling for its protective effects in bioreactors, it may prove to be a fruitful area of future research. Many mechanical stimuli sensed by cells converge on the Hippo signaling pathway, which controls nuclear localization of the transcriptional coactivators YAP (yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif). Mechanical stimuli from fluid forces block YAP activity and promote differentiation (129), whereas exogenous YAP expression can induce stemness in tissue-specific progenitor cells (130). Unpicking upstream regulators of Hippo signaling, or using LATS1/2 inhibitors to boost YAP activity (131), might also serve as a tool to modulate growth inhibitory or pro-differentiation cues induced by culture under turbulent flow conditions. There is therefore promise in leveraging fundamental cell biological insight to improve the physical stress tolerance of cells and complement engineering advances in low-shear bioreactor design.

7. GENETIC ENGINEERING FOR CULTURED MEAT

Aging of cells in vitro dramatically limits their proliferative and differentiative capacities, reducing protein and fat yields. For primary cell–based bioprocesses, there is a need to repeatedly sample new starting material from donor animals. Differences between samples and/or donor animals also hinder the generation of robust cell culture models for cultured meat research and development. Genetic engineering tools therefore offer significant advantages with respect to the establishment of optimized cell lines for production of cultured muscle and fat, but must be balanced against consumer and regulatory concerns, particularly with respect to genetically modified organisms (GMOs).

As discussed (Section 3), cellular life span is a major bottleneck in the use of primary cells for cultured meat bioprocesses. Proliferation requires phosphorylation of the retinoblastoma protein by cyclin-dependent kinases (CDKs) that drive cell cycle progression (132). However, DNA damage originating from telomere shortening and other stresses, including reactive oxygen species, leads to the accumulation of CDK inhibitors including p16 and p21, ultimately resulting in senescence and cell cycle arrest (40, 133). Abolishing this phenomenon is referred to as immortalization and offers significant advantages for cultured meat with respect to the robust, cost-efficient

scale-up of production processes without the need to sample new starting material. Whereas some approaches employ spontaneously immortalized cells (92, 134), other strategies involve the overexpression of telomerase, alone or in conjunction with the activation of cell cycle drivers (e.g., CDK4) or inactivation of cell cycle inhibitors (such as p16). The combination is particularly crucial for muscle, as telomerase alone is insufficient to immortalize muscle precursors (135, 136). Because immortalization could impede complex cellular pathways, such as myogenesis, one challenge will be to ensure that engineered cell lines still differentiate robustly. Here, initial reports found that immortalized SCs are transcriptionally and myogenically similar to their parental cells (137, 138), but also that differentiation potential decreases with age (139, 140). Importantly, spontaneously immortalized cells are not considered genetically modified, a benefit from regulatory and consumer acceptance standpoints (1, 141), although genetic drift is a potential concern (142). Alternatively, novel precision tools, including CRISPR base and prime editing (143), allow the alteration of single nucleotides to knockout or overexpress particular factors (such as p16, CDK4, or telomerase) via alterations in their respective coding sequences or promoters (144). In many jurisdictions, these cells will also not be considered GMOs, paving the way for novel food applications in GMO-skeptical geographies.

Alongside immortalization, genetic engineering can help bypass various cell biological shortcomings for cultured meat applications. Countless patents have now been filed in this direction, often aimed at reducing production-associated costs or improving final product quality. Some projects have focused on reducing cellular dependence on exogenous supplementation via genetic modification, including the generation of self-sufficient cells expressing a combination of growth factors (including insulin-like growth factor, fibroblast growth factor, insulin, and/or albumin) and the tuning of metabolic pathways to decrease the frequency of medium exchange during culture, for example, via overexpression of glutamine synthase (see **Table 1**). As discussed previously (Section 6), proliferation at industrial scales requires culturing cells in large bioreactors. For these vessels, cells that grow in suspension or as aggregates are ideal, whereas adherent cells may require microcarriers, adding extra layers of complexity to bioprocess upscaling. For this reason, iPSCs are a popular choice, because these can be grown in suspension and later differentiated into the desired cell type. Generating iPSCs requires genetic reprogramming of somatic cells, whereby transcription factors are ectopically expressed to achieve a pluripotent cell state (145). Alternatively, trans-differentiation methods, whereby differentiated cells are directly converted into another cell type (146), could allow the formation of multiple tissue types from a single cell line, simplifying bioprocess design and increasing efficiency.

Unsurprisingly, numerous patents have also been filed relating to the ability to control and promote differentiation by modulating expression of key myogenic or adipogenic transcription factors in various cell types (**Table 1**). Introduction of an inducible cassette driving the expression of MYOD1 is the method of choice to improve muscle differentiation in most cases. Transcription factor expression can also be modulated in a transient fashion, e.g., via transfection methods, although these are currently not feasible for large-scale applications due to protocol complexity and high cost. Engineered cell lines that produce collagen or other ECM factors for large-scale applications are another instance where genetic modification might help reduce upscaling costs. Recently, expression of myoglobin sequences derived from extinct mammoth in cultured ovine cells generated widespread interest.

Genetic engineering can thus help solve the majority of challenges in upscaling cultured meat production, particularly with respect to robustness and cost reduction. A major hurdle, however, will be to gain acceptance of genetic engineering similar to that seen in biomedical research, where several gene and cell therapies [such as chimeric antigen receptor T cells (147)] are approved and used in patients. Although there is no evidence of DNA transfer from food into the human

Table 1 Genetic engineering of cell lines for cultured meat production

Goal	Strategy	Example	Company/patent application
Life span extension/ immortalization	Cell-cycle inhibitor inactivation	CRISPR-based knockout of p16	Upside Foods (US20220251550A1)
	Cell-cycle driver activation	CDK4 overexpression	Upside Foods (US20220251550A1)
	Telomerase activation	TERT overexpression	Upside Foods (US20220251550A1), GOOD Meat (US20220183316A1)
	Reprogramming	iPSCs	Meatable (US20190338309A1[a])
Controlled differentiation	Lineage and differentiation specification	MYOD1-driven skeletal muscle differentiation	Upside Foods (US20210171912A1), Meatable (US20190338309A1[a]), WildType (US20200140821A1), Gourmey (WO2023281114A1)
Proliferation in carrier-free/suspension cultures	Induction of anchorage-independent growth	iPSCs	Meatable (US20190338309A1[a]), SCiFi Foods
Reduced dependence on medium composition	Medium recycling	Glutamine-synthetase-producing cells	Upside Foods (EP3638777A4)
	Growth factor production	IGF-producing cells	Tufts University (WO2022104373A1), Upside Foods (EP3638777A4)
	ECM protein production	Collagen-producing cells	Aleph Farms (WO2022043994A1)

[a]Meatable is licensing the technology described in this patent. Genetic engineering can be employed to immortalize cells or extend the life span of primary cells, control and improve differentiation, generate aggregate or suspension cultures, and reduce medium complexity.
Abbreviations: CDK4, cyclin-dependent kinase 4; CRISPR, clustered regularly interspaced short palindromic repeat; ECM, extracellular matrix; IGF, insulin-like growth factor; iPSC, induced pluripotent stem cell; MYOD1, myogenic differentiation 1; TERT, telomerase.

genome, the increased precision of modern gene editing tools might help to achieve this acceptance (148). Epigenetic editing and other techniques that can be used to engineer cells without genome alterations also offer promise in this respect (149). The US Food and Drug Administration's recent approval of Upside Foods' first product, including genetically modified chicken cells, offers a promising outlook for the future of genetic engineering for cultured meat (150).

8. CONCLUSION AND PERSPECTIVES

The development of cultured meat presents a promising solution for addressing major global challenges, including environmental sustainability, food security, and animal welfare. However, it is a complex and multifaceted problem that requires the solution of numerous interconnected challenges throughout the bioprocess, from cell isolation to tissue harvesting. To overcome these obstacles, a detailed understanding of the cell biology involved, along with its interface with other disciplines, such as tissue engineering, bioprocessing, and metabolomics, is essential (151). Although many remain, the pace of recent advances suggests that most scientific hurdles can be overcome through a combination of smart experimentation and empirical testing. Widespread adoption of cultured meat requires further development to achieve products that can compete with traditional meat in terms of price and taste, and achieving this goal will require significant focus on mimicry and cost optimization in the context of upscaled bioprocesses. Besides significant scientific progress, a host of other regulatory and commercial challenges must also be addressed. Overall, this enticing combination makes the field of cultured meat a compelling area of research at a crucial juncture.

DISCLOSURE STATEMENT

All authors were employees of Mosa Meat B.V., a company aiming to commercialize cultured meat, at the time of writing. All authors declare no other competing interests.

AUTHOR CONTRIBUTIONS

B.M., A.B., R.G.J.D., M.A.G., R.H., L.M., T.M., J.Pa., J.Pi., D.R., L.S., S.S., and J.E.F. researched and drafted the manuscript and designed the figures. B.M. referenced the final text. B.P.B. and J.E.F. edited the manuscript.

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