Prospects and challenges for cell-cultured fat as a novel food ingredient

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Abstract

Background

In vitro meat production has been proposed as a solution to environmental and animal welfare issues associated with animal agriculture. While most academic work on cell-cultured meat has focused on innovations for scalable muscle tissue culture, fat production is an important and often neglected component of this technology. Developing suitable biomanufacturing strategies for adipose tissue from agriculturally relevant animal species may be particularly beneficial due to the potential use of cell-cultured fat as a novel food ingredient.

Scope and Approach

Here we review the relevant studies from areas of meat science, cell biology, tissue engineering, and bioprocess engineering to provide a foundation for the development of *in vitro* fat production systems. We provide an overview of adipose tissue biology and functionality with respect to meat products, then explore cell lines, bioreactors, and tissue engineering strategies of potential utility for *in vitro* adipose tissue production for food. Regulation and consumer acceptance are also discussed.

Key Findings and Conclusions

Existing strategies and paradigms are insufficient to meet the full set of unique needs for a cell-cultured fat manufacturing platform, as tradeoffs are often present between simplicity, scalability, stability, and projected cost. Identification and validation of appropriate cell lines, bioprocess strategies, and tissue engineering techniques must therefore be an iterative process as a deeper understanding of the needs and opportunities for cell-cultured fat develops.

Keywords: cell-cultured meat, adipose tissue, lipids, meat science, food manufacturing, cellular agriculture

Declarations of interest: none

INTRODUCTION

The emerging field of cellular agriculture aims to apply cell culture, tissue engineering, fermentation, and other biotechnologies to produce foods like meat, eggs, and dairy that are traditionally obtained from animals. The use of cellular agriculture as an alternative paradigm to conventional livestock production has the potential to improve the sustainability of protein production by reducing the environmental and animal welfare impacts of current animal-based systems. Development of cell-cultured meat (meat produced *in vitro* through cell culture and tissue engineering) is a central goal within this field and requires research and innovation across four key domains: establishment of cell lines from agriculturally-relevant species, culture media development for cost reduction and growth optimization, bioprocess engineering to improve cell culture efficiency and scalability, and tissue engineering to form 3D meat products that mimic their animal-derived counterparts. To date, most of the experimental and theoretical work on cell-cultured meat has focused on muscle cells and tissue, which accounts for the majority of biomass in common meat products (Kadim et al., 2015). While fat typically comprises a smaller fraction of total meat content, it is a key determinant of flavor, texture, nutrition, and visual appearance, all of which are correlated with consumer preference and willingness to pay (Font-i-Furnols & Guerrero, 2014). Thus, production and incorporation of biomanufactured fat in cell-cultured meat products is essential to ensure favorable consumer responses and market success (Mehta et al., 2019).

Large-scale *in vitro* cultivation of fat tissue requires the establishment of adipogenic cell lines with several key characteristics from agriculturally relevant species. First, cell lines must have sufficient proliferative capacity to scale up from primary isolation through commercial production. An immortal line is ideal but not essential, as it may be possible to perform regular isolations to provide fresh cells for a seed train, provided robust validation procedures are used to ensure consistency. Second, cell lines must be adaptable to low-cost culture media without compromising performance. Lastly, cell lines must be capable of efficient, food-safe differentiation into adipose or adipose-like tissue and must retain that capacity after many population doublings. Once a suitable adipogenic cell line has been established, high-density bioreactor systems can be used to expand the cells to industrial scale.

Several opportunities exist for incorporation of these cultured cells into food (Figure 1). One option is to differentiate the adipogenic cells into mature adipocytes and use them as an ingredient in plant-based meat alternatives. Plant-based meats have grown in popularity in recent years due to concerns about the sustainability of animal-based meat production. A variety of plant-based protein sources have been used successfully for production of meat analogues, but mimicking the flavors and mouthfeel of animal fat remains a challenge (Joshi & Kumar, 2015). Coconut oil has emerged as the fat source of choice for many plant-based meat alternatives, with sunflower oil and canola oil also used in leading products. The challenge of encapsulating lipids from these fat sources has been an important factor limiting the similarity of existing plant-based meats to their animal-derived counterparts. The use of cultured fat cells along with plant-based protein may thus significantly improve the quality and consumer perception of plant-based meats without compromising sustainability. Cultured adipocytes can similarly be used as an ingredient in conventional animal-based meat products. *In vitro* cultivation may allow control of lipid profiles, enabling development of fat supplements for processed meats that are optimized for taste, nutrition, mouthfeel, juiciness, and other qualities relevant to human health and consumer perception (Jiménez-Colmenero, 2000). The use of cultured adipocytes as an ingredient in both plant-based and animal-based meats is appealing because relatively small quantities can improve the quality of existing products and reduce dependence on animal agriculture as a production system. Longer term, *in vitro* adipose tissue will also be an important component of meat products produced entirely through cell culture and tissue engineering. Development of such products will require cultivation of muscle and connective tissues in addition to adipose tissue, as well as 3D tissue engineering to mimic the appearance, structure, and texture of animal-based meats.

A key advantage of *in vitro* fat production, and cellular agriculture in general, is the potential alleviation of environmental and animal welfare issues caused by the intensification of animal agriculture (Bhat et al., 2017). There are various mechanisms through which the commercialization of cell-cultured fat for food may improve animal welfare. For instance, the incorporation of cell-cultured fat as an ingredient in predominantly plant-based meat analogs could lead to sufficient improvements in eating quality such that more consumers would be willing to substitute plant-based options in place of conventional meats, thereby reducing demand for products from high-density, low-welfare farms. Additionally, the use of *in vitro* fat and muscle for the development of cell-cultured meats that are biologically and culinarily indistinguishable from their conventional counterparts would help provide a more ethical meat supply without requiring a change in consumer behavior. These mechanisms may also ameliorate the environmental impacts of the meat industry, as both plant-based and cell-cultured meat production are projected to use fewer natural resources and reduce greenhouse gas emissions relative to animal agriculture (Sabaté & Soret, 2014; Tuomisto & Mattos, 2011). Ideally, development and commercialization of cell-cultured fat and other *in vitro* foods will work synergistically with policies that hold conventional producers to higher standards of animal welfare and environmental stewardship (Santeramo et al., 2019).

In this paper, we present relevant aspects of adipose tissue biology and explore the role of fat tissue in meat products. We then review work on adipogenic cell types, bioprocess engineering, and tissue engineering relevant to the large-scale *in vitro* growth of adipose tissue for cell-cultured meat applications and discuss the advantages and constraints of various production strategies. Past efforts in the fields of regenerative medicine and meat science have contributed to a foundational understanding of adipose tissue in meat and a repertoire of *in vitro* techniques that can be applied to accelerate innovation for cell-cultured fat production. These bodies of work provide a platform from which to pursue further research that is tailored to the unique needs of cellular agriculture.

ADIPOSE TISSUE BIOLOGY

Basic Functions of Adipose Tissue

Adipose tissue is composed of adipocytes, fibroblasts and macrophages as well as nerves and vasculature. This loose connective tissue is found beneath the skin (subcutaneous), around organs (visceral), in the bone marrow (yellow bone marrow) and within skeletal muscle tissue (intermuscular, intramuscular) and serves numerous physiological roles in metabolism, immune functions, and the endocrine system (Trayhurn & Beattie, 2001). Adipose tissue is most notably responsible for energy storage. When energy intake exceeds expenditure, adipocytes undergo expansion as they accumulate lipids and cholesterol (lipogenesis); when expenditure exceeds intake, adipocytes undergo mobilization, releasing fatty acids and glycerol for glucose metabolism (lipolysis) (Vázquez-Vela et al., 2008). White adipose tissue (WAT) is also involved in thermal insulation, mechanical support, inflammation, glucose homeostasis and endocrine signaling (Trayhurn & Beattie, 2001). White adipogenesis plays a key role in the development of intermuscular and intramuscular fat content, also known as marbling, which is an important aspect of red meat quality that it is strongly correlated with flavor, juiciness, tenderness and positive consumer perception (Hausman et al., 2014). Intramuscular fat content is determined by the size and population of intramuscular adipocytes. However, excess accumulation of non-marbling (e.g., subcutaneous, visceral) fat in meat animals reduces meat production efficiency and is typically considered undesirable. Subcutaneous and visceral adipocyte formation occurs in mid to late fetal development and early weaning, whereas intramuscular adipocyte formation occurs in late fetal-neonatal development and young animal age (Huang et al., 2012). While adipogenic gene expression patterns of intramuscular and subcutaneous adipocytes are similar, subtle differences point to a more mature differentiation state in subcutaneous cells (Grant et al., 2008; Hausman & Poulos, 2004). Intramuscular fat content is dependent

on a matrix of factors including species, muscle type, sex, genetics, age, feeding and material nutrition (Hocquette et al., 2010).

Fatty Acid Biosynthesis and Classifications

The lipids that accumulate in adipose tissue and drive important organoleptic properties of meat come both from fats in the animal's diet and from intracellular anabolism. Endogenous biosynthesis of fatty acids occurs through successive additions of two-carbon groups to Acetyl CoA and occurs with the help of fatty acid synthase, a protein complex with all enzymatic activities necessary for fatty acid formation. This process is used to produce a 16-carbon fatty acid, which can then be elongated, desaturated, or otherwise modified (Wakil, 1989). Fatty acids are often classified as saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs), and poly-unsaturated fatty acids (PUFAs) based on the number of double bonds present in their chain structure. Unsaturated fatty acids can be further classified as cis or trans fats by the conformation of hydrogen atoms around the double bonds. Lipid numbers are commonly used to describe fatty acids and follow the format "(number of carbon atoms in the chain):(number of double bonds along the chain)". For example, palmitic acid, a 16-carbon saturated fatty acid, can be denoted as 16:0. Another notation is used to describe the location of double bonds in unsaturated fatty acids: "n-x" or "omega-x", where x is the location of the first double bond, counting from the methyl terminus of the backbone). For example, an omega-3 fatty acid is any unsaturated fatty acid where the third carbon-carbon bond is a double bond. These biochemical differences are culinarily relevant, as the classes of fatty acids present in meat products (and the ratios between them) vary greatly and are key determinants of both nutritional and organoleptic properties (Table 1, Figure 2b). By tuning the lipid composition of *in vitro* adipose tissues for food, it may be possible to leverage the biochemical properties of different fatty acids to develop products with optimal flavor and nutrition.

Adipogenesis

Adipocytes originate from the mesoderm during development, with WAT arising from Myf5-negative progenitor cells and increasing in prevalence with age. Interestingly, adipose tissue is the only tissue capable of unlimited growth during adult life (Scherer, 2006). Adipose tissue expansion can be a result of hyperplasia (generation of adipocytes from precursor cells) or hypertrophy (individual adipocyte enlargement). During adipogenesis, cells undergo adipocyte determination, followed by adipocyte differentiation in response to regulation by a variety of signaling cascades and transcription factors (Figure 2a). Insulin signaling promotes differentiation into adipocytes, while the hedgehog and Wnt signaling pathways both inhibit adipogenesis. Following initial induction, the transcription factors CCAAT/enhancer-binding protein beta (C/EBPβ) and C/EBPδ act as the main early regulators of adipogenesis, and are themselves regulated by a host of other factors. C/EBPβ and C/EBPδ target the promoters of C/EBPα and PPARγ (the master controller of adipogenesis), which activate each other in a positive feedback loop and drive expression of genes that trigger lipogenesis and other phenotypic changes associated with adipogenic differentiation. The central feedback loop between C/EBPα and PPARγ is regulated by additional factors, including signal transducer and activator of transcription 5 (STAT5), sirtuin 1 (SIRT1), SIRT2, and foxhead box protein 01 (FOX01) (Lowe et al., 2011). Adiponectin—a protein secreted from WAT, low levels of which are linked to obesity-related disorders—plays a role in triggering more rapid adipocyte differentiation and greater lipid accumulation (Fu et al., 2005). While core features of this control system are generally conserved, expression patterns vary by species. For instance, cells from different species of pig display differential expression of lipogenic genes such as fatty acid synthase (FASN), sterol regulatory element binding protein 1 (SREBF1), PPARγ, and C/EBPα during adipogenesis (Li et al., 2012). Such species differences in

relevant gene expression patterns may become important considerations in attempts to generalize and optimize bioprocess strategies for *in vitro* production of fat tissue.

IMPORTANCE OF FAT IN MEAT PRODUCTS

A key goal in the production of cell-cultured meats is to replicate, and ultimately improve upon, the sensory and nutritional qualities of conventional meat products. The role of fat in shaping these properties makes it a priority area for research and development. To produce *in vitro* adipose tissue that contributes to accurate mimicry of animal-based meats, it is necessary to understand the role of fat in conventional products. Here we review the prevalence of fat relative to other macronutrients in meat and discuss it as a driver of nutrition, taste, and texture. The mechanisms presented can be applied to inform ongoing research for cell-cultured meat applications and guide the development of products that align with consumer preferences for various organoleptic properties.

Prevalence of Fat in Meat

Muscle, connective, and adipose tissues in meat provide the protein and fat that determine texture, flavor, and caloric value. While the composition of macronutrients in most meat products typically ranges from 70–75% water, 20–25% protein, and 1–10% fat, the relative amount of these nutrients varies widely with factors such as species, age, feeding regime, cut, and preparation (Wood, 2017). The most variable of these components is fat. The average intramuscular fat content of common species (averaged across several cuts) ranges from 1.6% in turkeys to 8% in sheep (Institute of Food Research, 2014). This variability is also found within species, with common cuts of beef offering fat content ranging from 2.0–12.7% (Williams, 2007) (Table 1). Specialized breeds and feeding regimes can push these values even higher, with Japanese Wagyu beef reaching over 30% fat (Corbin et al., 2015). These compositional values are for intramuscular fat (marbling); however, many retail cuts maintain a significant level of intermuscular (dissectible) fat, up to 30% in sheep loin steaks, that contributes to the flavor, nutrition, and culinary functionality of these products (Wood, 2017).

The amount of fat considered desirable by consumers varies greatly from product to product, making fat content an important economic consideration in meat production. Consumers have shown a general preference toward leaner meats, though the ideal level varies by both species and product type (e.g. ground meat vs. unprocessed cuts) (Koistinen et al., 2013; Lusk & Parker, 2009; Unnevehr & Bard, 1993). There are, however, important exceptions to this trend, such as the positive correlation between intramuscular fat content and beef quality/consumer preferences (Gotoh et al., 2018). For example, Japanese Wagyu beef sells at premium prices and is world-renowned in large part due to its intensive marbling and the quality of its fat composition (Motoyama et al., 2016). Given the spectrum of fat content in different products and the associated economic impacts, *ex vivo* cultivation of fat tissue may prove especially valuable by allowing tighter control over fat composition and a more efficient system for investigating and optimizing qualities of consumer importance.

Fat and Nutrition

Fat in meat is nutritionally important for several reasons, including high energy density, provision of essential fatty acids, and storage of fat-soluble vitamins A, D, E, and K7. Fat is the most energetically dense nutrient in meat, providing around 37 kJ/g compared to 17 kJ/g for protein (Wood, 2017). This energetic density has contributed to the emergence of dichotomous perspectives on meat and nutrition. On one hand, the protein and nutritional content of meat has historically been a boon to the human diet and continues to offer an effective means for improving nutrition in the developing world. On the other hand,

the developed world is increasingly aware of meat's association with surplus energy intake and diet-related diseases like obesity, raising concerns about public health impacts of excessive meat consumption (You & Henneberg, 2016). The specific lipid profiles of fat content in meat have been associated with several health and nutrition outcomes. Substantial research efforts have investigated the importance of dietary cholesterol, SFAs, PUFAs, MUFAs, and trans fats in human health, often yielding complex and convoluted results. For instance, a meta-analysis of 40 studies showed that while dietary cholesterol caused an increase in serum low-density lipoprotein (LDL) cholesterol, commonly considered "bad" cholesterol, it did not result in any increase in cardiovascular pathologies (Berger et al., 2015). This ambiguity is often attributed to confounding dietary factors. Thus, despite the uncertainty, a reduction in dietary cholesterol is often recommended for improved health. This is relevant when considering meat products, which tend to offer a significant proportion of the recommended daily intake for cholesterol (300 mg / day) (US Institute of Medicine, 2003).

As with cholesterol, the nutritional and health impacts of fatty acids in meat are complex. While SFAs in meat have previously been suggested as a potential health risk, there is conflicting evidence to support this claim, and meta-analyses have suggested that SFA intake is not associated with mortality or diseases for which it was historically implicated (de Souza et al., 2015). In contrast, a consensus has developed around the importance of the ratio of n-6/n-3 PUFAs and the impact of trans-fats. Specifically, an n-6/n-3 ratio below 4/1 is recommended to reduce several pathologies as well as all-cause mortality (Simopoulos, 2002). A reduction of trans fat consumption is a similarly well-established means for improving health (Forouhi et al., 2018). Trans fats are produced by bacterial metabolism of PUFAs in the rumens of cows, sheep, and other ruminants, and are then absorbed by the intestine and accumulate in tissues (Stender et al., 2008). They are therefore found in relatively high levels in ruminant products such as beef and lamb (~2–10% of total FA), but at negligible levels (<1% of total FA) in non-ruminant products such as pork and chicken (Aro et al., 1998). Interestingly, the synthesis of trans fats by ruminal bacteria means that meat produced through cell culture may have little or none of these fats.

Fat and Taste

Fat contributes substantially to meat flavor through both aroma and taste compounds. Indeed, species-specific differences in flavor can largely be attributed to lipid-derived compounds, while muscle-derived compounds contribute the shared "meaty" taste to all meats (Mottram, 1998). Flavor compounds from fat generally come from lipid oxidation, and include volatile aldehydes (the most prevalent volatile produced during the cooking of meat), lactones, ketones, alcohols, furans, hydrocarbons, acids, and esters (Flores, 2017). Additionally, lipid oxidation products interact with products of the Maillard reaction between amino acids and sugars, thus generating heterocyclic aroma compounds that contribute significantly to meat flavor (Whitfield, 1992). While the exact relationship between fatty acids, their derivative volatiles, and the flavor profile of meats is immensely complex, some broad features have been elucidated. For instance, intramuscular fat between 3% and 7.3% is generally considered ideal for flavor and acceptability, and myristic (14:0), palmitic (16:0), palmitoleic (16:1), and oleic (18:1) acid content are positively associated with favorable palatability (Calkins & Hodgen, 2007; Teye et al., 2006). In contrast, a high PUFA content is often associated with undesirable flavors (Calkins & Hodgen, 2007).

Fat and Texture

As with flavor, fat contributes to meat texture and can be a major factor in consumer perception. For instance, sensory analysis of pork fed varying diets found positive correlations between tenderness and myristic (14:0), palmitic (16:0), palmitoleic (16:1) and oleic (18:1) acids, while negative correlations were

found for tenderness and linoleic (18:2) acid or long chain PUFAs (Teye et al., 2006). These findings combine synergistically with the flavor results, where a high PUFA content can lead to off-flavors and suggests that high PUFA content in meats can reduce quality across a range of metrics. Interestingly, studies investigating the effects of total fat on tenderness have offered conflicting results. For instance, a quantitative study with meat from sheep showed that a 3–4% increase in intramuscular fat produced a 3.9 N increase in shear force (Starkey et al., 2016). Other studies, however, have suggested only limited correlations between fat characteristics of meat and meat tenderness, or have even suggested the opposite: that intramuscular fat disorganizes intramuscular connective tissue and decreases toughness (Fiems et al., 2000). As the levels of fat in meat are tied to many other factors that affect texture, such as protein content, tissue organization, and connective tissue content, more studies are needed to fully elucidate the mechanisms through which fat content affects meat texture and tenderness.

CELL SOURCES

Various cell types are capable of adipogenic differentiation *in vitro;* however, it is not yet clear which will serve as the optimal source for producing cell-cultured fat for human consumption. Characteristics of an ideal cell source include high proliferative capacity, a simple and high-efficiency differentiation process, low media requirements, homogeneity, stability, and resistance to fluctuations in environmental conditions. Tradeoffs are often present between these characteristics, so the selection of a cell source for cell-cultured fat production will require determining which phenotypes are most essential to meet the needs of a given production paradigm. Here we review several possible sources of cells and discuss advantages and limitations of each (Table 2).

Pluripotent Stem Cells

One option for producing fat tissue for use in cell-cultured meat is to culture pluripotent stem cells and differentiate them into mature adipocytes. Pluripotent stem cells have traditionally been obtained through the isolation of embryonic stem cells (ESCs) from the inner cell mass of blastocysts, and are an attractive cell source due to their unlimited proliferative capacity, homogeneity, and ability to differentiate into cell types from all three germ layers (Evans & Kaufman, 1981). ESCs have been successfully isolated from a variety of species, including humans (Thomson et al., 1998), mice (Nagy & Vintersten, 2006), chicken (Aubel & Pain, 2013), fish (Hong et al., 1996), and more recently, cows (Bogliotti et al., 2018). Despite the generation of select lines for these species, the isolation of ESCs from livestock for agricultural research has been an ongoing challenge. Ethical concerns may further complicate the application of ESCs, as their isolation requires destruction of an embryo. Fortunately, it is also possible to reprogram somatic cells to a state of pluripotency through expression of a set of defined transcription factors to generate induced pluripotent stem cells (iPSCs) (Okita et al., 2007). This technique has been used to generate pluripotent lines from mammalian farm animals, and is particularly useful for species like pigs, for which no ESC lines have been established (Ezashi et al., 2009). ESCs and iPSCs are both capable of differentiation into adipocytes and all other cell types relevant to the production of cell-based meat. However, adipogenic differentiation of pluripotent cells is often a complex, multi-step process (Hafner et al., 2016), and efficiency of differentiation varies between stem cell types. ESCs are capable of robust maturation into adipocytes, while iPSCs tend to differentiate at low efficiency when guided through the adipogenic pathway (Cuaranta-Monroy et al., 2014; Hannan & Wolvetang, 2009; Mohsen-Kanson et al., 2014). Furthermore, pluripotent stem cells are sensitive to media composition and other growth conditions and can be challenging to maintain in an undifferentiated, proliferative state, complicating the path to large-scale production (Ogorevc et al., 2016).

Mesenchymal Stem Cells

Adipocytes can also be obtained through differentiation of mesenchymal stem cells (MSCs), which are commonly isolated from bone marrow and fat. MSCs are multipotent adult stem cells and are capable of differentiating into cell types of mesodermal lineages, including adipocytes, osteoblasts, and myocytes. However, the plasticity of MSCs can vary between species, tissue sources, and passage numbers (Zomer et al., 2015). To obtain MSCs from adipose tissue, a tissue sample is typically digested with collagenase and centrifuged to separate the buoyant mature adipocytes from the heterogenous stromal vascular fraction, then the pelleted fraction is resuspended and plated onto tissue culture plastic to select for the adherent MSCs (also known as adipose-derived stem cells (ADSCs) (Bunnell et al., 2008). Additional sorting techniques, such as fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS), can be used to obtain higher purity cultures. With regard to nutritional requirements, MSCs can be grown in simple media relative to those needed for pluripotent stem cells (i.e. basal media supplemented with fetal bovine serum) (Jung et al., 2012). However, unlike pluripotent stem cells, MSCs have a limited proliferative capacity, typically halting proliferation at or before 40 population doublings. Of particular concern for the production of fat tissue is the finding that adipogenic differentiation potential decreases as these cells approach their proliferation limit (Wagner et al., 2008). Large-scale production using MSCs would thus depend on immortalization (either deliberate or spontaneous) or recurring isolations.

MSCs have been isolated from bovine (Hill et al., 2019), porcine (Bharti et al., 2016), avian (Adhikari et al., 2019), and ovine (Niemeyer et al., 2010) sources, as well as from other species of agricultural relevance (Lee et al., 2014). Thorough characterizations are necessary to assess the suitability of each existing or novel cell line for cellular agriculture applications, as the ease and efficiency of isolation, expansion, and differentiation can vary by species and/or source tissue (Javazon et al., 2004). In general, differentiation of MSCs into adipocytes is a straightforward process, most commonly achieved using a three-component hormonal cocktail of insulin, dexamethasone, and isobutylmethylxanthine (IBMX). A PPARγ agonist, such as rosiglitazone, is often included as a fourth component, and is essential in certain cases (Pu & Veiga-Lopez, 2017). This approach to differentiation is problematic for the production of fat tissue for meat products due to the acute oral toxicity of IBMX and steroid nature of dexamethasone. Fortunately, a wide variety of cocktails have been used to induce lipid uptake and adipogenesis, some of which do not require IBMX (Scott et al., 2011). A differentiation strategy for bovine preadipocytes involving induction by free fatty acids has been reported and was designed specifically to address the needs of cellular agriculture (Mehta et al., 2019). When developing additional adipogenic differentiation protocols for cell-based meat applications, it will be necessary to consider both the safety and cost of the components used, as well as potential species differences in the requirements for induction.

Dedifferentiated Fat Cells

Another potential cell source for cultured fat tissue is dedifferentiated fat (DFAT) cells, obtained through the dedifferentiation of mature adipocytes. Following enzymatic digestion of adipose tissue, adipocytes are separated from the stromal vascular fraction by centrifugation and then cultured on the ceiling of inverted tissue culture flasks. Over 2 to 3 weeks in culture, the cells gradually lose their lipid stores, adopt a fibroblast-like morphology, and resume proliferation (Zhang et al., 2000). The resulting DFAT cells are multipotent and capable of differentiation into osteoblasts, chondrocytes, myocytes, and adipocytes, although they are heterogenous in their differentiation potential and generally less plastic than MS cells (Matsumoto et al., 2008). DFAT cultures have shown long-term proliferative capacity, and can be readily differentiated into adipocytes through methods similar to those used for MS cell differentiation. Cultures of DFAT cells have previously been established from a variety of species, including humans (Kishimoto

et al., 2018), rats (Akita et al., 2016), and mice (Yagi et al., 2004), as well as agriculturally relevant animals like cows and pigs (Peng et al., 2015; Wei et al., 2013). With proliferative capacities comparable to MSCs and a high degree of culture homogeneity, DFAT cells could provide a stable cell source for scalable adipose tissue culture. A key limitation is their dependence on high serum concentrations (often 15-20%) to maintain a proliferative state *in vitro*. To date, no serum-free media has been reported for proliferation or differentiation of DFAT cells.

Satellite Cells

Satellite cells are a promising cell source for the muscle component of cell-cultured meat due to their ease of isolation and differentiation into myotubes, and may also be useful for fat production. Satellite cells *in vivo* reside beneath the basal lamina on the surface of muscle fibers and are responsible for growth and repair of myofibers throughout the lifespan. Simple protocols involving dissection, enzymatic digestion, centrifugation, and trituration can be used to isolate these cells from a broad variety of species, and pre-plating, FACS, or MACS techniques can be incorporated when enriched cultures are needed. While generally considered committed to myogenesis, transdifferentiation of satellite cells into adipocyte-like cells is possible through inhibition of Wnt signaling, high glucose exposure, or culture in adipogenic media (Starkey et al., 2011). However, adipogenicity is often highly heterogenous, with only small percentages of satellite cells accumulating lipids upon induction (Aguiari et al., 2008). Generation of adipose tissue through this process is unlikely to be efficient enough for a fat-focused production process, but it may be of use in future efforts to create complex meat products that include structured muscle tissue and marbling. An additional limitation of satellite cells is the heterogeneity and loss of stemness observed in *in vitro* cultures. After as few as three passages, cultures of high initial purity (>90% expression of the satellite cell marker paired box protein 7 (Pax7)) can drop to near 50% expression, indicating rapid loss of stemness and culture heterogeneity (Syverud et al., 2014). These Pax7-negative cells have converted from quiescent stem cells to activated myoblasts, which can still be transdifferentiated into adipocytes; however, activated myoblasts have significantly reduced proliferative capacity, so the loss of quiescence could frustrate large-scale cell expansion (An et al., 2017). Potential scale up of satellite cell cultures is thus predicated on further innovations to improve consistency and proliferative capacity.

Immortalized Adipogenic Cell Lines

A variety of immortalized preadipose cell lines are commercially available, including 3T3-L1, 3T3-F442A, and OP9. These lines have several characteristics well-suited for production of cell-cultured fat for food, including high proliferative capacity, robust differentiation into adipocytes, homogenous cell populations, simple maintenance protocols, and thorough characterization. However, the majority of such cell lines are from murine sources, limiting their utility for cell-cultured meat research and development (Ruiz-Ojeda et al., 2016). The 3T3-L1 cell line, for example, was derived from mouse embryonic fibroblast cells, and has been in use for more than four decades as a model of adipose tissue biology and obesity (Green & Meuth, 1974). OP9 is also murine derived and useful as a stromal line capable of rapid, high-efficiency lipid accumulation (Wolins et al., 2006). There is precedent for the establishment of adipogenic cell lines from agriculturally-relevant species, as evidenced by the porcine PSPA line and the bovine BIP lines reported in the literature, but none are commercially available (Aso et al., 1995; Nakajima, 2015). The development and commercialization of similar lines from common meat-producing species would provide an attractive source for fat production for food applications. In the meantime, existing lines are valuable research tools and can be leveraged to deepen understanding of cellular characteristics relevant to biomanufacturing of adipose tissue.

BIOPROCESSING

Creating cell-cultured fat at a scale and price point competitive with conventional products will require rapid, low-cost, ongoing production of massive quantities of cells. The type of bioreactor or bioprocessing

system used is a key determinant of both the scale and cost of biomanufacturing, and is thus an important consideration for the development of a production paradigm (Allan et al., 2019). There is typically an inverse correlation between the ease of adaptation and use of a bioprocess system and the ease and efficiency of scale-up (Figure 3), so selecting a system for use in culture fat production will require balancing these considerations or innovating around the relevant constraints. The need for both proliferation and differentiation stages in the biomanufacturing process presents additional challenges unique to fat production, including the problematic buoyancy of differentiating adipocytes. Here we review common bioprocess systems used in biomanufacturing, discuss their suitability for efficient and scalable production of cell-cultured fat, and highlight emerging technologies that may overcome current challenges (Table 3).

2D Adherent Culture Systems

Due to anchorage dependence, adipogenic cells are typically grown and differentiated in 2D adherent culture systems, such as petri dishes, T-flasks, or multi-well plates (Figure 3a, 3b). While convenient, these culture formats are labor-intensive and cost-inefficient to expand beyond bench scale, due in large part to their low surface area to volume ratio (Panchalingam et al., 2015). Several variations on traditional 2D culture systems have been developed to improve scalability, including roller bottles and multilayer flasks. These systems increase the available surface area for cell growth for a given footprint and workflow complexity, and have been used successfully for moderate scale-up of adipogenic cells (Jung et al., 2012). However, roller bottles and multi-layer flasks still require cells to grow as monolayers on 2D surfaces, and are thus unlikely to provide sufficient biomass production capacity to be useful for cell-based meat at a commercially-relevant scale.

Fixed-bed and Hollow-fiber Bioreactors

As an alternative to monolayer culture, anchorage-dependent adipogenic cells can be grown in fixed-bed or hollow-fiber bioreactors (Figure 3c, 3d) (Panchalingam et al., 2015). These systems provide substrate for cell attachment while dramatically increasing surface area for cell growth by expanding into three dimensions. Fixed-bed bioreactors consist of a column with an immobilized scaffold in which cells are seeded and fed by gradual raising and lowering of the media level (Rodrigues et al., 2011). In contrast, hollow-fiber bioreactors are used to grow cells on parallel arrays of porous capillaries in a cylindrical cartridge. Cells can be seeded into the intracapillary or extracapillary space and are fed by nutrient diffusion through the fiber pores as media is perfused through the chamber. Fixed-bed and hollow-fiber bioreactors both provide low-shear stress environments for high-density cell growth without compromising oxygen exchange, suggesting potential utility for scalable production (Mizukami & Swiech, 2018). Hollow-fiber systems in particular have been proposed as a viable means of achieving efficient, large-scale culture of mammalian cells for biomanufacturing (Whitford & Cadwell, 2011). Notably, a hollow-fiber bioreactor has been shown to support the proliferation of adipose-derived stem cells and their subsequent differentiation into mature adipocytes to form 3D adipose tissue (Gerlach et al., 2012). In order to apply this system for production of fat for cell-based meat, it will be necessary to develop efficient cell harvest strategies or produce fibers from edible or biodegradable materials that can remain with the cultured tissue as part of the final product.

Suspension Bioreactors

The most common system for large-scale biomanufacturing with mammalian cells is single-cell suspension culture in stirred-tank bioreactors (Mizukami & Swiech, 2018). These reactors typically consist of a cylindrical culture vessel mixed by a central impeller, and range in working volume from 15 mL to 10,000 L (Chu & Robinson, 2001). Key benefits of stirred-tank reactors are their versatile modes of operation (batch vs. fed-batch vs. continuous media replacement and harvest), their ease of transition from benchtop to industrial scale, and their ability to produce large quantities of cells in a single vessel under homogenous conditions (Panchalingam et al., 2015). Air lift bioreactors are also used for suspension culture of mammalian cells and have recently been proposed for use in cell-cultured meat production (Li

et al., 2020). However, the difficulty of adapting anchorage dependent cells to survive, proliferate, and maintain phenotype in single-cell suspension is a barrier to the broad utility of suspension bioreactor systems. Various strategies have been developed to transition cells from anchorage dependence to monosuspension, but the techniques are often time consuming and labor intensive and their efficacy varies greatly between cell lines (Caron et al., 2018).

Pseudo-suspension strategies have been developed for scalable culture of cells that are unable to adapt to single-cell suspension. These strategies include growing cells as aggregates or on microcarriers in suspension bioreactors (Jossen et al., 2018). For aggregate culture, anchorage dependent cells can be induced to form spheroids in the dynamic suspension environment, or in a static environment prior to the transition to suspension (Egger et al., 2018). In addition to enabling the use of suspension bioreactors for scalable production of adherent cell lines, this technique also promotes an *in vivo*-like environment with extracellular matrix and intercellular signaling interactions that are advantages to cell health (Hookway et al., 2016). Alternatively, adherent cells can be grown in suspension bioreactors using microcarriers: small beads (typically 100–300 μm in diameter) that can be maintained in suspension and allow attachment and high-density proliferation of anchorage-dependent cell lines. The use of microcarriers for anchorage-dependent cell culture can increase the surface area to volume ratio by an order of magnitude compared to standard 2D culture flasks (Panchalingam et al., 2015). Both these techniques have proven useful for scalable expansion of stem cells without jeopardization of their plasticity. ESCs, iPSCs and other adipogenic cell types have all been cultured successfully as suspension aggregates and on microcarriers (Ashok et al., 2016; Badenes et al., 2015). These bioprocess strategies could thus be useful for scalable proliferation of adipogenic cells for food production. However, the buoyancy of mature adipocytes may complicate or preclude adipogenic differentiation in suspension.

Alternative Bioprocess Strategies

To achieve the large-scale, low-cost cell growth necessary for commercial production of fat for cell-based meat, novel bioprocess strategies may need to be developed or adopted from other emerging industries. One technology that has shown early promise is the use of 3D thermoreversible hydrogel scaffolds for scalable proliferation and differentiation of pluripotent stem cells and other adipogenic cell types (Lei et al., 2014). Initially developed for use in the field of cell therapy, this approach has potential to dramatically drive down biomanufacturing costs for cell-based meat by enabling efficient, ultra-high-density production of relevant cell types. A hydrogel-based system may prove particularly useful for production of mature adipocytes, as immobilization of the cells in a 3D matrix allows sufficient nutrient diffusion and protection from shear forces while preventing cell migration due to buoyancy upon differentiation. Another alternative strategy involves the use of specialized 2D culture surfaces for continuous adhesion, proliferation, self-detachment, and harvesting of cells (Miotto et al., 2017). Originally developed for biomanufacturing of corneal stromal cells, this system allows continuous low-cost, low-maintenance cell production and avoids the challenge of adapting cells to non-adherent formats. While promising, further work will be necessary before considering these or other technologies as viable options for commercial-scale biomanufacturing of cell-based meat.

The Challenge of Scale Up

It remains unclear which bioprocess strategy or strategies will ultimately be used for commercial production of fat and other components of cell-based meat. Regardless of the manufacturing paradigm and bioreactor configuration, scale-up is likely to be a major barrier to the widespread use of *in vitro* meat production. Thus, it may be prudent to explore decentralized strategies that distribute production across geographic regions rather than attempting to concentrate in centralized factories. This approach would limit the extent to which biomanufacturing technologies must be scaled beyond current capacities, thereby smoothing the path toward high-volume production and distribution. However, a scale-out strategy would also limit the role of economies of scale in driving down production costs, which is another important hurdle that must be overcome in order to bring economically viable cell-cultured meat to consumers.

TISSUE ENGINEERING

Following proliferation and differentiation *in vitro*, adipocytes can be used in structured or unstructured cell-cultured meats, or as ingredients in plant-based meat alternatives or processed meats from animal sources. These latter applications will require minimal downstream processing, as the final products are not dependent on the adipocytes for structure. However, it will ultimately be desirable to incorporate cell-cultured adipocytes along with muscle and connective tissue to produce cell-cultured meats that mimic the appearance, taste, and texture of structured products like steak—a task that will require 3D tissue engineering. Traditionally, the goals of adipose tissue engineering have been to create *in vitro* models for drug screening and disease research and to generate adipose tissue for surgical procedures. Techniques developed for these medical applications are relevant to production of cell-based meat, since the overarching goal is similar: use cells produced *in vitro* to accurately recapitulate *in vivo* tissues.

A core challenge in efforts to produce large 3D tissues *in vitro* is achieving sufficient nutrient exchange and gas diffusion to support cells inside the tissue construct. *In vivo*, the vast majority of cells are located within 100 μm of a capillary to ensure adequate access to oxygen and nutrients (Alberts et al., 2002). Progress has been made toward the successful incorporation of vascular structure in engineered adipose tissue through co-culture with endothelial cells (Kayabolen et al., 2017); however, such vascular structures can take days or weeks to form, they have not been subjected to rigorous functional testing, and the level of complexity required for production is poorly suited to cellular agriculture applications (Morin & Tranquillo, 2013). This approach would also require sourcing endothelial cells from the relevant animal species, adding a level of complexity. A more promising alternative is the use of hollow-fiber bioreactors, or similar perfusion systems, which artificially mimic the functionality of vascularization and provide nutrient and oxygen exchange to support dense adipose tissues (Gerlach et al., 2012). Development of edible fibers for use in these systems may allow production of structured 3D adipose tissue that can be directly incorporated into meat products for human consumption (Allan et al., 2019).

Tissue engineering requires providing cells with surface area for attachment and proliferation in 3D space, more closely mimicking a cell's *in vivo* environment than 2D surfaces. This biomimetic 3D environment can be achieved by inducing cells to self-assemble into 3D structures such as spheroids, or by growing cells in a hydrogel or porous 3D scaffold. Relative to 2D culture systems, 3D adipogenesis improves both the efficiency and the extent of differentiation. 2D protocols typically yield only multilocular lipid accumulation, whereas 3D systems can produce the large, unilocular lipid droplets characteristic of mature adipocytes. Induction in 3D also results in a higher overall percentage of differentiated cells compared to monolayer cultures. For both 2D and 3D systems, maximum lipid accumulation has been observed after 3 to 5 weeks of differentiation (Gerlach et al., 2012; Hsiao et al., 2016).

Various scaffold materials have been used successfully for adipose tissue engineering and may be viable options for cell-cultured meat production. Scaffolds for cell-cultured meat must be food safe, able to mimic sensory attributes of meat even after thermal changes related to cooking, and either edible or degradable prior to consumption. Compressive modulus values can be used as a basis for rough mechanical comparisons, though the species-dependence of adipose tissue moduli and further variability as a function of strain rate and other factors makes direct comparisons from literature values difficult (Young's modulus of adipose tissue can vary between ≈1 kPa at low strain rates up to ≈3 MPa at high strain rates) (Comley & Fleck, 2010). The impact of scaffold materials on texture and taste before and after cooking must also be considered. Below, we highlight biomaterials that have been used for adipose tissue engineering with properties indicative of suitability for cell-cultured meat (Table 4), and discuss scaffold-free tissue engineering techniques of potential relevance. Other materials that have not yet been validated for production of adipose tissue, such as cellulose, starch, soy protein, and lentil protein may also be worth exploring and have been reviewed elsewhere (Shit & Shah, 2014).

Synthetic Polymers

Synthetic polymers are popular biomaterials used in many areas of tissue engineering, as they are known to be inexpensive, with tunable mechanical characteristics and degradation rates. In adipose tissue engineering, scaffolds made of poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their combined poly(lactic-co-glycolic acid) (PLGA) are especially common (Patrick et al., 1999). PLGA is often paired with other materials to address certain deficiencies, such as solubility and a lack of specific cell adhesion sites such as RGD. As PLA/PGA/PLGA can be tuned to break down after several weeks or months, it may be possible to use them as degradable scaffolds for cell-cultured meat. In their stable form, porous PLGA scaffolds show a range of mechanical properties (compressive moduli from ≈0.1 MPa to >10 MPa). While generally higher than reported values for adipose tissue, broad tunability as a function of pore size, shape, and porosity suggests possibilities for further mechanical optimizations (Pan & Ding, 2012). Conveniently, the products of the degradation reactions of these compounds are lactic acid and/or glycolic acid, which are commonly present in food (Crowley et al., 2013). As a potential benefit, it would be useful to determine the impact of low molecular weight acid hydrolysis products from these polymers on tissue stability and on antimicrobial features of the foods generated. Despite low cost, tunability, and food safety, synthetic polymers must be further assessed to determine their impact on flavor and texture of cell-cultured meat products at varying stages of degradation, processing, and preparation (e.g., cooking).

Native/Animal Extracellular Matrices

Native extracellular matrices (ECMs) make attractive scaffolds, as animal cells are naturally adapted to attach, proliferate and differentiate on these biomaterials. Fibrin and collagen have shown particular promise for use in generating 3D adipose tissue, and scaffolds from both materials can be mechanically tuned to match the properties of adipose tissue (Duong et al., 2009; Flynn & Woodhouse, 2008; Ghodbane & Dunn, 2016). 3D culture of adipose cells in native ECMs induces cells to efficiently accumulate lipids, express adipogenic markers and adopt morphologies similar to *in vivo* adipose tissue. From a food safety perspective, use of these products should not be a concern, as they are already present in conventional meats. Degradation of the scaffolds prior to consumption is also a possibility. However, scaffold degradation in adipose tissue engineering has historically been associated with problematic volume loss, an issue that will likely need to be mitigated in product development (Cimpean, 2014). A significant drawback of native ECMs as scaffolds is that they are animal-derived, contributing to high costs and batch-to-batch variability. Recombinant production methods may eventually alleviate these issues, but are unlikely to provide a near-term solution due to high costs.

Alginate

Alginate is a natural polymer derived from algae. It is commonly used as a scaffold material for tissue engineering and as a gelling agent, stabilizer, emulsifier, meat binder, and thickener in food products. Notably, alginate scaffolds have been shown to support 3D culture of both porcine and bovine adipocytes, confirming suitability for production of adipose tissues from agriculturally-relevant species (Kim et al., 2010; Mehta et al., 2019). Broad mechanical tunability of alginate gels has also been demonstrated as a function of calcium and polymer concentrations (Kuo & Ma, 2001). Due to its prevalence in the food industry, there is ample data on the sensory perception of alginate in food. Importantly, alginate seems to pair well with high-fat meat products, having been shown to significantly improve the sensory quality of pork patties (Wanstedt et al., 1981). Additionally, veal steaks reconstructed in 0.3–0.5% alginate solutions experienced increased meat binding without significant impacts on taste and texture (Raharjo et al., 1994). Some tasters noted off flavors, but this was attributed to incomplete homogenization of alginate into the meat. Other studies have explored the use of alginate as chicken skin substitute, and one sensory panel even found alginate films soaked in chicken stock more palatable than chicken skin itself (MedCrave, 2018). This capacity for improving sensory quality of meats, combined with its established functionality as a tissue engineering scaffold, makes alginate an attractive candidate for cell-cultured meat production, though functionalization may be required to promote cell adhesion.

Chitosan

Chitosan is another natural polymer used for both tissue engineering and food industry applications. Derived by chemical treatment of chitin isolated from crustacean shells or mushrooms, chitosan is affordable, easily accessible, edible, well-characterized as a biomaterial, and compatible with a broad variety of cell and tissue types (Croisier & Jérôme, 2013). Both hydrogels and porous scaffolds made from chitosan can be mechanically tuned to match a broad range of tissues, including soft tissues like fat (Dash et al., 2011). While pure chitosan has not been validated for use in creating 3D adipose tissue, collagen-chitosan hydrogels have been used for adipose tissue engineering, and chitosan scaffolds supported the attachment and growth of various adipogenic cell lines (Wu et al., 2007). Furthermore, chitosan scaffolds that mimic structural features of many meat products and support growth and differentiation of muscle cells have previously been developed for cell-cultured meat applications (Jana et al., 2013; Rubio et al., 2019). In the conventional meat industry, chitosan has been used as a preservative coating or additive due to its antimicrobial properties and has been shown to improve sensory attributes of various meat products (Darmadji & Izumimoto, 1994). Together, this functionality suggests promise for chitosan as a cell-cultured meat scaffold, although further work is needed to confirm suitability for production of mature 3D adipose tissue.

Scaffold-free Techniques

To form 3D adipose tissue for cell-cultured meat, it may be possible to forgo scaffolds and exploit the natural tendency of adipose cells to aggregate when no attachment surface is present. Conventional hanging drop techniques are sufficient to induce spheroid formation of preadipocytes, and cells grown in such spheroids robustly accumulate lipids, adopt adipose tissue morphology, and secrete adiponectin (Klingelhutz et al., 2018). This approach has been used successfully for *in vitro* bovine adipose tissue generation (Ma et al., 2018). In cellular agriculture, growing adipose tissue without scaffolds may circumvent the challenges of scaffold degradation prior to consumption and potential negative effects of biomaterials on sensory attributes. However, the extent to which scaffold-free *in vitro* adipose tissue is capable of mimicking the nutritional and sensory quality of native tissues has not been fully elucidated. Other potential issues with spheroid culture are scale and time. Generally, individual spheroids are less than 1 mm in diameter and can take weeks to form, which is problematic for expanding production. It may be possible to mass produce individual spheroids and aggregate them into larger constructs after differentiation, but this approach has yet to be validated. Alternatively, adipose spheroid culture has been performed with endothelial cells that self-assemble into vascular structures for each spheroid (Muller et al., 2019). Spheroids produced with this method were still under 1 mm in diameter, but further research in this area may enable production of larger tissue structures.

REGULATION AND CONSUMER ACCEPTANCE

In addition to the technical challenges of generating and scaling a production system for cell-cultured fat tissue for food, consumer acceptance and regulatory considerations may present additional barriers to commercialization. A systematic review of studies about consumer acceptance found conflicting reports of consumer willingness to try cell-cultured meat or substitute it in place of conventional meat (Bryant & Barnett, 2018). In all studies, a portion of the sample group was averse to the possibility of cell-cultured meat, typically citing reasons such as perceived unnaturalness, health concerns, food safety, and anticipated price (Hocquette et al., 2015; Slade, 2018; Wilks & Phillips, 2017). Other studies investigated the factors that drive these concerns and found that provision of information on potential benefits of cell-cultured meat and non-technical descriptions of the production system increased consumer acceptance (Siegrist et al., 2018; Verbeke et al., 2015). Trends in consumer perceptions of the prospect of cell-cultured meat vary significantly by region. In Europe, increasing attention is being devoted to the environmental, sustainability, and animal welfare issues of meat production, though it is unclear to what extent this attention will shape consumer habits or receptivity to meat alternatives (Santeramo et al.,

2018). In a direct comparison of consumers in India, China, and the USA, India and China showed greater familiarity with the concept of cell-cultured meat, which was positively correlated with purchase likelihood (Bryant et al., 2019). Familiarity with novel foods in general, and cell-cultured meat in particular, is associated with receptivity among consumers (Bekker et al., 2017; Santeramo et al., 2018). Use of cell-cultured fat tissue as an ingredient in products consumers are already accustomed to could therefore be leveraged as a means of normalizing the use of cultured cells in food to pave the way for acceptance of later products.

The regulatory pathway for cell-cultured meat products in the US was uncertain until 2019 when the USDA and FDA announced a joint oversight agreement (*USDA and FDA Announce a Formal Agreement to Regulate Cell-Cultured Food Products from Cell Lines of Livestock and Poultry*, 2019). Under the agreement, the FDA will have regulatory jurisdiction for components of the production process upstream of cell harvest and product formation (i.e. cell line isolation, selection, and banking; proliferation; and differentiation of cells into relevant tissue types). The USDA will oversee process elements downstream of harvest (i.e. product testing, inspections, labeling, and safety evaluations). While details of this regulatory system remain to be finalized, it provides an initial framework through which companies can pursue formal approval of their products and production processes for commercial deployment. In the EU, cell-cultured meat will be evaluated under the EU Novel Food Regulation program, requiring an application process and safety assessment by the European Food Safety Authority (EFSA). Regulatory bodies in other countries have been slower to establish clear pathways through which cell-cultured meat products can obtain approval for sale and consumption.

The potential use of genetic modification in the development of cell-cultured fat and other tissue types may create additional consumer acceptance and regulatory challenges. Genetic engineering is a common approach to cell line development, and may be a useful tool for overcoming key limitations related to proliferation capacity, contact inhibition, nutritional requirements, and differentiability of primary cell lines for production of cell-cultured fat. Particularly in the EU, use of genetic modification in food products likely precludes regulatory approval (Fresco, 2013). Even in regions where regulation of genetically engineered foods is not as stringent, consumers are often averse to such products as a result of health concerns, even when scientific consensus indicates no elevated risks (Wunderlich & Gatto, 2015). Thus, reliance on genetic modification, while attractive from a technical perspective, may create downstream challenges for the approval and acceptance of cell-cultured meat by both consumers and relevant regulatory bodies.

Price is another factor likely to play a significant role in shaping consumer acceptance of cell-cultured meats and other products of cellular agriculture. Meat is among the most price elastic of food categories, meaning that demand is significantly affected by price changes (Andreyeva et al., 2010). Demand for high-fat foods is particularly sensitive to changes in price and consumer income (Santeramo & Shabnam, 2015). Given these trends, cost-reduction efforts for production of cell-cultured fat will be essential for market success. Initial product releases will likely feature small-batch, specialty foods at a high price point, but consumers are unlikely to adopt such products as part of their regular purchasing habits until cost-competitiveness with existing options is achieved. The central issue in reducing production costs for cell-cultured meats or ingredients is the challenge of achieving efficient, low-input, large-scale cell cultivation. Radical innovations in cell line development, bioprocess engineering, and cell culture media development will likely be essential to enable production of sufficient cell mass at low enough cost to make cell-cultured foods commercially viable.

CONCLUSIONS

As the global population expands and meat consumption rises, alternative food production strategies are necessary to curb the environmental and animal welfare concerns associated with conventional food

systems (Willett et al., 2019). Cellular agriculture is a promising paradigm for sustainable and ethical production of foods like meat, eggs, and dairy that are typically derived from animals. The application of cell culture and tissue engineering technologies to create cell-cultured meat is a particularly important effort and has been a focus of media attention, investment, and research since the debut of a prototype cell-cultured hamburger in 2013. While most innovation in this space has focused on muscle tissue, the development of scalable fat production systems will be essential for replicating sensory and nutritional qualities of conventional meat products. Possibilities for incorporation of cell-cultured fat tissue as an ingredient in predominantly plant-based and animal-based meats make this an exciting area of development, since improving the quality of plant-based alternatives and partially displacing animal-based products may allow positive economic and environmental impacts before pure cell-cultured meats are ready for market.

Production of cell-cultured fat requires stable cell lines capable of extended proliferation and robust differentiation into adipocytes, scalable low-cost bioprocess strategies, and tissue engineering approaches to create edible constructs that replicate features of *in vivo* tissues. Satellite cells, DFAT cells, MSCs, iPSCs, and ESCs are all capable of differentiation into adipocytes or adipocyte-like cells and may be useful for fat culture for food. None of these cell types are perfectly suited for scalable production, as tradeoffs are present between plasticity, proliferation capacity, homogeneity, and sensitivity to media and culture conditions. The cell line or lines selected for use will depend to a significant degree on features of the broader production system and may require cell line engineering or strategic adaptation to meet key needs. The landscape of relevant bioprocess strategies is analogous. Various techniques, ranging in scale from adherent monolayer culture in T-flasks to high-density monosuspension in stirred-tank reactors, have been used successfully for *in vitro* proliferation of various adipogenic cell types, but the challenge of adaptation to highly scalable systems limits direct application for growth and differentiation of primary cells for cell-cultured meat production. Given these constraints, exploration of novel and alternative bioprocess systems should be prioritized alongside the application and optimization of existing strategies. For tissue engineering, edible, low-cost, sustainably produced biomaterials like chitosan and alginate have been identified that are capable of supporting cell growth and differentiation and may support development of 3D adipose tissue constructs that mimic features of native tissues. In the development of scaffold-based approaches, it will be necessary to perform material characterization and optimization to match structural, mechanical, and textural properties of animal-based meats. Scaffold-free approaches to tissue generation have also shown promise, but extensive engineering will be necessary to increase the scale of such systems.

The development of production systems capable of generating adipose tissue at the scale and price-point necessary for commercial viability will require investment and innovation in multiple biotechnology disciplines. The body of work presented here draws from adjacent industries and provides a starting point for various research paths relevant to the pursuit of cell-cultured fat for food. Our perspective on the needs and opportunities for cell-cultured fat is largely consistent with reports on the broader prospects of cell-cultured meat development (Post, 2012; Specht et al., 2018). However, our conclusions contrast with claims that the development of cell-cultured meat and its successful integration into the marketplace are inevitable, and that the myriad technologies necessary for *in vitro* production of relevant tissues have already been developed and validated. While expert reports in the peer-reviewed literature present relatively balanced viewpoints, industry groups in the space have made unsubstantiated claims about the state of relevant technologies and timelines to commercialization. To ensure a productive future for cellular agriculture research and development, the breadth and depth of academic work and open collaborations in the space must be increased. This will serve to provide a stronger foundation upon which commercial ventures can be based, as well as more accurate measures of progress that can be used to inform industry predictions and anticipate future needs. If such efforts are successful, *in vitro* production of meat and other animal products may ameliorate the environmental impacts of industrial animal

agriculture, address concerns about food system sustainability and scalability, and improve animal welfare.

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KF conceived and planned the project and wrote the manuscript. JY, AS, and NR researched and wrote the Tissue Engineering, Importance of Fat in Meat Products, and Adipose Tissue Biology sections, respectively. AS and DK provided major revisions and feedback on the complete manuscript. JY and NR provided minor revisions and feedback.

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Figure 1. Process overview for cell-cultured fat production. a) Adipogenic cells are isolated from the desired species and established as a cell line in culture. A seed train is used to expand the cells into a scalable bioreactor system for commercial production. b) Single cells are induced to differentiate inside the bioreactor or following initial harvest, and accumulate lipids. These cells can then be used as an ingredient to improve the quality of plant-based and animal-based meat products. c) Alternatively, cells harvested from the bioreactor are seeded into an edible or biodegradable biomaterial scaffold and differentiated to form 3D adipose tissue that can be combined with culture muscle and connective tissue to create structured meats *in vitro*.

Figure 2. a) Signaling pathways and transcription regulation of adipogenesis. The insulin (Ins) and insulin like growth factor 1 (IGF 1) pathways promoted lipid accumulation by inhibiting peroxisome proliferator-activated receptor gamma (PPARγ), the master regulator of adipogenesis. The Wnt and hedgehog (HH) pathways conversely inhibit this process. CAAT/enhancer binding protein beta (C/EBPβ) and C/EBPδ activate PPARγ and C/EBPα, which in turn form a positive feedback loop and promote expression of genes that drive the adipocyte phenotype. This pathway is regulated by a variety of other factors, including signal transducer and activator of transcription 5 (STAT5), sirtuin (SIRT 1 and 2), and foxhead box protein 01 (FOX01) (Lowe et al., 2011). b) Structure of fatty acids prevalent in consumer meat products.

Figure 3. Overview of bioprocess strategies of potential utility in the production of cultured fat tissue, across the negatively correlated spectrums of scalability and ease of adaptation. a) Standard T-flask. b) Multi-layer flask. c) Hollow-fiber bioreactor vessels, d) Fixed bed bioreactor. e) Stirred-tank bioreactor that can be used for single-cell suspension culture, microcarrier-based culture, or aggregate culture.

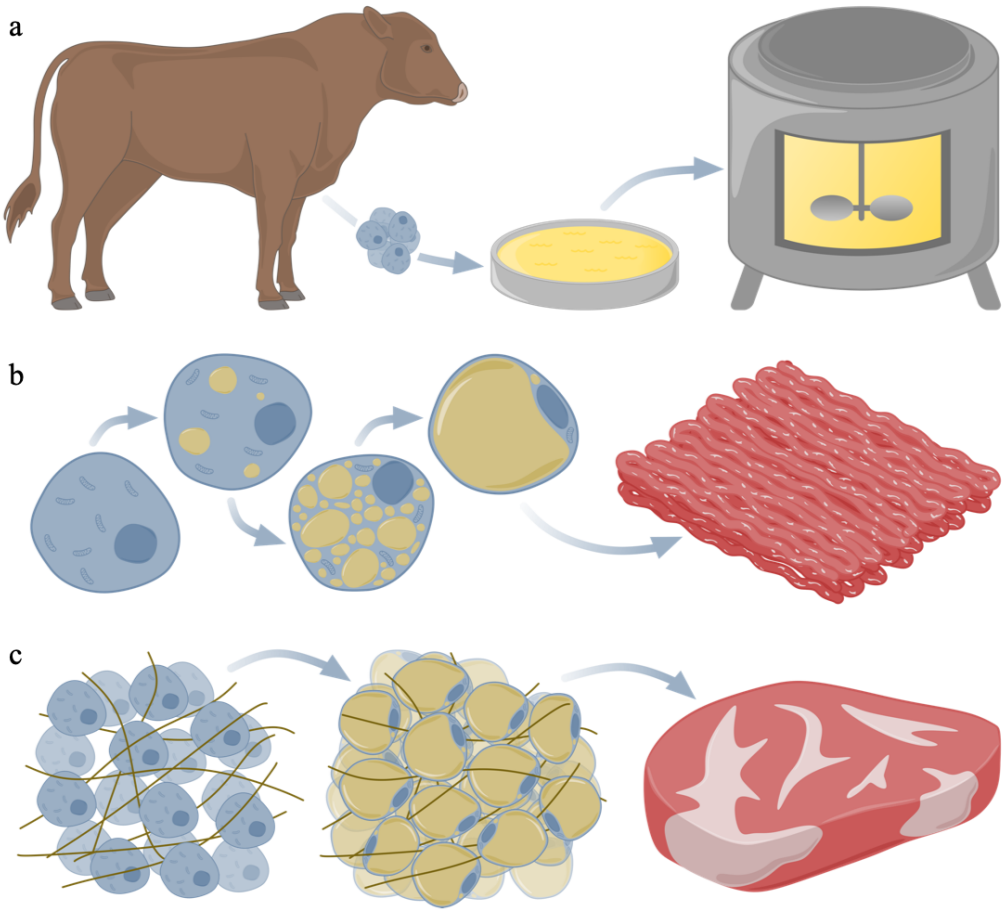

Figure 1.

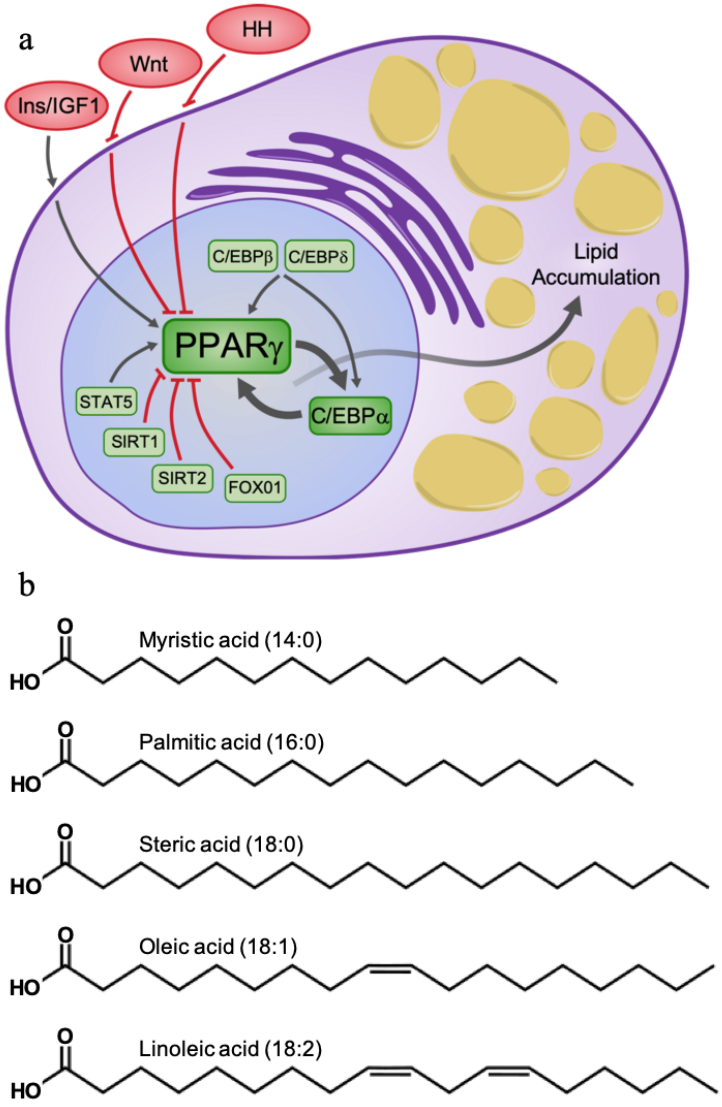
a
HH
Wnt
Ins/IGF1
C/EBPβ
C/EBPδ
Lipid
Accumulation
PPARγ
STAT5
C/EBPα
SIRT1
SIRT2
FOX01
b
Myristic acid (14:0)
Palmitic acid (16:0)
Steric acid (18:0)
Oleic acid (18:1)
Linoleic acid (18:2)

Figure 2.

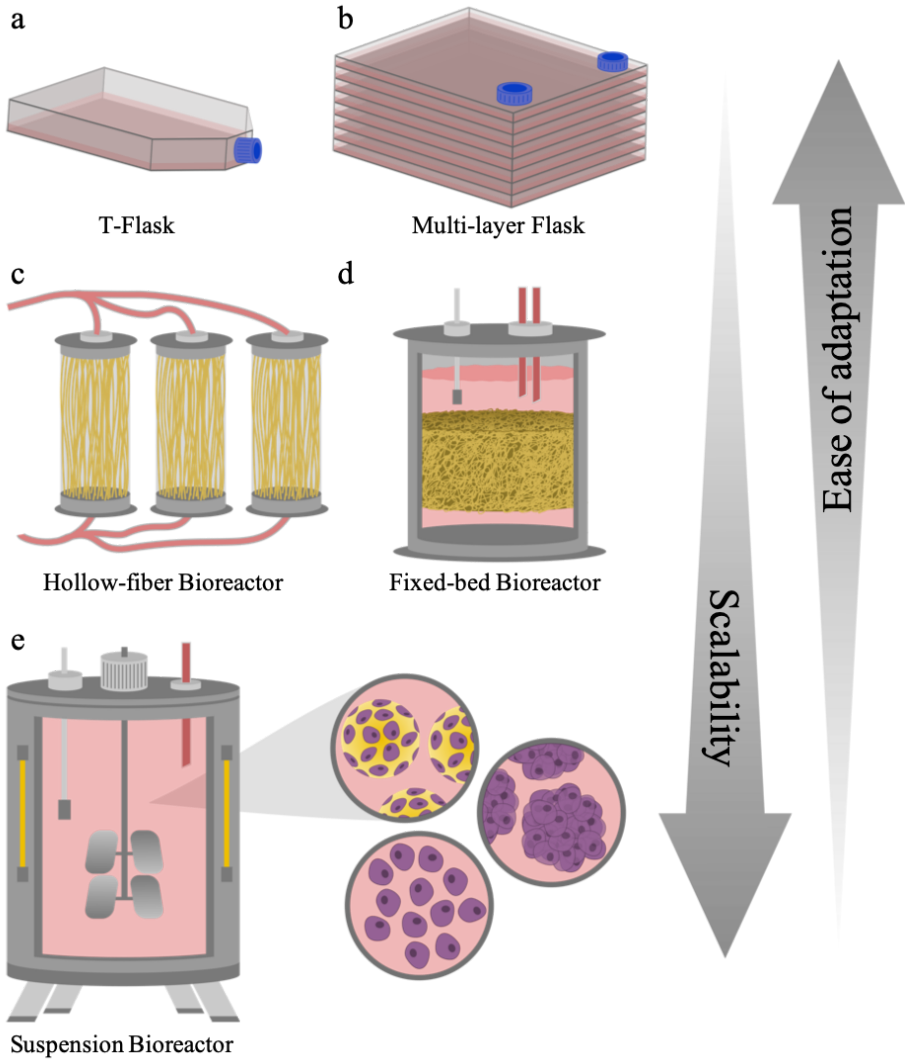
a
b
T-Flask
Multi-layer Flask
c
d
Hollow-fiber Bioreactor
Fixed-bed Bioreactor
e
Suspension Bioreactor
Scalability
Ease of adaptation

Figure 3.

Type	Animal Source	Product (Diet)	% IMF	% total fat			n-6/n-3
				SFA	MUFA	PUFA	
Meat	Porcine	Longissimus Lomborum; Loin (+ Beef tallow) (Mitchaothai et al., 2007)	3.03 +/- 0.5‡	37.93 +/- 2.79‡	46.55 +/- 3.52‡	12.93 +/- 4.42‡	9.82 +/- 1.2‡
		Longissimus Lomborum; Loin (+ Sunflower oil) (Mitchaothai et al., 2007)	2.97 +/- 0.6‡	35.32 +/- 2.83‡	41.63 +/- 2.63‡	21.07 +/- 4.75‡	27.01 +/- 6.57‡
		Longissimus Dorsi (+ Palm-kernel oil) (Teye et al., 2006)	2.458 +/- 0.252†	35.6 +/- 0.58†	45.15 +/- 1.14†	14.35 +/- 1.08†	8.25**
		Longissimus Dorsi (+ Soybean oil) (Teye et al., 2006)	2.271 +/- 0.252†	34.90 +/- 0.58†	42.88 +/- 1.14†	17.70 +/- 1.08†	9.52**
	Hircine	Longissimus Lomborum; Loin (Ad libitum diet) (Lopes et al., 2014)	2.2 +/- 0.24†	47.5 +/- 1.25†	45.6 +/- 1.08†	3.03 +/- 0.42†	3.94 +/- 0.28†
		Longissimus Lomborum; Loin (50% restricted diet) (Lopes et al., 2014)	2.0 +/- 0.24†	55.6 +/- 1.25†	30.2 +/- 1.08†	4.39 +/- 0.42†	1.70 +/- 0.28†
	Bovine	Longissimus Lomborum; Loin (Pasture) (Coleman et al., 2016)	3.7 +/- 0.3†	48.88 +/- 0.56†	45.72 +/- 0.67†	5.39 +/- 0.39†	1.07 +/- 0.02†
		Longissimus Dorsi; Ribeye (Feedlot) (Freitas et al., 2014)	3.76 +/- 0.28†	45.84 +/- 0.349†	44.11 +/- 0.349†	9.98 +/- 0.295†	5.82 +/- 0.171†
		Longissimus Dorsi; Ribeye (Pasture) (Freitas et al., 2014)	3.65 +/- 0.25†	45.66 +/- 0.33†	43.86 +/- 0.318†	10.13 +/- 0.259†	3.65 +/- 0.150†
	Avian (Chicken)	Breast (Soybean) (Sirri et al., 2010)	1.08 +/- 0.04†	32.1 +/- 0.29†	28.7 +/- 0.56†	37.5 +/- 0.48†	5.34 +/- 0.17†
		Breast (Faba bean) (Sirri et al., 2010)	1.06 +/- 0.04†	32.8 +/- 0.29†	30.3 +/- 0.56†	35.3 +/- 0.48†	5.74 +/- 0.17†
		Thigh (Soybean) (Sirri et al., 2010)	3.48 +/- .15†	29.9 +/- 0.25†	33.2 +/- 0.41†	35.1 +/- 0.52†	8.48 +/- 0.13†
		Thigh (Faba bean) (Sirri et al., 2010)	2.99 +/- 0.15†	31.5 +/- 0.25†	34.4 +/- 0.41†	32.2 +/- 0.52†	8.03 +/- 0.13†
	Piscine (Whitefish)	Fillet (Lake Bolsena) (Orban et al., 2006)	1.85 +/- 0.79‡	30.65 +/- 1.38‡	29.12 +/- 4.32‡	35.23 +/- 5.41‡	0.27 (0.26-0.29)#
		Fillete (Lake Bracciano) (Orban et al., 2006)	3.00 +/- 1.11‡	29.09 +/- 1.23‡	32.4 +/- 2.18‡	33.16 +/- 3.39‡	0.36 (0.34-0.38)#
Full Fat	Porcine	Fatback (Mitchaothai et al., 2007)	N/A	38.01 +/- 3.21‡	44.8 +/- 2.56‡	14.23 +/- 3.23‡	9.46 +/- 4.10‡
	Ovine	Mutton tallow (Sreenivasan, 1968)	N/A	43.5**	49**	7.5**	n.g.
	Bovine	Beef tallow (Sreenivasan, 1968)	N/A	64.9**	31.9**	2.3**	n.g.
	Avian	Duck fat (Sreenivasan, 1968)	N/A	27.4**	57.6**	14.9**	n.g.

Table 1. Fatty acid composition of various meat products.

† Variance given as SEM, ‡ Variance given as SD, # Variance given as the range of measurements, ** No variance information available

IMF: Intramuscular fat, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, n-6/n-3: ratio of omega-6 to omega-3 fatty acids

Table 2. Relevant characteristics of adipogenic cell types for cell-cultured fat production.

Cell Type	Ease of Isolation	Plasticity	Proliferative Capacity	Sensitivity to Culture Conditions	Ease of Adipogenic Differentiation
ESCs	Very Difficult	High	Unlimited	High	Difficult
iPSCs	Difficult	High	Unlimited	High	Difficult
MSCs	Easy	Moderate	High	Low	Species specific
DFAT cells	Easy	Low	Moderate	Low	Easy
Satellite cells	Moderate	Low (disputed)	Low	Moderate	Uncertain

ESCs: Embryonic stem cells, iPSCs: Induced Pluripotent Stem Cells, MSCs: Mesenchymal stem cells, DFAT cells: Dedifferentiated fat cells

Table 3. Comparison of bioprocess strategies for *in vitro* adipocyte production.

Bioreactor System	**Mode of Operation**	**Cell Densities** (Allan et al., 2019; Ellis et al., 2005)	**Working Volume for 1 kg of Meat** (Allan et al., 2019)	**Main Advantages**	**Main Disadvantages**
T-Flasks	Batch	1 x 10^5 cells/mL	2,900 L	• Ease of cell adaptation • Ease of use	• Lack of scalability • Inconsistency
Multi-layer flasks	Batch	1 x 10^5	2,900 L	• Improved scalability compared to T-flasks	• Scalability limited by surface area to volume ratio
Hollow-fiber bioreactors	Batch (perfusion)	10^8-10^9	1.4 L	• High density culture • *In vivo*-like tissue structure	• Difficult cell harvests • Not validated at industrial scales
Packed bed bioreactors	Batch (perfusion)	2.93 x 10^6	110 L	• High density culture • 3D growth of adherent cells	• Difficult cell harvests • Not validated at industrial scales
Suspension bioreactors	Batch, Fed-batch, or Continuous	2 x 10^6	570 L	• Ease of scale-up from benchtop to factory • Moderate-high culture densities	• Challenge of suspension adaptation • Large volumes required

Scaffold Material	Source	Mechanical Properties	Max Thermal Stability	Relevant Past Use	Main Advantages	Main Disadvantages
Synthetic Polymers (PLGA, PLA, PGA)	Chemical synthesis	≈0.1 MPa to >10 MPa	250-300 °C	Differentiation of murine preadipocytes on PLGA polymer discs (Patrick et al., 1999)	• Consistency • Broadly tunable mechanics and degradation	• Uncertain effects on organoleptic properties
Native ECMs	Various animal sources	<1 kPa to ≈5 kPa	280-315 °C (Collagen)	Adipogenesis from human MSCs in directional collagen scaffold (Hume et al., 2018)	• Natural substrates for cell growth	• Animal derived • Expensive • Batch-to-batch variability
Alginate	Brown seaweed	≈10 kPa to >400 kPa	215-285 °C	Differentiation of bovine and porcine adipose-derived stem cell in alginate hydrogels (Kim et al., 2010; Mehta et al., 2019)	• Low cost • Validated with food-relevant adipose tissue • Positive impacts on sensory qualities	• Lack of native cell attachment sites • Chemical processing likely required for functionalization
Chitosan	Crustacean exoskeletons and fungal mycelia	Broadly tunable	300-350 °C	*In vivo* and *in vitro* adipogenesis from murine preadipocytes on collagen-chitosan hydrogels (Wu et al., 2007)	• Common food additive • Previous use for cell-cultured meat applications • Antimicrobial properties	• Lack of validation for adipogenesis on pure chitosan • Chemical conversion from chitin often required • Batch-to-batch variability for crustacean sources

Table 4. Comparison of scaffolding materials for food-safe engineering of adipose tissues.
PLGA: poly(lactic-co-glycolic acid), PLA: poly(lactic acid), PGA: poly(glycolic acid), ECMs (extracellular matrices)