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Perspectives on Scaling Production of Adipose Tissue for Food Applications

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Abstract

With increasing meat consumption and significant environmental impact during production, it is important to develop sustainable alternatives to meat. Since fat is an important contributor to meat flavor, recapitulating this component in meat alternatives such as plant-based and cell cultured meats is important. Here, we discuss the topic of cell cultured or tissue engineered fat, growing adipocytes *in vitro* that could imbue meat alternatives with the complex flavor and aromas of animal meat. We outline potential paths for the large-scale production of *in vitro* cultured fat, including adipogenic precursors during cell proliferation, methods to proliferate and adipogenically differentiate cells at scale, as well as strategies for converting differentiated adipocytes into 3D cultured fat tissues. We showcase the maturation of knowledge and technology behind cell sourcing and scaled proliferation, while also highlighting that adipogenic differentiation and 3D adipose tissue formation at scale need further research. We also provide some potential solutions for achieving adipose cell differentiation and tissue formation at scale based on contemporary research and the state of the field.

Keywords

Adipose, cell culture, tissue engineering, food, cellular agriculture, cultured fat, cultured meat

Introduction

Raising livestock has been calculated to be a significant contributor to human impact on the environment, comprising an estimated 15%-18% of anthropogenic greenhouse gas (GHG) emissions and 44% of methane emissions [1,2]. For comparison, this is higher than the 14% attributed to GHG emissions attributed to transportation, with livestock emissions projected to continue to rise [3]. Currently, animal farming uses at least one-third of available land and fresh water [4]. Animal agriculture also generates wastes such as manure, which contributes to groundwater and local ecosystem contamination as well as eutrophication [5]. Meat production is the largest driver of livestock production, and concerns over the two extend beyond environmental and ecological impacts, affecting human health and playing a role in the growth of antibiotic resistance and emergence of pathogenic diseases; high levels of red meat consumption have increasingly been linked with the development of chronic and acute conditions such as stroke, diabetes and coronary heart disease [2,6]. Increased risks of prostate cancer, colorectal cancer, and pancreatic cancer are also linked to red meat consumption, especially processed red meat [7,8]. The emergence of antimicrobial resistance has been associated with antibiotic use in animal agriculture systems, causing significant health and food safety complications through human contact with contaminated food, water or manure and the horizontal gene transfer of antibiotic resistance genes amongst microbes [9–12]. The emergence and spread of zoonotic diseases have been connected to livestock farming practices, with over half of all human pathogens classified as zoonotic and 73% of emergent pathogens identified as zoonotic [13,14]. Recently, livestock processing plants posed a public health risk in the United States, being associated with 236,000 to 310,000 cases of coronavirus disease 2019 (COVID-19) in the United States during the first half of 2020 [15].

As meat production is predicted to increase 1.8-fold by 2050 from 2005/2007 levels, the impact of meat consumption on human health and the environment are at risk of further increasing [16]. Due to this, alternatives to conventional meat production that incur fewer negative impacts on human health and the environment are needed. Alternatives to conventional animal meat should initially be comparable in taste, texture, and sensory properties in order to satisfy the same consumer demand that drives meat consumption. Plant based meat utilizes plant or other non-animal components to mimic animal meat, bypassing the low efficiency feed to food conversion ratios encountered when raising livestock for meat (3% for beef for calories and protein) [17]. Beyond Meat produces plant-based meat alternatives on a large-scale, including a burger patty calculated to involve 90% less GHG emissions and land use, requires less than half the energy and over 99% less water use when compared to traditional livestock-derived beef [18]. Similarly, the Impossible Burger claims to require 87% less water, 96% less land, 89% less GHGs and 92% less aquatic pollutants than its beef equivalent [19].

Cultured meat (also called *in vitro*, cultivated, lab grown meat) is another alternative to traditional animal agriculture that aims to produce the skeletal muscle and adipose tissues that normally comprise animal meats, except using *in vitro* tissue and bioengineering techniques [20,21]. By directly growing meat (muscle and fat) *in vitro*, energy and nutrients can be more efficiently focused on the outcome [20,22]. The time frame to generate cultured meat tissues *in vitro* is also thought to be faster compared to animal agriculture, only requiring several weeks as opposed to months or years for pork and beef [23–25]. Moreover, the tight control over the cell biology during tissue cultivation, as well as the overall production process, allows for the fine tuning of parameters such as nutrition, where muscle and fat cells can be engineered to

produce vital nutrients that would otherwise not be found (or only at low concentrations) in conventional animal meat [26]. Taken together, these factors suggest that cultured meat production systems can offer healthier, more efficient and more environmentally compatible options to traditional animal sourced meats. Further, opportunities to improve food safety at all levels of need also derive from the cultured meat production process.

To date, the focus of plant based and cultured meat alternatives has been placed on the recapitulation of muscle components of meat [21,27,28]. However, fat is also a crucial component, contributing to sensory and textural attributes [29]. For example, an increase in fat content improved the juiciness and tenderness ratings of Japanese black steer samples, with maximum evaluation scores attributed to samples containing 36% crude fat [30]. Beef samples with an intramuscular fat content over 10% had significantly higher amounts of lipid-derived flavor volatiles, implying that the adipose content of meat improves texture as well as flavor [31]. The lipid component of meat is also responsible for the complex, undefined species-specific flavor that is present in meat from different animals, meaning that the inclusion of specific adipocytes (and/or other cell types) may be important for plant-based and cultured meats that aim to fully recapitulate the sensory profile of conventional meat [32,33]. For plant-based meats, this could mean directly supplementing cultured adipocytes to generate a plant-cultured hybrid meat product. In addition to species specific flavors, the addition of adipocytes to plant meats could generally aid in incorporating an animal “essence”, or general meat specific flavor. Hundreds of volatile compounds comprise the aroma of cooked animal meat, with the majority of them originating from the degradation of lipids upon heating [34,35].

Since fat plays a crucial role in the consumption of animal meat, cultured adipocytes are hypothesized to be key to sensory qualities in cultured and plant-based meats. This review contains a guide to the biology and engineering surrounding larger scale adipocyte cell cultures, with consideration for culturing fat for food applications. We provide an evaluation of the cell sources that may be used to produce cultured fat, followed by techniques for proliferating these cells at scale. We also discuss efficient induction of adipogenesis of the cells after large-scale proliferation, as well as how cultured adipocytes might ultimately be converted from cells into edible, macroscale fat tissue.

Cell Sources for Scaling Adipocyte Culture

Interest in large-scale adipocyte culture has centered on the potential use of human cells in personalized medicine [36]. With the recent emergence of cultured meat products, attention has expanded to include adipocytes from non-human animal sources that can be used to augment cell-based meats. Various cell sources (adipogenic precursor cells) can be utilized for cultured fat, related to proliferative capacity and lipid accumulation. Senescence is an issue that also has to be considered in the process. Some common cell types used in adipose tissue engineering applications, including pluripotent stem cells (PSCs), can be passaged indefinitely, while maintaining some proliferative and adipogenic capacities. Both natural and engineered cells are reviewed here as sources for large-scale production of adipose tissue for food. Here, we designate dedifferentiated fat (DFAT), PSCs, embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), adipose derived stem cells (ADSCs), and preadipocytes as natural cells and highlight their potential as cell sources for large-scale adipocyte generation. In the following section, we discuss cells, such as MSCs, will senesce, and their adipogenic capacity is lost.

However, primary cells, including MSCs, ADSCs, and preadipocytes (i.e., stromal vascular fraction-isolated cells) show improved lipid accumulation and differentiation potential compared to most PSCs. Though it is unlikely that livestock-derived PSCs will replace murine PSCs in most biomedical research, they have varied and timely applications in animal welfare, and the emerging field of cellular agriculture. We focus on some of the most promising livestock PSC sources and discuss future steps to improve their proliferative and adipogenic capacities. These cells are generally free from genetic manipulation or mutagenesis, or they can also be bioengineered to increase proliferative capacity or prevent senescence (i.e., immortalization) [37]. Bioengineered cells, including induced pluripotent stem cells (iPSCs), transdifferentiated cells, and genetically engineered cells are also considered as potential sources for food outcomes. A summary of cell sources reviewed here is provided in **Table 1**.

Primary/Unaltered/Natural Cells

Dedifferentiated fat (DFAT) cells

DFAT cells have emerged as a cell source for engineered fat applications [38]. DFAT cells are derived from lipid-containing (mature) adipocytes, which possess the ability to symmetrically or asymmetrically proliferate, replicate, and redifferentiate or transdifferentiate. Though adipocytes were previously thought to be terminally differentiated, their ability to proliferate into large numbers of daughter cells was documented nearly 50 years ago [39]. The origins and characteristics of DFAT cells have been reviewed elsewhere [38], and more recent studies have elucidated some of the mechanisms by which DFAT cells maintain both proliferative and differentiation capacities [40]. Briefly, DFAT cells are distinguishable from other adipocytes via the expression of the stem cell markers cluster of differentiation (CD)34, Stem cells antigen (Sca)1, CD90, and CD45, as well as octamer binding transcription factor (Oct)3/4, sex-determining region y box 2 (Sox2), myc proto-oncogene (c-Myc), and Krüppel-like factor 4 (Klf4). Under specific culture conditions, DFAT cells are multipotent; forming adipocytes, chondrocytes [41], osteoblasts [42], skeletal myocytes [43], cardiomyocytes [44], and smooth muscle cells [45]. Despite their multipotency, DFAT cells readily undergo adipogenic differentiation when culture media containing a mix of Dulbecco's modified Eagle medium/Nutrient Mixture F-12 (DMEM/F12) and 10% horse serum (HS) or fetal bovine serum (FBS) and is used. Overall, DFAT cells are promising for large-scale engineered fat production in terms of both proliferative capacity and adipogenic potential.

Porcine DFAT cells maintain proliferation and adipogenicity over at least 37 passages [46]. DFAT cells accumulated more lipid that stromal vascular-isolated preadipocytes, MSCs, and ADSCs, highlighting their enhanced potential for use in large-scale cultured fat production [46]. Porcine DFAT cells also grew and accumulated lipids over 60 days in culture, and consistently expressed adipocyte genes Peroxisome proliferator-activated receptor gamma (PPARγ), adipocyte protein 2 (aP2), lipoprotein lipase (LPL), and adiponectin [47]. Additionally, these cells maintained the capacity for myogenic differentiation when the culture medium was changed to include galectin-1. Interestingly, flow cytometric analysis showed that the porcine DFAT cells displayed similar cell-surface antigen profiles to MSCs, including markers CD44, CD29, and CD90, suggesting that DFAT cells maintain their high proliferative capacity during long-term culture at least in part by reverting to a stem-like state. The mechanisms by which this reversion takes place are yet to be elucidated, but improving understanding of these cellular processes will be a key factor in scaling up the cell-based production of adipose tissue. Overall,

DFAT cells are promising for large-scale engineered fat production in terms of both proliferative capacity and adipogenic potential. The mechanisms by which this reversion takes place are yet to be elucidated, but improving the understanding of these cellular processes will be a key factor in scaling up the cell-based production of adipose tissue.

Bovine DFAT cells have also been explored as a potential cell source for engineered adipose tissue. Bovine DFAT cells were not as adipogenic or proliferative as porcine cells, possibly due to the culture parameters evaluated [48]. Prior work has optimized and improved adipogenesis conditions for human, murine, and porcine cells, which share commonalities in terms of their biology. There remains a need to develop culture parameters for adipogenesis from bovine cells, and some recent work has focused on addressing this gap. Wagyu-intramuscular fat (IMF) DFAT cells cultured in flat plates versus flasks had increased viability, and more of the subpopulations of cells were adipogenic, compared to cells cultured in standard flasks [48]. High confluence promoted re-differentiation from a stem-like multipotent state to mature adipocytes. Despite the limitations compared to porcine DFAT cells, bovine DFAT cells can be returned to a proliferative state, and recent work has demonstrated simple and optimized culture conditions for improving this process [49]. A mix of DMEM/F12 and 10% fetal bovine serum (FBS) was sufficient to de-differentiate the cells when combined with inverting the culture flasks [49]. Taken together, existing work illustrates that improved culture conditions and media formulations should improve the proliferative and adipogenic capacity of bovine DFAT cells to improve yields of both proliferative and differentiating DFAT for scaling adipocyte generation.

Pluripotent Stem Cells (PSCs)

Mouse embryonic stem cells are considered the “original” PSC, and have helped illuminate many of the conditions favorable to adipogenesis [36]. While their role in deciphering potential adipogenic mechanisms has been invaluable, mouse cells are largely not appropriate for uses in regenerative medicine and cultured meat application. Instead, several livestock sources of ESCs have been developed in the last decade, and are reviewed comprehensively elsewhere [50].

Though much research has focused on the potential uses of porcine DFAT cells for cultured adipose tissue, a surprising number of studies have attempted to generate and characterize porcine PSCs. This is partially due to the high applicability of porcine cell and tissue models in human medicine, as well as the constant search for improved gamete generation and breeding schemes of farmed animals [51]. An optimized method for obtaining porcine PSCs has yet to be developed, though recent findings are promising. One study found that using culture medium containing basic fibroblast growth factor (FGF)2 and leukemia inhibitory factor (LIF), and inhibitors of mitogen activated protein kinase (MAPK)14, MAPK8, transforming growth factor beta (TGFβ)1, MAP2K1, glycogen synthase kinase (GSK)3A and bone morphogenetic protein (BMP), lentiviral-reprogrammed porcine PSCs were transformed into naïve-state stem cells [52]. The cells were viable for over 45 passages and expressed the pluripotency-related endogenous genes pPOU5F1 and pSOX2. RNA-sequencing (RNAseq) data suggested that the key mechanism was blocking TGFβ1 and BMP signaling. Many compounds can block these pathways, making this finding an important future direction to explore in the generation of adipocytes for cultured fat. It is worth noting that a significant limitation of PSCs is that the cells must be first be differentiated into MSCs, and then

adipogenically differentiated into fat [53]. These complex processes take time and result in some off-target effects, such as osteogenic or chondrogenic differentiation [54]. Direct conversion of PSCs to adipocytes or to readily accumulate lipid (i.e., become adipocyte-like cells), would make this process a more viable option. Despite this concern, PSCs remain a promising cell source for large-scale cultured fat production.

Mesenchymal Stem cells (MSCs), Adipose-Derived Stem Cells (ADSCs), and Preadipocytes

Other cell sources such as MSCs, ADSCs, and preadipocytes merit continued investigation with similar proliferative and adipogenic capacity to DFAT cells and PSCs [55]. In addition to specialized culture conditions, primary cells may require the selection of specific adipogenic clusters from the isolated population. In this case flow cytometry can be used to identify the presence of several adipogenic markers within a subpopulation of cells before these cells are differentiated. While MSCs can be obtained from adipose tissues, MSCs are frequently derived from bone marrow (BM-MSCs). In the context of this review, we refer to adipose-derived MSCs as ADSCs, while MSCs are multipotent stem cells derived from other sources, mainly bone marrow, but potentially also from cord blood or neural tissue [56]. While several decades of research have determined that MSCs have excellent osteogenic and adipogenic capacities [57][58], MSCs have yet to be successfully grown at the scales necessary for cultured fat production. We briefly examine recent work to optimize culture conditions and clone selection in order to enhance the adipogenic potential of MSCs for cultured adipose production at scale.

Long-term adipogenesis of MSCs may be enhanced via immunomodulation, as inflammation impacts ectopic fat formation, as abnormal adipogenesis is a persistent symptom of chronic inflammatory diseases [59]. Immunomodulation for enhancing lipid accumulation is promising for cultured fat applications, as the inflammatory factors that drive adipogenesis can be removed once the desired fat has developed. However, scaling up this approach remains a challenge. Under inflammatory conditions, human MSCs were no longer able to suppress neutrophil adhesion, while changes in interleukin (IL)-6 and TGFβ1 signaling resulted in increased adipogenesis [60]. Native stromal cells, adipocytes derived from native stromal cells, and mature adipocytes from adipose tissue were immunoprotective, and did not undergo increased adipogenesis in response to changes in IL-6. Increased inflammation appeared to cause abnormal adipogenesis, suggesting that mimicking inflammation, and particularly the observed increases in IL-6 and TGFβ1, may enhance the adipogenic potential of MSCs. Despite the increased adipogenesis in small numbers of cells, it remains unclear whether large amounts of fat can be generated by growing MSCs in culture conditions that mimic inflammation. In addition to immunomodulation, other culturing methods can be used to enhance the adipogenic potential of MSCs. A chondrogenic aggregate culture method was utilized to prepare 3D adipogenic cultures. The MSCs differentiated rapidly, and the aggregates could be handled and processed for histologic and biochemical assays [61]. The assessment of cell senescence showed that the aggregates maintained some proliferative capacity, though long-term viability over multiple passages was not assessed.

Unlike BM-MSCs, ADSCs are MSCs obtained from fat tissues [62–64], though with some differences in CD expression and genetic profiles [63]. ADSCs may be proliferative enough such that immortalization is not explicitly required for large-scale production [65]. Simple additives such as glutathione (GSH) and melatonin prevent ADSC senescence and preserve

stemness and multidirectional differentiation potential over at least 9 passages [66]. ADSCs are also readily available and easy to isolate, and several protocols exist to optimize harvest and expand [62,65]. Additionally, ADSCs may suppress rather than drive inflammation [67], making them a better cell source than MSCs for certain therapeutic applications. Finally, ADSCs can be effectively grown in animal-derived antigen-free media [68].

Despite their promise, growing large quantities of fat from ADSCs has proven challenging. Indeed, most prior large-scale expansions of MSCs have generated quantities of cells sufficient for clinical applications, but not cultured meat applications. An additional challenge is maintaining MSC viability once 3D cultured fat constructs reach a usable size. Aggregate culture offers a possible bypass for this problem. Aggregate culture is able to produce 3D constructs for osteogenic and chondrogenic differentiation of ADSCs [61], and robust lipid accumulation occurs when human ADSCs were cultured in 3D. However,3D adipogenic differentiation may also result in cell senescence or necrosis at the center of the constructs.

Recently characterized transcriptional profiles and single cell RNA-seq data may also offer clues for maintaining the proliferative and adipogenic potential of ADSCs [69]. ADSCs isolated from human fat and positive for CD34 and CD 90 differentiated into adipocytes when placed in culture, and were capable of generating a complete adipose tissue *in vitro* [70]. Differentiated adipocytes expressed several lipid-specific molecules, including adiponectin and peroxisome proliferator-activated receptor gamma. In addition, fibroblasts (approximately 10% of the whole sorted-cell population) secreted an extracellular matrix (ECM) after 60 days that was positive for type I collagen and fibronectin. After long-term culture (4 months), an adipose tissue with collagenic fibers and vessels formed, and was comparable with adult human adipose tissue. Overall, ADSCs may have enhanced regenerative capacity compared to MSCs [71], and other recent evidence suggests their adipogenic capacity may be comparable to DFAT cells [72]. More work is needed to assess the long-term potential of ADSCs for cultured fat. ADSCs derived from livestock animals, rather than human or murine sources, are highly desirable for non-clinical applications, such as adipose tissues for use in cultured meat.

ADSCs have been isolated from bovine [73], porcine [74,75], and chicken [76] sources. All three have multilineage potential, and while not immortalized, they possess significant proliferative and self-renewal capacities. Bovine and porcine ADSCs have been expanded for at least 25 and 28 passages, respectively, while chicken ADSCs were still viable after 37 passages [76]. Additionally, these livestock-derived cells expressed surface markers indicative of ADSCs. Porcine ADSCs expressed the surface markers CD29, CD44, CD71, CD73, CD90, CD105, and CD166, as well as vimentin, and were successfully differentiated into osteoblasts, adipocytes, epithelial cells, neural cells, and hepatocyte-like cells [74]. Bovine ADSCs expressed high levels of β-integrin, CD44, and CD73 [73], and chicken ADSCs expressed CD29, CD31, CD44, CD71 and CD73, though notably, CD31 was not expressed on the surface, indicating some species-specific differences in surface marker expression.

Preadipocytes

Variations in adipogenic and proliferative capacities between ADSCs and preadipocytes have only recently been explored in detail [77]. Preadipocytes are ceiling culture-derived cells that can be harvested from subcutaneous adipose tissue, and display a similar multilineage

potential to ADSCs [78]. The abundance of subcutaneous adipose tissue makes it an attractive cell source [79]. However, little is known about the epigenetic differences between ADSCs and preadipocytes, which may contribute to differences in osteogenic potential, between these cell types. One concern is that the osteogenic potential of preadipocytes is too high to use them in large-scale cultured fat applications. Recent work exploring the epigenetics of osteogenic potential and underlying epigenetic status of human adipose-derived stem cells and ceiling culture-derived preadipocytes found that human ceiling culture-derived preadipocytes showed greater osteogenic differentiation ability than ADSCs. Specifically, the osteogenic regulator genes Runt-related transcription factor (RUNX)2, osterix (SP7), activating transcription factor (ATF) 4, and bone gamma-carboxyglutamate protein (BGLAP) were more likely to be highly expressed in ceiling culture-derived preadipocytes, suggesting that ADSCs are a better choice for generating adipose tissue. Conversely, other studies have demonstrated that preadipocytes have high adipogenic potential, though the location from which they are harvested may impact their propensity to accumulate lipids. Overall, more work is needed to determine if culture conditions can be modulated to enhance the adipogenic or inhibit the osteogenic potential of preadipocytes.

Preadipocytes have also been isolated from livestock animals, and prior work has identified culture conditions to drive adipogenesis. In one study, bovine preadipocytes were efficiently differentiated into mature adipocytes using media supplemented with insulin, dexamethasone, isobutylmethylxanthine (IBMX), octanoate, and 2% Intralipid [80]. The differentiation media also increased mRNA expression of fatty acid binding protein-4, peroxisome proliferator-activated receptor gamma (PPARγ), and acetyl-CoA carboxylase alpha (ACC-alpha), compared with undifferentiated control cells. Another study achieved greater adipogenesis by co-culturing bovine preadipocyte alongside mature adipocytes [81]. While effective protocols exist for bovine preadipocyte isolation [82], more work is needed to scale up the culture procedures to the quantities of fat needed for lab-grown meat. Ultimately, highly proliferative and adipogenic cells are extremely promising for cultured fat applications. Studies highlighted in this section have demonstrated that cell lines exist that can proliferate over dozens of passages and remain adipogenic, though to varying degrees. Until now, most cell lines presented here have been characterized as “proof of concept” studies, with few cells proliferated and adipogenically differentiated at scale. Thanks to this prior work, a framework exists using both natural and engineered cells. Industrial and commercial proliferation of cells for cultured fat can rely on this framework as the demand for large-scale adipose tissue engineering increases, for both cultured meat and biomedical uses.

Table 1. Summary of natural cell sources for large-scale adipose production.

Cell Type	Species	Main outcome(s)	Reference(s)
DFAT	Porcine	Cells expressed stemness markers CD34, Sca1, CD90, and CD45; cells also expressed mature adipocyte markers Oct3/4, Sox2, c-Myc, and Klf4	[40]
	Porcine	Cells remained viable over at least 27 passages	[46]
	Porcine	Cells viable over 60 days in culture; expressed adipocyte genes PPARγ, aP2, LPL, and adiponectin	[47]
	Bovine	Improved cell growth in plates versus flasks; Wagyu-IM DFAT cells used	[49]
	Bovine	Improved adipogenesis with appropriate media formulation, but adipogenesis not as efficient as with porcine cells	[48]

PSCs	Porcine	Using culture medium containing FGF2 and LIF, and inhibitors of MAPK14, MAPK8, TGFB1, MAP2K1, GSK3A and BMP results in lentiviral-reprogrammed PSCs becoming naïve-state stem cells; cells were viable > 45 passages, expressed pPOU5F1 and pSOX2	[52]
	Porcine	Cells required differentiation to MSCs and then adipocytes	[53]
	Porcine	Off-target differentiation of PSCs into bone or cartilage; can be avoided by differentiating without turning into MSCs first	[54]
MSCs	Human	Increased inflammation may be adipogenic; increases in IL-6 and TGFβ1can be used to induce MSC adipogenesis	[60]
	Human	3D aggregate culture improved adipogenesis compared to suspension culture; some proliferative capacity maintained	[61]
ADSCs	Human	Changes in CD-expression profile compared to MSCs	[63]
	Human	Immortalization may not be necessary to maintain proliferation and achieve large-scale production of certain ADSCs	[65]
	Murine	GSH and melatonin prevent senescence, maintain differentiation potential of cells over at least 9 passages	[66]
	Human	ADSCs reduced inflammation compared to MSCs, lowering risk of unwanted adipogenesis	[67]
	Human	ADSCs successfully expanded in animal serum-free media	[68]
	Human	Cells expressed CD34 and CD90; large-scale *in vitro* adipogenesis demonstrated; cells secreted adipogenic ECM after 60 days in culture	[70]
	Human, murine	ADSCs showed enhanced regenerative capacity compared to MSCs	[71]
	Bovine	Cells successfully isolated, cultured over 25 passages; expressed high levels of β-integrin, CD44, and CD73	[73]
	Porcine	Cells successfully isolated, cultured over 28 passages	[74]
	Avian (Chicken)	Cells successfully isolated, cultured over 37 passages; cells expressed CD29, CD31, CD44, CD71 and CD73	[75]
Preadipocytes	Human	Ability to differentiate into multiple cell types including fat	[78]
	Human	Osteogenic regular genes (RUNX2, SP7, ATF4, BGLAP) more likely to be expressed in preadipocytes than ADSCs; ADSCs may be better cell source for fat	[77]
	Human	Preadipocytes have good adipogenic potential; potential varies based on harvest area	[83]
	Bovine	Adipogenic differentiation was effective when media was supplemented with insulin, dexamethasone, isobutylmethylxanthine, octanoate, and 2% Intralipid	[80]
	Bovine	Bovine preadipocytes co-cultured with mature adipocytes resulted in improved adipogenesis	[81]

	Bovine	Isolation methods for bovine preadipocytes exist; additional methods are necessary for future applications	[82]

Engineered cells for robust expansion and adipogenesis

Along with primary or native cell populations, engineered and adapted cell lines offer significant opportunities for scaling cultured fat production. For instance, immortalized somatic cell lines offer advantages in terms of proliferative capacity and control over cell fate and could therefore improve the dependability and scalability of cultured fat production. Precedent exists for this in food-relevant species, such as chicken, where primary preadipocytes have been immortalized through overexpression of Telomerase Reverse Transcriptase (TERT) alone or in concert with telomerase RNA (TR) to generate immortalized chicken preadipocytes (ICP1)[84]. This work showed that ectopic TERT alone could overcome the population doubling limits of primary preadipocytes, and that co-expression with TR could drastically improve proliferation rates (a ~30% rate increase compared to TERT alone). These preadipocytes maintained adipogenic potential and underwent robust lipid accumulation following long-term expansion. Alternatively, genetic immortalization of murine preadipocytes has been achieved through the conditional expression of the simian virus 40 (SV40) T-antigen during proliferation [85]. Application of these systems to various livestock species could offer promising options for cultured fat production. In addition to immortalized preadipocytes, the immortalization of alternate cell types with subsequent induction of adipogenesis could offer scalable solutions for cultured fat. For instance, adipogenesis in immortalized DF-1 chicken fibroblasts was demonstrated by treatment with fatty acids, insulin, and retinoic acid [86]. Immortalization of bovine and porcine fibroblasts was demonstrated with overexpression of TERT, cyclin-dependent kinase 4 (CDK4) and cyclin D1. Taken together, these results indicate that the immortalization of numerous cell types could help provide scalable solutions for cultured fat production.

In addition to engineering cells to generate immortalized somatic cell lines, spontaneously immortalization of cells can be pursued through routine subculturing or treatment with mutagenic agents such as radiation or chemical mutagens [87]. Under these conditions, some percentage of random mutations will induce an escape from cellular senescence, thereby immortalizing the cells. Indeed, spontaneous immortalization through subculture was the method through which the DF-1 chicken fibroblast cell line was established, as well as the commonly-used mammalian adipogenic fibroblast line—murine 3T3-L1's [88,89]. While these spontaneously generated cell lines may not be considered engineered, per se, neither are they unaltered, due to the selection for cells that have undergone specific mutations. However, it is possible that spontaneous immortalization could offer regulatory advantages compared with genetically engineered cells, particularly in jurisdictions that disallow any genetic engineering in food production [90]. Similarly, various methods for transiently inhibiting cellular senescence could offer non-genetically modified organism (GMO) opportunities for "immortalizing" cells. For instance, human fibroblasts treated with mRNA encoding TERT showed a 10^{12}-fold increase in total cell number compared with untreated cells, without requiring any permanent genetic modification [91]. Such transient genetic strategies could overcome regulatory issues facing GMOs, while offering the bioprocess benefits of genetically immortalized cells. Fully non-genetic methods can be explored as well. For instance, small molecule inhibition of the PAPD5 polymerase—which halts endogenous TERT activity by triggering degradation of the telomerase RNA component—can recover TERT activity in stem cells, extending proliferative capacity [92].

Additionally, antioxidant treatment has been shown to reduce senescence in adipose-derived stem cells while maintaining adipogenic potential [66].

Along with immortalizing somatic cells, adult cells can be reprogrammed to a stem cell state by the overexpression of Yamanaka factors (Oct3/4, Sox2, Klf4, and c-Myc), which are naturally expressed in high levels in embryonic stem cells [93]. These cells—called induced pluripotent stem cells (iPSCs)—are capable of indefinite expansion and differentiation into a range of cell types, including lipid accumulating adipocytes [94]. iPSCs offer a range of potential advantages for cultured fat production: They are capable of expansion in suspension bioreactors with rapid growth rates (~20 hour doubling times for porcine iPSCs in suspension culture) [95]; they can be obtained through entirely non-invasive means, such as from keratinocytes from hair follicles [96]; they can be generated through transient mRNA delivery, thus potentially avoiding regulatory hurdles facing fully engineered GMOs [97]; and they have been successfully generated for many livestock species, including pigs, cattle, chicken, sheep, and goats, as reviewed extensively by others [50,98–100]. Despite these advantages, iPSCs offer their own challenges for cultured fat production. For instance, unless produced through transient mRNA methods—and potentially even still—iPSCs could face regulatory hurdles as genetically controlled cells, as the specific regulatory status of transgenic, gene-edited, and transiently altered cells for cultured meat is yet to be determined. Additionally, iPSCs often require challenging or complex differentiation processes. For instance, differentiation into lipid accumulating adipocytes can be less robust in iPSCs than ADSCs, and often involves a transition through an MSC state, which can complicate the differentiation process [53,94]. Lastly, iPSC generation from livestock species have to-date offered some unique challenges compared with human or murine iPSCs, such as requiring additional genes (e.g., TERT) to be expressed or relying on continued Yamanaka factor expression, potentially indicating incomplete reprogramming [98,100–102]. Despite these challenges, the stated advantages of iPSCs warrant extensive exploration of their use for cultured fat and could allow for highly scalable production systems.

Lastly, along with engineering efforts that are directly related to the immortalization or reprogramming of cells, genetic strategies can be used to engineer cell lines towards optimal properties for large-scale fat production. For instance, engineering the previously mentioned immortalized chicken preadipocyte (ICP1) cells to overexpress BMP4 increased cell proliferation, which could improve process dynamics and economics [103]. Additionally, ICP1 cells engineered to overexpress perilipin 1 (PLIN1) or retinoid X receptor α (RXRα) showed improved lipid accumulation and adipogenic differentiation [104,105]. In both iPSCs and fibroblasts, overexpression of PPAR-gamma can be used to improve adipogenic differentiation and lipid accumulation [53,106]. In fibroblasts, commensurate overexpression of the CCAAT/enhancer-binding protein alpha (CEBPα) can further improve adipogenesis [106,107]. Taken together, these results demonstrate how immortalization or reprogramming and subsequent genetic engineering strategies can generate cell lines that are capable of both robust proliferation and differentiation to enable the large-scale production of high-quality cultured fat. A summary of engineered cell sources for large-scale adipose production is provided in **Table 2**.

Table 2. Engineered cell sources for large-scale adipose production.

Cell Type	Species	Main outcome(s)	Ref.

Immortalized cells with myogenic capacity	Avian (chicken) preadipocytes	Expression of Telomerase reverse transcriptase (TERT) and/or telomerase RNA enable >100 doublings of preadipocytes, while maintaining adipogenicity.	[84]
	Murine preadipocytes	Conditional expression of the SV40 T antigen imparts immortality during proliferation, and cessation of SV40 expression allows adipogenic differentiation.	[85]
	Human preadipoctyes	Combined expression of TERT with the papillomavirus E7 oncoprotein immortalized preadipocytes while maintaining adipgenicity.	[108]
	Avian (chicken) fibroblasts	Adipogenesis with treatment with fatty acids and insulin or all-*trans* retinoic acid.	[86]
	Porcine fibroblasts	Immortalized fibroblasts from peripheral blood. Adipogenesis in media supplemented with dexamethasone, 3-isobutyl-1-methylxanthine, indomethacin, and insulin.	[109]
	Bovine fibroblasts	Immortalized bovine embryonic fibroblasts. Ectopic expression of PPARγ2 induces adipogenesis in adipogenic media.	[110]
	Murine fibroblasts	Adipogenesis with increased serum concentration in the media.	[89]
Reprogrammed cells from food-relevant species*	Porcine iPSCs	Doxycycline-inducible expression of human Oct4, Sox2, Klf4, c-Myc, Nanog and Lin28 in primary ear fibroblasts result in reprogramming and the ability to differentiate into all three germ layers. At time of publication showed stability over 41 passages.	[111]
	Bovine iPSCs	Retroviral insertion of bovine Oct4, Sox2, Klf4, c-Myc, Lin28, and NANOG into embryonic fibroblasts resulted in the ability to differentiate into all three germ layers. At time of publication showed stability over 16 passages.	[112]
	Leporine (Rabbit)	Lentiviral insertion of human Oct3/4, Sox2, Klf4 and c-Myc into liver cells resulted in the ability to differentiate into all three germ layers. At the time of publication showed stability over 50 passages.	[113]
	Ovine	Lentiviral insertion of doxycycline-inducible mouse Oct4, Sox2, Klf4, and c-Myc into fetal fibroblasts resulted in the ability to differentiate into all three germ layers, though this capacity was unstable over more than two passages.	[114]
	Avian (Chicken)	Lentiviral insertion of mammalian Oct4, Sox2, Klf4, and c-Myc into embryonic fibroblasts resulted in the ability to differentiate into all three germ layers. At the time of publication, showed stability over 20 passages.	[115]
	Piscine (Zebrafish)	Lentiviral insertion of mammalian Oct4, Sox2, Klf4, and c-Myc into embryonic fibroblasts resulted in the ability to differentiate into all three germ layers. Stability over >5 passages was not described.	[115]

*First publication of iPSCs from these species listed here. For more in-depth review, the reader is referred to [116]

Large-scale Cell Proliferation Techniques for Cultured Fat

After an appropriate adipogenic cell source is selected, methods of proliferating the cells to produce a sufficient biomass for food applications will be required. Many scalable cell production systems exist from the biotechnology and pharmaceutical spaces and should be applicable to cultured fat cell production for food purposes. However, there may be unique challenges associated with adipocyte culture due to cell buoyancy during lipid accumulation as well as costs associated with process controls and growth factors. Scalable cell proliferation techniques are grouped here into two approaches: suspension-based and adherent cell-based culture strategies.

Suspension Culture

One promising avenue for large-scale adipose culture is via suspension culture. Suspension culture is the process of growing single cells or cell aggregates in growth medium with agitation, allowing for proliferation without attachment to a culture flask. Adaptation of adherent cells to suspension culture has been widely explored in the literature, and prior to inoculation in a suspension system cells are generally adapted to serum-free medium [117,118]. Although adherent culture can achieve high cell density (discussed further below), suspension culture is widely used in various biotechnology applications and large-scale infrastructure already exists that could be easily transferred to fat-relevant cell types [119–121]. Suspension culture is also favored for scale-up compared with adherent culture because cell growth is limited by cell concentration as opposed to surface area. Furthermore, suspension cultures are less labor-intensive, provide a more homogenous culture environment, and require fewer resources (culture flasks, enzymes to passage adherent cultures, etc.) [122].

Suspension bioreactor types

Many different bioreactor types have been explored to facilitate suspension cell culture (**Figure 1A**). The most common suspension bioreactor is a stirred suspension bioreactor (SSB), comprised of a cylindrical culture vessel and mechanism to stir the media to prevent excessive cell aggregation [123]. SSB are typically characterized by the media replenishment mechanism involved in the process: batch (no replenishment, proliferation until cell harvest), fed-batch (media replacement in batches), or continuous (constant media replacement). Other relevant suspension bioreactors are wave (disposable flexible systems on an undulating surface to gently mix) and airlift (gas is injected from the bottom of the culture vessel) [124–126]. Cell types relevant to fat culture have been grown in SSBs (bovine ADSCs, avian fibroblasts, porcine iPSCs) and wave bioreactors (avian fibroblasts) [95,123,127]. Computational fluid dynamic models suggest that airlift bioreactors may be the most realistic and efficient production method for large-scale cultured meat production (although specific cell types/species were not directly modeled) [128]. While these studies present promising evidence for the production of cultured fat-relevant cell types in suspension, future work will be necessary to optimize bioreactor type, as well as media and culture conditions to ensure efficient, cost-effective, fat culture scale-up depending on the cell type used. Despite the limited literature on livestock adipose suspension culture, a large volume of work has been published using suspension culture to scale-up human and rodent fat-relevant cell types and can serve as a base for the generation of large-scale fat

production systems. Human MSCs, ESCs, and iPSCs have been expanded in SSBs and maintain proliferative potential [129–133]. Notably, human iPSCs and ADSCs retained their ability to differentiate while still in suspension. This could be a promising route to simple large-scale fat production where livestock stem cells are expanded in suspension and adipogenic differentiation is induced after sufficient cell mass has been produced [134]. 3D adipose spheroids generated using pre-adipocytes and hADSCs in suspension using low attachment tissue culture plates show increased differentiation efficiency and more accurately represent the 3D microenvironment [134,135]. One potential explanation for this is that cell shape has been shown to regulate stem cell fate, and cells that are allowed to remain circular (such as in suspension as opposed to flattening during adherent culture) will favor adipogenesis over osteogenesis [136].

Many cell types self-organize into aggregates that proliferate, retain differentiation potential, and more accurately recapitulate a natural 3D environment than single cell suspensions [131]. Culturing cell aggregates in suspension eliminates the complexity of designing edible microcarrier/hydrogel scaffolds or removing inedible materials, produces higher cell numbers, and can theoretically be applied to many different cell types (iPSCs, MSCs, hADSCs, preadipocytes, etc.) [130,134,135,137]. One important consideration in aggregate cell culture is the formation of a necrotic core when cell density exceeds the limit where nutrients can reach the central cells [138]. While this is essential to consider when designing systems, engineering controls can be enacted to either harvest or passage spheroids into single cell suspensions before they become necrotic. This challenge could also be addressed by co-culturing fat-relevant cell types with endothelial cells to transport nutrients throughout the aggregate [139]. For example, a previous study generated vascularized human subcutaneous white adipose tissue spheroids that retained the ability to differentiate into uniocular adipocytes [140].

Overcoming Adipocyte Buoyancy in Suspension

While aggregate cell culture could be a promising route for simple and efficient fat scale-up, the inherent nature of lipid-producing cells will likely present unique challenges. As lipid accumulation increases cells become buoyant and float to the surface of culture systems, interfering with nutrient/oxygen flow in the bioreactor and decreasing yield [139]. One proposed method to circumvent this issue is called ceiling culture, where mature adipocytes that become buoyant in a static culture are allowed to adhere to a floating glass surface at the top of the culture vessel and retain adipocyte-specific functions [141]. While this is a good method for studying adipocyte cell biology, it not clear if it would be feasible for large-scale fat production because once the cells attach, growth is again constrained by surface area [142]. Another possibility could be utilizing heavy/dense microcarrier systems, however as previously mentioned a scaffold-based system will have a lower yield compared to aggregate systems.

Perhaps the most promising avenue to overcome buoyancy issues would be to adopt specialty suspension bioreactors for fat accumulation. Rotating wall vessel bioreactors (RWVBs) initially developed by NASA to simulate a microgravity environment produce rotational forces that draw cells towards the center of the bioreactor chamber, and may thus work for cells that sink downwards and float upwards (see “Rotating Wall Vessel” in **Figure 1A**) [143]. RWVBs comprise a rotating cylindrical culture vessel surrounded by a membrane to allow oxygenation are favored for cell types that are sensitive to shear stress [144,145]. In contrast to SSBs,

RWVBs do not require an internal mixing device and instead are completely filled with culture medium, while cells and nutrients are mixed by gentle rotation of the entire vessel. Two types of RWVBs exist: High Aspect Ratio Vessels (HARV, developed by NASA to stimulate a microgravity environment) and Slow-Turning Lateral Vessels (STLVs) [146]. HARV bioreactors have been used to culture lipid-containing 3T3-L1 aggregates and lead to increased adipogenesis in MSCs [147,148]. Hollow fiber membranes can also be added to RWVBs to enhance efficiency/cell health [149]. Alternatively, a system designed to draw floating adipose cells back into the media (e.g., a modified Vertical Wheel Bioreactor) could enable efficient culture of buoyant cells [137,150]. Another potentially relevant bioreactor type is the random positioning machine (RPM) bioreactor, which rotates a culture vessel over multiple axes to ultimately create a microgravity environment. RPMs require a large footprint relative to RWVBs however, and may be more difficult to scale up [151,152].

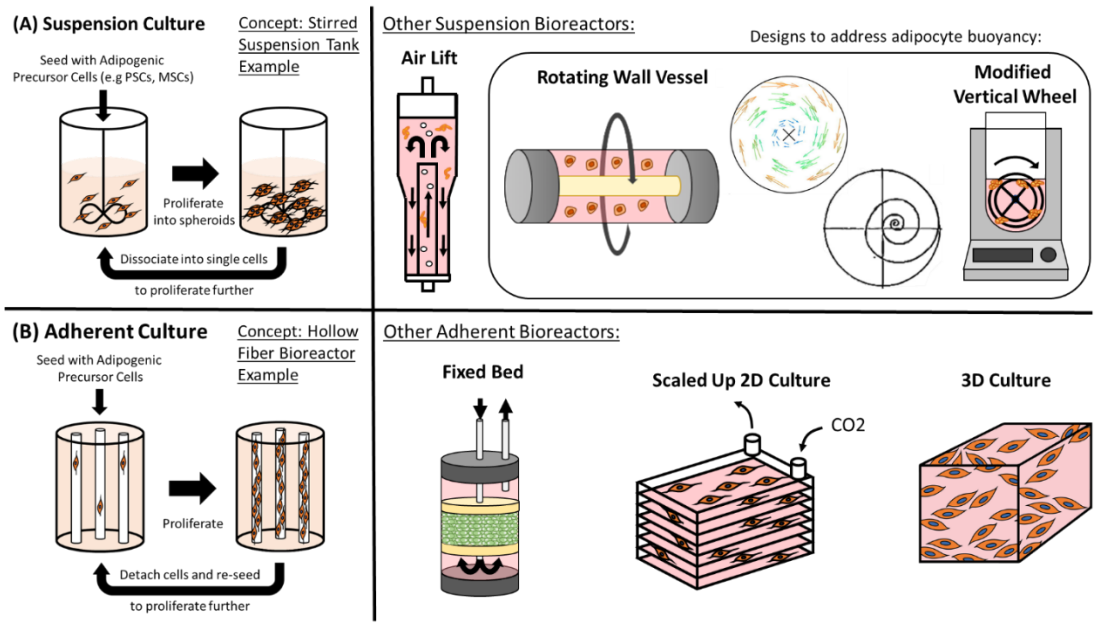

Figure 1. Overview of **(A)** suspension and **(B)** adherent culture-based bioreactors for the large-scale proliferation of adipogenic precursor cells in order to obtain large numbers of cells for the downstream generation of adipocytes. The fluid velocity tracking diagram (short arrows) for rotating wall vessel bioreactors is reproduced from [153], while the particle tracking diagram (spiral line) is from [154].

Adherent Culture

Using adherent cell culture strategies is another approach towards upscaling adipocyte cultures (**Figure 1B**). While suspension culture offers some advantages by foregoing the need to fabricate and utilize scaffolds, there are potential advantages to scaling the growth of adipocytes using adherent cell culture strategies. For example, the accumulation of lipid droplets during adipocyte differentiation increases cell buoyancy, often resulting in adipocytes floating to the top of bioreactor vessels [142]. This phenomenon creates a challenge in achieving proper oxygen and nutrient mixing, as well as reduces the available surface area for adipocyte cell culture. Thus, one benefit of growing adipocytes in adherent bioreactors would be

to prevent this occurrence by either having cells attached to an immobile 2D surface or adding weight to cell cultures via the use of microcarriers. In addition, ADSCs and adipocytes are sensitive to mechanical cues. For example, adult ADSCs undergo adipogenesis by simply tuning the substrate stiffness to physiologically relevant levels of adipose tissue (~2 kPa) [155]. In addition, adipocytes differentiated on more compliant substrates exhibited higher levels of lipid accumulation when compared to fat cells grown on less deformable substrates [156]. Thus, the use of adherent cell culture strategies would allow for the tuning of mechanical environments, which could be harnessed to improve the growth of adipocyte precursors and lipid accumulation of differentiated adipocytes. There is also potential to utilize cell adherence onto substrates to apply mechanical cues and thus stimulate adipogenesis in stem cells.

Next, we will discuss current technological approaches towards upscaling adherent cell cultures with specific examples highlighted towards progress with adipocyte-relevant cell types. For a more extensive review on general approaches towards upscaling adherent cell cultures, we refer the reader to a recent review [157].

Adherent Bioreactor Types

Given that cell culture techniques are best characterized and established in 2D, one approach would be to iterate and improve upon the efficiency of conventional 2D culture techniques. For example, 2D multilayered flasks offer a scale out approach for the growth of adherent cells. While the use of multilayered flasks is not feasible with static media at a larger scale, one study found that the incorporation of active gas ventilation resulted in >95% cell viability of hiPSCs cultured within 10 layers. Additionally, a 44% increase in cell proliferation of hiPSCs was observed when compared to cells grown in static media[158]. Using the same culture system, gas transport could be further enhanced with oxygen-permeable layers, similar to culture vessels which are commercially available such as Corning® HYPERflask or HYPERstack. Though concerns remain regarding the occurrence of adipocytes lifting off from culture substrates due to an increase in cell buoyancy upon differentiation, surfaces can be treated accordingly to maintain cell attachment, for example with the use of elastin-like peptides [159]. Thus, considerations towards improving upon traditional 2D culture methods can be considered, however, the ultimate scale out using these approaches will require additional innovations. Specifically, it will be important to address the technical challenge of achieving proper nutrient and oxygen mixing, particularly as culture vessels increase in volume.

The use of microcarriers grown in suspension is currently a promising approach with great potential for use in the upscale of adherent adipocyte cell culture [160]. The utilization of microcarriers offers the advantages of adherent cell culture techniques with the benefit of growing cells in suspension to increase scalability. Microcarrier beads are widely used to increase surface area for adherent cell attachment and to increase yield efficiency in cells that are unable to form spheroids or aggregates in suspension [161]. However, this process can add complexity to the culture process if synthetic carriers are used given that production facilities must separate cells from the carriers. Alternatively, microcarriers could be edible and engineered to match the mechanical properties of adipose tissue to increase cell differentiation efficiency [156]. However, it will be important to consider the effects of integrating scaffolds on the taste, texture, and nutritional profiles of the final adipose tissue culture product.

Another approach towards upscaling adipocyte culture is through the use of perfusion bioreactors, where media is passed through immobile cell cultures, such as hollow fiber bioreactors (HFBs). HFBs can be designed to have multiple oxygen and nutrient permeable tubing running in parallel, which provides a substrate for adherent cells to grow on. Using HFBs, researchers have demonstrated its ability to support scalable proliferation with well-characterized proliferation and differentiation of MSCs, ADSCs, and iPSCs [162–166]. Using a similar perfusion approach, fixed-bed bioreactors (FBBs) can be utilized. Here, cells are immobilized within macroporous carriers, packed within a cylindrical bioreactor, and perfused with media. The approach provides a higher surface area to volume ratio, and thus, less cell passaging is required [167,168]. It also provides a low shear stress environment for mammalian cells. Importantly, its ability to support the proliferation of adipocyte precursors, such as MSCs, has been demonstrated at laboratory scale [169]. Further innovations among perfusion-type bioreactors are underway, for example, with the recent development of an interchangeable production line system which incorporates feeder cells for the supplementation of growth factors and allows for adjustments in media circulation flow. Thus, media can flow to deliver essential growth factors to adherent cells and be reversed for other essential processes such as for media recycling. Though the process is currently geared towards muscle cell proliferation, the approach could be tuned towards adipocyte cell culture provided that the feeder cells are producing the appropriate growth factors needed [170].

Overall, although there is strong potential towards the scale-up of adipocyte cultures using scaffold suspensions or perfusion bioreactors, nutrient and oxygen mixing is key and this becomes more challenging as these systems are scaled [167]. Additionally, there are concerns over the use of expensive enzymes for cell passaging and harvesting when growing cells on 2D substrates. To address this challenge, innovative approaches are being developed which are designed to have cells proliferate and self-detach via cleavable peptides [171]. This approach would allow for additional cells to grow on newly unoccupied substrates as well as increase the ease of cell harvesting, while also reducing costs. It is important to note that this approach is technically not limited to 2D culture and could be applied to 3D culture techniques as well.Lastly, the potential effects on adipocyte precursor proliferation and ultimate differentiation when culturing in 2D must be considered given that studies have shown differences in cell behavior and gene expression when compared to 3D cultures [159,172,173]. For example, primary mouse adipocytes expressed native phenotypes such as the presence of unilocular lipid droplets when grown in 3D compared to 2D [174]. Thus, methods for upscaling adipocytes in 3D adherent cultures should be considered.

To replicate 3D environments similar to native adipose tissue, one approach to consider is the encapsulation of adipocytes within hydrogels. Though there has been extensive research regarding hydrogel encapsulation for tissue engineering applications, more recent innovations in biomaterial development have improved culturing efficiency. For example, the culture of cells using Mebiol Gel has been shown to support the growth of high cell densities (~20 million cells/mL using hMSCs) with the convenience of thermoreversibility, providing increased ease in cell harvesting [175]. Importantly, encapsulation of adipocyte precursors within hydrogels would protect cultures from high shear stress in the presence of tank mixing or fluid flow in a perfusion bioreactor.

Another option is the culture of adipocytes within porous scaffolds [176]. However, using a top-down approach in 3D tissue engineering is a current challenge given diffusion limitations of nutrients and oxygen. Here, 3D tissue cultures are limited to a tissue thickness of ~100 μm –

1 mm when cultured in static media and in the absence of vasculature [177]. As a result, there is potential to use a modular, or bottom-up approach in which smaller microtissues can be cultured as building blocks for larger 3D structures [178]. For example, this can be achieved using biodegradable microcarriers which assists in the formation of adipocyte cell aggregates in suspension and does not remain in the final tissue culture product. However, in this scenario, it will be important to use food-grade scaffold materials for cultured meat applications [179]. Another approach towards engineering adherent adipocyte spheroids is through surface chemistry treatments on 2D substrates. For example, the introduction of surface charge induces spheroid formation while surface conjugation with appropriate peptides results in consistent cell attachment to the substrate [159]. Using this approach provides the benefits of growing adipocyte precursors in 3D, while avoiding issues of adipocytes lifting off during differentiation.

Culture Media Considerations

Since the goal of plant based and cultured meat is to act as an alternative to animal agriculture, culture media used to grow adipocytes and their precursors should ideally be free of animal products such as serum from blood. For the cultivation of cultured fat cells, three types of culture media formulations are usually considered (**Figure 2A**): A proliferation medium that supports and promotes the expansion of adipogenic precursor cells to achieve a desired amount of biomass, followed by an adipogenic induction medium (also called differentiation medium) that initiates adipogenesis within precursor cells. Adipogenic differentiation is typically accompanied by cell cycle arrest, where cell division no longer takes place [180]. Once the adipogenic program has been sufficiently activated, cultured cells are often switched to a pared down lipid accumulation medium (also called maintenance medium) containing fewer adipogenic compounds at concentrations sufficient to maintain adipogenic gene expression [181].

For proliferation media, numerous serum free formulations have been devised for the adipogenesis-competent cells that we have covered in this review, albeit still comprising of animal components such as growth factors, extracellular matrix components and other signaling molecules [127,129,130,182–188]. Individual animal components are often produced recombinantly, but the question then shifts to cost and commercial viability. Low cost recombinant protein production may be feasible when performed at scale though; and simple serum free formulations only requiring a few recombinant factors exist for certain cell types such as MSCs [183,189–191]. For adipogenic differentiation and maintenance media, there have been numerous reports across species of serum free adipogenesis, as well as some cases documenting how the exclusion of serum can even lead to improved adipocyte lipid accumulation and gene expression [187,188,192–195]. It is important to note though, that adipogenesis media formulations may not be readily interchangeable across species due to differences in adipose biology between livestock species [48,196–198]. For example, there has been success with the adipogenic differentiation of human, mouse and porcine DFAT cells, but similar differentiation media formulations do not induce adipogenesis to the same degree in bovine DFAT cells [48].

During the large-scale production of cultured meat, culture media is one of the largest contributors to cost [199,200]. As such, media recycling may be an important consideration for boosting efficiency and extending culture media utility. The buildup of metabolic waste products is often the limiting factor that requires culture media changes in conventional cell culture, often

limiting cell growth as other nutrients and growth factors remain present at sufficient amounts. For example, iPSC culture media to remove ammonia and lactic acid waste products while simultaneously replenishing glucose levels [201]. The retention of unused nutrients via culture media recycling ultimately reduced TGF-β1, insulin, transferrin, ascorbic acid and selenium requirements 2 to 3-fold during the culture period (bFGF requirements were unchanged due to thermal instability). Extending culture media has also been explored through the co-culture of mammalian cells with algae, where lactate and ammonia levels were observed at lower concentrations in co-culture groups when compared to mono-culture controls. It was hypothesized that algae-produced oxygen promoted aerobic respiration, while ammonia was metabolized by co-cultured algae to produce amino acids [202,203].

Metabolic and genetic engineering approaches are additional options for reducing culture media costs. Glutamine synthetase overexpression is an option for reducing glutamine requirements in culture media, while also providing a method of reducing NH_3 levels as it is consumed by the enzyme during glutamine synthesis [204]. Chinese hamster ovary (CHO) cells engineered to overexpress tyrosine biosynthesis pathways gained the ability to grow in culture media devoid of tyrosine, while also producing fewer growth inhibitory compounds associated with bottlenecks in tyrosine synthesis [205]. Branched chain aminotransferase knock-out cells produced fewer inhibitory byproducts, improving proliferation [206]. Metabolic and genetic engineering approaches are additional options for reducing culture media costs. Glutamine synthetase overexpression is an option for reducing glutamine requirements in culture media, while also providing a method of reducing NH_3 levels as it is consumed by the enzyme during glutamine synthesis [204]. CHO cells engineered to overexpress tyrosine biosynthesis pathways gained the ability to grow in culture media devoid of tyrosine, while also producing fewer growth inhibitory compounds associated with bottlenecks in tyrosine synthesis [205]. Branched chain aminotransferase knock-out cells produced fewer inhibitory byproducts, improving proliferation [206].

Methods to Induce Adipogenic Differentiation and Lipid Accumulation at Scale

We have outlined how studies has been performed on scaled proliferation of stem cells such as MSCs and PSCs. Many studies have demonstrated the feasibility of using stirred suspension tanks or hollow fiber bioreactors for large-scale cell production. These cells are then differentiated using traditional 2D culture techniques (well plates, petri dishes) [95,130,131,207]. Unless specifically using a 2D culture system designed for large-scale production, this approach creates a bottleneck in the production process where many adipogenic precursor cells are generated, only to be limited by high surface area and labor intensive requirements associated with traditional 2D culture during the subsequent conversion of stem cells into adipocytes [157]. Thus, scalable methods of inducing adipogenesis in proliferated precursor cells are needed.

In situ Adipogenesis

One method to induce adipogenesis at scale would be to perform an *in situ* differentiation, where cells proliferated in a bioreactor are differentiated in place via a change in culture media or the addition of adipogenesis-inducing factors (**Figure 2B**). This has the advantage of eliminating transfer from proliferation bioreactors for differentiation, to reduce complexity, cost and the risk of contamination. For hollow fiber bioreactors, such an *in situ* adipogenesis approach has been reported using polyethersulfone (PES) fibers, where cells

were expanded for 2-3 weeks and differentiated via a change to adipogenic media [208,209]. After 2 weeks of differentiation, cultured MSCs on the hollow fibers had differentiated better than 2D cultured adipocytes. One method to induce adipogenesis at scale would be to perform an *in situ* differentiation, where cells proliferated in a bioreactor are differentiated in place via a change in culture media or the addition of adipogenesis-inducing factors. This has the advantage of eliminating transfer from proliferation bioreactors for differentiation, to reduce complexity, cost and the risk of contamination. For food applications, it may be possible to detach PES hollow fiber-grown adipocytes (e.g. proteolytically with enzymes such as TrypLE, Accutase and TrypZean) for further downstream processing [164,210–212]. Alternatively, edible hollow fibers could be used for process simplicity and to avoid obstacles such as hollow fiber clogging during cell detachment, while also preserving adipocyte extracellular matrix (ECM) components that would typically be degraded when using proteolytic enzyme-based cell detachment techniques [213,214].

For suspension cultures, *in situ* adipogenic differentiation was demonstrated using stirred suspension tank and rotating wall vessel bioreactors, where adipogenically differentiated MSC spheroids accumulated more lipid when compared to 2D controls [148,215]. ESC spheroids have also been adipogenically differentiated, but only in static suspension cultures [216]. While promising, these studies on MSCs and ESCs were performed using preformed spheroids, which in terms of cell expansion is less efficient and more complicated to scale up when compared to suspension cultures that are inoculated with single cells [150]. Nonetheless, the fact that other studies have demonstrated MSC and ESC spheroid formation from single cell inoculations suggests that an efficient expansion of MSCs and ESCs – followed by their *in situ* differentiation into adipocytes – should be possible [129,207]. Outside of stem cells, single cell inoculated 3T3-L1s in a rotating wall vessel bioreactor formed spheroids and accumulated lipid [147]. 3T3-L1 spheroids were also differentiated into fat upon deliberate induction with adipogenic media, which is important for preventing cells from spontaneously differentiating during proliferation and exiting the cell cycle [139]. For cultured fat purposes, it may thus be useful to develop adipogenic fibroblast cell lines similar to 3T3-L1s using livestock species.

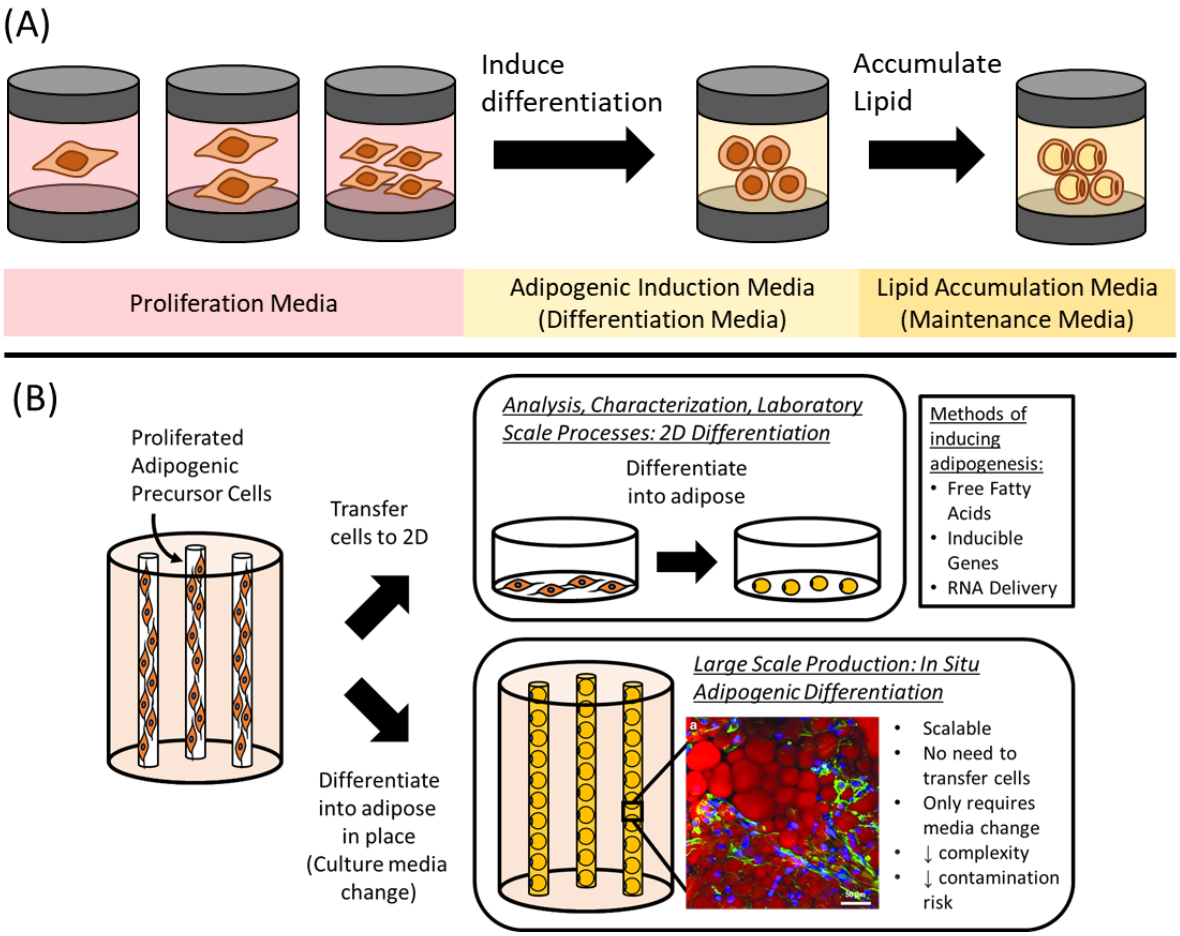

Figure 2. (A) Biological sequence of events for adipogenic differentiation, where proliferated adipogenic precursor cells are induced towards an adipogenic cell fate by adipogenic induction medium (differentiation medium), followed by a switch to medium to foster lipid accumulation (maintenance medium) after the cultured cells become adipogenic. **(B)** *In situ* adipogenic differentiation using a hollow fiber bioreactor as an example - leveraging the same proliferation bioreactor environment to enable differentiation of adipogenic precursor cells with only culture media changes. This is juxtaposed with the conventional practice of taking cells out of the proliferation bioreactor and differentiating them on 2D cell culture substrates. Inlay of hollow fiber differentiated adipose from [208] stained with AdipoRed, scale bar is 50 μm.

Methods to Induce and Enhance Adipogenic Differentiation

While the *in situ* adipogenic differentiation of cells in proliferation bioreactors has been demonstrated as an attractive option for large-scale cultured fat production, the formulations of differentiation media typically used to induce adipogenesis often contain ingredients that may be questionable for a food production process. Conventional adipogenic differentiation media commonly contains ingredients that are not approved for food use (e.g., 3-isobutyl-1-methylxanthine), or compounds (indomethacin, rosiglitazone, dexamethasone) that are used as medical drugs [217]. Although the use of such compounds may only be required

transiently during the initial induction of adipogenesis – and may thus be diluted to negligible levels by media exchanges during extended cell culture – here we cover alternative options for inducing adipogenesis that may be relevant to large-scale food production scenarios (**Figure 2B**).

Free Fatty Acid Differentiation

Certain free fatty acids (FFAs) have been characterized as agonists of PPARγ and have thus been investigated as adipogenesis-inducing compounds [218]. For livestock cells, bovine stromal vascular cells from adipose tissue have been induced to differentiate using a cocktail of monounsaturated and branched chain fatty acids [82]. In experiments of FFAs added to conventional differentiation media for bovine stromal vascular cells, oleic and linoleic acid were found to be the most efficacious for promoting lipid accumulation [219]. For bovine muscle satellite cells, oleic acid was found to promote adipogenic gene expression and lipid accumulation [220]. FFA-based adipogenesis has been demonstrated with porcine preadipocytes using oleic acid, linoleic acid and to a lesser extent α-linolenic acid [221]. For chicken, oleic acid alone has been shown to be sufficient for inducing lipid accumulation in chicken preadipocytes [222,223]. Preadipocyte cell lines such as 3T3-L1s have also been differentiated with FFAs [224].

FFA-based adipogenesis appears to have not been reported for PSCs, however the differentiation path of PSCs to adipocytes typically routes through an MSC or MSC-like cell phase, followed by adipogenic induction via a conventional differentiation media designed for MSC adipogenesis [53,54,225]. The fact that the final stage of PSC to adipocyte differentiation is the same as MSC adipogenesis suggests that it may be possible to substitute conventional differentiation media formulations with an FFA-based protocol, as has been done with cow, pig and chicken MSC or MSC-like cells. While FFAs have shown to have PPARγ activity and FFA differentiation has been shown to be sufficient in many cases, experiments have shown that FFAs coupled with conventional PPARγ agonists (e.g. rosiglitazone, ciglitazone) can yield superior results versus treatment with FFA alone [220,223]. Thus, the most optimal food-safe strategies for adipogenic differentiation will likely require pairing FFA treatment with other supplements. Relevant supplements that may enhance adipogenesis include biotin, pantothenate, as well as conventional growth factors such as BMP4 [181,217,226,227]. Glucose levels also influence adipogenesis [228].

Nonetheless, the discovery of oleic acid and linoleic acid as effective FFAs in the induction of livestock cell adipogenesis presents a favorable opportunity for large-scale cultured fat production, as these FFAs are present at high concentrations in plant based oils such as olive and soybean oil [229,230]. The direct use and efficacy of soybean oil in promoting adipogenesis has been explored through its use in Intralipid, a medical soybean oil emulsion that has found use in adipose cell cultures [231–233]. With a demonstrated adipogenic effect in livestock cells and the ready availability of plant-based sources, FFAs may be an appealing, food safe and cost-effective option for inducing adipogenesis during large-scale cultured fat production.

Genetic Engineering: Inducible Expression of Adipogenic Genes

When using engineered cell lines, it may be possible to include genes known to initiate adipocyte differentiation and pair them with inducible promoters to selectively activate them when differentiation is desired during the cultured fat production process. A selection of inducible promoters (also called conditional promoters) has been published and a list of adipogenic gene targets is provided below (**Table 3**) [234].

Table 3. List of genes that have been ectopically expressed to induce adipogenesis in various adipogenic precursor cells.

Adipogenic Gene	Species	Cell Types	Reference(s)
Peroxisome proliferator-activated receptor gamma (PPARγ)	Human, Mouse, Chicken, Cow, Goat, Pig	PSCs/MSCs, Myoblasts, Fibroblasts	[53,106,235–241]
CCAAT/enhancer-binding protein alpha and beta (C/EBPα, β)	Mouse, Chicken, Goat	Myoblasts, Fibroblasts, Preadipocytes	[235,237,239,242–244]
Sterol regulatory element-binding protein-1 (SREBP-1)	Mouse, Chicken	Fibroblasts	[237,245]
Fatty Acid Binding Protein 4 (FABP4)	Cow	MSCs	[246]
Zinc finger protein 423 (Zfp423)	Cow	MSCs	[247,248]
Zinc finger and BTB domain containing 16 (ZBTB16)	Cow	DFAT cells	[249]
Perilipin 1 (PLIN1)	Cow	Preadipocytes	[250]
Kruppel-like factor 13 (KLF13)	Pig	MSCs	[251]
Phosphoenolpyruvate carboxykinase 1 (PCK1)	Buffalo	Preadipocytes	[252]
Early B-Cell Factor (Ebf) 1-3	Mouse	Fibroblasts	[253]
Runt-related transcription factor 1 (RUNX1)	Mouse	MSCs	[254]
Cyclin-Dependent Kinase 4 (CDK4)	Mouse	Fibroblasts	[255]

RNA Delivery: Genetically Modified Organism (GMO)-Free Alternatives

DNA-based genetic engineering is a powerful tool in modulating protein and gene expression to direct cell differentiation of large-scale proliferated cells towards an adipogenic fate. However, it is also viewed unfavorably by certain consumers and governing bodies [256,257]. RNA-based technologies such as mRNA delivery present an alternative approach to modulating adipogenic genes without modifications to the cell genome. Here, mRNA of an adipogenic gene is delivered to a cultured cell, where it is then translated into a protein to ultimately promote adipogenesis [258]. The delivered mRNA does not interact with the cell's genome, so no genetic modification takes place. Delivered RNA is also transient and eventually degraded by the cell, which reduces the risk of exposure when consuming the final food product. Biologically, there are also multitudinous barriers to RNA exposure, such as RNAse and nuclease presence in saliva and the GI tract, as well as the low pH of the stomach [259]. For a commercial cultured fat process, the close to 100% transfection efficiency of mRNA delivery also means that a majority of proliferated cells could be converted into adipocytes,

which is advantageous for yield and cost [260,261]. There have been no reports of using mRNA to express adipogenic genes and generate adipocytes from precursor cells. However, there is precedent for the use of mRNA in modifying cell types, as the approach has found wide usage in the high efficiency generation of iPSCs from cells such as fibroblasts, as well as in the differentiation of iPSCs into other cell types such as neurons and muscle cells [97,262–265]. The temporary nature of mRNA-based gene expression may also be fitting for adipogenic differentiation, as it has been reported that a transient expression of PPARγ and C/EBPα (using DNA) is sufficient for adipogenic differentiation in MSCs [266]. It has also been reported that a prolonged C/EBPα expression could suppress adipogenesis through the upregulation of Sirtuin 1 (SIRT1) [267]. Outside of mRNA delivery, numerous long noncoding RNAs (lncRNAs) that promote adipogenesis have also been characterized [268,269].

RNA-based approaches also enable adipogenesis through the repression of anti-adipogenic genes. Numerous microRNAs (miRNAs) in human, rodent and livestock species have been characterized for their ability to repress anti-adipogenic genes and could be delivered exogenously to cultured cells [270,270–277]. Epigenetic approaches have also been explored with CRISPR inhibition (CRISPRi), where muscle satellite cells were transdifferentiated into adipocytes through the epigenetic repression MyoD [278]. Without an actual modification to the underlying DNA code, the dCas9 proteins used for CRISPRi could be delivered using mRNA in order to achieve high rates of transfection during epigenetic silencing.

With recent developments in RNA vaccine production, RNA production has been shown to be scalable and low cost [279,280]. Moreover, the use of technologies such as self-amplifying mRNAs (saRNAs) stand to further reduce cost by reducing the number of RNA molecules required during transfection by orders of magnitude, while also extending their duration of efficacy [281,282]. RNA delivery could also be used to execute epigenetic strategies using tools such as CRISPR activation (CRISPRa), which has been shown to yield longer lasting gene expression when compared to the direct insertion of a target gene [283,284]. Ultimately, with RNA delivery one can modulate the same adipogenic genes targeted during direct DNA-based genetic engineering, but without affecting the host genome and potentially avoiding a genetically modified organism (GMO) designation.

3D Culture

The formation of 3D culture environments promotes more robust adipogenesis in cultured cells. Bovine cells stromal vascular cells only undergo limited lipid accumulation when differentiated using a conventional adipogenic induction medium (insulin, dexamethasone, IBMX), whereas the same medium was effective in mice and porcine cells. However, cultivation of the bovine cells under 3D conditions via spheroid culture caused a significant increase in lipid accumulation and adipogenic gene expression, rescuing adipogenesis [285]. 3D spheroid conditions have also been shown to improve adipogenesis in human MSCs in multiple reports [148,159]. During 3D spheroid culture, adipose stromal vascular cells have even been reported to robustly accumulate lipid while also self-organizing to form a network of capillary vessels [140]. Other forms of 3D culture (sponge scaffolds, hydrogel encapsulation) have also been shown to promote robust adipogenesis in cultured cells [172,286–288]. 3D cultures of 3T3-L1s in embedded in agarose gels enabled their adipogenic differentiation without the need for an adipogenic induction medium, which might imply that a 3D cell environment is inherently favorable for adipogenesis [289].

Strategies to Form Macroscale Cultured Fat Tissue

After proliferating cells at scale and subsequently differentiating them to induce lipid accumulation, the adipocytes need to be transformed into actual macroscale adipose tissue that can be tangibly picked up, chewed and eaten. Here, we discuss two major strategies to forming cultured fat tissue: 1) Growing macroscale adipose tissue using 3D culture techniques and sustaining the tissue using vascularization or perfusion techniques, or 2) Growing small adipocyte tissue constructs, adipocyte aggregates or individual adipocytes and aggregating them at the end of the culture process to mimic macroscale 3D adipose tissue.

Macroscale 3D Culture: The Need to Address Nutrient Diffusion

One method of generating tangible, macroscale fat tissues is through bulk 3D tissue culture, where 3D constructs are formed instead of the cell monolayers that are produced from 2D culture (**Figure 3D**) [290]. However, large macroscale tissue cultures would require a method of delivering oxygen and nutrients to cells in the tissue interior, as these compounds can typically only diffuse several hundred microns deep into 3D tissue [291,292]. In the body this is addressed by the circulatory system, where blood vessels interspersed throughout our tissues replenish cell nutrients while removing metabolic waste products. Without a system to transport nutrients and waste, *in vitro* grown tissues are often limited to thicknesses under 1 mm [293,294]. For macroscale tissue engineering, the limitations of mass transport can be solved by manually incorporating perfusion channels into cultured tissues to permit culture media flow to the tissue interior. This has been explored in numerous ways, such as via 3D printing of sacrificial channel templates, incorporation of porous tubes, laser ablation of 3D tissues, as well as casting of cell-laden hydrogels around a wire which is taken out after gel polymerization [231,295–297]. However, the close channel spacing required to limit nutrient diffusion distances to several hundred microns necessitates a very dense packing of perfusion channels, increasing the cost and complexity of macroscale 3D culture. Each channel also needs to be small, as otherwise a large volume of the cultured tissue will ultimately consist of empty channels. Due to these limitations, there are few examples of perfusion culture systems scaled up to generate large amounts of 3D tissue. In one example, only 21% of the volume of a hollow fiber bioreactor growing 3D skeletal muscle actually consisted of cultured tissue, as the majority of space had been taken up by 480 μm wide channels and the tight spacing required to limit cell-to-channel distances [298]. For channel-perfused 3D cultures, more work is required to develop scalable methods of incorporating small, dense arrays of channels in cultured tissues in order for this approach to be viable.

Another solution to nutrient diffusion limitations may be to vascularize tissues via co-culture with endothelial cells. In 3D culture, endothelial cells spontaneously form perfusable capillary-like blood vessels that resemble *in vivo* microvasculature [299]. Cell to cell communication from endothelial co-culture has also been reported to be beneficial for adipogenic differentiation, and there have been many reports of vascularized adipose tissue formation in the literature [140,286,300–304]. However, vasculature from cultured endothelial cells is not formed immediately at the beginning of culture, possibly leaving cells deprived of nutrition for too long before perfusion is established [305]. Additionally, the limited volume of media perfusable through capillary sized vessels may not deliver a sufficient amount of nutrition to sustain a high number of cells residing in very large tissue constructs [306,307]. Interestingly,

the limitations of manually created perfusion channels and self-assembled endothelial capillaries appear to be antithetical – capillaries form small and dense channels that are difficult to achieve using engineered channels, while larger engineered channels enable sufficient nutrient flow into macroscale tissue constructs. It is possible that a hybrid system consisting of both engineered perfusion channels and capillaries from co-cultured endothelial cells might be advantageous in addressing nutrient diffusion during large macroscale 3D culture. However, to date no systems readily applicable to cost effective scale-up of bulk 3D culture in general are available, or able to provide densely packed, metabolically demanding adipose cells.

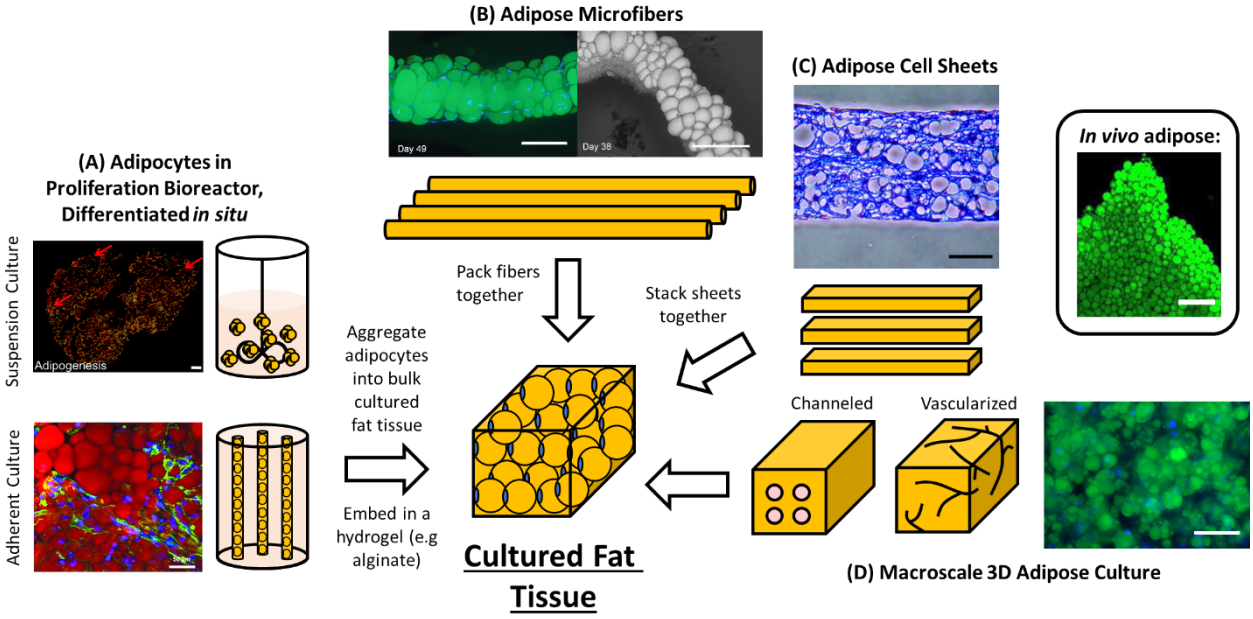

Figure 3. Strategies for producing cultured fat, either via adipose aggregation or macroscale 3D culture. **(A)** Cultured fat produced by differentiating adipocytes in the bioreactor used to proliferate them, then aggregating the cells and binding them together with a food-safe binder to form a cultured fat tissue construct. The Oil Red O stained adipose spheroid is from [139] and has a 100 μm scale bar. AdipoRed stained hollow fiber adipocytes are from [208] and has a scale bar of 50 μm. **(B)** Unilocular adipocytes in alginate-fibrin-collagen microfibers after extended cell culture [308]. Microfibers can be packed together to form macroscale cultured fat tissues. Scale bars 100 μm. **(C)** Adipose cell sheets prepared from differentiated MSCs [309]. Stacked sheets spontaneously fuse to form larger tissues. Scale bar 50 μm. **(D)** Macroscale 3D tissue culture of adipocytes could be performed to form bulk cultured fat constructs. These 3D tissues would require channels to ensure culture media access to the cells on the interior to avoid necrosis. The DAPI (nuclei, blue) and BODIPY (lipids, green) stained image is of 3T3-L1 MBX (5 million cells/ml) cultured under 3D conditions in a 3 mg/ml fibrin hydrogel for 20 days (unpublished data). Scale bar 100 μm. The image of in vivo adipose from newborn mouse was also stained with BODIPY [310]. Scale bar 200 μm.

Aggregated Adipose

Unlike the muscle component of meat, which comprises an elaborate structural hierarchy of muscle fibrils bundled to form muscle fibers, native white adipose tissue is largely a dense aggregation of adipocytes, with the fat cells occupying a majority of the tissue volume and ECM proteins holding them together [310,311]. Thus, it may be possible for a simple aggregation of individually grown adipocytes, small adipose spheroids, or miniature 3D tissue constructs to

produce an *in vitro*-grown tissue that is organoleptically comparable to native fat. Growing cells or small tissues sidesteps the obstacle of delivering nutrients to the interior of large *in vitro* tissues and allows nutrient and waste to diffuse across shorter distances of dense tissue, which is important for enabling strong cell growth and for obtaining highly differentiated and lipid-laden adipocytes [291,292,312]. Proper access to nutrition is especially important in this case for adipose, as its biological function is to act as a store of excess energy. One method in which this has been approached has been through the culture of 3D adipose microfibers. In this system, MSCs were suspended in two-phase microfibers comprising of a protective alginate shell with a fibrin-collagen core, after which the fibers were cultured in adipogenic induction and maintenance media to produce 3D microfiber tissues containing large unilocular adipocytes that resembled *in vivo* fat tissue. After adipogenic differentiation, alginate shells of the microfibers were degraded and the remaining adipose fibers were packed in molds overnight, where the fibers fused into a single tissue (**Figure 3B**) [308]. Alginate-based microfibers have also been successfully differentiated into adipose using FFAs [82]. Aggregated adipose would also pair well with a cell sheet engineering approach, which has been successfully used with MSCs to form sheets containing lipid-laden adipocytes that spontaneously fuse with each other when pressed together [309,313–315]. During cultured fat production, individual sheets of adipocytes and their secreted ECM could be stacked together to build large tissues (**Figure 3C**).

Another approach to achieve aggregated adipose would be to build upon the concept of *in situ* adipose differentiation discussed previously (**Figure 3A**). After proliferating and differentiating large numbers of adipocytes in scalable bioreactors such as a hollow fiber bioreactor, adipocyte clusters could be removed from the reactor and packed together to recapitulate denser cell arrangements observed in native adipose. While simple and scalable, a potential downside to this approach of packing adipocytes would be a deemphasis of the native ECM that holds adipocytes together in native fat tissue. However, the major contributors to meat flavor consist of lipids and water-soluble compounds [316]. As such, cultured fat formed from aggregated adipocytes may still be suitable for food purposes. Moreover, in aggregated adipose the ECM component of fat tissue would still be partially recapitulated by ECM that is autologously deposited during *in vitro* culture, especially when using techniques such as spheroid-based cultures where ECM is secreted to hold cell aggregates together [148,317,318]. Abundant ECM deposition has also been reported during 2D cell culture [319]. Upregulation of ECM deposition during *in vitro* cell culture has also been shown through the supplementation of ECM building blocks (e.g. hydroxyproline) in the culture media, as well as through co-culture strategies (e.g. adding fibroblasts) [320]. As such, cultured fat formed from aggregated adipocytes may still be suitable for food purposes. Moreover, in aggregated adipose the ECM component of fat tissue would still be partially recapitulated by ECM that is autologously deposited during *in vitro* culture, especially when using techniques such as spheroid-based cultures where ECM is secreted to hold cell aggregates together [148,317,318]. Abundant ECM deposition has also been reported during 2D cell culture [319]. Upregulation of ECM deposition during *in vitro* cell culture has also been shown through the supplementation of ECM building blocks (e.g. hydroxyproline) in the culture media, as well as through co-culture strategies (e.g. adding fibroblasts) [320].

In lieu of fully formed adipose ECM, a hydrogel scaffold or binder with characteristics similar to fat could be employed to encapsulate aggregated adipocytes. Scaffold requirements for aggregated cultured fat would be different from typical tissue engineering as it only needs to hold cells together - aggregation already achieves the final cultured fat product and no further cell culture is required. Ideally, a scaffold used in aggregated adipose would be unobtrusive in

terms of flavor, similar to adipose tissue in terms of texture and animal-free. Numerous gelling ingredients such as alginate and carrageenan have been used in the food industry as fat replacers and may thus be good options for binding aggregated adipose (**Table 4**).

Table 4. Possible gelling and binding ingredients to use with aggregated adipose forms of cultured fat.

Gelling or Binding Ingredient	Ingredient Source/Type	References
Alginate	Seaweed	[321–323]
Carrageenan	Seaweed	[321,322]
Cellulose	Plant Fiber	[172,324]
Corn Zein	Plant Protein	[325]
Guar Gum	Plant	[326,327]
Hyaluronic Acid	Animal ECM; Microbially Produced	[172]
Inulin	Plant Fiber	[328,329]
Konjac	Plant Fiber	[330]
Oat Bran	Plant Fiber	[323]
Pea Proteins	Plant Protein	[325]
Pectin	Plant Fiber	[329,331]
Soy Protein Isolate	Plant Protein	[325,332]
Starches	Plant	[333]
Wheat Gluten	Plant Protein	[325]
Xanthan Gum	Microbially Produced	[327,334]

Characterization of Sensory Properties and Nutrition in Cultured Fat

While it may be possible to produce 3D cultured fat tissues, to date no nutrition nor sensory evaluation data on cultured fat has been published. After safety studies are conducted, animal testing and sensory panels should be explored to elucidate cultured fat properties such as nutrition, texture, taste, and aroma. In addition to conventional methods such as sensory evaluation panels, MS-based analysis is one of the fastest growing and most widely used analytical methods in food science today [335]. For cultured fat, mass spectrometry (MS)-based analyses could be used to enable comparisons between animal-derived and *in vitro*-grown fats through the screening of various compounds present in tested samples. Lipidomics can be performed using liquid or gas chromatography-MS (LC-MS and GC-MS) to obtain fatty acid composition data and thus inform on its nutritional, as well as textural qualities [336]. For example, analysis of fatty acid compositions using GC-MS revealed that linoleic acid supplementation in cultured adipocytes enabled a corresponding increase of the fatty acid in the cells, increasing the unsaturated to saturated fat ratio when compared to un-supplemented adipocytes [337]. The change in lipid composition also demonstrated the tunability of cultured fat, analogous to how different feeds in cattle can impact their fatty acid and phospholipid make-up [338–340]. In the same study, profiles of phospholipid content in the cultured adipocytes were also obtained, which could be juxtaposed with native adipose to potentially shed light on how their flavor may differ, as phospholipids are thought to be a major contributor to flavor within the lipid fraction of meat [341,342]. Additionally, GC-MS based headspace analyses of the volatile organic compounds (VOCs) released by cultured and native fat tissues upon heating would enable the characterization of aromas that are released during cooking [343,344]. The breadth of MS analyses could also shed light on human health. For example, LC-MS/MS lipidomic analysis was used to explore how different types and amounts of dietary fat led to adipogenic and metabolic dysfunction [345]. In the context of cultured fat, the lipid

content of cultivated adipocytes could thus be tuned for optimal health through culture media optimization, or even genetic enhancement [26,337]. Ultimately, the comprehensive, in depth characterizations that come from omics modalities empower producers to fine tune their *in vitro* fats and ever more accurately recapitulate native adipose tissue.

Conclusions and Future Perspectives

With increasing consumption and impact of meat production on the environment, developing quality alternatives to livestock-derived meat is of growing significance. Fat is an important component of flavor, aroma and texture in meat, thus reproducing these features without animal agriculture should contribute to the sensory and textural experience when consuming meat alternatives such as plant-based and cell-cultured meats. In this review we outline various considerations and potential paths for the large-scale production of *in vitro* cultured fat, including adipogenic precursors during cell proliferation, methods to proliferate cells at scale, methods to differentiate cells at scale and strategies for converting differentiated adipocytes into tangible 3D cultured fat tissues at scale. For large-scale cultured fat production, various cell source options with proliferative and adipogenic capacities exist, with and without the use of genetic modifications. Adipose biology surrounding certain livestock species may need to be further elucidated to enable more efficient large-scale adipose production. Methods, infrastructure and bioreactor systems exist for the large-scale proliferation of adipogenic precursor cells, owing to previous developments in biopharmaceutical and biotechnology fields. However, specific considerations (e.g., cell buoyancy) for adipocyte cell culture may require adaptations of existing systems. Current solutions for adiopgenic differentiation involve compounds not approved for use in humans, while FFAs, RNA and genetic engineering offer potential methods of differentiation that are likely to be scalable. While much research has done on scalable cell proliferation, and promising options exist for adipogenic differentiation, research on the conversion of adipocytes into fat tissue is scarce. 3D cell culture approaches promote adipogenesis and could inherently produce macroscale tissues, but tissue perfusion in large 3D tissues remains to be further developed before this approach can be used efficiently to create bulk adipose tissues for food applications. An alternative strategy to forming macroscale adipose tissue is to aggregate smaller, separately grown adipose tissue constructs, which could circumvent the need for perfusion. . Lastly, while data on the nutrition and taste of cell cultured fat are not available, numerous analytical tools exist to characterize lipid and volatile fractions of adipose tissue samples. With comprehensive tools for characterizing cell cultured fat, future producers of cell cultured fat should enable control over the taste, nutrition and health of their *in vitro*-grown adipose at levels that are unattainable with traditional livestock-sourced meats.

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List of Abbreviations

ADSC, adipose derived stem cell;

aP2, adipocyte protein 2;

BM-MSCs, bone marrow mesenchymal stem cells;

BMP, bone morphogenetic protein;

CD, clusters of differentiation;

CHO, chinese hamster ovary

c-Myc, myc proto-oncogene;

COVID, coronavirus disease 2019;

DFAT, dedifferentiated fat;

DMEM, Dulbecco's modified Eagle medium;

ESC, embryonic stem cell;

F12, Nutrient Mixture F-12;

FBB, fixed-bed bioreactor

FBS, fetal bovine serum;

FGF, fibroblast growth factor;

GC-MS, gas chromatography-mass spectrometry

GHG, greenhouse gas;

GSK, glycogen synthase kinase;

HARV, high aspect ratio vessel

HFB, hollow fiber bioreactor

HS, horse serum;

IBMX, isobutylmethylxanthine;

ICP1, immortalized chicken preadipocytes;

iPSC, induced pluripotent stem cell;

Klf4, Krüppel-like factor 4;

LC-MS, liquid chromatography-mass spectrometry

LIF, leukemia inhibitory factor;

LPL, lipoprotein lipase;

MAPK, mitogen activated protein kinase;

MS, mass spectrometry

MSC, mesenchymal stem cell;

Oct, octamer binding transcription factor;

PPARγ, peroxisome proliferator-activated receptor gamma;

PSC, pluripotent stem cell;

RNAseq, RNA-sequencing;

Sca, stem cells antigen;

Sox2, sex-determining region y box 2;

STLV, slow-turning lateral vessel

SV40, simian virus 40;

TERT, telomerase reverse transcriptase;

TGFβ, transforming growth factor beta;

TR, telomerase RNA

VOC, volatile organic compound

Data Availability - This is a review article, thus, primary data is associated with the literature cited.

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