Studying Brown Adipose Tissue in a Human *in vitro* Context

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New treatments for obesity and associated metabolic disease are increasingly warranted with the growth of the obesity pandemic. Brown adipose tissue (BAT) may represent a promising therapeutic target to treat obesity, as this tissue has been shown to regulate energy expenditure through non-shivering thermogenesis. Three different strategies could be employed for therapeutic targeting of human thermogenic adipocytes: increasing BAT mass through stimulation of BAT progenitors, increasing BAT function through regulatory pathways, and increasing WAT browning through promotion of beige adipocyte formation. However, these strategies require deeper understanding of human brown and beige adipocytes. While murine studies have greatly increased our understanding of BAT, it is becoming clear that human BAT does not exactly resemble that of the mouse, highlighting the need for human *in vitro* models of brown adipocytes. Several different human brown adipocyte models will be discussed here, along with the potential to improve brown adipocyte culture through recreation of the BAT microenvironment.

Keywords: brown adipose tissue (BAT), 3D culture, HPSC model, obesity, thermogenic adipocyte differentiation, brown adipogenesis, human adipocytes, beige adipocyte

THE OBESITY PANDEMIC

Obesity, defined as excessive fat accumulation, has reached pandemic proportions. Among adults, more than 650 million were obese in 2016 (BMI $\geq$ 30), with over 340 million obese or overweight (BMI $\geq$ 25) children and adolescents (1). Obesity is associated with the metabolic syndrome, which comprises a cluster of metabolic abnormalities including insulin resistance, obesity, dyslipidemia, and hypertension, which are risk factors for cardiovascular disease and diabetes (2). Furthermore, obesity is a risk factor for cancer and musculoskeletal disorders, including osteoarthritis, among other diseases (3, 4). Thus, obesity places a substantial burden on society and healthcare systems and despite extensive research into the mechanisms controlling energy balance, body weight, and appetite, safe and effective measures to treat and prevent obesity are lacking.

BROWN ADIPOSE TISSUE

Brown adipose tissue (BAT) has become a focus area for weight loss therapies. This tissue, evolved to help maintain core body temperature, is able to burn nutrients as heat, thereby increasing total energy expenditure (5). This process is known as non-shivering thermogenesis and is mediated by the mitochondrial uncoupling protein1 (UCP1), and BAT activation leads to increased energy expenditure and decreased metabolic efficiency.

BAT has been extensively studied in the mouse, and these studies have revealed an important role for BAT in the control of energy balance and whole-body metabolism. BAT activation has, for instance, been shown to positively influence plasma triglyceride and cholesterol levels, atherosclerosis development, glucose tolerance, insulin sensitivity, and hepatic steatosis, in addition to promoting weight loss (6–9).

BEIGE ADIPOSE TISSUE

Another type of adipose tissue (AT), which has received considerable attention for the treatment of obesity, is beige AT, which arises from white adipose tissue (WAT) browning. WAT browning is characterized by the presence of multilocular, brown adipocyte (BA)-marker expressing adipocytes within WAT—so-called beige or brite adipocytes. Beige adipocytes can appear in WAT, mainly subcutaneous (sc)WAT, after cold exposure, hypercaloric diet, β-adrenergic stimulation, and exercise (10), and are believed to influence metabolism similarly to BAT (11, 12). Although beige adipocytes show high expression of UCP1 (13), they are believed to originate from different lineages than BAs (14), although this is still debated.

BAT IN HUMANS—BEIGE OR BROWN?

In humans, BAT mass and prevalence have been found to correlate negatively with BMI, diabetes status, and glucose plasma levels (15), and cold-induced BAT activation also had beneficial metabolic effects and induced weight loss (16, 17), suggesting that human and murine BAT may have similar metabolic roles. It has been estimated that adult lean men may have around 330 ml of cold-inducible BAT, with lower volumes (around 130 ml) found in obese men (18). Furthermore, seasonal changes in WAT thermogenic gene expression have been described (19), suggesting that human WAT has the capacity for browning, although the impact of browning on whole-body metabolism is unclear.

Based on this ability of BAT to increase energy expenditure and improve metabolic function, BAT seems a likely target for the development of obesity therapeutics. Importantly, the efficacy of these therapeutics depends on whether activation of BAT in adults is able to significantly influence energy expenditure. Cold exposure in adults is able to increase energy expenditure varying from 20 kcal/day (20) to more than 100 kcal/day (17), and this can be increased further through chronic cold adaptation or increased BAT mass (17, 18).

A growing body of research is focusing on identifying genes and molecules able to influence BAT biology; however, one question is how well the findings in mice translate to humans. First, there is debate around whether human BAT resembles murine brown or beige AT more closely. Analyses of human neck fat have revealed that on a molecular level, deep neck fat mostly resembles that of murine canonical BAT, whereas the superficial neck fat may be more white or beige (21). In a study characterizing beige adipocytes, Wu et al. (13) generated murine brown and beige adipocyte gene expression signatures.

Comparing these signatures with human supraclavicular BAT, they found that human BAT is closer to murine beige than brown AT. A similar study comparing murine AT gene signatures with human BAT from multiple anatomical locations came to the same conclusion (22). In contrast, a recent study suggests that when mice are physiologically humanized, meaning housed at thermoneutrality, fed a "Western diet," and examined at 9–11 months old, their BAT becomes comparable with human supraclavicular BAT (23), although this conclusion has raised some discussion (24). This debate may likely be solved as the composition of human and murine brown and beige AT is better characterized with the help of more sophisticated techniques such as single-cell RNA sequencing. Owing to discrepancies between mRNA and protein expression levels of certain genes, including *UCP1* (25), purely transcriptomic analysis of different BA populations may not be adequate to fully characterize their identity and thermogenic potential. To this end, protein analysis and functional characterization, including β-adrenergic response and bioenergetics, are required.

Second, whereas treatment with β_3-adrenergic receptor (β_3AR) agonists in mice is able to selectively stimulate BAT and protect against diet-induced obesity, these effects are not seen in humans at doses low enough to prevent cardiovascular side effects (26, 27). Interestingly, a recent study found that the β_1AR may be the predominant βAR in human BAT (28), highlighting another confounding issue when translating murine BAT studies to human biology. Other attempts to selectively activate thermogenesis through sympathomimetic drugs or mitochondrial uncouplers have not been successful (26). Thus, to achieve selective and safe activation of BAT in humans, we may need to look beyond adrenergic stimulation or uncoupling mechanisms and instead focus on alternative pathways to target BAT biology. Specifically, rather than focusing exclusively on increasing the activation of existing BAT, other strategies could include increasing BAT mass through differentiation of progenitors, or increasing thermogenic activity in WAT through WAT browning.

STRATEGIES FOR THE TARGETING OF HUMAN BAT

Increased energy expenditure in humans through BAT thermogenesis could be achieved by (a) increasing functional BAT mass, (b) increasing BAT activity, and (c) increasing WAT browning.

Increased BAT Mass/Recruitment

Increased BAT mass could be achieved through the recruitment of BA progenitors. As mentioned earlier, decreased BAT mass is found in obese individuals—the very individuals the BAT therapeutics are intended for—and thus even if a method to selectively activate BAT was found, this may be of limited use to obese recipients. However, BAT activity can be enhanced through cold acclimation even in obese individuals (29), suggesting the presence of BA precursors, the differentiation of which may be promoted given appropriate stimulation. The development of

targeted approaches to induce differentiation of BA progenitors requires in-depth knowledge of the developmental mechanisms of human BAT, which currently does not exist. Furthermore, newly differentiated BAs would still require activation through sympathetic or alternative pathways.

Increased BAT Activity

Increased BAT activity, as discussed earlier, has been challenging to achieve without cardiovascular side effects. However, several molecules have been identified as possible therapeutic targets for the activation of BAT, reviewed in (30). First, members of the bone morphogenetic protein (BMP) family have been reported to play roles in BAT or WAT browning, mainly using murine models. These include, among others, BMP4, which may promote browning of WAT (31, 32), BMP7, which can promote BAT development and activation (33) as well as white adipocyte (WA) browning (31), and BMP8B, which was found to increase the adrenergic response of BAT and induce scWAT browning (34, 35). In addition, factors released from heart, muscle, liver, and immune cells have been shown to regulate thermogenesis (30). Although these studies reveal several candidate targets, most of them have been conducted in murine models or *in vitro*, and tangible therapeutics are still far from a reality.

Increased WAT Browning

Finally, increased WAT browning is a strategy which may have substantial potential given the high abundance of WAT compared with BAT. However, as for BAs, the safe activation of beige adipocytes likely requires identification of specific molecular pathways to induce WAT browning that do not rely directly on adrenergic or uncoupling actions. These include fibroblast growth factor 21 (FGF21), which may regulate browning of scWAT through activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) (36), as well as through UCP1-independent mechanisms (37). Another potential molecule is irisin, which may activate UCP1 in scWAT via p38-MAPK/ERK pathways (38), although the levels of circulating irisin and its metabolic effects in humans are debated (39, 40). In addition, more studies on human WAT browning are necessary to determine whether this process is able to influence whole-body energy expenditure in a notable manner.

The three strategies presented earlier are all limited by the lack of knowledge regarding human thermogenic adipocyte development and function. This lack of knowledge stems principally from the lack of robust *in vitro* models of human BAs.

IN VITRO MODELS OF HUMAN BAT

Models of human BAs have been developed by several different groups and from starting material such as primary BAT, fibroblasts, muscle cells, adult stem cells, and pluripotent stem cells, among others. Some of these models are discussed later and represented in **Figure 1**.

Primary Cell-Derived BA Models

The study of human primary BAT is limited by the scarceness of the disperse tissue and the need for invasive procedures to obtain biopsies. Nonetheless, several *in vitro* BA models derived from primary human BAT do exist. If one does have access to primary BAT, preadipocytes from non-viable human fetal interscapular BAT can be cultured and differentiated *in vitro* (41); however, these are not stable cell lines and are thus not a viable long-term solution for the study of human BAT. To this end, immortalized human BA lines may be of more use. One of these is the PAZ6 cell line from immortalized infant BAT vascular stromal cells (42). These cells readily differentiate to BAs and respond to β-adrenergic stimulation; however, the differentiation potential of these cells declines with increasing passage number, significantly limiting their experimental value. Similarly, subcloned immortalized BA-like cell lines have been generated from adult supraclavicular AT stromal vascular fraction (SVF) (43) and neck AT SVF (44). In addition, a more heterogeneous BA-like cell model was created from adult deep neck AT SVF without subcloning (45). These BA cell models demonstrated expression of *UCP1*, lipolytic response to cAMP (43), and increased oxygen consumption in response to β-adrenergic stimulation (44, 45), and two of the lines were maintained for at least 20 passages (44, 45). These cell lines thus represent useful models for the study of human BAT. However, their generation does require access to primary tissue, and they, therefore, come from limited starting material, which means that these cell lines will deplete eventually.

Rather than using primary BAT, BA-like cells can be directly generated from human dermal fibroblasts, eliminating the need for invasive biopsies. One study demonstrated that overexpression of CCAAT/enhancer binding protein beta (*CEBPB*) and avian myelocytomatosis viral oncogene homolog (*C-MYC*), coupled with an adipogenic cocktail, induced conversion of human fibroblasts to BA-like cells (46). Another study demonstrated the direct conversion of primary fibroblasts into BA-like cells with only chemical compounds, without using gene transfer (47). These compounds included rosiglitazone, which is an agonist of peroxisome proliferator-activated receptor gamma (PPARγ), and known to promote browning (48).

Alternatively, BA-like cells were shown to be differentiated from fetal and adult muscle progenitor cells expressing CD34, a marker of hematopoietic progenitors as well as vascular endothelial progenitors (49), thus supporting the common lineage of skeletal muscle and BAT. This technique does require invasive biopsies; however, muscle tissue may be more accessible than BAT.

In addition to primary tissue access, a limitation of the aforementioned models is the inherent risk of cell depletion. Thus, to circumvent this limitation, cell lines can be generated from human stem cells.

Generation of Adipocytes From Stem Cells
Multipotent Stem Cell–Derived BAs

Multipotent mesenchymal stem cells (MSCs) are adult stem cells able to differentiate into a range of mesodermal cell types including adipocytes, and can be isolated from several tissues
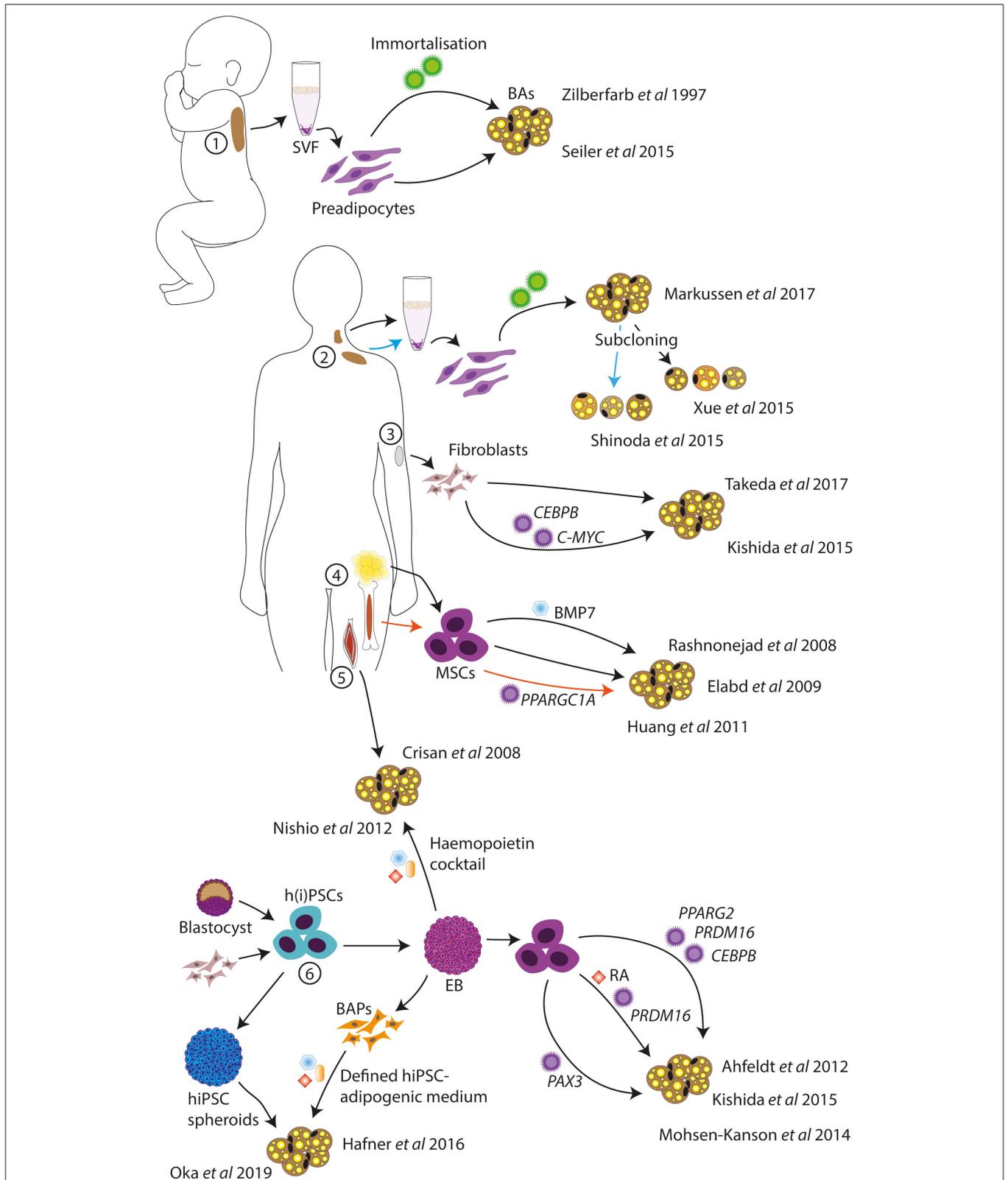
FIGURE 1 | Schematic of *in vitro* human BA models. Human BA models have been generated from infant BAT (1), deep neck and supraclavicular BAT (2), dermal fibroblasts (3), MSCs from AT and bone marrow (4), skeletal muscle progenitor cells (5), and hPSCs (6). Standard adipogenic cocktails or variations were used for the differentiations unless otherwise indicated. SVF, stromal vascular fraction; BAs, brown adipocytes; MSCs, mesenchymal stem cells; h(i)PSCs, human (induced) pluripotent stem cells; BAPs, BA progenitors; EB, embryoid body; RA, retinoic acid.

such as bone marrow, blood, and AT (50). MSCs isolated from human AT (termed human multipotent adipose-derived stem (hMADS) cells) could be differentiated to BA-like cells when stimulated with a PPARγ agonist on top of an adipogenic cocktail (51), and with the addition of BMP7 (52). Similarly, bone marrow–derived MSCs may also be able to differentiate to BA-like cells when overexpressing *PPARGC1A* (53). Thus, MSCs are a useful tool for the generation of BA-like cells, and the self-renewal ability of MSCs gives unlimited starting material, at least theoretically. As an alternative to MSCs, human pluripotent stem cells (hPSCs) have been used for the generation of BA cell models.

Pluripotent Stem Cell–Derived BAs

hPSCs are advantageous for the generation of cell models for several reasons, including that they are relatively easy to genetically engineer. Because of their pluripotency and the fact that protocols exist to differentiate hPSCs into a wide range of cell types (54), it is possible to generate isogenic disease models of cells from all three embryonic germ layers. Furthermore, the use of human induced (hi)PSCs, which are reprogrammed from human somatic cells (55), enables the generation of disease models with patient-specific genetic backgrounds.

Some studies have demonstrated the generation of adipocytes from hPSCs. Mature white and brown adipocytes were generated from hPSCs through transfection with lentiviral constructs to overexpress *PPARG2* or *PPARG2*, *CEBPB*, and *PRDM16*, respectively (56). In this study, hPSCs were first differentiated into MSCs using embryoid bodies (EBs), after which transgene expression was induced and an adipogenic cocktail was given. A similar method was used to generate BAs from hiPSCs through transduction of *PRDM16* (46). Here, EBs were generated in the presence of retinoic acid (RA), to obtain more myoblast-like cells (57), accounting for the reduced transgene requirement. Overexpression of transgenes in hPSCs is an efficient method for the generation and study of mature cell types (58–60), although it can be argued that this method of differentiation may not follow natural developmental steps and may instead shunt some of these pathways. Thus, this technique may not be optimal for the generation of a human BA model for the study of BA development.

Another group demonstrated the differentiation of BA progenitors from hiPSCs through EB formation and adipogenic cocktails (61). The differentiation efficiency was reported to be low but could be improved through overexpression of paired box 3 (*PAX3*), a marker of BA progenitors (61). A similar method was used to generate BA progenitors from hiPSCs without gene transfer, although this study reports higher differentiation yield, possibly due to inhibition of TGFβ signaling (62). TGFβ signaling has been shown to induce the conversion of endothelial cells to a more mesenchymal phenotype (63), and these findings, therefore, suggest an important role of endothelial cells in the adipogenic niche. The generation of BA-like cells from hPSCs using EB formation and a hemopoietin cocktail has also been reported (64), which may suggest that the developmental origin of BAs is more diverse than previously believed. Finally, BA-like cells were generated from hiPSCs in cytokine-free medium through the

formation of spheroids (65), although the molecular mechanisms of this differentiation method remain unclear.

Although hPSCs can, in theory, give rise to any cell type of the human body, their immature/embryonic state means that it can be difficult to generate mature cell types from hPSCs without forward programming. This issue does not arise when using BA models derived from primary adult AT. As an alternative, hPSC-derived BAs can be matured *in vivo* by transplantation into mice, which is also a method of functional validation of the generated BAs (46, 64, 65). Currently, transplantation *in vivo* may be the best technique for recapitulation of the physiological BAT microenvironment; however, advances are also being made toward recreating BAT *in vitro* using 3D cell cultures.

RECAPITULATING THE BA MICROENVIRONMENT *IN VITRO*

Long-term culture of adipocytes *in vitro* can be difficult because of the buoyancy of mature, lipid-laden adipocytes, which makes their attachment to 2D cell culture plates difficult (66). Furthermore, true understanding of BAT biology requires recapitulation of the *in vivo* BA microenvironment—the BA niche. *In vivo*, BAT is a highly vascularized and innervated tissue, containing not only BAs but also endothelial cells, fibroblasts, progenitor cells, and immune cells (5, 30). Furthermore, the extracellular matrix (ECM) of the AT is vital as a structural scaffold and to facilitate adipocyte differentiation, AT expansion, mechanotransduction, and biomolecule signaling (67–69).

Culturing BAs in 3D

A first step toward recapitulating BAT *in vitro* may be to use 3D cultures, which can provide mechanical support, allow physiological cell organization, and prevent loss of mature adipocytes (70). 3D culture techniques are successful for WA culture (71), allowing *in vitro* culture of unilocular primary mature human WAs (72, 73). Unilocular WAs can also develop *in vitro* when differentiated in spheroids (74–76).

Fewer 3D culture systems have been tested on BAs, potentially because the focus is still on the generation of useful BA cell models rather than their further development. Using hanging drop spheroid formation followed by culture in ultra-low attachment plates, Klingelhutz et al. (76) demonstrated improved differentiation of murine BAT SVF compared with 2D. Alginate microstrands have also been used for the differentiation of BAs from murine embryonic stem cells and brown preadipocytes (77). Using 3D printed hyaluronic acid/gelatin gels, Kuss et al. (78) showed that whereas immortalized human WA progenitors prefer soft gels, immortalized human BA progenitors prefer stiffer gels, suggesting that developments within WA 3D culture methods cannot necessarily be directly translated to BA cultures.

ECM and Vascularization

The next step toward recapitulating BAT *in vitro* is the addition of ECM and supporting cell types. It is possible to generate 3D scaffolds from ECM proteins such as collagens and glycoproteins (71), or to incorporate ECM components into synthetic hydrogels (72, 79). However, to recreate the human BAT ECM

in vitro, its composition must first be fully characterized *in vivo*, in both physiological and pathophysiological states.

For the generation of functional models of AT, vascularization is an important parameter, which can also aid engraftment *in vivo* during transplantation studies, as shown (74). Angiogenic signals, including VEGF (vascular endothelial growth factor), play important roles not only in BAT function but also in the development of BAs (80–83). For instance, VEGFA is able to trigger local angiogenesis in BAT, leading to brown adipogenesis and upregulation of *Ucp1* and *Pgc1α* (81). Vascularized (white) adipocyte spheroids can be generated using AT SVF, which contains endothelial cells as well as preadipocytes, or by coculturing adipocytes with endothelial cells (74, 75). For the generation of vascularized human BA models, it may be advantageous to focus on coculture methods, to avoid the need for primary tissue. Similar challenges exist regarding the integration of immune cells into the BA cultures.

DISCUSSION

Our understanding of BAT in mice and humans has increased significantly over the last decade, and with this understanding, we are moving closer to the development of therapeutics targeting BAT for obesity treatment. One of the main limitations in the field is the limited understanding of human BAT, including its development and molecular signature. These questions are steadily being tackled through in-depth characterization of primary human BAT and the development of human *in vitro* BA models. Human BA models can be generated from immortalization of primary human BAs, from multipotent stem cells or hPSCs, among others, and each of these methods has distinct advantages and limitations. For instance, *in vitro* models generated from adult BAT may represent the most mature BA models, whereas hPSC-derived BAs may be less mature unless forward programming is employed. In contrast, hPSCs are useful for disease modeling involving genetic engineering or patient-specific genetic backgrounds. Without a defined molecular signature for human BAT, lacking due to the heterogeneity and disperse anatomical distribution of BAT depots, it may, however, be challenging to determine which *in vitro* model is the best. Furthermore, as BAT does not exclusively consist of BAs, true recapitulation of this tissue involves modeling of the BAT microenvironment, including physiological BA organization in 3D, crosstalk with supporting cells such as immune cells, vascularization, and an ECM to facilitate BA differentiation and cell–cell signaling.

The road toward the development of obesity therapeutics targeting human BAT thus has plenty of challenges. However, with continued development of BA models and characterization of the *in vivo* depots, these challenges may soon be overcome.

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