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Opportunities and Challenges in Three-dimensional Brown Adipogenesis of Stem Cells

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Abstract

The formation of brown adipose tissue (BAT) via brown adipogenesis has become a notable process due to its ability to expend energy as heat with implications in the treatment of metabolic disorders and obesity. With the advent of complexity within white adipose tissue (WAT) along with inducible brown adipocytes (also known as brite and beige), there has been a surge in deciphering adipocyte biology as well as *in vivo* adipogenic microenvironments. A therapeutic outcome would benefit from understanding early events in brown adipogenesis, which can be accomplished by studying cellular differentiation. Pluripotent stem cells are an efficient model for differentiation and have been directed towards both white adipogenic and brown adipogenic lineages. The stem cell microenvironment greatly contributes to terminal cell fate and as such, has been mimicked extensively by various polymers including those that can form 3D hydrogel constructs capable of biochemical and/or mechanical modifications and modulations. Using bioengineering approaches towards the creation of 3D cell culture arrangements is more beneficial than traditional 2D culture in that it better recapitulates the native tissue biochemically and biomechanically. In addition, such an approach could potentially protect the tissue formed from necrosis and allow for more efficient implantation. In this review, we highlight the promise of brown adipocytes with a focus on brown adipogenic differentiation of stem cells using bioengineering approaches, along with potential challenges and opportunities that arise when considering the energy expenditure of BAT for prospective therapeutics.

Keywords

Adipogenesis; Brown adipocytes; Brown adipose tissue; Brown fat; Stem cells; 3D culture; Differentiation; Tissue engineering; Energy; Obesity

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1. Introduction

Excessive adipogenesis plays a critical role in energy imbalance, which is the leading cause of obesity and metabolic disorders. The knowledge of complete cellular processes is beneficial for the advancement of understanding adipogenesis and therefore treating disease. One method for gaining insight into these processes is by studying paths of cellular differentiation. Pluripotent stem cells, such as embryonic stem cells (ESC) and induced pluripotent stem cells (iPSCs) have the ability to differentiate into all three germ layers and are therefore an excellent starting point for understanding cellular processes (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998; Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Adipogenesis, the differentiation of preadipocytes or progenitor cells into adipocytes (fat cells), has been copiously studied as a model for cellular differentiation (Lee and Ge, 2014; Rosen and Spiegelman, 2000; Studzinski, 2001).

Beneath the umbrella of adipogenesis are two main types of adipocytes: white adipocytes and brown adipocytes. As distinct cell types, white and brown adipocytes develop into their own specialized tissues. White adipose tissue (WAT) functions to store energy in the form of triglycerides, and acts as a vital endocrine and immune organ (Dani et al., 1997; Ahfeldt et al., 2012; Townsend and Tseng, 2012). However, brown adipose tissue (BAT) is specialized to expend energy as heat by uncoupling respiration with its unique mitochondrial membrane embedded protein UCP1, a process known as nonshivering thermogenesis (Golozoubova et al., 2001; Selye and Timiras, 1949; Townsend and Tseng, 2012; Zhang et al., 2010). The rediscovery of functional brown adipocytes in human adults (Cypess et al., 2013; van Marken Lichtenbelt et al., 2009; Nedergaard et al., 2007; Virtanen et al., 2009) has inspired interest in the field and led to a paradigm shift in understanding the role of brown adipocytes in energy balance and obesity.

The balance of energy within cells and therefore the human body is critical for maintaining overall health, especially for the long term (Galgani and Ravussin, 2008; National Heart Lung and Blood Institute 2013). Therefore, the imbalance of energy has potential to cause a myriad of health issues, including obesity. The main cause of obesity, and thus obesity-related disease, is an energy imbalance where energy intake exceeds energy dissipation (Chen and Tong, 2013; Cypess et al., 2009; Tseng et al., 2010). Therefore, the expended thermal energy from brown adipocytes introduces a potential balance solution and also a critical need for understanding brown adipocyte biology. This comprehension has implications in potential obesity treatments, which is vital because obesity initiates a domino effect of health complications.

In order to effectively study brown adipocyte biology and understand the implications of BAT in the clinical setting, in vivo-like brown adipocyte constructs fabricated using tissue engineering approaches would be ideal, as this mimics the stem cell and/or adipogenesis microenvironment or niche. Hydrogels are a plausible material of choice for this type of growth platform due to their compressive strength and certain structural similarities to the native extracellular matrix (ECM) and adipose tissue (Geckil et al., 2010; Lee and Mooney, 2001; Tibbit and Anseth, 2010). Differentiation of stem cells into brown adipocytes within a

hydrogel construct would fulfill the need to understand early events in brown adipogenesis in addition to the encapsulation of brown adipocytes for any future potential implantation.

In this review, we highlight the current state-of-the-art of brown adipocytes for use in fighting obesity with a focus on the potential of brown adipogenic differentiation of pluripotent stem cells. Since bioengineering brown adipocytes is still in its infancy, we summarize cutting-edge research regarding microenvironmental cues for adipogenesis in general in addition to (white) adipocyte tissue engineering, as well as provide insights of 3D brown adipogenesis of pluripotent stem cells for better understanding brown adipocyte biology and as an implantable construct to combat obesity and metabolic disorders.

2. Health consequences and clinical relevance of energy imbalance

During the past few decades, there has been a substantial increase in obesity in the United States, resulting in about 34.9% of US adults (greater than 20 years of age) and 17.0% of US children (2 to 19 years of age) becoming obese (Ogden et al., 2014). These alarming statistics have led the American Medical Association to classify obesity as a disease with the term “obese” currently referring to individuals with a body mass index (BMI) of 30 kg/m² or greater (Breymaier, 2013; National Heart Lung and Blood Institute, 2012; Obesity Prevention Source Harvard School of Public Health, 2014; Ogden et al., 2010). The increase in obesity is of major concern due to the direct causation of cardiovascular disease, stroke, type 2 diabetes, metabolic disorders such as non-alcoholic hepatic steatosis, and certain cancers (Cohen et al., 2011; Kahn et al., 2006; Montague and O’Rahilly, 2000; Ogden et al., 2014). Battling obesity is increasingly critical as cardiovascular disease is the leading cause of death in the United States with stroke not far behind as the fourth (National Center For Chronic Disease Prevention and Health Promotion and Division for Heart Disease and Stroke Prevention, 2013; Centers for Disease Control and Prevention Media Relations, 2014). As a result, there is a sense of urgency to research obesity causes and potential treatments at the cellular level.

The energy imbalance that causes obesity, for example, by overconsumption of calorie-rich foods and poor exercise habits, is well understood. However, this imbalance translates into an overwhelming accrual of body fat compared to lean tissue (Campos et al., 2014). The most dangerous culprit in this imbalance is visceral WAT, which surrounds the body’s organs and is suspected to be heavily involved in insulin resistance (Yamashita et al., 1996). This involvement traces back to plasminogen activator inhibitor 1 (PAI-1), which is associated with visceral adipose tissue (Lindeman et al., 2007). The health risks associated with this factor belong to a direct correlation between increased levels of PAI-1 and cardiovascular risk, insulin resistance, and therefore, type 2 diabetes (Bremer et al., 2011; Lindeman et al., 2007). This is one example of how an abundance of WAT can in fact become a detriment to the quality of life. On the contrary, BAT is found to be present in adult humans and upon cold activation, fluorodeoxyglucose (FDG) uptake is inversely correlated to BMI (Cypess et al., 2009; Saito et al., 2009). In fact, cold-activated BAT has been shown to increase plasma clearance of triglycerides in mice (Bartelt et al., 2011). Moreover, glycated hemoglobin, blood glucose, total and LDL-cholesterol levels were reduced in a BAT-positive group in a recent human study (Matsushita et al., 2013).

Therefore, it is imperative to understand adipocyte biology, in particular, the role of brown adipocytes in potentially treating obesity and related deficiencies.

3. Brown adipocyte biology

3.1. Adipocyte biology

Adipose tissue plays an important role in energy homeostasis within the human body, and therefore, is critical for survival. Adipocytes, or fat cells, are one of the comprising factors within adipose tissue and are capable of initiating the expansion of adipose tissue by increasing in size (hypertrophy) and number (hyperplasia) (Jo et al., 2009). When either growth process becomes over abundant without proper energy expenditure, the scales tip in favor of stored energy and thus can have metabolic consequences.

Traditionally, WAT was thought to function primarily as a fat storage organ, yet over the last several decades, it has become apparent that not only does adipose tissue store and release fat as needed, but it also operates as an endocrine organ (Zhang et al., 1994; Mohamed-Ali et al., 1998). In fact, WAT is now considered to have a leading role in both healthy and dysfunctional metabolism (Owens, 2014; Peirce et al., 2014). The complexity of the tissue has reached such lengths that a systems biology approach has been used to understand the metabolic and signaling networks (Manteiga et al., 2013). To add to this complexity, the divergence of brown and white adipocytes has also led to the discovery of a subpopulation of brown-in-white adipocytes entitled beige, brite, or inducible adipocytes in WAT (Wu et al., 2012a). Adipocyte categories, location and cell origin are summarized in Table 1.

3.2. Brown versus white adipocytes

Although white, brown (also referred to classical brown) and beige adipocytes exist within the same tissue type, the differences outweigh the similarities (Figure 1). As mentioned in Table 1, the apparent difference between the two adipocytes is their function, white to store energy and brown to expend energy. The cause of this distinction stems from their lineage variance, as outlined in Figure 2. Classical brown adipocytes are derived from a Myf5 muscle lineage (Cannon and Nedergaard, 2008; Timmons et al., 2007), yet there has been debate on any relation between white adipocytes and muscle (Timmons et al., 2007; Seale et al., 2008; Schulz et al., 2011). Schulz and Tseng (2009) efficiently summarized characteristic differences between BAT and WAT in terms of color, vascularization, fat storage, size, nucleus location, and unique markers. Since brown adipocytes are responsible for energy expenditure, it comes as no surprise that they are rich in mitochondria (as white adipocytes are not), which contributes to their brown coloring (Figure 1). In addition, the vascularization of brown adipocytes contains multiple capillaries as opposed to the arteriole vascularization in white adipocytes. In terms of fat storage, brown adipocytes have multilocular fat droplets as opposed to the unilocular fat droplet in white adipocytes. This large fat droplet in white adipocytes contributes to their generally larger size ranging from 50–105 μm when compared to brown adipocytes between 38–45 μm (Cavallini et al., 2006). Another cellular difference is the nucleus placement, as the nucleus lies in the periphery in white adipocytes and is central in brown adipocytes (Schulz and Tseng, 2009). In addition,

there are unique markers for both adipocytes: leptin for white (relatively) and UCP1 for brown adipocytes (Lin and Klingenberg, 1980; Zhang et al., 1994) as well as newly identified brown adipogenic genes, potassium channel K3 (KCNK3) and mitochondrial tumor suppressor 1 (MTUS1) (Shinoda et al., 2015). Additionally, certain adipocyte markers such as PGC1 α , PRDM16, FGF21, and FOXC2 (Cederberg et al., 2001; Seale et al., 2007; Hondares et al. 2011; Fisher et al., 2012) are expressed at higher levels in brown than white adipocytes. Along with overall appearance, gene expression of these markers helps to distinguish brown from white adipocytes at the molecular biology level.

3.3. Brown adipocytes, energy expenditure and therapeutic potential

Classical BAT does not expend energy at a constant rate. Instead, the tissue must be activated in order for energy to be dissipated as heat, also known as non-shivering thermogenesis. This activation is initially triggered sympathetically either by exposure to cold temperatures or overfeeding, but it is important to take note that this type of thermogenesis is not inherent in all obese animals (Himms-Hagen, 1976, 1985; Rothwell and Stock, 1979; Seydoux and Girardier, 1977; Trayhurn et al., 1982). Once the sympathetic nervous system is activated, norepinephrine is released and binds to β 3-adrenoreceptors located on the surfaces of brown adipocytes. These receptors then mediate the activation of adenylate cyclase through the action of G proteins in order for adenylate cyclase to catalyze the formation of cAMP. cAMP is then able to migrate into the nucleus and bind to the promoter of the deiodinase enzyme gene to convert thyroxine (T4) into triiodothyronine (T3). T3 also enters the nucleus and binds to the promoter of the gene for UCP1, which ultimately inserts itself into the inner mitochondrial membrane and uncouples oxidative phosphorylation (Nicholls et al., 1978; Nicholls and Locke, 1984). When UCP1 is inserted into the inner mitochondrial membrane, the protons from the gradient formed as electrons move through the respiratory chain now have another possible entry point into the mitochondrial matrix besides ATP synthase (Nicholls et al., 1978). Therefore, when the protons move through UCP1, ADP is not converted to ATP and energy is released as heat. This expenditure of energy has therapeutic potential with the ability to tip the scales of energy balance in favor of energy output compared to input.

3.4. Beige or inducible brown adipocytes and therapeutic potential

A subtype of WAT has also been induced into brown-like adipose tissue, and as such has been titled beige, brite or inducible adipose tissue (Petrovic et al., 2010; Lidell et al., 2013; Schulz et al., 2013). We will use the term “beige” for the remainder of this review. Overall appearance as well as anatomical location of beige adipocytes is compared with white and brown adipocytes in Figure 1. Beige adipose tissue is titled as “brown-like” (Lee et al., 2014c) because it lacks the muscle-related lineage of classical BAT (Cohen et al., 2014). However, PGC1 α expressing myocytes and the exercise-induced myokine irisin have been shown to induce browning of subcutaneous white adipose tissue (Boström et al., 2012; Lee et al., 2014b; Zhang et al., 2014). Similar to BAT, beige adipose tissue has a thermogenic capacity upon induction by cold or pathways that increase intracellular cAMP (Vitali et al., 2012; Wu et al., 2012a). In addition, mice without functional beige fat are susceptible to obesity and obesity-related diseases when exposed to a high fat diet (Cohen et al., 2014). The induced thermogenic capacity and ability to affect mice physiology shows promise for

beige adipose tissue as an alternative obesity therapeutic (Chen and Tong, 2013; Nedergaard and Cannon, 2013; Wu et al., 2012). This would harness existing and potentially abundant WAT and focus on transdifferentiation into beige adipose tissue. This is especially interesting since there have been reports that beige adipocytes arise from a subgroup of precursors existing within WAT (Wang et al., 2013). However, it is worth noting that this process has been determined to be reversible if dependent upon environmental conditions. Therefore, a more intrinsic transdifferentiation is needed (Rosenwald et al., 2013). This notion is consistent with the fact that mature adipose tissue has the ability to dedifferentiate into progeny cells known as dedifferentiated fat cells (Wei et al., 2013). A more tightly controlled transdifferentiation could be achieved by studying this process using a 3D bioengineered microenvironment. This method not only has the ability to recreate certain conditions observed in vivo, but also is less invasive and less expensive than an animal model. Moreover, studying the bioengineered microenvironment could impact the maintenance of transdifferentiation by controlled release of specific soluble factors and modulation of cell-matrix and cell-cell interactions. By combining this approach with stem cells, early events as well as transdifferentiation could be better understood.

4. Brown adipogenesis of stem cells

4.1. Lineage progenitors of white and brown adipocytes

The lineage disparities between brown and white adipocytes have led to much research into developmental adipocyte origins. Before birth, BAT forms in order to maintain a newborn's body temperature whereas most WAT development begins moments after birth (Tang et al., 2008). As mentioned previously, BAT shares a Myf5+ (myogenic) lineage with skeletal muscle whereas the precursor for WAT has been assumed to be Myf5-, but more recent lineage tracing studies have suggested otherwise (Sanchez-Gurmaches et al., 2012; Timmons et al., 2007). In addition, early white adipocyte progenitor cells such as CD34(+), CD24(+), and Sca-1(+) have been found in vivo (Rodeheffer et al., 2008). Along with lineage variations the location of tissue formation within the mesoderm is different between WAT and BAT with BAT proceeding from a precursor within the paraxial mesoderm and WAT from the lateral mesoderm (Park et al., 2014). As expected, terminal differentiation of precursors into WAT and BAT involve varying factors. Adipogenic differentiation relies heavily on PPAR γ as well as the C/EBP family with particular emphasis on the α , β , and δ members (Rosen and Spiegelman, 2000; Taura et al., 2009). In contrast, for myogenic or BAT differentiation the members of this group involved are C/EBP β and α in addition to BMP7, PRDM16, PPAR γ and PGC1 α (Tseng et al., 2008; Kajimura et al., 2009). Therefore, it is apparent that although WAT and BAT are both types of adipose tissue, they are very distinct from one another from early developmental stages. In general, at the molecular level, white preadipocytes can commit to terminal white adipocyte differentiation with growth factors (e.g., BMP2, BMP4) and transcriptional factors (e.g., C/EBP α , β and PPAR γ) as mentioned (Bowers et al., 2006; Gustafson et al., 2013; Gustafson and Smith, 2012). In the presence of BMP7, PRDM16, PPAR γ , C/EBP β , and PGC1 α , brown preadipocytes are induced to brown adipocytes (Scimè et al., 2005; Seale et al., 2007, 2008; Tseng et al., 2008; Kajimura et al., 2009).

4.2. Differentiation of mesenchymal stem cells to white and brown adipocytes

The formation of white adipocytes from multipotent stem cells, e.g., bone marrow-derived mesenchymal stem cells (MSCs), adipose-derived stem cells (ASCs), has been widely established (Janderová et al., 2003; Menssen et al., 2011; Pittenger et al., 1999; Sekiya et al., 2004; Tseng et al., 2008). Brown adipocyte differentiation from MSCs and ASCs are outlined in Table 2 (Tseng et al., 2008; Elabd et al., 2009; Pisani et al., 2011). MSCs are adult multipotent stem cells capable of differentiating into bone, cartilage, tendon, ligament, marrow stroma, adipose, dermis, muscle and connective tissues (Caplan, 1991). Therefore, MSCs have the capacity to differentiate into both white and brown adipocytes; with pathways that involve similar transcription factors yet remain distinct.

The initial divergence occurs when MSCs commit to either a Myf5+ or Myf5- lineage, as described in Figure 2. From this point, the precursors can become either white or brown pre-adipocytes by treatment with BMP2/BMP4 and BMP7/PRDM16, respectively (Tseng et al., 2008; Kajimura et al., 2009). Further browning of white adipocytes can be induced by treating with irisin, FGF21 or apelin among other factors (Pyrzak et al., 2015; Than et al., 2015). It has also been shown that transient overexpression of the PPAR γ 2 and C/EBP α genes in MSCs would lead to the formation of adipose tissue that expresses the unique brown adipocyte marker, UCP1, in vitro and in vivo (Sheyn et al., 2013). MicroRNAs have recently received a lot of attention in both white and brown adipogenesis as well as transdifferentiation. For example, miR-17-5p and miR-106a, promote adipogenesis of ASCs by targeting BMP2, and therefore, decreasing osteogenic genes and increasing adipogenic C/EBP α and PPAR γ (Li et al., 2013). MicroRNAs have also been shown to regulate brown adipogenesis positively including miR-193b-365 and miR-196a (Mori et al., 2012; Sun et al., 2011), negatively with miR-27 and miR-133 (Sun and Trajkovski, 2014; Trajkovski et al., 2012), and in between, such as miR-155 where expression increases during proliferation of brown preadipocytes and declines when the cells are induced for complete differentiation (Chen et al., 2013). Additionally, long noncoding RNAs (lncRNAs) have been studied and confirmed to be involved in adipogenesis, including brown adipogenesis (Sun et al., 2013; Zhao et al., 2014; You et al., 2015).

Although it is clear that multipotent stem cells can be differentiated into white and brown adipocytes, it is important to note that these types of stem cells have limitations in proliferation and continuous differentiation potential (Rosenbaum et al., 2008). For example, long-term in vitro expansion may not affect the cell proliferation of ASCs until passage 5, but has a negative effect on adipogenic and chondrogenic differentiation while potentially favoring osteogenesis (Zhao et al., 2012b). Additionally, the long-term culture of MSCs could cause cellular senescence, resulting in growth arrest and reduction in differentiation (Cheng et al., 2011). Therefore, a pluripotent stem cell population would be more beneficial to overcome these limitations.

4.3. Differentiation of pluripotent stem cells to white and brown adipocytes

ESCs serve as a good model system for understanding early events in development (Martin, 1981; Reubinoff et al., 2000; Risau et al., 1988). ESCs have the potential to differentiate into all three germ layers (endoderm, mesoderm, and ectoderm) (Evans and Kaufman, 1981;

Martin, 1981; Thomson et al., 1998). In addition to being pluripotent, ESCs have the potential for extended and even unlimited self-renewal giving them a vast therapeutic potential (Blanpain et al., 2004; Watt, 2000).

Spontaneous differentiation of ESCs into adipocytes usually occurs as an infrequent event (Phillips, 2003). Directed differentiation was initially established by Dani et al. (1997) using a short treatment of all-*trans*-retinoic acid (RA) to commit mouse embryoid bodies (EBs) into a white adipogenic lineage. The concentration of RA is critical because if it is too high, the ESCs may commit to a neural lineage (Okada et al., 2004). Additionally, parthenogenetic stem cells have demonstrated the potential to be differentiated into white adipocytes (Liu et al., 2014).

A more detailed example of directed brown adipogenic differentiation of hESCs is shown in Table 2. Ahfeldt et al. (2012) accomplished this by first differentiating the hESCs into mesenchymal progenitor cells (MPCs) using previously established protocols (Barberi et al., 2005; Trivedi and Hematti, 2009). The MPCs were then transduced with combinations of doxycycline-inducible lentiviral constructs that encoded for PPAR γ 2, CEBP β , and PRDM16. These transcription factors were chosen based upon previous work showing that PPAR γ 2 is a key regulator of adipogenesis (Rosen et al., 1999), PRDM16 can convert mouse myoblasts into brown adipocytes (Seale et al., 2008) and a combination of CEBP β and PRDM16 can convert mouse cells and human fibroblasts into brown adipocyte-like cells (Kajimura et al., 2009). It was determined that any transcription factor combination that included PPAR γ 2 exhibited a greater induction of the brown fat marker protein UCP1 (Ahfeldt et al., 2012). In addition, the human ESC-derived brown adipocytes were injected subcutaneously in vivo and analyzed after 4–6 weeks with the transplanted cells staining positively for UCP1 expression. Moreover, ¹⁸FDG uptake followed by PET-CT showed that the transplanted hESC-derived brown adipocytes had considerable ability to uptake FDG (Ahfeldt et al., 2012). This is consistent with the notion that brown adipocytes act as a ‘glucose sink’ in that these cells are able to uptake large quantities of glucose as contribution to their vast metabolic capacity (Ahfeldt et al., 2012).

Although the potential to advance healthcare is vast with ESCs, there has been much ethical debate about the use of human embryos within the last few decades. Therefore, the reprogramming or induction of somatic cells into pluripotent stem cells became an appealing method to make stem cell treatment a reality. Not only does this approach address the ethical issues surrounding ESCs but also provides a method of using the patient’s own somatic cells to avoid potential transplantation complications (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Factors used to reprogram somatic cells, such as fibroblasts, into iPSCs include Oct3/4, Sox2, Klf4, c-Myc, etc. either in combination or with some omitted from the program (Takahashi and Yamanaka, 2006; Nakagawa et al., 2008).

PSCs represent a promising new source for white and brown adipogenesis (Elefanty and Stanley, 2012; Hafner, 2014). Adipogenic differentiation of hiPSCs has been accomplished similarly to ESCs by firstly allowing cells to form EBs and then exposing them to RA. Cells were then induced for 10 days using a medium containing DMEM-F12, 10% KnockOut Serum Replacement (KSR), and an adipogenic cocktail composed of 0.5 mM 3-isobutyl-1-

methylxanthine (IBMX), 0.25 μ M dexamethasone, 1 μ g/ml insulin, 0.2 mM indomethacin, and 1 μ M pioglitazone (Taura et al., 2009), resulting in iPSC-differentiated adipocytes confirmed by lipid accumulation and expression of adipocyte markers such as C/EBP α , PPAR γ 2, leptin and aP2. It has also been shown that differentiation of hiPSCs into classical brown adipocytes is most effective when exposing the cells to a specific hematopoietic cytokine cocktail because from previous iPSC differentiation studies, cells morphologically resembling brown adipocytes were observed surrounding hematopoietic areas (Nishio et al., 2012a; Nishio and Saeki, 2014). These examples have revealed the potential of differentiating hiPSCs into brown adipocytes as an alternative to using hESCs, which stir ethical issues as mentioned. On the topic of ethics, one potential alternative to studying brown adipogenesis in animal models is by culturing stem cells in a synthetic 3D microenvironment.

5. 3D microenvironments to control adipogenesis of stem cells

5.1. Advantages of 3D adipogenesis in comparison to 2D culture

Conventional 2D culture fails to recapitulate the in vivo adipose tissue complexity and physiology due to the lack of 3D architecture, complex cell-cell interactions and cellular signaling. Compared to 2D culture, 3D culture for adipogenesis in general could: 1) deposit an extensive ECM network (Grayson et al., 2004), 2) recapitulate adipose stem cell microenvironments (Yang et al., 2010), 3) enhance adipogenic differentiation, exhibiting more mature adipogenesis of stem cells (Gerlach et al., 2012), efficient lipid accumulation and in vivo-like organogenesis (Daquinag et al., 2013), expression of adipocyte-specific markers (Hong et al., 2005; Neubauer et al., 2005; Stacey et al., 2009) as well as VEGF (Girandon et al., 2011), and increase secretion of adipokines such as leptin (Kang et al., 2005), 4) be more sensitive to insulin-stimulated glucose uptake and drug treatment (Brännmark et al., 2014; Turner et al., 2014), 5) allow long-term survival (Neuss et al., 2008a), and 6) be feasible to manipulate cellular microenvironments for adipogenesis (Daya et al., 2007).

5.2. Stem cell microenvironments for adipogenesis

An important component in differentiating stem cells is to recreate their native microenvironment or niche. The niche defines an anatomic location that is capable of regulating how stem cells engage in tissue generation, maintenance, and repair (Scadden, 2006). The stem cell niche is composed of soluble factors, ECM, and neighboring cells (Mitsiadis et al., 2007). In addition to regulating stem cell differentiation, the niche provides protection from apoptotic stimuli and overproduction (Scadden, 2006; Mitsiadis et al., 2007). It is also worth mentioning that the cell microenvironment is different for different cell types and it is 3D in nature, making it beneficial to culture and differentiate cells in a 3D microenvironment as opposed to traditional 2D methods (Lund et al., 2009; Zhan et al., 2011). Moreover, 3D microenvironments also provide cells with increased surface area for growth.

As far as adipogenesis is concerned, biochemical, mechanical and topographic cues as well as neighboring cells in cellular microenvironments play an important role in regulating

adipogenic differentiation (Figure 3). During adipogenesis, preadipocytes rearrange their microfilaments in order to accommodate lipid accumulation within growing lipid vacuoles (Gregoire et al., 1998). A decrease in cytoskeletal protein biosynthesis and adoption of rounded cell morphology are identified to be associated with preadipocyte differentiation (Spiegelman et al., 1982; Spiegelman et al., 1983). Surface chemistry, mechanical property, and topography of the substrates could change cytoskeleton rearrangement and cell shape, and therefore, regulate adipogenesis.

5.2.1. Surface biochemistry, cell adhesion and adipogenesis—Surface chemistry and biochemistry affects cell adhesion that is found to influence white adipogenesis in both 2D and 3D. Under low adhesion conditions, such as rounded and poorly diffuse hMSCs (McBeath et al., 2004), ASCs cultured in suspension (Tholpady et al., 2005), and cells encapsulated in nonadhesive crosslinked hyaluronic acid (HA) (Flynn et al., 2007), differentiation into adipocytes was strengthened. On the contrary, while cells grown on adhesive surface such as fibronectin matrices, adipogenic differentiation was inhibited or dedifferentiation into precursor cells occurred (Spiegelman and Ginty, 1983; Tholpady et al., 2005). In addition, an RGD-modified alginate hydrogel stimulated adipogenesis by promoting 3D cell adhesion and formation of round cell aggregates (Kang et al., 2011). It has been shown that in conditions favoring adipogenesis, cells were usually associated with a cytoskeleton configuration composed of less organized and less-stiff actin cytoskeletons, fewer stress fibers and/or less focal adhesions (Mathieu et al., 2012; McBeath et al., 2004).

bFGFs promote adipogenesis of stem cells (Song et al., 2014). Substrates immobilized with bFGFs, exhibited more lipid accumulation than fibronectin-coated surfaces (Kang et al., 2012), which inhibited cell adhesion by using heparin to disrupt focal adhesions (Luo et al., 2008). In addition, surface chemistry affects adipogenic differentiation by influencing the activity of biological agents that induce adipogenesis as well (Lee et al., 2011). Recently, macromolecular crowding has been used to mimic the physiological crowding of the microenvironment and promote the expression of early and late adipogenic markers as well as adipocyte maturation (Ang et al., 2014).

5.2.2. Geometric regulation of adipogenesis—Cell shape influences adipogenesis as this characteristic affects cytoskeletal configuration. As mentioned previously, MSCs with spheroidal shape exhibited more adipogenic potential than protruded cells. Topographic micropatterning has been used to geometrically constrain the cell shape so that MSCs cannot spread, and therefore, adipogenic differentiation is enhanced (McBeath et al., 2004). Micropatterned squares, on which MSCs are confined, promote increased adipogenic differentiation when compared to micropatterned rectangles (Kilian et al., 2010). The effect of geometry on adipogenic potential has been further verified using different pentagonal symmetries, including a star shape (with concave edges and sharp points at the vertices), pentagon shape (with straight lines for the edges), and flower shape (with large convex curves along each edge). MSCs grown on micropatterns, ranging from star to pentagon to flower, show a decrease in contractile cytoskeleton (James et al., 2008) and an increase in percentage of differentiated adipocytes (Kilian et al., 2010). Additionally, microfabricated patterns have been used to generate 3D spheroid cultures to promote adipogenic

differentiation (Miyagawa et al., 2011, 2013; Wang et al., 2009). Furthermore, nanotopography has been used to regulate adipogenesis, which enhanced adipogenic differentiation by regulating the intracellular cytoskeletal network and stiffness (Ahn et al., 2014; Wang et al., 2012).

The effect of nanotopographical features on the differentiation of both human and rat MSCs has also been studied. Ahn et al. (2014) fabricated nanoposts composed of polyurethane with varying densities and examined how the changes amongst the various topographies contributed to adipogenic versus osteogenic differentiation of hMSCs. From this study, it was determined that adipogenic differentiation of hMSCs was biphasic with a clear relationship between the regulation of cell morphology (spreading) and the decision of cell lineage with a 2.4 μm post-to-post distance exhibiting maximal adipogenesis (Ahn et al., 2014). Osteogenesis occurred on both the flat control and the lowest nanopost density (5.6 μm post-to-post distance). To this point, Wang et al. (2012) examined how grooved topographies fabricated from polystyrene with varying width to depth ratios at the nanoscale affected the differentiation of rMSCs into osteoblasts, adipocytes and myoblasts. Adipogenesis was determined to be significantly ($p < 0.001$) amplified by all of the grooved surfaces compared with the flat substrate (Wang et al., 2012). However, osteogenesis was not significantly regulated by the grooved substrates in comparison with the flat control and myogenesis was enhanced by the grooved topographies but with a dependence on time and groove size. Overall, the in-depth analysis by these two research groups represents how the differentiation of stem cells, particularly in adipogenesis, is directed by the surfaces on which they are cultured, especially at the nanoscale.

5.2.3. Mechanical regulation of adipogenesis—Adipocytes and adipose tissue in vivo are physiologically exposed to mechanical stimuli such as tensile, compressive and shear stresses, caused by a weight-bearing force (Linder-Ganz et al., 2007; Slomka et al., 2009). Moreover, it has been demonstrated that adipocytes are mechanosensitive and mechanoresponsive (Shoham and Gefen, 2012). To be more specific, mechanical stimulations are capable of influencing the process of adipose conversion. As such, it has been reported that static stretching accelerates adipogenesis of mouse 3T3-L1 preadipocytes (Levy et al., 2012; Shoham et al., 2012). On the contrary, dynamic loading such as cyclic stretching inhibits adipogenesis of mouse 3T3-L1 preadipocytes through the MAPK/ERK pathway (Tanabe et al., 2004), of MSCs via the β -catenin signaling pathway (Sen et al., 2008, 2009, 2011; Case et al., 2010), and of human umbilical cord perivascular cells through TGF β 1/Smad signaling pathway (Turner et al., 2008). Additionally, both oscillatory and low frequency mechanical stimulations are able to suppress adipogenic differentiation (Khayat et al., 2012). Static compressive force also suppresses adipogenesis of human preadipocytes through COX-2 mediated PPAR γ 2 downregulation (Hossain et al., 2010). The inhibition of adipogenesis through mechanical stimulations has been observed not only in animal studies, but human as well (Shoham and Gefen, 2012a). In addition, mechanical cues have the potential to induce or enhance alterations in adipose function, e.g., lipolysis, fibro-inflammation (Pellegrinelli et al., 2014; Tanabe et al., 2008). Therefore, mechanical stimulations that cause increased cytoskeletal organization, can reduce adipogenesis (Au-Yeung et al., 2010; Pfeiler et al., 2008).

The biomechanical properties of primary adipocytes have been measured to be 0.9 ± 0.8 kPa (elasticity) and 0.61 ± 0.54 kPa (relaxing modulus) using atomic force microscopy (AFM) (Darling et al., 2008). To give perspective, the elasticity of adipocytes is much lower than osteoblasts (6.5 ± 2.7 kPa) as well as stem cells, e.g., ASCs (2.5 ± 1.2 kPa), MSCs (3.2 ± 2.2 kPa). In fact, the decrease in elasticity of hMSCs during adipogenic differentiation has been observed (Yu et al., 2010). Interestingly, AFM measurements determined the stiffness ratio of the lipid droplet in 3T3-L1 differentiated adipocytes to the nucleus to be 0.83 ± 0.14 and that of the lipid droplet to the cytoplasm ranging from 2.5–8.3. It indicates that the lipid droplets are stiffer than cytoplasm, and therefore, mechanically distort their intracellular environment (Shoham et al., 2014).

As a major component of the MSC microenvironment, the ECM elasticity regulates MSC lineage determination (Engler et al., 2006). Soft substrates (2–3 kPa) promote adipogenesis of adult stem cells (Parekh et al., 2011; Guvendiren and Burdick, 2012) even in the absence of adipogenesis induction cocktails (Young et al., 2013) and exhibit an elasticity similar to native adipose tissue (Comley and Fleck, 2010). Stiffer substrates, such as hydrogels with an elasticity of 59 kPa or 11–30 kPa, promote osteogenic differentiation of MSCs (Parekh et al., 2011; Shih et al., 2011) rather than adipogenic differentiation. It has also been shown that the elasticity during adipogenic differentiation of adult stem cells is controlled by the restriction of the cell aspect ratio (Young et al., 2013). To this point, stochastic modeling on the effect of mechanical stretching on preadipocyte monolayers has shown that in this state, cells exhibited enhanced growth and therefore adipogenesis (Shoham and Gefen, 2012b). Although the knowledge of these effects is very helpful for adipogenesis in general, it is not very clear how mechanical stimulations and mechanical properties of substrates affect brown adipogenesis not only in 2D but also in 3D. It is also important to remember that the mechanical attributes of the native tissue are influenced by its heterogeneous nature.

5.2.4. Co-culture to enhance adipogenesis—Adipogenesis is affected not only by mechanical properties, but also by neighboring cells. Adipose tissue in vivo is composed of multiple cell types, such as preadipocytes, differentiated adipocytes, stem cells, adipose stromal cells/fibroblasts, endothelial vascular cells, and perivascular support cells, embedded within an extensive 3D ECM network. Therefore, 3D multi-cellular culture has the potential to more efficiently recapitulate the complex stem cell and/or adipose tissue microenvironment to mimic the physiology and pathology of adipocytes (Hammoudi et al., 2012; Rinker et al., 2014; Sorrell et al., 2011).

As a whole, adipose tissue can secrete adipogenic differentiation factors (Wu et al., 2012). The paracrine interactions between adipocytes and preadipocytes are further confirmed by an in vitro microfluidic gradient chamber study, indicating that enlarged adipocytes could release adipogenic factors to induce the differentiation of preadipocytes in a size-dependent manner (Lai et al., 2012). Moreover, mature adipocytes derived from rat adipose tissue have been shown to promote preadipocyte differentiation, although the level of differentiation was lower than that induced by the conventional induction cocktail (Shillabeer et al., 1989). The effect of mature adipocytes on adipogenic differentiation of human preadipocytes was further amplified in 3D culture (Stacey et al., 2009). This co-culture effect has also been examined with mature adipocytes inducing adipogenic differentiation of bovine ASCs,

which might be mediated by Wnt signaling (Zhao et al., 2012). Although differentiation in this instance was to a lesser extent than that of an exogenous induction cocktail, a more homogeneous adipogenic differentiation response occurred. This has also been the case for the differentiation of ASCs into chondrocytes and osteoblasts co-cultured with the mature cell population, respectively (Zhao et al., 2012a). Purified human mature adipocytes incite ASC proliferation and adipogenic differentiation in vitro as well (Doornaert et al., 2012). On the contrary, it has been reported that co-culture with human mature adipocytes could not induce the differentiation of hASCs into adipocytes (Song et al., 2012) and even inhibited the differentiation of primary human preadipocytes (Janke et al., 2002). This may be due to the size and lipid content of the adipocytes being too low to produce an adequate amount of adipogenic differentiation factors (Lai et al., 2012).

The co-culture of hASCs with human umbilical vein endothelial cells (HUVECs) on 3D aqueous-derived silk scaffolds promoted adipogenic differentiation, exhibiting lipid accumulation and vascularization (Kang et al., 2009) as well as adipocyte function, including lipolysis and insulin sensitivity (Choi et al., 2011; Choi et al., 2010). Additionally, 3D co-culture with endothelial cells on porous silk protein matrices could maintain the structure and function of ASC-differentiated adipocytes for at least 6 months (Bellas et al., 2013). In this type of co-culture, there is a mutually enhanced effect between the adipocytes and endothelial cells, which mimics in vivo cell-cell interactions and supports improved adipocyte function, endothelial cell proliferation and capillary network formation (Aubin et al., 2015; Yao et al., 2013a). Moreover, an in vivo study demonstrated the long-term stability of volume and weight of the injected HUVEC-adipocytes within collagen/alginate microspheres, indicating that vascularization facilitates the formation, maturity, and maintenance of the adipose tissue (Yao et al., 2013b). Macrophages have also been shown to be a key player in neovascularization and adipogenesis as shown in vitro where macrophage depletion resulted in a lack of new vasculature and adipose tissue formation (Debels et al., 2013). Additionally, macrophages as well as neurons sustain adaptive thermogenesis via sympathetic nerve control (Harms and Seale, 2013).

6. Potential bioengineering approaches to 3D brown adipogenesis

6.1. Learning from white adipose tissue engineering

Although brown adipose tissue engineering is still in its infancy, the engineering of (white) adipose tissue in vitro and in vivo (referred as adipose tissue engineering) has provided the foundation for using this technique for bioengineering brown adipocytes, as summarized in Figure 4. Formation of adipocytes in a synthetic 3D microenvironment and/or co-culture has been accomplished using various techniques including microfibrous matrices, spheroid models, levitation culture systems, to name a few (Table 3). Biomaterials ranging from decellularized ECM from adipose tissue to inorganic substrates or organic polymers, have been used and shown to increase lipid accumulation and expression of adipogenic genes (Turner et al., 2012; Venugopal et al., 2012). Other modern techniques such as laser direct write (Dias et al., 2014) and 3D printing are starting to be used to form adipose tissue in vitro (Lee et al., 2014). The formation of 3D adipose in vitro provides a platform that can be used in conjunction with in vivo models to engineer adipose tissue.

The culturing of progenitors or stem cells in micro- or nanofabricated biomaterials such as collagen, fibrin glue, and porous sponge/silk protein, amongst other examples, has led to the engineering of adipose tissue in in vivo models (Table 4). hASCs cultured in decellularized adipose tissue microparticles not only grew more rapidly but formed more in vivo-like adipose tissue in subcutaneous nude mice (Wang et al., 2013). Longevity is also an advantage of these types of techniques, for example, adipose tissue constructs formed from porous sponge and silk were able to retain their volume for 18 months (Bellas et al., 2013a). The bioengineering of adipose tissue in this way shows great promise for future clinical applications involving the implantation of BAT.

6.1.1. Micro-/nanofibrous scaffolds for adipogenesis—Micro-/nanofibrous scaffolds have been used as adipogenesis culture systems in order to mimic the in vivo 3D tissue architecture. For example, electrospun PCL micro-/nanofibrous scaffolds support the differentiation of mouse preadipocytes and ESCs into mature adipocytes (Kang et al., 2007; Kang et al., 2005). PCL nanofibrous scaffolds have also been used to differentiate MSCs into adipocytes, representing the potential of this particular polymer scaffold for adipogenesis (Li et al., 2005). Other micro-/nanofibrous scaffolding materials that have been used for adipogenesis include bacterial nanocellulose, silk fibroin, poly(lactic-co-glycolic) acid (PLGA), glycol chitosan and chondroitin sulfate, amongst several others (Cheung et al., 2014; Krontiras et al., 2015; Mauney et al., 2007; Patrick et al., 1999). These studies represent the prospects for utilizing a synthetic scaffold in the micro- and nano regimes to induce 3D adipogenesis.

Although there is currently no report to show brown adipogenesis on nanofibrous matrices/scaffolds, these nanofiber-based matrices have considerable potential to be tuned to resemble the structural, biochemical, and mechanical properties of ECMs in a brown adipogenic microenvironment, leading to efficient brown adipogenesis.

6.1.2. Micropatterned surfaces for adipogenesis—Within the field of tissue engineering there have been concerns regarding uniformity and tuning mechanical properties of potential growth platforms. One method to address this issue is micropatterning. This technique has been done using biocompatible polymers such as PCL, that has been modified with nanowires in order to improve mechanical properties and serve as a pattern for proliferation and maintenance of ASCs (Trujillo and Popat, 2014). Poly (vinyl alcohol) (PVA) has also been micropatterned onto polystyrene plates to facilitate the adipogenesis of MSCs and to test the effect of cell density in this system (Lu et al., 2009). In this case, it was determined that the cell density did not affect adipogenic differentiation, contrary to previous reports. Wang et al. (2013) expanded on this by manipulating the sizes of the central dots within the micropattern as well as the protrusion lines in order to assess the spreading and protrusion of MSCs, and any effects on adipogenic differentiation. This group was able to observe single MSC adhesion and notice that areas with lower spreading promoted adipogenic differentiation as opposed to those with higher degrees of spreading. A less defined relationship was determined for cellular protrusion but generally, adipogenic differentiation was influenced with a smaller (30 μm) micropatterned feature (Wang et al., 2013).

As far as brown adipogenesis is concerned, the micro-/nanoscaled features to promote brown adipogenesis needs to first be identified, including the size, shape, and distance of the features to mimic the brown adipogenic microenvironment. In addition, the feature design needs to consider the restriction of stem cell spreading, and therefore, facilitate brown adipogenesis as well as resemble the biomechanical properties of brown adipocytes.

Although micropatterned surfaces have been shown to promote adipogenesis, it is imperative to introduce mimics of the ECM in order to effectively engineer a tissue. One approach could be the fabrication of micro-/nanopatterned structures followed by coating with electrospun nanofibers, or making micro-/nanopatterned structures on electrospun nanofibers. Alternatively, incorporating ECM mimics by infusing a cell-laden pattern or cells alone with a hydrogel is a promising approach to brown adipose engineering.

6.1.3. Implantation of 3D adipose tissue in vivo—Adipose tissue implantation is widely done because it is expendable and the largest tissue in the human body, allowing autologous transplantation to be easily achievable (Patrick, 2000). This is especially useful for procedures or conditions that result in the loss of adipose tissue such as correction of maxillofacial defects, mastectomy or tumor resection (Clauser et al., 2014; Patrick, 2000). The issue with transplantation, although autologous, is that the tissue undergoes a 40–60% reduction in graft volume resulting in deficient revascularization (Lee et al., 2000; Patrick et al., 1998). This has been attributed to the low viability and intolerance to ischemia of adipocytes as well as incomplete foundational blood supply following transplantation (Tanzi and Farè, 2009). At the microscale, there is more success with transplantation of adipocytes but at the macroscale, most of the cells are outside of the oxygen diffusion limit from blood vessels (Tanzi and Farè, 2009). Therefore, these obstacles shed light on tissue engineering as a potential alternative to traditional adipose tissue transplantation.

Injectable hydrogels have been developed to not only serve as fillers but also promote in vivo adipogenesis for adipose tissue engineering (Young and Christman, 2012). Examples include freeze-dried human ECM powder (Choi et al., 2009), crosslinked decellularized ECM (Wu et al., 2012), decellularized human adipose tissue microcarriers (Turner et al., 2012; Wang et al., 2013), fibrin glue (Aoyagi et al., 2011, 2012), HA/adipic acid dihydrazide (Shoham et al., 2013), collagen/alginate microspheres (Yao et al., 2012), HUVEC-adipocytes/collagen/alginate microsphere co-cultures (Yao et al., 2013b), and alginate/chitosan-nano fibrin composite hydrogels (Jaikumar et al., 2014). These examples demonstrate the suitability of injectable hydrogels for in vivo adipocyte delivery and adipose tissue regeneration.

6.2 Recreation and characterization of bioengineered 3D brown adipose tissue

As learned from bioengineering (white) adipose tissue in general, these various techniques mentioned hold great therapeutic potential. Therefore, it is appealing to bioengineer brown adipocytes in a similar fashion. A hydrogel culture system has great potential to serve as the foundation for bioengineering BAT, but it is important to remember the functional properties of the tissue need to be maintained. Characterization of 3D bioengineered BAT formulations will address this need and make therapeutic applications a reality.

6.2.1. The potential of a hydrogel approach to bioengineering 3D brown adipose tissue from pluripotent stem cells—

Hydrogels have been of great interest in the field of tissue engineering due to their similarities to the native ECM, biocompatibility, ability to form complex structures, and 3D properties (Tibbitt and Anseth, 2009; Geckil et al., 2010). Hydrogels, such as alginate, collagen, gelatin, fibrin, PEG-DA and HA, are attractive not only for engineering adipose tissue but also for increasing cell survival when implanted in vivo. It has been suggested that hydrogels might serve as an adipose-tissue-like material in terms of biocompatibility and mechanical behavior similar to the native adipose tissue (Shoham et al., 2013). With the knowledge of the biochemical and mechanical properties of brown adipocyte microenvironments, encapsulation of preadipocytes or stem cells in hydrogels during brown adipogenesis could provide a method for increasing cell density, reducing cytoskeletal organization and allowing efficient culture medium exchange that would permit the induction of brown adipogenic differentiation via an adipogenic cocktail.

More recently, a mixing-induced two-component hydrogel has been used to encapsulate cells and support cell growth, leading to enhanced adipogenic differentiation (Greenwood-Goodwin, et al., 2014). This particular hydrogel involved co-encapsulation of hASCs with alginate microspheres to deliver FGF1 and BMP4 for adipogenic differentiation. It is noticed that the dual-stage delivery of these factors in 3D increased UCP1 gene expression when compared to growth media alone, suggesting a brown-like adipogenic differentiation (Greenwood-Goodwin et al., 2014). A more straightforward encapsulation technique is the formation of alginate hydrogel microstrands using a microfluidic approach. Alginate is an intriguing material because it is a naturally occurring polysaccharide that has great potential to reconstruct the cell microenvironment in 3D. Alginate not only shares similar characteristics of chemical structure and biosynthesis of glycosaminoglycans (GAGs) (Smidsrod et al., 2001) but can also recapitulate a proteoglycan's ability to form a hydrogel with more than 95% of water molecules inside (Wee and Gombotz, 1998). This ability leads to the assertion that an alginate hydrogel can mimic the compressive strength component of the microenvironment. In addition, the negative charge of alginate allows it to be easily modified by collagen, laminin, fibronectin, polylysine, chitosan, gelatin, etc. (Dhoot et al., 2004; Lim and Sun, 1980; Mosahebi et al., 2003; Prang et al., 2006; Wang et al., 2006). The importance of these chemically modified alginate hydrogels are such that they have the ability to mimic the heterogeneous chemical structure of the ECM.

Although the chemical mimicry of alginate is pronounced, without biocompatibility it would be very difficult to use for tissue engineering. The biocompatibility of alginate is such that it has been employed and widely used for cell culture, tissue engineering and cell therapy (de Vos et al., 2006; Liu et al., 2012; Orive et al., 2003; Prang et al., 2006; Wang et al., 2006; Wee and Gombotz, 1998). In fact, alginate hydrogels are capable of forming layers on chips (Plouffe et al., 2009), microbeads, microcapsules (Lim and Sun, 1980; Wang et al., 2006), microstrands (Raof et al., 2011), microfibers (Onoe et al., 2013) and nanofibers (Bhattarai et al., 2006), and has the potential to provide versatile building blocks to encapsulate or assemble cells for tissue engineering and regenerative medicine purposes.

Our preliminary work utilizes the capacity of alginate as an encapsulation material by culturing mouse ESCs in alginate microstrands, with a microfabrication technique. An SU-8 filter of defined size and capillary action was initially used to create the microstrands. Mouse ESCs were cultured within the strands in both a liquid and gel interior microenvironment. The cells were shown to grow more freely and self-assemble within the liquid microenvironment, whereas cells within the gel system they exhibited a more branched structure (Raof et al., 2011). ESCs within the aqueous microstrands showed preference towards endoderm and mesoderm lineages based upon the compact microtissues formed (Raof et al., 2011). The gelled microstrands were shown to promote cells into an ectoderm and mesoderm lineage (Raof et al., 2011). The general conclusions from this work indicated that the interior conditions within the hydrogel microstrands affected the differentiation of the stem cells in terms of germ layer determination. Our unpublished results show the feasibility of differentiating pluripotent stem cells into brown adipocytes in hydrogel microstrands. Alginate encapsulation of bioengineered BAT could protect the tissue during this process while maintaining overall tissue shape. Therefore, an in vitro brown adipogenic differentiation of pluripotent stem cells within alginate hydrogel microstrands might provide useful insight into future clinical applications, especially in terms of in vitro assay development for the biological study of brown adipogenesis and drug screening, and in vivo implantation, as outlined in Figure 4.

6.2.2. Implications of bioengineered 3D brown fat depots in vivo—Animal studies in mice have indicated that when implanted with both brown and beige adipose tissue, they are less likely to become obese, and therefore, diabetic (Almind et al., 2007; Lowell et al., 1993). The potential implantation of bioengineered brown fat depots could provide a platform to either increase endogenous or introduce exogenous BAT. Endogenous BAT could refer to classical BAT or even white to brown conversion (beige). The latter has been a major point of interest in current research, with a number of small molecules, microRNAs, and transcription factors determined to contribute to “browning” of WAT (Cereijo et al., 2014; Jimenez et al., 2013; Moisan et al., 2014). This would be especially useful for individuals who need to regulate energy balance, as there is typically less BAT mass and therefore function (Cypess et al., 2009).

The high metabolic demand of BAT highlights the importance of vascularization. Therefore, to promote the survival and maintain function of implanted 3D brown fat depots in vivo, vascularization will be necessary. According to in vivo adipose tissue engineering, the use of adipose tissue or adipose-derived stem cells (Yoshimura et al., 2011) as well as in combination with 3D scaffolds, tissue engineering chambers or adipose tissue extracts, could promote vascularization of tissue engineered adipose implants. For example, freshly isolated lipoaspirate seeded on silk scaffolds forms denser pockets of large, mature adipocytes and attracts more vascularization than scaffolds alone or hASC-scaffold constructs (Bellas et al., 2013a). In support of this point, rabbit adipose tissue grown on electrospun nanofibers exhibits angiogenic potential in vitro (Panneerselvam et al., 2012). 3D cell-scaffold constructs containing human adipose tissue-derived precursor cells or stromal-vascular fraction (SVF) has been shown to enable angiogenesis and adipose tissue formation in vivo (Wiggenhauser et al., 2012; Wittmann et al., 2015). Additionally, cell-free or cell-containing

human adipose tissue extracts could promote adipogenesis and angiogenesis of hASCs in vitro (Sarkanen et al., 2012) and/or in vivo (Li et al., 2014a), respectively.

6.2.3. Evaluation of 3D bioengineered adipose tissue—There are both challenges and opportunities in monitoring the behavior and function of bioengineered adipose tissue in vitro and in vivo. New approaches and existing technologies have been developed or extended to non- or less-invasive detection of adipose tissue, which could apply to the assessment of 3D adipocyte-scaffold constructs. Adipogenic differentiation of MSCs has been monitored by impedance profiling that exhibits shallower initial slopes and eventually declining profiles compared to proliferating MSCs or osteogenic MSCs, corresponding to more compact adipocytes with lipid droplets (Angstmann et al., 2011). Considering that vitamin D3 and its metabolites are located in adipocyte lipid droplets and that lower levels are observed in obesity, measurement is made possible using time-of-flight secondary ion mass spectrometry (TOF-SIMS) (Malmberg et al., 2014). In addition, multimodal microscopy that combines coherent anti-Stokes Raman scattering (CARS) microscopy and multiphoton imaging can be used in the study of lipid droplets. By tuning the Stokes and pump beams (1064 and 817 nm, respectively) to excite the Raman peak at 2845 cm^{-1} (663 nm), corresponding to the CH_2 stretch in lipids, it is possible to monitor lipid accumulation, rounded morphology changes and increase in cell size (Mouras et al., 2011; Mouras et al., 2012). The calculated volumes of the lipid droplets are in agreement with total intracellular triglyceride content, indicating the coupling of adipocyte metabolism with lipid droplet morphology (Sims et al., 2014). Magnetic resonance (MR) elastography is also an interesting technique to measure local mechanical properties of a tissue through shear stiffness maps generated by coupling a sonic mechanical actuator with the tissue of interest and recording the shear wave propagation with an MR scanner (Curtis et al., 2012). Moreover, magnetic resonance imaging (MRI) has been used to visualize soft adipose tissues and assess graft volume retention, resorption rates and vascularization of engineered adipose tissues in vivo (Proulx et al., 2015; Torio-Padron et al., 2011). Additionally, micro-computed tomography (CT) can be used for 3D reconstruction through cubic-spline interpolation and volumetric analysis of fat graft in vivo (Chung et al., 2013). Most of these technologies are developed to characterize WAT or adipose tissue in general, yet to evaluate the brown adipocyte function, noninvasive approaches to assess the mitochondrial function are in need.

As mentioned previously, ^{18}F FDG PET-CT imaging has been used to evaluate the glucose uptake of BAT in vivo, and is the method that gained momentum for brown adipogenesis in adult humans (Cypess et al., 2009). Although the analysis of thousands of whole body scans and samples from surgical procedures has led to this vital conclusion, invasive means were taken to get there. However, one potential noninvasive method to assess BAT function is by taking advantage of the heat production asserted by brown adipocytes. Sato et al., (2014) developed a noninvasive biomaterial microcantilever system that can measure changes in brown adipocyte temperature from the bending of the microcantilever upon norepinephrine stimulation. This process is capable of measuring the increase in the cell temperature of $<1\text{ K}$ with a slow increase in temperature over several hours. During this time, the cells consumed fatty acids and lost their spherical shape, consistent with lipid droplet

decomposition (Sato et al., 2014). The use of this technique represents the importance of harnessing the heat expenditure native to brown adipocytes and the potential for treating obesity and other metabolic disorders.

7. Conclusions and perspective remarks

Although there is great potential for BAT to correct the energy imbalance in obese individuals, there are still unmet needs in clinical applications. The main issue with using free fat is that upon transplantation, cell necrosis often occurs causing poor formation of microvascular networks and also poor graft resorption (Patrick, 2000; Tanzi and Farè, 2009). It has been shown that an optimal seeding density exists for enhancing the survival of human free fat grafts. For example, fat grafts containing platelet-rich plasma and $10^5/\text{ml}$ ASCs retained significantly higher fat volume and dramatically increased capillary formation (Li et al., 2014). Encapsulation of preadipocytes or stem cells during brown adipogenesis could provide a method for avoiding necrosis, increasing local cell density and thereby facilitate self-assembly. Not to mention, an encapsulation technique could provide a more efficient method for BAT implantation. In particular, hydrogel microstrands, such as those made from alginate hydrogels, have great potential to differentiate pluripotent stem cells into functional brown adipocytes, providing useful insight into understanding of brown adipocyte biology, drug discovery and future clinical applications.

The use of 3D culture of pluripotent stem cells for BAT tissue engineering has several advantages. As a whole, stem cells represent a therapeutic arena that could lead to personalized medicine (Guzman and Allan, 2014; Kim et al., 2014; Li et al., 2014). The 3D adipogenesis platform has the potential for increasing the differentiation efficiency of stem cells by enhancing self-assembly mechanisms and could thus serve as an individualized anti-obesity drug-testing platform for personalized medicine development. Current challenges with 3D adipogenesis of pluripotent stem cells include: achieving high differentiation efficiency, maintaining the tissue structurally for long periods of time, conserving phenotypic expression, effectively mimicking tissue porosity, and vascularization, and integration with the 3D constructs in vivo.

By addressing these challenges in adipogenesis, the design of 3D culture models for bioengineering brown adipogenesis, should consider the biochemical and biomechanical cues within the native tissue as well as incorporate neighboring cells that originate in brown adipogenic microenvironments. As such, there is a need to efficiently mimic the ECM and therefore mechanical properties of brown adipocytes and the corresponding tissue. As a heterogeneous tissue, 3D co-culture systems are of high interest not only for investigating brown adipogenesis but also towards the study of brown-white adipocyte interactions and interconversion, leading to in vivo therapeutics.

There are several major challenges to be overcome before this vision is realized. The challenge for the development of 3D brown adipocyte constructs follows: 1) high efficiency and purity of brown adipogenic differentiation of stem cells or preadipocytes, 2) brown fat survival and stability, 3) selection of biomaterials (*e.g.*, natural versus synthetic scaffolds, chemistry, geometrical properties, biomechanical properties), and 4) construction of a 3D

adipogenic microenvironment to maintain brown fat function. Additional challenges of *in vivo* implantation of 3D brown adipocyte constructs include survival, stability, and vascularization as well as innervation of BAT by the sympathetic nervous system. Overcoming these challenges will lead to functional 3D brown adipocytes, which have the potential to remedy obesity and other metabolic disorders, perhaps by individualized tissue engineering since these diseases vary from patient to patient. With advancements in brown adipocyte biology, stem cell adipogenesis, biomaterials and soft tissue engineering, 3D functional brown adipocytes, which recapitulate the form and function of brown fat *in vivo*, will be constructed as an *in vitro* model for assay development and drug screening or *in vivo* implants for therapy.

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Abbreviations

ASCs	Adipose-derived stem cells
AFM	atomic force microscopy
bFGFs	basic fibroblast growth factors
BMI	body mass index
BMP	bone morphogenetic protein
BAT	brown adipose tissue
C/EBP	CCAAT/enhancer-binding protein
cAMP	cyclic adenosine monophosphate
COX-2	cyclooxygenase-2
EBs	embryoid bodies
ESCs	embryonic stem cells
ECM	extracellular matrix
FGF	fibroblast growth factor
FDG	fluorodeoxyglucose
PET-CT	positron emission tomography—computed tomography
FOXC2	forkhead box protein C2
hESCs	human embryonic stem cells
hASCs	human adipose-derived stem cells
hMSCs	human mesenchymal stem cells
IBMX	3-isobutyl-1-methylxanthine

iPSCs	induced pluripotent stem cells
KCNK3	potassium channel K3
KSR	KnockOut Serum Replacement
MPCs	mesenchymal progenitor cells
MRI	magnetic resonance imaging
MSCs	mesenchymal stem cells
MTUS1	mitochondrial tumor suppressor 1
Myf5	myogenic factor 5
PAI-1	plasminogen activator inhibitor 1
PPAR γ	peroxisome proliferator-activated receptor γ
PCL	poly caprolactone
PRDM16	PR domain containing 16
PGC1α	peroxisome proliferator-activated receptor γ coactivator 1 alpha
PVA	poly (vinyl alcohol)
RA	all- <i>trans</i> -retinoic acid
SVF	stromal-vascular fraction
UCP1	uncoupling protein 1
WAT	white adipose tissue

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Figure 1. Schematic representation of the morphology and anatomical location of brown, beige and white adipocytes

Brown adipocytes are located in the neck, supraclavicular, perirenal/adrenal, and paravertebral region around major blood vessels. Beige adipocytes are located in most if not all locations of WAT, yet there is debate as to whether some human supraclavicular and neck depots are beige or brown fat. White adipocytes are found throughout the body but are most densely located subcutaneously as well as around the internal organs of the chest and abdomen. Other locations include but are not limited to pericardial, perirenal, gonadal, intramuscular, omental, mesenteric regions of the body.

Figure 2. Schematics of brown, beige, and white adipogenesis of pluripotent stem cells
 Pluripotent stem cells first differentiate into mesenchymal stem cells that can become CD24⁺ and PPAR γ ⁺ white preadipocytes and Myf5⁺ brown preadipocytes. White preadipocytes can mature into white adipocytes with exposure to PPAR γ , BMP4, BMP2, and FGF10, to name a few factors. Brown preadipocytes can become brown adipocytes through treatment with PRDM16, FGF16, FGF19, and BMP7, among many more. White adipocytes can also form beige adipocytes upon further induction by factors including PPAR γ , PRDM16, PGC1 α , FGF21, irisin, apelin, Cox2, mir196a, and mir28.

Figure 3.

The brown adipogenic microenvironment comprised of ECM, soluble factors and neighboring cells. BAT secretes soluble factors in the microenvironment, such as FGF2, IGF1, VEGF, and NGF, etc. Stem cells and brown preadipocytes can be differentiated into brown adipocytes through factors including but not limited to BMP7, FGF16 and FGF19. Adipose stromal cells/fibroblasts can secrete angiogenic and anti-apoptotic factors (e.g., VEGF, FGF2, IGF1) to promote cell proliferation and increase brown adipocyte density. Additionally, brown adipocytes require vascularization and sympathetic innervation to maintain functionality, which can be made possible by interacting with endothelial vascular cells through VEGF and with neurons and immune cells through NGF.

Figure 4.

Approaches to bioengineering brown adipocytes. By culturing stem cells within biomaterials, they can be differentiated into 3D brown adipose tissue. Potential biomaterials include nanofibers, nanofabricated scaffolds and hydrogels, which have tunable biochemistry, topography, and biomechanics similar to native brown adipose tissue. This 3D brown adipocyte system could be used for *in vitro* assay development for biological studies and drug discovery, as well as *in vivo* transplantation.

Table 1

Comparison among Brown, Beige and White Adipocytes

Features	Brown Adipocytes	Inducible Brown (Beige) Adipocytes	White Adipocytes
Development	Before birth	After birth when exposed to cold or β 3-adrenergic factors	Shortly after birth
Anatomical location	Cervical-supraclavicular, perirenal/adrenal, paravertebral regions around major vessels	Supraclavicular, most locations of white adipocytes and intramuscular fat	Subcutaneous, visceral, pericardial, perirenal, intramuscular, gonadal, mesenteric, and omental fat
Mitochondria volume	Abundant	High	Few
Mature lipid droplet	Multilocular	Unilocular/Multilocular	Unilocular
Nucleus position	Central	Central	Peripheral
Signature biological markers	UCP-1, EVA1, PGC-1 α , DIO2, PRDM16, ZIC1, and EBF3	Cd137, Tbx1, TMEM26, CITED1, and SHOX2	Leptin, adiponectin, lipoprotein lipase (LPL), and resistin
Tissue microenvironment	Highly vascularized with high density of noradrenergic nerve fibers	Increased vascularization and innervation	Surrounded by loose connective tissue Some vascularization and innervation
Central function	Energy dissipation through heat	Adaptive thermogenesis	Energy storage, endocrine regulation

Table 2**Stem Cell Differentiation into Brown Adipocytes in vitro**

Stem Cell Population	Differentiation Scheme	Results	References
Mouse MSCs	1 C3H10T1/2 cell growth with BMP7 until confluence (3 days)	• Exhibited multilocular lipid formation and UCP1 expression;	Tseng et al., 2008
	2 Brown adipocyte differentiation: cells exposed to adipogenic cocktail for 48 hours and then placed into growth medium containing insulin and T3 (4–5 more days)	• Mitochondrial biogenesis indicated by PGC1 α , NRF1, TFAM, and cytochrome C expression at expected timepoints.	
hASCs	1 hMADS-2 cell growth with media containing hFGF2 until confluence	• Increased UCP1, CIDEA, and CPT1B expression and increased total respiration and oligomycin-sensitive respiration	Elabd et al., 2009 Mattsson et al., 2011 Pisani et al., 2011
	2 Brown adipocyte differentiation: 2 days post-confluence cells treated with an adipogenic cocktail (3 days) and then treated with a medium containing rosiglitazone with dexamethasone and IBMX removed (up to 17 more days)	• Increased UCP1 expression in response to isoproterenol and CL316243 (β - and β 3-agonists, respectively) • Responsive to β 1 and β 3 agonists but not β 2	
hESCs	1 EB formation: hESCs + growth medium in suspension with growth medium (7 days)	• More greatly induced UCP1 by PPAR γ 2-C/EBP β and PPAR γ 2-C/EBP β -PRDM16 than C/EBP β -PRDM16	Ahfeldt et al., 2012 Lee and Cowan, 2014
	2 Mesenchymal progenitor cell (MPC) formation: replated EB's + growth medium until confluency (5 days) and then replated + MPC growth medium. MPCs were split 1:3 prior to further differentiation.	• Multilocular lipid accumulation in programmed brown adipocytes in both PPAR γ 2-containing transcription factor combinations	
	3 Brown adipocyte formation: lentiviral transduction of MPCs containing combinations of PPAR γ 2, C/EBP β , and PRDM16 + adipogenic differentiation medium with (14 days) and without doxycycline (until day 21 or later)	• Exhibited functional properties with high oxygen consumption and extracellular acidification rates	
hiPSCs	1 Sphere formation: hiPSCs + differentiation medium + hematopoietic cytokine cocktail I (8 days)	• Induced brown adipocyte differentiation by hematopoietic cocktail regardless of BMP7	Nishio et al., 2012
	2 Brown adipocyte formation: replated spheres + differentiation medium + hematopoietic cytokine cocktail II (including BMP7 for several days)	• Formed multilocular lipid droplets • Increased UCP1 and PRDM16 expression upon β -adrenergic receptor agonist	
hiPSCs	1 EB formation: hiPSCs in suspension (10 days, with and without RA added from days 3–5)	• Increased UCP1, Dio2, and PGC1 α expression upon β -adrenergic stimulation	Cedex et al., 2014
	2 MSC formation: replated EB outgrowths + mesenchymal cell growth medium (7 days)	• Resulted in low UCP1 expression by RA treatment prior to brown AP formation	
	3 Adipocyte progenitor (APs) formation: collection and expansion of cells being able to form clones 10 days after replating	• Displayed higher clonogenic potential by brown APs than white APs	
	4 Brown AP formation: APs transduced with C/EBP β /LAP-expressing	• Enriched Pax3 expression in brown APs compared with white APs	

Stem Cell Population	Differentiation Scheme	Results	References
	retroviral vector + induced with adipogenic medium		

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Table 3

Construction of 3D adipose tissue

Methods	Biomaterials	Cell types	Major results	References
Microfibrinous matrix	Polyethylene terephthalate	Mouse 3T3-L1 preadipocytes	Acquired morphology and biological characteristics of mature adipocytes	Kang et al., 2005
3D levitation adiposphere co-culture system	Magnetic nanoparticles	Mouse 3T3-L1 preadipocytes and bEND.3 endothelial cells	Enabled 3D lipogenesis and vascularization; Recapitulated in vivo-like cellular interactions	Daquinag, Souza, & Kolonin, 2013
Surface-tethered spheroid model	Elastin-like polypeptide (ELP) and a synthetic polymer, polyethyleneimine (PEI)	Mouse 3T3-L1 preadipocytes	More sensitive modeling technique than 2D	Turner et al., 2014
Hydrogel	Collagen	Primary preadipocytes	Remained confined within the matrix and intact during biochemical analysis	Daya et al., 2007
Hydrogel/3D co-culture	Diacrylated poly(ethylene glycol) (PEG-DA) and methacrylated hyaluronic acid (HA-MA)	Human preadipocytes and adipocytes	Significantly elevated adipogenic marker expression in 3D compared to 2D; and stimulated greater adipogenesis under co-culture conditions	Stacey et al., 2009
Hydrogel	Collagen matrix and gelatin sponge	Uncultured human adipose SVF	Confirmed lipid accumulation in mature adipocytes and marker gene expression (GAPDH, PPAR- γ , and LPL)	Lin et al., 2011
Custom-made scaffolds	PLGA	hMSCs	Enhanced adipogenic differentiation by bFGF	Neubauer et al., 2005
Hydrogel	PEG-DA hydrogel	hMSCs	Retained predefined shape and dimensions for in vivo soft tissue augmentation	Alhadlaq et al., 2005
Hydrogel	Gelatin hydrogel	hMSCs	Accumulated lipid droplets and increased GAPDH activity	Hong et al., 2005
Hydrogel	Collagen	hMSCs	Long-term survival and differentiation into adipocytes and osteoblasts	Neuss et al., 2008
Hydrogel	Collagen	Marmoset bone marrow-derived MSCs	Generated dense and evenly distributed fat cells that accumulated lipid droplets and expressed adipogenic genes	Bernemann et al., 2011
Hydrogel	Decellularized human placenta and crosslinked hyaluronan (XLHA) gels	hASCs	Enhanced 3D adipogenesis in non-adhesive XLHA gels	Flynn et al., 2007

Methods	Biomaterials	Cell types	Major results	References
3D co-culture	Silk	hASCs and HUVECs	Exhibited lipid accumulation and vascularization	Kang et al., 2009
3D co-culture	Silk	hASCs and endothelial cells	Maintained adipose-like outcomes for over 6 months	Bellas et al., 2013
Native ECM scaffolds	Decellularized adipose tissue	hASCs	Provided an inductive microenvironment for adipogenesis without the need of exogenous induction	Flynn, 2010
Microcarriers	Decellularized ECM from adipose tissue	hASCs	Exhibited high level of intracellular lipid accumulation and adipogenic gene expression (PPAR γ , C/EBP α , and LPL) and GPDH activity	Turner et al., 2012
3D spheroid culture	—	ASCs	Differentiated into adipocytes in a microtissue model	Naderi et al., 2014
Hydrogels	Human platelet-poor plasma, alginate/fibrin gel/collagen sponge	hASCs	Showed highest expression of adipogenic markers and VEGF in alginate	Girandon et al., 2011
Hydrogel	Alginate, alginate/gelatin	hASCs	Obtained high ratio of evenly distributed adipocytes	Yao et al., 2012
Hydrogel	Fibrin	hASCs	Exhibited higher number of blood vessels, less severe inflammatory response and higher level of adipocyte gene expression than scaffold-free spheroids	Verseijden et al., 2012
Hydrogel	Adipose-derived matrix (ADM)	ASCs	Differentiated into adipocytes with high efficiency (>90%) in vitro and exhibited long-term adipogenesis in vivo (8 weeks)	Poon et al., 2013
Hydrogel	Gelatin/HA	Porcine ASCs	Demonstrated good proliferation and adipogenic differentiation in vitro and in vivo adipose tissue and new capillary formation	Chang et al., 2013
Hydrogel	Gelatin, alginate, and polyacrylamide (G-A-PAA)	hASCs	Accumulated lipid droplets and expressed perilipin	Dinescu et al., 2014
Hydrogel	Alginate	hASCs	Increased lipid accumulation, upregulation of key markers of brown-like adipocytes	Greenwood-Goodwin et al., 2014

Methods	Biomaterials	Cell types	Major results	References
Biphasic calcium phosphate (BCP)	Hydroxyapatite and α -tricalcium phosphate	Rat ASCs	Presented multilocular adipocyte-like cells in vitro and	Venugopal et al., 2012
Lipid templating porous scaffolds	PLGA	Rat adipose-derived stromal cells	Supported cell proliferation and adipogenic differentiation	Ambrosch et al., 2012
3D hollow fiber-based bioreactor	Poly(ether sulfone) (PES) capillary systems	hASCs	Exhibited similar expression of mature adipocyte marker of <i>FABP4</i> to the native fat tissue; Feasibility of testing the metabolic activity and insulin sensitivity of 3D fat	Gerlach et al., 2012
3D printing	PCL and PEG	ASCs	Demonstrated in vitro tissue formation from the separately printed chondrocytes and adipocytes for ear regeneration	Lee et al., 2014
Aligned electrospun nanofibers	PCL	hASCs	Increased lipid accumulation and sensitivity in insulin-stimulated glucose uptake	Brännmark et al., 2014
Electrospun nanofibers	PCL	Mouse ESCs	Exhibited adipocyte morphology, marker expression and function	Kang et al., 2007

Table 4

Examples of in vivo adipose tissue engineering

Cells	Biomaterials	Model systems	Results	References
hASCs	Collagen	Grafted nude mouse model	Improved cell survival	Mojallal et al., 2011
Ceiling culture-derived adipocytes	Fibrin glue	Subcutaneous mouse model	Increased cell survival and secretion of transgene product	Aoyagi et al., 2012
Human adipose tissue-derived stromal cells with or w/o HUVECs	Collagen microcarriers/fibrin matrix	Implanted under the skin of SCID mouse model	Led to functional stable vascular networks by co-transplantation of HUVEC with no significantly change the fat tissue volume	Frerich et al., 2011
ASCs isolated from subcutaneous fat	Porous PLGA scaffolds	Rabbit dorsal laminectomy model	Formed a continuous linear adipose tissue regenerated along the spinal cord at 24 weeks	Xu et al., 2012
hASCs and rat ASCs	Decellularized human adipose tissue microcarriers	Subcutaneous Wistar rat model	Confirmed injectability and stable volume retention over 28 days; Enhanced cellularity and angiogenesis	Turner et al., 2012
hASCs	Decellularized human adipose tissue extract microparticles	Subcutaneous nude mice model	Proliferated faster and formed more in vivo-like human fat tissue than on small intestine submucosa microparticles	Wang et al., 2013
Freshly isolated lipoaspirate	Porous sponge silk protein matrix	Adult, male athymic T-cell deficient RH-rnu rat model	Lipo-silk constructs regenerated subcutaneous adipose tissue and retained the original implanted volume for 18 months	Bellas et al., 2013