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3D brown adipogenesis to create "Brown-Fat-in-Microstrands"

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ABSTRACT

The ability of brown adipocytes (fat cells) to dissipate energy as heat shows great promise for the treatment of obesity and other metabolic disorders. Employing pluripotent stem cells, with an emphasis on directed differentiation, may overcome many issues currently associated with primary fat cell cultures. In addition, three-dimensional (3D) cell culture systems are needed to better understand the role of brown adipocytes in energy balance and treating obesity. To address this need, we created 3D "Brown-Fat-in-Microstrands" by microfluidic synthesis of alginate hydrogel microstrands that encapsulated cells and directly induced cell differentiation into brown adipocytes, using mouse embryonic stem cells (ESCs) as a model of pluripotent stem cells, and brown preadipocytes as a positive control. Brown adipocyte differentiation within microstrands was confirmed by immunocytochemistry and qPCR analysis of the expression of the brown adipocyte-defining marker uncoupling protein 1 (UCP1), as well as other general adipocyte markers. Cells within microstrands were responsive to a β-adrenergic agonist with an increase in gene expression of thermogenic UCP1, indicating that these "Brown-Fat-in-Microstrands" are functional. The ability to create "Brown-Fat-in-Microstrands" from pluripotent stem cells opens up a new arena to understanding brown adipogenesis and its implications in obesity and metabolic disorders.

1. Introduction

The thermogenic capacity of brown adipose tissue (BAT) has changed the general conception of fat [1,2]. Originally, BAT remained "under the radar", as it was primarily associated with rodents, hibernating animals, and human infants where activated BAT is responsible for nonshivering thermogenesis during cold exposure [3] and diet-induced thermogenesis [4,5]. However, BAT has become redefined by the recent confirmation of its existence and physiological significance in human adults [6–14]. This rediscovery has led to an influx of research concerning the role of BAT on the regulation of metabolic thermogenesis and thereby body fat content and triglyceride clearance [15,16]. Moreover, it is the innate ability of BAT that generates heat via a tightly controlled and extremely energy-expensive process. As a result, BAT provides an enormous impact on energy balance, which presents a clear therapeutic target, especially for treating obesity and related metabolic disorders, such as type 2 diabetes and non-alcohol hepatic steatosis [17–20].

The main cause of obesity, and thus obesity-related disease, is an energy imbalance in which energy intake exceeds energy dissipation [6,17,18]. Thus, it is critical to understand adipocyte biology for the development of potential obesity treatment strategies. During the development of obesity, adipocytes can increase in size (hypertrophy) or in amount (hyperplasia) [21,22]. Both cell conditions are known to occur in adolescents. However, adipocyte turnover is maintained at a steady state in adults [22]. The two main types of adipose tissue are white and brown adipose tissue [23]. White adipose tissue (WAT) is composed of unilocular white adipocytes, functions to store energy in the form of triglycerides, and acts as a vital endocrine and immune organ [24–27]. On the contrary, BAT is composed of multilocular brown adipocytes containing abundant mitochondria, and is specialized to expend energy as heat by uncoupling the synthesis of ATP from the electron transport chain with its unique mitochondrial membrane embedded protein, uncoupling protein 1 (UCP1) [24,28–32]. A new type of thermogenic adipocytes, known as beige, brite (brown in white), or inducible brown adipocytes, have been recently identified within white fat depots, named as such because they can be induced into brown-like adipocytes [9,33–38], thereby representing the plasticity of adipocytes [39]. Brown and beige fat can be activated by

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stimulation through cold exposure, receptor activation, exercise, hormone treatment (e.g., irisin, FGF21) or microRNA networks [40–54]. Similar to BAT, beige adipocytes express UCP1 and possess thermogenic capacity upon induction [18,36,55,56].

Although there is great potential for BAT to correct the energy imbalance in obese individuals, there are still many challenges to overcome prior to clinical applications. These include the need for an adequate cellular model system of human adipose tissue, as well as an effective implantation method. Although primary culture and differentiation of human adipocyte precursors have been used to understand adipocyte biology, these cells are difficult to obtain, culture, and expand *in vitro* [1], and fail to fully recapitulate human adipocyte development [57] and metabolic processes [58]. Pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells, provide a good model system for understanding early events in development [59–61] as well as an unlimited source of white, brown, and beige adipocytes [25,62–64]. The feasibility of generating brown or white adipocytes from human pluripotent stem cells has been demonstrated with up to 85–90% differentiation efficiency through cellular programming and transplantation techniques [25,58]. However, this approach includes multiple steps and relies on transferring exogenous genes to derive adipocytes from pluripotent stem cells. For instance, human pluripotent stem cells are first differentiated into mesenchymal progenitor cells through embryoid body (EB) formation, followed by replating of EBs on gelatin-coated tissue culture dishes. Then, these mesenchymal progenitor cells are replated again and transduced with a lentivirus constitutively expressing the regulator genes of white or brown adipogenesis, respectively, followed by the addition of adipogenic factors such as insulin, dexamethasone, and rosiglitazone. In order to differentiate human pluripotent stem cells into functional, classic brown adipocytes without gene transfer, a specific hematopoietic cytokine cocktail has been used [63,65]. Differentiation in this manner also includes the formation of EB-like spheres as the very first step, and replating of these spheres on gelatin-coated tissue culture plates thereafter. Taken together, data from these techniques suggest that it would be beneficial to recreate a three-dimensional (3D) microenvironment for pluripotent stem cell differentiation and adipogenesis *in vitro* [66], including BAT formation. Additionally, although BAT transplantation has been demonstrated for decades [67], cell necrosis often occurs upon transplantation of free fat, resulting in poor formation of microvascular networks and graft resorption [68,69]. Altogether, there is a great need for a 3D culture system that could recreate the microenvironment for BAT differentiation from pluripotent stem cells, recapitulating BAT function during *in vitro* culture, and provide a new vehicle to improve the stability and engraftment efficiency during *in vivo* BAT transplantation.

We envision that cell encapsulation in alginate hydrogel microstrands could offer an effective 3D culture solution to address the needs for BAT differentiation and transplantation. Alginate is an FDA-approved biomaterial that has been demonstrated to be safe for drug delivery, stem cell culture, tissue engineering, and cell therapy [70–73]. The long tubular structure and small diameter (200 μm) of alginate hydrogel microstrands can easily overcome the diffusion limitation that challenges the use of hydrogel microbeads for cell implantation [74], which allows for more efficient signaling, nutrient and oxygen exchanges, and support for high cellularity of stem cells grown in the tubular structure [75,76]. Additionally, these microstrands are easy to be handled for *in vivo* delivery by injection or implantation while maintaining their structural integrity. Moreover, alginate hydrogel microstrands exhibit great potential for reconstituting intrinsic morphologies and functions of living tissues [77,78]. The current approaches to fabricate hydrogel microstrands include utilizing coaxial flow and a microfluidic chip [79], flowing through a microfabricated SU-8 filter by a variety of techniques, including capillary force [75,76], wet spinning [80], and composite techniques [81]. Here, we present a new microfluidic approach for cell encapsulation in alginate hydrogel microstrands, by simply driving an alginate solution to flow consistently into a calcium solution.

In this study, we create "Brown-Fat-in-Microstrands" by encapsulating brown preadipocytes and pluripotent stem cells in 3D alginate hydrogel microstrands, and directly differentiating them into functional brown adipocytes. Mouse embryonic stem cells (ESCs) are used as model of pluripotent stem cells to test the feasibility of 3D brown adipogenesis in alginate microstrands. Mouse WT-1 brown preadipocytes are also grown within the same system to serve as a positive control. We culture cells within the alginate hydrogel microstrand system, and then expose them to a brown adipogenic differentiation scheme. We then assess the expression of characteristic brown adipogenesis markers, compare to 2D differentiation, and test for functional responsiveness. This bioengineered "Brown-Fat-in-Microstrands" has great potential to serve as an *in vitro* culture system for understanding brown adipogenesis, an *in vitro* assay for testing the efficacy of treatment, and as a stable, functional brown fat depot for *in vivo* implantation.

2. Materials and methods

2.1. ES cell culture

The mouse CCE ES cell line was provided by StemCell Technologies, Inc. (Vancouver, Canada) [82,83]. Cells were cultivated in gelatin-coated tissue culture flasks and maintained in an undifferentiated state using maintenance medium. This maintenance medium consisted of Dulbecco's Modified Eagle's Medium (DMEM 4.5 g/l D-glucose), enhanced with 15% (v/v) fetal bovine serum (FBS), 10 ng/ml murine recombinant leukemia inhibitory factor (LIF; StemCell Technologies), 0.1 mM non-essential amino acids, 0.1 mM monothioglycerol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, and 1 mM sodium pyruvate (Sigma–Aldrich, St Louis, MO).

2.2. WT-1 preadipocyte culture

Murine WT-1 preadipocytes (Joslin Diabetes Center, Harvard Medical School Affiliate, Boston, MA) were cultured following a previously established protocol [84,85]. Briefly, WT-1 preadipocytes were propagated in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. When the cells were ready to be split for experimentation, 0.1 μg/mL insulin and 10^{-8} μM T3 were added to the maintenance medium to begin the differentiation process on day 1 (this medium will be referred to as differentiation medium). To induce differentiation into brown adipocytes, at 4-day postconfluence, cells were treated with DMEM containing 10% FBS, 125 μM indomethacin, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 0.1 μg/ml insulin, and 10^{-8} μM triiodothyronine (T3) (Sigma–Aldrich). The cells were incubated in this induction medium for 5 days, and then refed with the differentiation medium on days 6 and 8 to be ready for characterization by day 10.

2.3. Fabrication of alginate microstrands containing ESC and WT-1 cells

One million mouse ESCs were suspended in 1 mL of 1.5% alginate (made from sodium alginate from brown algae with a viscosity of 100–300 cP in 2% solution at 25 °C, Sigma–Aldrich) in physiological saline solution at 37 °C. This ESC-alginate suspension was then loaded into a syringe in a pump set to dispense 0.5 mL/h. A