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Long term perfusion system supporting adipogenesis

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ABSTRACT

Adipose tissue engineered models are needed to enhance our understanding of disease mechanisms and for soft tissue regenerative strategies. Perfusion systems generate more physiologically relevant and sustainable adipose tissue models, however adipocytes have unique properties that make culturing them in a perfusion environment challenging. In this paper we describe the methods involved in the development of two perfusion culture systems (2D and 3D) to test their applicability for long term *in vitro* adipogenic cultures. It was hypothesized that a silk protein biomaterial scaffold would provide a 3D framework, in combination with perfusion flow, to generate a more physiologically relevant sustainable adipose tissue engineered model than 2D cell culture. Consistent with other studies evaluating 2D and 3D culture systems for adipogenesis we found that both systems successfully model adipogenesis, however 3D culture systems were more robust, providing the mechanical structure required to contain the large, fragile adipocytes that were lost in 2D perfused culture systems. 3D perfusion also stimulated greater lipogenesis and lipolysis and resulted in decreased secretion of LDH compared to 2D perfusion. Regardless of culture configuration (2D or 3D) greater glycerol was secreted with the increased nutritional supply provided by perfusion of fresh media. These results are promising for adipose tissue engineering applications including long term cultures for studying disease mechanisms and regenerative approaches, where both acute (days to weeks) and chronic (weeks to months) cultivation are critical for useful insight.

1. Introduction

Adipose tissue engineering is a multifaceted research initiative that will address current clinical needs related to filling various adipose tissue defects and for study disease pathologies *in vitro*. Defects in adipose tissue are caused by congenital abnormalities, traumatic injuries and tumor resections in various areas of the body and require better tissue engineering strategies for repair [1,2]. Physiologically relevant and sustainable adipose models will also enhance our understanding of cell biology and provide novel experimental systems *in vitro* to study metabolism and metabolic disease mechanisms, such as obesity and type II diabetes [3].

In general, cells require and rely on fluid flow for mechanotransduction and nutrient supply *in vivo*. Similarly, perfusion systems provide control of the physiological environment *in vitro* (i.e. mechanical signals, pH, temperature, nutrient supply, oxygen tension, waste removal) and play a significant role in useful tissue engineering outcomes by improving the quality of engineered tissues, correcting for insufficient nutrient and metabolite transport present in static culture, improving mass transport, and generating more homogenous cell distributions [4–9]. The key cells in adipose tissue (adipocytes), however have unique properties that make culturing more challenging in a perfusion environment. Adipocytes are buoyant and their lipid laden morphology makes them fragile [10,11]. Additionally, differentiating adipocyte cell lines requires lengthy culture times (at least 3 weeks) which needs to occur within the cultivation system [12,13] or differentiated cells need to be perfused into the device [14]. Finally, adipocytes secrete hydrophobic metabolites that can accumulate in microscale devices blocking fluid flow [11].

To create more physiologically relevant tissues we sought to create a perfusion system for enhancing adipogenic potential in long term culture. Two dimensional (2D) culture systems have utility when compared to 3D culture systems, including direct visualization of cells and the lack of a complex three dimensional (3D) matrix. However, cells respond differently in 2D versus 3D environments [15]. In particular, 3D culture systems improve adipogenic differentiation [16,17], including increased secretion [16] and production [18] of relevant proteins and gene expression patterns

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which are more similar to *in vivo* patterns [19]. Therefore, we describe the development of two perfusion culture systems (2D and 3D) to evaluate their applicability for long term *in vitro* adipogenic culture. Long term culture is particularly relevant for adipose tissue systems to create fully differentiated tissues [20] and will be required to generate an adequate supply of tissue mass required to implant *in vivo*. Additionally, diseases that affect adipose dysfunction, such as obesity and type II diabetes, are chronic conditions that develop *in vivo* over weeks to months [21], suggesting long term cultures will be required to create accurate *in vitro* models.

It was hypothesized that silk protein scaffolds would provide a 3D framework that in combination with perfusion flow would establish more physiologically relevant sustainable adipose tissue engineered systems. Silk is a naturally occurring and clinically acceptable biocompatible polymeric biomaterial that has tunable mechanical strength, low inflammatory and immunogenic responses, an absence of cell-specific signaling domains, and can be tailored to degrade slowly to support the needs of long term cultures [22–24]. Additionally, silk as a biomaterial scaffold has demonstrated compatibility with adipose tissue engineering applications [25–30].

2. Material and methods

2.1. Materials

Bombyx mori silkworm cocoons were obtained from Tajima Shoji Co (Yokohama, Japan). Unless otherwise noted all cell culture supplies were purchased from Invitrogen (Carlsbad, CA). Additionally, phalloidin, 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), Picogreen kits and collagenase type I were purchased from Invitrogen. Human recombinant insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, indomethacin, laminin and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO). AdipoRed was purchased from Lonza (Walkersville, MD). Triglyceride and Glycerol quantification kits were purchased from BioAssay Systems (Hayward, CA). A Lactate Dehydrogenase (LDH) Assay Kit was purchased from Abcam (Cambridge, MA). Polyethylene molds (Catalog number: 03-338-1E), 5 mm biopsy punches (Catalog number: NC9151828), needle-free valves (Catalog number: NC0332521), 4 way stopcocks (Catalog number: NC9052592), hydrophobic filters (SLFGL25BS), hydrophilic filters (SLMPL25SS), Polydimethylsiloxane (PDMS, Catalog number: NC9644388), tridecafluoro-1,1,2,2-tetrahydrooctyl trichlorosilane and 60 mL syringes were purchased from Fisher Scientific (Waltham, MA). Tubing (Catalog number: 5054K304) and male and female adaptors were purchased from McMaster Carr (Robbinsville, NJ). Uncoated glass bottom 96 well plates were purchased from MatTek Corp (Ashland, MA). 3D Kube perfusion chambers were obtained from Kiyatec (Greenville, SC).

2.2. Isolation and expansion of preadipocytes

Subcutaneous adipose tissue was obtained from elective abdominoplasty procedures (Tufts University IRB Protocol #0906007, first patient – 30 year old female with a BMI of 28, second patient – 50 year old female with a BMI of 27, both non diabetic) and was processed the same day of surgery as described previously [31] with some modifications. The adipose tissue was separated from the skin and the fascia of Scarpa by blunt dissection. The remaining adipose tissue was liquefied in a blender by successive short pulses to break up the tissue. An equal volume of pre-warmed phosphate buffered saline (PBS) was added to the blended adipose tissue and the resulting solution was allowed to phase separate for 3–5 min at room temperature. The lipid laden tissue floated above the aqueous phase and the infranatant (lower liquid phase) was aspirated. This wash step was repeated until the infranatant was clear of blood (~2 times). An equal volume of warm collagenase (PBS+ 1% BSA+ 0.1% collagenase type I) was then added, and the tubes were incubated for 1 h (37 °C, 5% CO_2). Following the incubation step, the solution was centrifuged at 300×*g* for 5 min at room temperature. The stromal vascular fraction pelleted at the bottom of the tube and the supernatant containing oil, primary adipocytes (yellow layer of floating cells) and the collagenase solution was aspirated. The pellet was resuspended in warm PBS with 1% BSA and was centrifuged again (300×*g* for 5 min at room temperature). The pellet was resuspended in maintenance media (DMEM/F12, 10% fetal bovine serum, 1× Antibiotic–Antimycotic) and centrifuged for the final time (300×*g* for 5 min at room temperature) and the cells were plated in flasks so that 0.16 mL of the original liquefied tissue was plated per cm^2. Forty-eight hours after plating, the media was aspirated and the flasks were washed with pre-warmed PBS before adding maintenance media. The media was changed every 2–3 days until cells were 80–90% confluent. Cells were then released from the plastic with trypsin, counted, and used for 2D and 3D experiments at passage 2. As the cells were obtained from patients on different days, all of the experiments were independently repeated at different times.

2.3. 2D device fabrication and cell seeding

An epoxy mold was fabricated as shown in Fig. 1. First, a thin film (1 mm) of polydimethylsiloxane (PDMS or Sylgard® 184) was formed by mixing the silicon elastomeric base with the curing agent at a 10:1 ratio. The solution was cured for 6 h at 60 °C. The desired feature of the device, a circular chamber with one inlet and outlet, was cut from the PDMS film using a 5 mm biopsy punch and a sharp blade. In this study, the large chamber size (5 mm) was chosen based on the concept that adipocytes secrete hydrophobic metabolites which may be difficult to transport through microscale PDMS-based devices [11]. Additionally, 5 mm was chosen to match the diameter of the glass bottom 96 well plates used as a static control. After treating both surfaces with an oxygen plasma gun (Rotaloc: Littleton, Colorado) for 30 s the PDMS film was bonded to another layer of PDMS. The bond was cured for 2 h at 60 °C. The surface of the PDMS mold was modified using a silanizing agent (tridecafluoro-1,1,2,2-tetrahydrooctyl trichlorosilane) to facilitate the de-molding process for the next step. Next, the design was transferred to an epoxy to create a master mold for the device. This process was essential for creating multiple devices with a consistent design. To create an epoxy mold, a two component fast curing epoxy – Smooth-Cast® 321 – was mixed at a 1:1 ratio and blended thoroughly to avoid bubbles in the plastic disposable container. The mixture was then poured over the PDMS mold and cured for 40 min at room temperature. The epoxy mold was then used to create PDMS devices by casting PDMS over the mold and allowing the solution to cure for 6 h at 60 °C. The PDMS was then peeled off from the epoxy mold and inlet/outlet holes were punched out of the PDMS. The PDMS mold was bonded to a glass substrate by treating both surfaces (PDMS and Glass) with the oxygen plasma gun for 30 s. Devices were annealed for 2 h at 60 °C and autoclaved. Prior to cell seeding the devices were coated with laminin (2 μg/cm^2) for 2 h. The devices were rinsed with PBS and seeded with cells. An approximate density of 9000 cells/cm^2 [32] was seeded in the 2D devices and static culture (96 well glass bottom plates coated with laminin). Cells were incubated in the device for 2 h before perfusion was initiated. Both static and perfused 2D cultures were grown in maintenance media to confluence and then differentiated for the remainder of the culture in differentiation media which consisted of DMEM/F12 supplemented with 10% fetal