A protocol for the isolation and cultivation of brown bear (*Ursus arctos*) adipocytes

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Received: 18 September 2015 / Accepted: 9 December 2015 / Published online: 8 February 2016
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Abstract Brown bears (*Ursus arctos*) exhibit hyperphagia each fall and can become obese in preparation for hibernation. They do this without displaying the physiological problems typically seen in obese humans, such as Type 2 diabetes and heart disease. The study of brown bear hibernation biology could therefore aid in the development of novel methods for combating metabolic diseases. To this end, we isolated mesenchymal stem cells from subcutaneous fat biopsies, and culture methods were developed to differentiate these into the adipogenic lineage. Biopsies were taken from 8 captive male (N = 6) and female (N = 2) brown bears, ages 2–12 years. Plastic adherent, fibroblast-like cells were proliferated and subsequently cryopreserved or differentiated. Differentiation conditions

were optimized with respect to fetal bovine serum content and time spent in differentiation medium. Cultures were characterized through immunostaining, RT-qPCR, and Oil red O staining to quantify lipid accumulation. Adiponectin, leptin, and glycerol medium concentrations were also determined over the course of differentiation. The culturing protocol succeeded in generating hormone-sensitive lipase-expressing, lipid-producing white-type adipocytes (UCP1 negative). Serum concentration and time of exposure to differentiation medium were both positively related to lipid production. Cells cultured to low passage numbers retained similar lipid production and expression of lipid markers PLIN2 and FABP4. Ultimately, the protocols described here may be useful to biologists in the field investigating the health of wild bear populations and could potentially increase our understanding of metabolic disorders in humans.

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Keywords Bear · Adipocyte · Cell culture · Lipid metabolism

Introduction

Brown bears (*Ursus arctos*) have developed unique adaptations to cope with seasonal changes in food availability, undergoing dramatic seasonal weight gain and loss each year. Distinct phases within this cycle include summer lean mass gain, fall fat accumulation,

and weight loss during hibernation, with 81 % of fall mass increase due to fat gain (Hilderbrand et al. 1999). Maximizing fat accumulation during the active season is therefore an essential survival and fitness mechanism. Indeed, fatter females produce larger cubs with higher survival rates than do leaner females, creating heavy selection for large appetites and “morbidly obese” phenotypes (Dahle et al. 2006; Derocher and Stirling 1996; Robbins et al. 2012a). Despite their massive weight gain each year, brown bears do not appear to experience health problems typically associated with obesity in humans. Thus, brown bear physiology represents an example of extreme metabolic adaptation to a seasonal environment.

Although some studies in other hibernators have described alterations in metabolic function (Wang et al. 1997), these species differ from bears in the genus *Ursus* by exhibiting periodic arousals that restore euthermic metabolic function for brief periods. Only the members of *Ursus* exhibit a continuous hypometabolic state for the entirety of hibernation (Pagès et al. 2008; Tøien et al. 2011). The mechanisms underlying these seasonal transitions in brown bears have not been fully elucidated. Furthermore, the use of anesthetics in whole animal studies, which is often essential with ursids, potentially makes interpretation of metabolic parameters difficult (Kamine et al. 2012b; Nelson and Robbins 2015). Therefore, the development of an in vitro method to study brown bear physiology could provide a way to avoid this problem. Because fat serves such an important function in hibernators, it is essential to understand how fat-borne signals are utilized by the body, and conversely, how fat cells respond to metabolic cues. Two adipocyte-produced hormones, leptin and adiponectin, vary seasonally in several *Ursus* species with peaks occurring just prior to hibernation (Hill 2013; Hissa et al. 1998; Tsubota et al. 2008). However, the role of these adipokines in hibernation and in seasonal fat metabolism has yet to be firmly established.

Understanding fat cell metabolism is also critical to improving human health. Obesity rates in humans are increasing globally (Seidell and Halberstadt 2015; Swinburn et al. 2011). Obesity is associated with numerous comorbidities such as hypertension, Type 2 diabetes mellitus, and coronary heart disease (Malnick and Knobler 2006; Pi-Sunyer 2002). Many studies have used 3T3-L1 and 3T3-F422A mouse cells or primary human adipocytes to study the metabolic changes that

occur in individuals suffering from obesity-related illnesses (Farmer 2006; Lefterova and Lazar 2008; Ntambi and Kim 2000). Although both types of cells are vital tools for research in the field of fat cell metabolism, brown bear cell cultures would provide an in vitro model that attempts to capture the unique seasonal adaptations found in these animals. Fink et al. (2011) cultured adipose-derived mesenchymal stem cells (MSCs) from brown bears and differentiated them into the adipogenic, osteogenic, and chondrogenic lineages. In this study, we focused on optimizing adipogenic culture methods and confirming the identities of both MSCs and mature adipocytes.

We describe a cell culture protocol for the isolation and expansion of brown bear adipocytes to lay the groundwork for further study of adipocyte function. The use of brown bear cell cultures will enable investigation into the effects of numerous physiological and environmental variables on lipid metabolism and potentially the health of brown bear populations. Additionally, our understanding of human health could be greatly improved by research on this natural and reversible model of healthy obesity. Study of the adaptations bears use to survive extended fasts and seasonal obesity may provide a reverse translational approach for identifying novel mechanisms to treat human metabolic disorders.

Materials

Solutions for cell culture

1. Collagenase solution: 1 mg Type 1 collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA, LS004196) per 1 ml 1× Hank’s balanced salt solution (HBSS) (Sigma, St. Louis, MO, USA, H1641), filtered with a 0.22 µm filter
2. Erythrocyte lysis buffer: a solution of 0.154 mM NH₄Cl, 10 mM K₂HPO₄, and 0.1 mM EDTA in ultrapure water (pH 7.3)
3. Reconstruction buffer: a solution of 2.2 g of NaHCO₃ and 4.77 g of HEPES in 100 ml of 0.05 N NaOH (Sugihara et al. 1988)
4. Collagen gel: one volume of reconstruction buffer and one volume 10× Ham’s F-12 was mixed with eight volumes Collagen Type I (Invitrogen, Carlsbad, CA, USA, #A1048301) (Sugihara et al. 1988)

5. Preadipocyte medium: a solution of 89 % DMEM/F-12 containing GlutaMAX™ (Life Technologies, Carlsbad, CA, USA, #10565), 10 % MSC-qualified fetal bovine serum (FBS) (Thermo Fisher, Waltham, MA, USA, #12662), and 1 % 100× antibiotic/antimycotic (Sigma, #15240) (final concentrations 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B)
6. Differentiation medium: a solution of preadipocyte medium containing 86.1 nM insulin (Sigma, #I9278), 1 nM 3,3',5-triiodo-L-thyronine (T₃) (Sigma, #T2877), 125 mM indomethacin (Sigma, #I7378), 5.1 µM dexamethasone (Sigma, #D1756), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma, #I5879), and 0.5 µM rosiglitazone (Sigma, #R2408)
7. Maintenance medium: a solution of preadipocyte medium containing 86.1 nM insulin, 1 nM T₃, and 0.5, 1 µM, or no rosiglitazone; the amount of rosiglitazone added depends on the amount of time that has passed since differentiation. See differentiation timetable (Fig. 1)

5. Blocking buffer: PBS with 5 % donkey serum and 0.1 % Triton-X
6. Antibody dilution buffer: 1× PBS containing 1 % BSA and 0.3 % Triton-X
7. Diluted phalloidin: PBS containing 2.5 % phalloidin
8. Diluted BODIPY: BODIPY diluted 1:1000 in 1× PBS

Supplies

1. Twenty-four well cell culture plates (Sigma, #M8812)
2. Trypsin/EDTA solution: 0.25 % trypsin/2.21 mM EDTA (Zenbio Inc., Triangle Park, NC, USA, #TRP-100)
3. Cryopreservation medium (Zenbio Inc., #FM-1-100)
4. Millicell EZ SLIDE 8-well glass slides (Millipore, Billerica, MA, USA, #PEZGS0816)
5. Rabbit anti-HSL antibody (Cell Signaling Technology, Danvers, MA, USA, #4107)
6. Donkey anti-rabbit immunoglobulin G (IgG) conjugated to Alexa Fluor 594 (Jackson ImmunoResearch Inc., West Grove, PA, USA, #711-586-152)
7. Bovine serum albumin (Sigma, #A7906)
8. Normal donkey serum (Jackson ImmunoResearch Inc., #017-000-121)

Solutions for immunostaining

1. 3.7 % paraformaldehyde in PBS (pH 7.4)
2. 1× PBS at room temperature and at 37 °C
3. 1 % bovine serum albumin (BSA, Sigma, #A7906) in PBS
4. 0.1 % Triton-X 100 (Sigma, #X100) in PBS

Fig. 1 A timetable of the differentiation process from preadipocytes through maturation. Days are relative to the initiation of differentiation

9. Alexa Fluor 568 Phalloidin (Life Technologies, #A12380)
10. BODIPY 493/503 (Life Technologies, #D-3922)
11. ProLong Gold AntiFade mountant with DAPI (Life Technologies, #P36931)

Procedures

We collected adipose samples from a total of 8 captive brown bears housed at the Washington State University Bear Education, Conservation and Research Center. All procedures were approved by the WSU Institutional Animal Care and Use Committee (IACUC). Bears included four 2-year-old males (bears A, B, C, D), two 11-year-old males (bears E, F), and two 10-year-old females (bears G, H). Different combinations of individuals were used in each experiment. Bears were anesthetized using the protocol described in Ware et al. (2012). Biopsy sites were shaved and surgically prepared using chlorhexidine and 70 % ethanol. Samples of subcutaneous adipose tissue were obtained from the right and left gluteal depots using 6 mm punch biopsies (Miltex, York, PA, USA, #33-36). Adipose tissue was processed to isolate MSCs using a modified version of the protocol described by Lee and Fried (2014).

After the dermis was removed from each biopsy sample, the remaining adipose was submerged in 6 ml $1\times$ HBSS with 1 % antibiotic/antimycotic and transported to the laboratory at room temperature. Samples were weighed and then rinsed over a fine mesh stainless steel strainer using $1\times$ PBS to remove any debris. Then, each tissue sample was placed in an HBSS-filled 35 mm culture dish (Corning, Corning, NY, USA, #353001) and dissected into ~ 30 mg pieces using surgical scissors. Visible debris, including blood vessels and any remaining dermis, was removed and discarded. Adipose tissue chunks were then rinsed again as described above and placed into a new culture dish filled with fresh HBSS, where they were further dissected into smaller (~ 5 mg) pieces. The adipose pieces were then transferred to a sterile 15 ml centrifuge tube containing 2 ml collagenase solution per g adipose and incubated undisturbed and loosely capped at 37 °C and 5 % CO₂ for 15 min. The tube was then tightly closed and placed in a water bath at 37 °C and shaken at 100 rpm for 30–60 min until the tissue became soupy. Digested tissue was rinsed with 5 ml preadipocyte medium through a Pierce 250 μ m tissue strainer (Thermo Fisher, #87791) into a fresh

15 ml tube, and the mesh was scraped if clogged by tissue. The tube was then centrifuged at $500\times g$ for 5 min. The fat cake that formed at the top of the solution was removed and discarded, leaving the infranatant and pelleted stromal vascular fraction (SVF) in the tube. The infranatant was plated for expansion whereas the pelleted SVF was resuspended in 2–4 ml erythrocyte lysis buffer, depending on the number of erythrocytes present. The SVF was then incubated undisturbed for 15 min at room temperature and centrifuged at $500\times g$ for 5 min. The supernatant was discarded and the SVF pellet was resuspended in 3–5 ml preadipocyte medium. Viability and yield were determined via acridine orange/propidium iodide (AO/PI) staining using an automated cell counter (Nexcelom, Lawrence, MA, USA, K2). At this point cells were either plated (see below) or cryopreserved at 1×10^6 cells/ml in Zenbio cryopreservation medium according to the manufacturer's instructions. A CoolCell LX (BioCision, San Francisco, CA, USA, #BCS-405) was used to control the rate of cooling to -80 °C prior to storage in liquid nitrogen.

Cells were plated in 24-well plates at a density of 1×10^4 cells/well (5.2×10^3 cells/cm²) in 1 ml preadipocyte medium and maintained at 37 °C in a humidified incubator containing 5 % CO₂. Medium changes of 0.5 ml occurred 24 h after plating and every 48 h thereafter until cells reached confluence (Fig. 2a), at which point cells were passaged, cryopreserved, or differentiated.

To induce differentiation of cells derived from the SVF, all preadipocyte medium was first removed and replaced with 1 ml differentiation medium for 2 days. The differentiation medium was then removed and the cells were maintained in 0.5 μ M rosiglitazone maintenance medium for an additional 2 days. This medium was changed to 1 μ M rosiglitazone maintenance medium for 4 days, with a 0.5 ml medium change on the second day. Cells were then maintained in maintenance medium without rosiglitazone for 2 days. At this point the cells were considered mature as evidenced by the more rounded phenotype and the development of large numbers of lipid droplets (Fig. 2b). All figures were generated using Prism 6 (Graphpad, Inc., La Jolla, CA, USA) unless otherwise specified.

Real-time quantitative PCR

For gene expression analysis we extracted total RNA from cells using QIAzol (QIAGEN, Valencia, CA,

Fig. 2 **a** Undifferentiated cells isolated from the SVF of a juvenile male bear and cultured in medium containing 10 % FBS. **b** Mature lipid-containing adipocytes from this bear on day 10 post differentiation. Scale bar 50 µm

USA, #79306). Total RNA was then purified using an RNeasy Micro Kit (QIAGEN, #74004). This process was automated using the QIAcube system (QIAGEN, #9001292) and the QIAcube DNase I digestion program. Two micrograms of total RNA were then reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA, #4368814). Real-time quantitative PCR was performed using a ViiA 7 Real-Time PCR System (Applied Biosystems, #4453535) and TaqMan probes. Probe specificity was first confirmed with a BLASTn search of the National Center for Biotechnology Information database comparing canine or human TaqMan probe sequences to the polar bear (*U. maritimus*) genome (Table 1), as the polar bear is the closest relative of the brown bear that has a sequenced genome (Welch et al. 2014), and

subsequently by the appearance of abundant PCR product within 40 cycles. For all RT-qPCR we used RPS18 as a reference gene to normalize expression.

Optimization of culturing conditions

Cells from 2 juvenile males (bears A, B) were collected in mid-October during the hyperphagic season; cells from 2 adult males (bears E, F) were obtained from biopsies taken early in the active season (late May). All cells were at or below passage 1. Cells were grown according to the protocol described above and then differentiated. At the time of differentiation, wells were divided into treatment groups of 6 wells per bear grown in medium supplemented with 0, 0.5, 1, 3, 5, or 10 % FBS. Each treatment group was exposed to differentiation medium for 2, 4, 6, or 8 days. Thus, each serum group was cultured in differentiation medium for each of the aforementioned time periods with cells from each individual. Cells were washed twice with PBS and fixed with 10 % formalin for 60 min on day 10 post differentiation. They were stained with 0.5 % Oil red O for 30 min followed by hematoxylin (Vector Laboratories, Burlingame, CA, USA, #H-3404) for 1 min. Cells were washed with deionized water and stored at 4 °C until analyzed. Five images were taken per well with a Carl Zeiss Axiovert 40 CFL microscope using a Canon Powershot A620 with a Soligor adapter tube at a 200× magnification. Amount of staining was quantified using ImageJ software based on protocols described in Deutsch et al. (2014). Images were first standardized by using the auto levels function. Next, color thresholding was applied to isolate the individual lipid droplets. After confirming that the selection included ± 95 % of all lipid droplets, the selection was converted to a binary image. Once the image scale was calibrated, the area of the lipid droplets (μm^2) was automatically determined by the software. The procedure was repeated for each image.

Oil red O staining and quantification as described above was also used to examine differences in lipid accumulation in cells collected during hibernation (January) and the active season (May). All cells were at or below passage 2. Biopsies were taken from juvenile (bears A, B) and adult male bears (E, F), and cells were cultured using the standard 2-day differentiation protocol with 10 % FBS.

To examine the effects of differing storage conditions on adipose viability and yield, we biopsied 2

Table 1 Probe names, expected values, and predicted polar bear homology for the genes used in this study. Homology and expected values were determined using a BLASTn search of the National Center for Biotechnology Information database

Gene	TaqMan Probe ID	Species	Interrogated RefSeq	<i>U. maritimus</i> RefSeq	E value	Identity (%)
CD44	Cf02693346_m1	Canine	XM_533152.2	XM_008686804.1	$1 e^{-59}$	94
FABP4	Hs01086177_m1	Human	NM_001442.2	XM_008689088.1	$3 e^{-39}$	93
PLIN2	Cf02629676_g1	Canine	XM_531946.2	XM_008689860.1	$4 e^{-34}$	92
PPARG	Cf02625640_m1	Canine	NM_001024632.2	XM_008697869.1	e^{-41}	96
RPS18	Cf02624915_g1	Canine	NM_001048082.1	XM_008693439.1	$1 e^{-44}$	95
THY1 (CD90)	Cf02651285_g1	Canine	XM_546483.2	XM_008689968.1	$1 e^{-27}$	97
UCP1	Cf02622091_m1	Canine	NM_001003046.1	XM_008685422.1	$4 e^{-47}$	90

male (bears E, F) and 2 female bears (G, H) in May. Small (<0.1 g) pieces of adipose tissue were stored in 1 ml $1\times$ HBSS in 2 ml microcentrifuge tubes following the rinsing and mincing stages of the cell isolation protocol. Tubes were stored in the dark at either 4 or 22 °C for 24 or 48 h, then digested in collagenase and processed as described above.

Culturing and staining protocols for fluorescence microscopy

Undifferentiated and mature cells were obtained from 4 adult bears (E, F, G, and H) and 2 juvenile males (A, B) during the active season (May). All cells used were at or below passage 1. Percent differentiation was determined using 6 images from 2 wells from each of bears F, G, and H by calculating the ratio of hormone-sensitive lipase (HSL) stained cells, assumed to be differentiated adipocytes (Ailhaud et al. 1992), to total stained (DAPI) nuclei.

For these procedures the cells were cultured in wells on Millicell glass slides (5×10^3 cells/well, 7.14×10^3 cells/cm²). With the exception of the culture chamber, the culturing and differentiation protocols described above were used. Confluent undifferentiated or fully differentiated cells were rinsed gently with warm (37 °C) $1\times$ PBS and fixed in 3.7 % paraformaldehyde in $1\times$ PBS for 10 min. Cells were then rinsed twice with $1\times$ PBS. All incubations were performed at room temperature unless otherwise specified.

A. Undifferentiated cells

After fixation, each well was incubated in 75 μ l antibody dilution buffer for 30 min. The buffer was then removed and 75 μ l dilute phalloidin was added. Twenty minutes later, the phalloidin was

gently removed and 75 μ l BODIPY was added (negative control). Following a 30 min incubation, the cells were rinsed 3 times with $1\times$ PBS and coverslipped using ProLong Gold AntiFade with DAPI. Cells were visualized and photographed using a fluorescent microscope (Zeiss, Oberkochen, Germany, Axioplan 2IE) fitted with an INFINITY2-3C digital CCD color camera (Lumenera Corporation, Ottawa, ON, Canada) at 200–400 $\times$ magnification. Fluorescent microscopy images were generated using Adobe Illustrator (Adobe Systems, Inc., San Jose, CA, USA).

B. Differentiated cells

Mature adipocytes on glass slides were fixed as above and then incubated in 300 μ l blocking buffer for 60 min. The buffer was removed and replaced with 75 μ l HSL antibody diluted 1:100 in antibody dilution buffer. The slides were then incubated in the dark at 4 °C for 36 h. Cells were then gently washed 3 times with $1\times$ PBS and incubated in secondary antibody diluted 1:100 in antibody dilution buffer for 90 min. After removal of the secondary antibody, the cells were washed an additional 3 times in PBS. They were then incubated in BODIPY (positive control) for 30 min. Each well was rinsed 3 times and coverslipped and imaged as described for undifferentiated cells.

Characterization of cells in culture

A. Undifferentiated cells

In order to better define the SVF-derived cell population, we performed RT-qPCR on undifferentiated cell cultures at or below passage 2 to test

for the expression of the stem cell markers CD44 and CD90 (bears F, G, H) (Ghaffari et al. 1997; Haynesworth et al. 1992) as well as for peroxisome proliferator-activated receptor gamma (PPAR γ) (bears E, F, G, H), which stimulates lipogenesis and is critical for differentiation (Chawla et al. 1994; Morrison and Farmer 2000). Undifferentiated passage 1 cells from 2 juvenile male bears (A and B) originating from the active season and hibernation were stained with alizarin red and alcian blue after reaching confluence to assess multipotency.

B. Differentiated cells

To further characterize the adipocyte cultures, we performed qPCR for PPAR γ (bears E, F, G, H) and the brown fat marker uncoupling protein 1 (UCP1) (bears A, B, E, F, H) using cells at or below passage 2. Cells obtained from 2 juvenile male bears during the hyperphagic season were assayed for adipokine (bears B, C) and glycerol concentrations (A, B) in medium collected throughout the differentiation process in passage 1 cells. Medium was collected to assay for glycerol production as an indication of lipolysis from 4 wells on days 2, 6, and 10 post differentiation, then stored at -80°C . Samples were run in duplicate using a Glycerol Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA, #10010755). Medium was also collected from 4 wells during each medium change post differentiation (day 2, 4, 6, 8, and 10) and stored at -80°C for leptin and adiponectin measurements. Medium samples (1 ml) were first lyophilized (Labconco Corp., Kansas City, MO, USA, Free-Zone 6) and then reconstituted in 100 μl ultrapure water to produce concentrated medium. These concentrated samples were assayed in duplicate using a Canine Leptin ELISA (Millipore, #EZCL-31 K) and a Mouse/Rat Adiponectin ELISA (B-Bridge International, Santa Clara, CA, USA, #UM-100201) according to instructions provided by the manufacturers.

We also grew tissue explants in collagen gel matrix as described in Sonoda et al. (2008) to examine adipokine production. Adipose samples were taken during the hyperphagic season from four 2-year-old male bears (A, B, C, and D). Collagen was mixed with reconstruction buffer and Ham's F-12 and stored on ice. To create collagen disks, 1 ml of this solution was mixed with 0.1 g finely minced adipose per well in a

12-well Corning Costar cell culture plate (Sigma, CLS3513). The plate was incubated in a humidified environment at 37°C and 5 % CO_2 for 20 min to allow the collagen to harden. Then 3 ml Zenbio subcutaneous adipocyte maintenance medium (Zenbio, #AM-1) was added to each well and the plate was returned to the incubator. Medium changes of 1 ml occurred 24 h after plating and then every 48 h thereafter. These 1 ml samples of conditioned medium were stored at -80°C until assayed as described above. Explants in collagen disks were maintained for 35 days, then transferred to cassettes and fixed in 10 % formalin with agitation for 60 min. Disks were paraffin embedded, sectioned, and mounted on glass slides. Paraffin sections were then stained with hematoxylin and eosin according to standard procedures. Images of collagen cultures were generated using Adobe Illustrator (Adobe Systems, Inc.).

Passaging effects

Cells isolated from 3 juvenile male bears (bears A, B, D) during the hyperphagic (October) season were removed from liquid nitrogen and cultured according to the above protocols. Upon reaching confluence, cells were either stimulated to differentiate into mature adipocytes or passaged and reseeded at 1×10^4 cells per well. To passage cells, cultures were washed twice with $1 \times$ PBS and treated with 0.25 % trypsin/2.21 mM EDTA (Zenbio, #TRP-100) according to the manufacturer's instructions. Trypsin/EDTA was neutralized with preadipocyte medium at 0.1 ml/cm², and cells were counted and viability measured. Cells were trypsinized and replated to passage 3. Mature adipocytes from passages 0, 1, 2, and 3 were fixed on day 10 post differentiation and stained with Oil red O in order to determine effects of passaging on lipid content. Images were analyzed as described above. Quantitative PCR was also performed using samples from each passage to determine the effect of passaging on the lipogenic markers fatty acid binding protein 4 (FABP4) and perilipin 2 (PLIN2).

Statistical analysis

Data are presented as mean $\pm$ SEM. Effects of serum concentration and time of exposure to differentiation medium on lipid production were analyzed using a

two-way ANOVA. A one-way ANOVA was performed on Oil red O staining values from cell passages. For measurements of gene expression in passaged cells, fold change in PLIN2 and FABP4 was calculated relative to passage 0 and analyzed with a one-way ANOVA. A Kruskal-Wallis test was used to analyze fold change in PPAR γ , which was calculated with respect to expression in undifferentiated cells. Unpaired *t* tests were performed to assess differences in lipid accumulation in hibernation and active season cells and to compare medium adiponectin concentrations from monolayer and collagen cultures. Differences with $P \leq 0.05$ were considered statistically significant.

Results

Optimal culturing conditions

We found both FBS concentration and time in differentiation medium had significant positive effects on Oil red O staining [two-way ANOVA; effect of serum concentration, $F(5,60) = 4.373$ ($P = 0.0018$); effect of time of differentiation, $F(3,60) = 6.140$ ($P = 0.0010$)]. Adipocytes grown in 10 % FBS had the greatest amount of lipid accumulation (Fig. 3). Within the 10 % serum medium group, maximum lipid was found in cells grown in differentiation

Fig. 3 Mature adipocyte culture stained for neutral lipid with Oil red O. The cells were grown in medium supplemented with 10 % FBS which was changed every 48 h. Cells were grown to confluence at which time differentiation medium was added. Cultures were fixed in 10 % formalin and stained 10 days post differentiation. Scale bar 100 μm

medium for 8 days (Fig. 4). No differences were found in lipid accumulation between cells from the active season ($19,640 \pm 7126 \mu\text{m}^2$) and cells from hibernation ($19,213 \pm 7318 \mu\text{m}^2$) [unpaired *t* test, $t(5) = 0.04097$ ($P = 0.9689$)].

Neither storage time (24 vs 48 h) nor temperature (4 vs 22 °C) had any significant effect on cell number [two-way ANOVA; effect of time, ($P = 0.3$); effect of temperature, ($P = 0.48$)] or viability [effect of time, ($P = 0.2$); effect of temperature, ($P = 0.53$)] when samples were stored under the two different conditions for the aforementioned time periods (Fig. 5).

Characterization of cells in culture

Undifferentiated cells collected during the early active season and hibernation from 3 adult bears (F, G, and H) were all found to express stem cell markers CD44 and CD90. Alizarin red and alcian blue staining indicated the presence of chondrogenic cells and osteogenic nodules in undifferentiated cultures, further confirming the multipotent nature of these cells (Fig. 6).

Immunostaining of differentiated adipocyte cultures revealed lipid vacuoles of varying sizes concentrated in the center of the cell body with the majority of the cell body expressing HSL (Fig. 7a–c). Staining affirmed the cell structure observed in live cultures. Control wells lacking primary antibody showed little to no staining, confirming the specificity of the

Fig. 4 Lipid accumulation expressed as area stained with Oil red O (μm^2) in adipocyte cultures grown in medium supplemented with 10 % FBS and exposed to differentiation medium for varying amounts of time. Treatment groups include cells grown in differentiation medium for 2, 4, 6, or 8 days before being switched into the described maintenance medium formula

secondary antibody (Fig. 7d). Undifferentiated cells incubated in BODIPY showed minimal staining of lipid droplets, indicating a lack of lipid production in these cells. Phalloidin staining of undifferentiated cells allowed for visualization of the cell structure (Fig. 7e, f). Immunostained slides of mature adipocytes were used to determine percent differentiation of SVF cells stimulated to differentiate under the previously described protocol. Cells grown under these conditions had a $75 \pm 8.2\%$ differentiation rate into mature adipocytes. Gene expression of PPAR γ was significantly higher in differentiated cells than in undifferentiated cells from both the active season and hibernation, increasing approximately 17-fold upon induction to the adipogenic lineage (Kruskal–Wallis

test; $P < 0.0001$). No amplification was detected using the UCP1 probe for samples obtained from the active season and hibernation biopsies, suggesting the differentiation protocol used was specific for producing white adipocytes.

Both adiponectin and leptin production increased throughout differentiation with adiponectin production reaching a maximum of approximately 50 ng/ml at day 10 post differentiation (Fig. 8). Leptin and adiponectin followed similar profiles with production increasing slowly from day 2 to 4 and more rapidly from day 4 to 8. Adiponectin was found at comparatively much higher concentrations in the culture medium than leptin, the latter reaching a maximum of >1.5 ng/ml on day 10. Glycerol production also increased from day 2 to day 10 with the highest rate of increase from days 2 to 6 in culture (Fig. 9).

Fig. 5 Percent viability and number of live cells for the SVF isolated from subcutaneous fat biopsies taken from 4 adult bears (2 males, 2 females) during the active season. Tissue was stored for 24 and 48 h at 4 and 22 °C prior to processing. Neither storage time nor temperature had a significant effect on viability [two-way ANOVA; effect of time, $F(1,12) = 1.819$ ($P = 0.2$); effect of temperature, $F(1,12) = 0.41$ ($P = 0.53$)] or cell yield [effect of time, $F(1,12) = 1.18$ ($P = 0.3$); effect of temperature, $F(1,12) = 0.52$ ($P = 0.48$)]

Fig. 6 **a** Alizarin red and **b** alcian blue staining of undifferentiated MSCs confirming the multipotent (i.e., chondrogenic and osteogenic) cell lineages in SVF cells obtained from brown bear adipose. Scale bar 100 μm

Fig. 7 **a–d** Differentiated cells stained for expression of HSL. BODIPY was used to stain for lipid droplets and nuclei are stained with DAPI. HSL—red, BODIPY—green, DAPI—blue;

d no HSL primary antibody. **e, f** Undifferentiated cells stained with phalloidin. Phalloidin—red, DAPI—blue; scale bar 100 μ m (**b, c, d, f**); 200 μ m (**a, e**)

Visualization of collagen cultures revealed clusters of large mature adipocytes with smaller preadipocytes proliferating and expanding into the surrounding collagen matrix (Fig. 10). Assay of the culture medium confirmed the presence of significantly greater adiponectin concentrations (101.06 ± 6.55 ng/ml) compared to monolayer cultures [41.5 ± 8.01 ng/ml, $t(13) = 5.7546$ ($P < 0.0001$)].

Evaluation of passaging effects

In cells cultured to passage 3 and stained with Oil red O, there were no significant differences in neutral lipid staining between passages [one-way ANOVA;

$F(3,39) = 0.9556$, $P = 0.4233$] (Fig. 11). There were also no significant differences in expression of PLIN2 or FABP4 between passages [one-way ANOVA; PLIN2 $F(2,15) = 2.678$, $P = 0.1013$; FABP4 $F(2,15) = 0.1277$, $P = 0.881$] (Fig. 12).

Discussion

We describe a robust and reliable method for the isolation, storage, expansion, and differentiation of adipose-derived MSCs from brown bears. This technique could be used to advance our understanding of hibernation physiology and potentially gain insight

Fig. 8 Adipokine concentrations in medium of adipocyte cultures derived from the hyperphagic season and grown in medium supplemented with 10 % FBS. Medium was collected and adiponectin and leptin measured on days 2, 4, 6, and 10 post differentiation

Fig. 9 Glycerol concentrations in culture medium collected on days 2, 6, and 10 post differentiation. Cultures were derived from the hyperphagic season and grown in medium supplemented with 10 % FBS

into the treatment of human metabolic disorders. Our optimization procedures focused primarily on lipid production as this is unique to the adipogenic lineage under normal conditions. We demonstrate that lipid content was positively related to length of exposure to differentiation medium and was maximal at 10 % FBS concentration. These results corroborate previous findings that indicated increased growth rates in cells cultured with higher serum concentrations (Green and Meuth 1974; Ozturk and Palsson 1991).

Cells from hibernation and the active season accumulated similar amounts of lipid. Differentiated cells from both seasons expressed dramatically higher levels of PPAR γ than undifferentiated cells. Further

Fig. 10 Mature adipocytes cultured in a 3-dimensional collagen matrix. Collagen disks were fixed in 10 % formalin 35 days post differentiation, sliced, and mounted on glass slides. Cells were stained with hematoxylin and eosin, *asterisk* mature adipocytes, *c* collagen matrix, *arrows* preadipocytes; **a** scale bar 1000 μ m; **b** scale bar 250 μ m

Fig. 11 Lipid accumulation expressed as area stained with Oil red O (μ m²) in passaged cells grown in medium supplemented with 10 % FBS. Cultures were exposed to differentiation medium for 2 days and fixed and stained on day 10 post differentiation

Fig. 12 FABP4 and PLIN2 mRNA expression in passages 1–3 expressed as fold change relative to passage 0. A one-way ANOVA revealed no significant differences between passages for either gene. RPS18 was used as a reference gene

characterization included immunostaining for HSL and qPCR for expression of UCP1, a negative control for white adipocytes. Together, our results indicate that the culturing protocol is selective for producing cells of the white adipocyte lineage, rather than brown or beige/brite adipocytes. This is not surprising given that brown fat has only been described in one unverified report (Davis et al. 1990) that was later refuted by Jones et al. (1999). Undifferentiated cells were plastic adherent and expressed mesenchymal stem cell markers CD44 and CD90. The multipotent capacity of these cells was further demonstrated by positive staining for osteogenesis and chondrogenesis in some cultures that were maintained in preadipocyte medium after reaching confluence. This is similar to findings of spontaneous differentiation in adipose-derived MSCs from young brown bears (Fink et al. 2011). These results suggest a broad application of the

described protocols, as the SVF isolated from subcutaneous fat biopsies in brown bears can be used to study multiple cell lineages. However, we did not optimize culture conditions for other cell lineages and thus additional work is required to characterize the physiology of those cells.

Our findings of increasing adipokine concentrations and glycerol release over the course of differentiation indicate commitment to the adipogenic lineage and confirm that brown bear adipocyte differentiation is similar to in vitro adipogenic differentiation in other mammals (Rodriguez et al. 2005). Adiponectin production is specific to mature adipocytes (Körner et al. 2005) while leptin is produced in several tissues with greatest expression in adipocytes (Margetic et al. 2002). Leptin expression was found to increase throughout differentiation (MacDougald et al. 1995). Adipose tissue stores triacylglycerol during periods of energy excess and glycerol release is indicative of lipolysis. Lipolysis and the expression of enzymes involved in glycerol formation and release were found to increase in cells upon differentiation into adipocytes, consistent with our findings (Dani et al. 1989, 1997; Dicker et al. 2007; Dolinsky et al. 2003).

Analysis of neutral lipid staining and RT-qPCR for several adipocyte-specific genes revealed no differences between cells in passages 0–3, indicating that subculturing to low passage numbers preserves cellular characteristics close to the in vivo condition. The viability and number of cells per gram of adipose did not differ between small (<0.1 g) adipose tissue fragments stored in HBSS at 4 and 22 °C and processed 24 or 48 h post biopsy. This finding could provide flexibility to researchers in the field who may not be able to immediately process tissue samples. Adipose tissue obtained from biopsy darts could be used for functional and mechanistic studies assessing nutritional status, reproductive fitness, and metabolic state preceding hibernation. The use of remote biopsy darts to collect adipose tissue has already been validated in polar bears (McKinney et al. 2014; Pagano and Peacock 2013). Thus, studies of wild bears in different environmental conditions could provide valuable information about the factors affecting bear biology and facilitate the analysis of trends within and between populations.

Despite the critical role that adipose plays in bear physiology and survival, investigation into the mechanisms regulating their seasonal cycles has been

primarily limited to in vivo studies. Bear hibernation behavior and numerous aspects of their physiology have been described (Craighead and Craighead 1972; Hellgren 1998; Nelson 1973, Nelson et al. 1983; Nelson and Robbins 2010; Ware et al. 2012). Information on foraging behavior (Farley and Robbins 1995; Lopez-Alfaro et al. 2013; Robbins et al. 2012b) and the distinct phases of weight change throughout the year was presented by Nelson et al. (1983), Hilderbrand et al. (1999), and Schwartz et al. (2003). Tøien et al. (2011) documented a reduction in metabolism during hibernation to 25 % of basal rates independent of lowered body temperature, suggesting other endogenous factors may be involved in triggering the physiological changes accompanying hibernation. Studies of the processes regulating hibernation revealed a higher proportion of plasma unsaturated fatty acids during winter than during summer months, and increased leptin concentrations directly before hibernation corresponding to maximal fat content (Hissa et al. 1998; Tsubota et al. 2008).

More recently, Japanese black bears (*U. thibetanus japonicus*) were found to undergo changes in peripheral insulin sensitivity and glucose tolerance upon entering hibernation (Kamine et al. 2012a). This supports prior results from insulin tolerance tests in American black bears (*U. americanus*), which showed delayed glucose responses to insulin injections (Palumbo et al. 1983). However, studies at the cellular level are needed to further explore the mechanistic attributes of seasonal biology in bears including changes in gene expression, protein activation, and lipolytic capacity. Further investigation into factors responsible for seasonal changes in fat metabolism could be accomplished through manipulation of culture conditions using brown bear adipocytes collected during different seasons. These methods could be used to determine how bears are able to exhibit healthy obesity and transition from a carbohydrate to a lipid-based metabolism each year. The in vitro model we describe could therefore yield important insights into metabolic disorders in humans given the unique seasonal and reversible nature of bear physiology.

Acknowledgments Funding was provided by Amgen Inc., the Interagency Grizzly Bear Committee, the Raili Korkka Brown Bear Endowment, the Bear Research and Conservation Endowment, and a National Science Foundation Graduate Research Fellowship (KSR, 1347943). We thank the scientists at Washington State University's Department of Integrative

Physiology and Neuroscience including Jamie Gaber and Marina Savenkova for their mentorship and technical expertise. We also thank Danielle Rivet, Joy Erlenbach, and the other dedicated researchers at WSU's Bear Research, Education, and Conservation Center for their assistance in data collection and captive bear care.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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