

# Region-specific effects of oestradiol on adipose-derived stem cell differentiation in post-menopausal women

Kimberly A. Cox-York <sup>a</sup>, Christopher B. Erickson <sup>b</sup>, Rocio I Pereira <sup>c, d</sup>, Daniel H. Bessesen <sup>c, d</sup>,  
Rachael E. Van Pelt <sup>b, \*</sup>

<sup>a</sup> Department of Nutrition, Colorado State University, Fort Collins, CO, USA

<sup>b</sup> Department of Medicine, Division of Geriatric Medicine, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

<sup>c</sup> Department of Medicine, Division of Endocrinology, Metabolism and Diabetes, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

<sup>d</sup> Denver Health and Hospital Authority, Denver, CO, USA

Received: July 28, 2016; Accepted: September 18, 2016

## Abstract

The goal of this study was to determine the effect of acute transdermal 17 $\beta$ -oestradiol (E<sub>2</sub>) on the adipogenic potential of subcutaneous adipose-derived stem cells (ASC) in post-menopausal women. Post-menopausal women ( $n = 11$ ; mean age  $57 \pm 4.5$  years) were treated for 2 weeks, in a randomized, cross-over design, with transdermal E<sub>2</sub> (0.15 mg) or placebo patches. Biopsies of abdominal (AB) and femoral (FEM) subcutaneous adipose tissue (SAT) were obtained after each treatment and mature adipocytes were analysed for cell size and ASC for their capacity for proliferation (growth rate), differentiation (triglyceride accumulation) and susceptibility to tumour necrosis factor alpha-induced apoptosis. Gene expression of oestrogen receptors  $\alpha$  and  $\beta$  (ESR1 and ESR2), perilipin 1 and hormone-sensitive lipase (HSL), was also assessed. In FEM SAT, but not AB SAT, 2 weeks of E<sub>2</sub> significantly ( $P = 0.03$ ) increased ASC differentiation and whole SAT HSL mRNA expression ( $P = 0.03$ ) compared to placebo. These changes were not associated with mRNA expression of oestrogen receptors  $\alpha$  and  $\beta$ , but HSL expression was significantly increased in FEM SAT with transdermal E<sub>2</sub> treatment. Adipose-derived stem cells proliferation and apoptosis did not change in either SAT depot after E<sub>2</sub> compared with placebo. Short-term E<sub>2</sub> appeared to increase the adipogenic potential of FEM, but not AB, SAT in post-menopausal women with possible implications for metabolic disease. Future studies are needed to determine longer term impact of E<sub>2</sub> on regional SAT accumulation in the context of positive energy imbalance.

**Keywords:** adipose tissue • oestrogen • stem cells • menopause • adipogenesis

## Introduction

Loss of endogenous ovarian hormones is thought to play a role in menopause-related increases in subcutaneous adipose tissue (SAT) accumulation, particularly shifts in SAT distribution from the femoral (FEM) to abdominal (AB) region. This gynoid-to-android redistribution is proposed to contribute to the rise in cardiometabolic disease risk observed in women at the time of menopause [1, 2]. The decline in endogenous sex hormones (in particular 17 $\beta$ -oestradiol, E<sub>2</sub>) likely impacts SAT accumulation indirectly through central mechanisms that regulate energy intake and expenditure [3], but there may also be a direct effect on SAT expandability at the cellular level.

Subcutaneous adipose tissue hypertrophy is determined by the capacity for committed adipocytes to store triglycerides, while the

capacity for SAT to expand *via* hyperplasia is dependent on the proliferation and differentiation (adipogenic potential) of adipocyte precursor cells (adipose-derived stem cells; ASC) [4, 5]. Adipose-derived stem cells isolated from discrete human adipose tissue regions (visceral, omental, subcutaneous) and maintained in culture appear to vary in their capacity for adipogenesis, [6–8] where proliferation is reportedly greater in SAT than visceral adipose tissue (VAT) and differentiation is either greater or not different in SAT relative to VAT.

Animal and cell models support E<sub>2</sub>-mediated regulation of adipogenic potential. *In vitro* E<sub>2</sub> treatment decreased lipid accumulation in adipocyte precursor cells derived from rat SAT [9, 10] and mouse bone marrow cells [11], as well as in 3T3 mouse fibroblasts [10]. Human ASC treated *in vitro* with E<sub>2</sub> also exhibited altered adipogenic potential [12, 13], and 3 months of E<sub>2</sub> treatment altered the expression of genes central to lipogenesis (*e.g.* SCD1, FAS, ACC1) in SAT

\*Correspondence to: Rachael E. VAN PELT, Ph.D.  
E-mail: rachael.vanpelt@ucdenver.edu

from post-menopausal women [14]. Administration of *in vivo* compounds has previously been shown to modulate *ex vivo* adipogenic potential of ASC, suggesting that ASC maintain the attributes of their origin [15]. However, to our knowledge, the adipogenic potential of ASC isolated from human participants treated with E<sub>2</sub> has never been studied.

We previously reported that in post-menopausal women, acute E<sub>2</sub> treatment resulted in decreased cell size and increased cell number (hyperplasia) in FEM, but not AB SAT [16]. We postulated that this difference may be driven by the adipogenic potential of the ASC. We employed a randomized, cross-over design to treat post-menopausal women with transdermal E<sub>2</sub> or placebo to study the capacity for proliferation, differentiation and susceptibility to apoptosis in primary ASC isolated from FEM and AB SAT.

## Materials and methods

### Volunteers

Study volunteers were: (i) aged 45–60 years; (ii) post-menopausal (no menses  $\geq$ 12 months or history of bilateral ovariectomy and FSH  $>$ 30 IU/l); (iii) with body mass index  $>$ 20 and  $<$ 30 kg/m<sup>2</sup>; (iv) weight stable ( $\pm$ 2 kg in past 2 months); (v) non-smokers; (vi) sedentary to moderately active; and (vii) not using any type of hormone therapy or oral contraceptives. Exclusion criteria included: diabetes, hypertriglyceridaemia (TG  $>$ 400 mg/dl), medications known to affect lipid metabolism, elevated resting blood pressure ( $>$ 140 mmHg systolic,  $>$ 90 mmHg diastolic) and any conditions in which E<sub>2</sub> treatment would be contraindicated (*e.g.* increased risk of blood clots, stroke, heart disease and breast cancer). All participants provided written informed consent to participate in the study, in accordance with the approved guidelines of the Colorado Multiple Institutional Review Board.

### Body composition assessment

Total and regional (trunk, leg) fat mass and fat-free mass were assessed at baseline by dual-energy x-ray absorptiometry (DXA, Hologic Discovery W, software version 11.2; Hologic, Inc., Marlborough, MA) as previously described [17].

### Energy balance

Prior to enrollment and throughout the testing, participants were required to maintain their current body weight  $\pm$ 2 kg and to refrain from exercise for the 3 days leading up to the biopsy visit.

### E<sub>2</sub> administration

Each participant acted as their own control. Participants were randomly assigned to wear either identical placebo (no hormone) or E<sub>2</sub> (0.15 mg;  $3 \times 0.005$  mg ea.) transdermal patches for 2 weeks. Patches were placed on the flank, just above the buttocks. Following a 6–8 week

wash-out period, participants were switched to the alternate treatment. Subcutaneous adipose tissue biopsies were obtained at the end of each treatment from the FEM (thigh) and AB (lateral umbilicus) regions as described below.

### Blood work

Fasting serum samples were stored at  $-80^{\circ}\text{C}$  and analysed in batch. Glucose was determined enzymatically (Beckman Coulter; Brea, CA); insulin, leptin by radioimmunoassay (EMD Millipore; Darmstadt, Germany); and oestradiol and sex hormone-binding globulin by chemiluminescence (Beckman Coulter; Brea, CA). Sensitivity and precision details can be found at <http://cctsi.ucdenver.edu/Research-Resources/CTRCs/Pages/Assays.aspx>.

### Adipose tissue biopsies

Aspiration biopsies of subcutaneous AB and FEM SAT were collected in the fasting state at both visits, and performed with a mini-liposuction technique as previously described [18]. Approximately 0.5–1.5 g of tissue was obtained from each biopsy. This was sufficient material to complete cell sizing, mRNA expression and cellular dynamics studies for most participants ( $n = 11$ ) as indicated in figure legends.

### Mature adipocyte size

Immediately after collection, 50 mg of SAT was collagenase digested. Mature adipocytes were stained and cell size determined with Cell Counting Analysis Program (Mayo Clinic, Rochester, MN, USA) as previously described [19, 20].

### Adipose-derived stem cell isolation and analysis

Adipose-derived stem cells were isolated from AB and FEM biopsy samples as described previously [21]. Briefly, fresh SAT (0.5–1.5 g) was collagenase digested, filtered, then centrifuged to pellet the stromal vascular (SV) fraction. The SV was treated with erythrocyte lysis buffer, then seeded in growth medium ( $\alpha$ -MEM with 10% foetal bovine serum (FBS), penicillin: 100 U/ml, streptomycin: 100  $\mu\text{g/ml}$ ). Media was changed after 24 hrs. Once the cells reached  $\sim$ 80% confluence, they were detached, passaged twice and frozen in an isopropanol container before storing in liquid nitrogen. Cells were revived from frozen and analysed in batch—both depots and both study days were analysed together for each participant. This method has been shown to yield 70% adipogenic precursor cells (CD34<sup>+</sup> CD31<sup>-</sup>), and only a small percentage of monocytes/macrophages (CD11b/c<sup>+</sup>) and endothelial cells (CD34<sup>+</sup> CD31<sup>+</sup>) after an initial culture of 3 hrs [22]. The overnight culture yields an even more pure population [23].

### Differentiation

Frozen cells (passage 3–4) were revived from frozen in fresh growth medium and plated in triplicate 12-well plates (5000 c/cm<sup>2</sup>). At

confluence, the medium was switched to either low glucose maintenance medium (controls; DMEM-low glucose, 10% FBS, 2 mM L-glutamine, 5 µg/ml gentamycin) or commercial differentiation-inducing medium (differentiated; Stem-Pro Adipogenesis Differentiation kit, cat. A10070-01; Life Technologies; Carlesbad, CA). After 12–14 days, control and differentiated cells were stained for lipid with Oil Red O for 15 min, followed by multiple rinses with ddH<sub>2</sub>O as previously described [24]. Oil Red O was then extracted with isopropanol and absorbance measured at 569 nm. Differentiation was also quantified using AdipoRed Assay Reagent (Lonza, Walkersville, MD) per manufacturer's instructions and fluorescence was detected at (485<sub>EX</sub>/572<sub>EM</sub>). Differentiation values are reported as a percent of undifferentiated, control cells.

## Proliferation

Proliferation was assessed *via* bioreduction of a novel tetrazolium compound by metabolically active cells at several time-points across the growth phase using CellTiter 96 reagent (cat. G3580; Promega Corp.; Madison, WI). Briefly, cells were seeded in triplicate in a 96-well plate (1000 cells/well; 3000 c/cm<sup>2</sup>). After overnight incubation the proliferation reagent was added, the plate was incubated at 37°C for 1 hr, and absorbance was read at 490 nm. Proliferation was monitored every 24 hrs in continuously cultured cells up to 72 hrs after plating. Values are presented as daily growth rate (absorbance). Proliferation plateaued between 48 and 72 hrs, therefore the slope from 0 to 48 hrs was calculated for each sample.

## Apoptosis

Apoptosis was quantified relative to total viability using the ApoLIVGlo kit (cat. G6410; Promega Corp.), a duplexed assay designed to measure viability (live-cell protease activity) and apoptosis (caspase-3/7 activation) in the same well. Cells were plated at 10<sup>4</sup> cells/cm<sup>2</sup> in opaque, 96-well plates. After an overnight incubation in growth medium, cells were treated with tumour necrosis factor (TNF)-α (10 nmol/l) and cyclohexamide (10 µg/ml) to induce apoptosis. After 6 hrs of treatment, the viability reagent was added, incubated for 30 min at 37°C, and fluorescence read at 400<sub>EX</sub>/505<sub>EM</sub>. Subsequently, apoptosis reagent was added to the same wells, incubated at room temperature for 30 min and luminescence was measured.

## mRNA quantification (qPCR)

Total RNA was isolated from 50 to 100 mg of whole adipose tissue using the RNeasy Lipid Mini Kit (Qiagen Inc.; Hilden, Germany). One microgram of RNA was (i) analysed and quantitated using Experion (Bio-Rad; Hercules, CA), (ii) reverse transcribed using iScript (Bio-Rad), and (iii) analysed by qPCR in duplicate using iQ SYBR Supermix (Bio-Rad) on an iQ5 Real-Time PCR Detection System (Bio-Rad) along with a no-template control per gene. Reference gene ribosomal protein L13a (RPL13A) was used for normalization. The efficiencies of target and reference genes were approximately equal, and RPL13A expression was not different between groups (overall standard deviation 0.49). Primers (Bio-Rad) sequences can be found in Table 1. The relative change in mRNA expression in response to E<sub>2</sub> treatment was calculated as 2<sup>-ddCt</sup>.

**Table 1** Subject characteristics (n = 11)

Variable	Placebo	Oestradiol
Age (years)	57 ± 4	
Years past menopause	7 ± 6	
Weight (kg)	64.4 ± 9.1	
BMI (kg/m <sup>2</sup> )	25.1 ± 2.8	
Fat-free mass (kg)	41.0 ± 5.4	
Fat mass (kg)	23.7 ± 5.7	
Trunk fat mass (kg)	11.3 ± 4.1	
Leg fat mass (kg)	9.0 ± 1.7	
Fasting glucose (mg/dl)	87.0 ± 1.9	84.4 ± 2.2
Fasting insulin (µU/ml)	10.7 ± 1.1	9.6 ± 1.0
Oestradiol (pg/ml)	14.3 ± 2.3	115.6 ± 17.2**
Sex hormone-binding globulin (nm/l)	50.8 ± 5.1	53.6 ± 5.1
Leptin (ng/ml)	14.9 ± 3.3	17.4 ± 3.8*
Adiponectin (µg/ml)	17.7 ± 4.3	17.7 ± 3.1

Mean ± SD; \**P* = 0.02, \*\**P* < 0.001 compared to placebo.

## Statistical analyses

All data were examined using descriptive statistics (mean, median, S.D., range) and graphical summaries (boxplots, scatterplots). Paired *t*-tests were used to evaluate changes in response to E<sub>2</sub> compared with placebo. Depot-specific differences were analysed using unpaired *t*-tests. Analyses were done using IBM SPSS Statistics (v22; IBM, Inc.; Armonk, NY).

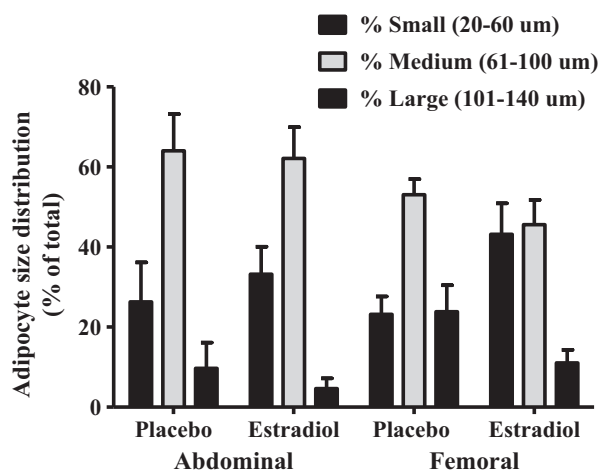
## Results

### Participant characteristics

Study participants were healthy, non-obese (BMI 20–29 kg/m<sup>2</sup>) post-menopausal women (aged 51–59 years) ranging from 1 to 15 years since their final menses (Table 1). Compared to placebo, 2 weeks of transdermal E<sub>2</sub> treatment significantly increased plasma E<sub>2</sub> (14.3 ± 2 *versus* 115.6 ± 17 pg/ml; *P* < 0.001). Plasma leptin was also significantly increased with E<sub>2</sub> relative to placebo (*P* = 0.02).

### Adipose tissue cellularity

There were no differences in cell diameter or cell size distribution between AB and FEM SAT under placebo-treated conditions (Fig. 1).



**Fig. 1** Change in adipocyte size. Change (mean  $\pm$  S.E.M.) in proportion of small (20–60  $\mu\text{m}$ ;  $P = 0.09$ ), medium (61–100  $\mu\text{m}$ ,  $P = 0.07$ ) and large (101–140  $\mu\text{m}$ ;  $P = 0.07$ ) in femoral (FEM) and abdominal AB SAT following 2 weeks of transdermal oestradiol ( $E_2$ ) compared with placebo treatment;  $n = 8$  AB, 11 FEM.

Short-term  $E_2$  treatment did not significantly change mean adipocyte size in the FEM SAT depot ( $61 \pm 19 E_2$  versus  $76 \pm 14 \mu\text{m}$  placebo;  $P = 0.09$ ), however, there was a trend towards an increase in the proportion of small (20–60  $\mu\text{m}$ ;  $P = 0.07$ ) and decreased proportion of large (100–140  $\mu\text{m}$ ;  $P = 0.07$ ) adipocytes. Adipocyte size in the AB SAT depot did not change in response to  $E_2$  ( $P = 0.51$ ).

## Adipogenic potential

In the placebo condition, ASC from FEM SAT underwent significantly less differentiation than ASC from AB SAT (Fig. 2A;  $P = 0.02$ ). Compared to placebo,  $E_2$  treatment significantly increased differentiation in ASC isolated from FEM SAT ( $P = 0.03$ ), but not those isolated from AB SAT ( $P = 0.98$ ). Proliferation was not different between SAT depots, or between  $E_2$  and placebo conditions (Fig. 3). While FEM SAT, compared to AB SAT, tended ( $P = 0.06$ ) to be less susceptible to TNF- $\alpha$ -induced apoptosis under control conditions, there was no effect of  $E_2$  treatment in either region (Fig. 2B).

## Gene expression

Transdermal  $E_2$  treatment did not significantly change mRNA expression of ERS1 and ERS2 in SAT from either FEM or AB regions (Fig. 4A). Expression of perilipin (PLIN1) mRNA was also unchanged, however, relative hormone-sensitive lipase (HSL) expression (dCt) was significantly greater ( $P = 0.03$ ) in FEM SAT after transdermal  $E_2$  treatment than after placebo treatment (Fig. 4B).

## Discussion

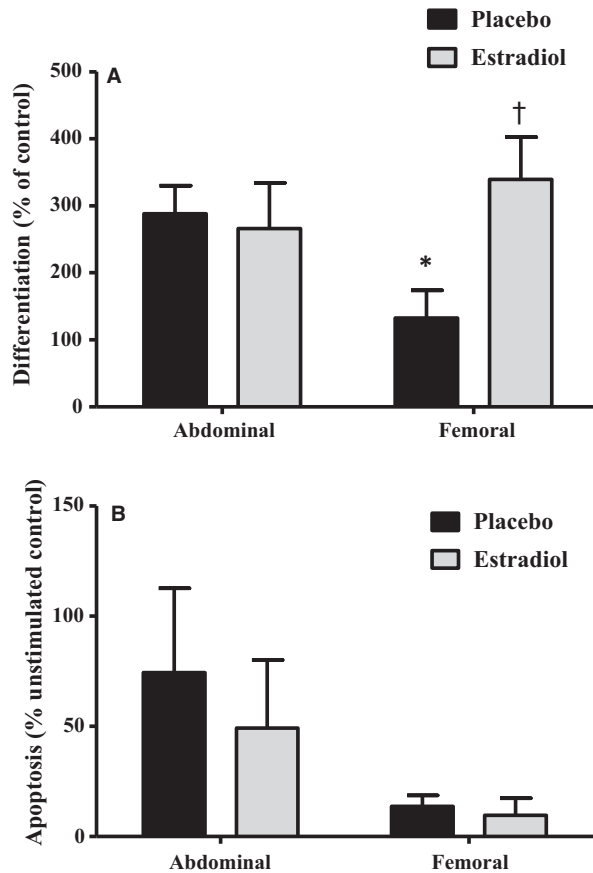
This study demonstrated that, compared with placebo, 2 weeks of transdermal  $E_2$  in post-menopausal women increased FEM, but not AB, ASC differentiation. This change was accompanied by a modest decrease in FEM adipocyte size and increased HSL mRNA expression, while rates of ASC proliferation and apoptosis were not altered by treatment with  $E_2$  compared with placebo. These preliminary observations suggest that  $E_2$  may increase the adipogenic potential of SAT in a regionally specific manner to influence SAT expansion.

Lower body SAT is associated with decreased risk for cardiometabolic disease [25] and smaller adipocytes are generally recognized as being more insulin sensitive and capable of expansion [26–29]. We recently reported that pre-menopausal women have smaller adipocytes in FEM SAT than post-menopausal women, and that 2 weeks of  $E_2$  treatment in post-menopausal women resulted in a reduction in mean adipocyte size in FEM SAT [16]. In the current study, transdermal  $E_2$  treatment also resulted in a consistent trend towards smaller adipocytes in FEM SAT. This trend did not reach significance, perhaps because of the smaller participant number ( $n = 11$ ) and relatively wide range in time since menopause. We did not observe a change in AB SAT cell size, indicating depot-specific  $E_2$  regulation of adipose tissue cellularity.  $E_2$  treatment may mediate SAT cell size, and thereby SAT expansion by modifying aspects of lipolysis [3, 30] and/or adipogenesis.

Adipose tissue expansion is largely dependent on the adipocyte progenitor population to proliferate (multiply) and differentiate (take up and store lipid) into mature adipocytes (adipogenesis). In our *ex vivo* model of ASC adipogenesis, we did not observe a difference in ASC proliferative capacity in either AB or FEM SAT collected from post-menopausal women treated with  $E_2$  and placebo. These results are in contrast to the increased proliferation of AB ASC observed

**Table 2** mRNA analysis; qPCR primer sequences

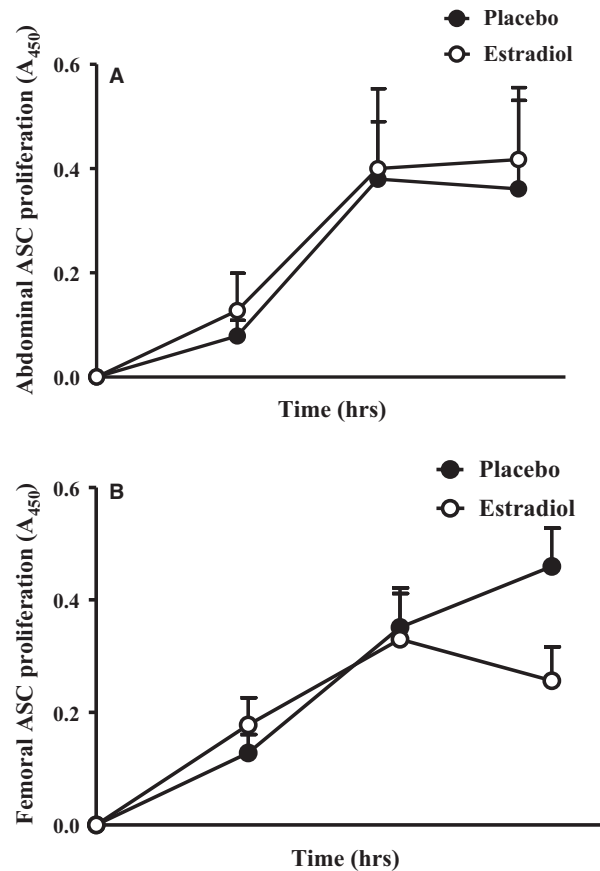
GenBank no.	Gene	5' primer	3' primer
NM_002666	PLIN1	GGAAGAATTGGAGACTGAGGAG	GGTCTTCTGCAGGGTATGTG
NM_005357	HSL	AACCAGTGCTCGGAATCACAGACA	AGTCACCAGCGACTGTGTCATTGT
NM_000125	ER $\alpha$	AGATCTTCGACATGCTGCTGGCTA	AGACTTCAGGGTGCTGGACAGAAA
NM_001437	ER $\beta$	TTGGTTTGGGTGATTGCCAAGAGC	ATGTTGAGCAGATGTTCCATGCC
NM_012423	RPL13A	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTTATTGTCAA



**Fig. 2** ASC differentiation and apoptosis. **(A)** Adipose-derived stem cell (ASC) differentiation [triglyceride accumulation;  $n = 6-8$  abdominal (AB), 8–10 femoral (FEM)]. \* $P = 0.02$  FEM versus AB SAT in placebo condition; † $P = 0.03$  FEM SAT placebo versus  $E_2$ . **(B)** ASC susceptibility to  $TNF\alpha$ -induced apoptosis ( $n = 5$ /group) in FEM and AB subcutaneous adipose tissue following 2 weeks of transdermal oestradiol ( $E_2$ ) compared to placebo.

when cells from pre- and post-menopausal women are treated with  $E_2$  *in vitro* [12, 13]. On the other hand, removal of endogenous estrogen through ovariectomy in ASC-specific ER knock-out mice was shown to increase adipocyte progenitor proliferation [31]. Together these results highlight potential differences in ASC proliferation when  $E_2$  is given *in vivo* versus *in vitro* and based on prevailing tissue ER expression.

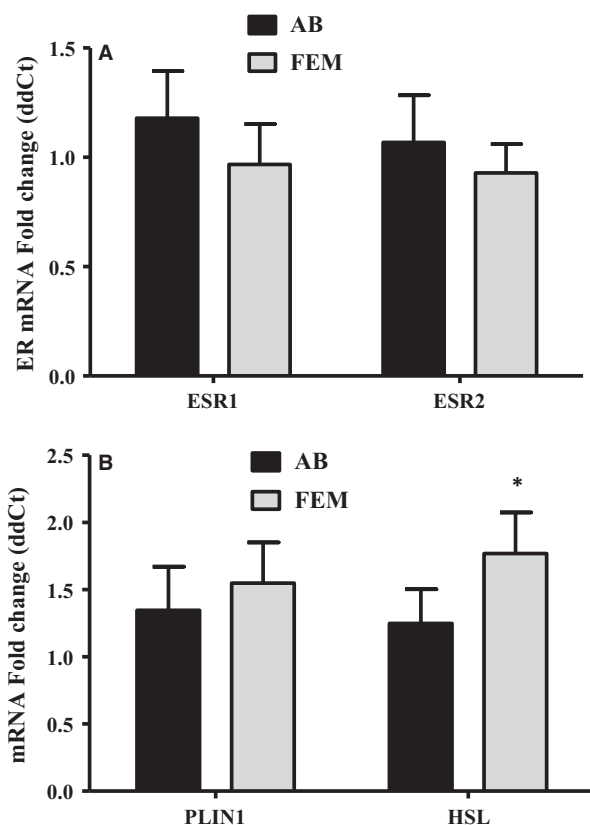
Compared to age-matched men, pre-menopausal women have a greater number of immature (early differentiated;  $aP2^+/CD68^-$ ) adipocytes, particularly in the FEM compared to AB depot, supporting a role for sex hormones in regional adipocyte recruitment [23]. We found that 2 weeks of transdermal  $E_2$ , compared with placebo, increased FEM, but not AB, ASC differentiation in post-menopausal women. We are unaware of other human *ex vivo* studies with which to compare our results. However, one study of *in vitro*  $E_2$  treatment demonstrated that AB ASC isolated from a single female participant



**Fig. 3** ASC proliferation. **(A)** Abdominal (AB) and **(B)** femoral (FEM) subcutaneous adipose-derived stem cell (ASC) proliferation ( $A_{450}$ ) following 2 weeks of transdermal oestradiol ( $E_2$ ) or placebo;  $n = 10$  AB,  $n = 9$  FEM.

resulted in a dose-dependent increase in ASC differentiation [32]. Cell line and animal studies are mixed in this regard, with some reporting increased [9, 32] and others decreased [11, 33–35] differentiation with  $E_2$  treatment. As with proliferation rates it is likely that the effect of  $E_2$  on differentiation depends on whether it is administered *in vivo* or to cells *in vitro* and the ER expression profile. We observed lower differentiation in FEM, compared to AB ASC in the placebo treated condition. This regional difference has been observed previously [23, 36, 37] and is retained in cells passaged for multiple generations [6]. Recent evidence suggests that precursors from different regions express variable levels of developmental genes (*i.e.* HOX) and have different developmental origins (reviewed in [38]).

In addition to proliferation and differentiation, adipose tissue mass also depends on progenitor cell death. Regional differences in susceptibility of adipocyte precursors to apoptosis have been observed [21], but unlike differentiation, there do not appear to be sex differences [23]. The effect of  $E_2$  on apoptosis has not been well studied, but Luo *et al.* recently showed that *in vitro* treatment of FEM ASC from pre-menopausal women decreased serum starvation-induced



**Fig. 4** mRNA expression. (A) ESR1 (ER $\alpha$ ) and ESR2 (ER $\beta$ ). (B) Perilipin 1 (PLIN1) and hormone-sensitive lipase (HSL) mRNA relative expression (2-ddCt) in abdominal (AB) and femoral (FEM) SAT with 2 weeks of transdermal E<sub>2</sub> relative to placebo. \*P<0.05

apoptosis [39]. In contrast, we found no differences in the susceptibility of ASC to TNF- $\alpha$ -induced apoptosis in response to acute E<sub>2</sub> treatment *in vivo*. This discrepancy is likely due to differences in the experimental design, i.e., *in vitro* application of E<sub>2</sub> versus *ex vivo* susceptibility to apoptosis with *in vivo* E<sub>2</sub> administration. It is also possible that age and menopausal status contributes to differential susceptibility of ASC to apoptosis as the participants in the *in vitro* study were young women (aged 22–30 years).

Oestrogen regulates lipolysis *in vitro* and *in vivo* in part through actions on the lipolytic proteins, PLIN1 and HSL [40, 41]. We measured the mRNA expression of PLIN1 and HSL in whole SAT samples and while there was no difference in the expression of PLIN1, HSL expression was significantly greater in FEM SAT following E<sub>2</sub> treatment relative to placebo. This suggests that decreases in cell size in the FEM region may be a result of a combination of increased recruitment of immature adipocytes (differentiation) and increased lipolysis.

Leptin was significantly increased in the E<sub>2</sub> condition relative to placebo. Body composition scale weight (by DXA) was only measured at baseline, but did not change over the 2-week treatment period. It is therefore not likely that there were significant changes in adipose

tissue mass that would explain an increase in leptin. There are data in humans, animals and cells supporting oestrogen regulation of leptin. Plasma leptin concentrations vary over the menstrual cycle and the phases of pregnancy [42, 43] and oestrogen has been shown to modulate leptin production and sensitivity in rodents and humans [43, 44]. In omental adipose tissue collected from non-obese women, *ex vivo* treatment with oestradiol resulted in increased leptin secretion, which was not observed in explants from men [45]. It is well accepted that oestrogen acts on total adiposity *via* interactions with its canonical receptors (ER $\alpha$  and ER $\beta$ ) in whole adipose tissue and on mature adipocytes [46–49]. Further, studies in 3T3-L1 adipocytes have demonstrated bidirectional communication between the leptin and oestrogen receptors [50]. While we did not find differences in ESR1 and ESR2 expression between E<sub>2</sub>-treated and placebo conditions in either AB or FEM SAT, it is possible that ER $\alpha$  and ER $\beta$  protein were affected. Oestrogen may also have indirect effects on leptin secretion, as leptin secretion is induced within 9 days of *in vitro* differentiation initiation in ASC [51]. The increase in plasma leptin in our participants could be indirect *via* an increase in newly differentiating adipocytes in response to transdermal E<sub>2</sub>.

The current pilot study included a relatively small number of participants, limiting our ability to detect treatment and regional differences. Moreover, given that the physiological actions of the exogenous E<sub>2</sub> treatment likely depend on time since menopause and duration of oestrogen deficiency, the fact that there was a wide range in time since menopause among our women likely added to the variability in responses. Nevertheless, these preliminary studies add novel data to the literature. We were unable to collect enough SAT in our lean participants to complete protein and activity analysis for the ERs, PLIN1 and HSL; these analyses will be required to further elucidate the effect of short-term E<sub>2</sub> treatment on these proteins.

There have been multiple studies investigating the effects of E<sub>2</sub> on the adipogenic potential of various cell lines (*e.g.* bone marrow-derived stem cells [11, 33], 3T3-L1 fibroblasts [10] and animal tissues [9, 10]). However, studies of human adipose tissue, comparing different depots and administering E<sub>2</sub> *in vivo* are lacking. Our analysis of ASC isolated from the AB and FEM tissues of post-menopausal women treated short term with transdermal E<sub>2</sub> begins to fill this void and provides proof-of-concept for *ex vivo* analysis of clinical modulation of SAT. Future studies will include a larger number of participants and control for time since menopause.

## Conclusion

Greater femoral adiposity is associated with an advantageous metabolic profile (glucose, insulin, triglycerides and HDL-C) [2], whereas abdominal fat accumulation is associated with insulin resistance and lipotoxicity [27, 52]. After menopause women begin to store less fat in the femoral region and more fat abdominally [53, 54]. It is therefore important to understand the mechanisms driving regional fat distribution. Our results demonstrate a role of E<sub>2</sub> in the regulation of femoral adipogenic potential, specifically in increasing ASC differentiation. This would suggest that the loss of endogenous oestrogens at menopause may reduce the ability of FEM SAT to recruit progenitor

cells to triglyceride-accumulating cells, while AB SAT remains unchanged in this regard. Such regional changes in adipocyte lipid storage could contribute, in part, to shifts in regional adiposity to a more android distribution, resulting in the morbidities associated with menopause.

## Acknowledgements

We thank our participants, the staffs of the UCD-Anschutz Medical Campus Clinical Translational Research Center (CTRC), and Energy Balance and Molecular and Cellular Analytical (Rachel Janssen; RT-PCR) Cores of the Colorado Nutrition and Obesity Research Unit (NORC) for their assistance in conducting this study. We also thank the members of our research group for carrying out the day-to-day activities of the project. The following awards from the National Institutes of Health supported this research: R01

DK077992, R01 DK088105, T32 AG000279, P50 HD073063, P30 DK048520, UL1 TR000154.

## Conflicts of interest

Authors have no competing financial interests to declare.

## Author Contribution

KCY and REV conceived of the experiments and wrote the manuscript. KCY performed cell culture work and assays. CBE processed biopsies and did cell sizing. RIP and DHB performed biopsies and RIP edited the manuscript.

## References

1. Ferrara CM, Lynch NA, Nicklas BJ, *et al*. Differences in adipose tissue metabolism between postmenopausal and perimenopausal women. *J Clin Endocrinol Metab*. 2002; 87: 4166–70.
2. Van Pelt RE, Evans EM, Schechtman KB, *et al*. Contributions of total and regional fat mass to risk for cardiovascular disease in older women. *Am J Physiol Endocrinol Metab*. 2002; 282: E1023–8.
3. Clegg DJ. Minireview: the year in review of estrogen regulation of metabolism. *Mol Endocrinol*. 2012; 26: 1957–60.
4. Gesta S, Tseng YH, Kahn CR. Developmental origin of fat: tracking obesity to its source. *Cell*. 2007; 131: 242–56.
5. Kirkland JL, Tchkonja T, Pirtskhalava T, *et al*. Adipogenesis and aging: does aging make fat go MAD? *Exp Gerontol*. 2002; 37: 757–67.
6. Tchkonja T, Giorgadze N, Pirtskhalava T, *et al*. Fat depot-specific characteristics are retained in strains derived from single human preadipocytes. *Diabetes*. 2006; 55: 2571–8.
7. Van HV, Rohrig K, Hauner H. Comparison of proliferation and differentiation capacity of human adipocyte precursor cells from the omental and subcutaneous adipose tissue depot of obese subjects. *Metabolism*. 2004; 53: 632–7.
8. Baglioni S, Cantini G, Poli G, *et al*. Functional differences in visceral and subcutaneous fat pads originate from differences in the adipose stem cell. *PLoS ONE*. 2012; 7: e36569.
9. Dieudonne MN, Pecquery R, Leneuve MC, *et al*. Opposite effects of androgens and estrogens on adipogenesis in rat preadipocytes: evidence for sex and site-related specificities and possible involvement of insulin-like growth factor 1 receptor and peroxisome proliferator-activated receptor gamma2. *Endocrinology*. 2000; 141: 649–56.
10. Jeong S, Yoon M. 17beta-Estradiol inhibition of PPARgamma-induced adipogenesis and adipocyte-specific gene expression. *Acta Pharmacol Sin*. 2011; 32: 230–8.
11. Okazaki R, Inoue D, Shibata M, *et al*. Estrogen promotes early osteoblast differentiation and inhibits adipocyte differentiation in mouse bone marrow stromal cell lines that express estrogen receptor (ER) alpha or beta. *Endocrinology*. 2002; 143: 2349–56.
12. Anderson LA, McTernan PG, Barnett AH, *et al*. The effects of androgens and estrogens on preadipocyte proliferation in human adipose tissue: influence of gender and site. *J Clin Endocrinol Metab*. 2001; 86: 5045–51.
13. Roncari DA, Van RL. Promotion of human adipocyte precursor replication by 17beta-estradiol in culture. *J Clin Invest*. 1978; 62: 503–8.
14. Lundholm L, Zang H, Hirschberg AL, *et al*. Key lipogenic gene expression can be decreased by estrogen in human adipose tissue. *Fertil Steril*. 2008; 90: 44–8.
15. Maneschi E, Vignozzi L, Morelli A, *et al*. FXR activation normalizes insulin sensitivity in visceral preadipocytes of a rabbit model of MetS. *J Endocrinol*. 2013; 218: 215–31.
16. Bessesen DH, Cox-York KA, Hernandez TL, *et al*. Postprandial triglycerides and adipose tissue storage of dietary fatty acids: impact of menopause and estradiol. *Obesity*. 2015; 23: 145–53.
17. Van Pelt RE, Jankowski CM, Gozansky WS, *et al*. Lower-body adiposity and metabolic protection in postmenopausal women. *J Clin Endocrinol Metab*. 2005; 90: 4573–8.
18. Coleman SR. Structural fat grafts: the ideal filler? *Clin Plast Surg*. 2001; 28: 111–9.
19. Di GM, Mendlinger S, Fertig JW. A simple method to determine fat cell size and number in four mammalian species. *Am J Physiol*. 1971; 221: 850–8.
20. Jackman MR, Steig A, Higgins JA, *et al*. Weight regain after sustained weight reduction is accompanied by suppressed oxidation of dietary fat and adipocyte hyperplasia. *Am J Physiol Regul Integr Comp Physiol*. 2008; 294: R1117–29.
21. Tchoukalova Y, Koutsari C, Jensen M. Committed subcutaneous preadipocytes are reduced in human obesity. *Diabetologia*. 2007; 50: 151–7.
22. Tchoukalova YD, Fitch M, Rogers PM, *et al*. In vivo adipogenesis in rats measured by cell kinetics in adipocytes and plastic-adherent stroma-vascular cells in response to high-fat diet and thiazolidinedione. *Diabetes*. 2012; 61: 137–44.
23. Tchoukalova YD, Koutsari C, Votruba SB, *et al*. Sex- and depot-dependent differences in adipogenesis in normal-weight humans. *Obesity (Silver Spring)*. 2010; 18: 1875–80.
24. Yu G, Floyd ZE, Wu X, *et al*. Adipogenic differentiation of adipose-derived stem cells. *Methods Mol Biol*. 2011; 702: 193–200.
25. Karpe F, Pinnick KE. Biology of upper-body and lower-body adipose tissue—link to whole-body phenotypes. *Nat Rev Endocrinol*. 2015; 11: 90–100.
26. Arner E, Westermark PO, Spalding KL, *et al*. Adipocyte turnover: relevance to

- human adipose tissue morphology. *Diabetes*. 2010; 59: 105–9.
27. **Virtue S, Vidal-Puig A.** Adipose tissue expandability, lipotoxicity and the Metabolic Syndrome—an allostatic perspective. *Biochim Biophys Acta*. 2010; 1801: 338–49.
  28. **Weyer C, Foley JE, Bogardus C, et al.** Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. *Diabetologia*. 2000; 43: 1498–506.
  29. **Yang J, Eliasson B, Smith U, et al.** The size of large adipose cells is a predictor of insulin resistance in first-degree relatives of type 2 diabetic patients. *Obesity*. 2012; 20: 932–8.
  30. **Gavin KM, Cooper EE, Raymer DK, et al.** Estradiol effects on subcutaneous adipose tissue lipolysis in premenopausal women are adipose tissue depot specific and treatment dependent. *Am J Physiol Endocrinol Metab*. 2013; 304: E1167–74.
  31. **Lapid K, Lim A, Clegg DJ, et al.** Oestrogen signalling in white adipose progenitor cells inhibits differentiation into brown adipose and smooth muscle cells. *Nat Commun*. 2014; 5: 5196.
  32. **Hong L, Colpan A, Peptan IA, et al.** 17-Beta estradiol enhances osteogenic and adipogenic differentiation of human adipose-derived stromal cells. *Tissue Eng*. 2007; 13: 1197–203.
  33. **Dang ZC, van Bezooijen RL, Karperien M, et al.** Exposure of KS483 cells to estrogen enhances osteogenesis and inhibits adipogenesis. *J Bone Miner Res*. 2002; 17: 394–405.
  34. **Heim M, Frank O, Kampmann G, et al.** The phytoestrogen genistein enhances osteogenesis and represses adipogenic differentiation of human primary bone marrow stromal cells. *Endocrinology*. 2004; 145: 848–59.
  35. **Lea-Currie YR, Monroe D, McIntosh MK.** Dehydroepiandrosterone and related steroids alter 3T3-L1 preadipocyte proliferation and differentiation. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol*. 1999; 123: 17–25.
  36. **Tchkonina T, Giorgadze N, Pirtskhalava T, et al.** Fat depot origin affects adipogenesis in primary cultured and cloned human preadipocytes. *Am J Physiol Regul Integr Comp Physiol*. 2002; 282: R1286–96.
  37. **Tchoukalova YD, Votruba SB, Tchkonina T, et al.** Regional differences in cellular mechanisms of adipose tissue gain with overfeeding. *Proc Natl Acad Sci USA*. 2010; 107: 18226–31.
  38. **White UA, Tchoukalova YD.** Sex dimorphism and depot differences in adipose tissue function. *Biochim Biophys Acta*. 2014; 1842: 377–92.
  39. **Luo S, Hao L, Li X, et al.** Adipose tissue-derived stem cells treated with estradiol enhance survival of autologous fat transplants. *Tohoku J Exp Med*. 2013; 231: 101–10.
  40. **Darimont C, Delansorne R, Paris J, et al.** Influence of estrogenic status on the lipolytic activity of parametrial adipose tissue *in vivo*: an *in situ* microdialysis study. *Endocrinology*. 1997; 138: 1092–6.
  41. **D'Eon TM, Souza SC, Aronovitz M, et al.** Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and nongenomic regulation of lipogenic and oxidative pathways. *J Biol Chem*. 2005; 280: 35983–91.
  42. **Hardie L, Trayhurn P, Abramovich D, et al.** Circulating leptin in women: a longitudinal study in the menstrual cycle and during pregnancy. *Clin Endocrinol (Oxf)*. 1997; 47: 101–6.
  43. **Shimizu H, Shimomura Y, Nakanishi Y, et al.** Estrogen increases *in vivo* leptin production in rats and human subjects. *J Endocrinol*. 1997; 154: 285–92.
  44. **Clegg DJ, Brown LM, Woods SC, et al.** Gonadal hormones determine sensitivity to central leptin and insulin. *Diabetes*. 2006; 55: 978–87.
  45. **Casabiell X, Pineiro V, Peino R, et al.** Gender differences in both spontaneous and stimulated leptin secretion by human omental adipose tissue *in vitro*: dexamethasone and estradiol stimulate leptin release in women, but not in men. *J Clin Endocrinol Metab*. 1998; 83: 2149–55.
  46. **Davis KE, Neinast MD, Sun K, et al.** The sexually dimorphic role of adipose and adipocyte estrogen receptors in modulating adipose tissue expansion, inflammation, and fibrosis. *Mol Metab*. 2013; 2: 227–42.
  47. **Barros RP, Gustafsson JA.** Estrogen receptors and the metabolic network. *Cell Metab*. 2011; 14: 289–99.
  48. **Dieudonne MN, Leneuve MC, Giudicelli Y, et al.** Evidence for functional estrogen receptors alpha and beta in human adipose cells: regional specificities and regulation by estrogens. *Am J Physiol Cell Physiol*. 2004; 286: C655–61.
  49. **Wend K, Wend P, Krum SA.** Tissue-specific effects of loss of estrogen during menopause and aging. *Front Endocrinol (Lausanne)*. 2012; 3: 19.
  50. **Yi KW, Shin JH, Seo HS, et al.** Role of estrogen receptor-alpha and -beta in regulating leptin expression in 3T3-L1 adipocytes. *Obesity*. 2008; 16: 2393–9.
  51. **Mitterberger MC, Lechner S, Mattesich M, et al.** Adipogenic differentiation is impaired in replicative senescent human subcutaneous adipose-derived stromal/progenitor cells. *J Gerontol A Biol Sci Med Sci*. 2014; 69: 13–24.
  52. **Faubion SS, Kuhle CL, Shuster LT, et al.** Long-term health consequences of premature or early menopause and considerations for management. *Climacteric*. 2015; 18(4): 483–91.
  53. **Ley CJ, Lees B, Stevenson JC.** Sex- and menopause-associated changes in body-fat distribution. *Am J Clin Nutr*. 1992; 55: 950–4.
  54. **Gambacciani M, Ciaponi M, Cappagli B, et al.** Body weight, body fat distribution, and hormonal replacement therapy in early postmenopausal women. *J Clin Endocrinol Metab*. 1997; 82: 414–7.