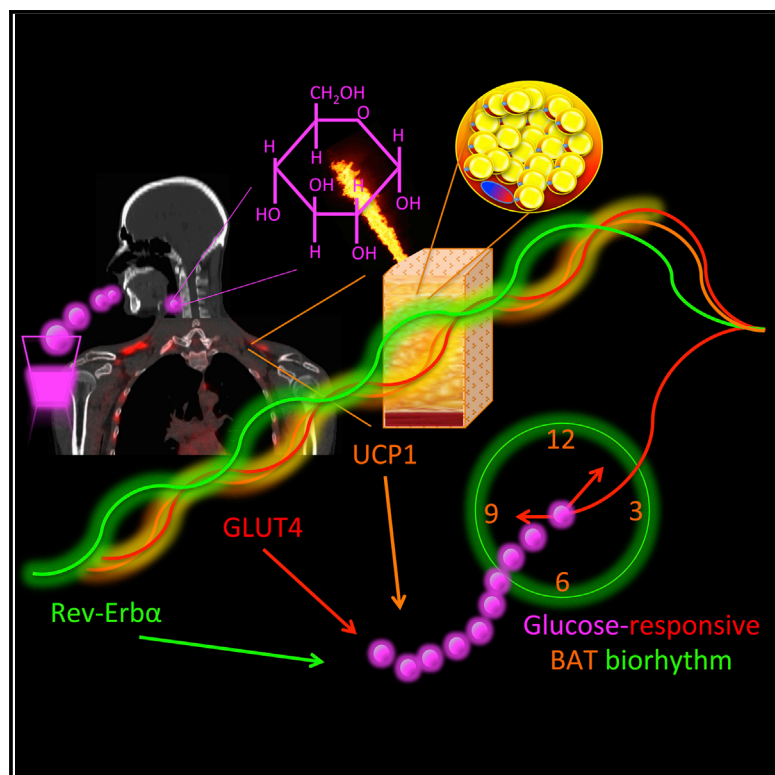


Cell Metabolism

Brown Adipose Tissue Exhibits a Glucose-Responsive Thermogenic Biorhythm in Humans

Graphical Abstract



Authors

Paul Lee, Ron Bova,
Lynne Schofield, ..., Matt A. Govendir,
Louise Emmett, Jerry R. Greenfield

Correspondence

p.lee@garvan.org.au

In Brief

Lee et al. reveal how glucose utilization by brown fat in humans is coupled with heat production in a circadian manner. Higher brown fat abundance correlates with lesser glycemia variability, suggesting that brown fat may help buffer glucose fluctuations and maintain whole-body glucose homeostasis over time.

Highlights

- Brown fat utilizes glucose as substrate fuel to produce heat in humans
- Human brown fat exhibits a thermogenic circadian rhythm
- Brown fat circadian rhythm is glucose responsive
- Low brown fat abundance is associated with greater glycaemic fluctuations



Brown Adipose Tissue Exhibits a Glucose-Responsive Thermogenic Biorhythm in Humans

Paul Lee,^{1,2,7,*} Ron Bova,³ Lynne Schofield,^{1,2} Wendy Bryant,⁵ William Dieckmann,⁶ Anthony Slattery,⁴ Matt A. Govendir,¹ Louise Emmett,⁴ and Jerry R. Greenfield^{1,2,5,7}

¹Diabetes and Metabolism Division, Garvan Institute of Medical Research, Sydney, NSW 2010, Australia

²Department of Endocrinology

³Department of Surgery

⁴Department of PET and Nuclear Medicine

⁵Diabetes Centre

St. Vincent's Hospital, Sydney, NSW 2010, Australia

⁶Department of Positron Emission Tomography, National Institutes of Health, Bethesda, MD 20892, USA

⁷Faculty of Medicine, University of New South Wales, Sydney, NSW 2052, Australia

*Correspondence: p.lee@garvan.org.au

<http://dx.doi.org/10.1016/j.cmet.2016.02.007>

SUMMARY

High abundance of brown adipose tissue (BAT) is linked to lower glycaemia in humans, leading to the belief that BAT may protect against diabetes. The relationship between BAT glucose utilization and systemic glucose homeostasis has not been defined. In this paper we have characterized glycaemic excursions and BAT thermogenic responses in human brown adipocytes, BAT explants, and healthy adults through supraclavicular temperature profiling, revealing their circadian coupling in vivo and in vitro, orchestrated by UCP1, GLUT4, and Rev-erb α biorhythms. Extent of glycated haemoglobin also correlated positively with environmental temperature among community-dwelling patients. These data uncover potential crosstalk between BAT and glucose regulatory pathways, evident on cellular, tissue, individual, and population levels, and provide impetus to search for BAT harnessing strategies for therapeutic purposes.

INTRODUCTION

Brown adipose tissue (BAT) generates heat by uncoupling oxidative phosphorylation through the action of mitochondrial uncoupling protein 1 (UCP1). In cold-exposed rodents, this process consumes remarkable quantities of glucose and lipids, far exceeding hepatic and skeletal muscle capacity (Bartelt et al., 2011). Accordingly, BAT induction reverses hyperglycaemia and protects animals against diabetes (Schrauwen et al., 2015).

Rediscovery of BAT in humans (hBAT) by ¹⁸F-deoxyglucose (FDG)-positron emission tomography (PET)/CT has driven global research interest into therapeutic potential of BAT recruitment as diabetes treatment (Spiegelman, 2013). BAT detection by cold-stimulated PET/CT builds upon high FDG uptake of hBAT to reveal its activity, supporting hBAT as a glucose-utilizing tissue. It is widely assumed that its high FDG uptake indicates its partic-

ipation in whole-body glucose metabolism, at least during cold exposure. This is corroborated by cross-sectional studies revealing lower glycaemia among adults with greater hBAT abundance (Lee et al., 2010; Matsushita et al., 2014), stimulation of glucose uptake in hBAT by cold and insulin (Orava et al., 2011), and improvement of insulin sensitivity following hBAT recruitment (Chondronikola et al., 2014; Lee et al., 2014b). However, direct evidence associating hBAT activity with glycaemic excursions is lacking. Whether hBAT activity relates to whole-body glycaemia in the absence of cold exposure is not known. This remains a fundamental question in the quest of BAT-based therapeutics.

In this study, we explored the relationship between hBAT and glucose metabolism in a series of whole-body and in vitro studies in humans. We hypothesized that hBAT regulates glucose fluxes and plays a determinative role in whole-body glucose homeostasis.

RESULTS AND DISCUSSION

Supraclavicular Skin Temperature Is a Surrogate for hBAT Status

Cold-stimulated PET/CT provides only a single snapshot of hBAT FDG uptake in time. In contrast, supraclavicular (SCV) skin temperature profiling, previously shown to reflect BAT activity (Boon et al., 2014; Jang et al., 2014; Lee et al., 2011), can be applied continuously and may be a feasible tool of capturing hBAT activity in real time. To validate this methodology, eighteen healthy volunteers (29 \pm 4 years old, 15 men, body mass index [BMI] 23 \pm 2 kg/m²) underwent cold-stimulated PET/CT scanning with concurrent measurement of skin temperature responses. hBAT was present in all volunteers by PET/CT criteria (96 \pm 11 g, mean standard uptake value [SUV] 3.4 \pm 0.8) (Figures 1A and 1B). Core temperature was unchanged, but mean skin temperature fell and SCV skin temperature increased upon cold exposure (Figure 1C), consistent with maintenance of thermal balance via activation of insulative (5% \pm 6%, p < 0.01) and BAT thermogenic (3% \pm 2%, p < 0.01) responses. Supraclavicular temperature response (STR) during cold exposure correlated positively with BAT activity (Figure 1D). These results

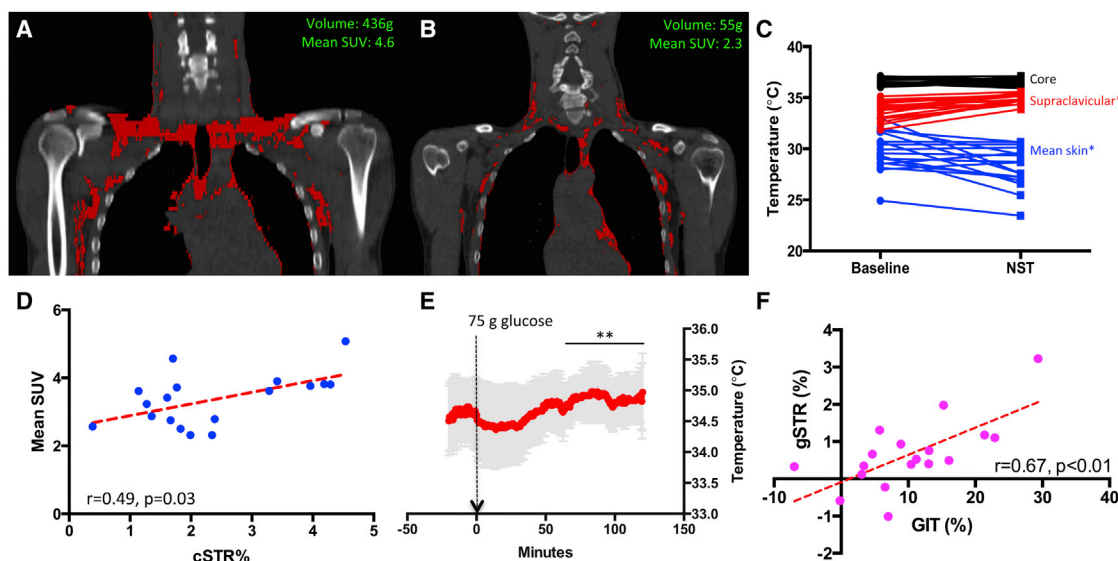


Figure 1. BAT, Thermogenic Response, and Glucose-Induced Thermogenesis

Cold-stimulated PET/CT scanning revealed BAT positivity in all volunteers. (A) and (B) show representative images of volunteers with high and low abundance, respectively. During personalized cold exposure, mean skin temperature decreased while supraclavicular temperature rose (C). Core temperature remained unchanged. Mean BAT activity (measured in standardized uptake value [SUV]) correlated positively with cold-stimulated supraclavicular temperature response (cSTR) (D). During oral glucose tolerance test (OGTT), BAT temperature progressively increased and mean temperature was significantly greater than baseline after 60 min (E). Supraclavicular temperature response (gSTR) correlated positively with glucose-induced thermogenesis (GIT) (F). * $p < 0.05$, ** $p < 0.01$.

thus validate STR profiling as a surrogate for hBAT status and provide a tool to explore relationship between STR (i.e., hBAT activity) and glucose metabolism in a real-time continuous fashion.

hBAT Thermogenesis Increases during Glucose Challenge

The same volunteers with known BAT status then underwent an oral glucose tolerance test (OGTT) on a separate study day at thermoneutrality (24°C). We hypothesized that STR would increase during OGTT if hBAT was indeed a glucose-utilizing organ. STR increased progressively during OGTT (Figure 1E), exceeding baseline by $0.2^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ at 120 min ($p = 0.04$) and correlated positively with glucose-induced thermogenesis (GIT) (Figure 1F). A positive correlation was also observed between STR during cold exposure and OGTT within subject ($r = 0.69$, $p < 0.01$), further supporting BAT as a common regulatory origin of the two processes. In contrast, no relationships existed between changes in GIT and/or STR with blood pressure or heart rate, indicating STR was not a phenomenon of general sympathetic arousal (see Table S1 available online). No relationships were observed between BAT abundance and prandial hormonal and insulin-related indices (Table S1).

BAT is a regulator of GIT in animals, but how GIT is controlled in humans has remained enigmatic (Himmels-Hagen, 1989, 1995; Isler et al., 1987). Our findings provide evidence linking hBAT activation to GIT response during glucose challenge. This is concordant with animal studies identifying BAT as a glucose-clearing organ (Bartelt et al., 2011). Overall BAT positivity among our healthy volunteers could have reduced the sensitivity in detecting relationships between BAT versus insulin-related parameters.

Glucose Utilization and Transport in Human Brown Adipocytes

To ascertain a causal relationship between BAT activity and glucose homeostasis, we characterized glucose utilization in primary human brown adipocyte culture, a hBAT model independent of systemic influences. Primary human brown (hBA) and white adipocyte (hWA) cultures were established from deep and subcutaneous neck adipose tissue, regions known to harbor brown and white adipocyte precursors, respectively (Jespersen et al., 2013). Differentiated hBA expressed brown/beige but not white fat genes (Figure 2A). Transcripts of *GLUT4* and *UCP1* mRNA were significantly more abundant in hBA than in hWA (Figure 2A). Accordingly, brown adipogenesis was accompanied by upregulation of *UCP1* and *GLUT4* (Figure 2B), but not *GLUT1* protein (Figure S1A). Glycolysis, glycolytic capacity, and fatty acid oxidation were all significantly greater in hBA compared to hWA (Figures S1B–S1E).

Although PET/CT demonstrates glucose/fatty acid uptake in human BAT (Ouellet et al., 2012), the extent of glucose/fatty acid-utilizing capacity of hBAT has remained elusive to date. Our findings reveal functional glucose uptake machinery in hBA sufficient to impact cellular bioenergetics and their substrate versatility between glucose and fatty acids. These results shed mechanistic insight on the close correlations between STR and GIT observed in healthy volunteers.

Human Brown Adipocytes Display Circadian Glucose Uptake

Coupling of glucose utilization and *UCP1* expression in hBA begs the question of whether hBA exhibits an intrinsic glucose-responsive rhythm. Recent studies have discovered circadian

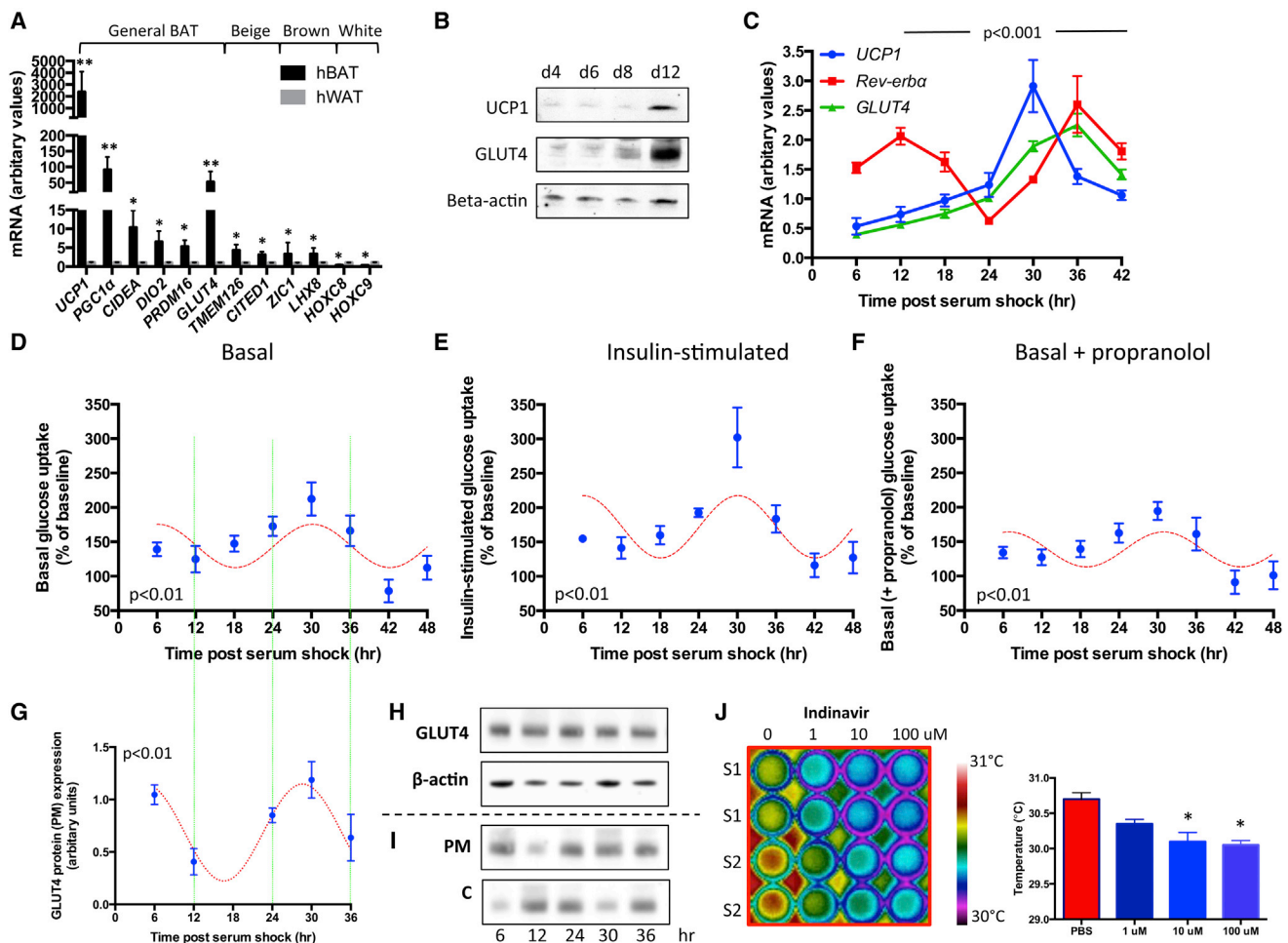


Figure 2. Human Brown Adipocytes Exhibit Glucose-Responsive Circadian Rhythm

(A) shows higher expression of general BAT, beige, and brown gene markers in differentiated human brown adipocytes (hBA) compared to white adipocytes (hWA). UCP1 and GLUT4 were progressively upregulated during brown adipogenesis (B). Circadian rhythmicity of *UCP1*, *Rev-erbα*, and *GLUT4* were observed in hBA following serum synchronization (C). Glucose uptake in hBA manifested circadian rhythm in basal state (D) and was augmented by insulin (20 nM) (E) but unaffected by propranolol (1 μM) (F). Changes in glucose uptake were mirrored by plasma membrane (PM) GLUT4 expression (G and I) but not whole-cell or cytosolic (C) GLUT4 level (H and I). Indinavir decreased hBA heat production in a dose-dependent manner, as shown by representative thermogram from 2 hBA specimens (S1 and S2) in duplicates (J). Graph next to thermogram represents mean temperature changes from four hBA specimens. * $p < 0.01$, ** $p < 0.001$, compared to hWA or PBS treatment. Data are presented as mean \pm SD.

thermogenic plasticity of rodent BAT under the control of nuclear receptor *Rev-erbα* (Gerhart-Hines et al., 2013). These led us to hypothesize the existence of a functional circadian rhythm entrained in hBA orchestrated by UCP1, *Rev-erbα*, and GLUT4 oscillations.

First, we profiled *UCP1*, *GLUT4*, and *Rev-erbα* excursions in hBA synchronized by serum shock. Figure 2C shows rhythmic *UCP1* expression antiphase to *Rev-erbα* in hBA, with *GLUT4* tracking *UCP1* expression ($p < 0.001$). As a result, the nadir of *Rev-erbα* preceded peaks of *UCP1* and *GLUT4*. Next, functional significance of rhythmic gene changes was explored by glucose uptake measurement. Basal glucose uptake demonstrated significant rhythmicity (Figure 2D) with doubling of its amplitude occurring at the fitted acrophase (~30 hr) (Table S3). These findings unveil the presence of a functional glucose-utilizing rhythm in human brown adipocytes.

Insulin and adrenergic agents are both known stimuli of brown adipose thermogenesis. We thus investigated if glucose-utilizing rhythms are insulin and β -adrenergic sensitive. Insulin augmented the observed circadian rhythm by $19\% \pm 4\%$ ($p < 0.01$) [Figure 2E]. However, coincubation with a pan- β antagonist had no effect on glucose uptake ($-3\% \pm 4\%$, $p = 0.45$) (Figure 2F). These results suggest the existence of nonadrenergic mediators capable of modulating hBA metabolic activities. This is consistent with reports showing greater BAT activation by cold exposure than pharmacological sympathetic stimuli (Cypess et al., 2012; Vosselman et al., 2012). Indeed, nonadrenergic BAT-activating agents, such as adenosine (Gnad et al., 2014), have been described recently. Future studies should dissect the contribution of adrenergic and nonadrenergic factors on hBA bioenergetics and glucose metabolism.

Because GLUT4 was significantly upregulated in mature hBA (Figure 2B), we hypothesized GLUT4 expression to be an effector of observed glucose uptake rhythmicity. As expected from its $t_{1/2}$, UCP1 protein did not change (data not shown) (Nedergaard and Cannon, 2013). Total cellular GLUT4 protein was also unchanged (Figure 2H). In contrast, plasma membrane GLUT4 abundance varied in a rhythmic fashion (Figures 2G and 2I), with peak and trough expression mirroring circadian glucose uptake (Figure 2D). Insulin treatment alone without serum synchronization abolished the observed rhythm (Figures S1G and S1H). Taken together, these results indicate circadian cytosolic-membrane GLUT4 trafficking as a mechanism contributing to cell-autonomous glucose uptake oscillations in hBA.

GLUT4 Upregulation Is Accompanied by Heat Production in Human Brown Adipocytes

As the primary function of BAT is heat production, we asked whether glucose utilization indeed couples with BAT thermogenesis. Heat production was quantified by high-sensitivity infrared thermography during brown adipogenesis. Mature hBA that expressed high abundance of UCP1 and GLUT4 (Figure 2B) produced the greatest amount of heat ($0.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$, $p < 0.001$) (Figure S1F). Following indinavir treatment, a GLUT4-inhibiting compound (Konrad et al., 2002), glucose uptake decreased (Figure S1I), which in turn repressed heat production in a dose-dependent manner (Figure 2J). These results thus underscore regulatory links between UCP1 and GLUT4 in fueling brown adipose functional thermogenic machinery.

Crosstalk between hBAT Thermogenesis and Circadian Glucose Homeostasis In Vivo

If hBA exhibits a cell-autonomous thermogenic rhythm, does a corresponding rhythm modulate systemic glucose homeostasis? We explored this possibility by performing simultaneous continuous STR and subcutaneous glucose monitoring over a 12 hr period in 15 volunteers (27 ± 4 years old, 12 men, BMI 23 ± 3 kg/m²). They were stratified into three groups based on BAT activity and differed only by BAT status (Table S2), thereby allowing assessment of STR-glycaemia relationships independent of anthropometric/hormonal confounders. Time-dependent cross-correlation ($R^2 = 0.58$, $p < 0.001$) coupled STR and glucose excursions among BAT positive volunteers as a whole (Figure 3A). No relationships were observed between STR, core, and/or abdominal/mediastinal skin temperatures (Table S1). Among individuals with the highest BAT abundance (BAT_{high}), a negative correlation ($R^2 = 0.31$, $p < 0.0001$) was detected between glucose and STR (Figure 3B), with STR leading glycaemic changes by three time periods (Figure 3D). In contrast, glucose correlated positively with STR ($R^2 = 0.10$, $p < 0.001$) among individuals with low BAT abundance (BAT_{low}) (Figure 3C), and glycaemic changes preceded STR evolution among these individuals (Figure 3F). No relationships were detected between STR or glucose excursions among volunteers devoid of any BAT (BAT_{neg}) (Figures S2A–S2D). BAT activity did not relate to maximal or mean glycaemia (Table S1) but correlated negatively with overall glycaemic variability (Figure 3E), which was greatest among BAT_{neg} individuals (Figures S2E and S2F).

Our real-time BAT-glucose profiling in healthy volunteers revealed an unexpected dichotomous relationship between ther-

mogenesis and glucose excursion dependent on BAT abundance. Among BAT_{low} individuals, BAT responds to systemic glycaemic fluctuation, conceivably functioning as a glucose sink (Kajimura et al., 2015). In contrast, BAT thermogenesis modulates glycaemia directly among BAT_{high} individuals. Since BAT activity was more than doubled in these volunteers with “superactive BAT” compared to BAT_{low} individuals, it is tempting to speculate an activity-dependent BAT threshold potentiating glucose-clearing dynamics. One cannot exclude the possibility that a yet-to-be-defined systemic signal mediates glycaemic changes in BAT_{high} individuals. Although no relationships were observed between total glucose output and BAT activity, possibly a result of the inclusion of a healthy euglycaemic cohort, high BAT abundance was associated with significantly lesser glycaemic variability, a protective factor against dysmetabolism (Blaak et al., 2012). Whether BAT_{neg} individuals, who exhibited the greatest glycaemic variability, are at risk of diabetes warrants further prospective investigations.

hBAT Retains *Rev-erb α* Circadian Patterning Ex Vivo

To definitively confirm that STR indeed represents an underlying BAT biorhythm, we repeated experiments using hBAT ex vivo. Freshly harvested hBAT explants (Figure 4B) were cultured and UCP1 expression studied in a circadian fashion (Figure 4A). The underlying hypothesis is that hBAT explants retained their intrinsic thermogenic rhythm entrained in vivo. We mimicked physiologic sleep-wake cycle by extracting mRNA throughout extrapolated day/night periods. A circadian rhythm of *UCP1*, peaking during the day and reaching a nadir at night, was observed, and *UCP1* expression rose at dawn, closely resembling in vivo STR responses (Figure 4C). *GLUT4* tracked *UCP1* rhythm closely, while *Rev-erb α* manifested a reciprocal relationship with *UCP1* and *GLUT4* (Figure 4C). Their imputed parameters from periodic regression analysis were concordant with those observed in primary brown adipocytes (Table S3).

The physiology of the “dawning” phenomenon is poorly understood. In rodents, BAT activity rises just before waking (Gerhart-Hines et al., 2013), conceivably a survival mechanism preparing animals for thermal defense during relatively cold-challenged wakeful hours. Hormones that rise at dawn, such as catecholamines and cortisol, have been regarded as signals prompting the waking response in humans. We therefore examined thermogenic genes in hBAT explants incubated with adrenergic or glucocorticoid antagonists to investigate their interdependence. Neither affected expression of *UCP1*, *GLUT4*, or *Rev-erb α* (Table S3). Taken together, BAT explants harbored the highest abundance of *UCP1* and *GLUT4* transcripts at hours equating to prewaking period unaltered by adrenocortical or sympathetic hormonal influences, further supporting the functionality of a bona fide BAT thermogenic axis in humans.

Study Limitations

We emphasize that STR is a surrogate measurement of BAT activity and does not represent a direct quantification of BAT thermogenesis per se. Confounding by skin perfusion/insulation is unlikely, given absent relationships between BAT and non-SCV skin temperatures. Although we captured *Rev-erb α* and *GLUT4* circadian rhythms in vitro and ex vivo, whether the

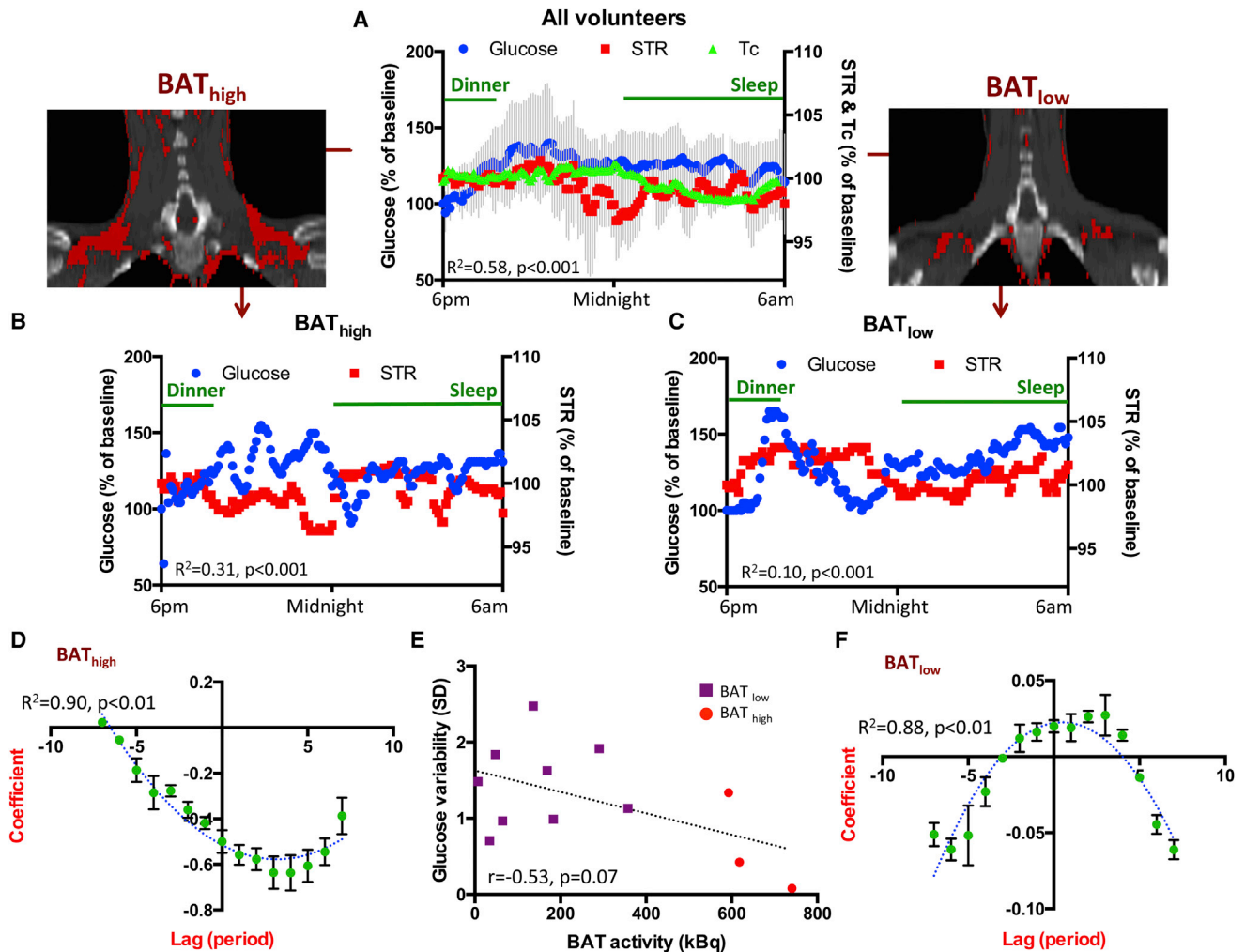


Figure 3. BAT Thermogenic and Glucose Circadian Rhythms in Healthy Volunteers

(A) shows 12 hr (6 p.m. to 6 a.m.) continuous tracing of supraclavicular temperature response (STR), core temperature (Tc), and subcutaneous glucose excursions in 12 healthy volunteers, stratified into BAT high (BAT_{high}) and low (BAT_{low}) status. Both BAT_{high} (B) and BAT_{low} (C) individuals manifested significant cross-correlations between STR and glucose. (D) and (F) are cross-correlation plots between glucose excursion and STR. A positive lag indicates that the first series (i.e., glucose) *leads* the second series (i.e., STR), while a negative lag indicates that the first series (i.e., glucose) *follows* the second (i.e., STR). In this regard, STR tracked glucose excursions among BAT_{low} volunteers as signified by weakly positive correlative coefficients (F). In contrast, STR led changes in glycaemia among BAT_{high} subjects, with strongly negative correlation coefficients (D). In other words, STR is a leading indicator among BAT_{high} individuals and predicts glucose level best three periods later. Total BAT activity correlated negatively with glucose variability (E). Data are presented as mean \pm SD.

same entrainment is present in vivo cannot be proven within the confines of clinical research.

An Evolutionary Perspective

In the survival hierarchy, core temperature defense trumps food/water. Our ancestors frequently faced cold/famine as simultaneous environmental challenges. Active BAT may be a survival organ, allowing defense against cold, minimization of shivering, and thus efficient food hunting/gathering in a sub-thermal environment. A hBAT-glucose biorhythm could represent a constitutive component of a fundamental survival mechanism ensuring protected BAT substrate utility independent of nutrient-related hormonal signals, as BAT activity restrained by nutrient scarcity could prove detrimental when thermal defense is life sustaining.

Clinical Implications

Modernization has moved humans indoors. Hypothermia is an infrequent threat. However, active BAT may equip the body with an intrinsic glycemic buffering system. Coupling of STR with glucose-induced thermogenesis led us to speculate that “BAT deficiency” could be a clinical stage potentially heralding fasting/prandial hyperglycaemia development. So *does BAT abundance affect glycaemia over time?* Because BAT is known to be most abundant in winter (Saito et al., 2009), we probed this question by examining the relationship between glycaemia and outdoor temperature in 65,535 patients who had blood tests throughout a 1-year period. Environmental temperature correlated positively with glycated haemoglobin, a measurement of overall glucose control (Figure S3). Whether this is a benefit of greater BAT abundance merits further studies.

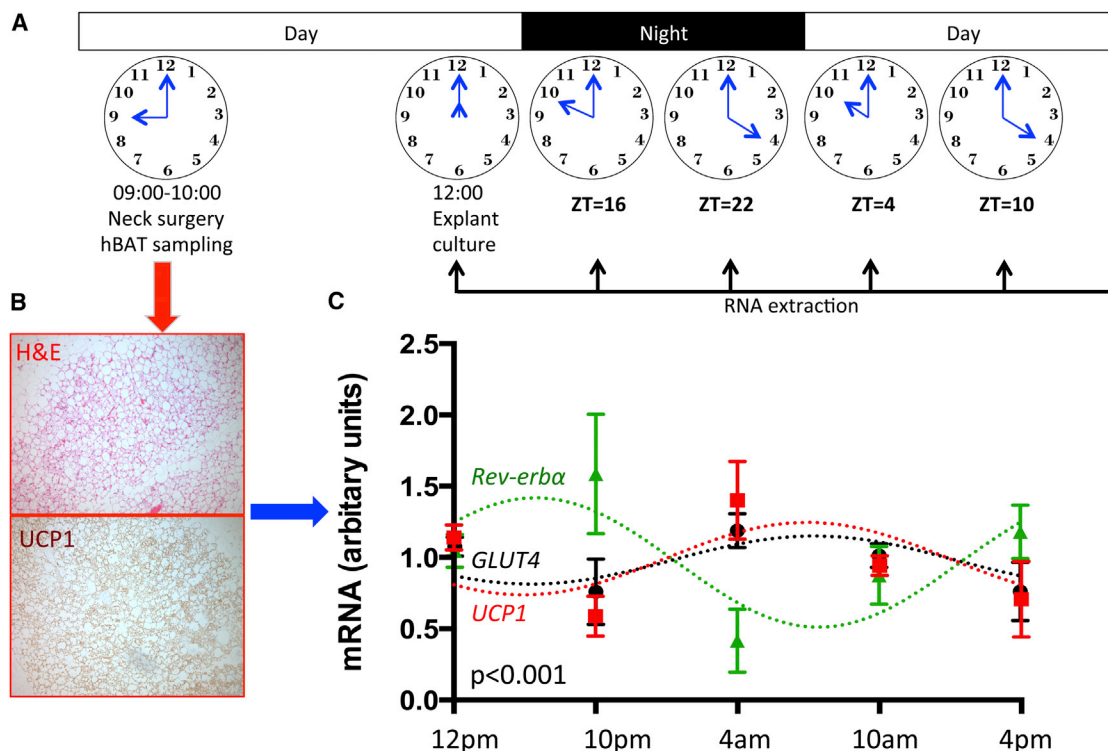


Figure 4. Human BAT Tissue Explant Exhibits Circadian *UCP1* Rhythmicity

Perithyroidal fat showed features of BAT with multilobulated lipid-filled adipocytes on H&E (20 \times) (B, top) and strong *UCP1* staining on immunohistochemistry (20 \times) (B, bottom). Explant tissue was established and cultured after surgical biopsies to allow circadian examination according to chronogram indicating specified time points corresponding to Zeitgeber time (ZT) 16, 22, 4, and 10, with 6 a.m. as ZT = 0 (A). Transcripts of *UCP1* and *GLUT4* mRNA showed significant rhythmicity reciprocating the trend of *Rev-erba* (C). Data are presented as mean \pm SD.

Summary and Conclusions

In a series of combined in vitro and in vivo experiments, we have simultaneously captured glucose and BAT thermogenic bio-rhythms, revealing their bidirectional modulatory relationships. BAT participates in glucose clearance, yet in high abundance it may be capable of dictating glycaemic excursions in healthy humans. Collectively, our report provides evidence supporting a therapeutically relevant glucose-modulatory role of active human BAT beyond cold-induced thermogenesis.

EXPERIMENTAL PROCEDURES

Clinical Studies

Eighteen healthy volunteers were recruited via local advertisement to participate in the Brown Adipose Tissue Thermogenesis in Humans (BATMAN) Study (ANZCTR: ACTRN1261500688583). The St. Vincent's Hospital Human Research Ethics Committee approved the study. All volunteers participated in cold-stimulated PET-CT scanning and main metabolic study. Twelve of the volunteers and an additional three BAT-negative volunteers also underwent overnight continuous glucose and BAT thermogenic monitoring.

Cold-Stimulated PET/CT Study

This study quantifies BAT abundance and allows assessment of relationship between metabolic profile and BAT status. Volunteers were studied after 6 hr of fasting. Each was exposed to 2 hr of personalized cooling to elicit maximal nonshivering thermogenesis response. All volunteers received a 50 MBq dose of 18 F-fluoro-deoxyglucose (FDG) for PET/CT scanning. In the circadian study, volunteers were stratified into those with high (BAT_{high}) and low (BAT_{low}) BAT status based on midrange BAT activity within the whole group

(Table S2). Further details on personalized cooling and PET/CT analysis are described in Supplemental Information.

Main Metabolic Test

Volunteers attended on a separate date after an overnight fast to undergo metabolic testing at thermoneutrality (24 $^{\circ}$ C). Twenty minutes of resting energy expenditure (EE) measurement was obtained by indirect calorimetry as baseline, after which volunteers were given 75 g of glucose (Carbotest) in a standard oral glucose tolerance test (OGTT). EE measurement continued throughout this period at 20 min interval for 2 hr. Supraclavicular temperature response (STR) was measured by iButtons (Maxim, San Jose, CA), as previously described (Lee et al., 2014a), and subcutaneous glucose levels by Dexcom continuous glucose monitoring system (CGMS). Fifteen volunteers wore iButtons and Dexcom for a continuous 12 hr period to study circadian BAT and glucose rhythms.

Laboratory Measurements

Blood samples were obtained during OGTT at -20 , 0, 10, 20, 30, 60, 90, and 120 min for hormone/substrate measurements. Plasma glucose was determined by the glucose oxidase method using a YSI glucose analyzer (model 2300 STAT PLUS 230V, YSI, Inc., Yellow Springs, OH). Prandial hormones were measured by commercially available immunoassays.

In Vitro Studies

Primary Adipocyte/Adipose Explant Culture

Primary adipocyte/explant cultures were established from 21 patients (12 women, 51 ± 12 years old, BMI 25 ± 3 kg/m 2) undergoing elective neck surgery (Graves' disease, multinodular goitre, thyroid adenoma/cancer). All patients were biochemically euthyroid.

Circadian Study

Serum synchronization was performed by incubating differentiated adipocytes with 50% horse serum for 2 hr. After PBS washes, cells were incubated in basal media supplemented with 0.5% FCS with/without 20 nM insulin or

1 μ M propranolol, followed by RNA/protein extraction and glucose uptake analysis at designated time points, as previously described (Yamamoto et al., 2011).

Gene/Protein Expression and Bioenergetics

Standard techniques were used for RNA/protein extraction and analysis by semiquantitative real-time PCR and immunoblotting. Cellular respiration was measured by XF24-3 extracellular flux analyzer (Seahorse Bioscience) and heat production by infrared thermography (FLIR systems).

Additional clinical/laboratory details are available in [Supplemental Information](#).

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as mean \pm SD. Comparisons between results during cold exposure/OGTT were performed using repeated-measure ANOVA with Bonferroni's correction. Kruskal-Wallis test was used for comparison between volunteers stratified to BAT status. Pearson and Spearman correlation coefficients were computed to examine relations between parametric and nonparametric variables, respectively. Areas under the curve were calculated using the trapezoidal rule. Circadian rhythm analysis was performed by fitting of a cosine function to data. Details on mathematical modeling are available in [Supplemental Information](#). An α error of 0.05 was considered the threshold for statistical significance.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, three tables, and Supplemental Experimental Procedures and can be found with this article at <http://dx.doi.org/10.1016/j.cmet.2016.02.007>.

AUTHOR CONTRIBUTIONS

Conceptualization, P.L. and J.R.G.; Methodology, P.L., R.B., L.S., W.B., W.D., A.S., M.A.G., L.E., and J.R.G.; Investigation, P.L., R.B., L.S., W.B., W.D., A.S., M.A.G., L.E., and J.R.G.; Writing – Original Draft, P.L.; Writing – Review & Editing, P.L., R.B., L.S., W.B., W.D., A.S., M.A.G., L.E., and J.R.G.; Funding Acquisition, P.L., R.B., L.E., and J.R.G.

ACKNOWLEDGMENTS

P.L. was supported by an Australian National Health Medical Research Council (NHMRC) Early Career Fellowship (APP1035997). This study was supported by Project Grant (APP1088123) NHMRC, Australia, Diabetes Australia Research Trust (DART) Grant, the Royal Australasian College of Physicians (RACP) Foundations Establishment Fellowship and Ramaciotti Establishment Fellowship. The authors thank Professor Don Chisholm for helpful discussions and Ms. Samarina Musaad from LabTests New Zealand for provision of glycosylated haemoglobin data.

Received: October 13, 2015

Revised: December 18, 2015

Accepted: February 12, 2016

Published: March 10, 2016

REFERENCES

- Bartelt, A., Bruns, O.T., Reimer, R., Hohenberg, H., Ittrich, H., Peldschus, K., Kaul, M.G., Tromsdorf, U.I., Weller, H., Waurisch, C., et al. (2011). Brown adipose tissue activity controls triglyceride clearance. *Nat. Med.* 17, 200–205.
- Blaak, E.E., Antoine, J.M., Benton, D., Björck, I., Bozzetto, L., Brouns, F., Diamant, M., Dye, L., Hulshof, T., Holst, J.J., et al. (2012). Impact of postprandial glycaemia on health and prevention of disease. *Obes. Rev.* 13, 923–984.
- Boon, M.R., Bakker, L.E., van der Linden, R.A., Pereira Arias-Bouda, L., Smit, F., Verberne, H.J., van Marken Lichtenbelt, W.D., Jazet, I.M., and Rensen, P.C. (2014). Supraclavicular skin temperature as a measure of 18F-FDG uptake by BAT in human subjects. *PLoS ONE* 9, e98822.
- Chondronikola, M., Volpi, E., Børsheim, E., Porter, C., Annamalai, P., Enerbäck, S., Lidell, M.E., Saraf, M.K., Labbe, S.M., Hurren, N.M., et al. (2014). Brown adipose tissue improves whole-body glucose homeostasis and insulin sensitivity in humans. *Diabetes* 63, 4089–4099.
- Cypess, A.M., Chen, Y.C., Sze, C., Wang, K., English, J., Chan, O., Holman, A.R., Tal, I., Palmer, M.R., Kolodny, G.M., and Kahn, C.R. (2012). Cold but not sympathomimetics activates human brown adipose tissue in vivo. *Proc. Natl. Acad. Sci. USA* 109, 10001–10005.
- Gerhart-Hines, Z., Feng, D., Emmett, M.J., Everett, L.J., Loro, E., Briggs, E.R., Bugge, A., Hou, C., Ferrara, C., Seale, P., et al. (2013). The nuclear receptor Rev-erb α controls circadian thermogenic plasticity. *Nature* 503, 410–413.
- Gnad, T., Scheibler, S., von Kügelgen, I., Scheele, C., Kilić, A., Glöde, A., Hoffmann, L.S., Reverte-Salisa, L., Horn, P., Mutlu, S., et al. (2014). Adenosine activates brown adipose tissue and recruits beige adipocytes via A2A receptors. *Nature* 516, 395–399.
- Himms-Hagen, J. (1989). Brown adipose tissue thermogenesis and obesity. *Prog. Lipid Res.* 28, 67–115.
- Himms-Hagen, J. (1995). Role of brown adipose tissue thermogenesis in control of thermoregulatory feeding in rats: a new hypothesis that links thermodynamic and glucostatic hypotheses for control of food intake. *Proceedings of the Society for Experimental Biology and Medicine*. Society for Experimental Biology and Medicine 208, 159–169.
- Isler, D., Hill, H.P., and Meier, M.K. (1987). Glucose metabolism in isolated brown adipocytes under beta-adrenergic stimulation. Quantitative contribution of glucose to total thermogenesis. *Biochem. J.* 245, 789–793.
- Jang, C., Jalapu, S., Thuzar, M., Law, P.W., Jeavons, S., Barclay, J.L., and Ho, K.K. (2014). Infrared thermography in the detection of brown adipose tissue in humans. *Physiol. Rep.* 2, 2.
- Jespersen, N.Z., Larsen, T.J., Peijs, L., Dagaard, S., Homøe, P., Loft, A., de Jong, J., Mathur, N., Cannon, B., Nedergaard, J., et al. (2013). A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. *Cell Metab.* 17, 798–805.
- Kajimura, S., Spiegelman, B.M., and Seale, P. (2015). Brown and beige fat: physiological roles beyond heat generation. *Cell Metab.* 22, 546–559.
- Konrad, D., Bilan, P.J., Nawaz, Z., Sweeney, G., Niu, W., Liu, Z., Antonescu, C.N., Rudich, A., and Klip, A. (2002). Need for GLUT4 activation to reach maximum effect of insulin-mediated glucose uptake in brown adipocytes isolated from GLUT4myc-expressing mice. *Diabetes* 51, 2719–2726.
- Lee, P., Greenfield, J.R., Ho, K.K., and Fulham, M.J. (2010). A critical appraisal of the prevalence and metabolic significance of brown adipose tissue in adult humans. *Am. J. Physiol. Endocrinol. Metab.* 299, E601–E606.
- Lee, P., Ho, K.K., Lee, P., Greenfield, J.R., Ho, K.K., and Greenfield, J.R. (2011). Hot fat in a cool man: infrared thermography and brown adipose tissue. *Diabetes Obes. Metab.* 13, 92–93.
- Lee, P., Linderman, J.D., Smith, S., Brychta, R.J., Wang, J., Idelson, C., Perron, R.M., Werner, C.D., Phan, G.Q., Kammula, U.S., et al. (2014a). Irisin and FGF21 are cold-induced endocrine activators of brown fat function in humans. *Cell Metab.* 19, 302–309.
- Lee, P., Smith, S., Linderman, J., Courville, A.B., Brychta, R.J., Dieckmann, W., Werner, C.D., Chen, K.Y., and Celi, F.S. (2014b). Temperature-acclimated brown adipose tissue modulates insulin sensitivity in humans. *Diabetes* 63, 3686–3698.
- Matsushita, M., Yoneshiro, T., Aita, S., Kameya, T., Sugie, H., and Saito, M. (2014). Impact of brown adipose tissue on body fatness and glucose metabolism in healthy humans. *Int. J. Obes.* 38, 812–817.
- Nedergaard, J., and Cannon, B. (2013). UCP1 mRNA does not produce heat. *Biochim. Biophys. Acta* 1831, 943–949.
- Orava, J., Nuutila, P., Lidell, M.E., Oikonen, V., Noponen, T., Viljanen, T., Scheinin, M., Taittonen, M., Niemi, T., Enerbäck, S., and Virtanen, K.A. (2011). Different metabolic responses of human brown adipose tissue to activation by cold and insulin. *Cell Metab.* 14, 272–279.
- Quellet, V., Labbé, S.M., Blondin, D.P., Phoenix, S., Guérin, B., Haman, F., Turcotte, E.E., Richard, D., and Carpentier, A.C. (2012). Brown adipose tissue oxidative metabolism contributes to energy expenditure during acute cold exposure in humans. *J. Clin. Invest.* 122, 545–552.
- Saito, M., Okamatsu-Ogura, Y., Matsushita, M., Watanabe, K., Yoneshiro, T., Nio-Kobayashi, J., Iwanaga, T., Miyagawa, M., Kameya, T., Nakada, K., et al.

(2009). High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* 58, 1526–1531.

Schrauwen, P., van Marken Lichtenbelt, W.D., and Spiegelman, B.M. (2015). The future of brown adipose tissues in the treatment of type 2 diabetes. *Diabetologia* 58, 1704–1707.

Spiegelman, B.M. (2013). Banting Lecture 2012: Regulation of adipogenesis: toward new therapeutics for metabolic disease. *Diabetes* 62, 1774–1782.

Vosselman, M.J., van der Lans, A.A., Brans, B., Wierds, R., van Baak, M.A., Schrauwen, P., and van Marken Lichtenbelt, W.D. (2012). Systemic β -adrenergic stimulation of thermogenesis is not accompanied by brown adipose tissue activity in humans. *Diabetes* 61, 3106–3113.

Yamamoto, N., Ueda, M., Sato, T., Kawasaki, K., Sawada, K., Kawabata, K., and Ashida, H. (2011). Measurement of glucose uptake in cultured cells. *Current Protocols in Pharmacology*, ed. S.J. Enna *Chapter 12*, Unit 12 14 11–22.