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Perspective

Emerging debates and resolutions in brown adipose tissue research

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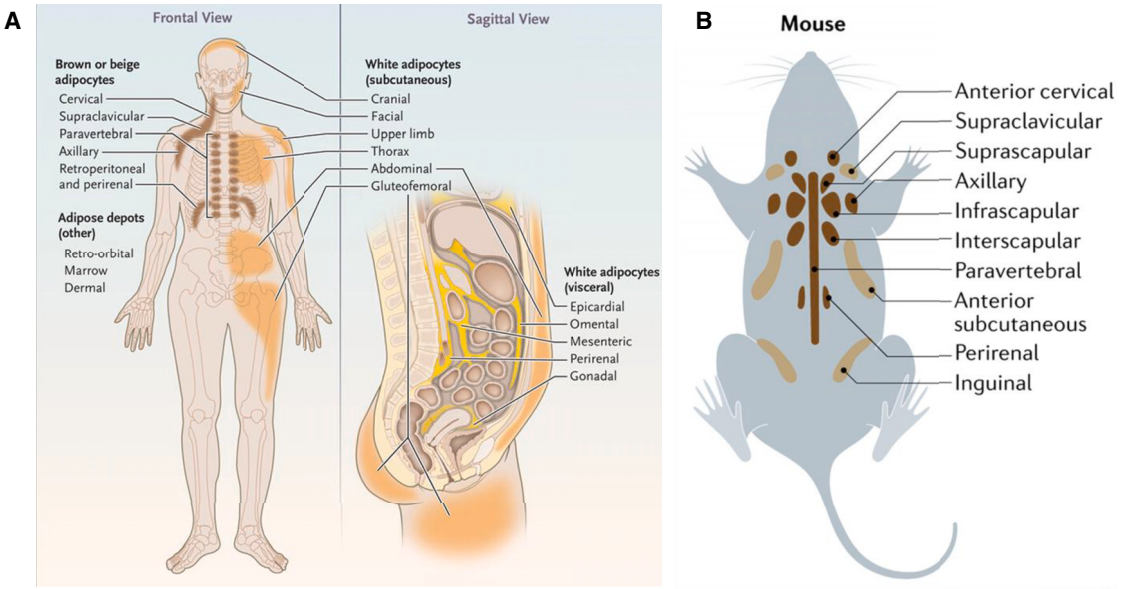
SUMMARY

Until two decades ago, brown adipose tissue (BAT) was studied primarily as a thermogenic organ of small rodents in the context of cold adaptation. The discovery of functional human BAT has opened new opportunities to understand its physiological role in energy balance and therapeutic applications for metabolic disorders. Significantly, the role of BAT extends far beyond thermogenesis, including glucose and lipid homeostasis, by releasing mediators that communicate with other cells and organs. The field has made major advances by using new model systems, ranging from subcellular studies to clinical trials, which have also led to debates. In this perspective, we identify six fundamental issues that are currently controversial and comprise dichotomous models. Each side presents supporting evidence and, critically, the necessary methods and falsifiable experiments that would resolve the dispute. With this collaborative approach, the field will continue to productively advance the understanding of BAT physiology, appreciate the importance of thermogenic adipocytes as a central area of ongoing research, and realize the therapeutic potential.

INTRODUCTION

Brown adipose tissue (BAT) is an extraordinary organ that can dissipate energy in the form of heat. In rodents, BAT thermogenesis is essential for chronic adaptation to cold environments, and its activation leads to resistance to obesity and protects against numerous other negative metabolic consequences.[1,2] The physiological relevance of BAT in adult humans became widely appreciated only two decades ago in a series of groundbreaking studies that showed it was metabolically active and thermogenic.[3–8] Subsequent years witnessed an ever-increasing series of manuscripts that demonstrated BAT's translational potential, as basic science experiments using cultured cells and animal models explained and predicted the discoveries in clinical trials. In turn, studies in humans uncovered unexpected features of mammalian BAT physiology, the mechanisms of which were then evaluated in the laboratory. Part of the success underlying this synergy stems from BAT's unique biology. BAT's primary physiological role is nonshivering thermogenesis, and the principal protein mediating this process is tissue-specific uncoupling protein 1 (UCP1) in the BAT mitochondria. This feature has been essential for studies designed to identify human BAT (hBAT), which is unique among solid organs for its unusual distribution: adult hBAT resides in anatomical locations distinct from those of infants, including the supraclavicular region—nearly all inaccessible without invasive procedures—where BAT can be found



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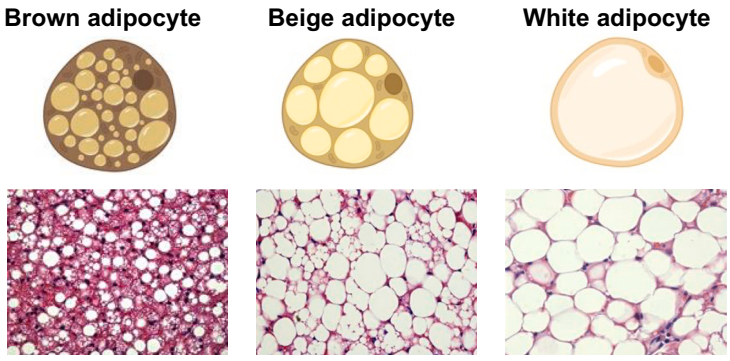

Thermogenic function	Thermogenic	Thermogenic	Energy storage
Mitochondrial contents	High	High	Low
Lipid droplets	Multi-locular	Multi-locular	Uni-locular
Location	Embryonic BAT (e.g., interscapular)	Sporadic in WAT (highly inducible)	WAT depots
Developmental timing	Embryonic	Adult (postnatal)	Embryonic and Adult

Figure 1. Anatomical locations and cellular characteristics of human brown and white adipose tissue

(A and B) (A) Anatomical locations of brown adipose tissue in humans and (B) (left) and mice (right).

(C) Cellular characteristics of embryonic brown adipocytes, beige adipocytes, and white adipocytes. Figures are modified from previous review articles.[14,17] (A) is modified from (New England Journal of Medicine, Cypess A.M., Reassessing Human Adipose Tissue, 386, 768–779, Copyright © 2022) Massachusetts Medical Society. Reprinted with Permission. (B) and (C) are modified from "The cellular and functional complexity of thermogenic fat," Paul Cohen et al., Nature Reviews Molecular Cell Biology, Springer Nature, March 23, 2021.

with widely varying amounts (Figure 1A). Rather, there are specific depots within the far more prevalent white adipose tissue (WAT) that have the capacity to induce the biogenesis of thermogenic adipocytes.[9–11] At the cellular level, the field has gained a deeper understanding of the inducible form of thermogenic adipocytes residing within WAT, referred to as beige adipocytes.[12,13] A large body of research has explored the developmental origins, regulation, and function, leading to an appreciation of the common functions as well as the distinct characteristics of brown and beige adipocytes[14] (Figure 1B). Intriguingly, recent studies have suggested that adipocyte browning was central to the evolution of endothermy prior to UCP1 acquiring its thermogenic function and pointed to a larger role for BAT in human physiology.[15,16]

Table 1. Topics of dispute in brown fat biology
Mechanisms of cellular thermogenesis
UCP1 only—Cannon and Nedergaard
Multiple futile cycle mechanisms—Kazak
Physiological relevance of human BAT
Yes—Tseng
No—Chang and Krakoff
Capacity for beiging in human WAT
Only specific depots—Schéele
All WAT depots—Boucher
Humanizing mouse BAT by warming housing temperature
Yes—Petrovic
No—Kajimura
Cooling protocol to activate human BAT
Fixed temperature—Blondin and Carpentier
Personalized—Virtanen
Pharmacological activation of human BAT
Via β_2-AR—Kooijman and Rensen
Via β_3-AR—Cero and Cypess

In order to continue advancing knowledge of BAT physiology over the next decades, it is helpful to understand the research within the context of the philosophy of science. The scientific revolution and the subsequent advancement of knowledge succeeded because of the professional community's commitment to its uniquely effective methodology. The philosophical framework is based on the principle of falsifiability,[18] which maintains that a scientific model is valid only if experiments can be done that could disprove it. However, two additional provisions must then be satisfied: there is consensus on basic experimental design, and colleagues agree on how to interpret experimental results. A laudatory example of this process comes from investigators studying BAT physiology: researchers in the field have devoted themselves to understanding its physiology with a particular interest in utilizing BAT activation to treat metabolic disease. To help the hBAT field advance in an organized way, the National Institutes of Health's Division of Diabetes, Endocrinology, & Metabolic Diseases (NIDDK) sponsored workshops designed to establish consensus points and experimental guidelines[19] as well as standards for reporting criteria for imaging studies.[20] The esprit de corps demonstrated through these publications and conferences led to an explosion in interest and productivity, going from fewer than 200 papers per year as recently 2006 to nearly 1,000 per year by 2022.

A diversity of discoveries has accompanied the clinical and translational research over the last two decades. The interpretation of many of these discoveries has achieved consensus, but others are still debated. In this perspective, we identified six debatable topics, ranging from the molecular to the clinical, that had clearly distinct, dichotomous positions (Table 1): the topics include how adipose tissue generates heat; what physiological roles hBAT has; what mouse models best recapitulate human thermal physiology; and by what mechanisms should one stimulate BAT metabolic activity for study and therapeutics. Each side contributed its understanding of each debate, cited the evidence, and, crucially, how each debate can be resolved using agreed-upon experimental design and methods.

MECHANISMS OF CELLULAR THERMOGENESIS

The best-known physiological role for BAT is thermogenesis. For decades, it was believed that UCP1-mediated uncoupling was the only source of nonshivering thermogenesis.[21] Curiously, some mammalian species, including pigs, lack the functional *UCP1* gene[22]; yet pig adipocytes retain thermogenic capacity.[23] Furthermore, marsupials possess beige fat-like adipose tissue but lack thermogenic UCP1.[16] Recent studies have offered additional pathways, including futile creatine cycling and Ca^{2+} cycling, as independent mechanisms of adipose tissue thermogenesis.[23–25] The contribution of UCP1 and UCP1-independent thermogenic pathways to thermoregulation and whole-body energy balance has been a topic of interest in the field. The proposed assessment shown in Figure 2 using new mouse models, such as the inducible UCP1 knockout (KO) mice and double/triple KO mice that lack UCP1 and UCP1-independent thermogenic pathways, will help resolve the dispute.

Only UCP1 can do it

Barbara Cannon and Jan Nedergaard

In 1964, our mentor Olov Lindberg returned to Sweden from Los Angeles after having been introduced to the fascinating—and then newly discovered—heat-producing ability of the, until then, scientifically rather neglected BAT. The basic question for him was then obviously: where does the heat come from?

Being a bioenergeticist, the first thing he did was measure the ATP-synthesizing capacity of the tissue: it was extremely low. And in the electron microscope, the mitochondrial inner membrane did not display the coating of "lollipops," the physical manifestation characteristic of the ATP synthase.[26] Thus, he immediately concluded that the heat production in the brown-fat mitochondria could not occur via any of the futile cycle mechanisms that at that time had been advocated for thermogenic processes in, e.g., WAT (lipid re-esterification,[27,28] glycerol kinase,[29] Na/K-ATPase[30,31]): there would simply not be enough ATP for any such process to occur in a quantitatively significant manner.

Time has shown that this absence of significant ATP-synthesizing capacity was not a preparation artifact. Instead, a fascinating story has evolved where, specifically in BAT, the P1 isoform of the c subunit demonstrates very low gene expression. All other ATP synthase subunits are highly expressed at the mRNA level, but the functional ATP synthase cannot assemble due to the lack of the essential c subunit membrane component.[32] This does not mean that BAT is entirely devoid of ATP synthase, but it means that it can only use a very small fraction of its extremely high respiratory capacity to combust lipids and sugar for ATP production, and it of course also needs this ATP for its own cellular life. Thus, any process that utilizes ATP can never be of major quantitative significance for thermogenesis in the tissue, as only ≈20% of the norepinephrine-induced thermogenesis is dependent upon ATP synthesis.[33] This does not mean that futile cycles do not exist in BAT (or in any other tissue), but the mere ability to identify enzymes that functionally are kinases and phosphatases for the same/any substrate does not

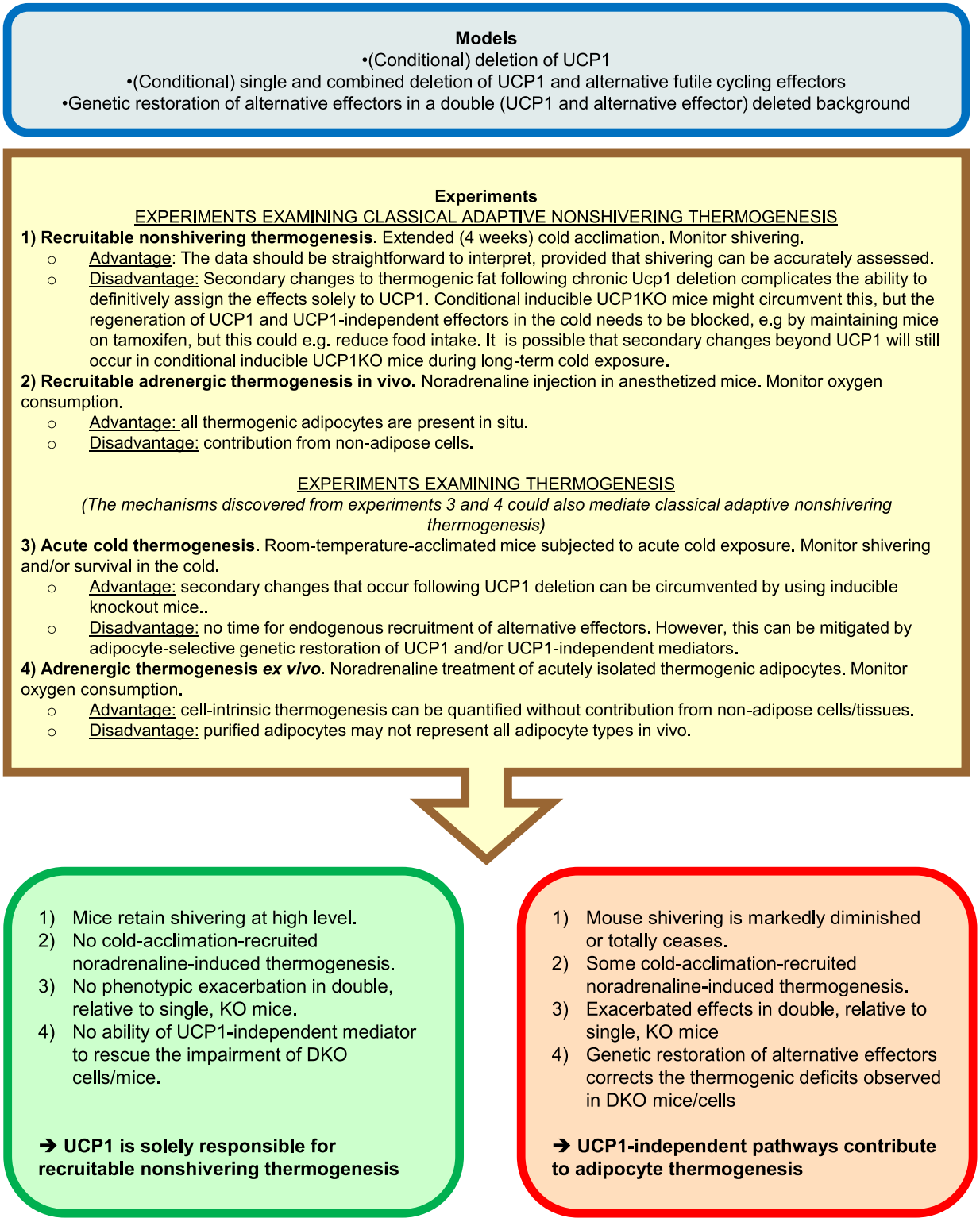

Figure 2. Proposed animal models (blue), experiments (yellow), and interpretation (green and red) in section "mechanisms of cellular thermogenesis"

in itself demonstrate that a futile cycle is physiologically ongoing—or, if it is, it may not have thermogenesis as its purpose. Perhaps, as has earlier been discussed, such futile cycles may yield some regulatory advantages.[34] And any of these kinases or phosphatases and their substrates may, of course, be essential for the normal functioning of BAT.

This absence of significant ATP-synthesizing capacity initiated a long series of biochemical investigations that ultimately resulted in the identification of UCP1. Thus, there was firstly a phenomenon, principally a high proton conductivity in isolated brown fat mitochondria,[35] and then a successive series of biochemical approaches that ultimately led to the identification of the protein responsible (i.e., UCP1).[36] Thus, we want to stress that UCP1 is a protein that was discovered in a physiologically orthograde manner: a specific physiological function found in its enzyme mediator. This may be contrasted with several alternative processes presently discussed for heat production, where in a physiologically retrograde manner, increased expression of certain genes in BAT (or other tissues) in cold-exposed mice has made them candidates for UCP1-independent thermogenic processes.

When mice or rats are exposed to the cold, they immediately (minutes) increase thermogenesis by intense shivering. However, with time in the cold (weeks), shivering successfully decreases, while the elevated metabolism is retained at the same high level.[37] This is thus the process of *adaptive* nonshivering thermogenesis, the cellular and molecular basis of which is discussed here. When UCP1 KO mice could finally be generated,[38] they indeed demonstrated the expected properties of an animal that lacked the ability to perform cold-induced adaptive nonshivering thermogenesis: they shivered incessantly in the cold, not only immediately but over weeks, while shivering disappeared in the wild-type mice.[39] This does, of course, not in itself demonstrate that UCP1 is the functional mediator of adaptive nonshivering thermogenesis; this was deduced from the biochemical experiments. However, if alternative mechanisms for thermogenesis existed, they should have been successfully recruited, and shivering should, with time, have ceased. This did not happen. Thus, UCP1 is the sole mediator of *adaptive* nonshivering thermogenesis: no other protein (including other so-called UCPs) or mechanism can do it.

As has been repeatedly suggested, adaptive nonshivering thermogenesis could occur or develop elsewhere in the mammalian body, particularly in muscle. However, if this would be the case, shivering in the cold would cease even in the UCP1-ablated mice, but present studies agree that this is not the case: shivering continues unremittingly in these mice in the cold.[24,39] To us, this remains *the critical experiment* in mammalian adaptive thermogenesis: any functional alternative UCP1-independent thermogenesis in brown fat, beige fat, muscle, or anywhere else in the body must manifest itself during prolonged cold exposure (weeks) by being able to successively replace or substantially diminish shivering in mice molecularly unaltered except for the UCP1 KO.

This critical experiment is undoubtedly valid if the proposed UCP1-independent extra heat would originate from muscle or beige adipose tissue. The objection has been forwarded that this type of experiment cannot exclude the presence of UCP1-independent *brown-fat-derived* thermogenesis in the UCP1 KO mice due to a profound general negative effect of the UCP1 ablation on brown fat mitochondria.[40] However, the negative effect may not generally be as profound as first reported, at least concerning mitochondrial structure and mitochondrial oxidative phosphorylation protein complexes,[41] and such effects may thus not invalidate the critical experiment defined here (using conditional UCP1 KO mice would seem to be advantageous as they do not show this problem[42]), but UCP1 returns in these mice within weeks.[42] Additionally, another interpretation of this brown fat-specific mitochondrial deterioration is that even this problem can be seen as an indication that all heat comes from UCP1. Had significant UCP1-independent thermogenesis been available, the organism (mouse) would be satisfied with the amount of heat produced and would not attempt to mount further heat production by overstimulating the tissue sympathetically, probably through this causing the mitochondrial problems.[43]

So, in conclusion, our mammalian ancestors that developed thermogenically functional UCP1 and true BAT gained the advantage of being able to be comfortably active in the cold through UCP1-mediated nonshivering thermogenesis. That became their successful evolutionary prerogative, and that is why we are here today. Only UCP1 could do it.

ATP-linked adipocyte thermogenesis

Lawrence Kazak

A major goal of the metabolic research community is to activate calorie burning in a safe and sustained manner to meaningfully impact nutrient utilization. For this, a comprehensive understanding of the heat-producing pathways within adipose tissue is critical.

The importance of thermogenesis by UCP1 has been inferred from studies in mice genetically lacking *Ucp1* from birth (germline *Ucp1*$^{-/-}$). While great advances in understanding thermogenesis have been made using this model, brown adipocytes in these mice exhibit a suite of molecular changes that extend beyond UCP1,[40] which may explain their general reduction in respiratory capacity.[44] Since these secondary changes might attenuate energy dissipation by any mechanism, the lowered thermogenic capacity of germline *Ucp1*$^{-/-}$ mice may result from a combined reduction in UCP1-dependent and UCP1-independent thermogenesis. Interestingly, when differentiated *in vitro*, *Ucp1*$^{-/-}$ brown adipocytes do not acquire the secondary changes that occur *in vivo*, and thermogenesis is fully ATP-dependent,[41] indicating that ATP-linked thermogenesis is quantitatively meaningful. Whether a thermogenic pathway running parallel to UCP1 is quantitatively important *in vivo* remains unresolved. UCP1-dependent and UCP1-independent thermogenesis are not mutually exclusive; the existence of one does not obviate a key thermogenic role for the other.

A physiological role for UCP1-independent thermogenesis has been questioned on the basis that ATP synthase levels are too low in brown adipocytes to be meaningful.[45] Yet, the fractional contribution of ATP synthesis, in relation to total electron transport chain activity, in BAT mitochondria is ~30% after severe chronic cold exposure[46] and ~60% at thermoneutrality,[47] the temperature purported to be required for human translational relevance. Furthermore, 30%–40% of noradrenaline-stimulated thermogenesis of acutely isolated brown adipocytes is

ATP-linked,[48,49] and coupled and uncoupled respiration is operational in human brown adipocytes.[50,51] Therefore, as pointed out by Prusiner over half a century ago, uncoupling of substrate oxidation from ATP synthesis in adipocyte mitochondria during thermogenesis is not an all-or-none response but one that is graded in nature.[52]

Strong conceptual models are internally consistent with a set of observations and support the construction of testable hypotheses. Energy dissipation by UCP1-independent futile cycles (coordinated metabolic reactions that progress in opposite directions) that accelerate ATP turnover rely on synchronized reactions of transporters and channels, lipases and acyltransferases, and kinases and phosphatases. Investigation into one of these pathways, called the futile creatine cycle, began with a bioenergetic phenomenon whereby creatine triggered a super-stoichiometric release of ADP.[24] This phenomenon was represented with a working model that guided genetic and biochemical studies in mitochondria, cells, and mice that led to the identification of the mediators of this process: creatine kinase b (CKB)[53] and tissue-nonspecific alkaline phosphatase (TNAP).[54] CKB and TNAP control 40% of noradrenaline-stimulated thermogenesis in acutely isolated brown adipocytes in an ATP synthase-dependent manner.[49]

Recently, temporal and cell-type-selective control over *Ucp1* inactivation has enabled the generation of a mouse model that can be studied independently of the multifaceted disruptions that occur in germline *Ucp1*$^{-/-}$ BAT.[42] Combining this model with inducible *Ckb* inactivation revealed that mice with both *Ckb* and *Ucp1* deleted in adipocytes exhibited an exacerbation of cold intolerance compared with single KOs.[42] This negative genetic interaction supports the idea that CKB influences thermogenesis alongside UCP1. Consequently, UCP1-dependent and UCP1-independent mechanisms are likely to coexist and complement each other rather than being mutually exclusive. However, the futile creatine cycle model proposes that CKB and TNAP act together within mitochondria to facilitate ATP turnover through creatine phosphorylation and phosphocreatine hydrolysis. Yet, CKB expression outside mitochondria raises the possibility of its involvement in broader adipocyte health maintenance. Therefore, despite the established functional link between CKB and UCP1, future experiments can be devised to further explore whether thermogenesis via the futile creatine cycle operates concurrently with UCP1.

To resolve the debate regarding whether thermogenesis relies solely on UCP1 or can also engage alternative pathways such as the futile creatine cycle, we propose several experiments that integrate genetic loss and rescue experiments. First, *Alpl* (encoding TNAP) would be co-deleted with *Ucp1* in an adipocyte-selective and inducible manner, followed by utilizing indirect calorimetry to measure cold-triggered thermogenesis. If this co-deletion exacerbates hypothermia similarly to what is observed with *Ckb* and *Ucp1* co-deletion, it will lend additional support to the idea that the futile creatine cycle operates alongside UCP1. Additionally, these experiments would help rule out the conventional pathways of creatine metabolism, such as the phosphocreatine circuit, as significant contributors to thermogenesis. Second, given the influence of shivering and heat conductance on whole-body thermogenesis, it will be crucial to assess the inherent thermogenic capacity of the futile creatine cycle in brown adipocytes using respirometry. To conduct these experiments, BAT from mice lacking native *Ucp1* and *Ckb* would be transduced with adeno-associated viruses (AAVs) expressing a mitochondrial-exclusive CKB variant. Successful genetic rescue, where mitochondrial CKB restores thermogenesis and alleviates hypothermia in BAT lacking native *Ucp1* and *Ckb*, would strongly indicate the physiological relevance of the futile creatine cycle. Moreover, if this thermogenic response is indeed mediated by the futile creatine cycle, it should be ATP-dependent and be reliant on TNAP. Similar loss-of-function and rescue experiments could be performed in mice lacking endogenous *Ucp1* and *Alpl*, utilizing AAVs expressing *Alpl* for rescue, to further elucidate the role of TNAP in this thermogenic process. Other proposed UCP1-independent thermogenic pathways[25] could be tested with these strategies.

PHYSIOLOGICAL RELEVANCE IN HUMANS

In rodents, compelling evidence demonstrates that BAT plays a central role in whole-body energy homeostasis and glucose/lipid metabolism. Whether or not hBAT, which comprises a much smaller percentage of total body mass, has any physiological relevance is a topic of debate. This debate stems from what is the primary readout of BAT-associated metabolic benefit: B1 considers a broader aspect of metabolic health beyond body weight, including systemic glucose and lipid metabolism, whereas B2 considers energy expenditure (EE) and adiposity as the primary outcome (Figure 3).

Yes to physiological relevance of hBAT

Yu-Hua Tseng

BAT, a unique type of fat tissue primarily contributing to thermogenic heat production, is initially thought to be functionally atrophic after infancy. However, this paradigm was challenged with the demonstration of metabolically active BAT, which can take up a notable amount of ^{18}F-fluorodeoxyglucose (^{18}F-FDG) in positron emission tomography/computed tomography (PET/CT) scans in adult humans.[3,4,6] Since then, numerous rodent studies have significantly increased our understanding of BAT in systemic metabolism and demonstrated a therapeutic potential to target this tissue and counteract obesity and its metabolic sequelae. However, given the relatively small amount of BAT in adult humans and the current limitation in precisely quantifying the mass and activity of hBAT, doubts about the likelihood of hBAT in physiological relevance exist.

Careful review of the literature indicates that BAT maintains physiological relevance in adult humans. Emerging data from multiple independent research groups have consistently pointed toward the metabolic significance of BAT, as supported by both retrospective and prospective clinical studies.

Retrospective studies have demonstrated an inverse correlation between BAT activity and BMI, highlighting its potential role in mitigating obesity-related complications.[4,6] Despite concerns that BAT-induced increases in EE on body weight might be counteracted by increased appetite, Becher et al. performed a large retrospective study with 134,529 in 52,487 individuals and strongly demonstrated people with detectable BAT have a significantly lower prevalence of cardiometabolic diseases, including type 2 diabetes, cardiovascular disease, and

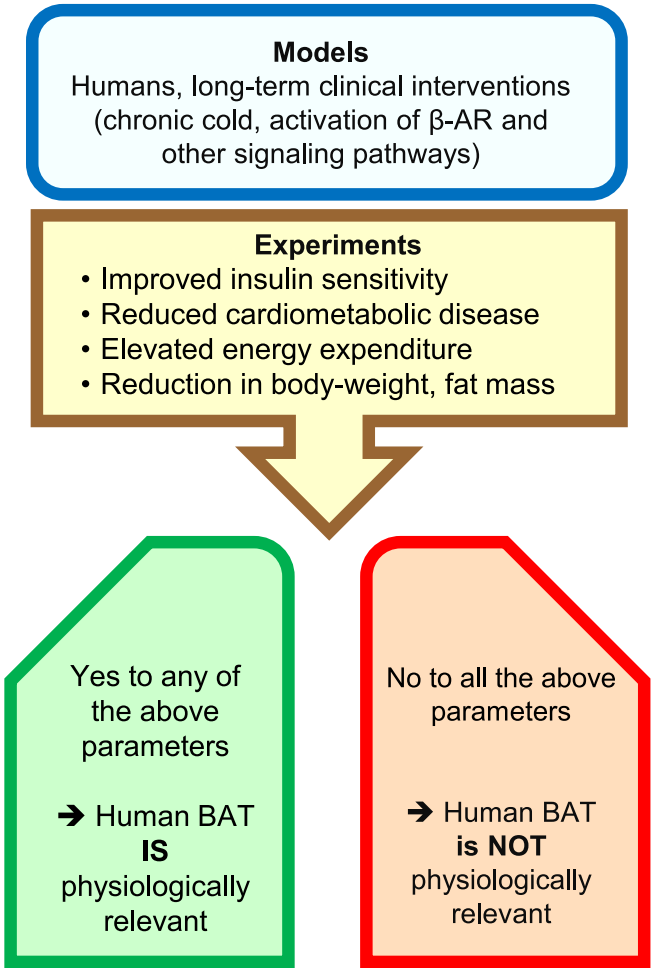

Figure 3. Proposed animal models (blue), experiments (yellow), and interpretation (green and red) in section "physiological relevance in humans"

dyslipidemia.[55] Prospective studies have further corroborated the link between BAT and glucose metabolism, insulin sensitivity, and diabetes, particularly in response to cold exposure. Cold has been shown to activate BAT, increase glucose disposal, promote EE,[11,56–58] and improve insulin sensitivity in diabetic subjects.[59] Using magnetic resonance imaging, Ahmed et al. showed that higher cold-induced BAT activity is associated with reduced non-alcoholic fatty liver disease,[60] pointing to a potential role of BAT activation in ameliorating hepatic disease. Pharmacological approaches targeting BAT activation, such as using the β_3-adrenergic receptor (β_3-AR) agonist mirabegron, have also shown improved metabolic profiles, including increased high-density lipoproteins (HDL) and plasma bile acids.[10,61] However, these studies employed high doses of mirabegron, which may activate receptors other than β_3-AR. Recently, a specific serotonin transporter was found to be highly expressed in human but not murine brown adipocytes, and the selective serotonin reuptake inhibitor (SSRI) sertraline could effectively dampen BAT activity in healthy individuals.[62] Clinically, SSRIs are known to induce weight gain and metabolic dysfunction, and thus, those effects could be mediated via its suppressive effects on BAT, underscoring the importance of BAT in these processes. Furthermore, some causative evidence supports these findings, such as the transplantation of brown-like or beige adipocytes into immunocompromised mice, which showed improvement in metabolic homeostasis and amelioration of high-fat diet-induced metabolic abnormalities.[63,64]

Several mechanistic studies provide additional evidence for the role of BAT on human metabolic health and suggest the metabolic impact of BAT in humans is beyond EE. First, BAT serves as a metabolic sink, effectively capturing and utilizing glucose, fatty acids, and branched-chain amino acids for thermogenesis and energy dissipation.[57,58,65] Recent research in tumor-bearing mice highlights BAT's capability to efficiently regulate glucose metabolism[66] and suggests a potential anti-cancer therapy via BAT activation. Moreover, the metabolic effects of BAT are also mediated by releasing various signaling molecules, such as peptides, metabolites, and extracellular vesicles, orchestrating systemic metabolic responses.[67] BAT-produced bioactive lipids, including 12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME),[68] 12-hydroxyeicosapentaenoic acid (12-HEPE),[69] and Maresin 2,[70] can regulate fatty acid and glucose metabolism and resolve inflammation in obesity. Notably, the levels of all these lipids are elevated in subjects receiving cold or mirabegron treatment and are negatively associated with BMI and insulin resistance. Altogether, these multifaceted mechanisms underscore the pivotal role of BAT in shaping human metabolic health.

In conclusion, the ongoing debate surrounding the physiological relevance of BAT in humans calls for rigorous efforts and advancements in several key areas. First, the field requires refined methods for detecting and quantifying BAT thermogenic capacity with precision and reliability. Also, establishing reliable biomarkers for BAT activation in humans is imperative. Identifying individuals genetically active or deficient in BAT will provide crucial insights into its role in metabolic regulation. Lastly, implementing long-term studies encompassing diverse populations will provide a comprehensive understanding of BAT's impact on metabolic health. By addressing these critical research fronts, we will unravel the full scope of BAT's physiological relevance in human metabolism and develop new therapeutic approaches to metabolic disorders.

No to the relevance of BAT

Douglas C. Chang and Jonathan Krakoff

The increased use of FDG PET-CT scan in the 2000s accompanied the recognition that many adults possess BAT.[3,4,6] Originally a nuisance for radiologists (who developed ways to suppress it), BAT has become a significant source of scientific inquiry about its metabolic and energetic implications.

The interest in BAT is due to its potential role in modulating EE to manage weight. The underlying assumptions are that human EE is an important contributor to weight change and BAT significantly contributes to EE. Both assumptions are suspect. Evidence linking decreased metabolic rate to weight gain in humans is inconsistent, with results showing increased,[71] decreased,[72] and no effects.[73] Even in populations where lower EE predicts weight gain, the effect is very weak.[71] These varying results may be due to population differences and variations in the conditions and methods for measuring EE. If EE is not a large

determinant of weight regulation, the targeting of EE is not likely to lead to anticipated benefits. While BAT activity may be correlated with EE changes, the overall small volume of hBAT and low blood flow to adipose tissue (relative to tissues with much higher metabolic rates) casts doubt on BAT's thermogenic capacity and thus its contribution to EE.[74] For example, BAT thermogenesis in humans accounted for only 15–25 kcal/day when stimulated by cold.[75] Although administration of the β_3 agonist mirabegron to men increases both BAT activation and EE,[76,77] the moderate increase in EE is likely due to off-target activation of other tissues. Though browning of WAT from adrenergic stimulation has been proposed, usual increases in oxidative activity are not observed with acetate tracers.[78]

Part of the interest in BAT stems from the concept that it leads to resistance to weight gain via stimulation of EE with overfeeding. While rats can double their EE in conjunction with BAT activation,[79] there is little and contrary evidence to support this in humans. In humans, doubling weight maintaining caloric intake increases EE by only ~10%.[80] BAT activation as measured by [^{18}F]-FDG uptake increases after a high-calorie, high-carbohydrate meal but is not associated with diet-induced thermogenesis.[81] In participants with known BAT, doubling caloric intake for 24 h resulted in a 7.5% increase in EE but no evidence of BAT activation.[82] These studies used [^{18}F]-FDG uptake to measure BAT activity, and it is unclear if results would have changed with alternative methods (e.g., acetate or fatty acid tracers).

The dearth of data for BAT as an explanation for resistance to weight gain or as an organ to be manipulated to facilitate weight loss has led investigators to cast a wider net for a functional role of BAT. BAT has now been linked to a wide range of cardiometabolic outcomes, including cardiovascular disease, type 2 diabetes, and fatty liver disease.[55,60] One of the causal factors driving this search for additional metabolic roles for BAT lies in prior research demonstrating improvements in metabolism (e.g., insulin sensitivity and improved glucose disposal) from cold. However, cold exposure drives a multi-organ response (e.g., increased muscle glucose uptake and energy expended by liver re-esterification of free fatty acids from WAT) that may be independent of BAT.[83] Given the low volume of BAT and the activation of other organs/tissues in cold that provide plausible physiologic explanations for improvements in metabolism, it is unlikely that BAT is causal in improved liver and cardiometabolic health.

If endogenous BAT does not play a substantial role in EE and weight regulation, would pharmacologic manipulation of BAT leading to more substantial EE increases hold promise? Even if BAT can be exploited to induce sizable EE changes, adaptive increases in food intake caused by disruption in energy balance might occur to blunt benefits,[84] as there is evidence for energy deficits driving energy intake.[85] Furthermore, past efforts in medications that increase EE have led to disappointing results with concerning outcomes. These uncertainties cast doubt on BAT's role as a target to control weight. To understand its role, BAT needs to be carefully measured alongside measurements of EE and food intake in longitudinal studies.

CAPACITY FOR BEIGING IN HUMAN ADIPOSE TISSUES

Rodents have defined adipose tissue organs: some depots, like interscapular BAT, are primarily composed of brown adipocytes, while others, such as visceral WAT, contain white adipocytes. With adrenergic stimulation, subcutaneous WAT depots of rodents adapt a thermogenic phenotype with active beige adipocyte biogenesis, *a.k.a.*, browning or beiging of WAT. It is a topic of debate whether all human WAT depots can support the growth of thermogenic adipocytes or if this occurs only in specific depots. This debate is attributed, in part, to the complex cellular heterogeneity and insufficient understanding of species differences in adipose tissue (Figure 4).

Location specific: Understanding the diversity and complexity of human thermogenic adipose tissue and where it resides

Camilla Schéele

Adipocytes exhibit specialized functions that correspond to specific anatomical locations within distinct adipose depots. In humans, dysfunction in these roles is linked to the onset of cardiometabolic diseases and overall adipose tissue malfunction. Consequently, it is highly important to understand the disparities between healthy and unhealthy adipocytes within each distinct depot. Progenitor cells isolated from distinct adipose tissue depots maintain some of their phenotype during *in vitro* differentiation, suggesting that these progenitors are intrinsically programmed to differentiate into a specific adipocyte subtype. In this respect, progenitor cells isolated from hBAT depots, such as the supraclavicular, deep neck, and perirenal areas, retain their thermogenic phenotype when cultured and differentiated *in vitro*,[86–89] demonstrating that these progenitors are determined for a thermogenic cell destiny.

With the impressive anti-obesity effects of BAT activation in mice, the research field has been focused on strategies for increasing the activity of BAT in humans. The discovery of browning/beiging in murine subcutaneous adipose tissue, converting WAT into a thermogenic phenotype, was therefore a huge breakthrough, and numerous investigations have since then been made to assess this phenomenon in human subcutaneous adipose tissue. However, in my opinion, current evidence for beiging of human subcutaneous adipose tissue is not convincing, and data rather points to that in humans, the visceral adipose tissue is a more promising target for this kind of transformation.

In vitro, it has been shown that administration of peroxisome proliferator-activated receptor γ (PPARγ) agonists during differentiation or overexpression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) increase thermogenic gene expression in human subcutaneous adipocytes.[90,91] However, inducing thermogenic gene expression is just one part of BAT activation, and functional evidence, such as increased uncoupled respiration, is, to my knowledge, still lacking with this model. Importantly, in a comparison between *in vitro* differentiated adipocytes derived human from perirenal BAT versus subcutaneous WAT, where all cells were differentiated in presence of a PPARγ agonist, only the perirenal adipocytes responded with increased uncoupled respiration when stimulated with norepinephrine.[89]

In vivo, a study of experienced young, healthy winter swimmers demonstrated no signs of increased thermogenic gene expression in subcutaneous WAT when biopsies obtained before and after acute cooling were compared. In fact, UCP1

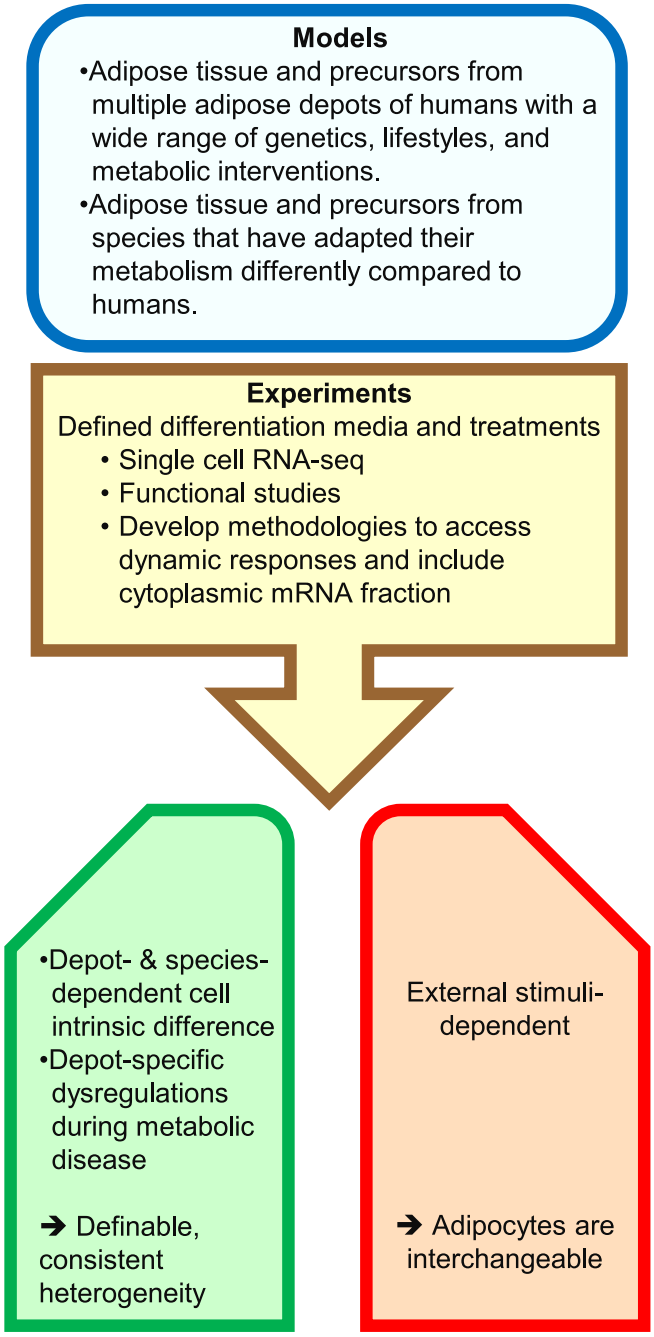

Figure 4. Proposed animal models (blue), experiments (yellow), and interpretation (green and red) in section "capacity for beiging in human adipose tissues"

was even undetectable in these samples.[92] Interestingly, it has been suggested that patients with severe burn injury, as a response to losing heat, exhibit beiging in their subcutaneous WAT.[93] Lipid depletion in the subcutaneous WAT makes it morphologically resemble active BAT. UCP1 expression is also found to be upregulated in this tissue. However, this phenotype has likely nothing to do with an upregulation of a thermogenic phenotype. Severe burn injury is extreme physiological stress with high levels of circulating norepinephrine that could explain the upregulation of UCP1 but is not evidence of thermogenesis. The burn injury condition activates a hypermetabolic and catabolic state, with whole-body accelerated onset of lipolysis, proteolysis, and futile substrate cycling, resulting in extreme energetic needs for maintaining body weight.[94] Hence, the phenotype observed in subcutaneous fat is thus likely more related to organ stress and catabolism rather than WAT beiging.

By contrast, the evidence is more convincing for beiging to occur in the visceral adipose tissue. Whereas visceral adipose tissue was not analyzed in the burn victims, beiging has been observed in this depot *in vivo* in pheochromocytoma patients, where a tumor in the adrenal gland results in an overproduction of norepinephrine.[95–97] FDG-PET/CT scans and adipose tissue analysis of these patients clearly demonstrate beiging in the visceral adipose depot, with massive glucose uptake in the classical brown as well as the visceral adipose tissue[97] and with subpopulations of multilocular, UCP1 positive adipocytes.[95,96] Importantly, the thermogenic phenotype was limited to visceral adipose, with no effect on the subcutaneous adipose tissue. The conclusion that beiging in humans is more likely to occur in the visceral adipose depot is further emphasized by comparisons between visceral and subcutaneous adipose tissue at a single-cell resolution, demonstrating that a thermogenic adipocyte subtype was present in visceral but not subcutaneous adipose tissue.[98]

To understand the complex function of adipose tissue, it is important to take evolutionary-driven differences between species metabolism into account. Substantial differences exist between humans and mice in terms of thermogenic needs and energetic storage capacity, and adipose tissue depots have likely evolved differently to fulfill these needs. Human BAT and BAT-derived cell gene expression differ from murine BAT.[86,87] At a molecular level, a human-specific long non-coding RNA, without murine counterpart, has been found to regulate BAT function in humans,[99] and further exploration of the adipose-specific non-coding genome is expected to reveal additional differences.

Single-cell technologies have allowed for understanding the differences between distinct adipose depots at high resolution, delineating several subtypes of adipocytes.[100] Intriguingly, a common denominator was identified during early differentiation of human adipocytes across four different depots.[101,102] It was found progenitors from any of these depots differentiated into either adipogenic cells or the extracellular matrix producing, multipotent so-called structural Wnt-regulated adipose tissue-resident (SWAT) cells. Important tasks to solve in the future include delineating the *in vivo* function of SWAT cells, identifying what regulates the balance between the lipid-storing adipogenic cell destiny and the structural SWAT cell destiny, and their dysregulations during cardiometabolic disease.

In conclusion, adipose depots are determined for distinct functions and are not necessarily directly comparable with adipose depots in rodents. However, this does not exclude that beiging might also occur in humans, and there are clear indications that this could be induced in the visceral adipose depot. Thus, with the purpose of rewiring dysregulated adipocytes into a healthy state, we should tailor the efforts to each depot. To define these efforts, we must delineate the adipose

depot-dependent intrinsic programming of progenitor cells, the cell-type-specific adaptations to metabolic cues, and the species-dependent adaptations of adipose tissue biology. This will require researching adipose tissue from several adipose depots and from patients with distinct metabolic conditions. Ultimately, additional model systems than mice must be studied, allowing for new insights from species that have solved metabolic challenges and adapted their adipose tissue in alternative ways.

All depots can do: Unlocking the thermogenic potential of WAT

Jeremie Boucher

Activating and/or increasing the amount of thermogenic adipocytes represents an attractive strategy for the treatment of metabolic diseases. Our understanding of the mechanisms involved in brown fat activation and its role in normal and pathophysiological conditions in humans has increased considerably over the past two decades. However, this progress has not yet translated into treatments being tested in the clinical setting. BAT exists in discrete depots in humans (such as the supraclavicular and perirenal depots), and BAT mass and activity are decreased in older, obese, and diabetic subjects. In addition, it does not appear possible to robustly activate the existing BAT depots with β-AR agonists without triggering unwanted effects on the cardiovascular system.[76,103] Wanting to convert dormant cells from these small and discrete BAT locations into thermogenic adipocytes seems like a difficult task with our limited understanding of the molecular mechanisms at play and the difficulty to access these tissues. To unlock the thermogenic potential of adipose tissue, we perhaps should not look where brown adipocytes are present within adipose depots, but rather where they are absent. Indeed, adipose tissue possesses an extraordinary plasticity, and under certain circumstances, thermogenic adipocytes can be found in WAT depots, even when BAT is absent.[104] The potential of human white adipocytes to convert into brown-like or thermogenic cells has been demonstrated: overexpression of PGC-1α in freshly isolated human mature subcutaneous white adipocytes leads to an increase in expression of brown fat markers.[91] Pharmacological treatment with tesaglitazar, a PPARα/γ dual agonist, has also been shown to induce robust browning of freshly isolated human subcutaneous white adipocytes and of differentiated preadipocytes.[90] *In vivo*, a white to brown-like phenotype switch was observed in severely burned patients, who lose heat due to the extent of their injuries.[93,105] Similarly, a switch from white to brown-like fat occurs in cancer-associated cachexia and is associated with increased EE.[106] Overall, these and other studies convincingly demonstrate that human WAT has the capacity to acquire brown-like features *ex vivo* or *in vivo*.

However, several key questions remain to be addressed to better understand and capture the thermogenic potential of WAT:

(1) Are all white adipocytes and preadipocytes equal in regard to their browning capacity? It is likely that different adipocyte and preadipocyte cell populations have different propensities to turn on a thermogenic program, as there is important inter- and intra-depot heterogeneity.[98,107] The conversion capacity of mature adipocyte and adipocyte precursors from different human fat depots should be assessed. The advancement of new techniques allowing the culture of mature adipocytes or the formation of adipose spheroids will help facilitate those studies in physiologically relevant systems.[91,108,109] Likewise, the impact of obesity, age, and diabetes should be assessed and compared with adipocytes from lean, young, and healthy individuals.

(2) Do human adipocytes have the same thermogenic potential as mouse adipocytes? Here again, likely not, as human and mouse adipose tissue display significant differences.[110,111] There is abundant literature on the browning of white fat in rodents, but too rarely are the main findings tested in human cells. With the increasing access to human material from hospitals, commercial vendors, or the generation of immortalized human adipocyte cell lines, key rodent findings should systematically be tested in human systems.[50]

(3) Can we convert enough white adipocytes into thermogenic adipocytes for it to be therapeutically relevant? The level of brown fat markers after browning of WAT reported in most studies far from reaches the amount found in true brown fat. However, WAT depots are significantly more abundant than brown fat depots, especially in the obese situation. The conversion of a proportion of white adipocytes into thermogenic adipocytes could therefore quantitatively represent a higher number of cells than the number of bona fide brown adipocytes and have a meaningful impact. It is nevertheless important to establish quantitative comparisons to true brown fat.

(4) Will activation of newly formed thermogenic adipocytes also be needed? Beige adipocytes, like brown adipocytes, require activation for energy dissipation to occur. The increased sympathetic tone reported in obese conditions, the occasional cold exposure and physical exercise, the diet-induced or the lipolysis-induced activation, may all contribute to overall thermogenic adipocyte activation. However, in all likelihood, a combination therapy consisting of one agent inducing browning of WAT and another activating brown-like adipocytes would have synergistic beneficial effects. It is also important to remember that the benefit of BAT activation or browning of WAT extends far beyond its UCP1-mediated weight loss potential. UCP1-independent thermogenic pathways have been described, and so have weight loss-independent metabolic improvements, at least in part via the secretion of beneficial secreted factors.[112]

(5) Can we identify novel pharmacological agents capable to induce robust browning of white fat? Phenotypic screens should be performed in physiologically relevant human adipocyte models to identify novel targets and compounds, with UCP1, other brown fat markers, oxygen consumption, or omics as potential readouts.

WAT represents a large reservoir of potential thermogenic adipocytes that remain to be unlocked. Drugs from the GLP1 class were recently approved for the treatment of obesity and

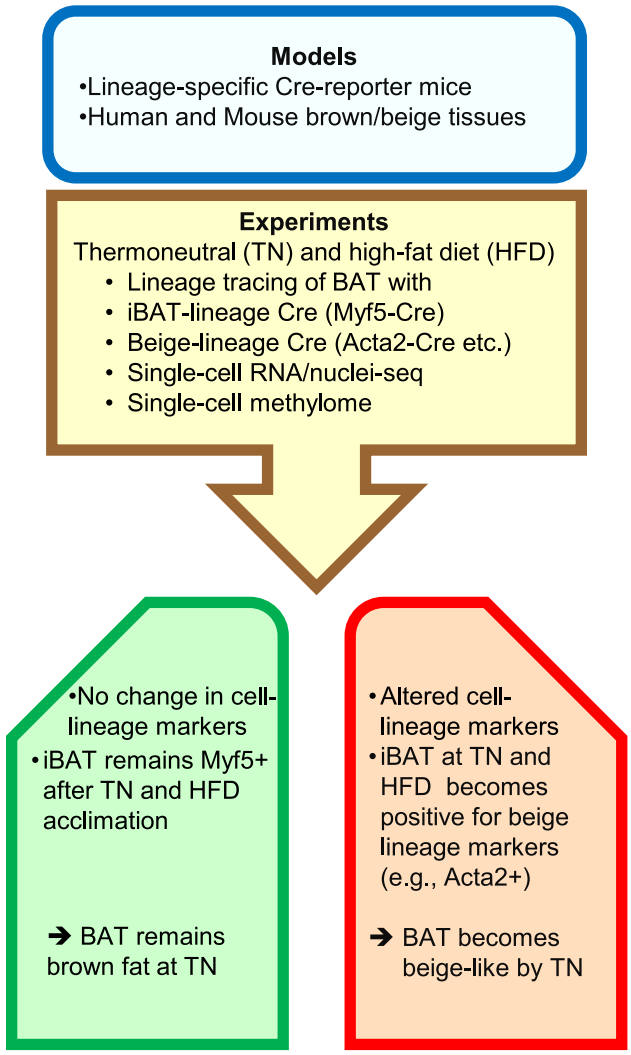

Figure 5. Proposed animal models (blue), experiments (yellow), and interpretation (green and red) in section "humanizing mouse BAT by warming housing temperature"

diabetes. Their impressive weight loss is mediated mostly via decreased food intake.[113] A combination with a drug stimulating EE via browning of white fat and/or activating BAT would undoubtedly provide additional or synergistic effects on weight loss and metabolic improvements.

HUMANIZING MOUSE BAT BY WARMING HOUSING TEMPERATURE

In general, mice are a well-accepted animal model for studying metabolism and energy homeostasis; however, interspecies differences between mice and humans in terms of size, life span, and thermoneutral temperature range make it challenging to determine which experimental conditions best model human BAT biology. Although both sides agree that mice will not fully replicate human physiology, a debate is whether or not warming housing temperature in mice on a high-fat diet can recapitulate human BAT (Figure 5).

Yes—On the validity of physiologically humanized mouse model

Natasa Petrovic

In similarity to many other mammals, the mouse has innately high anatomical, physiological, and genetic similarity to humans, and, given the specific possibilities for genetic manipulations, the mouse has become an invaluable primary animal model in translational research. However, the mice routinely employed in the majority of biomedical studies are young (some 6–10 weeks, corresponding to young teenagers), in reality housed under constant cold stress conditions (as in "standard" housing conditions, i.e., at about 20°C), and fed a boring chow diet.

Notably, a series of studies have unveiled noteworthy effects, resulting especially from the cold-induced metabolic stress experienced by these mice when maintained at room temperature (as compared with mice housed at thermoneutrality). These effects span a wide range of research areas, including metabolism,[114–117] exercise,[118] cancer,[119,120] cardiovascular function,[121,122] and immunity.[116,123]

Therefore, the central question that arises pertains to whether standard mice, subjected to continuous cold-induced stress, can truly serve as physiologically relevant models for humans. Humans predominantly live under thermoneutral conditions thanks to buildings and clothes and are often chronically exposed to enticing diets.

In our opinion, translational metabolic research (e.g., concerning obesity) should aim to diminish apparent "species differences" by conducting experiments under conditions that closely resemble those relevant for humans. To address this, we have introduced the concept of "physiologically humanized mice"—middle-aged mice that live under conditions approaching both human thermal conditions (thermoneutrality, where the need for heat production to combat heat loss is eliminated) and human nutritional conditions (exposure to an energy-rich diet) for an extended period (about 6 months, similar to human middle age).

The experimental conditions employed in biomedical research can profoundly affect not only the experimental outcomes but also the interpretation of the data and consequently may undermine the translation of findings in mice to insights into human (patho)physiology. An example that continues to be a topic of discussion is the characterization of brown fat in humans, specifically whether it exhibits "classical brown" or "beige" character. The initial studies,[13,124] based on elegant molecular analyses, concluded that beige rather than classical brown fat was the preferred mouse model for human brown fat. However, when we conducted a similar analysis using physiologically humanized mice,[125,126] the results suggested that the mouse equivalent of human brown fat was classical brown fat. Our observation was supported by analyses at a molecular level and also by a high degree of anatomical similarity between human and mouse brown fat under these physiologically humanized conditions.

From a translational perspective, it is particularly important to note that human brown fat but not beige fat from the physiologically humanized mice was predicted, through an *in silico* analysis,[127] to have fully preserved browning capacity. This prediction was fully validated experimentally[126]—beige fat was incapable of undergoing browning, while brown fat from physiologically humanized mice, despite substantial macrophage infiltration[128] retained full competence to attain the greatest possible

recruitment state. This differential response to cold stimulation demonstrates that adipocytes found in beige and brown adipose depots maintain distinct characteristics even after a long "humanization" process, indicating the persistence of the beige and classical brown adipocyte lineages in their respective depots. Furthermore, this observation strongly supports the notion that human brown fat and mouse classical brown fat are equivalent tissues and implies that in humans exposed to analogous physiological or pharmacological stimuli, classical brown fat depots would be capable of being recruited and transformed into tissue with high thermogenic activity, potentially leading to clinically relevant outcomes. While we are confident that human brown fat is of classical brown fat character, a definitive assessment will only be possible once specific marker genes that differentiate between classical brown and beige adipocytes are identified.

To further improve the translational applicability of biomedical research, mouse experiments can be conducted under conditions that even more closely mimic human conditions, e.g., by introducing variable temperatures during active and inactive phases,[129] providing a wider range of diets, and adjusting circadian conditions. However, implementing these refinements would inevitably lead to a significant increase in the cost of biomedical research. Thus, a careful balance must be struck between gaining more translatable data and managing costs. It is important to note that even with these refinements, mice will not fully replicate human physiology. However, they would represent a step closer to achieving more translationally reliable research outcomes.

No—High-fat feeding at thermoneutrality does not humanize mouse BAT

Shingo Kajimura

There is a report that BAT depots in 9-month-old mice kept under a thermoneutral condition (at 30°C) on a high-fat diet (45%) for over 30 weeks become physiologically "humanized" based on the whitened BAT morphology and the bulk tissue transcriptional profile.[126] In my opinion, this claim is overstatement and lacks mechanistic depth. This misunderstanding may, in part, be a lack of consensus and/or understanding of brown and beige adipocytes.

First, the original characterization of beige adipocytes is based on the distinct developmental lineage from embryonic brown adipocytes residing in the interscapular region of mice (iBAT).[130] Embryonic thermogenic adipocytes were initially called "classical" brown adipocytes, although the terminology may be inappropriate after over a decade of research because mouse iBAT depots also contain heterogeneous adipocyte populations.[131,132] Beige adipocytes are considered a distinct cell type from embryonic brown adipocytes in iBAT as some beige adipocytes do not originate from the *Myf5*$^{+}$-derived lineage,[130] and the transcriptional regulation of *Ucp1* in the inguinal WAT is distinct from that in iBAT.[133] It is worth noting that a subset of beige adipocytes arise from *Myf5*$^{+}$[134] or *MyoD*$^{+}$ lineage,[135] depending on external stimuli and location. Given the high heterogeneous nature, subsequent transcriptome studies in clonal populations showed the relevance of beige adipocytes to adult human BAT.[13,87,124,136] The field has learned more about the shared and distinct features of brown and beige adipocytes in terms of molecular regulation and function, which have been discussed recently (reviewed in Ikeda et al.[137]).

Second, adipose tissue morphology does not reflect the cellular lineage: even if BAT morphology becomes "whitened" after chronic acclimation to a thermoneutral condition and high-fat diet, these factors would not alter adipocyte lineages.[138] In this regard, epigenome profiling is useful: a recent work demonstrated that the chromatin architecture of whitened BAT remained unchanged even when BAT lost its multilocular morphology at a thermoneutral condition.[139] Thus, warming housing temperature and high-fat diet do not change the developmental lineage and "humanize" mouse BAT. Conversely, humans housed at 22°C would not adopt a mouse-like BAT phenotype. The only way to humanize mouse brown fat will be to reconstitute mouse tissues with human-derived cells.

Third, mice chronically fed a high-fat diet for 10 weeks or longer develop adipocyte hyperplasia, pro-inflammatory responses, and insulin resistance in the adipose tissues. It is also important to note that a high-fat diet in mice is not physiologically relevant to human diet. These changes substantially impact the adipose tissue function and cellular composition, such as immune cell infiltration, mitochondrial dysfunction, reduced sympathetic nerve tones, and lipolysis. After 30 weeks of high-fat diet feeding, adipose tissues are at a pathological but not physiological state. Similarly, aging negatively impacts brown and beige fat biogenesis, mitochondrial fuel oxidation and respiration, and thermogenesis.[140,141] At 6 months old, BAT oxidation capacity starts to decline due to reduced mitochondrial protein lipoylation and iron-sulfur cluster assembly factors.[142] Furthermore, the sympathetic nerve tone to the BAT is reduced in aging.[143] Thus, aged BAT from >9-month-old mice does not represent healthy adult human BAT, even though morphologically aged BAT adapts a beige-like, whitened phenotype.

Lastly, many humans do not always live at thermoneutrality. Indeed, human BAT exhibits dynamic seasonal changes, with higher glucose uptake in winter than in summer.[144,145] Of note, UCP1 has been extensively used as a molecular marker of brown and beige adipocytes, but we can expect new data showing that many adipocytes in human BAT lack UCP1 protein expression. This is intriguing as recent studies demonstrate that UCP1 is dispensable for a large part of metabolic benefits associated with active brown/beige fat, including glucose and lipid metabolism.[23,146] Furthermore, UCP1 null mice were previously reported to develop obesity at a thermoneutral condition[114]; however, this was challenged by recent studies using independently developed UCP1 null mice.[42,147]

Together, we as scientists should be aware of the limitations of mouse studies (e.g., body size, thermogenic demand, cellular composition, and location) rather than overestimating the human relevance. Nonetheless, the following experiments are helpful to rigorously examine the contribution of brown and beige fat to energy balance.

(1) Mouse metabolic studies should be performed both at room temperature and thermoneutrality (28°C–30°C). A benefit of mouse metabolic phenotyping at thermoneutrality is to reduce the contributions of adaptive thermogenesis by muscle shivering and BAT to whole-body

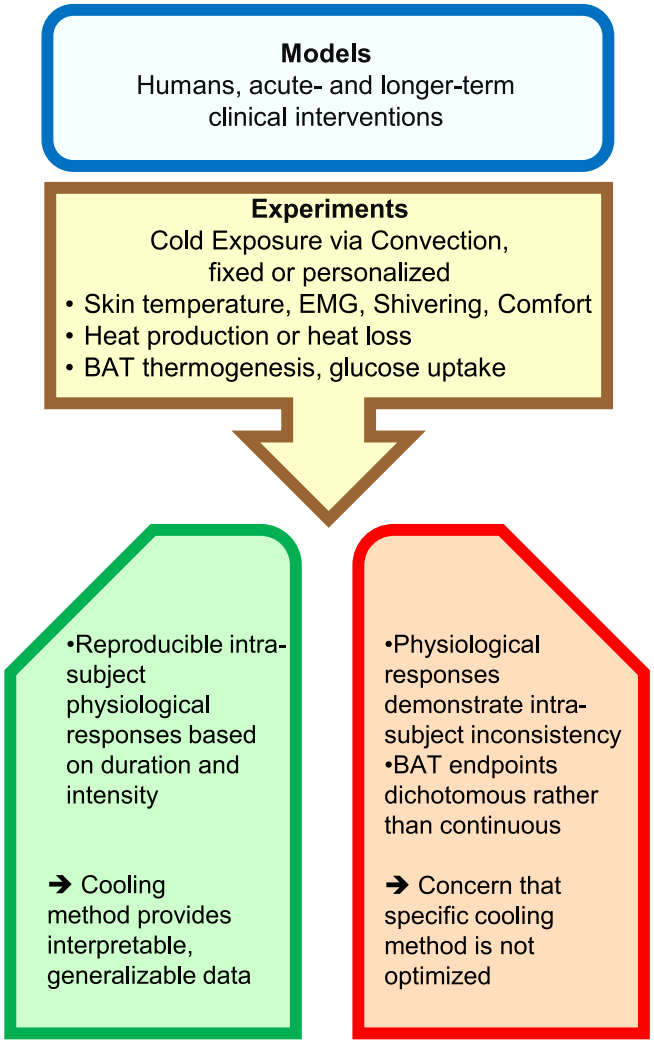

Figure 6. Proposed animal models (blue), experiments (yellow), and interpretation (green and red) in section "cooling protocols to activate human BAT"

energy balance, as the contribution of adaptive thermogenesis is much greater in mice than in humans. However, it is important to recognize that warming housing temperature influences numerous aspects of biology other than BAT thermogenesis, such as mouse behavior.[118] In this regard, a short-term acclimation to 28°C (for a few days) but not long term is helpful even though it is impossible to separate the contribution of muscle shivering from brown/beige fat. Under a short-term thermoneutral condition, indirect calorimetry is helpful in assessing the effect of β_3-AR agonists (e.g., CL316,243) on whole-body EE. In addition, the analysis of covariance (ANCOVA) is a well-accepted analysis of indirect calorimetry data if mice with different body weights are compared.[148]

(2) A feasible experiment to test the "humanized" BAT theory is to perform lineage tracing of mouse BAT using *Myf5*-Cre (embryonic brown fat) and beige fat-lineage markers (e.g., inducible *Acta2*-Cre, *Pdgfrb*-Cre, *Cd81*-Cre) under chronic high-fat diet and thermoneutrality conditions.[149,150] Single-cell assay for transposase-accessible chromatin (ATAC) analysis would be instrumental in determining if acclimation to thermoneutrality on a high-fat diet alters the developmental lineage of BAT.

(3) It is useful to target a gene of interest by using Adipo-Cre or UCP1-Cre. It is worth noting, however, that the efficiency of UCP1-Cre to target beige adipocytes is low,[151] and UCP1-Cre is ectopically expressed in non-adipose tissues.[152] Thus, validations using independent Cre lines, such as inducible Cre lines, would be useful.

(4) It would be insightful to examine the cell-intrinsic function in human adipocytes through gain- or loss-of-function approaches. While there is no perfect system, one should consider the advantages of cultured human cells versus the disadvantages of cultured studies outside of the physiological context, depending on the scope and question.

COOLING PROTOCOLS TO ACTIVATE HUMAN BAT

Mild cold exposure is the physiological mechanism that stimulates BAT EE and thermogenesis. Cold exposure is necessary for visualizing human BAT via non-invasive imaging. However, two distinct approaches have been used to induce cold exposure: a fixed temperature and a personalized cooling protocol. A current debate is about which protocol is appropriate to activate human BAT, while the proposed studies in Figure 6 will help reach a consensus.

Fixed temperature—Cool to heat up BAT

Denis P. Blondin and André C. Carpentier

Given its primary function as a thermoregulatory organ, the most potent stimulus to increase BAT thermogenesis remains cold exposure. Consequently, when investigating BAT thermogenesis, careful consideration is needed to determine the best medium, temperature, and duration of cold exposure to apply to meet the study objectives. We posit that both cold air and full-body liquid-conditioned suits (LCSs) are the ideal cooling modalities to study cold-stimulated BAT thermogenesis and that either the fixed temperature or personalized cooling approaches serve their respective purposes, so long as they can be reproduced across laboratories and elicit a comparable cold stress across individuals or cohorts.

The cooling medium: quantifying cold-stimulated BAT thermogenesis in humans using PET or magnetic resonance (MR) imaging is only feasible using cold air exposure or a LCS. Given the higher convective flow from circulating water in a full-body LCS, the same temperature will elicit a greater thermogenic response when circulating cold water through a suit versus being exposed to the same air temperature.[153] Some have also intermittently immersed participants' feet in cold water or placed them on blocks of ice[5,154] to simulate BAT thermogenesis. This approach not only stimulates cold-sensitive receptors in the skin but also stimulates pain receptors (noxious cold) that can inhibit or blunt cold-evoked activation of thermal effectors, including BAT[155]

Fixed temperature versus personalized cooling: Among the most debated issues in human BAT research is the methodological approach used to expose individuals to cold. Ultimately, two

approaches have emerged. The first relies on exposing everyone to the same temperature (e.g., 18°C), whether with cold air or a LCS.[156,157] Here, the aim is to elicit the same cold-stimulated increase in whole-body EE to investigate the thermogenic contribution of shivering and BAT thermogenesis. The second aims to personalize the cooling stimulus by cooling individuals to a threshold, often the presence of visually determined overt shivering, then followed by slight rewarming.[57] This personalized cooling approach, as currently applied, presents several critical limitations. First, the presence of overt shivering is highly subjective and does not account for the increased tonic muscle contraction that often precedes it, particularly by postural muscles.[158] Second, this approach systematically results in a dichotomous response, with one group of individuals, often identified as "BAT-positive" or "high-BAT," having a nearly 2-fold greater cold-induced increase in whole-body EE[154,159] and/or exposure to colder water temperature[160] compared with the individuals categorized as "BAT-negative" or having "low-BAT." Obviously, this difference in BAT metabolic activity could be attributable to a difference in the degree of cold exposure and creates an apparent heterogeneity that is driven by methodology rather than inter-individual biological differences. If investigators choose to personalize the cold stimulations, they should be standardized according to a measurable outcome based on either a fixed heat loss or a fixed heat production. The former has recently been performed and showed the same variability as a fixed temperature.[161] The variability in thermogenic responses using these more reproducible methods is largely explained by inter-individual differences in body morphology. Given the prolonged debate between the fixed temperature versus "personalized cooling" approaches, a critical experiment would require a head-to-head comparison that examines the variability and reproducibility in BAT thermogenesis between a fixed temperature (18°C) and a personalized cooling approach individualized on the basis of whole-body heat loss (skin temperature) or heat production. A second critical experiment should also examine whether below a minimal temperature threshold, BAT thermogenesis exhibits a graded temperature-dependent response or follows the all-or-nothing principle (i.e., activated or not).

Personalized cooling protocol rather than a fixed temperature

Kirsi A. Virtanen

Cold exposure is an effective way to stimulate hBAT function, while cooling may be carried out in several ways. The first experiments in humans were done using fixed temperature cooling protocol: precooling in an air-conditioned room (19°C) and intermittently putting the legs on an ice block (4 min every 5 min).[5] Following similar approach in the beginning of our experiments,[3,154] we used precooling the volunteers in a room with 17°C–19°C (63°F–66°F) and then placing one foot into ice-cold water (5°C–10°C) with 5 min intervals during the PET/CT scanning. At the same time, a Dutch group utilized precooling in climate chambers (16°C) 2 h prior to PET/CT scanning and following the skin temperature and shivering sensation of the volunteers.[4] A more controlled way of cooling was introduced by the Canadian group,[58] utilizing a liquid-conditioned tube suit perfused with water (18°C). Water temperature and flow of the suit are continuously controlled, thus allowing minimal shivering observed by electromyography (EMG), along the reduction in skin temperature. More recently, we have also conducted cooling using perfusable cooling blankets with adjustable water temperature.[162,163] Water immersion, which is regarded as the strongest way of cooling, is not adaptable in an experimental setting if at the same time PET/CT or PET/MR scanning are conducted.

The term "personalized cooling" was introduced by the Dutch investigators once they identified significant differences in cold sensation and hBAT activation between volunteers, especially between men and women.[57] Also, the effects of cold exposure on hBAT activation are less in obesity[4,6] and later decades of age.[164] Personalization allows adjusting the power of the cold according to either personal cold sensations (feeling of shivering) or skin temperature measurements. Cooling is carried out by decreasing step-wise the temperature, and once the volunteer reports cold and shivering, or skin temperature reaches the set point, the temperature (of the blanket or suit) is increased by a couple of degrees to avoid stronger shivering and muscle involvement in the whole-body thermogenesis.

Both approaches of cooling include questions, which are not simple to resolve. In terms of hBAT activation, fixed temperature protocol provides information on how a specific temperature is recognized and transmitted through the complex neural signal chain from the skin to brain and further to hBAT. Is it the density of cold-sensing receptors in the skin, neural signaling in the root ganglia, recognition of the signal in the deep brain areas, release of norepinephrine from the nerve endings, density of ARs in brown/beige adipocytes, or the intracellular signal cascade that makes the difference and high variation between the subjects? We are only able to measure the situation in the starting point (cold temperature) and the result, hBAT activation. Personalized cooling protocol may resolve some of these differences with fixed temperature protocol, as cooling may be stronger for the volunteers with probable low hBAT activation, such as in obesity. However, it is not completely clear whether the cooling should be stronger (colder) or should the protocol be longer, allowing more time for the organism to respond.

A clear answer to the question of whether to choose personalized cooling or fixed temperature protocol for activation of hBAT does not yet exist. If the question concerns the physiological responses of winter outdoor temperature, fixed protocol may be recommended. Instead, if similar response in hBAT activation and the individual variation to be "clamped" are searched for, personalized cooling protocol may be the choice. In the latter, it is noteworthy that measure to be clamped (for example, skin temperature) is selected to be reliably measurable one. Reporting of shivering may be regarded as not reliable.

PHARMACOLOGICAL ACTIVATION OF HUMAN BAT

Besides cold exposure, human BAT can be activated using adrenergic agonists targeting the β-ARs. A central methodological and therapeutic question is which β-AR to target, the β_2-AR or the β_3-AR (Figure 7).

Via b2-AR: β_2-AR is the dominant AR in human BAT

Sander Kooijman and Patrick C.N. Rensen

Although the role of β-ARs in promoting fatty acid release from WAT and fatty acid oxidation in BAT has been studied for

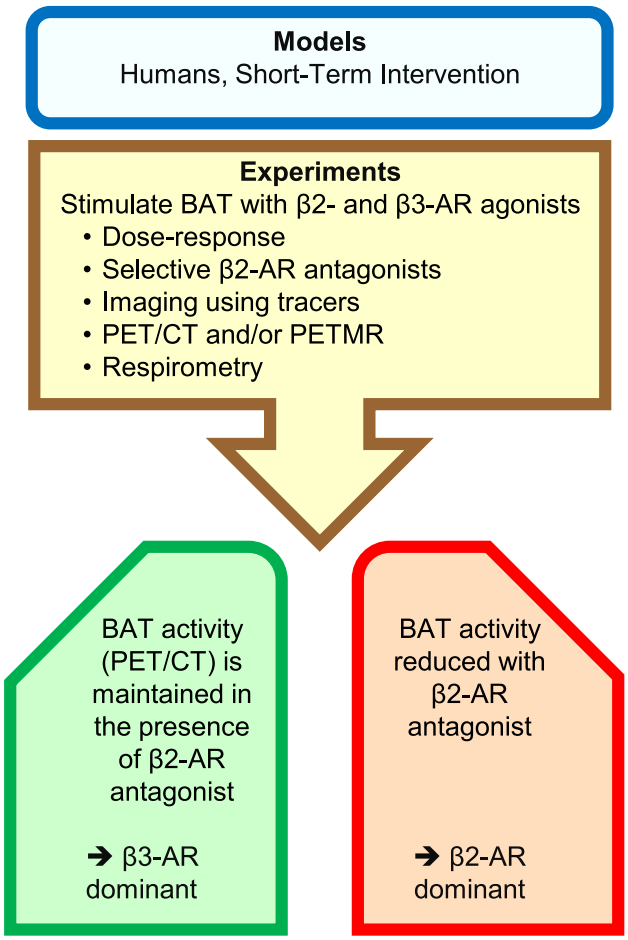

Figure 7. Proposed animal models (blue), experiments (yellow), and interpretation (green and red) in section "pharmacological activation of human BAT"

many decades,[165] still much controversy exists on the specific roles of the three β-AR subtypes (i.e., β_1-AR, β_2-AR, and β_3-AR) in humans.

The β_3-AR clearly is most abundant in murine BAT,[166] and the potent and highly selective β_3-AR agonist CL316,243 activates BAT and produces a pronounced increase in EE in mice.[167] In 2011, Bartelt et al. identified BAT as a lipid-combusting organ.[168] By using APOE*3-Leiden.CETP mice, a well-established model for human lipoprotein metabolism and atherosclerotic cardiovascular disease, we subsequently demonstrated that prolonged selective β_3-AR agonism specifically increases the uptake and oxidation of triglyceride-rich lipoprotein (TRL)-derived fatty acids by BAT and browned WAT, resulting in the generation of cholesterol-enriched TRL remnants that are avidly taken up by the liver. As a consequence, β_3-AR agonism lowers circulating triglycerides and cholesterol,[169] and in addition increases HDL levels to increase reverse cholesterol transport,[170] collectively attenuating the development of atherosclerosis (reviewed in Ying et al.[171]).

In 2015, Cypess et al. were the first to show that an oral dose of 200 mg of the β_3-AR agonist mirabegron increases metabolic activity of BAT in young, healthy male volunteers, evidenced by more uptake of ^{18}F-FDG in the supraclavicular area and increased EE.[76] However, a more recent study by Blondin et al. demonstrated that 50 mg mirabegron, the effective dose approved for the treatment of overactive bladder, does not increase net glucose uptake or oxidative metabolism.[172] One explanation might be that the effects of the higher dose are not mediated by the β_3-AR but rather reflect a generalized β-AR stimulation. In line with this notion, we found high expression of the β_2-AR, while expression of the β_3-AR was almost undetectable in human BAT biopsies. In addition, mobilization of fatty acids by mirabegron or norepinephrine in human brown adipocyte cell cultures was fully prevented when combined with the selective β_2-AR antagonist ICI118,551.[172] Based on these findings, we performed a randomized double-blinded crossover trial in young, healthy male volunteers to compare the effects of a single intravenous dose of 250 μg of the β_2-AR agonist salbutamol without and with the β_1/β_2-AR antagonist propranolol on glucose uptake by BAT. Salbutamol, compared with salbutamol and propranolol, increased net uptake of glucose by BAT as well as EE, and the net glucose uptake positively correlated with the increase in EE.[103] These data collectively suggest the β_2-AR to be essential in human BAT lipolysis, glycolysis, and thermogenesis. Interesting enough though, Cero et al. showed that *in vitro* silencing of β_3-AR expression in human brown adipocytes abolished the stimulating effects of mirabegron on lipolysis and thermogenesis,[173] which does point to a role for the β_3-AR in conserving thermogenic capacity of human brown adipocytes.

To unequivocally answer the question of whether 200 mg mirabegron acutely activates the metabolic activity of human BAT via the β_3-AR or another β-AR subtype, its effect on the metabolic activity of BAT should be assessed without or with propranolol. Either way, however, the doses of the β_3-AR and β_2-AR agonists that have been shown to activate BAT also increase heart rate and blood pressure,[103] and the β_2-AR is additionally expressed in pulmonary airways and skeletal muscle.[174,175] Specific targeting of β-AR agonists to adipose tissue is, therefore, likely to be key, and the feasibility of adipose targeting has recently been demonstrated using liposomes provided with a homing peptide as identified by *in vivo* phage display.[176] Alternative approaches include combining systemic β_2-AR agonism intervention with selective β_1-AR antagonism to minimize postsynaptic activation of the heart or performing more extensive dose-response studies to identify the lowest dose needed to activate BAT with minimal side effects. Timing of dosing should also be considered, given the strong circadian rhythmicity of BAT activity in mice[177] and probably also in humans.[178] Studying the role of β_3-AR in conserving thermogenic capacity of BAT would require dedicated long-term studies.

Beyond pinpointing the β-AR activating human BAT, we need better tools to study BAT in humans, and in particular its association with cardiometabolic health.[55] Ideally, PET-CT-compatible lipid-based tracers should be developed to evaluate effects of β-AR agonism on TRL kinetics.

Via b3-AR: Targeting the β_3-AR is the optimal approach to activating human BAT

Cheryl Cero and Aaron M. Cypess

The discovery two decades ago of functional human BAT led to a paradigm shift in the understanding of human physiology. If

activating human BAT could achieve any of the numerous metabolic benefits seen in rodent models, then there would be an innovative opportunity to treat obesity and the related metabolic diseases. Mild cold exposure was the first approach that successfully activated human BAT thermogenesis and led to promising metabolic responses.[59] Though physiologic, limitations of cold include discomfort, inaccurate dosing, lack of target organ specificity, and the infeasibility of conducting large, prospective studies. The critical need for a pharmacological approach to acute activation of human BAT has been met using mirabegron,[76] a selective β_3-AR agonist (Myrbetriq) approved to treat overactive bladder at a maximum dosage of 50 mg daily. Chronic mirabegron treatment increased detectable BAT metabolic activity and led to improvements in several metabolic parameters related to glucose and lipoprotein metabolism,[10,61] suggesting that targeting the β_3-AR could be used to treat metabolic disease in humans. Intriguingly, this scenario was complicated by two clinical-translational studies where the authors maintained that mirabegron increased human BAT thermogenesis by stimulating β_2-AR instead.[103,172] This perspective addresses two related questions regarding the roles of the β_2-AR and β_3-AR in human BAT thermogenesis and metabolism: (1) which receptor(s) is mirabegron binding to and (2) what class of drugs is optimal to clinically to activate BAT?

The binding inhibition constant Ki reflects the affinity of a drug for its receptor. For mirabegron, the Ki for the human β_1-, β_2-, and β_3-AR are 4,200, 1,300, and 40 nM, respectively.[179] With a Cmax of 106 ± 37 nM in men dosed at 200 mg,[180] mirabegron should be binding almost exclusively to β_3-AR with little activation of the β_2-AR. *In vitro* studies of Chinese Hamster Ovary (CHO) cells expressing each of the three β-AR show mirabegron's negative logarithm of the half maximal effective concentration (pEC_{50}) for cAMP accumulation is 6,900 nM for the β_2-AR and 4.8 nM for the β_3-AR.[181] In primary human brown adipocytes, knocking down the β_3-AR lowed expression of genes essential for thermogenesis, fatty acid metabolism, and mitochondrial mass, and mirabegron was unable to stimulate lipolysis and thermogenesis.[173] These *in vitro* and *in vivo* studies demonstrate that mirabegron, even at supraphysiological levels, uses the β_3-AR to stimulate human BAT thermogenesis. The central point can be framed as when mirabegron is administered at higher dosages—100–200 mg orally—what proportion of the thermogenesis comes from the β_2-AR versus the β_3-AR?

The paradigm for resolving the dispute is dose-response studies with pharmacokinetic and pharmacodynamic measurements of both β_2-AR and β_3-AR actions. Mirabegron, or the even more selective β_3-AR agonist vibegron,[182] would be administered, with measurement of drug plasma levels. Simultaneously, BAT thermogenesis would be determined via dynamic PET/CT or PET/MR. Tracers could be ^{11}C-acetate or ^{15}O-O_2 to directly measure oxidative phosphorylation or ^{18}F-FDG for higher sensitivity given that glucose uptake correlates directly with BAT thermogenesis.[183] β2-AR activation would be measured via spirometry since bronchodilation is a known, receptor-specific effect. The β_3-AR agonist would be compared with a selective β_2-AR agonist such as formoterol or salmeterol. Complementary studies could include coadministration with β_2-AR- or β_3-AR-specific antagonists, such as butoxamine and SR 59230A, respectively; however, neither of these drugs is currently approved in humans, complicating their use. Ultimately, the best oral drug to activate human BAT is going to be the one that produces metabolic activation and thermogenesis without adverse effects, particularly tachycardia. At this time, the evidence supports using β_3-AR agonists such as mirabegron as the safest and most effective way to activate human BAT and treat obesity-associated metabolic disease.

CONCLUSIONS

Two decades ago, one of the principal debates within the field was whether humans had any functional BAT at all. Thanks to a broad commitment to collaboration, discussion, and reconsideration, this and many of the previous controversies were resolved. As anticipated, these have been succeeded by a newer series of disagreements at the forefront of the field. In this perspective, we all endeavored to identify the areas of disagreement, provide supporting evidence, and, importantly, describe how the debate can be resolved using agreed-upon experimental design and methods. Over the next two decades, we anticipate that this framing, along with a commitment by investigators to this process, will lead to a resolution of the dichotomous positions and the identification of yet new, intriguing questions to debate and address. This confidence comes because, despite any differences, all of the authors share the same conviction that research to understand thermogenic adipocytes and BAT requires continued attention and resources. We hope that this perspective will be useful both for those curious about the field as well as those who devote their energies to understanding this exceptional organ.

DECLARATION OF INTERESTS

D.P.B. is the GlaxoSmithKline Research Chair in Diabetes of Université de Sherbrooke. A.C.C. is the Canada Research Chair in Molecular Imaging of Diabetes. S.K. serves as a scientific advisory board member of Moonwalk Bioscience Inc.

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