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Review

Mitochondrial efficiency: lessons learned from transgenic mice

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Abstract

Metabolic research has, like most areas of research in the life sciences, been affected dramatically by the application of transgenic technologies. Within the specific area of bioenergetics it has been thought that transgenic approaches in mice would provide definitive proof for some longstanding metabolic theories and assumptions. Here we review a number of transgenic approaches that have been used in mice to address theories of mitochondrial efficiency. The focus is largely on genes that affect the coupling of energy substrate oxidation to ATP synthesis, and thus, mice in which the uncoupling protein (Ucp) genes are modified are discussed extensively. Transgenic approaches have indeed provided proof-of-concept in some instances, but in many other instances they have yielded results that are in contrast to initial hypotheses. Many studies have also shown that genetic background can affect phenotypic outcomes, and that the upregulated expression of genes that are related to the modified gene often complicates the interpretation of findings.

Keywords: Uncoupling protein; Oxidative phosphorylation; Thermogenesis; Brown adipose tissue; Energy expenditure; Mouse; Obesity

1. Introduction

The application of transgenic technologies in life science research has become widespread. Within the field of bioenergetics and metabolism it has been expected that their application would provide definitive evidence for many longstanding metabolic hypotheses and theories. The expectation is generally founded on one of the prevailing benefits of transgenic technologies – the ability to decipher the importance of a specific protein under physiological conditions, in vivo. While this is clearly an advantage over more traditional in vitro cellular and molecular approaches, the latter approaches still remain necessary for the characterization of metabolic pathways under normal and diseased states. It is also important to recognize that most metabolic diseases have polygenic origins and are affected often by environmental factors (e.g. diet), and thus their elucidation will only be advanced in part by transgenic approaches. Interestingly, in many instances, rather than providing the proof for longstanding theories, transgenic approaches have produced results that call into question some well established metabolic concepts.

1.1. Aim and definitions

The aim in this review is not to detail transgenic technologies, but to review what useful findings have

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emerged from their application to the study of mitochondrial energetics. Specifically, we will address the question: how has the application of transgenics improved our understanding of the factors affecting the efficiency of mitochondrial energetics? The term 'mitochondrial efficiency' is used in the context of the efficiency of energy transduction. In itself, it conveys the now well recognized fact that there is indeed significant variation in the degree to which mitochondria produce ATP from a given amount of energy substrate, originating from dietary fat, carbohydrate and protein. The major mechanisms affecting mitochondrial efficiency comprise uncoupling protein (UCP)-mediated proton leaks, and proton leaks having other origins. Both have been proposed to decrease the efficiency of mitochondrial ATP synthesis (oxidative phosphorylation), but the degree to which the two are distinct is currently a much debated topic, as discussed by others in this monograph. The control and regulation of oxidative phosphorylation will also be described by others within this monograph, as will the pathologic and more rare mechanisms underlying variation in mitochondrial energetic efficiency, e.g. mitochondrial cytopathies.

Before proceeding however, some fundamental concepts should be reviewed. Regardless of the specific mechanism, the uncoupling of oxidative phosphorylation results in the release of energy in the form of heat. Heat production (thermogenesis) results directly from substrate oxidation, and is a normal and advantageous byproduct of mitochondrial energetics, particularly in endothermic species. When oxidative phosphorylation is well coupled, thermogenesis is positively correlated to the amount of ATP synthesis, and to the rate of substrate oxidation (and thus tissue oxygen consumption). However, during uncoupled oxidative phosphorylation, thermogenesis correlates only with the rate of energy substrate oxidation. Because the route of proton return to the matrix bypasses ATP synthase in uncoupled states, oxygen consumption is clearly unrelated to (uncoupled from) ATP synthesis.

1.2. *Implications of uncoupling for mammalian energy metabolism – a historical perspective*

At the level of the whole animal, uncoupling affects both thermoregulation and fuel efficiency. Thermoregulation can be defined as the ability of the animal to adjust its heat production and heat loss in accordance with changes in environmental temperature to maintain its body temperature. Fuel efficiency at the level of the whole animal refers to the proportion of dietary energy that is ultimately available for use by the animal [3]. Uncoupled oxidative phosphorylation is the hallmark feature of metabolism in brown adipose tissue (BAT) where the presence of UCP1, in concert with unique anatomical features of the tissue, allows a profound degree of thermogenesis [35,39]. As will be discussed below in the context of the Ucp1 knockout mouse, UCP1[1]-mediated uncoupling is necessary for the acute increase in thermogenesis on exposure to cold. Because of the high rates of fuel oxidation and heat production that occur when BAT is fully active, uncoupled energy metabolism affects energy expenditure, especially in small rodents where energy expenditure is often doubled or tripled when animals are cold-acclimated. In humans, BAT is present in large amounts in newborns and plays an important role in the adaptation to postnatal ambient temperatures, but by adulthood only small amounts remain [35,39].

A state of energy balance exists when an animal's dietary energy intake equals its energy expenditure. When energy intake chronically exceeds energy expenditure, the surplus is stored mainly as adipose triacylglycerol. The outcome of uncoupling is increased energy expenditure; the energy is simply released as heat. Thus uncoupling has implications for energy balance, and for this reason, there has been a longstanding interest in the role of uncoupled energy metabolism in the development, and the potential treatment, of obesity. That uncoupled energy metabolism in BAT could play a significant role in the development of [37] and resistance to [75] obesity was well demonstrated over 20 years ago. Recent studies using β-3-adrenergic agonists in rodents to stimulate brown adipose thermogenesis demonstrate that an acute dose can double resting energy expenditure, and that chronic treatment with agonist results in recruitment of Ucp1-expressing thermogenic brown adipocytes in white adipose tissue (WAT) de-

[1] Uppercase and lowercase letters will be used to denote the protein and the gene (or mRNA thereof), respectively.

pots that normally contain only white adipocytes [20]. There is much interest in the possibility that β-3-adrenergic agonists may be used to recruit thermogenic adipocytes in WAT of adult humans to treat obesity and mature-onset diabetes.

The extensive uncoupling and thermogenesis that can be achieved in BAT was described in scientific literature long before the identification of the protein thought to cause the uncoupling. In the early 1960s the thermogenic function of BAT was identified [79]. It was almost another 20 years before UCP (referred to as UCP1 following the identification of newer members of the gene family) was isolated [62,72] and cloned [5,6,42,71]. While a lesser degree of uncoupling was described for mitochondria from tissues other than BAT in animals not exposed to cold, the uncoupling was initially perceived as an artifact of mitochondrial isolation. However, in the late 1980s and early 1990s, Brand and colleagues showed that the uncoupling is physiologic, and is regulated by a number of factors known to affect total body energy metabolism (e.g. thyroid hormones) [31–33,63]. This process, referred to as mitochondrial proton leak, has been estimated to account for 20–50% of resting metabolic rate in rats [8,73,74]. The hypothesized mechanism for leak and factors affecting the regulation of leak will be reviewed by others in this monograph, and only the fundamental discoveries will be introduced here.

In the search for mechanisms underlying mitochondrial proton leak, a number of proteins that are homologous to UCP1 were identified. There are now five putative UCPs. Ucp2 is expressed in most tissues of humans and rodents [16,21]. Ucp3 is expressed preferentially in skeletal muscle of humans; in rodents it is also expressed in BAT [7,91]. UCP2 and UCP3 have considerable homology with UCP1 – roughly 57–59%. Ucp4 is expressed in the brain, and not in any of the other many tissues probed [55]. The fifth putative member is brain mitochondrial carrier protein 1 (Bmcp1), and it is expressed mainly in the brain, but also in other tissues to a minor extent [76]. The homologies of UCP4 and BMCP1 with UCP1 are approximately 34%. BMCP1 is increasingly being referred to as UCP5, and very recent findings correlate UCP5 expression with metabolic rate [1,93]. Despite the proliferation of studies on the novel UCPs (approximately 400 papers published since 1997) (reviewed in [7,38,78,82]), little is known about their true physiologic function. It has been expected that the application of transgenic methods (reviewed below) would shed considerable light on this area.

Prior to the development of transgenic methods, studies aimed at elucidating the physiological significance of uncoupled metabolism in BAT employed dietary, environmental temperature and pharmacologic methods to modify the activities of uncoupling processes. Overfeeding was shown to induce the expression of Ucp1 and increased uncoupling activity in BAT; this is referred to as diet-induced thermogenesis, and it was proposed as a metabolic mechanism favoring leanness [75]. The exposure to environmental cold is well known to result in increased UCP1 protein and uncoupling activity in BAT mitochondria, and in the appearance of Ucp1-expressing adipocytes in adipose tissue depots that normally contain only white adipocytes. Rodents that are housed at thermoneutrality (approximately 31°C for mice, and 28°C for rats) on the other hand have no need for thermoregulatory heat production, and it has been shown that UCP1 expression and activity are low under such conditions. A number of studies of the thermogenic effects of β-3-adrenergic agonists, and studies of specific hormone and disease states (thyroid hormones; pheochromocytoma) have corroborated the generally well accepted opinion that uncoupled BAT metabolism, at least in rodents, was important with respect to obesity and thermoregulation [35]. It is generally well accepted that noradrenaline, secreted by the sympathetic nerves that innervate BAT, is responsible both for the acute thermogenic response of the brown adipocytes and for the hypertrophy of BAT that occurs in response to chronic stimulation. This hypertrophy involves proliferation of precursor cells and then differentiation, marked mitochondriogenesis in new and old brown adipocytes and a concomitant upregulated expression of Ucp1. The thermogenic capacity of BAT increases to the extent that it can sustain in mice a 3.5-fold increase in whole body heat production at a temperature of 5°C in the absence of shivering [65]. In the warm acclimated mouse, an increase in whole body heat production can still occur, to almost the same extent as in the cold-acclimated mouse, but in this case the increased heat production occurs in the shivering skeletal muscles, as well as, to a much less-

er extent, in BAT. Because of the clear importance of BAT towards energy expenditure in rodents there was substantial support for the hypothesis that UCP1-mediated uncoupling is relevant to obesity prevention.

1.3. Transgenic approaches

Transgenic approaches have a particular advantage over traditional approaches in that they sometimes allow unequivocal answers to questions of the importance of a specific protein under physiological conditions, in vivo. The term 'transgenic' is used here to describe mice in which recombinant DNA technology is used to remove a gene (or part of a gene), or in which a gene is overexpressed through the transfer of a foreign gene construct. The resulting mice are commonly referred to as either 'knockout' or 'overexpression' mice, respectively, for a particular gene. Several good reviews of mammalian transgenic technologies are available [9,51,53].

One of the difficulties encountered with the use of 'knockout' mice in the elucidation of a specific protein's physiological function is that another gene may encode a protein having a homologous function, and the expression of the homologue may be upregulated as a result of the removal of the targeted gene. The outcome may be partial, or even complete compensation, and the conclusions drawn may then underestimate the true physiological importance of the targeted gene. Another fundamental and common problem is that the removal of the gene product may interrupt normal embryonic development, precluding postnatal life.

Transgenic mice in which genes are overexpressed through the transfer of a foreign gene construct also have inherent advantages and disadvantages. 'Overexpression' mice can be produced relatively quickly, but are associated with the unpredictable integration of the transgene into the genome. As a result, multiple copy numbers of the transgene can be incorporated, and there is often no control over the integration site in the genome.

In summary then, the main advantage of transgenic approaches over the traditional in vitro cellular and molecular approaches is that the former provide information about the importance of a specific protein at the level of the whole animal. However, as will be elaborated upon during the discussion of specific transgenic models, studies of transgenic mice are replete with complexities stemming predominantly from redundancies, or 'back-up' mechanisms within metabolic systems. Consequently, mouse phenotypes need to be interpreted with an integrative understanding of metabolism and physiology. Despite the above described limitations and potential problems, the application of transgenic technologies has generated resourceful information on mitochondrial energetics.

2. Transgenic alterations in mitochondrial proteins affecting the efficiency of energy transduction

A number of transgenic approaches have been used to study mitochondrial efficiency. First to be considered will be the approaches used to modify genes that are linked to mitochondrial uncoupling through direct effects on mitochondrial proteins. There are also several examples of transgenic modification of extramitochondrial factors which impinge on mitochondrial efficiency; these will be discussed afterwards. Table 1 outlines, within these categories, the specific transgenic models that will be described here.

2.1. Removal or relative absence of UCPs

2.1.1. Transgenic ablation of cells expressing UCP1

Since it had not been feasible using traditional metabolic approaches to assess the function of the recently identified UCPs in their physiological environment, it was hoped that studies of transgenic mice that lack the UCPs might clarify their physiological function. The first attempt was in the early 1990s and used transgenic ablation of Ucp1-expressing adipocytes in mice with a FVB/N genetic background [52]. Cell ablation was achieved by linking the expression of diphtheria toxin A (DTA) to the Ucp1 promoter; thus Ucp1-expressing cells were killed. Based on the extensive literature describing the importance of UCP1 in rodents for energy balance and thermoregulation, it was anticipated that these mice would be obese and cold-intolerant. The mice, referred to as UCP-DTA mice, were indeed obese and, surprisingly, hyperphagic. It was also surprising that the

UCP-DTA mice were not cold-intolerant. Despite a blunted increase in total body oxygen consumption in response to an acute dose of β-3-adrenergic agonist, the mice were able to tolerate the exposure to cold (4°C).

Interestingly, the ablation of Ucp1-expressing cells in UCP-DTA mice was not complete; only 60% of the UCP1-expressing cells were removed. Thus the obesity observed is intriguing – particularly when one considers the metabolic phenotypes of the Ucp1 knockout mouse, and the Ucp3 knockout mouse (described below). The obesity in the UCP-DTA mouse is accompanied by the common metabolic sequellae of obesity, and include high circulating leptin levels, leptin resistance of feeding, insulin resistance, and hyperlipidemia [17,18,28,29]. The regulation of hypothalamic neuropeptides by leptin appears fairly normal and some other defect in control of food intake is suggested [54,88]. A role for BAT in a leptin-independent control of food intake has been suggested on other grounds [57,58]. These studies with UCP-DTA mice suggested that the relative lack of UCP1 function might lead to hyperphagia as well as obesity. It must however be remembered that the ablation of Ucp1-expressing cells would also ablate other UCPs that are also present in UCP1-expressing brown adipocytes. The possibility that the transgene may have been inappropriately expressed, or inserted into a gene associated with leptin signaling has also been discussed [47]. Consistent with the latter possibility is the fact that another line of UCP-DTA mice that was described in the original report (Ucp176 line; [52]) was not hyperphagic, and showed a transient obesity. Further investigations using this mouse model of obesity are needed.

2.1.2. The Ucp1 knockout mouse

The metabolic characteristics of UCP-DTA mice suggested that the complete removal of Ucp1 through gene knockout technologies might lead to profound obesity in mice. However, the Ucp1-deficient, or 'knockout', mouse showed that this, surprisingly, is not the case [14]. The targeted inactivation of the Ucp1 gene was achieved through homologous recombination. The mice have no mRNA for Ucp1 of normal size, as assessed by Northern blot analysis, or by reverse transcription-PCR. As expected though, the mice are cold-intolerant, as demonstrated by their inability to maintain normal body temperature when exposed to 4°C. Consistent with the latter was the observed blunted response in total body oxygen consumption to the administration of an acute dose of a β-3-adrenergic agonist.

The finding that Ucp1 knockout mice show no enhanced susceptibility for obesity even when fed a high fat (58 kcal%) diet is remarkable, particularly in light of their defective thermogenesis. In contrast to the UCP-DTA approach described above, in the Ucp1 knockout mouse the UCPs other than UCP1

Table 1
Some transgenic mouse models relevant to studies of mitochondrial efficiency

Cellular location	Transgenic modification	References[a]
(A) *Mitochondrial*		
(i) Removal or reduction	Ucp1 toxigene (Ucp-DTA)	[17,18,28,29,47,**52**,57,58]
	Ucp1 knockout	[**14**,47,56,59]
	Ucp3 knockout	[**23**,**90**]
	Ucp1–Ucp3 double knockout	[**23**]
	Ucp2 knockout	–
	Ant1 knockout	[15,**25**]
(ii) Overexpression or ectopic expression	aP2-UCP1 ectopic	[**45**,46,81]
	hUcp3 overexpression	[**10**]
(B) *Extramitochondrial*		
(i) Removal or reduction	PKA-RIIβ knockout	[2,**11**,66]
	Hormone-sensitive lipase knockout	[**64**,92]
	β-3-Adrenergic receptor knockout	[27,40,69,**85**]
	Dopamine β hydroxylase knockout	[86,**87**]
(ii) Overexpression	Glut4 overexpression	[**89**]

[a]Reference numbers in bolded font indicate the first publication reporting the transgenic mouse model.

would remain intact. It is important to also note that these initial experiments were conducted on mice having a mixed 129/Sv Pas and C57BL6J genetic background. Genetic background appears to have significant consequences on the resulting phenotypes. Kozak and colleagues have now placed the targeted allele on several defined genetic backgrounds, such as C57BL6, 129/SvImJ and BALB/cBy [47]. Their as yet unpublished results show profound effects of genetic background on cold-intolerance in the different lines of Ucp1 knockouts. Studies relating to possible effects of background on the susceptibility to obesity are in progress.

A number of groups have begun to examine the implications of the absence of UCP1 for bioenergetics at the level of isolated mitochondria and cells from BAT. The first was Monemdjou et al. [59] who showed that proton leak in mitochondria isolated from BAT of Ucp1 knockouts is insensitive to the purine nucleotide, GDP, that very effectively inhibits UCP1 activity. Monemdjou et al. show that GDP inhibits proton leak in mitochondria isolated from control mice by approximately 50%; under the same incubation conditions, GDP has no effect on leak in mitochondria from Ucp1 knockout mice. Because BAT expresses Ucp2, Ucp3, and Ucp5 [93], as well as Ucp1, their results show that any proton leak that is caused by any of these UCPs is insensitive to GDP. It is also interesting to note that despite the 5-fold increase in level of message for Ucp2 (reported by Enerbäck et al., [14]), and no change in the levels of mRNA for Ucp3 (Kozak, unpublished findings), there is no difference in the amount of proton leak between knockouts and controls when mitochondria are incubated in the presence of saturating concentrations of GDP. Thus the level of message for Ucp2 is incongruent with the level of its putative function, proton leak as assessed under their experimental conditions (see discussion below).

A provocative series of experiments on the fatty acid induction of proton leak in BAT mitochondria from Ucp1 knockout and control mice have recently been conducted by Nedergaard and Cannon and colleagues [56]. The experiments were predicated upon the widely accepted notion that fatty acids liberated from noradrenergic stimulation of lipolysis were the intracellular activators of uncoupling in brown adipocytes [67,68]. This hypothesis was bolstered over many years by the results of numerous types of studies, such as those showing that fatty acids and fatty acyl CoA esters could relieve purine nucleotide inhibition of uncoupling activity (e.g. [12,70,83,84]). Moreover, experiments using mitochondria isolated from yeast that were transfected with Ucp1 (wild type or mutated Ucp1) have suggested that a cysteine residue near the C-terminal of UCP1 plays an important role in fatty acid activation of uncoupling [24]. Other reports describe distinct sites for fatty acid activation and nucleotide binding sites in studies on isolated and liposome-reconstituted UCP1 [43].

The conclusion of Matthias et al. [56] that UCP1 is not directly involved in the uncoupling effect of fatty acids in brown fat mitochondria was thus surprising. The authors investigated the fatty acid-induced changes in mitochondrial membrane potential and oxygen consumption in BAT mitochondria from Ucp1 knockout and control mice. Short and long chain fatty acids were shown to decrease mitochondrial membrane potential, and increase oxygen consumption in mitochondria from both Ucp1 knockout mice and from controls. The substrate provided to the mitochondria in these studies was glycerol-3-phosphate, and rotenone was used in the incubations to inhibit the oxidation of any endogenous substrate through complex 1. Thus it cannot be argued that fatty acids were being used as substrates for mitochondrial oxidation. Because their findings show that fatty acids uncouple oxidative phosphorylation in Ucp1 knockout mitochondria just as well as they do in wild type control mitochondria, their findings thus support the conclusion that free fatty acids are not the intracellular activator of UCP1. Their report is indeed provoking further studies on this specific question, and on the identification of the true physiological activators of other UCPs [60].

It must be noted that the initial studies by Monemdjou et al. [59] on the kinetics of mitochondrial proton leak were conducted on mitochondria that were isolated and incubated in the presence of 0.5% defatted bovine serum albumin (BSA). Given more recent findings on the effects of fatty acids and purine nucleotides, it is possible to re-examine these findings, and reflect further on the results of Matthias et al. [56]. Relevant are the recent results of Jaburek and colleagues [41] who expressed UCP2 and UCP3 in *Escherichia coli* and reconstituted the

proteins into liposomes. Based on the results of their ion flux studies they conclude that purified UCP2 and UCP3 behave identically to UCP1. All three catalyze electrophoretic flux of protons and alkylsulfonates. They also show that flux induced by each of the three UCPs has an obligatory requirement for fatty acids, and that there are large differences in the K_i values for nucleotide inhibition of the UCPs. While the K_i for GDP inhibition of UCP1 activity is 17 μM, that for UCP2 and UCP3 are approximately 1.2 mM and 1.0 mM, respectively. The possible implications of these findings for the earlier results of Monemdjou et al. [59] and Matthias et al. [56] are as follows. An obligatory requirement for fatty acids would explain the low rates of leak-dependent oxygen consumption observed by Monemdjou et al. [59] since their mitochondria were studied in the presence of 0.5% defatted BSA. The fact that Monemdjou et al. [59] used a GDP concentration of 1.0 mM would mean that while UCP1 activity would be fully inhibited, the activity of UCP2 and UCP3 might only be partially inhibited. While nucleotides could be an in vivo regulator of UCP1 activity, it is unlikely that they regulate the activities of UCP2 and UCP3 given the high concentrations needed to inhibit them. Thus, UCP2 and UCP3 could be active under conditions where there are relatively high concentrations in vivo of ADP, such as near state 4 respiration, when a cell has very little need for ATP. UCP1 would not be active under these conditions given its lower K_i for purine nucleotides. In fact the phosphorylation ratio in BAT cells would seem to have little bearing on the activity of UCP1; this is evident in the flux control coefficients for proton leak between Ucp1 knockout and control mitochondria [59].

The results of Matthias et al. [56] are interpreted as showing that the fatty acid activation of uncoupling in BAT mitochondria is the same in mitochondria regardless of the presence or the absence of Ucp1. However, once again it must be noted that it is not simply the absolute presence versus the absence of Ucp1 that was studied. We know that the genetic removal of Ucp1 induces an upregulated expression of UCP2 mRNA; it is likely that there are changes in the expression level of a number of other inner membrane proteins. It is also known that other members of the mitochondrial carrier protein family, such as the adenylate carrier, can also catalyze fatty acid-induced uncoupling [77]. It thus seems that in order to accept the idea that fatty acids do not directly activate UCP1 activity, further corroborative results are needed.

2.1.3. The Ucp3 knockout mouse

Two groups have simultaneously reported on the creation and metabolic characteristics of transgenic mice lacking Ucp3. Based on the high homology of Ucp3 with Ucp1, and on its high levels of expression in skeletal muscle and BAT, and its ability to diminish mitochondrial protonmotive force, Ucp3 was hypothesized to play an important role in energy balance and thermoregulation. The finding that thyroid hormones increase the expression of Ucp3 [22,49,50] is consistent with this hypothesis. Inconsistent with this concept is the observation that fasting results in increased expression of Ucp3. However, this has been described as indicating a possible role for UCP3 in the metabolism of the fatty acids liberated during fasting. Another putative beneficial role for the recently discovered UCPs is the decreased production of reactive oxygen species (ROS), as has been described for UCP2 [61].

Lowell's group produced knockouts by electroporating the targeting vector into J1 embryonic stem cells and the targeted clones were then injected into C57BL6 embryos [90]. Reitman and colleagues used a 129/SvC embryonic stem cell line and the chimeric mice that were heterozygous for the targeted allele were crossed to Swiss Black mice [23]. Both groups report that Ucp3 knockout mice are quite healthy, at least at the ages studied, and are not more predisposed to obesity than control mice. The most striking findings of Lowell's group were that skeletal muscle mitochondria lacking UCP3 are better coupled (improved respiratory control ratio (RCR)), and produce a significantly higher amount of ROS than control mitochondria. Based on the hypothesized role for UCP3 in the metabolism of the fatty acids, it was expected that the mice might demonstrate abnormal oxidation of lipids as assessed by indirect calorimetry, and perhaps by abnormal serum lipid profiles. The former was not observed in any of the mice under any condition (e.g. exercise, fasting, feeding a high fat diet), and the latter was only observed in older (18–24 weeks) mice when fed a high fat diet.

Their findings relating the absence of UCP3 in

muscle to the production of ROS are particularly interesting. Two independent methods were used to assess the production of ROS: lucigenin-derived chemiluminescence (to detect superoxide anion production), and mitochondrial aconitase activity (to assess in vivo ROS inhibition of this mitochondrial enzyme). As ROS are thought to underlie aging processes, it will be interesting to see if the UCP3 knockout mouse ages at an accelerated rate, and has a diminished maximum life span (see [30,80]). Potentially relevant are the findings of Harper and colleagues who have shown that mitochondrial proton leak is increased in hepatocytes of old compared to young mice [34]. There is also evidence that caloric restriction lowers mitochondrial proton leak in skeletal muscle from old rats (Harper, unpublished observations), and it is well demonstrated that caloric restriction minimizes damage from ROS, and extends maximum life span [80].

In the simultaneous report, Reitman and colleagues [23] similarly describe mice whose phenotype at the whole body level is unaffected by the absence of UCP3. An important finding from this group was that mitochondrial proton leak is greatly reduced in muscle, minimally reduced in BAT, and not altered in liver mitochondria. The findings suggest that UCP3 accounts for the majority of leak in skeletal muscle. The overall kinetics of muscle proton leak reactions are described by protonmotive force values that are significantly greater than those for control mitochondria – consistent with the absence of a protein that uncouples oxidative phosphorylation. Despite the reductions in muscle mitochondrial proton leak, no consistent phenotypic abnormalities at the whole body level were observed. The knockout mice were not obese and had normal serum insulin, triglyceride, FFA and leptin levels. Circadian rhythms for body temperature and motor activity were normal as were body temperature responses to fasting, stress, thyroid hormone, and cold exposure. Metabolic rate and respiratory quotients as assessed by indirect calorimetry were normal, and the responses in each to fasting, thyroid hormone and to a β-3-adrenergic agonist (CL 316,243) were normal; responses after feeding a high fat diet were not assessed. Because increases in basal metabolic rate in response to thyroid hormone treatment were the same in knockout and control mice, their findings show that thyroid thermogenesis does not require UCP3. Overall, while the results show abnormal bioenergetic characteristics in muscle mitochondria lacking UCP3, this does not apparently have a significant effect on metabolic rate.

Thus both groups provide evidence supporting the idea that mitochondrial proton leak, as it has been traditionally defined, is lower in skeletal muscle mitochondria of Ucp3 knockout mice. This should not however be interpreted as indicating that UCP3 is thus a proton translocating protein. Even the mechanism of uncoupling for UCP1 is a matter of debate, despite many years of study [19,44,47]. While it is possible that UCP3 may use the same mechanism for uncoupling as UCP1, it may cause electrophoretic transport of other metabolites such as those related to fatty acid oxidation. The latter would be consistent with the increased expression of UCP3 during fasting. Further studies utilizing skeletal muscle mitochondria from these mice aim at elucidating the transported moiety.

2.1.4. The Ucp1–Ucp3 double knockout mouse

To determine whether the phenotype of the Ucp1 knockout mouse would be made more severe by the genetic removal of Ucp3, the double Ucp1–Ucp3 knockout mouse was bred [23]. As described above, the Ucp1 knockout mouse has a reduced thermogenic response to a β-3-adrenergic agonist, and is cold-sensitive [14]. The double knockout mice however did not have any further reduction in their response to β-3-adrenergic stimulation, and were no more sensitive to cold compared to the Ucp1 knockout. Thus these results suggest that Ucp3 is not a modifier gene of the Ucp1 knockout phenotype. It would be interesting to see whether the tendency towards higher circulating levels of fatty acids on older Ucp3 knockout mice fed a high fat diet is accentuated in the Ucp1–Ucp3 double knockout mouse, given that fatty acid oxidation to support UCP1 activity is normally important, especially at colder environmental temperatures.

2.1.5. The Ucp2 knockout mouse

Reports on production of Ucp2 knockout mice are imminent from two laboratories. A discussion of the phenotypes of these models awaits the official publications.

2.1.6. The adenine nucleotide translocator 1 (Ant1) knockout mouse

To create a mouse model of tissue-specific mitochondrial disease, Wallace and colleagues generated mice that are deficient in Ant1 [25]. Mice normally have two Ant genes, Ant1 which is expressed in skeletal muscle, heart, and brain, and Ant2 which is expressed in all tissues except skeletal muscle. Thus, skeletal muscle of the Ant1 knockout mice is devoid of any known adenine nucleotide transporters, while brain and heart express the Ant2 isoform, and thus are only partly deficient in transporter activity. Ant1 knockout mice exhibit severe exercise intolerance, and the classical anatomical, histological, biochemical, metabolic and physiological features associated with the development of myopathy and hypertrophic cardiomyopathy. Biochemically, skeletal muscle mitochondria of knockout mice have defective ADP-stimulated (i.e. state 3) respiration (most substrates), consistent with the absence of ADP/ATP transport. State 4 rates were comparable between knockouts and controls. As a result of the lower state 3 rates, respiratory control ratios were 2–3-fold lower in knockouts than in controls. There was also a marked proliferation of mitochondria in skeletal muscle suggesting a compensatory proliferation of mitochondria.

Sequential studies revealed that mitochondria from skeletal muscle, heart and brain of the Ant1 knockout mice produce dramatically higher amounts of the ROS, hydrogen peroxide [15]. The increased ROS elicits manganese superoxide dismutase activity in muscle and heart. Glutathione peroxidase-1 activity is moderately increased in both tissues. Mitochondrial rearrangements in mtDNA occurred in knockouts and were higher in heart than in skeletal muscle, consistent with the lower degree of antioxidant defenses in the heart. Whether or not there are any changes in the levels of expression of other UCPs is not as yet known.

2.2. Overexpression or ectopic expression of UCPs

2.2.1. The aP2-Ucp(1) mouse

Before the identification of UCP2 and the other putative UCPs, Kozak and collaborators produced the aP2-Ucp mouse to test the idea that UCP(1) could provide defense against obesity if expressed not only in BAT, but also in WAT [45]. To do this they used the adipose-specific promoter aP2 to direct the expression of Ucp1. The presence of transgenic UCP1 in BAT results in downregulation of the endogenous gene so that BAT becomes relatively deficient in Ucp1 expression under the control of its own promoter. The actual amount of UCP1 protein in BAT is relatively normal. As hypothesized however, the expression of the transgene in adipose tissues results in reductions in total body weight and subcutaneous fat stores in the genetically obese A^{vy} mouse. Further studies showed that diet-induced obesity in C57BL6J mice was also mitigated by the ectopic expression of Ucp1 in WAT [46]. In the latter, feed efficiency (weight gain per unit of dietary energy intake) was 50% of the efficiency of non-transgenic control mice. The expression of the transgene was however not sufficient to allow a normal thermogenic response to an acute dose of norepinephrine, or in homozygotes, to cold, and the dose of transgene was inversely correlated to the degree of thermogenic response [81]. The authors attribute the impaired heat production to the potentially detrimental effects of the early expression of Ucp1 during differentiation (controlled by the aP2 promoter in these transgenic mice); normally Ucp1 expression occurs later during differentiation. These findings, in conjunction with the findings from Ucp1-deficient mice (above), indicate that BAT bestows the unique function of adaptation to acute cold. The high levels of Ucp1 expression in BAT, plus the unique anatomical features of BAT (arterio-venous anastamoses, high degree of sympathetic innervation) make it an efficient producer and exporter of heat.

2.2.2. The human Ucp3 (hUcp3)-overexpressing mouse

The question of the physiological effects of the increased expression of Ucp3 has very recently been addressed by one group [10]. Clapham and colleagues generated a mouse line overexpressing hUcp3 in muscle of C57BL6×CBA mice in which transcription was driven from the mouse α-actin promoter (in order to restrict gene expression to muscle). By 8 weeks of age, transgenic males had significantly lower body weights, despite increased food intake, compared to wild type mice. Following the feeding

of a palatable diet (condensed milk diet) the transgenics still displayed lower body weight despite increased energy intakes. Mice overexpressing UCP3 were also more glucose-tolerant than wild type mice. These results provide exciting evidence supporting the idea that UCP3 can indeed affect the efficiency of mitochondrial oxidative phosphorylation, and whole body energetics. Further detailed analyses on skeletal muscle mitochondria will be of significant interest.

3. Transgenic modification of extramitochondrial factors affecting the efficiency of energy transduction

3.1. Removal or reduction of the activators/factors

3.1.1. The protein kinase A (PKA)-RIIβ knockout mouse

Cyclic AMP (cAMP) is a well recognized intracellular factor in the control of cellular energy metabolism. To study its significance, McKnight's group in 1996 generated mice in which the PKA-RIIβ subunit of PKA was genetically disrupted [2,11]. Because the effects of cAMP are mediated through cAMP-dependent PKA, and because the RIIβ subunit of PKA is abundant in BAT and WAT and the brain, it was anticipated that the genetic removal of this key regulatory element would clarify the physiological functions of PKA in energy metabolism. While their findings confirm the importance of the regulatory subunit, what is also very interesting is the compensatory switch to the use of the RIα isoform which almost entirely replaces the lost RIIβ isoform. As a result the holoenzyme in transgenic adipose tissue has a greater affinity for cAMP and is constitutively activated. The phenotypic outcome is decreased adiposity despite normal food intake, and protection against diet-induced obesity. Increased UCP1 expression and uncoupling activity are thus thought to explain at least partly the altered energy balance. However, Planas et al. [66] show that the lipolytic response to isoproterenol and CL 316,243 is deficient, thus indicating that the compensatory switch to the use of the RIα isoform is not fully compensatory.

3.1.2. The hormone-sensitive lipase knockout mouse

Another intracellular mediator widely considered as crucial for the activation of uncoupling in BAT is hormone-sensitive lipase, as it hydrolyzes triacylglycerol, releasing fatty acids which in turn have been considered as activators of UCPs. Hormone-sensitive lipase also is known to hydrolyze cholesterol esters in tissues including the adrenals, ovaries, testes and macrophages. It was anticipated that a hormone-sensitive lipase knockout mouse would thus have defective cold-induced thermogenesis, and steroidogenesis. Here, once again, is an example of findings that refute widely accepted notions on the mechanisms activating uncoupling. Notwithstanding the genetic inactivation of hormone-sensitive lipase, mice are neither obese nor cold-sensitive which must be interpreted as indicating alternate and as yet unknown mechanisms that cause the liberation of fatty acids to fuel BAT thermogenesis, and activate UCP1 activity [64,92]. (However, see also the discussion above on the results of Matthias et al. [56].) The only phenotypic abnormality in knockouts was sterility in males. Adipocytes in BAT and WAT were roughly 5-fold and 2-fold larger in knockouts compared with controls, suggesting that hydrolysis of triacylglycerol was to some extent affected. However, knockout mice retained approximately 40% of triacylglycerol lipase activity relative to controls. Overall their results indicate that hormone-sensitive lipase cannot, as earlier thought, be the sole lipolytic enzyme in adipose tissues.

3.1.3. The β-3-adrenergic receptor knockout mouse

The activation of β-1-, β-2- or β-3-adrenergic receptors leads to increased adenylate cyclase activity and cAMP levels. In adipose tissues, the latter activates PKA which then activates lipolysis in white adipocytes, and uncoupling in brown adipocytes [48]. The β-3-adrenergic receptor is expressed almost exclusively in adipose tissues (brown and white), but adipose tissues express all three types of receptors. Because the β-3-adrenergic receptor is relatively resistant to desensitization and downregulation [26], it was hypothesized that it might maintain signaling during periods of sustained sympathetic stimulation, such as that in BAT during chronic exposure to cold, or during diet-induced thermogenesis.

To examine the physiology and pharmacology of the β-3-adrenergic receptor, Lowell and collaborators produced mice with targeted disruption of the β-3-adrenergic receptor gene [85]. It was initially thought that these mice might be obese, and perhaps have defective thermoregulatory mechanisms. However, the mice were found to have only a modest increase in body fat. As observed in other gene knockout mice, here again there was increased expression of a related gene. β-1-Adrenergic receptor, but not β-2-adrenergic receptor mRNA levels were upregulated. However, their results show that the effects of the β-3 selective agonist, CL 316,243, such as adipocyte triacylglycerol lipolysis, whole body energy expenditure, and reduced food intake, are mediated exclusively by β-3-adrenergic receptors.

Later studies by this same group showed interestingly that a full thermogenic response to β-3-adrenergic receptor stimulation requires that receptors are present in both brown and white adipocytes. It seems, in other words, that both the 'furnace' (BAT thermogenesis) and the 'fuel tanks' (WAT triacylglycerol stores) must be activated [27]. For these studies, tissue-specific β-3-adrenergic receptor transgenic constructs were injected into mouse zygotes homozygous for the β-3-adrenergic receptor knockout allele.

It is interesting to note that β-3-adrenergic receptor knockout mice having a different genetic background from those used by Lowell's group (i.e. FVB/N, FVB/N×129/SvJ, and 129/SvJ) are predisposed to obesity. In mice having a 129/Sv×C57BL6J background, Revelli et al. [69] report a 41% increase in body fat content in knockouts compared to wild type controls despite slight (non-significant) increases in food intake. In contrast to the results of Susulic et al. [85], who report a compensatory increase in mRNA levels for the β-1-adrenergic receptors in BAT and WAT of knockout mice, Revelli et al. [69] observe a decrease in mRNA for the β-1-adrenergic receptor in BAT, and no change in levels in WAT.

Ito et al. [40] produced a transgenic mouse that expresses the human form of the β-3-adrenergic receptor rather than the murine form. This humanized mouse should be useful in the development of drugs that are effective in treating human obesity given the well known differences in the affinities of agonists between rodent and human forms of the receptor [4,13].

3.1.4. The dopamine β hydroxylase knockout mouse

Another mouse that was produced in order to study mechanisms of thermogenesis and energy balance is the dopamine β hydroxylase knockout mouse [87]. Dopamine β hydroxylase is the enzyme responsible for the synthesis of noradrenaline and adrenaline, the main effectors of the adrenal medulla, and the sympathetic nervous system, respectively. Because these effectors promote the catabolism of triglycerides and glycogen, activate thermogenesis in BAT, and alter heat loss by modulating peripheral vasoconstriction and piloerection, it was anticipated that this knockout mouse would be cold-sensitive and obese. As expected, the mice were cold-intolerant due to defective BAT thermogenesis, and peripheral vasoconstriction. However, they were not obese, in fact body weights were slightly lower in knockouts than in controls. Moreover, the knockouts consumed significantly greater amounts of dietary energy. Increased basal metabolic rate is reported. However, oxygen consumption data were divided through by the body weights of the animals (weights of the knockouts are approximately 20% lower than the controls); without knowing the percentage of lean body mass of the mice, it is difficult to appreciate the significance of this finding. (See also [36].) If knockouts are treated with injections of the synthetic amino acid precursor of norepinephrine, L-threo-3,4-dihydroxyphenylserine, levels of norepinephrine are restored in many tissues, and many phenotypic characteristics are restored (e.g. UCP expression, male fertility, hind-limb extension) [86].

3.2. Overexpression

3.2.1. The Glut4-overexpressing mouse

A recent report describes altered levels of expression of UCPs in transgenic mice in which the insulin responsive glucose transporter, Glut4, is overexpressed [89]. Tsuboyama-Kasaoka and colleagues created transgenic mice harboring Glut4 minigenes with differing lengths of the 5′-flanking sequence.

The expression of the Glut4 transgenes was found to alter not only the level of expression of Glut4, but also the level of Ucp3. In skeletal muscle, BAT, and WAT, the level of Glut4 expression was increased by 1.4–4.0-fold compared to control littermates. The level of Ucp3 expression was increased by 4.0- and 1.8-fold in muscle and WAT, respectively. However, the level of Ucp1 expression was decreased in BAT by approximately 38%. Glut4-overexpressing mice displayed a 16% increase in whole body oxygen consumption (ml/min), a 14% decrease in blood glucose concentration and a 68% increase in blood lactate concentration. There were no changes in the levels of circulating free fatty acids. Their results suggest that, in addition to increased fatty acids, increased glucose uptake by cells may result in increased expression of Ucp3 mRNA.

4. Summary and conclusion

While it may have been anticipated that the application of transgenics to the area of mitochondrial energetics would solve, or at least clarify, a number of controversies, there have been as many questions raised as there have been answers gained. The interpretation of results from studies of transgenic mice is often complicated by compensatory processes within and beyond gene families of the gene being modified. Given that mitochondria provide cellular energy conversion pathways that underpin both cell life and death, the presence of 'back-up mechanisms' is not surprising. Increasingly noted are the significant effects that genetic background has on the phenotypes of transgenic mice. The complex interactions between genes, genetic background, and the environment will likely prove to be important. Overall, the findings from transgenic approaches to the study of mitochondrial efficiency are best considered as valuable additional tools in metabolic research.

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