Cloned mice have an obese phenotype not transmitted to their offspring

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Mammalian cloning using somatic cells has been accomplished successfully in several species, and its potential basic, clinical and therapeutic applications are being pursued on many fronts. Determining the long-term effects of cloning on offspring is crucial for consideration of future application of the technique. Although full-term development of animals cloned from adult somatic cells has been reported, problems in the resulting progeny indicate that the cloning procedure may not produce animals that are phenotypically identical to their cell donor. We used a mouse model to take advantage of its short generation time and lifespan. Here we report that the increased body weight of cloned B6C3F1 female mice reflects an increase of body fat in addition to a larger body size, and that these mice share many characteristics consistent with obesity. We also show that the obese phenotype is not transmitted to offspring generated by mating male and female cloned mice.

Male and female animals have been generated using adult somatic cells in many species[1–8]. Several types of somatic cells have been successful in directing full-term development[9–15]. Although attention is now being directed toward application of this technique in basic and clinical sciences, rapid advances and escalating interest in the cloning field has also generated many questions related to its safety and limitations. Cloned offspring are not without health problems, which are in some cases lethal[2,3,16]. Whether these aberrations result from the cloning technique itself or from the use of somatic cell nuclei that must be 'reprogrammed' to re-establish zygotic state remains to be determined. Other reports indicate that cloned offspring are not always completely identical to their donors[17–24]. The proposed applications of this technology hinge on the reliability with which cloning can reproduce specific genotypes and phenotypes in resulting offspring.

We previously documented the postnatal growth and behavioral development of cloned mice[25]; although they displayed no gross behavioral abnormalities, their body weights were substantially higher than those of age-matched controls (Fig. 1). In addition, evaluation of five generations of adult clones (that is, clones derived from clones) showed that although each generation showed the same increased body weight phenotype, there was no evidence of an enhanced effect of cloning on body weight with each successive generation[20]. At birth, cloned mice and control offspring derived by natural fertilization followed by *in vitro* embryo culture have similar weights; however, both groups are heavier than background stock mice. Embryonic stem cell–derived clones have also been found to be heavy at birth[26,27]. However, embryonic stem cells that are exposed to long-term *in vitro* culture conditions may be more likely to acquire culture-induced modifications long before they are used in cloning. Perhaps analogous to these findings, cloned cattle and sheep reportedly have 'large offspring syndrome' characterized by increased birth weights[28], a phenotype that has recently been attributed to epigenetic modifications during embryo culture[29]. Mouse clones weigh more than *in vitro* cultured controls beginning at 8–10 weeks of age. Similarly, mice produced from cryopreserved embryos have increased body weight beginning in adulthood[30]. The consistency of these observations across species and methods indicates that either the technique used or the biological events involved in the cloning process itself produces offspring that are heavier than their 'parents'.

To differentiate these possibilities and to further characterize the increased body weight phenotype, we examined cloned mice of different background strains (B6C3F1 and B6D2F1) and used specific control groups and conditions to analyze the behavioral and metabolic characteristics of these mice as well as the heritability of the phenotype. We assessed several indicators of obesity in the mice, including carcass analysis and plasma concentrations of insulin and leptin to distinguish increments of body weight from obesity itself. We report here that adult cloned mice not only are heavier but also have a spectrum of behavioral and metabolic alterations consistent with obesity. We also examined the phenotype of offspring derived from natural mating of cloned B6C3F1 male and female mice and found that the obese phenotype of cloned mice is not transmitted to subsequent generations.

Body weight, food intake and response to food deprivation

At birth, clones and *in vitro* embryo-manipulated (IVEM) control mice, generated to match the *in vitro* manipulation and culture conditions of the cloned embryos did not differ in weight, but both groups were heavier than stock control mice (Fig. 2*a*). The clones and IVEM mice followed the same growth curves through approxi-

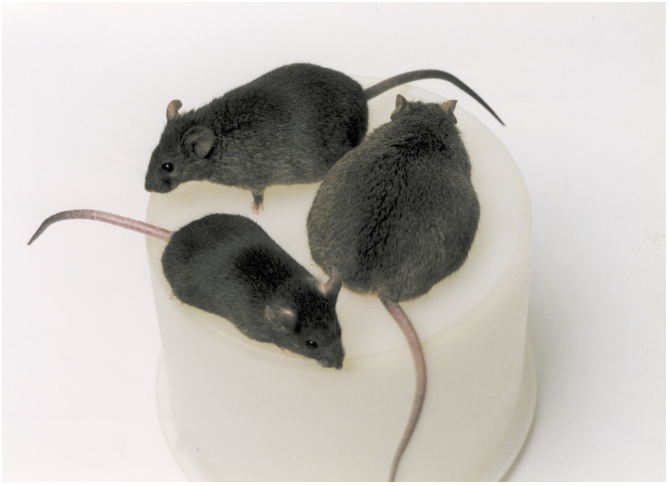

Fig. 1 Cloned and control mice. An adult cloned B6C3F1 mouse (right) and strain- and age-matched representatives of the two control groups of mice used in this study: stock control (foreground) and IVEM mouse (top left).

mately 8 weeks of age (Fig. 2*b*). After that, clones became significantly heavier than IVEM mice. Focusing on the period immediately after the divergence of the growth curves, we compared the body weights of cloned and IVEM mice with those of stock control mice and found that both the cloned and IVEM mice were heavier than normal stock control mice (Fig. 2*b*, inset). This relationship persisted throughout the experiment.

We measured the *ad libitum* food intake of the mice to determine if the increased body weight in clones results from hyperphagia. Although clones were significantly heavier than both stock and IVEM mice, they were not hyperphagic. Clones ate the approximately the same amount as stock mice and more than IVEM mice. However, as the clones were significantly heavier than stock and IVEM mice, we also calculated their food intake in terms of lean body mass, as body fat is metabolically inert. Clones ate significantly less per gram of lean body mass than did stock mice, and ate the same amount as IVEM mice (Fig. 3).

To assess the clones' response to an acute hypocaloric challenge, we deprived mice of food, but not water, for 24 hours. Mice in all three groups lost the same percentage of baseline body weight during the deprivation period. When food was returned, all groups compensated for the loss of body weight by increasing their intake (Fig. 3). The stock controls ate more food (on a g/g lean-body-weight basis) than either of the other groups during both *ad libitum* and refeeding conditions.

Response to peripheral administration of MTII and leptin

We next assessed the clones' response to a well-characterized suppressant of food intake, MTII, to determine whether a malfunctioning melanocortin signaling system is involved in the increased body weight of clones. MTII reduced food intake over the first 6 hours of the dark cycle in the clones (Fig. 4*a*). A smaller, nonsignificant decrease occurred in the IVEM and stock control mice. Similar evidence of the increased sensitivity of cloned mice to MTII was produced with extension of the food deprivation period to 18 hours before the administration of MTII (data not shown). We also examined the clones' response to an anorexigenic adiposity signal, leptin, and found that peripheral leptin administration decreased food intake more in clones than in IVEM and stock mice (Fig. 4*b*).

Carcass and plasma hormone analysis

To determine whether increased body weight could be attributable to higher body fat or lean body mass, we measured body composition of the clones by carcass analysis. Clones had significantly more carcass fat than IVEM or stock mice. Hence, cloned mice not only weighed more, they also had more body adiposity than both control groups (Table 1). In addition, we measured plasma leptin, insulin and corticosterone and found that clones had increased plasma leptin and insulin. However, in contrast to some models of obesity, plasma corticosterone concentrations in the clones did not differ reliably from that of the control groups.

Background strain influence on obesity in cloned mice

To determine the influence of background strain on the obese phenotype of clones, we also generated clones with a B6D2F1 background (derived from a C57Bl/6 female and DBA/2 male cross). The body weights of these clones were indistinguishable from those of stock B6D2F1 mice at 8 weeks of age (stock, 22 g (ref. 31) to 23 g (ref. 32); B6D2F1 clones, 22.9 ± 0.2 g). However, whereas the body weights of stock B6D2F1 mice peak at 30 g after 18 months of age[31], the clones averaged 42.4 ± 3.5 g at that age.

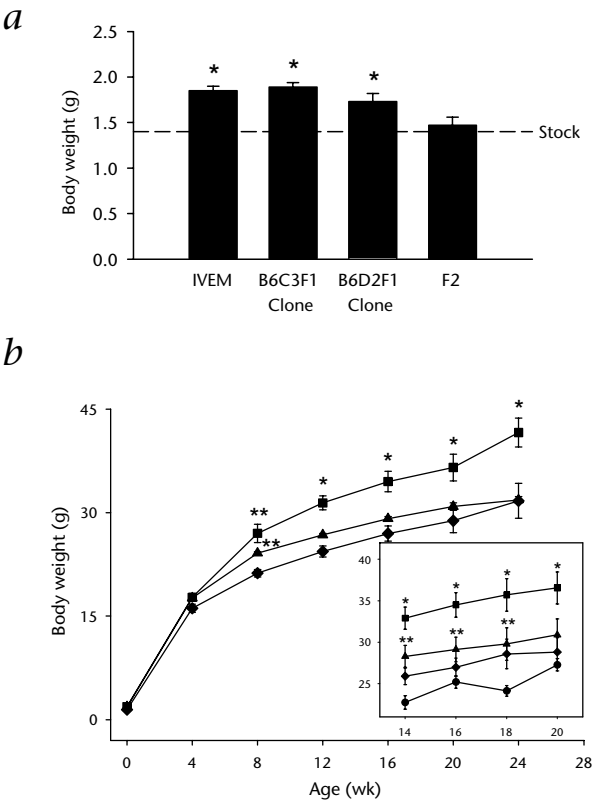

Fig. 2 Mouse body weights. ***a***, Birth weights of IVEM, cloned and F2 mice. *, $P < 0.05$, versus stock and F2. ***b***, Body weights of clones (■), IVEM (▲) and F2 mice (◆). Growth curves are identical through 8–10 wk of age, at which time clones begin gaining weight more rapidly than IVEM mice. Body weights of F2 offspring over the same time period are significantly less than those of clones. *, $P < 0.05$, versus IVEM and F2; **, $P < 0.05$, versus F2. Inset, comparison of clone (■), IVEM (▲) and F2 (◆) offspring with normally bred and raised age-matched stock mice (●) at 14–20 wk of age. Clones and IVEM mice are significantly heavier than female stock mice, but F2 offspring are of similar weight. *, $P < 0.05$, versus IVEM, F2 and stock; **, $P < 0.05$, versus stock.

Mating of cloned male with cloned female mice

To determine whether the obese phenotype of cloned mice is transmitted genetically through the germline, we mated male B6C3F1 clones derived from fibroblast cell nuclei with female B6C3F1 clones derived from cumulus cell nuclei. The average litter size ($n = 4$) was 5.8 ± 2.4 pups. Litter size may have been decreased, as mice were mated at 12–14 months of age to generate F2 offspring. Previous assessment of the fertility of cloned male and female mice at reproductive maturity showed no significant differences in fertility or litter size[2,3]. The body weights of offspring (F2) were lower than those of cloned mice (Fig. 2*b*) and placenta weights were within the normal range (T.W., unpublished observation). A comparison of F2 mice and age-matched stock mice is shown in the inset of Fig. 2*b*.

Discussion

The production of cloned animals using adult somatic cells has opened new avenues of investigation in basic and clinical sciences. The prospect of using cloning technology in humans for therapeutic and reproductive applications has enhanced the necessity for comprehensive studies of cloned animals. Although creating clones with various types of somatic cells has been successful[9–15], the safety and possible adverse biological effects remain to be investigated. Studies of cloned offspring in species other than the mouse face the challenges of obtaining sufficient sample size and controlling for factors involved in the cloning technique. Moreover, determining the biological effects during an animal's lifespan and over successive generations is an inherent obstacle in studies of cloned domestic species. Despite success in cloning large ungulates, including sheep[1], cattle[4], goats[33] and pigs[8], to our knowledge, only one study has examined a large number of adult cloned animals[34] and none has reported body fat content or other indices of possible obesity. Although comprehensive studies must ultimately be done in large-animal species to determine the long-term effect(s) of somatic cell cloning on the health and well-being of those animals, at present the mouse provides an ideal model in which to examine health implications of the short-term as well as the long-term effects of cloning.

The postnatal growth and behavioral development of mice cloned using adult somatic cells has been documented; although there were no gross behavioral abnormalities, body weights of cloned mice were significantly increased beginning at 8–10 weeks of age[25]. Here we have extended the examination of those cloned mice to provide a comprehensive characterization of their food intake and metabolic physiology. An increase in body weight does not necessarily indicate the existence of obesity and, conversely, a lack of change of body weight does not rule out an underlying obesity. We have shown here through food intake, physiological and body composition analyses that cloned mice are truly obese, and are not simply larger than controls. The higher body weight of cloned mice is directly attributable to increased adipose tissue, and the mice share several other characteristics with obese humans, including hyperinsulinemia and hyperleptinemia. Notably, the obese phenotype and enlarged placenta characteristic of clones was not genetically transmitted through the germline to offspring produced by natural mating of cloned mice.

Cloned mice of the B6C3F1 background had significantly greater weight at birth than the average weight of stock mice of the same strain, but were not different from IVEM mice in weight. However, as stock mice are born in litters that are larger than those of clones and IVEM mice, the difference in birth weight may reflect different litter sizes.

The significant increase in body weight of clones begins in adulthood. However, contrary to our expectation, the clones were not hyperphagic as adults. As food intake was measured at adulthood, after cloned mice had become obese, we cannot rule out the possibility that the mice were hyperphagic before the onset of accelerated body weight gain. In response to an acute hypocaloric challenge, the clones lost the same percentage of body weight and compensated for this by increasing their food intake to the same degree as control mice. The general activity of these clones was similar to that of IVEM mice at 2, 4 and 6 months of age[25], indicating that clones gain more weight than IVEM while maintaining the same amount of activity. Hence, the difference in body weight may be due to lower basal energy expenditure in clones.

Several mouse obesity models have deficiencies in melanocortin signaling. In particular, leptin reduces food intake and body weight by stimulating pro-opiomelanocortin-synthesizing neurons in the arcuate nuclei[35], and these neurons in turn project to the paraventricular nucleus (and other brain sites) and act on MC-4 melanocortin receptors[36,37]. Administration of MTII, a melanocortin agonist, reduced food intake in the B6C3F1 clones but not in the stock or IVEM mice. This result is striking, given that the clones weighed more than controls and received a lower dose of MTII on a g/g basis. Clones were also more sensitive to exogenous leptin. Hence, although some forms of mouse obesity show reduced sensitivity of the central melanocortin signaling system, obese cloned mice have, if anything, increased sensitivity in this system. Because the clones are hyperleptinemic, the implication is that the clone's endogenous leptin chronically suppresses appetite and body weight through the central melanocortin system. Hence, the cloned mice might be even more obese were it not for the catabolic action of their endogenous leptin. The obesity of cloned mice cannot, therefore, be attributed to a defect associated with the leptin–melanocortin system, and must be due to some other mechanism(s).

Cloned mice show several characteristics of obesity. They have a significantly higher percentage of body fat mass with a correspond-

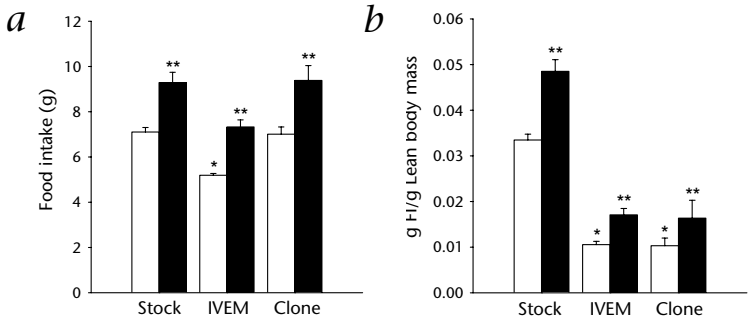

Fig. 3 Food intake. Food intake was measured daily for all groups as adults (1 y of age). Mean 24-h intake is expressed as grams of total intake (*a*) and gram/gram of lean body mass (*b*) on a typical day (*ad libitum* feeding; open bars) and after a 24-h food deprivation (filled bars). ***a***, Clones eat approximately the same amount of food as stock controls as adults. ***b***, After adjustment for lean body mass, cloned mice are shown to eat significantly less per gram of lean body mass than stock controls and the same amount as IVEM mice. After a 24-h food deprivation, all groups have lost the same percentage of body weight. Food intake over the subsequent 24-h period increased significantly for all groups, indicating that clones are capable of 'defending' body weight loss as well as IVEM and stock mice. *, $P < 0.05$, versus stock; **, $P < 0.05$, versus *ad libitum*.

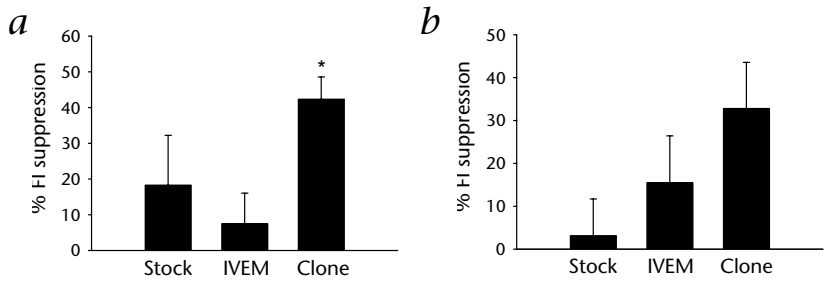

Fig. 4 Effect of MTII and leptin on food intake. ***a***. MTII (100 nM), a melanocortin agonist, significantly suppresses food intake (FI) of cloned mice (*, $P < 0.05$, $n = 7$-9). A smaller, nonsignificant decrease occurs in IVEM and stock mice. ***b***. Leptin (5 μg/g body weight) suppresses food intake more in clones than in IVEM and stock mice. Data represent percent suppression of food intake compared with vehicle over 6 h (MTII) and 4 h (leptin) after administration; $n = 7$-9.

ing lower percentage of body water mass compared with controls, and a percentage of body lean mass similar to that of IVEM mice. The clones are also hyperinsulinemic and hyperleptinemic, but have corticosterone amounts similar to those of controls. Finally, some clones generated on a different genetic background, B6D2F1, also had the phenotype of extremely increased body weight. Therefore, although there may be strain differences in the degree and time of onset of obesity in clones, cloning increases body weight independent of the strain of mouse used as the nuclear donor.

Consistent with previous reports[38], we found that the processes of *in vitro* culture of embryos result in significantly heavier offspring than do natural matings. As the IVEM embryos were produced as a result of natural mating followed by *in vitro* culture, this indicates that some aspect of the somatic donor cell used in nuclear transfer or the technique itself may be causally related to the subsequent development of obesity in cloned mice. The nucleus of a differentiated donor cell must be 'reprogrammed' and properly imprinted to initiate normal embryonic development. Aberrant DNA methylation due to faulty epigenetic reprogramming after somatic cell nuclear transfer will result in over- or underexpression of one or more genes, although the process by which epigenetic reprogramming occurs is not thoroughly understood[10,39]. Failure or deficiencies in these mechanisms may be a point at which abnormalities are introduced. Although some failures and deficiencies may be lethal, others may produce adult phenotypes that are different from those of the donor, such as obesity.

Table 1 Terminal parameters

	Stock ($n = 7$)	IVEM ($n = 7$)	Clone ($n = 9$)
Terminal body weight (g)	28.9 ± 0.7	39.3 ± 1.3*	49.7 ± 3.9**
% fat tissue	14.4 ± 1.4	19.5 ± 1.9	25.2 ± 2.8*
% lean tissue	27.5 ± 0.4	34.7 ± 1.9*	32.9 ± 2.1*
% water	58.1 ± 1.2	45.8 ± 1.4*	41.9 ± 2.8*
Plasma measures:			
Leptin (ng/ml)	2.1 ± 0.5	9.6 ± 1.7*	14.6 ± 1.7**
Insulin (pM)	12 ± 2	26 ± 2*	32 ± 5*
Corticosterone (μg/dl)	8.23 ± 3.20	3.83 ± 0.76	4.54 ± 0.72

Data are expressed as mean ± s.e.m. *, $P < 0.05$, compared with stock controls; **, $P < 0.05$, compared with stock and IVEM mice.

A recent study found that epigenetic marks on an imprinted gene (*Xist*) in adult mouse somatic cells can be reprogrammed during cloning[40]. Decreased expression of non-imprinted as well as imprinted genes in the placentas of cloned fetuses compared with that of controls has been reported, and it was suggested that the enlarged placental phenotype found in cloned mice may occur independently of changes in genomic imprinting[41]. In contrast, other studies have reported that tissue-specific genes are differentially methylated or unmethylated in full-term mouse fetuses cloned with adult cumulus cells[24]. Furthermore, cloned fetuses derived from embryonic stem cells show fetal overgrowth that may result from differences in imprinted gene expression[26,27]. It has also been suggested that reproductive technologies that use artificial embryo culture conditions may produce epigenetic modifications that affect fetal development[42] as well as growth after birth. As an example, an epigenetic change in the receptor for insulin-like growth factor 2 has been associated with embryo culture in sheep and is proposed to cause fetal overgrowth[29]. It remains unclear whether a mechanism such as this is directly responsible for the low success rate of cloning and/or the abnormal phenotypes found in adult cloned animals.

To assess whether the obesity of adult cloned mice could be attributed to a genetic or an epigenetic event, we used a biological assay, producing B6C3F2 offspring by mating B6C3F1 cloned male and female mice. These F2 offspring weighed less than F1 cloned mice and also weighed less than F1 IVEM mice for at least 6 months. Furthermore, F2 offspring did not have the enlarged placentas commonly found in cloned mice. Obesity observed in cloned mice, therefore, is not transmitted through the germline, indicating that epigenetic modifications that occur during the cloning procedure are eliminated or 'corrected' during gametogenesis. Further investigation will be required to determine the point at which these changes are introduced and whether methods can be developed to circumvent their occurrence. The donor age and cell source of the transferred nucleus may also result in different phenotypes in clones. Although many cell types from donors of different ages have been shown to be successful in producing mammalian clones[13], further comprehensive studies are needed to determine the consequences of using these cells for cloning.

In summary, we have characterized an obese phenotype in cloned mice, which has implications for the advancement of the cloning technique. Notably, although cloned mice are obese, they do not transmit this phenotype to their progeny generated through natural mating. This in turn indicates that an epigenetic modification, rather than a genetic change, may be responsible for the low success rate of cloning and/or for producing aberrant phenotypes in surviving cloned animals. As the success rate of cloning techniques using adult somatic cells regardless of the method used (for example, cell electrofusion or microinjection) is less than 3% (ref. 15), reproduction by natural mating may be recommended as soon as offspring with specific desired traits are produced by cloning.

Methods

Animals. All mice were individually housed in polycarbonate cages (18.5 x 29 x 13 cm) with *ad libitum* food and water, unless otherwise indicated. They were maintained in temperature- and humidity-controlled rooms with a 14-h

light–10-h dark cycle, with light onset at 5:00 am. The protocol of animal handling and treatment for this study was reviewed and approved by the Animal Care and Use Committees at the University of Hawaii and the University of Cincinnati.

Female cloned mice were produced by microinjection of the nucleus of an adult cumulus cell from a B6C3F1 mouse into an enucleated oocyte collected from a B6D2F1 mouse, as described before[2]. Nine female cloned mice were randomly selected for this study.

IVEM mice were generated with consideration of a number of possible confounding factors involved in the cloning technique: embryo manipulation, *in vitro* embryo culture, embryo transfer into pseudo-pregnant surrogate CD-1 mothers, reduced litter sizes, caesarian section delivery, and cross-fostering. Ten C57Bl/6 female and ten C3H/He male mice obtained from the National Cancer Institute were mated to generate B6C3F1 hybrid mice. To control for embryo manipulation and *in vitro* culture, pronuclear eggs were collected from the oviducts of mice on the day after mating and cultured *in vitro* for 20 h until they reached the two-cell stage. To control for litter size and use of surrogate mothers, 2–3 embryos were transferred to the oviducts of each of 20 pseudo-pregnant CD-1 surrogate mothers to allow only 1–2 embryos to implant in each mouse. At present, only one to two cloned mouse embryos reach term in each surrogate mother, and these litters are delivered by caesarian section, as parturition is usually not initiated naturally because of the small number of fetuses per litter. Therefore, IVEM pups (hybrid B6C3F1) were delivered on day 19.5 after conception by caesarian section and were placed with the litters of lactating CD-1 foster mothers to be raised. We randomly selected seven female mice from this group for our study (IVEM mice). Our second control group (stock controls) consisted of seven female B6C3F1 mice obtained from the National Cancer Institute. All experiments used mice 10–12 months of age, unless otherwise indicated. All mice were B6C3F1 strain, unless noted otherwise.

Body weight, food intake and response to food deprivation. Body weights were monitored weekly from birth until 8 wk of age and biweekly thereafter. Baseline food intake was determined over 4 d of *ad libitum* feeding. All mice were maintained on Laboratory Rodent Diet 5001 (PMI Feeds, Richmond, Indiana). To assess the response to food deprivation, mice were deprived of food but not water for 24 h. Food was then returned and was available *ad libitum*. Food intake and body weight were measured hourly for 6 h and at 24 h.

MTII administration. Mice were deprived of food for 4 h before the dark cycle began, followed by intraperitoneal administration of a 100-nM bolus of MTII (Phoenix Pharmaceuticals, Mountain View, California) in 100 µl sterile deoxygenated water or 100 µl vehicle alone at 30 min before the dark cycle. Food was returned at the onset of the dark cycle, and intake was measured hourly for the first 6 h and after 24 h.

Leptin administration. Leptin (5 µg/g body weight in physiological saline; Calbiochem, La Jolla, California) or vehicle was administered intraperitoneally using the same protocol used for MTII.

Carcass analysis and plasma assays. Mice were food deprived for 18 h before being killed by CO_2 inhalation. Blood was collected and centrifuged at 4 °C. Plasma insulin was measured by radioimmunoassay (RIA) using an antiserum recognizing rodent insulin[43]. Plasma leptin was determined using a commercial RIA (Linco, St. Charles, Missouri). Plasma corticosterone was measured by RIA using rabbit antiserum raised against corticosterone (B3-163; Endocrine Sciences, Tarzana, California). Standard procedures were used for carcass analysis. Carcasses were dehydrated in a lyophilizer (Labconco Freezone 18) until carcass weights differed by less than 0.1 g from the previous day's weight. Fat mass was then determined by petroleum ether extraction and water mass, fat mass, and (water-free) lean body mass were determined.

F2 generation mice. B6C3F2 mice were generated through natural mating of male and female B6C3F1 cloned mice. Body weights were monitored weekly until 8 wk of age and biweekly thereafter for 6 mo.

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Competing interests statement

The authors declare that they have no competing financial interests.

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