Phenotype of Cloned Mice: Development, Behavior, and Physiology

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Cloning technology has potential to be a valuable tool in basic research, clinical medicine, and agriculture. However, it is critical to determine the consequences of this technique in resulting offspring before widespread use of the technology. Mammalian cloning using somatic cells was first demonstrated in sheep in 1997 and since then has been extended to a number of other species. We examined development, behavior, physiology, and longevity in B6C3F1 female mice cloned from adult cumulus cells. Control mice were naturally fertilized embryos subjected to the same *in vitro* manipulation and culture conditions as clone embryos. Clones attained developmental milestones similar to controls. Activity level, motor ability and coordination, and learning and memory skills of cloned mice were comparable with controls. Interestingly, clones gained more body weight than controls during adulthood. Increased body weight was attributable to higher body fat and was associated with hyperleptinemia and hyperinsulinemia indicating that cloned mice are obese. Cloned mice were not hyperphagic as adults and had hypersensitive leptin and melanocortin signaling systems. Longevity of cloned mice was comparable with that reported by the National Institute on Aging and the causes of death were typical for this strain of mouse. These studies represent the first comprehensive set of data to characterize cloned mice and provide critical information about the long-term effects of somatic cell cloning. Exp Biol Med 228:1193–1200, 2003

Key words: somatic cell cloning; development; obesity; longevity; mouse

Early nuclear transfer experiments by Spemann (1) and Briggs and King (2) using newts and frogs clearly demonstrated that nuclei removed from early embryonic cells retain the ability to support the development of a complete animal. The fact that cloning becomes increasingly difficult as the developmental stage of the nucleus donor advances (3) led to the conclusion that it is impossible to generate another animal from terminally differentiated adult somatic cells (3). In 1996, Campbell *et al.* (4) cloned the first mammal from cultured inner cell mass cells. A year later, Wilmut *et al.* (5) cloned a sheep named "Dolly", the first mammal cloned from an adult somatic (mammary gland) cell.

Since that first report, somatic cell cloning has been demonstrated in several other mammalian species (6–11). Success in generating cloned animals has opened new avenues of investigation and is a valuable tool that basic scientists have employed to study complex processes such as genomic reprogramming, imprinting, and embryonic development (12–15). In addition, the possible agricultural and clinical applications that are being explored include reproductive cloning of farm animals and therapeutic cloning for human cell, tissue, and organ replacement (16–18).

Prenatal and perinatal death rates are significantly higher in clones compared with controls. Despite the high mortality rate before and at birth, a number of apparently healthy cloned animals survive to adulthood. Specifically in the mouse, we have observed high embryo implantation rate (57%–71%) but low fetal (5%–16%) and very low full-term (2%–3%) development rates following nuclear transfer using adult somatic cells (7, 19). Perinatal and postnatal complications include developmental deficiencies and respiratory distress (8, 20, 21). However, the mortality rate after the immediate postnatal period is low.

Systematic studies of cloned animals that have been generated by nuclear transfer of adult somatic cell nuclei are critical in assessing the utility of somatic cell cloning in other applications. This is not a simple task given the low

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success rate of nuclear transfer techniques, a value that currently stands at approximately 1% to 3% using adult somatic cells (22). Obtaining sufficient numbers of animals to conduct comprehensive studies is a challenge in many species. However, despite this technical obstacle, several groups have conducted follow-up studies of cloned animals and indicate that clones are not always phenotypically identical to their somatic cell donors (23–29). These studies focused on behavioral and physiological measures to assess the consequences of cloning. Other studies have examined telomere length (30–35), genomic imprinting and DNA methylation (26, 36), and mitochondrial DNA genotype (37). All of these reports are consistent with the possibility that there are aberrations in cloned animals that could have detrimental future consequences in cloned offspring. The number of conflicting results among current reports, and the lack of consistency in the possible side effects resulting from the cloning process, are cause for concern and clearly indicate that the technique is still unpredictable, highlighting the need for comprehensive longitudinal studies of cloned animals. A limited number of longitudinal studies have been conducted to examine long-term consequences of cloning. For example, cattle (25) are being monitored longitudinally, but these animals have a long lifespan and the long-term consequences will not be known for years. The only species that has been systematically followed throughout the entire lifespan with a sufficient number of animals is the mouse (38).

Possible applications of cloning technology are currently being explored, and studies that use nuclear transfer are escalating at a remarkable pace. Government attempts to regulate or ban cloning research using human cells is a clear indication that studies to determine the possible adverse consequences in cloned animals is of utmost importance. Without useful information about undesirable side effects of cloning, governmental regulations may impede progress in basic and clinical research and prevent scientists from capitalizing on this potentially powerful technique. Comprehensive studies of the phenotype of cloned animals have begun but are far from being complete. At the moment, the long-term consequences of mammalian cloning remains poorly characterized and data available thus far suggest that we should limit use of this technology until numerous questions are answered.

In this article, we review data from studies that we have conducted in cloned mice from birth through senescence and death. The advantages of using a mouse model are clear. Their short generation time and short lifespan facilitate completion of longitudinal studies in a few years compared with more than a decade for studies in cloned livestock such as cattle and sheep. The ability to generate control groups to account for the numerous manipulations involved in the cloning process and to closely monitor and control animal husbandry after birth also makes the mouse a desirable model to determine the long-term effects of mammalian cloning. We examined development, behavior, and physiology of these interesting animals. To date, our experiments represent a comprehensive set of studies aimed at characterizing mice cloned from adult somatic cells. In this article, we also provide an overview of current and future studies to further phenotype cloned mice.

Animals

Cloned Mice. The cloning method is described in detail elsewhere (7). Briefly, mouse clones were generated by microinjection of cumulus cell nuclei from adult (8–10 weeks old) B6C3F1 (C57BL/6 × C3H/He) hybrid mice into enucleated oocytes collected from adult (8–10 weeks old) B6D2F1 (C57BL/6 × DBA/2) mice. Preimplantation embryos were transferred into pseudo-pregnant CD-1 surrogate mothers (7). Pups were delivered at 19.5 days post coitum (d.p.c.) by Caesarean section and placed with the litters of lactating CD-1 foster mothers to be raised. Animals were weighed weekly until 8 weeks of age and biweekly thereafter.

Control Mice. The cloning procedure bypasses several processes involved in natural reproduction including meiosis and fertilization. In addition, cloning includes several artificial techniques such as micromanipulations (enucleation of oocytes and microinjection of somatic cell nuclei), chemical activation of reconstructed oocytes, *in vitro* embryo culture, and embryo transfer into surrogate mothers. To control for this, we generated a group of mice that underwent comparable *in vitro* embryo manipulation (designated "IVEM"). We allowed C57BL/6 female mice (National Cancer Institute (NCI)) to mate with C3H/He male mice (NCI) to generate B6C3F1 embryos, the same hybrid as cloned mice. The following day, pronuclear eggs were collected from the oviducts of females and cultured *in vitro* for 20 hours until they reached the 2-cell stage. To control for the small litter size (1–2 pups) typical of cloned mice, only two or three embryos were transferred to the oviducts of pseudo-pregnant CD-1 surrogate mothers to generate 1 to 2 pups per litter. Pups were delivered by Caesarean section at 19.5 d.p.c. and were placed with litters of lactating CD-1 foster mothers to be raised. A second control group consisted of normally fertilized and reared mice (National Cancer Institute) that were of the same hybrid strain and age-matched to the clones (designated "STOCK").

Housing and Care. All animals, both clones and controls, were individually housed in polycarbonate cages (18.5 × 29 × 13 cm) with food (Purina Rodent Diet 5001) and water available *ad libitum*. They were maintained in temperature and humidity controlled rooms under a 14 hour light/10 hour dark cycle with light onset at 0500 hr. The animals were maintained in accordance with the guidelines of the Laboratory Animal Service at the University of Hawaii, the Laboratory Animal Medical Services at the University of Cincinnati, and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources of the National Research Council. Our protocol of

animal handling and treatment was reviewed and approved by the Institutional Animal Care and Use Committees at the University of Hawaii and the University of Cincinnati.

Behavior

We examined the postnatal development of cloned mice according to established criteria (24) and found no differences between IVEM and cloned mice with the exception of negative geotaxis, ear twitching, and eye opening (24). Both groups still fell well within the normal range reported for mice, and our subsequent behavioral tests did not reveal any deficits or long-term consequences in the clones that may be related to delay in development of these milestones. The milestones and behaviors included in our examination are expressed at different time points during the first 21 days of life and thus provide a measure of development spanning the entire postnatal pre-weaning period (39).

Cloned mice have normal diurnal activity patterns as measured by home cage activity that does not differ from that of IVEM control mice at any of the time points examined. Additionally, we assessed motor skills and abilities of the clones and did not find any deficits in motor coordination, muscle strength, or balance. In sum, cloned mice appear to develop reflexes and other behaviors at the same ages as normal mice, and to have normal motor control and coordination.

To evaluate learning and memory of cloned mice, we used a well-characterized and widely used task, the Morris water maze (40). Both clones and IVEM controls successfully completed the task, finding the submerged platform with a shorter latency over consecutive days of testing. Additionally, there were no differences between the groups, suggesting that both groups were able to use information obtained from previous trials to find the platform faster on subsequent days. Our results also found that both groups were employing a spatial learning strategy as indicated by the increased amount of time spent in the quadrant where they had been trained to find the platform. Furthermore, when the position of the submerged platform was changed, the cloned mice navigated to the new platform position and found with a shorter latency than during the initial acquisition trials. Together these results suggest that cloned mice are capable of completing a spatial learning task and do not have deficits in learning and memory, at least through 6 months of age (24).

Overall, our behavioral data suggest that cloned mice are not significantly different from control mice from birth through 6 months of age. We have also examined multiple generations of cloned mice and have not noted any significant deficiencies in any of the developmental or behavioral measures described previously (30). Further longitudinal studies of behavior over the entire lifespan of cloned mice have yet to be conducted. It is possible that cloned animals will develop deficits later in life and indeed some studies have reported indications of premature aging (sheep) (31) and shorter lifespans (mice) (38) in clones.

Aging and Longevity

Aging is a complex process, and whether it occurs normally in cloned animals is a question that remains to be answered. Indirect measures that have been used include telomere length and telomerase expression. However, these studies have generated conflicting results. Dolly, the sheep cloned from adult mammary gland cells, was found to have short telomeres that were comparable to the age of the cell donor from which she was cloned and shorter than age-matched control sheep suggesting that she may have been prematurely aging (31). She also developed arthritis early and died at the young age of 6 years, about half the 12-year lifespan of a normal sheep. Tian *et al.* (33) reported normal telomere length in cloned cattle while Lanza *et al.* (32) found that even using "senescent" fibroblasts ("aged" by *in vitro* culture) produced clones with telomeres that are longer than those of age-matched controls. Wakayama *et al.* (30) cloned mice to four and six generations in two independent lines and found that mice cloned over 5 generations (clones of clones) did not have shorter telomeres with each successive generation. The mice were generated from adult cumulus cells that were found to express telomerase, the enzyme that is responsible for telomere elongation. Therefore, the possibility that the cumulus cells used to produce mouse clones had long telomeres at the outset cannot be ruled out. The reasons for the wide range of findings in telomere studies are unclear; however it is possible that resulting telomere length in cloned offspring may depend upon the cell type of the nucleus donor (41).

The first mouse clones were produced in 1997, and we now have been able to follow a sufficient number of clones over their lifespan to examine their longevity. The short lifespan of the mouse provides an ideal model organism with which the consequences of cloning on the aging process can be addressed in cloned animals.

A study by Ogonuki *et al.* (38) suggests that mice cloned by nuclear transfer of immature Sertoli cells from 3- to 10-day-old male mice die earlier than controls; the authors attribute the early mortality to organ dysfunction, specifically hepatic and immune system failure. There are several factors to consider before extrapolating their report to all mice or to other species of cloned animals. First, the age and type of donor cell may have an influence on the health of the resulting cloned animal. Ogonuki *et al.* used immature Sertoli cells from mice that were 3 to 10 days old. It may be possible that immature cells such as these harbor defects that would normally cause the cell to undergo selective apoptosis during maturation. While the defects or deficiencies apparently do not preclude full-term development of cloned mice, it may result in adverse effects such as hepatic failure and immune incompetence later in life, as was reported by Ogonuki *et al.* (38). Rather than implying that the clones are necessarily aging prematurely, it may

suggest that they die early due to increased incidence of organ dysfunction.

We monitored the lifespan of two strains of female cloned mice, B6C3F1 and B6D2F1 (Fig. 1). The mice were cloned from adult cumulus cells (average age of the female donor mouse 6–8 weeks) using the technique of Wakayama *et al.* (7). The longevity of female mice cloned from B6C3F1 adult cumulus cells was comparable with animals followed by the National Institute on Aging (NIA) [776 ± 76 days vs 890 days (42)]. Likewise, longevity of female mice cloned from the B6D2F1 strain was comparable with NIA animals [817 ± 41 days vs 850 days (42)]. Histopathology at the time of death indicated that most of the cloned mice died of conditions associated with normal aging. The apparent cause of death of our cumulus cell clones included leukemia, myocardial degeneration, adenocarcinoma, and lymphoma. It is interesting to note that the first mouse cloned from an adult cell, Cumulina, lived for 2 years and 7 months, a very long time for a mouse that has a 2-year expected lifespan (43). Aside from a benign skin tumor that developed when she was about 2 years old, she was reportedly active and healthy until her death.

The lifespan of mice cloned from cumulus cells is, therefore, not different from that reported for the same gender and strain by the NIA. It is important to note also that cumulus cell clones that were examined did not develop the conditions reported by Ogonuki *et al.* A larger cohort of clones and control mice must be followed systematically. Our study provides information about female cloned mice generated from adult cumulus cells. Other cell types have been used as nuclear donors for somatic cell cloning (20), and the consequences of using those cells must also be investigated to determine whether there may be cell type-specific differences in longevity.

Body Weight and Obesity

Although our studies indicate that pre-weaning development, behavior, and longevity of cloned mice appear to be normal, we observed that they gained significantly more body weight as adults than controls (24). We found that clones and IVEM control mice followed similar growth curves from birth through 8 weeks; however, as they reached adulthood at 8 to 10 weeks of age, clones gained more weight than controls for the remainder of the experiment. Interestingly, IVEM controls were heavier than age-matched STOCK mice, suggesting that *in vitro* embryo culture alone was sufficient to result in increased body weights in the IVEM mice (Fig. 2) (27). The fact that clones weighed more than IVEM mice indicates that specific characteristics of the somatic donor cell (cumulus cell) or some aspect associated with the cloning process itself produces a heavier animal.

Increased body weight does not necessarily imply that an animal is obese. Body composition analysis revealed that clones had a significantly higher percentage of body fat (almost twice as much) compared with control animals (Fig. 3) (27), while the percentage of lean body mass did not differ among the groups. In addition, clones also are hyperleptinemic and hyperinsulinemic. These data therefore demonstrate that the increased body weight in cloned mice can be associated with an increased percentage of body fat and that cloned mice are indeed obese and exhibit hormonal characteristics consistent with this condition (27).

Obesity in cloned mice could be attributable to any of several possible mechanisms, one of the most obvious being increased food intake. Cloned mice as adults, however, eat the same amount of food as IVEM controls and even less than STOCK controls (27), such that if anything, they ap-

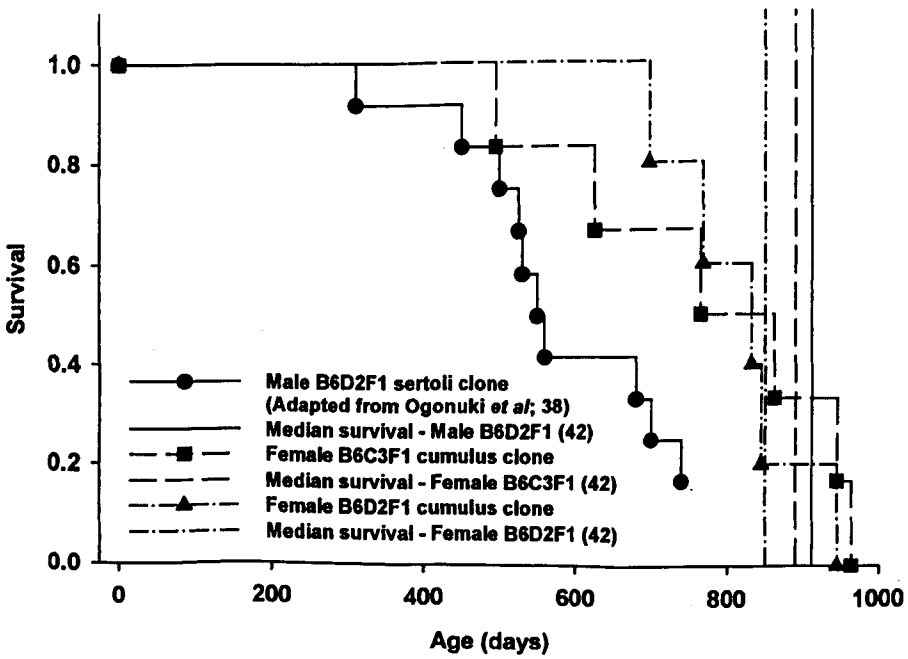

Figure 1. Survival rate of female B6C3F1 and B6D2F1 mice cloned from adult cumulus cells. Male B6D2F1 mice were cloned from fetal Sertoli cells by Ogonuki *et al.* (38).

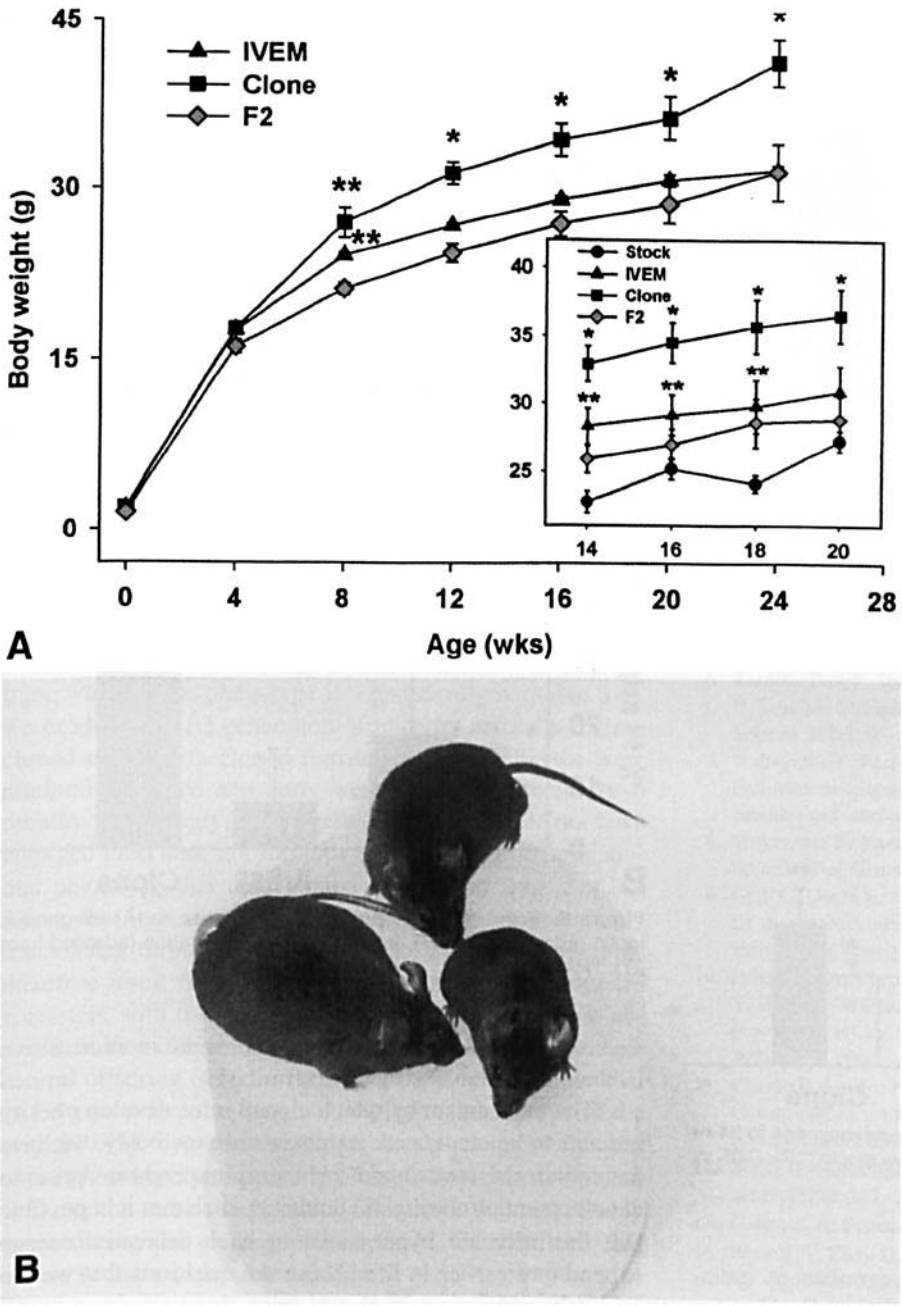

Figure 2. (A) Body weight of cloned mice, IVEM, and F2 offspring (adapted from 27). $^{*}P < 0.05$ vs IVEM and F2; $^{**}P < 0.05$ vs F2. (Inset) comparison of body weight of clones, IVEM, and F2 to age-matched background stock mice at 14 to 20 weeks of age. $^{*}P < 0.05$ vs IVEM, F2, and stock; $^{**}P < 0.05$ vs stock. (B) An adult cloned female B6C3F1 mouse (bottom left) with age- and strain-matched IVEM (top middle) and stock (bottom right) control mice.

pear to be attempting to reduce their body weights. Additionally, clones respond to 24-hour food deprivation comparably as both control groups, implying that altered food intake in adulthood is not responsible for increased body weight in cloned mice (Fig. 4) (27). It is certainly possible that the clones were hyperphagic before adulthood when the increased body weight and obesity had already manifested, and we are currently investigating this possibility.

Alterations in body weight regulatory systems may underlie the obese phenotype of cloned mice. We assessed the function of two such systems, the melanocortin and leptin signaling pathways. Leptin is produced by adipose cells, is secreted in proportion to body fat, and also functions to suppress food intake (44, 45). Cloned mice are heavier and the increased body weight is attributable to increased percentage of body fat. This implies that they should also have higher circulating leptin levels, and they do. In light of this, it is possible that the clones are insensitive to their own leptin signal. Contrary to this, we found the opposite to be true. Cloned mice are actually hypersensitive to exogenous leptin and suppress their food intake even more than both control groups (Fig. 5A) (27). This also suggests that, if anything, the clones are mounting responses to reverse their obese state.

The brain's melanocortin signaling system is recognized as an important controller of energy homeostasis (44, 45). Melanocortins such as endogenous α-melanocyte stimulating hormone (α–MSH) or the synthetic analog,

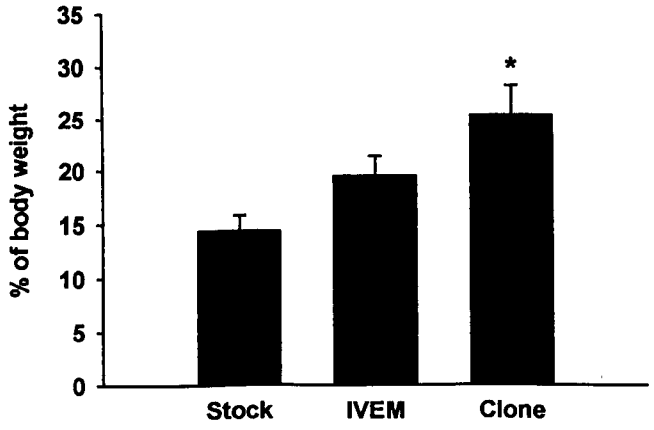

Figure 3. Percent body fat in clones and controls (adapted from 27). *$P < 0.05$ compared to stock control mice.

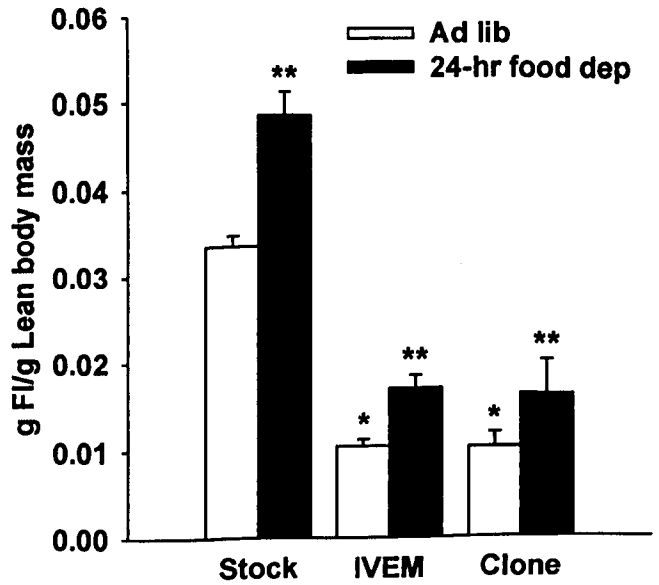

Figure 4. Twenty-four hour *ad lib* food intake and response to 24-hr food deprivation (adapted from 27). *$P < 0.05$ vs stock; **$P < 0.05$ vs *ad libitum*.

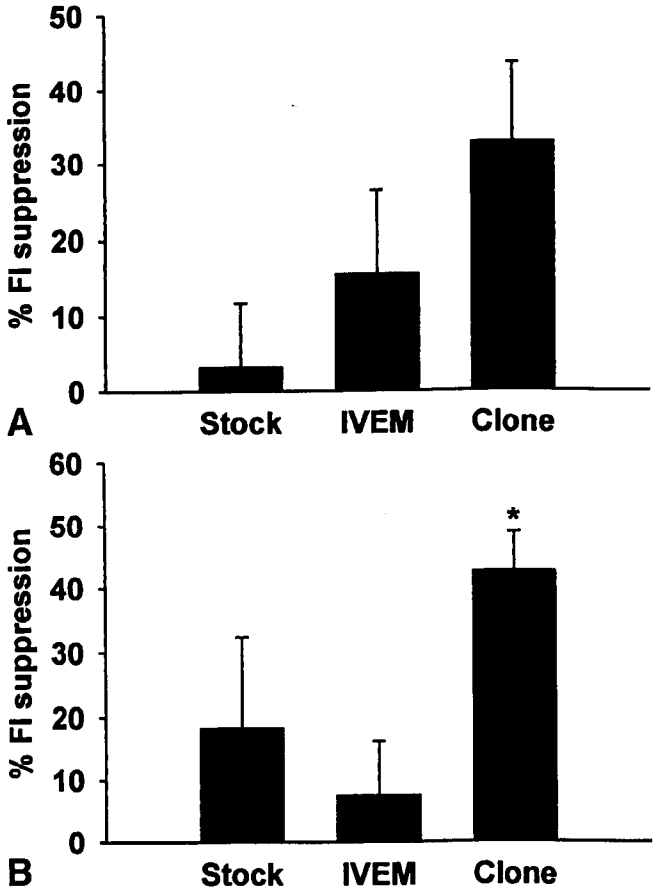

Figure 5. Food intake suppression in response to (A) exogenous leptin (adapted from 27) and (B) MTII administration (adapted from 27). *$P < 0.05$.

MTII (Phoenix Pharmaceuticals), are agonists at melanocortin-3/4 receptors and act there to suppress food intake. A possible explanation for the obesity of the clones is therefore a central insensitivity to melanocortin agonists. We evaluated this by administering MTII peripherally and observed that cloned mice in fact have a hypersensitive melanocortin system, suppressing their food intake significantly more than controls (Fig. 5B) (27). Because we administered a constant amount of MTII to each animal regardless of body weight (100 nMol of MTII), and since the clones weighed more than controls, they in fact received a lower dose of MTII per gram of body weight than controls. In spite of this, they suppressed their food intake more than control mice that received a higher dose of MTII. Taken together, all of these data indicate that the clones, if anything, would be much heavier if these systems regulating body weight were not functioning to oppose the obesity. The exact reason that these two systems are more sensitive in the clones remains to be determined.

The mechanism by which cloned mice develop obesity remains to be elucidated. As discussed previously, we have not monitored food intake or energy expenditure prior to development of obesity at adulthood, such that it is possible that the mice are hyperphagic or have decreased energy expenditure earlier in life. These are questions that we are currently addressing in cloned mice that have been monitored since weaning at 3 weeks of age. In addition, other systems involved in growth and development may be affected by the cloning process. As an example, the insulin-like growth factor (IGF) system has been implicated in the "large offspring syndrome" observed in sheep cultured as embryos. The expression of the IGF-2 receptor is attenuated in offspring exhibiting the large animal phenotype at birth (46), and we are currently evaluating the IGF system in cloned mice. We are also assessing expression of hypothalamic neuropeptides involved in body weight regulation, including proopiomelanocortin, agouti-related peptide, and melanocortin-4 receptor, to determine whether their regulation might be disrupted resulting in higher body weights.

All of the studies summarized were conducted in female B6C3F1 mice. It is important to note, however, that

the obese phenotype is independent of donor cell type, gender, or background strain. Male mouse clones have been generated using fibroblasts (8), Sertoli cells (47), and fetal neurons (48) as donor cells. Although the focus of this article is to review our studies and other existing literature about the phenotype of mice cloned from *adult somatic cells*, it is important to point out that embryonic stem (ES) cells have also been successfully used in cloning and there are reports that resulting offspring also exhibit the large fetus phenotype at birth (21). However, to our knowledge, there has been no subsequent follow-up studies performed on these mice.

Multiple generations of cloned animals have not been reliably generated in species other than the mouse (30). The success rate in generating clones from clones decreased with increasing generations and the two independent lines ultimately generated clones only up to the fourth and sixth generations (30). It was interesting to note that although these clones of clones are also obese, the phenotype was not *enhanced* in subsequent generations. That is, cloning of clones did not produce progressively larger mice. To determine whether this phenotype is a genetically heritable trait, we produced an F2 generation of mice by naturally mating cloned males with cloned females. Female F2 mice were randomly selected and body weight was monitored for 6 months. Intriguingly, the F2 generation of mice did not have enlarged placentas, nor did they exhibit the same large animal phenotype that their cloned parents did (Fig. 2) (27). These data indicate that the phenotype was not genetically transmitted through the germline to offspring and must therefore result from an epigenetic alteration. Our data are consistent with data from other studies indicating that aberrant traits exhibited by clones are not inherited by their natural offspring (49). Furthermore, there is one report that an F3 generation also does not display the phenotype of the original (49). Together these data strongly suggest that the obese phenotype, and possibly other aberrant phenotypes as well, are the result of epigenetic alterations that presumably occur during the cloning process itself. Exactly how this occurs remains to be determined.

Conclusion

It is clear that the consequences of adult somatic cell cloning are unpredictable and far from being completely understood. We have reviewed data what we have accumulated in an attempt to phenotype cloned mice generated mainly from cumulus cells, but have also discussed data from mouse clones derived from other donor cell types, generated on different background strains, and produced from both genders. The most obvious aberrant phenotype displayed by cloned mice is obesity, which we have begun to systematically examine at multiple levels using behavioral, physiological, and molecular approaches. Although obesity is the most easily recognizable aberrant phenotype in cloned mice, many other problems resulting from the cloning process may not be as readily apparent. Further studies over the entire lifespan and on larger numbers of cloned animals are necessary before we can confidently extend this technology into general applications.

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