

## A New ‘Crowded Uterine Horn’ Mouse Model for Examining the Relationship Between Foetal Growth and Adult Obesity

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**Abstract:** Obesity is an increasing health problem, not only in developed countries but also all over the world. In addition to the focus on food intake and energy expenditure, current studies suggest two other important influences on adult body weight: birth weight and postnatal rate of growth. A common procedure in laboratory animal studies to examine the relationship of low birth weight and adult obesity is maternal nutrient restriction, but maternal undernutrition is not the basis for the majority of obese individuals in developed countries. We have thus developed a new mouse model for human obesity referred to as ‘the crowded uterine horn model’. By removing one ovary from a female CD-1 mouse, the female produces a litter of about 13 pups in one uterine horn, resulting in crowding and a 4-fold difference in placental blood flow among fetuses in a litter. Restricted placental blood flow results in intrauterine growth restriction (IUGR); these animals show a 2-fold increase in body weight during the week after weaning, while macrosomial fetuses that go through a very small amount of growth during the same postnatal period. Male mice categorized as IUGR or macrosomic at birth both are obese in adulthood. This pattern of changes in body weight throughout life in male mice mirrors findings from epidemiological studies of human fetuses with IUGR and macrosomia who become obese, and thus may provide a new model that reflects the condition of people in developed countries who become obese.

As the incidence of obesity in developed countries increases, there is an increasing need for animal models to study and develop treatments for human obesity and other associated diseases referred to as ‘metabolic syndrome’. While additional diseases and conditions are being added to the list of obesity-associated diseases, the list currently includes cardiovascular disease, type II diabetes mellitus, arthritis, sleep apnoea, high cholesterol, hypertension, asthma and a number of cancers [1–3]. While epidemiological studies can reveal relationships between diseases, establishing causality and determining molecular mechanisms is greatly facilitated by animal models. A concern is whether current animal models are relevant to the aetiology of obese people living in developed countries and/or less developed regions where obesity and related metabolic diseases are also an increasing health problem [4].

Current epidemiological studies show two induction points for adult obesity, foetal and postnatal rates of growth, and these two points must both be considered in order to study the ontogeny of obesity. The first of these two factors has led to a new field of science (and a scientific society), known as the developmental origins of health and disease (DOHaD),

to focus on the issue of developmental induction of disease. The premise regarding DOHaD, specifically with regard to metabolic diseases, is that alterations in the foetal environment that cause a change in normal birth weight can have a detrimental outcome throughout the life of the individual. This is true whether the change is an increase or decrease relative to the median birth weight. DOHaD also applies to other diseases and syndromes, such as the testicular dysgenesis syndrome [5] and the foetal oestrogenization syndrome, as shown by the adverse effects of exposure to the oestrogenic drug diethylstilboestrol during foetal life [6–8]. The ‘programming’ of metabolic systems during foetal life by nutrients, environmental chemicals or other factors such as maternal stress is predicted to be due to epigenetic modification of cytosine bases together with modification of associated histone proteins [9,10].

The first factor of the dual induction points for obesity, the rate of foetal growth, is considered critical as it sets the conditions that will affect the entire life of the individual. The growth states that are specifically of concern are intrauterine growth restriction (IUGR) and macrosomia. IUGR occurs when a foetus develops in an environment that is nutrient deprived, although the causes of this altered growth are not always clear. While there are many factors that promote IUGR (fig. 1), it is thought that reduced placental transport is related to the decreased concentrations of nutrients and oxygen in IUGR fetuses. In the case of babies categorized as showing macrosomia at birth, many of the factors

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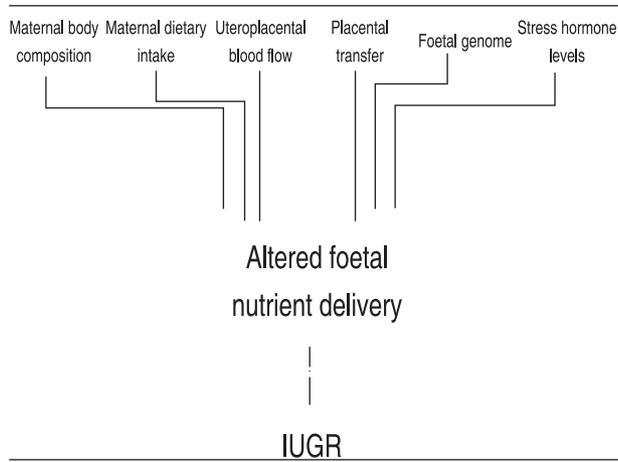


Fig. 1. Proposed factors influencing induction of intrauterine growth restriction (IUGR). There are a number of factors that have been proposed to affect development of IUGR and thus obesity in human beings and animals.

that initiate IUGR are reversed. Macrosomia is believed to be caused by foetal over-nutrition. One hypothesis is that the placenta acts as a sensor that adjusts the transport of essential nutrients from mother to foetus in response to levels of nutrients and regulatory molecules, such as leptin, insulin-like growth factors (IGF) and insulin, in the maternal circulation [11]. However, this hypothesis is only partly supported by existing data.

Nutrients do not passively cross the placenta. For example, there are a number of placental amino acid active transport systems, with some being expressed only on the basal membrane (foetal side) of the syncytiotrophoblast cells in the placenta, others only on the apical membrane (maternal villous side of the syncytiotrophoblast), and others on both membranes [12]. The activities of some of these transport systems are altered in the placenta of an IUGR foetus; for example, the transport systems for taurine, lysine and leucine all show reduced transporter activity [11]. It has also been shown that system A, which is major amino acid transport system and is responsible for the placental transport of neutral amino acids, is down-regulated in IUGR [13].

An issue related to 'foetal programming' of obesity is that it is common for obese people who try to lose weight return to what appears to be an abnormal 'set point' of body weight. This has led to the hypothesis that it is possible that metabolic systems are subject to 'programming' during foetal life [14], with the result that once the 'set point' for body weight is established, it is not easily altered. If this hypothesis is correct, the consequence would be that, similar to other physiological processes that are subject to foetal 'programming', obesity related to abnormal foetal growth would not easily be altered by simple solutions, such as just reducing food intake or increasing energy expenditure, which are the two major approaches that are the current focus of treating obesity in medicine [15]. The foetal programming hypothesis would explain why there are individuals in a population

who do not develop obesity while consuming the same types and amounts of foods as their obese counterparts. The concept of foetal programming of obesity is consistent with the 'thrifty phenotype' hypothesis that IUGR babies are 'metabolically programmed' for nutrient deprivation, and when faced with what would be a normal caloric intake for someone without IUGR, the IUGR individual becomes obese [14].

The second major factor in the aetiology of adult obesity is the rate of postnatal growth prior to adulthood. Current epidemiological studies show that while birth weight is important, the rate at which an individual grows during infancy and childhood is also a critical factor in obesity [16–18]. An increased growth rate during childhood is a predictor for adult obesity, and, as noted above, once a person becomes obese, the likelihood of returning to a 'normal' weight is low [19–25].

Studies showing that rapid postnatal growth increases the risk of adult obesity have led to a change in approach to treating pre-term and IUGR infants. The classical approach by pediatricians was to attempt to get IUGR infants to go through a phase of catch-up growth so that they would be the same size as normal birth weight babies. Now studies are indicating that rapid, catch-up growth in IUGR babies predisposes the infants to greater risk for subsequent obesity and other metabolic diseases.

The deviations from normal body weight in adulthood due to abnormal foetal growth may be caused by predictive adaptive responses to the environment. Predictive adaptive responses are defined as the foetus using an environmental cue to alter the expression of certain genes in a manner that predicts a similar food-restricted adult environment [14]. As discussed below, an alteration of nutrient availability from this predicted restricted environment can have dramatic metabolic consequences.

### Animal models of obesity

Prenatal protein restriction models to induce IUGR in the rodent have shown that raising the postnatal nutrition level to normal for a protein-restricted foetus causes rapid growth that is associated with subsequent obesity [26]. While this finding is intriguing, maternal protein restriction animal models are not relevant to the aetiology of human obesity in developed countries such as the USA, due to the fact that mothers in developed countries are typically not severely protein-deprived but the incidence of obesity is rapidly increasing. This provided the basis for our decision to explore other animal models for obesity that did not involve severely limiting maternal nutrient intake, either through protein restriction or total caloric restriction.

Other animal models of adult obesity draw on the use of treatments designed to make all individuals overweight in adulthood. Some of these methods, in addition to maternal caloric restriction and maternal low-protein diets, include streptozocin-induced gestational type 2 diabetes mellitus, post-implantation surgical manipulation (to restrict blood flow to foetuses) and glucocorticoid exposure [27]. It is

important to recognize that there are several different ways in which foetal undernutrition can occur, which is why there are a number of different animal models for obesity. As shown in fig. 1, there are many factors that affect the nutrient demands of the foetus and the ability of the placenta to supply those nutrients [28]. While all of these factors may not operate in any one IUGR foetus, changing only one of them creates the potential for producing IUGR. In other words, which factor an animal model focuses on likely determines that subpopulation of IUGR offspring it includes, thus potentially limiting the application to human IUGR offspring.

Although some IUGR offspring may be produced by malnourished mothers in the USA, the mother's body composition and factors that influence placental blood flow appear to be better candidates as causal agents of IUGR. Our new model provides a natural model for examining the consequences of differential placental blood flow.

### The 'crowded uterine horn' model

We will describe findings from ongoing studies using a novel mouse model that shows that very low birth weight (IUGR) predisposes the individual to the induction of the metabolic syndrome cascade of increased weight and glucose intolerance. However, aspects of the phenotype of these obese individuals are different from the phenotype of obese mice that were in the top 5th percentile for body weight at birth, a condition referred to as macrosomia (the exact percent body weight at birth used to assign individuals to these categories is not always consistent). The possibility that both IUGR and macrosomia might result in obesity was initially dismissed, but is now supported by a considerable amount of evidence [11].

### Materials and methods

*Animal husbandry.* CD-1 mice (*Mus domesticus*) were purchased from Charles River Breeding Laboratories (Wilmington, MA, USA) in December 2004, and were maintained as an outbred colony since that time. The mice were housed in 18 × 29 × 13-cm polypropylene cages on corn cob bedding. Pregnant and lactating mice were fed Purina mouse breeder chow 5008 (soy-based, Purina-Mills, St. Louis, MO, USA). After weaning, offspring were fed Purina standard laboratory chow 5001 (soy-based). Water was provided *ad libitum* in glass bottles and was purified by ion exchange followed by a series of carbon filters. Rooms were maintained at 25 ± 2°C under a 12-hr light:dark cycle, with the lights on at 10:30 a.m.

*General methods.* To induce a crowded uterine horn, post-partum 2-month-old female mice were hemi-ovariectomized. Following a 5-day healing time, mice were timed mated and pregnancy confirmed by the presence of a vaginal plug. On gestation day 0, body weights were taken and pregnancy is verified by an increase of 3 g body weight by gestation day 10. On gestation day 19, dams were allowed to deliver vaginally. Postnatal day 1 weights were recorded at 1 p.m. Following weights taken on postnatal day 1, mouse pups were left unhandled until postnatal day 22, when the mice were weaned, and weighed. Male mice were singly housed, and their food weighed so that intake could be monitored over the following 7 days. Following weaning, mice were weighed once every 7 days through the remainder of their life. Animals were challenged with a glucose tolerance test at

4 months of age. Following glucose challenge, animals were killed by asphyxiation and blood was collected by decapitation. The blood was centrifuged, and serum was stored for hormone analysis. In addition to blood, the following organs were collected and weighed: liver, kidney, gonadal fat, inguinal fat, adrenal fat, heart, right epididymis, spleen, right gastrocnemius and both testes.

The data presented were obtained from an experiment with 57 litters. These 57 litters produced a total of 605 offspring, 585 of which survived until weaning, resulting in a 3.3% pre-weaning mortality rate. Twenty-five per cent of all pre-weaning deaths were of IUGR animals, while only 0.05% were macrosomic animals. The sex of the offspring was 297 females and 308 males. For the purpose of this experiment, IUGR was defined as the bottom 5th percentile, and macrosomia as the top 5th percentile. Within this experiment, the weight cut-offs for these two categories were <1.25 g and >2.01 g, respectively. Sixteen animals were selected from each of these groups, as well as 16 males at the median for birth weight, for collection of tissues in adulthood.

### Results and discussion

Our mouse model shows that IUGR animals can be produced without changing maternal food intake, maternal drug treatment or altering the nutrition levels of the dams' food. Using this model, adult obesity is related to differential birth weight and differential postnatal growth. Our ongoing studies reflect the human condition in developed countries, because both the birth weight and postnatal growth interact to determine adult body weight.

Our mouse model illustrated in fig. 2 is based on the finding that there is a difference in placental blood flow as a function of where foetuses are located within the uterus; the location of the foetuses is a random event [29]. As a result of bidirectional blood flow into the uterine loop artery from both the descending aorta (at the ovarian end) and internal iliac (at the cervical end), foetuses located in the middle of the uterus have reduced placental blood flow and are lighter at birth relative to foetuses at either end of a uterine horn, as previously reported in rats [30] and mice [31]. This phenomenon is exaggerated if an adult female rodent is hemi-ovariectomized prior to pregnancy, which results in compensatory ovulation by the remaining ovary, which ovulates the number of ova that would have been produced by both ovaries reviewed in [32]. The consequence is a crowded uterine horn (which in the mouse are completely independent, each with a cervix leading to the vagina) and a greater effect of uterine location on placental blood flow and foetal growth relative to an uncrowded normal pregnancy.

Preliminary findings from ongoing studies suggest that placental glycine transport is correlated with placental blood flow (unpublished findings). In summary, the data in fig. 2 show that this crowded uterine horn model produces offspring with markedly different rates of placental blood flow and nutrient transport without nutritionally depriving or surgically manipulating females during pregnancy. IUGR in human beings is related to reduced placental blood flow and placental active transport of specific nutrients [11].

Figure 3 shows the average birth weight and postnatal growth rates for median (50th percentile), IUGR (bottom 5th percentile) and macrosomic (top 5th percentile) male

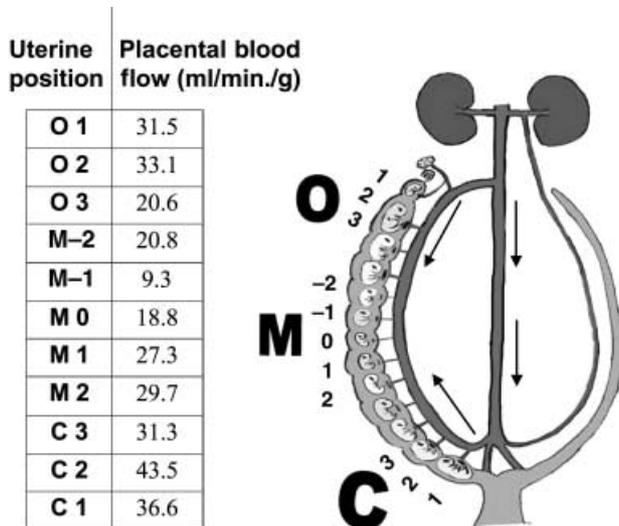


Fig. 2. A schematic of the 'crowded uterine horn' model. In this model, weaning female mice are paired with a stud male and allowed to give birth, after which they are hemi-ovariectomized and remated. Hemi-ovariectomy causes a compensatory increase in ovulation from the remaining ovary, resulting in a litter of normal number in one uterine horn. There is differential growth of the pups due to a decreased blood flow to the pups in the middle (M) of the uterine horn. This is due to bidirectional flow of blood (indicated by arrows) into the loop uterine artery from both the ovarian (O) and cervical (C) ends of the uterine horn. The pup nearest the ovary may also be growth restricted if its placental artery is a branch from the smaller ovarian artery rather than the larger uterine artery. The data shown are placental blood flow measurements taken from a pregnant female CD-1 mouse on gestation day 18 injected with radiolabelled microspheres, as described in Even et al. [30].

mice in a study that involved examination of offspring from 57 hemi-ovariectomized female CD-1 mice. For males categorized as IUGR at birth, there was a dramatic increase in post-weaning growth rate that resulted in what has been referred to as 'centile crossing', where the IUGR animals catch-up to and surpass animals with a 'normal' birth weight. The consequence is that the IUGR males ended up as adults with the same significantly increased body weight (similar to that of macrosomic males) relative to 'normal' males. This model thus produces significantly heavier adult animals that had experienced two very different foetal growth rates, IUGR and macrosomia, with both of these groups of males being heavier than males born at the median (or mean) birth weight. The female siblings of these males were also examined and showed the same inverse relationship between body weight at birth and weeks 3–4 weight gain as did the males. However, unlike males, females in all body weight at birth categories were virtually identical in body weight in adulthood (data not shown).

In addition to increased adult body weight, IUGR male CD-1 mice that exhibited a rapid postnatal growth phase during the first week after weaning had an impaired ability to metabolize glucose (unpublished data) relative to males at the median for body weight at birth. The animals with the highest postnatal growth rate had a serum glucose concen-

tration peak 40 mg/dl higher after glucose challenge than those that did not show a rapid postnatal growth rate. They also did not clear as much of the glucose dose in the given 2-hr time frame.

A potential application of this model is to compare the effect of IUGR as opposed to macrosomia identified at birth in terms of gene expression in adipocytes, because our initial findings suggest that the two different routes to adult obesity for these two populations could result in differences in adipocyte gene activity. For example, we collected the bilateral epididymal fat pads from adult male mice that had been categorized as IUGR or macrosomic at birth and had become obese. Differences in adipocyte gene expression between the IUGR and macrosomic animals were examined by microarray analysis (Affymetrix Mouse Genome 430 array) and revealed differential expression of hundreds of genes. Although we have numerous genes of interest, this analysis is still ongoing, and we are confirming expression differences for these genes by quantitative reverse transcriptase–polymerase chain reaction. These preliminary findings suggest that there are significant differences in adipocytes from these two sub-populations of obese mice, those that were categorized as macrosomic at birth and those that were categorized as IUGR and then experienced rapid post-weaning weight gain.

### Conclusions

Obesity and related morbidities are a rapidly increasing health problem throughout the world. Where the uncertainty lies is in our understanding of the causes for the rapid rate of increase in obesity that has occurred over the last two decades and effective methods to treat individuals who are obese. However, the intractable nature of obesity to current approaches in medicine has led to suggestions that the simplistic assumption that energy intake and expenditure explain obesity is misguided, and that obesity may be difficult to treat because it is a consequence of foetal programming of metabolic systems [15].

The finding that mouse foetuses that have restricted placental blood flow and are in the bottom 5th percentile for body weight at birth subsequently go through a rapid increase in body weight immediately after weaning and become as heavy as animals that are in the top 5th percentile for body weight at birth (which do not show a rapid post-weaning weight gain) is evidence that there are different paths to adult obesity that likely require different therapeutic approaches. It is our hope that animal models, such as the crowded uterine horn model, will provide useful information about the underlying mechanisms involved in different subtypes of adult obesity and will also provide clues regarding treatment and ultimately prevention.

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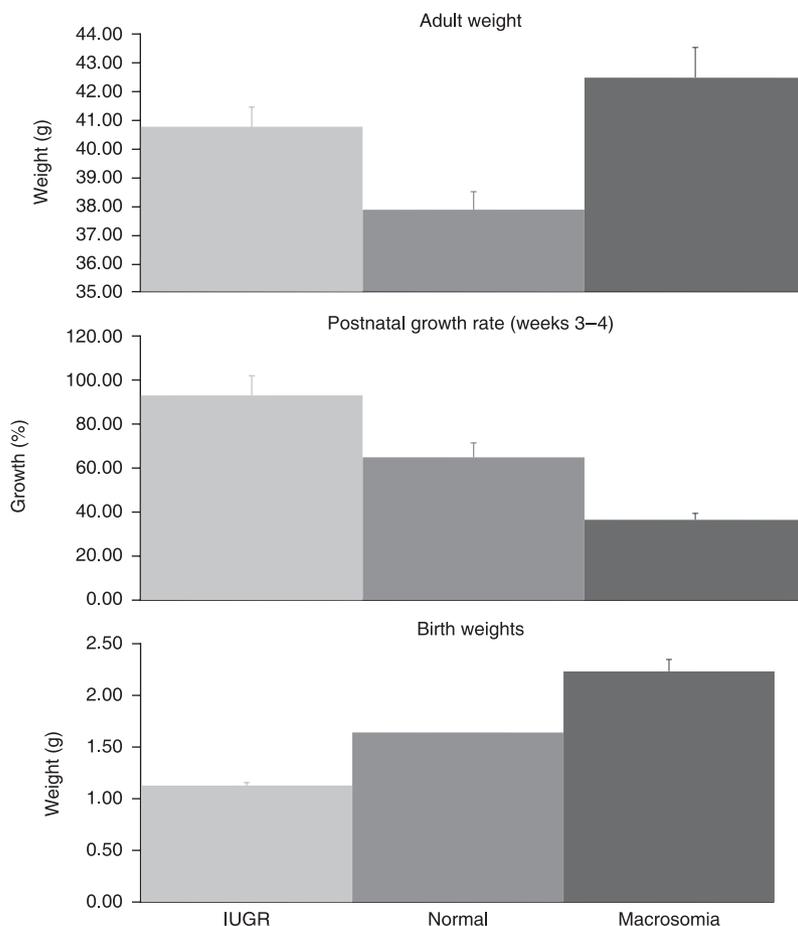


Fig. 3. Birth weight category, rate of post-weaning growth and adult body weight in male mice. Mean ( $\pm$ S.E.M.) birth weights, percent increase in body weight between postnatal weeks 3 and 4 (after weaning) by birth weight class and adult weights on postnatal week 14. We categorized mice as intrauterine growth restricted (IUGR) if they were in the bottom 5th percentile. Normal mice were at the 50th percentile, while macrosomia was defined as the top 5th percentile for body weight at birth.

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