The maternal environment programmes postnatal weight gain and glucose tolerance but placental and fetal growth are determined by fetal genotype in the Leprdb/+ model of gestational diabetes

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Abstract

Mice heterozygous for a signalling-deficient leptin receptor (Leprdb/+ (db/+)) are widely used as a model of gestational diabetes that results in poor fetal outcomes. This study aimed to investigate the importance of fetal genotype (db/+) relative to abnormal maternal metabolism on placental function and therefore fetal growth and offspring health.

Wild type (wt) and db/+ females were mated to db/+ and wt males respectively to generate litters of mixed genotype. Placentas and fetuses were weighed at E18.5; offspring weight, hormone levels, glucose tolerance and blood pressure were assessed at 3 and 6 months.

Pregnant db/+, but not wt, dams had impaired glucose tolerance. db/+ placentas and fetuses were heavier than wt but the maternal environment had no effect; wt placentas/fetuses from db/+ mothers were no bigger than wt placentas/fetuses carried by wt mothers. Postnatal growth, glucose metabolism and leptin levels were all influenced by offspring genotype. However maternal environment affected aspects of offspring health as wt male offspring born to db/+ dams were heavier and had worse glucose tolerance than the sex-matched wt offspring of wt mothers. Blood pressure was not affected by maternal or fetal genotype.

These data reveal that studies using the db/+ mouse to model outcomes of pregnancy complicated by gestational diabetes should be mindful of the genetically predisposed fetal/post-natal overgrowth. Although inappropriate for dissecting the effect of maternal hyperglycemia on the contribution of placental function to macrosomia, the db/+ mouse may prove useful for investigating mechanisms underlying in utero programming of suboptimal postnatal growth and glucose metabolism.

Keywords: placenta, birth weight, post-natal growth, GTT, leptin, blood pressure
Introduction

The incidence of obesity amongst women of child-bearing age has doubled over recent years [1]. Pre-pregnancy weight is associated with the development of gestational diabetes (GDM) and the frequency of this condition has also increased [2]. Pregnancy complicated by GDM is associated with increased fetal mortality and morbidity [3]. Fetal overgrowth - macrosomia – occurs in a third of babies born to such mothers [4]. These infants are more likely to experience birth injuries, asphyxia and postnatal metabolic disturbances [5]. Furthermore, *in utero* exposure to an adverse nutrient environment can perpetuate disease; long-term studies have demonstrated that macrosomic offspring have impaired glucose tolerance, increased adiposity and raised systolic blood pressure as children, and an increased risk of developing diabetes, obesity and cardiovascular disease as adults [6,7].

Maternal, and consequently fetal, hyperglycemia undoubtedly plays a role in fetal overgrowth. However, good maternal glucose control does not abolish macrosomia [8] suggesting that increased maternal-to-fetal transfer of other nutrients, for example lipids and amino acids, may contribute to fetal overgrowth and importantly, that instead of merely reflecting an increase in nutrient supply, macrosomia may be a consequence of abnormal placental function. Indeed, numerous studies have shown that nutrient metabolism and transport are altered in placentas from pregnancies complicated by GDM [9]. Furthermore, placental mass is increased [10], exacerbating augmented nutrient transport by increasing the surface area of the transporting epithelium (syncytiotrophoblast).

Interventions aimed at modulating placental function and thereby preventing fetal macrosomia could be used to halt the transgenerational cycling of diabesity and reduce the consequent global health burden. However, such advances are dependent upon the availability of appropriate models to aid understanding of the role of the placenta in GDM.
and to test potential therapies. Mice, like humans, have a haemochorial placenta and previous studies have suggested that a strain that is heterozygous for a signalling-deficient leptin receptor (C57BL/KSJ-Leprdb/+) is a good experimental model of GDM. Dams develop diabetes (impaired glucose tolerance and elevated HbA1c) only during pregnancy [11,12] and the offspring have significantly greater birth weights [11,13,14,15] and deranged metabolism [15] compared to the offspring of wild-type (wt) mothers. These poor outcomes have been attributed to the adverse maternal environment, however, the relative contribution and importance of the fetal genotype (db/) to placental function, and therefore fetal growth and programming, have not been evaluated. This study aimed to determine the usefulness of the db/+ mouse as a model for investigating placental function in pregnancies complicated by the abnormalities in maternal metabolism that occur in gestational diabetes.
Methods

All experimental procedures were conducted in accordance with the Home Office Animals (Scientific procedures) Act 1986 of the United Kingdom. All animals were maintained with free access to food and water. Wild type and db/+ female were mated at twelve weeks of age to db/+ and wt males respectively in order to generate litters of mixed genotype; day of plug was counted as E0.5. Some dams (10 wt; 9 db/+ ) were euthanised on day E18.5 to enable collection of placentas and fetuses (140 in total) which were weighed and then genotyped, using DNA extracted from tail snips, by sequencing PCR products obtained using primers flanking the Lepr mutation (F: 5′-CCCTCCCCCTCTCCAAGTGT-3′; R: 5′-CAGCAACCGTCACACCATT-3′) [16]. This analysis revealed that 58 of the placenta/fetus pairs were wt (28 from wt dams; 30 from db/+ mothers) and 82 were db/+ (40 and 42 from wt and db/+ mothers respectively). Other dams were allowed to deliver and pup genotype was determined by analysis of DNA extracted from ear punches obtained at weaning (21 days of age). The F1 offspring were maintained for up to 6 months.

Dams (day 18.5 of pregnancy) and F1 offspring (3 and 6 months) were subjected to a glucose tolerance test (fasted overnight, injected with 2g glucose/kg ip and tail vein blood samples collected at 0, 20, 30, 60, 90 and 120 minutes) before sacrifice. Glucose concentrations were measured using a glucometer (OneTouch Vita) and the 0 minutes sample was also used to measure insulin and leptin levels using mouse-specific ELISAs (Millipore and R&D Systems respectively).

The systolic and diastolic arterial pressure of the F1 offspring was measured at 6 months of age by tail-cuff volume pressure recording (CODA system, Kent Scientific Corporation, USA) as previously described [17], ensuring that mice were accustomed to the procedure before collecting the blood pressure readings (average of 5/animal).
Data are presented as mean (±SEM). Within-litter comparisons were made using a paired t-test. Data from different litters were analysed using an independent t-test; \( p<0.05 \) was considered significant.
Results

_db/+ dams have impaired glucose tolerance:_ _db/+_ dams had lower fasting insulin levels than wt mothers \((0.16(\pm 0.04)\text{ng/ml} \text{ versus } 0.31(\pm 0.03)\text{ng/ml}; p<0.05)\) and analysis of glucose levels confirmed impaired glucose tolerance during pregnancy (area under curve \(1339(\pm 85)\) versus \(987(\pm 16)\); \(p<0.05\)). _db/+_ mothers had higher circulating leptin levels \((760(\pm 50)\text{ng/ml})\) than wild-type mothers \((148(\pm 15)\text{ng/ml}; p<0.05)\), and both had significantly higher levels than their non-pregnant counterparts (33- and 19-fold respectively).

Effect of maternal diabetes on placental and fetal growth: There was no significant difference in the average litter size of wt and _db/+_ dams \((6.8\pm 0.6 \text{ versus } 8.0\pm 0.6 \text{ respectively})\), nor in the number of wt and _db/+_ fetuses within each litter. Consequently the total fetal and placental weight carried by wt dams \((7375\pm 583\text{mg} \text{ and } 558\pm 45\text{mg}, \text{respectively})\) was similar to that carried by _db/+_ mothers (fetal weight – 8534\pm 610\text{mg}; placental weight – 653\pm 46\text{mg}). However, after accounting for the weight of the fetal/placental unit, _db/+_ mothers were significantly heavier than their wt counterparts \((33.06\pm 0.75\text{g versus } 30.37\pm 0.72\text{g}; p<0.05)\). _db/+_ fetuses \((n=40)\) carried by wt dams exhibited a significantly higher (5%) birth weight than their wt littermates \((n=28, p=0.05; \text{see Figure 1 for individual pup data and Table I for mean litter weights}). _db/+_ fetuses \((n=42)\) from _db/+_ mothers were also bigger (3%) than their wt counterparts \((n=30, p=0.05; \text{Figure 1, Table I}).\)

Surprisingly, maternal genotype had no effect on progeny birthweight. _db/+_ fetuses carried by _db/+_ mothers were of similar size to those from wt dams \((\text{Figure 1,Table I}).\). Moreover, wt pups from _db/+_ mothers (offspring / dam combination that most closely models human gestational diabetes) were no bigger than wt fetuses carried by wt mothers \((\text{Figure 1, Table I}).\)

Similarly, placentas from _db/+_ fetuses \((n=82)\) were larger \((p<0.05)\) than those of wt fetuses \((n=58)\) irrespective of maternal genotype \((\text{Figure 1, Table I}).\). Consequently, the fetal to
placental weight ratio, commonly used as an indicator of placental efficiency, was the same in all animals (Figure 1).

**Effect of maternal diabetes on offspring growth, metabolic parameters and blood pressure:**
Initial analysis of F1 offspring weight suggested no difference between those from normal pregnancy (23.72±0.97g and 27.86±1.05g at 3 and 6 months respectively) and those born to dams with gestational diabetes (24.83±0.78g and 28.56±0.88g at 3 and 6 months). However, analysis of data accounting for offspring genotype and sex revealed that wt males born to db/+ mothers are heavier than wt males born to wt mothers (p<0.05) but the post-natal growth of female offspring is not affected by the maternal environment (Figure 2A). In keeping with our observations of the effect of the db/+ genotype on pre-natal growth, male and female db/+ offspring, from both normal and complicated pregnancy, are heavier (Figure 2A) than their wt littermates at both 3 and 6 months of age.

A comparison of all offspring born to wt and db/+ mothers showed that at 6 months of age, those from mothers with gestational diabetes had significantly lower fasting insulin levels (0.16(±0.02)ng/ml versus 0.21(±0.02)ng/ml in wt; p<0.05) and worse glucose tolerance (AUC 1603(±69) versus 1420(±41) in wt; p<0.05). Again there was an influence of sex and genotype as the glucose tolerance of wt males born to db/+ mothers was significantly worse than that of wt males from normal pregnancies at both 3 and 6 months (Figure 2B). db/+ offspring, both male and female, had impaired glucose tolerance, irrespective of the maternal environment, in comparison to their wt littermates (Figure 2B).

Offspring leptin levels were affected by genotype (17.5(+2.2)ng/ml in six month old db/+ animals versus 5.4(0.81)ng/ml in wt mice; p<0.05) rather than sex or maternal environment (Table II).
At six months of age, the systolic, diastolic and mean arterial (MAP) pressure of the male and female wt offspring from *db/+* mothers was similar to that of the sex-matched wt offspring from uncomplicated pregnancies (MAP 132±7 versus 148±5mm Hg respectively); none of the parameters measured were affected by offspring genotype (Table III).
**Discussion**

This study shows that the *db/+* mouse is not ideal for investigating the effect of GDM on placental function and therefore its contribution to fetal growth. However, the model may be useful for dissecting mechanisms underlying *in utero* programming as the male, but not female, offspring from *db/+* mothers were heavier and had impaired glucose tolerance at six months of age.

We demonstrate that fetal genotype influences both placental and fetal growth as the weight of *db/+* placentas and fetuses, carried by either wt or *db/+* mothers, was significantly greater than that of wt littermates. The leptin receptor is known to regulate leptin mRNA expression in an autocrine manner [18] thus *lepr* heterozygosity likely affects placental leptin production and consequently placental development and function, leading to increased fetal growth. Indeed, others have noted increased leptin levels [19] and cellular hypertrophy [13] in *db/+* placentas and in human placenta, leptin stimulates increased activity of the amino acid transporter, system A [20].

Crucially however, our data suggest that in this model, fetal genotype is more important than the maternal environment in determining placental and fetal growth as the placental and birthweight of wt fetuses carried by *db/+* and wt dams were similar. These data contrast with that of other studies which report that *db/+* mothers bear offspring with greater placental [13] and birth [13,14,15,21,22] weights than wt mothers. It is possible that differences in gestational age may have contributed to the discrepant findings as some studies [14,15] assessed fetal weight at a later time point (E19 versus E18). However, changes in placental growth usually precede changes in fetal growth [23] and, using the proxy measure of placental:fetal weight ratio, we found no evidence of altered placental function in *db/+* pregnancies. A more likely explanation lies in differences in experimental design. Previous studies either set up matings such that *db/+* pups were absent from wt pregnancies [21,22], or
compared all pups born from db/+ versus wt pregnancies without knowledge of pup genotype [13], or commented, without detailing the results, that there were no significant differences in placental and birth weight between wt and db/+ fetuses from the same litter, thus data from each litter were grouped [14,15]. Interestingly, the weight of fetuses from db/+ mothers is reported to be greater than that of pups from wt pregnancies even when maternal hyperglycemia was reduced by overexpression of GLUT4 [14]. Increased placental growth, and therefore transfer of nutrients, was mooted as an explanation of this finding, but the current study does not support this hypothesis. Moreover, administration of leptin to db/+ mothers during late pregnancy reduced their adiposity and circulating glucose levels, but fetal growth was not affected [21]. In that study [21], placental and fetal leptin levels were higher in db/+ compared to wt pregnancies which, together with our own data, point towards fetal genotype as the dominant regulator of placental and fetal growth in this model. Models of other pregnancy complications have also noted that fetal genotype contributes to pregnancy outcome [24], highlighting the need, where possible, to study mixed litters in order to truly appreciate the influence of the maternal environment.

The fact that the db/+ model does not mimic the placenta/fetal overgrowth often associated with gestational diabetes in women [4] is interesting and suggests that impaired maternal glucose tolerance is not necessarily detrimental to placental function. A study of trophoblast isolated from normal human placentas at term found that unlike elevated levels of non-esterified fatty acids, raised glucose levels had little effect on placental structure, metabolism and inflammation [25], leading the authors to postulate maternal dyslipidaemia as the key determinant of placental dysfunction in pregnancies complicated by diabetes. In our study, db/+ dams were heavier than wt dams at day18.5, which is in keeping with their reported hyperphagia [15,21], though we did not assess maternal adiposity or profile circulating lipids.
Mice carrying other single gene mutations, such as those that are heterozygous for the prolactin receptor or that lack the serotonin receptor also develop glucose intolerance during pregnancy, as do animals with conditional deletion of genes for transcription factors such as HNF-4α, FoxD3 and FoxM1 from pancreatic β cells [26]. However, these models have mainly been explored in relation to understanding the mechanisms underlying maternal pancreatic adaption to pregnancy and the pathogenesis of disease; further research is needed to determine their suitability for studying the effect of GDM on placental function and fetal growth. Alternative strategies include surgical removal or chemical (e.g. streptozotocin) destruction of pancreatic β cells, though neither model accurately reflects the aetiology of GDM and some clinical outcomes such as macrosomia are absent [26]. GDM induced by nutritional manipulation, for example feeding mice a diet high in saturated fat, has been reported to affect placental structure and function [27]; this supports the observations made in human placenta discussed above, however GDM in women is a heterogeneous condition and susceptibility is due to the combination of environmental and polygenic factors. It is unlikely that any currently available model will be suitable for all studies [26] and researchers must be careful to choose the most appropriate for their purpose.

Indeed male wt offspring from db/+ mothers were heavier and had poorer glucose tolerance than those from normal pregnancies in agreement with a previous study which reported differences in the weight of 8-week old wt male, but not female, offspring from db/+ and wt pregnancies [22]. However, differences observed in the leptin levels of such animals are not replicated herein; in our study, the levels of circulating leptin in 6 month old animals are related to genotype rather than the maternal environment. It is possible that the adverse maternal influence resolves with increasing age. Others have reported that at 6 months, the weight of wt offspring born to db/+ and wt mothers is similar in both sexes but that female offspring have increased body fat and insulin resistance [15]. Offspring adiposity was not
assessed in our study but we did not detect sex differences in fasting insulin levels. A sex difference in offspring outcomes, with males often faring worse, is a common observation in studies of *in utero* programming, especially in relation to glucose intolerance [28], and has been ascribed to differences in maternal investment of energy depending on fetal sex [29]. Data from this and other published studies suggest that future investigations of the *db/+* model should consider offspring sex when assessing outcomes.

db/db mice are known to have raised systolic, diastolic and mean arterial blood pressures in comparison to their *db/+* littermates [30] but a comparison of *db/+* and wt offspring, born to either wt or *db/+* mothers, has not been reported. In this study, all measures of blood pressure were similar between offspring. However, it is possible that more sensitive assessment, for example using radiotelemetry might reveal subtle effects of the maternal environment and / or offspring genotype or that a secondary stressor may not be as well tolerated. It will be interesting to uncover the mechanisms that can programme the postnatal health of male offspring from *db/+* dams in the absence of placental / fetal overgrowth. *In vitro* studies suggest that GDM could influence epigenetic programming [31,32] and more recently, genes involved in appetite control and energy metabolism have been shown to be epigenetically modified in placentas and cord blood of infants from pregnancies complicated by GDM [33,34]. Furthermore, a mouse model of GDM induced by administration of streptozotocin found that although the birthweight of F1 offspring was not affected, the male offspring had impaired glucose tolerance as adults and altered methylation of the imprinted genes *Igf2/H19* that are important for pancreatic islet development [35]. Altered nutrition in the perinatal period can also cause epigenetic changes that affect adult health [36,37]. Nothing is known about the quantity and quality of milk from *db/+* dams, thus cross-fostering experiments will be important to determine how maternal nutrient supply during this critical period of development contributes to the long-term health of the wt offspring born to *db/+* dams.
In summary, our study highlights the need to genotype offspring when interpreting the effect of the maternal environment on placental and fetal weight in genetic models of GDM. The \(db/+\) mouse may be most useful for investigating mechanisms underlying GDM programming of postnatal growth and glucose metabolism.
Figure Legends

Figure 1. Heterozygosity in a leptin receptor gene mutation predisposes to placental and fetal overgrowth. Ten wild type (wt) females at twelve weeks of age were mated to db/+ males and nine age-matched db/+ females were mated to wild type males in order to generate litters of mixed genotype. Day of plug was counted as E0.5 and fetuses and their corresponding placentas were collected for assessment of weight and genotype at E18.5. Offspring carrying the db/+ genotype (n=82) have increased body and placental weights in comparison to wt offspring (n=58), regardless of maternal genotype. The weight of each pup was divided by the weight of its corresponding placenta to give the fetal: placental ratio, commonly used as an indicator of placental efficiency, which was not affected by maternal or fetal genotype. Data points represent individual pups / placentas; bar represents mean. * - p<0.05.

Figure 2. Effect of maternal diabetes on postnatal weight gain and glucose tolerance. Offspring (male and female) from wild type (wt) ♀ / db/+ ♂ or db/+ ♀ / wild type ♂ crosses were weighed (A) or subjected to a glucose tolerance test (B) at 3 and 6 months of age. Data are shown as mean±SEM; AUC – area under curve; n – number of offspring analysed; * - p<0.05.
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<th>wt mothers (n=10)</th>
<th>db/+ mothers (n=9)</th>
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<td>wt offspring</td>
<td>db/+ offspring</td>
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<td><strong>Placental weight</strong></td>
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<td>83.80 (±1.91)&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><strong>Fetal weight</strong></td>
<td>1037 (±39.6)</td>
<td>1122 (±35.8)&lt;sup&gt;a&lt;/sup&gt;</td>
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**Table I.** Placental and fetal weights of day E18.5 litters. The mean placental and fetal weight of all the wt or db/+ offspring within each litter was calculated from ten wild type (wt) and nine db/+ mothers and are presented as mean±SEM. a – p<0.05 versus wt pups from wt dam; b – p<0.05 versus wt pups from db/+ dam.
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<td>leptin (ng/ml)</td>
<td>4.5 (±1.8)</td>
<td>7.9 (±3.0)</td>
<td>16.6&lt;sup&gt;a&lt;/sup&gt; (±2.5)</td>
<td>23.3&lt;sup&gt;a&lt;/sup&gt; (±6.5)</td>
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<td>3.1 (±0.7)</td>
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<td>16.3&lt;sup&gt;b&lt;/sup&gt; (±4.8)</td>
<td>17.8&lt;sup&gt;b&lt;/sup&gt; (±3.8)</td>
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**Table II.** Serum leptin levels (mean (±SEM)) of offspring from wild type (wt) and db/+ mothers measured at 6 months of age. <sup>a</sup> - p<0.05 versus sex-matched wt littermates from wt dam; <sup>b</sup> – p<0.05 versus sex-matched wt littermates from db/+ dam.
Table III. Systolic and diastolic pressure (mean (±SEM)) of offspring born to wild type (wt) and db/+ mothers measured at 6 months of age. Neither parameter was significantly affected by offspring sex, genotype or the maternal environment.
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Duality of Interests

The authors have nothing to declare.

Author Contributions

R.N. - researched data, reviewed/edited manuscript. M.R.D. - researched data, reviewed/edited manuscript. C.P.S. - contributed to experimental design and discussion, reviewed/edited manuscript. P.N.B. - contributed to experimental design, reviewed/edited manuscript. S.T.D. - contributed to experimental design, reviewed/edited manuscript. J.M.G. - contributed to experimental design, reviewed/edited manuscript. J.D.A. - contributed to experimental design and discussion, reviewed/edited manuscript. M.W. - contributed to experimental design and discussion, researched data, performed data and statistical analyses, wrote manuscript.
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