Maternal obesity heritably perturbs offspring metabolism for three generations without serial programming

S A Eaton, A J Aiken, P E Young, J W K Ho, J E Cropley, C M Suter

Cite this article as: S A Eaton, A J Aiken, P E Young, J W K Ho, J E Cropley, C M Suter, Maternal obesity heritably perturbs offspring metabolism for three generations without serial programming, International Journal of Obesity accepted article preview 6 October 2017; doi: 10.1038/ijo.2017.247.

This is a PDF file of an unedited peer-reviewed manuscript that has been accepted for publication. NPG are providing this early version of the manuscript as a service to our customers. The manuscript will undergo copyediting, typesetting and a proof review before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers apply.

This is the author’s version of a work that was accepted for publication. Changes introduced as a result of publishing processes such as copy-editing and formatting may not be reflected in this work. For a definitive version of this work please refer to the published source available online at: http://dx.doi.org/10.1038/ijo.2017.247

Received 18 June 2017; revised 17 September 2017; accepted 24 September 2017; Accepted article preview online 6 October 2017

© 2017 Macmillan Publishers Limited. All rights reserved.
Maternal obesity heritably perturbs offspring metabolism for three generations without serial programming

Sally A. Eaton¹,², Alastair J. Aiken¹, Paul E. Young¹,
Joshua W.K. Ho¹,², Jennifer E. Cropley¹,²
and Catherine M. Suter¹,²

¹ Victor Chang Cardiac Research Institute, Darlinghurst NSW 2010, Australia
² Faculty of Medicine, University of New South Wales, Kensington NSW 2052, Australia

Correspondence:
Jennifer E Cropley
Victor Chang Cardiac Research Institute
405 Liverpool Street,
Darlinghurst NSW 2010
Australia
t: +61 2 9295 8619
f: +61 2 9295 8601
e: j.cropley@victorchang.edu.au

Funding support: JEC is supported by an Australian Research Council DECRA (DE120100723). CMS was supported by an Australian Research Council Future Fellowship (FT120100097). This study was supported in part by a Diabetes Australia Research Trust grant-in-aid.

Running title: Inheritance of programming by maternal obesity

Word count: Abstract 200 words, Main text 1513 words
Abstract

Maternal obesity can program offspring metabolism across multiple generations. It is not known whether multigenerational effects reflect true inheritance of the induced phenotype, or are due to serial propagation of the phenotype through repeated exposure to a compromised gestational milieu. Here we sought to distinguish these possibilities, using the A\textsuperscript{vy} mouse model of maternal obesity. In this model, F1 sons of obese dams display a predisposition to hepatic insulin resistance which remains latent unless the offspring are challenged with a Western diet. We find that F2 grandsons and F3 great-grandsons of obese dams also carry the latent predisposition to metabolic dysfunction, but remain metabolically normal on a healthy diet. Given that the breeding animals giving rise to F2 and F3 were maintained on a healthy diet, the latency of the phenotype permits exclusion of serial programming; we also confirmed that F1 females remained metabolically healthy during pregnancy. Molecular analyses of male descendants identified upregulation of hepatic \textit{Apoa4} as a consistent signature of the latent phenotype across all generations. Our results exclude serial programming as a factor in transmission of the metabolic phenotype induced by ancestral maternal obesity, and indicate inheritance through the germline, probably via some form of epigenetic inheritance.

Keywords

Fetal programming, maternal obesity, developmental origins of health and disease, transgenerational epigenetic inheritance, ApoA4.
Introduction

Maternal metabolism can program the metabolism of offspring. In particular, maternal obesity and/or diabetes during pregnancy results in offspring with an increased risk of metabolic dysfunction in adult life (1). It is increasingly recognised that such effects may manifest not only in immediate offspring, but also in subsequent generations (2). Such multigenerational programming of metabolism may be one driver of the present obesity epidemic, and so understanding the underlying mechanisms at play is important.

One explanation for the multigenerational maternal programming is epigenetic inheritance: the germline transmission of some induced non-genetic factor from one generation to the next. This idea has become very popular in the field, even though the putative heritable factors remain obscure (3). However, intergenerational transmission can also result from serial programming, in which the phenotype induced in offspring itself programs defects in second generation offspring, which may then in turn program defects in the third generation, et cetera (2).

In human epidemiology, and almost all animal models of maternal programming, it is difficult – if not impossible – to distinguish epigenetic inheritance from serial programming. It has been proposed that most, if not all, examples of multigenerational maternal programming are due to serial propagation of the induced phenotype (2). We have previously described a mouse model of programming by maternal obesity in which the induced metabolic phenotype is latent. Offspring of obese mothers remain healthy unless they are challenged by exposure to a Western diet, upon which they rapidly develop hepatic insulin resistance; control offspring do not (4). Here we capitalise on the latency of the induced phenotype to investigate whether multigenerational inheritance of a maternally programmed phenotype can occur without serial programming.
Methods

Detailed methods including of statistical analyses can be found in Supplementary Material 1.

Mouse breeding and diets

Breeding mice were fed NIH-31 diet *ad libitum* (5% w/w fat, 13.5 MJ/kg) (see Figure 1a for breeding scheme). Experimental *ala* males were weaned at 21 days; littermates were randomly assigned to either high fat, high sugar Western style diet (WD; 22% w/w fat, 19.4 MJ/kg) or matched control diet (CD; 6% w/w fat, 16.1 MJ/kg) as previously described (5), and culled at 12 weeks of age.

Metabolic profiling

For glucose tolerance testing, mice were fasted for six hours then given an intraperitoneal injection of 1.5g glucose/kg; blood glucose was measured at intervals over 90 minutes using an Alpha Trak 2 Meter (Zoetis). Serum insulin and leptin were measured using the Mouse/Rat Insulin or Leptin ELISA kits (Millipore).

Gene expression analysis

Total RNA from the liver of 12 week old mice was subject to microarray in quadruplicate, on a GeneChip® Mouse Transcriptome Assay 1.0 array (Affymetrix); data are available at the Gene Expression Omnibus under accession GSE103786. Data were normalised by Robust Multi-Array Average and differentially expressed genes identified using limma. Small RNA-seq was performed as previously described (5). *ApoA4* qRT-PCR was performed in triplicate using 10 ng cDNA per reaction.
Results

Maternal obesity programs a latent metabolic phenotype in F2 grandsons and F3 great grandsons

A\textsuperscript{vy}/\textit{a} mice provide a model of maturity-onset obesity and prediabetes (6), in which metabolic programming of offspring can be assessed by segregating away the obesogenic A\textsuperscript{vy} allele after the founder generation. We asked whether the latent predisposition to hepatic insulin resistance displayed by \textit{a} F1 sons of obese A\textsuperscript{vy}/\textit{a} mothers (4) could be transmitted to subsequent generations. We mated the F1 daughters of obese dams (MatObF1) to control males to generate MatObF2, and interbred F2 mice to generate MatObF3 (Figure 1a).

We found that, like F1 sons, both MatObF2 grandsons and MatObF3 great-grandsons exhibit a latent defect in glucose handling (Supplementary Table 1, Figure 1b-d). When maintained on a low-fat control diet (CD), MatObF2/F3 mice were indistinguishable from controls in a glucose tolerance test (Figure 1b), nor did they exhibit elevated insulin. But when weaned onto Western diet (WD), after three weeks both MatObF2 and MatObF3 males exhibited an impaired response to glucose (Figure 1c), and developed hyperinsulinemia by 12 weeks of age (Figure 1d); controls remained normal. Thus the latent predisposition to hepatic insulin resistance programmed by maternal obesity is maintained for three successive generations.

Transmission of the latent metabolic defects is independent of serial programming

The latency of the phenotype displayed by the descendants of obese dams suggests germline inheritance of the induced phenotype and argues against serial programming. MatObF2 and MatObF3 were generated from MatObF1 females mated to control males and maintained on control diet. Like male MatObF1, female MatObF1 are not obese (Supplementary Table 2)
and appear metabolically normal on a control diet. Female MatObF1 are indistinguishable from controls in a GTT (Figure 1e), are normoinsulinemic (Figure 1f) and normoleptinemic (Figure 1g).

We considered the possibility that stress of pregnancy might unmask a metabolic phenotype in MatObF1. We assessed glucose tolerance in MatObF1 females during pregnancy (at 14.5dpc), and found no difference from pregnant controls (Figure 1h).

Taken together, these results militate against serial programming by a repeatedly compromised metabolic state, and against germ cell exposure as an explanation for the phenotype in F3. Thus the latent metabolic defects in F2 and F3 appear result from true inheritance of an induced phenotype. Because F3 were generated by interbreeding of F2, we cannot dissect the individual contributions of the male and female germlines here.

*ApoA4* expression is a molecular signature of the latent phenotype

We next assessed whether inheritance of the induced phenotype could be linked to any molecular changes, by comparing the hepatic transcriptomes of MatObF2 and MatObF3 males relative to control males. We reasoned that the transcriptomes of Western-fed animals would be affected by the disease itself, so assessed control-fed animals in order to distinguish changes that potentially underlie the phenotype. We observed a positive correlation between the expression differences in MatObF2 vs control and MatObF3 vs control (Pearson correlation = 0.44, \( p < 0.01 \)), suggesting a transcriptome-wide trend in gene expression change that is consistent from F2 to F3 (Figure 2a). However, these changes were subtle at the individual gene level. We used generous thresholds (nominal \( p < 0.05 \), log\(_2\) fold change > 0.5) to identify 20 genes with altered expression in either MatObF2 or MatObF3 (Supplementary Table 3). Of these changes, only two (*Apoa4* and a miRNA transcript, *Mirlet7c*), were altered concordantly, being upregulated in both MatOb generations.
Using qRT-PCR on an expanded cohort of mice, we confirmed that Apoa4 is significantly and concordantly upregulated in all generations programmed by maternal obesity (Figure 2b).

We also performed small RNA-seq on the same hepatic RNA to obtain miRNA expression profiles and verify Mirlet7c expression. We found no significant differences in the expression of any hepatic miRNA (Supplementary Table 4), including miR-let-7c, suggesting that the expression difference detected by the microarray may reflect levels of the Mirlet7c pre-miR transcript and not the mature miRNA.

Thus, ApoA4 upregulation was the only abnormality in gene expression that we found to be consistently associated with multigenerational programming by maternal obesity.

**Discussion**

In this study we have demonstrated the inheritance of the effects of maternal obesity for three generations. The unique properties of our model allowed us to exclude serial programming by maternal obesity as a contributing factor, and thus establish the existence of true inheritance of a maternally programmed phenotype. The manifestation of the induced phenotype in the F2 generation could reflect persistent effects of germ cell exposure during the gestation of F1, but its penetrance into F3 indicates true epigenetic inheritance. Dissection of this mode of inheritance from serial transmission provides material support to the idea of epigenetic inheritance in maternal programming, as it implicates the germline transmission of information that is not encoded in the DNA itself.

Epigenetic gene regulation is a system that controls gene expression, so we sought gene expression changes as a signature of the heritable phenotype. The singular molecular correlate we found was elevation of ApoA4 transcription. Apoa4 is a lipid binding protein that
is involved in regulation of plasma lipid and glucose homeostasis. It is upregulated in response to a high-fat diet (7, 8) and has previously been among genes reported as responsive to maternal metabolic programming (9), as well as paternal metabolic programming (10). The mice in our study were on a low fat diet, with no indication of metabolic disturbance, but still exhibited upregulated ApoA4. Together these findings identify ApoA4 as a candidate for further investigation into the molecular basis of maternal metabolic programming.

Distinction between germline inheritance and serial propagation is also important in understanding how programming effects might be mitigated. In the case of serial programming, successful intervention is likely to be lifestyle based. But our results imply that second and third generation descendants of obese mothers are inherently prone to metabolic dysfunction even if the intervening generations maintain a healthy diet and normal metabolism. With germline inheritance of the predisposing factor, mitigation may require further (and possibly unique) interventions. At least in our model, two subsequent generations of healthy eating did not mitigate the risk for third generation descendants of obese mothers. It would be interesting to observe the effects of interventions such as maternal exercise (11) in this system.
Supplementary Information

Supplementary information is available on the International Journal of Obesity’s website.

Acknowledgements

JEC is supported by an Australian Research Council DECRA (DE120100723). CMS was supported by an Australian Research Council Future Fellowship (FT120100097). This study was supported in part by a Diabetes Australia Research Trust grant-in-aid.

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

SAE designed and performed experiments, analysed data and drafted the manuscript. AJA performed experiments. PEY performed experiments and analysed data. JWKH analysed data. JEC conceived and designed experiments, analysed and interpreted data and drafted the manuscript. CMS conceived and designed experiments, interpreted data and drafted the manuscript. All authors critically revised the paper and approved the final version.
References


Figure legends

**Figure 1. Metabolic defects programmed by maternal obesity are inherited into F2 and F3 in the absence of serial programming.** (a) Experimental schema. Obese yellow A^vy/a dams were mated to congenic lean a/a sires. For simplicity, only a/a offspring are shown; A^vy/a offspring were discarded. Female offspring were mated to control males to generate F2; F2 offspring were interbred to generate F3. A subset of F2 and F3 offspring were weaned onto Control diet (CD) or Western diet (WD) for metabolic testing. (b) GTT on six-week old CD-fed male mice. Ctrl n = 35, MatObF2 n = 18, MatObF3 n = 15. (c) GTT on six-week old WD-fed male mice. Ctrl n = 30, MatObF2 n = 14, MatObF3 n = 12. (d) Serum insulin in 12-week old male mice; CD: Ctrl n = 7, MatObF2 n = 7, MatObF3 n = 7; WD: Ctrl n = 9, MatObF2 n = 10, MatObF3 n = 7; * p < 0.05, ** p < 0.01. (e) GTT on six-week-old CD-fed F1 female mice. Ctrl n = 13, MatObF1 n = 8. (f) Serum insulin in 12-week old CD-fed female mice. Ctrl n = 7, MatObF1 n = 8. (g) Serum leptin in 12-week old CD-fed female mice. Ctrl n = 8, MatObF1 n = 8. (h) GTT on 14.5 dpc pregnant F1 females. Ctrl n = 10, MatObF1 n = 8.

GTT Error bars represent SEM. Box and whisker plots: box, median ± IQR; whisker, min/max data points.

**Figure 2. Hepatic Apoa4 is upregulated in three generations in response to maternal obesity.** (a) Correlation of the log$_2$ fold-change in hepatic gene expression between MatObF2 vs control (x-axis), and MatObF3 vs control (y-axis), in CD-fed 12-week male mice. (b) Hepatic Apoa4 expression by qRT-PCR, relative to Actb and normalised to control, in 12-week male mice on CD: Ctrl: n = 15, MatObF1: n = 12, MatObF2: n = 12, MatObF3 n = 12. Error bars represent SEM; * p < 0.05, ** p < 0.01.
Figure 1

(a) Congenic lean parents: male $a/a$ and female $a/a$; congenic sire: male $a/a$ and female $A^{vy}a$; obese, diabetic dam: female $A^{vy}a$. Male $a/a$ littermates randomly assigned to either a high fat Western diet (WD) or matched control diet (CD) at weaning.

(b, c) Blood glucose (mM) for Control Diet and Western Diet.

(b) Control Diet: Control vs. MatObF2, AUC ns; Control vs. MatObF3, AUC ns.

(c) Western Diet: Control vs. MatObF2, AUC $p < 0.05$; Control vs. MatObF3, AUC $p < 0.05$.

(d) Serum insulin (ng/ml) for Control and Western Diet.

(e) Control Diet: Control vs. MatObF1, AUC ns.

(f) Serum insulin (ng/ml) for Control vs. MatObF1.

(g) Serum leptin (ng/ml) for Control vs. MatObF1.

(h) Blood glucose (mM) for Pregnant: Control vs. MatObF1, AUC ns.
Figure 2

a

\[ \log_2(F3\text{-control}) \]

\[ \log_2(F2\text{-control}) \]

\[ \rho = 0.44, p < 0.01 \]

b

Relative Apoa4 expression

<table>
<thead>
<tr>
<th>MatOb</th>
<th>Ctrl</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
<td>2.5</td>
<td>2.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

© 2017 Macmillan Publishers Limited. All rights reserved.