THE UNIVERSITY of EDINBURGH

This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

- This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.
- A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.
- This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.
- The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.
- When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.

Intergenerational Effects of Early Life Programming:

The Role of Glucocorticoids and Maternal Obesity

Lincoln Liu

Doctor of Philosophy

The University of Edinburgh

2011

TABLE OF CONTENTS

LIST OF FIGURES VII

LIST OF TABLES IX

DECLARATION X

ACKNOWLEDGEMENTS XI

ABSTRACT XIII

LIST OF ABBREVIATIONS XV

PUBLICATIONS FROM THIS THESIS XVIII

CHAPTER 1 1

1.1 Introduction 2
1.2 Programming 5
1.2.1 Evidence of programming in humans 5
1.2.1.1 Programming of cardiovascular diseases 5
1.2.1.2 Programming of metabolic disorders 8
1.2.1.3 Programming of the brain and behaviour 10
1.2.2 Obesity in pregnancy 13
1.2.3 The role of post-natal growth 14
1.2.4 Animal models of programming 16
1.2.4.1 Prenatal glucocorticoid overexposure 16
1.2.4.1.1 Glucocorticoid actions 16
1.2.4.1.2 11β-hydroxysteroid dehydrogenase 19
1.2.4.2 The role of maternal nutrition 22
1.2.4.2.1 Maternal undernutrition 22
1.2.4.2.2 Maternal overnutrition 22
1.2.5 Importance of timing of insult exposure 26
1.2.6 The role of epigenetics in programming 29
1.2.7 Glucocorticoids and the epigenome 31
1.2.8 Transmission of programming effects to subsequent generations 32

1.3 Mechanisms to explain the transmission of programming effects 34
1.3.1 Maternal physiology 34
1.3.1.1 Maternal size 34
1.3.1.2 Maternal health 34
1.3.1.3 Maternal behaviour 35
1.3.1.4 Maternal exposure to drugs 36
1.3.2 Epigenetics and the transmission of programming effects 36
1.3.3 Genomic imprinting 38
1.3.4 Imprinted genes important in growth: 40
1.3.4.1 Insulin-like growth factor 2 (Igf2) 40
1.3.4.2 Grb10 41
1.3.4.3 Cyclin dependent kinase inhibitor (CDKn) 41
1.3.4.4 Pleckstrin homology-like domain family A (Phlda) 41
1.3.4.5 Mesoderm specific transcript homolog (Mest/PEG1) 42
1.4 The placenta 43
1.4.1 The role of the placenta in early life programming 43
1.4.2 Nutrient transporters 46
1.4.2.1 Glucose transporters (Glut) 46
1.4.2.2 System A amino acid transporters (Slc38) 48
1.5 The Insulin-like Growth Factor pathway 50
1.5.1 Insulin receptor substrate – 1 (IRS1) 53
1.5.2 Phosphoinositide 3-Kinase (Pi3k) 53
1.5.3 Akt 54
1.5.4 Mammalian target of rapamycin (mTOR) 54
1.5.5 Glycogen Synthase Kinase-3β (GSK3β) 55
1.6 Hypothesis and aims: 56
CHAPTER 2 57
2.1 Materials 58
2.1.1 General chemicals/solutions/kits 58
2.1.2 Molecular biology 59
2.1.2.1 Ribonucleic Acid 59
2.1.2.2 Western blotting 60
2.1.3 Equipment 61
2.1.4 Statistical analysis 62
2.2 Buffers and solutions 63
2.3 Animal maintenance 65

2.3.1 Rats 65
2.3.2 Mice 65
2.4 Rat 66
2.4.1 F1 offspring production 66
2.4.2 F2 offspring production 67
2.5 Mice 69
2.5.1 Production of F1 offspring 69
2.5.2 Production of F2 offspring 69
2.6 Biochemical assays 71
2.6.1 Measurement of plasma insulin concentrations 71
2.6.2 Measurement of plasma glucose 72
2.6.3 Measurement of plasma triglyceride 73
2.6.4 Measurement of plasma cholesterol 74
2.6.5 Measurement of glycogen in tissue 75
2.7 Molecular procedures 76
2.7.1 Extraction of total RNA from tissues 76
2.7.2 Extraction of total RNA from adipose tissue 76
2.7.3 RNA quantification and agarose gel electrophoresis 76
2.7.4 Reverse transcription of RNA 77
2.7.5 Real-time polymerase chain reaction 78
2.7.5.1 Rat self designed primers (UPL) 80
2.7.5.2 Rat Taqman® primer-probe set 81
2.7.5.3 Mouse self designed primers (UPL) 82
2.7.5.4 Mouse Taqman® primer-probe set 83
2.7.6 Detection and quantification of Igf2 promoter transcripts 84
2.7.6.1 SYBR® Green quantification of Igf2 promoter transcripts 84
2.7.6.2 Standard polymerase chain reaction for rat Igf2 promoters 86
2.7.7 Western blotting 87
2.7.7.1 Protein extraction and quantification 87
2.7.7.2 Protein gel electrophoresis 87
2.7.7.3 Gel transfer 88
2.7.7.4 Protein immunoblotting 89
2.7.7.5 Blot reading 89
2.8 Histological procedures: 91
2.8.1 Tissue collection: 91
2.8.2 Tissue sectioning 91
2.8.3 Histological staining: 91

2.8.4 Slides analysis: 94
2.9 Experimental diet constituents: 95

CHAPTER 3 96

3.1 Introduction 97
3.1.1 Introduction 97
3.1.2 Hypothesis 98
3.1.3 Aims 99
3.2 Methods 100
3.2.1 Animal maintenance, treatment and tissue collection 100
3.2.2 Metabolic experiments 101
3.2.3 Molecular experiments 101
3.2.4 Placental stereology procedures 102
3.2.5 Glycogen measurement in the placenta 102
3.2.6 Statistical analysis 102
3.3 Results 103
3.3.1 Mother (F0) and F1 offspring 103
3.3.2 F1 fetal liver gene expression 107
3.3.3 F1 placental labyrinth gene expression 110
3.3.4 F1-E20 placental stereology 114
3.3.5 F1 placental glycogen 115
3.3.6 Western blot analysis of proteins involved in the Igf2 signalling pathway in placental labyrinth 116
3.3.7 Western blot analysis of proteins involved in the Igf2 signalling pathway in fetal liver 118
3.4 Discussion 120

CHAPTER 4 127

4.1 Introduction 128
4.1.1 Introduction 128
4.1.2 Hypothesis 129
4.1.3 Aims 129
4.2 Methods 130
4.2.1 Animal maintenance, treatment, tissue collection, and offspring production 130
4.2.2 Metabolic experiments 131
4.2.3 Molecular experiments 131
4.2.4 Placental stereology procedures 132

4.2.5 Statistical analysis 132
4.3 Results 133
4.3.1 F1 cohort 133
4.3.2 F2 cohort 133
4.3.3 F2-E20 fetal liver gene expression 138
4.3.4 F2-E20 placental labyrinth gene expression 138
4.3.5 F2-E20 placental stereology 144
4.4 Discussion 146
CHAPTER 5 155
5.1 Introduction 156
5.1.1 Introduction 156
5.1.2 Hypothesis 157
5.1.3 Aims 157
5.2 Methods 158
5.2.1 Animal maintenance and diet 158
5.2.2 Metabolic experiments 159
5.2.3 Molecular experiments 159
5.2.4 Statistical analysis 160
5.3 Results 161
5.3.1 F0 maternal physiology and F1 offspring 161
5.3.2 F1 offspring plasma analysis and hepatic triglyceride 165
5.3.3 F1 offspring organ weight 171
5.3.4 F1 female offspring hepatic gene expression 174
5.3.5 F1 female offspring adipose tissue gene expression 174
5.4 Discussion 178
CHAPTER 6 183
6.1 Introduction 184
6.1.1 Introduction 184
6.1.2 Hypothesis 185
6.1.3 Aims 185
6.2 Methods 186
6.2.1 Animal maintenance, diet induction, and offspring production 186
6.2.2 Metabolic experiments 187
6.2.3 Statistical analysis 187
6.3 Results 188
6.3.1 F2 weights 188

6.3.2 F2 offspring plasma analysis ... 195
6.4 Discussion ... 198

CHAPTER 7 ... 205

7.1 Introduction ... 206
7.2 Multigenerational programming in the glucocorticoid programmed rat ... 206
7.3 Multigenerational programming as a consequence of maternal obesity ... 210
7.4 Public health implications ... 213
7.5 Future work ... 215

REFERENCES ... 216

List of Figures

Figure 1.1 Hypothalamic pituitary adrenal (HPA) axis schematic 18
Figure 1.2 Glucocorticoid activation and reduction in human and rodents 21
Figure 1.3 Proposed mechanisms of programming effects 25
Figure 1.4 Model showing mechanisms of transmission of programming effects across generations 33
Figure 1.5 Schematic of rodent and human placenta 45
Figure 1.6 Diagrammatic representation of the Igf signalling pathway 52
Figure 2.1 Schematic diagram of the glucocorticoid programmed rat experimental protocol... 68
Figure 2.2 Schematic diagram showing the duration of exposure to experimental diets in F0 mice and time points of experimental procedures in F1 and F2 offspring 70
Figure 2.3 Gel sandwich configuration for one or two gel transfer 88
Figure 3.1 Maternal weight gain during pregnancy and maternal weights at E20.. 104
Figure 3.2 F1 offspring fetal and placental weights and litter size 105
Figure 3.3 Maternal and fetal glucose and insulin levels at E20............................ 106
Figure 3.4 Gene expression in F1 fetal liver at E20 108
Figure 3.5 Expression of transcripts from Igf2 promoters in fetal liver 109
Figure 3.6 Expression of genes in E20 placental labyrinth 111
Figure 3.7 Expression of nutrient transporters in E20 placental labyrinth............. 112
Figure 3.8 Expression of transcripts from Igf2 promoters in placental labyrinth ... 113
Figure 3.9 Gel electrophoresis of Igf2 transcripts from P1, P2, P3, and P0 promoters in E20 fetal liver and placental labyrinth 113
Figure 3.10 Ratio of the volume of labyrinth and junctional zones in placenta at E20 114
Figure 3.11 Glycogen levels in placental labyrinth and junctional zones at E20.... 115
Figure 3.12 Placental labyrinth Igf2 pathway proteins 116
Figure 3.13 Representative blots of placental labyrinth Igf2 pathway proteins...... 117
Figure 3.14 Fetal liver Igf2 pathway proteins 118
Figure 3.15 Representative blots of fetal liver Igf2 pathway proteins 119
Figure 4.1 F2 weights of A) fetus, B) placenta, and C) at birth 136
Figure 4.2 Maternal and fetal glucose and insulin at E20 137
Figure 4.3 F2-E20 fetal liver gene expression 139
Figure 4.4 F2 fetal liver Igf2 promoter transcript levels and correlation 140

Figure 4.5 F2 placental labyrinth Igf pathway and imprinted gene expression 141
Figure 4.6 F2-E20 placental labyrinth nutrient transporter gene expression 142
Figure 4.7 F2 placental labyrinth Igf2 promoters' transcription level and correlation 143
Figure 4.8 Ratio of the volume of F2 placental labyrinth and junctional zones 144
Figure 4.9 Representative pictures of H&E stained placentas from each group..... 145
Figure 4.10 DNA methylation in germ cells of females and males 152
Figure 5.1 Weight of F0 pregnant females before and during pregnancy 162
Figure 5.2 Maternal weight change following introduction of male to cage 163
Figure 5.3 Plasma insulin and glucose levels at 3 months of age in F1 females 166
Figure 5.4 Plasma insulin and glucose levels at 6 months of age in F1 females 167
Figure 5.5 Plasma insulin and glucose levels at one year of age in F1 females 168
Figure 5.6 Plasma insulin and glucose levels at one year of age in F1 males......... 169
Figure 5.7 Hepatic gene expression in three months old F1 females...................... 175
Figure 5.8 Hepatic gene expression in six months old F1 females 176
Figure 5.9 Gene expression in adipose depots from six month old F1 females...... 177
Figure 6.1 F2 birth weights 189
Figure 6.2 F2 litter size 190
Figure 6.3 F2 weaning weights 191
Figure 6.4 F2 male and female offspring growth trajectory.................................... 192
Figure 6.5 Organ weights of F2 male offspring at six months of age 193
Figure 6.6 Organ weights of F2 female offspring at six months of age 194
Figure 6.7 Plasma insulin and glucose levels at six months of age in F2 males..... 196
Figure 6.8 Plasma insulin and glucose levels at six months of age in F2 females.. 197
Figure 6.9 Summary of findings in F2 mice 203
Figure 6.10 Schematic of F1 and F2 exposure to experimental diets 204
Figure 7.1 Summary of main findings in the glucocorticoid programmed rat model 209
Figure 7.2 Summary of findings in the mouse model of maternal obesity 212

List of tables

Table 1.1 Studies showing programming effects in humans 12
Table 1.2 Animal models of excess glucocorticoid 28
Table 2.1 Recipe for real time PCR using either Taqman® primer-probe mix or self designed UPL primers with corresponding UPL probe.............................. 79
Table 2.2 Recipe for SYBR® Green quantification of Igf2 promoter transcripts..... 85
Table 2.3 List of different rat Igf2 transcript primers 85
Table 2.4 Recipe for standard PCR reaction... 86
Table 2.5 Primary antibodies for immunoblotting of protein................................... 90
Table 2.6 Haematoxylin and Eosin staining protocol 93
Table 4.1 F0 maternal cohort 134
Table 4.2 F1 cohort for 8 litters per group... 134
Table 4.3 F2 cohort – pregnancy setup 135
Table 5.1 F0 maternal diet consumption during pregnancy from E1 – E18........... 163
Table 5.2 F1 offspring weights at birth, weaning, 2, 3, 6 and 12 months 164
Table 5.3 F1 plasma triglyceride, plasma cholesterol, and hepatic triglyceride levels 170
Table 5.4 F1 female offspring organ weights 172
Table 5.5 F1 male offspring organ weights at one year of age............................... 173

Declaration

I declare that the work and data presented in this thesis is a result of my original work. Where contributions from others are involved, every effort is made to indicate this clearly. The data presented in this thesis has not been submitted for any other degree.

____

Dr. Lincoln Liu (MBChB)

Edinburgh, Scotland, UK

Acknowledgements

It is my greatest honour to thank the many people who made this thesis possible.

It is impossible to overstate my deepest gratitude to my PhD supervisor, Dr. Amanda Drake. Her enthusiasm, her guidance, her motivation, her true inspiration, her immerse knowledge, and her forever patience has helped me through the past three years of my studies and throughout the thesis-writing. Her support, reassurance, and kind words have also helped me through the pre-viva period. This work would not have been made possible and I would have been lost without her. I am, forever, in debt to her.

I would also like to thank Prof. Jonathan R. Seckl for his insightful comments and guidance that has made this work possible. I would like to thank Dr. Rebecca M. Reynolds and Prof. Jane E. Norman for their advice and suggestions. I would like to thank Prof. Rudolf A. Riemersma and Dr. Richard B. Weller, without them, I would not have been able to be here in Edinburgh.

I would like to thank Mr. David Kerrigan for his technical assistance and guidance in the laboratory. His expertise in laboratory experiments has provided a useful source of troubleshooting when I am lost. I would also like to thank Mr. William Mungall, Mr. Jon Hendersson, Mr. Ian McCall, Mr. David Read, and Mr. Bill Smith at the Biomedical Research Facility for their expertise and help with animal works. I would like to thank Dr. Vicky King for breeding the mice for experiments; Ms. Rachel S. Dakin for advice, support and organising events full of unforgettable memories; and

Mr. James R. O'Reilly for advice and support. I would like to thank Dr. Kerry J. McInnes for the antibodies for western blot. I would like to thank Mr. Zhenguang Zhang for his technical help and cheerful talks throughout the years. I would like to thank my students, Ms. Mercy Danga and Ms. Fiona E. Graham for their work. I would also like to thank all the colleagues in the Endocrinology lab, Tommy's lab, and the other members of Team Drake that has made my past three years fun, exciting, and unforgettable.

I would also like to express my sincere gratitude to both my internal examiner, Prof. Richard M. Sharpe and external examiner, Dr. Susan E. Ozanne. They have provided in-depth and immerse knowledge and a fruitful discussion during my viva. I truly enjoyed the nice discussion and I thank both of them for their insightful suggestions.

I owe my gratitude to Tommy′s: the baby charity and the University of Edinburgh′s Scottish Overseas Research Students Awards Scheme. This work would not have been possible without their support.

Last but not least, I would like to thank my grandmother, Mrs. Shiang-Ge Yue for her full support throughout my PhD. I am truly grateful to my mother Mrs. Miao Chen for her utmost support and trust. I do not have suitable words to describe my sincere gratitude to her! Thank you mom! Most importantly, I would like to thank my brother, Dr. Donald Liu for always being by my side, supporting me, helping me and cheering me up when I encounter difficulties. Without him, I wouldn′t have enjoyed the past three years as much as I do. I am, forever, in debt to them! To them, I dedicate this thesis.

Abstract

Hypertension and type two diabetes mellitus (Type 2 DM) are serious chronic illnesses that impact on the lives of millions of people around the world. Various epidemiological studies have shown a relationship between early life events such as intrauterine growth retardation (IUGR) resulting in low birth weight and the development of these chronic illnesses in adult life. To explain the link between these two events, it has been suggested that an 'insult' at a critical time point of development can 'program' alterations in gene expression, organ size, and cell number. This has been termed "the early life origins of disease'. There is also evidence that these programmed effects are not limited to the first generation but can also be passed to subsequent generations.

With changes in lifestyle in modern society, the prevalence of obesity is increasing, in association with problems such as type 2 DM, hypertension, fatty liver, atherosclerosis and the metabolic syndrome. Obesity during pregnancy is linked to problems such as gestational diabetes, hypertension and early miscarriage as well as a higher risk of congenital malformations. Maternal obesity has also been recognised as one of the factors capable of 'programming' the offspring, increasing the risk of childhood and adult disorders such as obesity and hypertension.

In this thesis I have used two animal models to explore the underlying mechanisms of programming and its intergenerational effects: i) a rat model of prenatal glucocorticoid over-exposure (the dexamethasone-programmed rat) and ii) a mouse model of obesity during pregnancy.

Using the dexamethasone-programmed rat, I have shown that prenatal glucocorticoid overexposure reduces fetal and placental weight in the first generation (F1) offspring, in association with alterations in gene expression in placenta and liver. In addition, I have shown effects on fetal and placental weights and gene expression in the second generation (F2) offspring. The observed changes in gene expression in the F2 offspring differ from those in the first generation. Thus, although effects on fetal growth are seen in both generations, the underlying mechanisms appear to be different. We also observed marked parent of origin effects on fetal and placental growth and gene expression in the second generation.

In the mouse model of maternal obesity, birth weight was decreased in the F1 offspring. At weaning, the offspring of obese mothers were heavier than controls, however this difference in weight was not persistent. At three months of age, F1 female offspring of obese mothers showed altered expression of hepatic genes important in lipid regulation and metabolism. More striking changes were seen in the F2 generation in which there was an effect of paternal exposure to maternal obesity to decrease birth weight. There were also parent of origin effects on organ weights and insulin levels at six months of age.

These results provide evidence for the transmission of programming effects to a second generation in two different programming models and suggest that the mechanisms leading to these effects differ between generations.

List of abbreviations

11β-HSD1	11β-hydroxysteroid dehydrogenase type I
11β-HSD2	11β-hydroxysteroid dehydrogenase type II
5-hmc	5-hydroxymethylcytosine
5-mC	5-methylcytosine
9/11	September 11th
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AUC	Area under curve
Bet	Betamethasone
BMI	Body mass index
BRF	Biomedical Research Facility
BSA	Bovine serum albumin
CCD	Charge-coupled device
CDKn1c	Cyclin dependent kinase inhibitor 1c
cDNA	Complimentary DNA
CMACE	Centre for Maternal and Child Enquiries
CON	Control diet
Cort	Corticosterone
CpG	Cytosine-phosphate-Guanine
CV	Coefficient of variance
Dex	Dexamethasone
DIO	Dietary induced obesity
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferases
DW	Drinking water
E	Embryonic day
ELISA	Enzyme-linked immunosorbent assay

F1	Filial 1 / First generation
F2	Filial 2 / Second generation
GC	Glucocorticoid
GH	Growth hormone
Glut	Glucose transporter
GR	Glucocorticoid receptor
Grb10	Growth receptor bound protein 10
Grb2	Growth receptor bound protein 2
GRE	Glucocorticoid response element
GSK3	Glycogen synthase kinase 3
HAPO	Hyperglycaemic and Adverse Pregnancy Outcome
HC	Hydrocortisone
Hnf4α	Hepatic nuclear factor 4α
HPA	Hypothalamic-pituitary-adrenal
ICR	Imprinting control region
Igf	Insulin-like growth factor
Igf1	Igf type 1
Igf1r	Igf1 receptor
Igf2	Igf type 2
Igf2r	Igf2 receptor
IgfBP	Igf binding protein
IM	Intramuscular
IP	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
IQ	Intelligent quotient
IR	Insulin receptor
IRS1	Insulin receptor substrate 1
IUGR	Intrauterine growth restriction
IV	Intravenous
LG-ABN	Licking / grooming and arched back nursing
Mest	Mesoderm specific transcript homolog
MR	Mineralocorticoid receptor

mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
Na^+	Sodium
NAD^+	Nicotinamide adenine dinucleotide
ncRNA	Non-coding RNA
OP	Osmotic pump
p	Phosphorylated
PEPCK	Phosphoenolpyruvate carboxykinase
Phlda2	Pleckstrin homology-like domain family A member 2
Pi3k	Phosphoinositide 3-kinase
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PPARα	Proliferator-activated receptor α
PPARγ	Proliferator-activated receptor γ
PTSD	Post-traumatic stress disorder
RNA	Ribonucleic acid
rpm	Revolutions per minute
-RT	No reverse transcriptase sample
RT°	Room temperature
SC	Subcutaneous
SEM	Standard error of mean
Ser	Serine
siRNA	Small interfering RNA
Slc38	System A amino acid transporters
SNAT	Sodium-dependent neutral amino acid transporters
Thr	Threonine
Tyr	Tyrosine
UPL	Universal ProbeLibrary™
Veh	Vehicle
WHO	World Health Organization

Publications from this thesis

Full papers:

1) Drake, A J & **Liu, L** 2010. Intergenerational transmission of programmed effects: public health consequences. *Trends in Endocrinology & Metabolism,* **21,** 206-13

2) Drake, A J, **Liu, L**, Kerrigan, D, Meehan, R R & Seckl, J R 2011. Multigenerational programming in the glucocorticoid programmed rat is associated with generation-specific and parent of origin effects. *Epigenetics : official journal of the DNA Methylation Society,* **6,** 1334-43

Abstracts:

1) **Liu L**, Seckl JR, Drake AJ
Early life programming by maternal obesity in mice
Oral presentation at the Tommy's three centre meeting, Manchester, Sept. 2010

2) **Liu L**, Seckl JR, Drake AJ
Transgenerational programming
Oral presentation at the Edinburgh Taiwanese Students Society Symposia, Edinburgh, Dec. 2010

3) **Liu L**, Seckl JR, Drake AJ
Transgenerational programming: Generation-specific and parent-of-origin specific effects
Presented at the Scottish Society for Experimental Medicine meeting, Edinburgh, May 2011

4) **Liu L**, Seckl JR, Drake AJ
Early life programming
Oral presentation at the Paediatric Research in Progress meeting, Edinburgh, May 2011

5) **Liu L**, Seckl JR, Drake AJ
Multigenerational programming: Generation-specific and parent-of-origin specific effects
Presented at the American Endocrine Society Annual meeting, Boston, June 2011

CHAPTER 1

General introduction

1.1 Introduction

Since initial studies which were published in the late 20th century, a considerable amount of evidence, both from human and animal studies, has demonstrated that an ‘insult’ occurring at a critical time point in development can have lifelong consequences for health, a phenomenon which has been termed ‘the early life origins of disease’ (Barker 1990). Although the theory of ‘programming’ effects was advanced and popularized by Barker et al. in 1989 in their studies showing a link between low birth weight and an increased risk of ischaemic heart disease (Barker et al. 1989), studies showing evidence for ‘programming’ date back much further (Weismann 1875, Gilbert 2005). In 1875, August Weismann, a German biologist discovered that the wing colour of the butterfly *Araschnia levana* changed depending on the season of hatching. He performed an experiment in which he incubated caterpillars at different temperatures and successfully bred a spring coloured butterfly in summer (Weismann 1875, Gilbert 2005); this was one of the first clues that an alteration to the environment during a specific time point of development is able to ‘programme’ the offspring. Another example of ‘programming’ that occurs in nature involves the honey bee. It has been demonstrated that during the first three days of larval development, the larvae may either develop into a queen or a worker (Weaver 1955). Although it is known that the underlying genome is exactly the same between a queen and a worker bee; they differ in body size and organ development. The queen bee has functional reproductive organs and a larger body size whereas the worker bees are smaller and do not have functional reproductive organs (Weaver 1955). The main determinant of developing in to either a queen or worker is the nutritional status of the larvae during their first three days of life (Weaver 1955,

Haydak 1970). The queen larvae are fed with more nutritious royal jelly, whereas worker larvae are fed with less nutritious food (Weaver 1955, Haydak 1970). With this in mind, Weaver et al. conducted an experiment in the mid 1950s in which they transferred worker larvae into fresh queen larvae cells and allowed the worker larvae to grow in the nutritious royal jelly. They found that worker bee larvae raised in this environment grew into adults with queen-like features such as a larger body and ovaries (Weaver 1955). These observations give an indication that alterations in the environment, here either a change in temperature or nutrition, during a specific time point of development can permanently alter future growth and development.

Importantly, such programming effects are not exclusive to any single system or organ and studies in animal models and in humans have shown effects on a variety of organs, in association with chronic illnesses such as type two diabetes mellitus (Hales et al. 1991, Valdez et al. 1994), renal disease (Vikse et al. 2008), hypertension (Benediktsson et al. 1993), depression (Meaney 2001), schizophrenia (St Clair et al. 2005) and muscular hypotrophy (Bayol et al. 2005). Interestingly, the occurrence of low birth weight and adult onset chronic illnesses appears to be unrelated to age, gender, ethnicity and lifestyle such as smoking and alcohol consumption (Barker et al. 1989, Barker et al. 1993, Valdez et al. 1994, Rich-Edwards et al. 1997). Mechanisms which may account for these seemingly unrelated events of low birth weight and adulthood chronic illnesses include effects on gene expression (Nyirenda et al. 1998, Meaney 2001, Drake et al. 2005, Burdge et al. 2007), organ size (Ibanez et al. 2000), and cell number (Pladys et al. 2005).

With improvements in the lifestyle of modern society, obesity has become a

pandemic issue (WHO 2011). Obesity has been ascribed to an over-sufficient food supply and lack of exercise (WHO 2011) and has been linked to health related problems such as type two diabetes, hypertension, fatty liver, atherosclerosis, and the metabolic syndrome (WHO 2011). Obesity in pregnancy is known to be linked to problems such as gestational diabetes, hypertension, early miscarriage, as well as a higher risk of congenital malformations (Carmichael and Shaw 2000, Galtier-Dereure et al. 2000, Watkins et al. 2003, Samuelsson et al. 2008). Maternal obesity has also been proposed to be a factor capable of 'programming' the offspring, leading to an increased risk of childhood and adult obesity, hyperphagia, and hypertension (Bayol et al. 2005, Bayol et al. 2007, Samuelsson et al. 2008).

1.2 Programming

1.2.1 Evidence of programming in humans

Several retrospective epidemiological studies around the world have provided evidence that links early life events with subsequent adulthood diseases. These impacts are not restricted to physiological changes in the next generation but also to psychological and behavioural changes (Archer and Blackman 1971, Hernández-Martínez et al. 2008, Mueller and Bale 2008a). In this section I will discuss some of the large scale epidemiological studies providing evidence of programming effects in humans from different parts of the world and various ethnicities.

1.2.1.1 Programming of cardiovascular diseases

In 1944, during the World War II, following the landing of the allied forces on Omaha beach in northern France, the war was expected to end rapidly. However, the progress of the allied forces soon came to a halt after the failed mission of the operation Market Garden to take control over the river Rhine at Arnhem in the Netherlands. In order to help the allied forces, the Dutch government stopped all railways from functioning to prevent German forces from advancing. The Germans responded by discontinuing food supplies to the Dutch. The Netherlands then experienced a harsh winter and there was a major food shortage, known as the Dutch famine (Roseboom et al. 2001). Retrospective studies on the Dutch famine have shown that women who experienced famine during early gestation gave birth to offspring who were more prone to develop cardiovascular diseases in adulthood (Lumey 1992, Roseboom et al. 2000, Roseboom et al. 2001, Painter et al. 2005).

In contrast, retrospective studies which followed individuals who experienced a similar period during World War II, the siege of Leningrad, which occurred between 1941 and 1944, did not show a link between maternal malnutrition and offspring adulthood coronary heart disease and hypertension (Stanner et al. 1997). Suggestions made to explain the differences between the Leningrad and the Dutch famine studies include that the duration of famine experienced during the Leningrad siege was about four times longer than the Dutch famine (>2 years compared to 6 months); also, the time that it took for the resumption of a normal state of food supply was very different in that the Dutch food supply quickly returned to normal after the famine and it took much longer for Leningrad to resume a normal food supply (Roseboom et al. 2000). Thus, the offspring in the Leningrad study were probably exposed to famine after birth and with decreased nutrition in- and ex-utero, had 'adapted' to the environment of under-nutrition.

In another large scale retrospective study which was conducted on a non-war period of crop failure in Finland in 1866 – 1868, Kannisto et al. used groups of control individuals to compare with a group who were directly exposed to famine, a group which was born within five years before the famine started and another group which were born within five years after the famine. This study showed that there were no differences between survival rates in offspring from age 17 until 80 years in both males and females (Kannisto et al. 1997). This study suggests some 'adaptation' as in the Leningrad study, since both populations suffered from a scarce food supply even after the famine period, therefore being exposed to famine in- and ex-utero allowed these offspring to adapt to the environment and not show significant signs of programming.

Within the United Kingdom, Barker et al. demonstrated the link between low birth weight and an increased risk of developing ischaemic heart disease (Barker et al. 1989, Barker et al. 1993). Amongst more than 5600 adult males who were born in Hertfordshire, England between 1911 – 1930, those with a birth weight below 5.5lb were at a three-fold greater risk of developing coronary artery disease in later life (Barker et al. 1989). Additionally, a lighter weight at one year of age was also strongly linked to adult ischaemic heart disease (Barker et al. 1989), with the highest risk in men that had low birth weight and below average weight at one year of age (Barker et al. 1989, Osmond et al. 1993). Although this is not in line with other studies showing catch up growth (discussed in section 1.2.3) being problematic (Eriksson et al. 1999, Ozanne and Hales 2004), it supports the importance of the postnatal environment in further moderating programming effects. In another study on the Hertfordshire population, Osmond et al. showed that women born with lighter birth weight were also at increased risk of cardiovascular diseases; with the highest cardiovascular death rate in women who were born lighter but were above average weight at one year of age (Osmond et al. 1993).

Other studies from outside of Europe have also provided evidence of a link between low birth weight and cardiovascular disease risk in later adulthood. A retrospective study involving over 121,000 female nurses in the USA provided further evidence for a link between low birth weight and adult coronary artery disease and stroke; a link which appeared to be independent of current lifestyle factors such as smoking (Rich-Edwards et al. 1997). Another retrospective study also linked low birth weight with the development of adulthood hypertension in men (Curhan et al. 1996).

1.2.1.2 Programming of metabolic disorders

Other than programming of cardiovascular risk, studies have also suggested programming effects in other organs leading to an increased risk of obesity and type two diabetes. From the same population of the Dutch famine study described in section 1.2.1.1, the offspring, particularly young men, were found to be prone to obesity (Ravelli et al. 1976) and those experiencing famine in late gestation were prone to develop decreased glucose tolerance at age of 50 (Ravelli et al. 1998). In contrast again, the offspring exposed to famine during the siege of Leningrad did not display glucose intolerance or dyslipidaemia (Stanner et al. 1997). Possible explanations for these differences between the two studies have been discussed above in section 1.2.1.1.

In the United Kingdom, a large retrospective study conducted on over 600 individuals from Motherwell, Scotland has suggested that exposure to a high protein, low carbohydrate diet during pregnancy leads to programming effects in the offspring (Shiell et al. 2001). Between 1952 – 1976, pregnant women attending the maternity hospital in Motherwell were advised to consume a diet which was low in carbohydrate but extremely high in protein; containing at least one pound (~450 grams) of red meat and a moderate amount of fish, eggs and cheese per day (Reynolds et al. 2007). The idea behind this was to decrease the risk of developing preeclampsia. Studies have linked this dietary alteration during pregnancy to increased fetal adiposity, increased childhood obesity and increased blood pressure as well as other metabolic phenotypes (Shiell et al. 2001, Reynolds et al. 2007). Shiell et al. reported that women who had a greater consumption of meat and fish in the second half of pregnancy give birth to offspring who developed higher systolic blood

pressure in adulthood; and high maternal fish (but not red meat) consumption associated with increased offspring diastolic blood pressure (Shiell et al. 2001). It has been suggested that a high protein diet can stimulate the maternal hypothalamic-pituitary-adrenal (HPA) axis to secrete more cortisol, potentially resulting in overexposure of the developing fetus to glucocorticoids (Herrick et al. 2003). Indeed, studies have shown that offspring exposed to this unbalanced high protein diet during pregnancy had increased fasting cortisol levels and increased cortisol levels in response to stress in adulthood (Herrick et al. 2003, Reynolds et al. 2007).

In the United States, studies have also shown a link between low birth weight and metabolic risk factors. Rich-Edwards et al. showed that females that had a lower birth weight are at increased risk of developing type two diabetes in adulthood (Rich-Edwards et al. 1999), while Curhan et al. showed that this link is also present in men (Curhan et al. 1996). Furthermore, a heavier birth weight has also been shown to be linked to the development of obesity in adulthood (Curhan et al. 1996).

In Asia, during the ‘Great Leap’ of 1959 – 1961, various parts of China were exposed to famine. It was reported that during this period of famine, birth rate was dramatically decreased (St Clair et al. 2005); a similar observation to that in the siege of Leningrad (Antonov 1947). Yang et al. reported a significant increase in body mass index (BMI) in women born during the famine compared with control women in the same region (Yang et al. 2008); but there was no difference in men (Yang et al. 2008).

1.2.1.3 Programming of the brain and behaviour

Psychiatric disorders have also been shown to have a link with the prenatal environment. In the Dutch famine cohort, female offspring exposed to famine during early gestation have a relative risk of more than two of developing schizophrenia (Susser and Lin 1992).

Studies from the Chinese famine (discussed in section 1.2.1.2) also report a link between the intra-uterine environment and psychiatric disorders. Offspring that were exposed to the famine in utero were at a significantly greater risk of developing schizophrenia (St Clair et al. 2005). However, this study did not report on the gender difference that was seen in the Dutch famine study.

Studies of individuals involved in the more recent event of the September 11th 2001 incident in the United States have also revealed programming effects in the offspring (Yehuda et al. 2005). Following the collapse of the World Trade Centre on 9/11 the frequency of people developing clinical signs of post-traumatic stress disorder (PTSD) rose significantly (Neria et al. 2006). Women who were around the World Trade Centre during the 9/11 event who went on to develop PTSD had lower salivary cortisol levels (Yehuda et al. 2005). Interestingly, the babies of the mothers who developed PTSD from the 9/11 event also had low salivary cortisol levels at one year old, a finding which was particularly noted if the mother was exposed to the 9/11 event during the third trimester of pregnancy (Yehuda et al. 2005). These findings are consistent with previous studies looking at the offspring of the survivors from the Nazi holocaust which also showed lower cortisol level (Yehuda et al. 2000) as did their children (Yehuda and Bierer 2007, Zohar et al. 2007).

In addition, it has been shown that stress resulting from natural disasters may also have programming effects on offspring. A study of women that were pregnant when the most costly ice storm in the recent history of Canada struck Québec in 1998 (Laplante et al. 2008) showed that maternal stress, particularly during early gestation, had a clear relationship with the children's cognitive and language ability at two years of age (Laplante et al. 2004) and was associated with lower intelligence quotient (IQ) and poorer verbal skills at five years of age (Laplante et al. 2008).

Species	Country/City	Stressor	Gestation	References	Primary findings
Human	Amsterdam Netherlands	↓ global nutrient intake	Early	(Roseboom et al. 1999)	↑ Blood pressure
	Amsterdam Netherlands	↓ global nutrient intake		(Ravelli et al. 1976)	↑ Obesity especially in males
	Netherlands	↓ global nutrient intake		(Susser and Lin 1992)	↑ Risk of schizophrenia in males
	Québec Canada	Natural disaster		(Laplante et al. 2008)	↓ Intellectual performance
	Amsterdam Netherlands	↓ global nutrient intake	Late	(Ravelli et al. 1998)	↓ Glucose tolerance
	Motherwell Scotland	↑ protein ↓ carbohydrate		(Shiell et al. 2001)	↑ Systolic blood pressure
	Motherwell Scotland	↑ protein ↓ carbohydrate		(Reynolds et al. 2007)	↑ Cortisol secretion to stress
	Motherwell Scotland	↑ protein ↓ carbohydrate		(Herrick et al. 2003)	↑ Fasting plasma cortisol
	New York City United States	Post traumatic stress		(Yehuda et al. 2005)	↓ Salivary cortisol
	Leningrad Russia	↓ global nutrient intake	All	(Stanner et al. 1997)	No difference between intrauterine malnutrition and control
	Finland	↓ global nutrient intake		(Kannisto et al. 1997)	No difference between intrauterine malnutrition and control
	China	↓ global nutrient intake		(Yang et al. 2008)	↑ BMI in female
	Wuhu China	↓ global nutrient intake		(St Clair et al. 2005)	↑ Risk of schizophrenia
	-	Post traumatic stress		(Yehuda et al. 2000)	↓ Urinary cortisol
	Québec Canada	Natural disaster		(Laplante et al. 2008)	↓ Language ability

Table 1.1 Studies showing programming effects in humans

1.2.2 Obesity in pregnancy

The prevalence of obesity is increasing rapidly. Within the UK alone, the prevalence of obesity has increased by more than 300% between 1980 and 2002 (Rennie and Jebb 2005). Heslehurst et al. reported that in 2004, the prevalence of maternal obesity at the beginning of pregnancy was as high as 16% compared to 9.9% in 1999 (Heslehurst et al. 2007). Heslehurst et al. also predicted that by 2010, 22% of women would be obese at the beginning of pregnancy (Heslehurst et al. 2007). In the United States, the prevalence of obesity has continued to grow not only in adults, but also in adolescents and children (Ogden et al. 2006); Chu et al. reported that between 2004 – 2005, in 26 states, 23% of women were overweight and 18.7% of women were obese during pregnancy (Chu et al. 2009). Globally, the WHO has estimated that more than 20% of the world population are overweight (WHO 2011). As previously noted, obesity predisposes to various serious metabolic illnesses.

Obesity in pregnancy has been linked to programming effects in the offspring. Numerous studies from around the world have provided evidence that an increased maternal BMI is linked to an increased risk of childhood obesity (Whitaker 2004, Gale et al. 2007), increased fat mass (Gale et al. 2007, Reynolds et al. 2010), and metabolic illnesses (Catalano et al. 2009, Group 2010) in the offspring. These programming effects have been shown to be already apparent at birth (Catalano et al. 2009). A large scale international study conducted by the HAPO (Hyperglycaemic and Adverse Pregnancy Outcome) group on over 23,000 adult participants in nine countries has suggested that increased maternal BMI is linked to increased body fat in the offspring along with increased fetal hyperinsulinaemia, this appears to be independent of maternal glycaemia (Group 2010).

Taken together, these large scale retrospective population studies have provided convincing evidence for an effect of an altered intrauterine environment and the subsequent risk of disease in adulthood. This link is independent of gender, ethnicity, lifestyle factors, geographical distribution, and socio-economic status.

1.2.3 The role of post-natal growth

In the previous sections, the idea that in utero environmental alterations can lead to various programming effects has been discussed. However, studies have also suggested an important role of the post-natal environment in programming effects.

Programming is thought to reflect the adaptation of the fetus to a poor environment, resulting in some organs that are less important in survival to be less developed than other organs which may be critical for survival (Hales and Barker 1992). This change in organ development may be of critical importance in later development of chronic diseases. The highest risks of later diseases appear to be in offspring that had restricted growth in utero but had rapid catch up growth during infancy. Some have suggested that catch up growth, especially those that occurred within the first two years of life are at greater risk (Ong et al. 2000); while others suggested that less weight gain before 1 year of age followed by rapid weight gain at increased risk (Eriksson et al. 2001). This suggests that retarded fetal growth may be associated with effects on organ function and that the subsequent accelerated growth during the post-natal period, increasing metabolic demands on these under-functioning organs, may lead to an increased risk of diseases (Eriksson et al. 2000).

Human and animal studies have provided evidence that rapid postnatal growth may

be important in programming effects. Cardiovascular disease risk and death from cardiovascular events are related to reduced birth weight (Barker et al. 1989), with an even higher risk in children who displayed catch up growth (Eriksson et al. 1999). Children with catch up growth during early infant life have more central fat distribution and increased BMI (Ong et al. 2000), increased insulin secretion (Soto et al. 2003a), and increased blood pressure (Williams et al. 1992, Launer et al. 1993). In mice, restricted intrauterine growth and rapid post natal catch up growth has been associated with a shorter life span (Ozanne and Hales 2004) and in the rat, catch up growth has been linked to obesity and disruption in insulin and glucose homeostasis (Bieswal et al. 2006). Although it has been suggested that the increased cellular division and oxidative damage may occur in rodents with accelerated growth during early postnatal life leading to shortening of telomeres (Jennings et al. 1999); the exact mechanisms underlying the link between early nutritional status and adulthood diseases remain to be explored.

1.2.4 Animal models of programming

Various animal models of alterations in the fetal environment have been developed with the ultimate goal of achieving 'programming' in the offspring. These models facilitate understanding the underlying mechanisms that lead to such effects.

1.2.4.1 Prenatal glucocorticoid overexposure

In the past few decades, studies have suggested a connection between glucocorticoid exposure and low birth weight both in animal models and in humans (Reinisch et al. 1978, Nyirenda et al. 1998, French et al. 1999, Bloom et al. 2001, Drake et al. 2005). In Edinburgh, the hypothesis that fetal overexposure to glucocorticoids during a critical developmental period might underpin the epidemiological association between low birth weight and future adulthood disease has been explored (Benediktsson et al. 1993). In rodents, prenatal glucocorticoid overexposure has been associated with programming effects on the cardiovascular system including hypertension and effects on the vasculature (Hadoke et al. 2006, Tang et al. 2011), glucose-insulin homeostasis and behaviour (Nyirenda et al. 1998, Welberg et al. 2001, O'Regan et al. 2004). Similar programming effects of prenatal glucocorticoids have been shown in other species including sheep (Tangalakis et al. 1992) and non-human primates (Clarke et al. 1994, Koenen et al. 2002, de Vries et al. 2007).

1.2.4.1.1 Glucocorticoid actions

Glucocorticoids are steroid hormones that are synthesized in the adrenal cortex under the tight control of the HPA axis (Figure 1.1). Glucocorticoids exert their actions on nearly every tissue in the body, with many functions including roles in metabolism (Leung and Munck 1975, McEwen et al. 1986), immune responses and appetite

regulation (Simpson et al. 1974). Glucocorticoids exert their effects by binding to the glucocorticoid receptor (GR) and regulate gene expression, homeostasis, and cell signalling (Lu and Cidlowski 2004). Glucocorticoids also bind with high affinity to the mineralocorticoid receptor (MR) (Funder 1993, Seckl and Chapman 1997).

Glucocorticoids are used clinically as an anti-inflammatory agent for patients who suffer chronic illnesses such as asthma or rheumatoid arthritis and have long been used in pregnancy to promote acceleration of fetal lung maturation if preterm delivery is expected, as well as for the treatment of fetuses at risk of congenital adrenal hyperplasia (Seckl and Meaney 2004). Other then promoting lung maturation, glucocorticoid administration has also been shown to reduce the incidence of periventricular haemorrhage and necrotizing enterocolitis in preterm infants thereby increasing survival (Crowley et al. 1990). The role of glucocorticoids in epigenetic programming mechanisms is discussed in section 1.2.7.

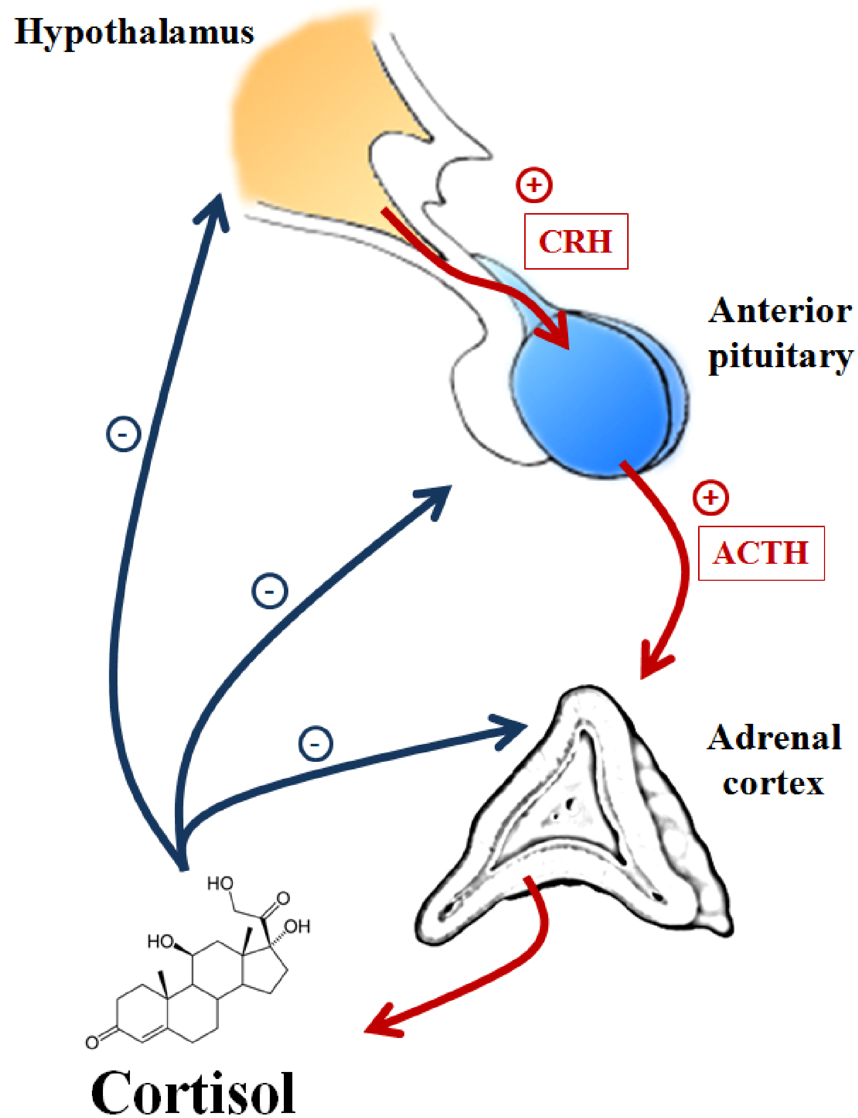


Figure 1.1 Hypothalamic pituitary adrenal (HPA) axis schematic

Red arrows = stimulatory effects; blue arrows = negative feedback; CRH = Corticotropin-releasing hormone; ACTH = Adrenocorticotropic hormone

1.2.4.1.2 11β-hydroxysteroid dehydrogenase

Under normal physiological conditions, maternal glucocorticoids do not readily cross the placenta into the fetal circulation (Seckl and Chapman 1997). This is due to the enzyme 11β-hydroxysteroid dehydrogenase type II (11β-HSD2), a NAD^+ dependent enzyme that is expressed mainly in MR rich tissues such as the kidneys (Roland and Funder 1996), colon (Whorwood et al. 1994), and salivary glands (Roland and Funder 1996), where it acts to restrict inappropriate activation of MR by glucocorticoids (Stewart et al. 1988, Roland and Funder 1996, Seckl and Chapman 1997, Deuchar et al. 2011). This enzyme also appears to be present in some non-mineralocorticoid receptor rich tissues such as blood vessels (Christy et al. 2003) and the placenta (Seckl and Chapman 1997) where it is relatively abundant in the trophoblast cells in humans (Krozowski et al. 1995, Stewart et al. 1995, Pepe et al. 1999), baboons (Pepe et al. 1999), sheep (Yang 1995, Clarke et al. 2002), rabbit (Hundertmark et al. 2001), rat (Waddell et al. 1998), and mouse (Brown et al. 1996). The enzyme acts to convert active cortisol into inactive cortisone in humans and from active corticosterone to the inactive 11-dehydrocorticosterone in rodents (Edwards et al. 1996, Seckl and Meaney 2004) (Figure 1.2).

In the placenta, the enzyme 11β-HSD2 acts as a barrier to prevent the fetus from overexposure to excess maternal glucocorticoid (Staud et al. 2006). This enzymatic barrier does not completely inactivate all glucocorticoid however, and it has been suggested that about 20% of glucocorticoids pass from the mother to the fetus unaltered (Benediktsson et al. 1997). In humans, it has been reported that reduced birth weight is associated with deficient level of placental 11β-HSD2 (Shams et al. 1998), supporting the importance of this enzyme in the placenta as a barrier

preventing fetal glucocorticoid overexposure.

In animal models, fetal glucocorticoid overexposure can be achieved by, i) administration of a synthetic glucocorticoid such as dexamethasone (Dex), which is a poor substrate for the 11β-HSD2 enzyme in the placenta (Benediktsson et al. 1993, Nyirenda et al. 1998, Drake et al. 2005), ii) administering an agent which inhibits 11β-HSD2 (e.g. carbenoxolone) (Edwards et al. 1996, Lindsay et al. 1996), iii) using a 11β-HSD2 genetic knockout model (Wyrwoll et al. 2009), and iv) exposing the pregnant mother to stressful events which increase natural maternal circulatory glucocorticoids and swamp the placental 11β-HSD2 barrier (Barbazanges et al. 1996).

In summary, glucocorticoids have diverse actions on multiple organs. Despite the important role of glucocorticoids in fetal growth and organ maturation, evidence suggests a role for fetal glucocorticoid overexposure in programming, and this has therefore become an important area of research.

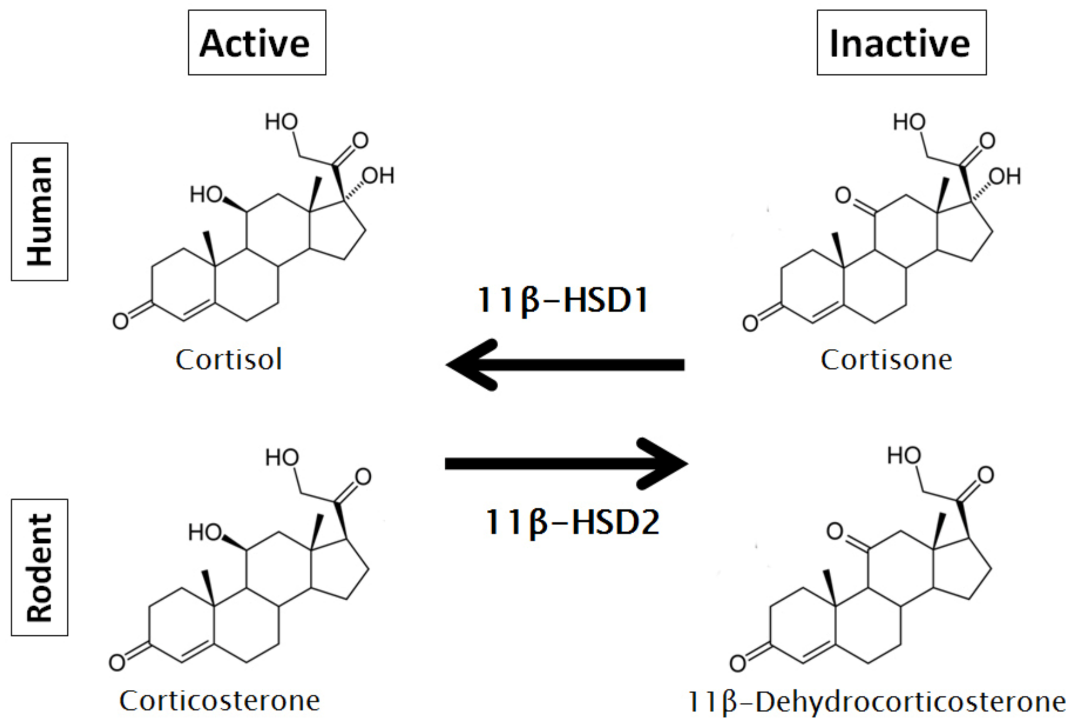


Figure 1.2 Glucocorticoid activation and reduction in human and rodents

1.2.4.2 The role of maternal nutrition

Evidence from the various large scale human cohort studies discussed previously, highlights the importance of the nutritional status of the mother during pregnancy. Similar programming effects are also seen as a consequence of early life nutritional alterations in animal models.

1.2.4.2.1 Maternal undernutrition

Various methods have been implemented to achieve the goal of undernutrition in animal models and to induce programming effects (Harding 2001). Programming effects on blood pressure (Langley Evans and Jackson 1994) and glucose tolerance (Langley et al. 1994, Jimenez-Chillaron et al. 2009) have been shown by restricting dietary protein in rats. In a rat model, the introduction of a low protein diet two weeks before pregnancy was associated with a significant decrease in birth weight as well as lowered placental 11β-HSD activity (Langley Evans and Jackson 1994, Langley-Evans et al. 1996) and the offspring subsequently developed hypertension. Other models of undernutrition including those in mice (Peixoto-Silva et al. 2011), guinea pigs (Persson and Jansson 1992) and sheep (Gardner et al. 2005) have also shown similar programming effects in offspring.

1.2.4.2.2 Maternal overnutrition

Animal models of dietary induced obesity (DIO) have been developed to explore the mechanisms by which maternal obesity in pregnancy causes programming effects in the offspring (Carmichael and Shaw 2000, Gale et al. 2007, Catalano et al. 2009). Various dietary content combinations have been used to achieve obesity; including high fat content alone, high sugar content alone, or a combination of both (Surwit et

al. 1988, Surwit et al. 1995a, Bayol et al. 2005, Sumiyoshi et al. 2006, Samuelsson et al. 2008, Surve 2008, Chechi et al. 2010). Studies using females exposed to an obesogenic diet prior to pregnancy, throughout pregnancy, and during lactation (Khan et al. 2003, Bayol et al. 2005, Samuelsson et al. 2008) have shown programming effects on offspring metabolic function, with offspring of obese mothers developing a metabolic and cardiovascular phenotype such as insulin resistance/hyperglycaemia (Han et al. 2005, Liang et al. 2009, Shankar et al. 2010), hypercholesterolaemia (Elahi et al. 2009), steatohepatitis (Bruce et al. 2009), hypertension (Khan et al. 2003, Samuelsson et al. 2008, Liang et al. 2009) or a change in the regulation of appetite and satiety in the hypothalamus (McMillen et al. 2005, Taylor and Poston 2007) therefore making them prone to develop obesity in adulthood (Bayol et al. 2005, Bayol et al. 2007, Bayol et al. 2008, Akyol et al. 2009). Unlike the glucocorticoid over-exposure model, in most DIO programming models the offspring are exposed to an already obese or over-nourished mother and it is difficult to determine the critical window of developmental programming (Drake and Reynolds 2010), however some studies have attempted to address the importance of timing. In one study, Shankar et al. showed that the offspring of obese female rats maintained on a standard chow diet during pregnancy still showed programming effects, suggesting that maternal obesity at conception is able to programme the offspring (Shankar et al. 2008). Some studies have shown that exposure to high fat diet for 7 – 10 days prior to conception and during pregnancy is sufficient to cause programming effects on blood pressure, insulin sensitivity, fat deposition and metabolism in the offspring (Guo and Jen 1995, Khan et al. 2005, Taylor et al. 2005). Finally, feeding a pregnant mother a ‘junk food’ diet from the day of conception till weaning was also associated with alterations in offspring gene expression changes as

well as a differential preference for ‘junk food’ (Bayol et al. 2007, Bayol et al. 2008).

Other than programming by ‘in utero’ exposure to an obesogenic environment, post-natal events (discussed in section 1.2.3) can also programme the animals (Francis et al. 1999, Meaney 2001). The role of early postnatal overfeeding has been explored using the “pup in a cup” model pioneered by Hall et al. (Hall 1975) which allows studies of the effects of overnutrition during the suckling period (Patel and Srinivasan 2002). Modifying milk constituents using this model can permanently alter the metabolism of these animals leading to hyperinsulinaemia and subsequent diabetes (Patel and Srinivasan 2002) suggesting the importance of early postnatal feeding in mediating programming effects. A summary of the proposed mechanisms of programming is presented in figure 1.3.

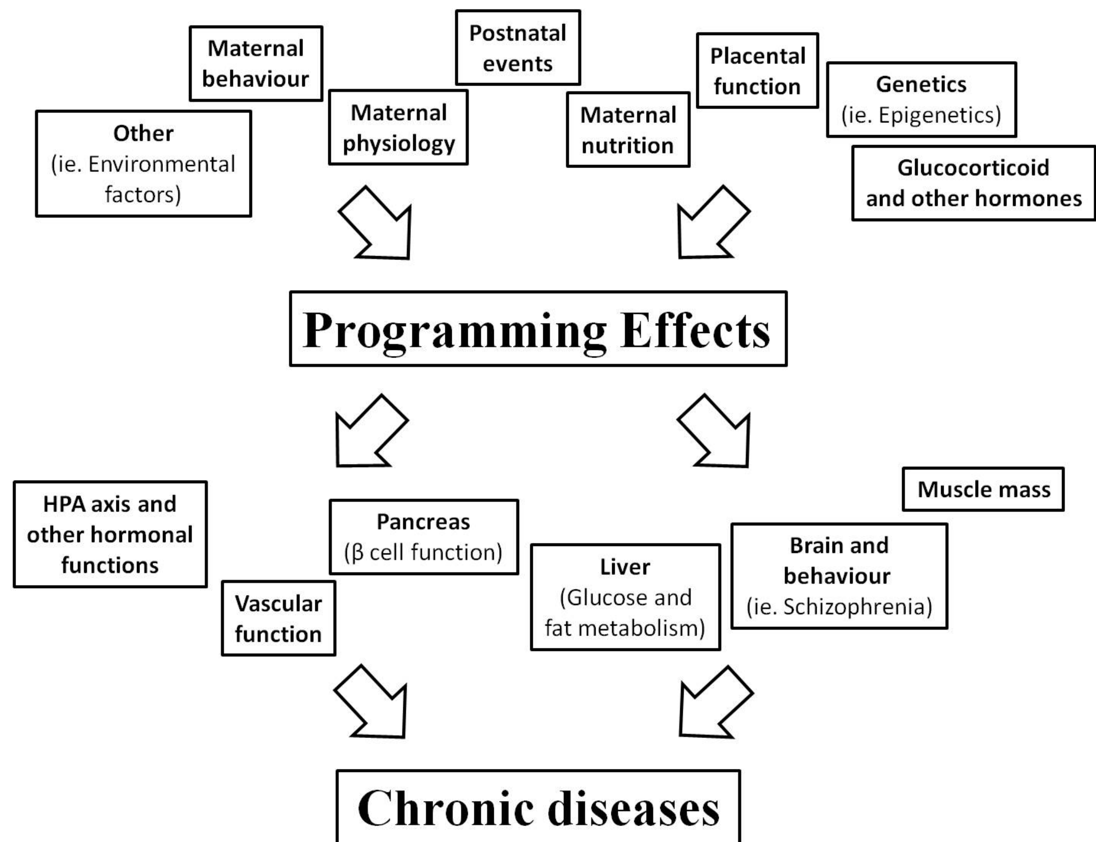


Figure 1.3 Proposed mechanisms of programming effects

1.2.5 Importance of timing of insult exposure

The timing of exposure to an insult appears to be a critical factor influencing programming outcome. In the Dutch Famine cohort, exposure to famine during early gestation shifts programming effects toward developing cardiovascular diseases (Roseboom et al. 2000), whereas exposure in late gestation leads to programming effects on metabolic phenotype in which decreased glucose tolerance is more evident (Ravelli et al. 1998). Similarly, women exposed to the 9/11 event during their third trimester of pregnancy have offspring that showed lower salivary cortisol level (Yehuda et al. 2005). Studies in animal models have also indicated the importance of the timing of exposure to an insult (Table 1.2). Excess glucocorticoid exposure during the last week of pregnancy in the rat leads to reduced birth weight, glucose intolerance (Nyirenda et al. 1998), hypertension (Levitt et al. 1996), whereas if the exposure to excess glucocorticoid was during the first or second week of gestation no effects were seen on glucose and insulin responses (Nyirenda et al. 1998). A further study suggests that two days of glucocorticoid excess during the last week of pregnancy was sufficient to alter renal development leading to hypertension (Singh et al. 2007). The timing of exposure is also important in determining programming effects on behaviour. Glucocorticoid exposure throughout the whole gestation, is associated with programming of anxiety-like behaviour and increased GR and MR mRNA expression in the amygdala (Welberg et al. 2001), whereas exposure during the last week of gestation in rodents has been linked to decreased cognitive function, increased stress responsiveness (Brabham et al. 2000) and alterations in GR and MR mRNA expression in the hippocampus (Levitt et al. 1996, Shoener et al. 2006).

Species	GC	Timing	Dose	References	Primary findings
Baboon	BET	E117-E131 (term E185)	IM 87.5μg/kg	(Koenen et al. 2002)	↑ blood pressure
		E134	IM 87.5μg/kg	(Antonow-Schlorke et al. 2003)	Structural alteration of neuronal cytoskeleton
Sheep	CORT	E127-E143 (term E145-150)	IV 4μg/hr for 5hr	(Wood et al. 1987)	↓ heart rate ↓ blood volume ↑ arterial pressure
	BET	E104, 111, 118, 125	IM 500μg/kg	(Moss et al. 2001)	↓ insulin sensitivity ↓ arterial pressure at 3 months
		E104, 111, 118	IM 500μg/kg	(Jobe et al. 1998b)	↓ birth and organ weight
		E104, 111, 118, 124	IM 500μg/kg	(Huang et al. 1999)	Retards fetal brain growth
Rat	DEX	Throughout pregnancy	SC 100μg/kg	(Welberg et al. 2001)	↑ anxiety ↑ GR/MR expression in amygdala
		Throughout pregnancy	SC 100μg/kg	(Hadoke et al. 2006)	↑ blood pressure ↑ mesenteric artery but not aortic contraction
		E1-7 E8-14 E15-21	SC 100μg/kg	(Nyirenda et al. 1998)	No effect No effect Hyperglycaemia and hyperinsulinaemia
		E8, 10, 12	IM 100μg/kg	(Dahlgren et al. 2001)	↓ glucose tolerance ↑ fat depots
		E15-delivery	SC 100μg/kg	(Levitt et al. 1996)	↑ blood pressure ↑ basal CORT
		E14-21	SC 100μg/kg	(O'Regan et al. 2004)	↑ hepatic PEPCK mRNA Hypertension Gender specific changes
		E14-21	SC 100μg/kg	(Holson et al. 1995)	↓ sexual performance Altered sexual differentiation
		E15-E21	SC 100μg/kg	(O'Regan et al. 2008)	Basal hypotension Hypertension to stress

		E15-21	SC 100μg/kg	(Drake et al. 2005)	Intergenerational effects in F2 and ↑ PEPCK activity
		E15-delivery	DW 2.5μg/ml	(Brabham et al. 2000)	↓ cognitive function ↑ stress response ↑ hippocampal GR mRNA
		E17-19	SC 50, 200, 800μg/kg	(Kauffman et al. 1994)	↓ cardiac function to hypoxia
	DEX/ BET	E8-delivery	IP 100μg/kg	(Emgård et al. 2007)	↓ cognitive function
	BET	E15-21	SC 50, 100, 200, 400, 600μg/kg	(McDonald et al. 2003)	Except lowest dose of 50μg/kg, all doses reduce birth weight No change in blood pressure
		E19-20	IM 200μg/kg	(Murthy and Moya 1994)	Transient decrease in cellular immunity
		E20	SC 170μg/kg Twice in 4 hr	(Scheepens et al. 2003)	Somatic growth retardation No difference in postnatal CORT level
	CORT	E14-15	IP 800μg/kg Twice daily	(Singh et al. 2007)	↓ nephron number ↑ arterial pressure
		E14-21	SC 7000μg/kg	(Holson et al. 1995)	Altered sexual differentiation
	HC	E17-19	SC 1500μg/kg	(Piffer and Pereira 2004)	Disturbed oestrous cycle and fertility in adulthood
Mouse	DEX	E20-delivery	OP 125μg/kg	(Dickinson et al. 2007)	↑ expression of genes involved in branching of tubules in the kidneys ↓ nephron number

Table 1.2 Animal models of excess glucocorticoid

Phenotypes presented are of mix ages and some may emerge only with ageing

GC = Glucocorticoids; E = Embryonic day; BET = Betamethasone; DEX = Dexamethasone; CORT = Corticosterone; HC = Hydrocortisone; GR = Glucocorticoid receptor; MR = Mineralocorticoid receptor; PEPCK = Phosphoenolpyruvate carboxykinase; mRNA = messenger ribonucleic acid; F2 = Second generation; SC = Subcutaneous; IM = Intramuscular; IV = Intravenous; IP = Intraperitoneal; OP = Osmotic pump; DW = Drinking water

1.2.6 The role of epigenetics in programming

There has been much recent interest in the role of epigenetic modifications as a mechanism for programming effects. The term “epigenetic” was first used by the Jewish Italian philosopher, Eugenio Rignano, in his ‘centroepigenetic hypothesis’ (Rignano 1911); however, it was the British developmental biologist, Conrad Hal Waddington (Waddington 1957), Professor of Animal Genetics at the University of Edinburgh who popularised this term. Professor Waddington first used this term to describe how a certain genotype may give rise to phenotypes (Waddington 1957). Now, the word “epigenetic” is most commonly defined as changes which alter the expression of a gene that cannot be accounted for by genetic variations (Richards 2006). This can be achieved by several mechanisms: a) DNA methylation, a phenomenon where a methyl group is attached to the DNA (on the 5’-position of the cytosine nucleotide on a CpG dinucleotide) to either allow transcription (e.g. Igf2) (Szabo et al. 2004) or silencing (e.g. CDKn1c) (Beatty et al. 2006) gene expression. The patterns of such methylation (5-methylcytosine/5-mC) are preserved relatively accurate through mitosis through the actions of DNA methyltransferases (DNMTs) (Bestor 2000, Dahl et al. 2011). Recently, interest has centred on an alternative type of DNA methylation, the hydroxylated form of 5mC, 5-hydroxymethylcytosine (5-hmC). The first discovery of 5hmC in mammalian cells dates back to 1972 when Penn et al. reported its presence in brain tissues of several species (Penn et al. 1972). Recently, 5-hmC has been detected in various mouse tissues including kidney, lung, muscle, heart, brain (Kriaucionis and Heintz 2009) and at lower levels in bladder, liver, and endocrine glands (Globisch et al. 2010). 5-hmC may be involved in active DNA demethylation pathways and DNA repair (Tahiliani et al. 2009); b) non-coding RNAs (ncRNA) which can impact on gene expression and are of particular

importance in the transcriptional silencing of retrotransposons (Chu and Rana 2007, Rana 2007). ncRNAs have a wide range of functions including RNA editing, splicing, and inhibition of gene translation (Mattick and Makunin 2006). Additionally, emerging evidence has linked these ncRNAs with epigenetic regulation through effects on chromatin and transcriptional silencing of genes (Bernstein and Allis 2005, Mattick and Makunin 2006). Lastly, c) histone modifications (including acetylation, methylation, ubiquitination and SUMOylation) can affect chromatin accessibility and impact on DNA transcription (Delcuve et al. 2009). As histones are condensed as a consequence of hypoacetylation, access of binding proteins to DNA for transcription is restricted; in contrast, hyperacetylation of histones can lead to chromatin decondensation allowing accessibility (Wang et al. 2001, Kouzarides 2002, Delcuve et al. 2009). Methylation status of histones has also been associated with activation (Noma et al. 2001, Kouzarides 2002) or silencing (Tachibana et al. 2008) of transcription depending on the site of methylation.

Various studies have suggested a role for epigenetic modifications as an underlying mechanism in programming. For example, Waterland et al. showed that with maternal dietary methyl donor supplementation in mice, an epigenetic modification (increased DNA methylation) occurred at the Agouti gene in the offspring, leading to a change in coat colour and metabolic phenotype (Waterland and Jirtle 2003). Similarly, Lillycrop et al. showed that offspring of rats fed a protein restricted diet during pregnancy had alterations in DNA methylation at the promoters of the hepatic glucocorticoid receptor and peroxisomal proliferator-activated receptor α (PPARα) in association with altered gene expression (Lillycrop et al. 2005); these alterations in methylation status affected specific CpG dinucleotides (Lillycrop et al. 2008).

Prenatal exposure to protein restriction has also been shown to alter histone configuration on the GR 1_{10} promoter by increasing acetylation (at H3K9 and H4K9) and methylation (at H3K4) which facilitated transcription (Lillycrop et al. 2007). Another recent report has also shown that a protein restricted diet given to rats during pregnancy alters epigenetic regulation of the hepatic nuclear factor 4α (Hnf4α) gene in the islets of the pancreas (Sandovici et al. 2011). Maternal behaviour has also been shown to lead to differences in the pattern of DNA methylation; offspring of mothers with higher rate of grooming and nursing of pups had decreased methylation at the glucocorticoid receptor promoter (Weaver et al. 2004). In addition, a model which uses uterine arterial ligation has also shown decreased expression of Pdx1, a gene important in pancreatic β cell growth regulation, by epigenetic modifications leading to alteration in glucose homeostasis (Park et al. 2008). Thus, epigenetic modifications may play an important role in programming of the offspring.

1.2.7 Glucocorticoids and the epigenome

Glucocorticoids may affect the epigenome. Glucocorticoids are known to affect the expression of epigenetically regulated imprinted genes such as Igf2 (Li et al. 1998) and glucocorticoid treatment of cultured fetal hepatocytes was associated with DNA demethylation, a phenomenon which persisted even after withdrawal of glucocorticoids, providing a ‘memory’ of the event (Thomassin et al. 2001). One recent report has shown that glucocorticoid overexposure during the last week of gestation is associated with decreased DNA methylation at one of the differentially methylated regions controlling the expression of Igf2 in the fetal liver (Drake et al. 2011). Thus, glucocorticoids may be linked to programming effects by epigenetic mechanisms.

1.2.8 Transmission of programming effects to subsequent generations

Increasing evidence suggests that programmed effects may be transmitted to subsequent generations (Anway et al. 2005, Drake et al. 2005, Anway et al. 2006a, Benyshek et al. 2006, Godfrey et al. 2007, Inawaka et al. 2009). In this thesis the terminology proposed by Skinner will be used (Skinner 2008). The term "multigenerational" will be used when the insult might affect several generations at once e.g. in situations where the pregnant F0 females are exposed to an insult, programming effects may be seen in F1 fetus(es) due to direct exposure to this insult. However the germ cells which will go on to form the F2 generation are already present in the developing gonad and are therefore potentially also exposed to the same insult (Figure 1.4) (Drake and Liu 2010). The term "transgenerational" will be used only when programming effects are seen in a non-exposed generation, such as the presence of programmed effects in a third (F3) generation (Skinner 2008).

Studies in humans have also suggested that programmed effects can be transmitted to subsequent generations, such as the Dutch Famine cohort study(Lumey 1992, Roseboom et al. 2000, Painter et al. 2005). Additionally, studies in Sweden linked the nutritional status of grandparents to the risk of adulthood diseases and longevity in grand-offspring (Kaati et al. 2002, Kaati et al. 2007). This study suggested that the male proband had a higher mortality rate if their grandfather had good nutritional supply during his prepubertal slow growth period, thus suggesting a transgenerational response down the male lineage, possibly through sperm (Kaati et al. 2007). Therefore, evidence from animal and human studies suggest programmed effects can be passed down through maternal (Emanuel et al. 1992, Drake et al. 2005) or paternal (Anway et al. 2006a, Kaati et al. 2007) lineages.

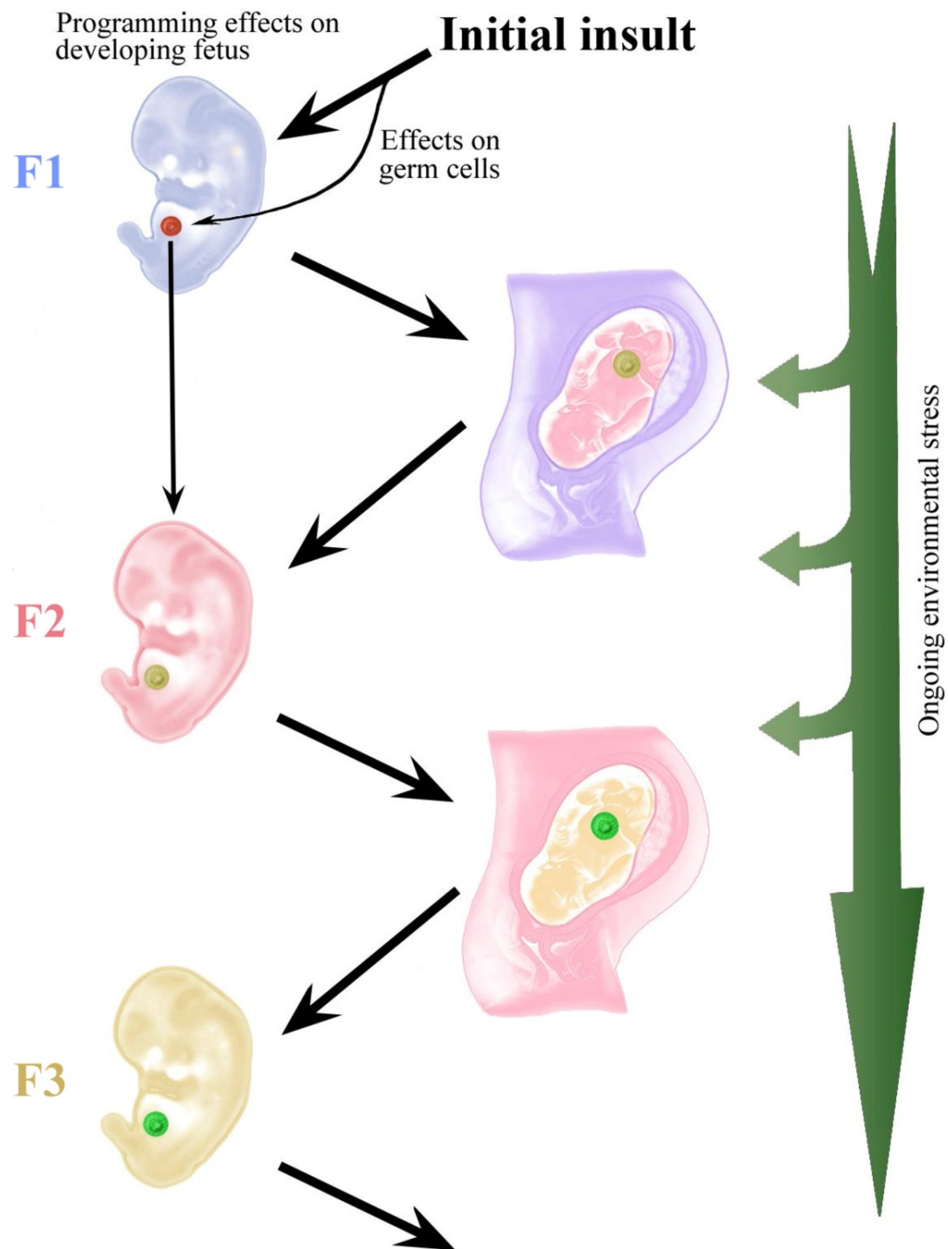


Figure 1.4 Model showing mechanisms of transmission of programming effects across generations

The initial insult may have direct programming effects on both the F1 and F2 generations, but at different stages of development. Meanwhile, ongoing environmental changes may induce programming effects in subsequent generations

1.3 Mechanisms to explain the transmission of programming effects

1.3.1 Maternal physiology

1.3.1.1 Maternal size

There is much evidence that maternal size correlates with offspring birth size. One classic example of this comes from the studies of Walton et al in which large Shire horses were crossed with smaller Shetland ponies. He concluded that size at birth is in direct correlation with maternal size rather than genotype (Walton and Hammond 1938). These findings have been replicated in other species such as horses (Allen et al. 2002), sheep (Hunter 1956), pigs (Gama and Johnson 1993), mice (Cowley et al. 1989), and humans (Ibanez et al. 2000). In humans, the height of the mother correlates strongly with the size of the uterus (Cogswell and Yip 1995). It has also been shown in ovum donation studies that infant birth weight correlates with the recipient mother's BMI rather than the donor's BMI (Brooks et al. 1995), stressing the importance of maternal factors in determining offspring birth weight. Therefore, these studies indicate the possibility that a smaller mother might give birth to a smaller baby, which may grow up to become a smaller mother and continue a cycle of lower birth weight.

1.3.1.2 Maternal health

It has also been suggested that maternal illnesses may cause programming effects in the offspring. For example, pregnant women with diabetes are prone to deliver babies that are large for gestational age (Ehrenberg et al. 2004). These offspring are thus more prone to develop obesity and metabolic syndrome in adulthood. Similarly, women with hypertension tend to have offspring that are small for gestational age (McCowan et al. 1996), thus more prone to cardiovascular disease risk in adulthood.

Similar effects have been demonstrated in animal studies (Aerts et al. 1990, Gauguier et al. 1990, Wichi et al. 2005). For example, reports suggested that diabetes in rats can lead to increased offspring blood pressure (Wichi et al. 2005) and higher blood pressure in pregnancy associates with lower offspring birth weight (Fang et al. 1999, Odell et al. 2006), thus potentially leading to cycle of multigenerational programming.

1.3.1.3 Maternal behaviour

Interestingly, maternal behaviour has programming effects. In humans, parental behaviour such as neglect, sexual and physical abuse associates with adulthood obesity (Lissau and Sorensen 1994), depression (Bifulco et al. 1991), anxiety (McCauley et al. 1997), diabetes and heart disease (Felitti et al. 1998), and also violence and aggression in the offspring (Doumas et al. 1994). In animals, similar results have been shown, for example in studies showing that postnatal handling of the young infant and separation from maternal care from birth decreases the physiological and psychological reaction to stress in adulthood (Levine et al. 1967). Also, differences in normal maternal care behaviour in rodents (licking/grooming and arched back nursing (LG-ABN)) in the first week post delivery has been related to neonatal neural development (Francis et al. 1999); where mothers who show high rates of LG-ABN have offspring that are less fearful and show milder HPA response to stress (Francis et al. 1999, Weaver et al. 2004). These effects are transmissible to future generations since female offspring of mothers who showed high rate of LG-ABN also display high rate of LG-ABN towards their offspring (Francis et al. 1999).

1.3.1.4 Maternal exposure to drugs

In animal models, the use of certain drugs, such as vinclozolin (an antifungal drug) has been shown to have programming effects on the offspring. Effects have been demonstrated on reproductive organs such as the testis (Anway et al. 2005), blood chemistry and red blood cell numbers (Nilsson et al. 2008), and behaviour (Skinner et al. 2008). It has been reported in some studies that programming effects induced by vinclozolin may be transmissible to subsequent generations through the male germline as far as the F4 generation (Anway et al. 2005, Anway et al. 2006a) and to the F3 generation through female germline (Nilsson et al. 2008). These findings were not recapitulated in a similar study (Inawaka et al. 2009) and it has been suggested that it may be due to different strain of animals used (Dolinoy et al. 2006).

1.3.2 Epigenetics and the transmission of programming effects

An increasing amount of evidence suggests that epigenetic marks may be passed to subsequent generations (Anway et al. 2005, Cropley et al. 2006, Burdge et al. 2007, Stouder and Paoloni-Giacobino 2010). In a rat model of maternal protein restriction, methylation in the promoter region of hepatic PPARα and $GR1_{10}$ was decreased in F2 as well as F1 males (Burdge et al. 2007). Similar multigenerational effects have been reported with methyl donor diet supplementation, in which the methylation of the agouti viable yellow (A^{vy}) gene was increased in both F1 and F2 offspring (Cropley et al. 2006).

Emerging evidence suggests changes in DNA methylation patterns (Anway et al. 2005, Franklin et al. 2010) and small non-coding RNAs (Rassoulzadegan et al. 2006, Lalancette et al. 2008) may be transmissible through the germline. Additionally,

histone modifications may be transmissible in sperm (Hammoud et al. 2009); about 5 – 15% of DNA in sperm is organised by histone packaging, and many of these are sperm variant specific (Zalensky et al. 2002, Hammoud et al. 2009, Oliva and Mateo 2011). The remainder of the sperm DNA is tightly packed around protamines (Oliva and Dixon 1991). Importantly, studies have suggested that packaging of genes either by histones or protamines is not random (Oliva and Dixon 1991, Wykes and Krawetz 2003, Hammoud et al. 2009) suggesting a crucial role for histone modifications in sperm. Prenatal vinclozolin exposure may disrupt the methylation pattern of imprinted genes in sperm (Oliva and Dixon 1991, Wykes and Krawetz 2003, Hammoud et al. 2009), which may explain the transmission of epigenetic changes through the male germline.

It is important to note that in mouse, epigenetic marks such as DNA methylation are reset during development at two developmental stages: i) erasure: which occurs at around embryonic day 12 – 13 and, ii) re-establishment: which occurs only after sex determination of the offspring. In males, the re-establishment of epigenetic marks occurs during late fetal development whereas in females, it occurs predominantly after birth (Kafri et al. 1992, Szabó and Mann 1995, Reik and Walter 2001, Lee et al. 2002, Sasaki and Matsui 2008). There is a further wave of epigenetic reprogramming which occurs after fertilisation, when the paternal and maternal genomes both undergo a process of epigenetic reprogramming. In the paternal genome, this process has been shown to occur within 6 – 8 hours following fertilization, whereas that in the female genome occurs after several cleavage divisions (Mayer et al. 2000b). Importantly, epigenetic marks at imprinted loci escape this period of epigenetic reprogramming following fertilisation. In humans, the exact time of epigenetic marks

being erased and reset is currently unknown, but one recent study has suggest it to be different from that of rodents and also to differ between genders (Wermann et al. 2010).

Nonetheless, it has been shown that under certain conditions, such as vinclozolin exposure during pregnancy, defective epigenetic marks may not be re-set, but may be carried through to subsequent generations (Anway et al. 2005, Stouder et al. 2009, Stouder and Paoloni-Giacobino 2010). The exact mechanisms of such resistance to reprogramming remains to be discovered (Youngson and Whitelaw 2008, Stouder and Paoloni-Giacobino 2010).

1.3.3 Genomic imprinting

Genomic imprinting is an epigenetic mechanism in which one allele of a gene is inactivated in a parent-of-origin specific manner. Imprinting of a gene is tissue specific, developmentally regulated, time point specific, and species specific (Fowden et al. 2006a). For example, the paternally expressed gene Igf2, is imprinted in most fetal tissue in mice and humans, but is bi-allelically expressed in brain (Tycko and Morison 2002); Igf2 has also been shown to be silenced in all somatic tissues in rodents by the time of weaning (Fowden 2003). Also, a maternally expressed organic cation transporter, Slc22a3, is imprinted in mice at embryonic day 11.5 in the placenta, but loses its imprinted status by day E15.5 (Zwart et al. 2001).

An interesting and unique feature of imprinted genes is that they are not randomly distributed across the genome, but are usually found in clusters (Reik and Walter 2001). For example, the two imprinted genes, CDKn1c and Phlda2 (discussed in

section 1.3.4.3 and 1.3.4.4 respectively) are both located on human chromosome 11p15 (mouse chromosome 7; rat chromosome 1) in the same locus as a number of other imprinted genes (Salas et al. 2004, Beatty et al. 2006). Each cluster of imprinted genes is regulated by a *cis*-element called the imprinting control region (ICR), which may itself be modulated by other elements (Edwards and Ferguson-Smith 2007).

Many imprinted genes are important in growth, either in the placenta or fetus or both (Moore and Haig 1991, Reik et al. 2002, Coan et al. 2005). According to the 'parental conflict' hypothesis (sometimes referred to as Haig's hypothesis or the kinship theory of imprinting), paternally expressed imprinted genes act to extract more nutrients from the mother to promote fetal growth (thus decreased expression of these may lead to IUGR); whilst maternally expressed genes act to restrict fetal growth, thus limiting nutrient supply to the fetus to protect the mother's own well-being (Moore and Haig 1991, Reik and Walter 2001). This theory has been backed up by evolutionary explanations (Haig 1993) suggesting that failure to regulate the distribution of the nutrient resources between the maternal needs and those of the fetus may have severe consequences for either, especially during periods when food is scarce. More nutrients taken by the fetus will result in a better chance of fetal survival, but this could cause harm to the mother; in contrast, if the mother preserves all the nutrients for herself, the chance of survival of the fetus decreases. However, the father in this case will not be harmed in either situation, therefore it is in the best interest of the father to promote growth of the fetus to increase the chance of fetal survival so that it can pass on the paternal genome (Haig 1993, Haig 2000). This theory has also been supported by the distribution of imprinted genes in specific

branches of the phylogenetic tree (Reik et al. 2002). It appears that genes that have been found to be imprinted in mammals are not imprinted in egg laying animals (monotremes and birds) (Reik et al. 2002). Further support for this theory can be derived from data showing that the disruption of the key paternally expressed imprinted gene insulin-like growth factor 2 (Igf2) causes growth deficiency (DeChiara et al. 1991, Constância et al. 2002), whereas disruption of the maternally expressed gene Igf2r (which sequesters Igf2) results in fetal overgrowth (Lau et al. 1994). Examples of some imprinted genes important in fetal and placental growth are discussed in detail below.

1.3.4 Imprinted genes important in growth:

1.3.4.1 Insulin-like growth factor 2 (Igf2)

Insulin-like growth factors (Igf) are probably the most well characterised genes in the development of the placenta and fetus. There are two main forms of Igf, namely Igf1 (which is not imprinted) and Igf2. Igf2 in particular plays an important role in placental and fetal development. It is a maternally imprinted gene that is expressed from the paternal allele in most fetal tissues and placenta, but is bi-allelically expressed in the brain (Tycko and Morison 2002). Igf2 is known to be imprinted during fetal life and gradually loses its imprinting status during ageing into adulthood. There are four promoters regulating the tissue-specificity of Igf2 expression (P0, P1, P2, and P3), with the P0 promoter being most important in the placental labyrinth (Moore et al. 1997, Constância et al. 2002). Decreased Igf2 expression results in placental and fetal growth restriction (Baker et al. 1993, Constância et al. 2002, Fowden et al. 2006a). Notably, the Igf2 gene in ovine liver has a glucocorticoid response element (GRE) on one of the promoter regions, suggesting that

glucocorticoids may regulate Igf2 transcription (Li et al. 1998).

1.3.4.2 Grb10

Grb10 (growth receptor bound protein 10) encodes a protein which interacts with receptor tyrosine kinases. Grb10 is a maternally expressed gene in most tissues including the placenta, however, it has been shown to be paternally expressed in the brain (Monk et al. 2009). The exact function of this gene is not clearly characterised, but it has been shown to cause placental and fetal overgrowth when disrupted, suggesting it has a role as a growth suppressant (Charalambous et al. 2003).

1.3.4.3 Cyclin dependent kinase inhibitor (CDKn)

Cyclin dependent kinase inhibitor 1c (CDKn1c) or $p57^{kip2}$ is a tumour suppressor gene that is maternally expressed. It is a cell cycle regulator and is known to arrest cells in the G1 phase when over expressed. In humans, CDKn1c disruption has been linked to various tumours and is also thought to be important in Beckwith-Wiedemann syndrome, a condition associated with prenatal overgrowth (Takahashi et al. 2000). CDKn1c deficient mice develop placentomegaly (Hatada et al. 1996), confirming its importance in the control of development of the placenta. A GRE is located about 5kb upstream of the promoter region of CDKn1c gene in humans (Fitzpatrick et al. 2002).

1.3.4.4 Pleckstrin homology-like domain family A (Phlda)

Phlda member 2 (Phlda2 or IPL = “imprinted in placenta and liver”) is a tumour suppressor gene expressed maternally in a region along with various other tumour suppressor genes (including CDKn1c). The expression of Phlda2 along with CDKn1c is controlled by DNA methylation in a region known as KvDMR1. The

KvDMR1 is an imprinting control region and methylation on the maternal allele is associated with expression of CDKn1c and Phlda2 from the maternal allele (Fitzpatrick et al. 2007, Shin et al. 2008).

Deletion of the Phlda2 gene has been shown to lead to the development of placentomegaly with specific enlargement of the junctional zone (Frank et al. 2002, Salas et al. 2004) and recent data suggests it has an important function in the regulation of glycogen storage in the junctional zone of the placenta (Tunster et al. 2010). Phlda2 over-expression is associated with a significant reduction in glycogen storage and a reduction in the size of the junctional zone (Tunster et al. 2010).

1.3.4.5 Mesoderm specific transcript homolog (Mest/PEG1)

Mest is a maternally imprinted gene (paternally expressed) that is abundantly expressed in mesodermal derivatives. The function of this gene is unknown, however, sequence similarities with the α/β-hydrolase fold family suggest an enzymatic role (Kaneko-Ishino et al. 1995). Although its function is largely unknown, studies have suggested a role for Mest in fetal growth (Lefebvre et al. 1998) and angiogenesis in the placenta (Mayer et al. 2000a).

1.4 The placenta

1.4.1 The role of the placenta in early life programming

The placenta connects the mother to the fetus, thereby providing the fetus with oxygen and nutrients, removing waste products, and acting as an immunological barrier between the mother and the fetus. Complications in placental development can lead to pregnancy loss and other pregnancy related problems such as preeclampsia (Kaufmann et al. 2003), as well as future offspring developmental problems (Soares et al. 1996, Fowden et al. 2006a). Birth weight is closely related to the morphology of and nutrient transport across the placenta (Fowden and Forhead 2004). Since low birth weight is linked to various adulthood diseases as discussed previously this highlights a potentially important role of the placenta during development.

The rodent placenta, like the human placenta, is hemochorial in formation (Figure 1.5) (Ain et al. 2006), meaning the fetal chorion is bathed in maternal blood by direct contact (Rossant and Cross 2001). It is formed by the fusion of two tissues, the allantois derived from extra embryonic mesoderm and the extra embryonic chorion (Rinkenberger and Werb 2000). Shortly after the fusion of these two tissues, blood vessels grow in from the allantois to form the fetal compartment of the placental vascular network (Rossant and Cross 2001). Thereafter, the trophoblast invades and further branching of the fetal blood vessels occurs, forming the labyrinth zone; this formation helps increase the area of contact with maternal blood (Peel and Bulmer 1977). This is similar to the chorionic villi in humans (Rinkenberger and Werb 2000). Although there are some similarities between human and rodent placentas, some fundamental differences exist. For example, human placentas only have two layers: a

syncytial layer plus a trophoblast stem cell layer; compared to the three trophoblast cell layers (trichorial) of the rodent placenta (Rossant and Cross 2001).

As described above, the rodent placenta is divided grossly into two layers, namely the junctional zone (also known as the spongiotrophoblast layer) on the maternal facing surface, and the labyrinth layer on the fetal facing surface. The labyrinth layer contains numerous important nutrient transporters, including the system A amino acid (e.g. Slc38a1, Slc38a2, Slc38a4) and glucose transporters (e.g. Glut1/Slc2a1, Glut3/Slc2a3). The precise function of the junctional zone is largely unknown, although it is involved in invasion (Soares et al. 1996), hormone secretion (such as prolactin-like hormones and placental lactogen), the production of cytokines which help maintain the secretion of progesterone by the corpus luteum (Coan et al. 2006, Tunster et al. 2010) and glycogen storage by glycogen cells (Tunster et al. 2010).

The placenta appears to play a critical role in programming. Examples of its importance include studies in sheep which show different programming effects of glucocorticoids depending on whether they are administered to the mother or the fetus (Jobe et al. 1998a). Because of the close link between placental wellbeing and fetal health and growth, much recent interest has focused on the role of placental development in mediating programming effects.

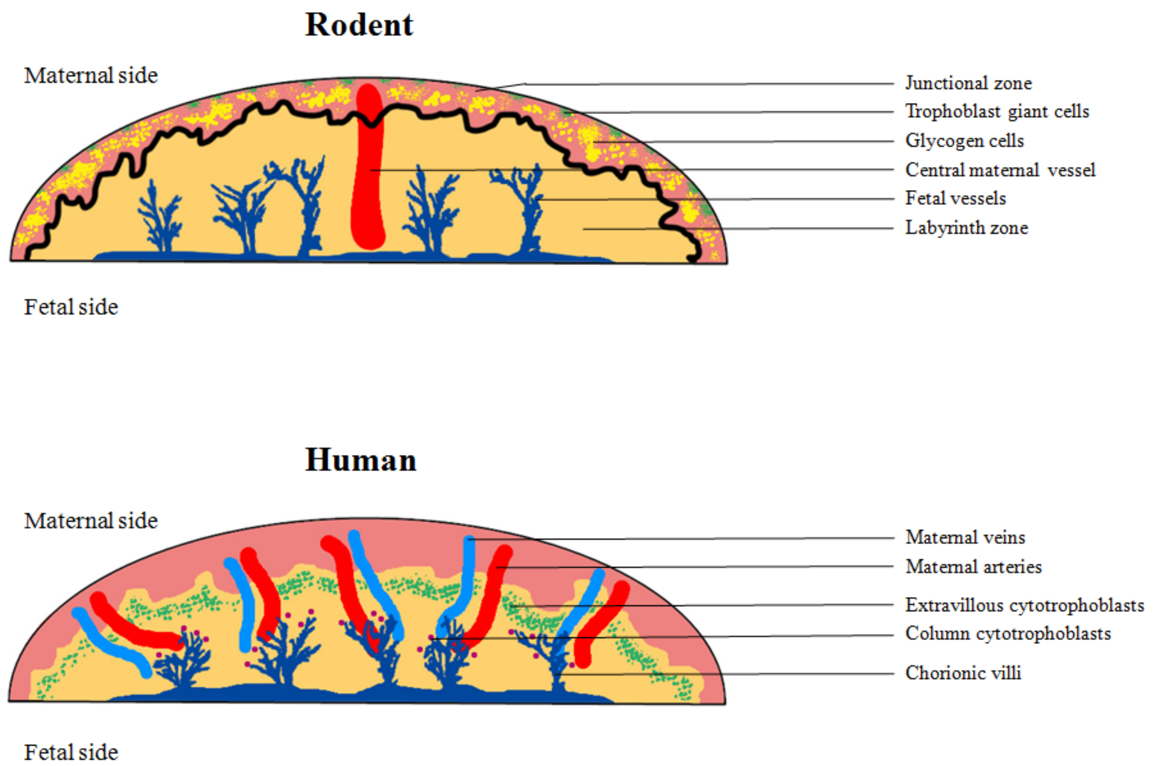


Figure 1.5 Schematic of rodent and human placenta

This shows the similarity and differences between a rodent and human placenta

1.4.2 Nutrient transporters

Nutrients transported across the placenta are directly related to the growth of the fetus. As pregnancy progresses towards the end of gestation, the fetus grows at a rate much faster than the placenta (Sands and Dobbing 1985), therefore, it is reasonable to assume that the nutrient transporting mechanisms may be up-regulated towards the end of pregnancy (Desforges and Sibley 2010). The majority of nutrients pass through the placenta by passive diffusion, which is broadly divided into the lipophilic route by which lipid soluble molecules cross the placenta and the hydrophilic route by which lipid insoluble molecules cross the placenta (Desforges and Sibley 2010). Because hydrophilic molecules do not pass easily through the lipid bilayer (Dobson and Kell 2008), hydrophilic molecules are also transported through specialised membrane transporters. These transporters may transfer nutrients across the plasma membrane by means of passive diffusion or against the concentration gradient (Desforges and Sibley 2010). However, it should be noted that other determinants such as blood flow, contact surface area of placenta and maternal blood spaces, hormones, pH, and oxygen tension are also important in the regulation of nutrient transport (Jones et al. 2007, Desforges and Sibley 2010).

1.4.2.1 Glucose transporters (Glut)

Glucose oxidation is a major source of energy in living organisms. Glucose is transported into a cell by one of two ways: down the concentration gradient or against the concentration gradient by Na^{+}- dependent transporters (such as the Gluts), a number of which have been identified. Glut1 is mainly found at the blood-tissue barrier such as the blood-brain-barrier (Kalaria et al. 1988) or the placental barrier (Takata et al. 1992) but numerous studies have identified its presence in different

tissues (Gould and Holman 1993). Glut3 has been suggested to be found mostly in tissues with a large glucose demand, such as the brain, but it is also present in the placenta (Gould and Holman 1993). In the rodent placenta, immunohistochemistry studies have shown the presence of both Glut1 and Glut3 in the placental labyrinth (Takata et al. 1994, Shin et al. 1997), but only Glut1 in the junctional layer (Shin et al. 1997). In the human placenta, Glut1 is seen mainly in syncytiotrophoblast cells at term and Glut3 is present at lower amounts in both syncytiotrophoblast cells and non-syncytial cells (Jansson et al. 1995). Although both Glut1 and Glut3 act as glucose transporters, their action seems to differ in the placenta. Zhou et al. showed that in the placenta, Glut1 acts mainly to provide glucose to the placenta itself from the maternal circulation as fuel, whereas Glut3 acts mainly to provide glucose to the fetus from the placenta (Zhou and Bondy 1993).

Interestingly, despite the known importance of Gluts in glucose transport, the expression and activity of Gluts are unaltered in human placenta from growth restricted fetuses (Jansson et al. 1993, Jansson et al. 2002, Desforges and Sibley 2010). In contrast, in human fetal overgrowth, transport of glucose is shown to be increased in the basement membrane but unchanged in the microvillous membrane (Jansson 2001, Desforges and Sibley 2010).

In rodent models of glucocorticoid exposure, Dex treatment in late gestation increases the expression of Glut1 and 3 (Langdown and Sugden 2001), whereas, treatment by triamcinolone leads to decreased expression (Hahn et al. 1999). In a model of maternal high fat feeding and obesity the activity of the glucose transporters was found to be significantly enhanced (Jones et al. 2009).

1.4.2.2 System A amino acid transporters (Slc38)

Amino acids are essential for the growing fetus, in which they help in laying down protein as well as being a major source for energy (Battaglia and Meschia 1978). The system A amino acid transporters are also known as Na^+- dependent neutral amino acids transporters (SNAT). The main purpose of these transporters is the transport of non-essential neutral amino acids such as alanine, glycine, and glutamine (Jansson et al. 2002, Desforges and Sibley 2010). mRNA encoding the three SNATs (Slc38a1, Slc38a2, and Slc38a4) has been shown to be present in the rodent placenta and immunohistochemistry studies have shown both Slc38a1 and Slc38a2 to be present in the placental labyrinth in the final third of gestation (Novak et al. 2006). Studies in rats have shown that fetuses of mothers fed protein restricted diets have a decreased fetal concentration of amino acids despite normal levels of amino acid in the mother, suggesting a decrease in the amino acid transport through the placenta (Malandro et al. 1996). Whilst some studies in humans have shown reduced expression of SNATs in the placenta of small for gestational age and IUGR babies (Jansson et al. 2002, Shibata et al. 2008); studies in mice suggest that expression is up-regulated in smaller placentas, suggesting that the placenta can enhance its nutritional transfer capability to meet fetal demands (Coan et al. 2008a). In the 11β-HSD2 knockout mouse, which has decreased fetal and placental size, expression of SNATs was also found to be up-regulated (Wyrwoll et al. 2009). *In vitro* studies suggest that Dex exposure in human placental villous tissues does not increase the mRNA expression of SNATs but does result in increased function along with syncytiotrophoblast differentiation and maturation (Audette et al. 2010). In mice, Dex treatment at midgestation had no effect on SNAT mRNA expression in placental labyrinth, but decreased system A mediated transport at the end of gestation (Audette et al. 2011).

In a rodent model of high fat feeding leading to maternal obesity, SNAT transport capacity was found to be increased (Jones et al. 2009). These studies suggest the importance of SNATs in the placenta and fetal development and demonstrate that manipulation of the prenatal environment can impact on placental amino acid transport. One of the SNATs, Slc38a4, has been shown to be imprinted and paternally expressed in mice.

1.5 The Insulin-like Growth Factor pathway

Insulin-like growth factors 1 and 2 play crucial roles in promoting fetal and placental growth and may have a role in programming effects. Insulin-like growth factors 1 and 2 are single chain polypeptides which can be found normally in circulating plasma and at a slightly reduced level in tissues (Humbel 1990). It has been suggested that most of the circulating Igfs are produced from the liver, whereas Igfs found in the tissues are suggested to be paracrine/autocrine in action (Humbel 1990, DeChiara et al. 1991). Throughout development, growth hormone (GH), Igf1 and Igf2 play crucial role in growth, from the fetus to adulthood. Igf2 is produced at a higher levels during fetal life, these levels gradually decrease postnatally; in contrast, Igf1 is produced in a much lower level during fetal life and gradually increases postnatally (DeChiara et al. 1991). During fetal life, GH may have very little influence on fetal growth (Randhawa and Cohen 2005).

The actions of both Igf1 and Igf2 are exerted by binding to the insulin-like growth factor 1 receptor (Igf1r) (Forbes and Westwood 2008). Thereafter, signalling through a cascade of downstream proteins results in cellular growth, translation of genes, and inhibition of apoptosis (Figure 1.6). Igf1 binds exclusively to Igf1r (a cellular membrane tyrosine kinase receptor) whereas Igf2 binds to both Igf1r and the insulin-like growth factor 2 receptor (Igf2r), binding with greater affinity to the latter. Once bound to Igf2r, Igf2 is thought to be inactivated (Louvi et al. 1997, Randhawa and Cohen 2005) although studies have also shown that when Igf2 binds to Igf2r, it can exert its effects on trophoblast cells to stimulate migration and invasion (McKinnon et al. 2001) and on blood vessels to stimulate angiogenesis and vascular remodelling (Herr et al. 2003). Additionally, during embryogenesis in mice, Igf2 can

act in part through the insulin receptor (IR) and therefore exert its effect to promote growth (Louvi et al. 1997). Although both Igf1 and Igf2 interact with Igf1r to exert their effects, these effects may differ. Igf1 infusions in pregnant rodents resulted in an increase in fetal growth but no difference in placental growth (Gluckman et al. 1992). As Igf1 does not cross the placenta (Davenport et al. 1990), it has been suggested that Igf1 exerts its effect on the nutrient transport across the placenta and affects the fetus indirectly. In contrast, Igf2 given to guinea pigs during early to mid-gestation increased placental size, thereby providing more nutrition to the developing fetus and enhancing growth (Sferruzzi-Perri et al. 2006).

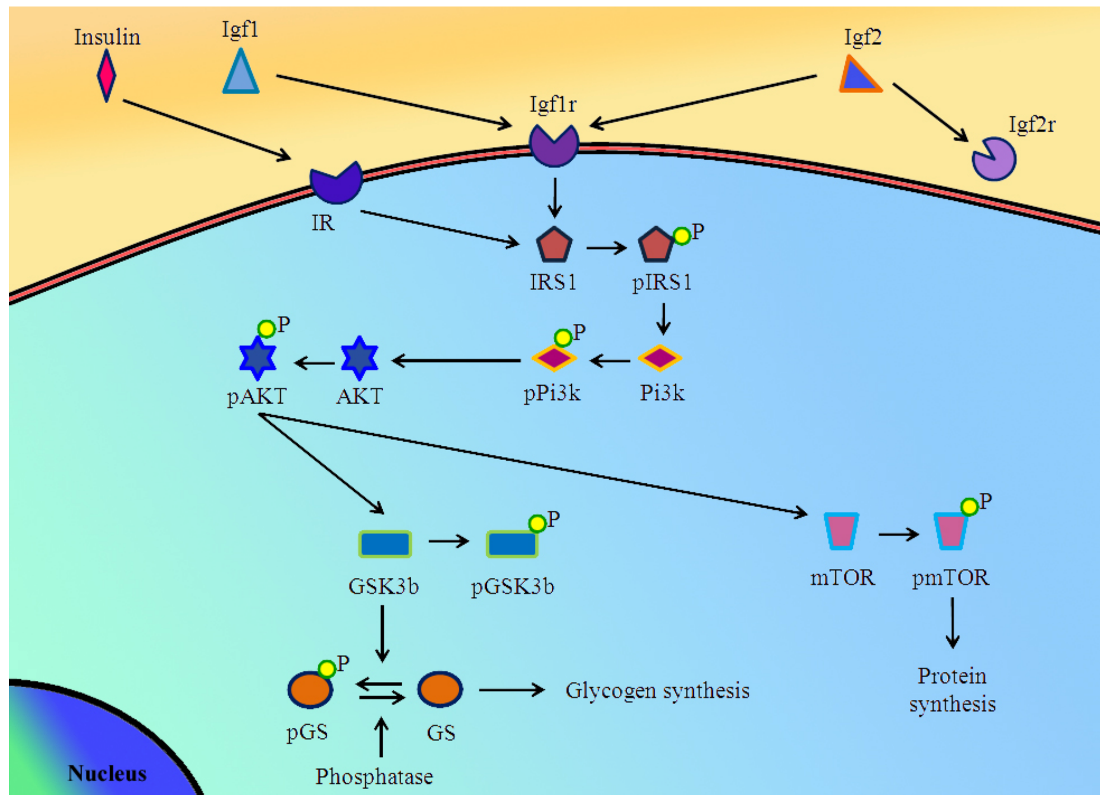


Figure 1.6 Diagrammatic representation of the Igf signalling pathway

Igf1 = Insulin-like growth factor 1; Igf2 = Insulin-like growth factor 2; Igf1r = Igf1 receptor; Igf2r = Igf2 receptor; P = Phospholation; IR = Insulin receptor; IRS1 = Insulin receptor substrate 1; pIRS1 = Phosphorylated IRS1; Pi3k = Phosphoinositide 3-kinase; pPi3k = Phosphorylated Pi3k; GSK3β = Glycogen synthase kinase-3β; pGSK3β = Phosphorylated GSK3β; mTOR = Mammalian target of rapamycin; pmTOR = Phosphorylated mTOR; GS = Glycogen synthase; pGS = Phosphorylated GS

Circulating Igfs are mostly bound to insulin-like growth factor binding proteins (IgfBP). There are six known IgfBPs (IgfBP1 – IgfBP6) which have higher affinity for the Igfs than Igf1r (Hwa et al. 1999, Forbes and Westwood 2008). When Igfs are bound to IgfBPs, their effects may either be inhibited or enhanced depending on the role of IgfBP in the tissue as well as its molecular status (eg. phosphorylated vs non-phosphorylated) (Firth and Baxter 2002). IgfBPs serve not only to transport Igfs in the circulation, but also prolong Igf half-life (Forbes and Westwood 2008).

1.5.1 Insulin receptor substrate – 1 (IRS1)

After Igf1 or Igf2 binds to Igf1r, or following stimulation of the insulin receptor by insulin, a tyrosine phosphorylation reaction occurs at the IRS1 protein leading to its activation. IRS1 is a protein with a size of about 165 – 185kDa which has at least ten potential tyrosine phosphorylation sites (Sun et al. 1991, Saad et al. 1992). IRS1 acts primarily as a docking protein between the receptor and a variety of Src homology-2 (SH2) containing proteins, such as phosphoinositide 3-kinase (Pi3K) and growth factor receptor bound protein 2 (Grb2) (Saad et al. 1992). However, only specific site (Tyr612) phosphorylation on the IRS1 protein can lead to Pi3K activation and growth (Esposito et al. 2001).

1.5.2 Phosphoinositide 3-Kinase (Pi3k)

After activation of IRS1, Pi3k catalyses the production of phosphatidylinositol-3,4,5-triphosphate (PIP_3) which functions to co-ordinate cellular growth and survival (Cantley 2002). The Pi3K protein consists of two subunits; the regulatory subunit at the p85 position and the catalytic subunit at the p110 position (Carpenter et al. 1990, Backer et al. 1992). The activation of Pi3k by phosphorylation

results in the stimulation of downstream proteins, such as Akt and PDK1 through PIP_3 to stimulate cellular growth, protein synthesis and survival (Cantley 2002).

1.5.3 Akt

Akt (which is also referred to as protein kinase B (PKB)) is homologous to the PKA and PKC family of protein kinases (Franke et al. 1997). Akt plays a central role in the regulation of growth, inhibition of apoptosis, and protein synthesis in part through the factors that activate Pi3K (Franke et al. 1995, Andjelković et al. 1996). There are two main residues where Akt can be phosphorylated, namely: threonine 308 (Thr308) and serine 473 (Ser473) (Alessi et al. 1996). When phosphorylation of either of the residues occurs, the protein becomes activated (Alessi et al. 1996). There are debates as to whether the phosphorylation of one of the residues is more important than the other (Sarbassov et al. 2005); but evidence suggests that Ser473 phosphorylation may be more important that Thr308 phosphorylation (Scheid et al. 2002, Sarbassov et al. 2005).

1.5.4 Mammalian target of rapamycin (mTOR)

Mammalian target of rapamycin (mTOR) is a Ser/Thr protein kinase which is important in protein synthesis, nutrient and energy sensing (Dennis et al. 1999, Dennis et al. 2001). Two phosphorylation sites have been identified on mTOR which can be triggered by the Pi3K/Akt pathway activation: threonine 2446 (Thr2446) and serine 2448 (Ser2448) (Navé et al. 1999, Hay and Sonenberg 2004); as well as a site of autophosphorylation at serine 2481 (Ser2481) (Navé et al. 1999). Studies have suggested a role for placental mTOR in the regulation of nutrient transporters (Edinger 2007, Roos et al. 2009, Rosario et al. 2011). In fact, mTOR activity in the

placenta has been shown to be reduced in human IUGR (Roos et al. 2007, Yung et al. 2008), suggesting the importance of this protein in fetal growth.

1.5.5 Glycogen Synthase Kinase-3β (GSK3β)

Glycogen synthase kinase 3 (GSK3) has been known for decades to be involved in the synthesis of glycogen via its action on glycogen synthase (GS), the rate limiting enzyme of glycogen biosynthesis (Welsh et al. 1996). Unlike most other kinases, when GSK3 is phosphorylated, it becomes inactive (Welsh et al. 1996). Under normal conditions, non-phosphorylated GSK3 acts to inhibit GS by phosphorylation; however, under certain conditions such as increased insulin levels, GS may be dephosphorylated leading to synthesis of glycogen (Parker et al. 1983). This may either be a direct effect of insulin on GS or may be through an effect of insulin to inhibit GSK3 through phosphorylation of the serine residue at position 9 (Ser9) (Cross et al. 1995).

1.6 Hypothesis and aims:

In the dexamethasone-programmed rat model:

I hypothesise that overexposure to glucocorticoids in utero during the last week of gestation will induce programming effects in the developing offspring and that these programming effects may be passed down to the second generation but that the mechanisms of programming underlying the phenotype in the two generations will be different. I will therefore explore the effects of prenatal glucocorticoid overexposure on fetal and placental growth as well as on the expression of genes known to be important in fetal and placental growth in both F1 and F2 offspring. I will concentrate particularly on the expression of imprinted and various growth related genes as well as the developing placenta. I will also explore the Igf pathway in F1 placenta and liver.

In the maternal obesity mouse model:

I hypothesise that maternal obesity will program the offspring and grand offspring and that the programming effects will be generation and gender specific. I will describe programming effects on the F1 and F2 offspring, and explore the expression of genes that are important in metabolism.

This study will be aimed at exploring the transmission of programming effects to subsequent generations in two different animal models.

CHAPTER 2

Materials and methods

2.1 Materials

Unless otherwise stated, all general chemicals and solutions were purchased from Sigma-Aldrich Company Ltd, Dorset, UK.

Room temperature (RT°) is defined as 18-22°C.

2.1.1 General chemicals/solutions/kits

Supplier	Product
VWR International, Leicestershire, UK	Ethanol
	Methanol
Sigma-Aldrich Ltd., Dorset, UK	Neutral Buffered Formalin (10%)
	Harris Haemotoxylin
	Eosin-Y
	Ponceau-S solution
Promega Ltd., Southampton, UK	Reverse transcription enzymes
	Bovine serum albumin
Thermo Fisher Scientific, Leicestershire, UK	Infinity™ glucose hexokinase
	Infinity™ triglyceride reagents
	Infinity™ cholesterol reagents
	Ammonia solution
	Xylene
Starstedt, Leicester, UK	Microvette® CB-300 EDTA tubes
Alpha Laboratories, Hampshire, UK	NEFA kit
Crystal Chem Inc., Illinois, USA	Insulin ELISA kit

2.1.2 Molecular biology

2.1.2.1 Ribonucleic Acid

Qiagen, West Sussex, UK	RNeasy® mini kit
	QIAzol® lysis reagent
	RNase-free DNase set
Invitrogen, Paisley, UK	Primers
Applied Biosystems, Warrington, UK	TaqMan™ primers and probes
Promega Ltd., Southampton, UK	1kb DNA ladder
	PCR Master Mix
Roche Diagnostics Ltd., West Sussex, UK	SYBR® Green I Master
	LightCycler® 480 probes master
Lonza, Maine, USA	Agarose powder
Biotium Inc., California, USA	Gel-Red™ nucleic acid stain

2.1.2.2 Western blotting

Supplier	Item
Roche Diagnostics Ltd., West Sussex, UK	Protease inhibitor cocktail tablet
Bio-Rad, Hertfordshire, UK	Protein assay solutions
	Kaleidoscope™ protein ladder
	Blotting grade blocker non fat dry milk
GE Healthcare, Buckinghamshire, UK	Hybond™-C Extra nitrocellulose membrane, 0.45µm
Whatman Ltd, Kent, UK	Filter paper, 3mm
Invitrogen, Paisley, UK	NuPAGE® Novex 4-12% Bis-Tris gel
	NuPAGE® MOPS SDS running buffer (20x)
	NuPAGE® transfer buffer (20x)
	NuPAGE® sample reducing agent (10x)
	NuPAGE® LDS sample buffer (4x)
	NuPAGE® antioxidant
	Alexa Fluor® 680 goat anti-rabbit IgG

2.1.3 Equipment

Supplier	Equipment
Thermo Fisher Scientific, Leicestershire, UK	ND1000 Nanodrop quantification spectrophotometer
	Microtome blade MB35
UVItec, Cambridge, UK	DBT-08 gel documentation system
Molecular Devices Ltd., Berkshire, UK	OPTImax plate reader
Eppendorf, Cambridge, UK	5810R Bench centrifuge for microplates
	5415D Bench centrifuge for RT°
	5415R Bench centrifuge for other temperatures
Biochrom Ltd., Cambridge, UK	Microplate washer
Grant Instruments Ltd., Cambridge, UK	SS40-2 Shaking water bath
PRO Scientific, Connecticut, USA	PRO200 Homogeniser
Electrothermal, Essex, UK	Paraffin section mounting bath
Leica Microsystems Ltd., Buck, UK	Rotary microtome
Invitrogen, Paisley, UK	XCell Surelock™ western blot tank
Photometrics Ltd., Buckinghamshire, UK	CoolSNAP CCD camera
CRi, Massachusetts, USA	Micro*Color RGB crystal filter
LI-COR Biosciences LTd., Cambridge, UK	Odyssey® Infrared Imaging system
G-Storm Ltd., Somerset, UK	GS1 Thermocycler
Roche Diagnostics Ltd., West Sussex, UK	LightCycler® 480

2.1.4 Statistical analysis

The data were analysed using SPSS® (IBM Ltd., New York, USA) and GraphPad® (GraphPad Software, California, USA). A Student's *t*-test was used when the data is normally distributed and has one variable. A Mann-Whitney *u*-test was used when the data has one variable but not normally distributed as tested by skewness. A two-way ANOVA was used when two individual variables were present. A Chi-square test was used to determine the statistical significance of an expected and observed outcome. A Pearson's correlation was used when determining the statistical correlation between two variables. Results were considered significant if $p<0.05$.

2.2 Buffers and solutions

10x TBE Buffer: Tris base (108g), boric acid (55g), and 40ml of 0.5M EDTA were dissolved in 800ml of Milli-Q water. pH was adjusted to 8.0 by adding 10M KOH and thereafter, the volume was adjusted to 1L with Milli-Q water. The buffer was autoclaved and kept at RT°.

0.5x TBE Buffer: 50ml of 10x TBE was diluted in 950ml of Milli-Q water and stored at RT°.

10x TBS Buffer: Tris base (30g), KCl (2g), and NaCl (88g) were dissolved in 800ml of Milli-Q water. The pH was adjusted to 7.4 with 37% HCl, thereafter, volumes were adjusted to 1L with Milli-Q water and stored at RT°.

1x TBS Buffer: 10ml of 10x TBS buffer was diluted with 90ml of Milli-Q water and stored at RT°.

1x TBST Buffer: 100ml of 10x TBS was diluted in 900ml of Milli-Q water and 1ml Tween-20 was added, the solution was mixed gently and stored at RT°.

2x Protein lysis buffer: 50ml of 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), NaCl (8g), $CaCl_2$ (0.15g), $MgCl_2$ (0.2g), NaF (0.42g), EDTA (0.37g) and sodium pyrophosphate ($Na_2H_2P_2O_7$, 2.29g) were dissolved in 1L of Milli-Q water and stored at 4°C.

1x Protein lysis buffer: 5ml of 2x protein lysis buffer, 1ml NP40 (10% v/v), 1ml glycerol, 0.2ml $NaVO_4$ (100mM), 1 protease inhibitor cocktail tablet were dissolved in 2.8ml of Milli-Q water. This buffer was prepared immediately prior to use.

5% Blocking milk: 2.5g of blotting-grade blocker, non-fat, dry milk powder were dissolved in 50ml of 1x TBST and stored at 4°C. This was prepared immediately prior to use.

5% BSA: 0.5g of bovine serum albumin (BSA, Fraction V) was dissolved in 10ml of 1x TBST and stored at 4°C. This was prepared immediately prior to use.

1x Running Buffer: 50ml of 20x NuPAGE® MOPS-SDS running buffer was diluted in 950ml of Milli-Q water.

1x Transfer Buffer:

For 1 gel transfer: 25ml of 20x NuPAGE® transfer buffer were diluted in 425ml of Milli-Q water and 50ml of methanol. 500µl of NuPAGE® Antioxidant was added prior to use.

For 2 gel transfer: 25ml of 20x NuPAGE® transfer buffer was diluted in 375ml of Milli-Q water and 100ml of methanol. 500µl of NuPAGE® Antioxidant was added prior to use.

Alkaline tap water: 3 drops of ammonia were added to 400ml of tap water.

2.3 Animal maintenance

All animal procedures were carried out under the Project License number 60/3962 and personal licence number 60/12181 under the terms of the Animals (Scientific Procedures) Act 1986. William Mungall and Jon Hendersson were responsible for the daily maintenance of rats and mice respectively in the BRF Little France facility; Ian McCall, David Read, and Bill Smith were responsible for the daily maintenance of mice in the BRF Western General Hospital facility. Animals were maintained in a controlled environment with set humidity (55%), temperature (22°C), and light/dark cycle (lights on 12 hours per day from 07:00 to 19:00) in both units. Animals were kept at a maximum of five per cage and cages were cleaned weekly.

2.3.1 Rats

Female Wistar rats were bought from Harlan, UK Ltd. Female rats were purchased for mating at a weight of 200-250g; in house male rats of equivalent age were used for mating. Standard BRF laboratory diets, CRM, were used and were supplied by Special Diet Services (Witham, Essex, UK). Filter water was used and animals were allowed *ad libitum* access to food and water.

2.3.2 Mice

In house wild type C57Bl/6 mice were used, and were maintained on standard BRF laboratory diets, RM1, unless otherwise stated. The standard laboratory diets were supplied by Special Diet Services. Experimental control (CON) and diet-induced obesogenic (DIO) diets were purchased from Research Diets (New Brunswick, NJ, USA). Filter water was used and animals were allowed *ad libitum* access to food and water.

2.4 Rat

2.4.1 F1 offspring production

Females were allowed to acclimatize for two weeks after arrival at the BRF. At mating, a female was singly housed with a wild type male of the similar age. The day when the expelled vaginal plugs was noted was designated as embryonic day zero of the pregnancy (E0). From this day on, Pregnant females were group housed with animals from the same group at a maximum of five animals per cage until last week of pregnancy after which females were singly housed. Between E15 and E21, pregnant females were weighed daily in the morning before 09:30 and given a subcutaneous injection of either 100μg/kg of dexamethasone mixed in 4% ethanol and 0.9% normal saline (Dex) or an equivalent volume of 4% ethanol mixed with 0.9% normal saline (Veh).

Groups of pregnant females were killed at E20 or left to litter naturally which occurred between days E21 – E23. At E20, females were sacrificed by carbon dioxide asphyxiation followed by cervical dislocation in the morning (between 09:20 – 12:00) at least one hour after the final injection. Food and water were not withdrawn prior to sacrificing. Fetal and placental tissues were dissected, weighed and snap frozen on dry ice immediately and were stored at -80°C until use. Some placentas were randomly selected from each litter to be placed in a glass bijou with 10% neutral buffered formalin (Sigma-Aldrich). The placentas were stored at 4°C overnight and were transferred into 70% ethanol and kept at RT° until use. Trunk blood was collected via the inferior vena cava or cardiac puncture with a 2ml syringe rinsed with 0.5mM EDTA, and then transferred to an Eppendorf™ tube and placed on wet ice. Fetal blood was collected using EDTA coated collection tubes

Microvette® CB-300 (Sarstedt) after decapitation. The collected blood was placed on wet ice and centrifuged as soon as possible at 5000rpm for 10 minutes at 4°C, the supernatant plasma was then transferred into a new Eppendorf™ tube and stored at -20°C.

For females which littered naturally, pups were weighed, sexed and culled back to eight per litter on the day of delivery with a similar number of males and females kept for each litter. Pups were reared by their mother until three weeks of age and were then weaned. Pups were then housed randomly with littermates of a same age from the same treatment group. Animals were selected at random and from as many different litters as possible for subsequent experiments.

2.4.2 F2 offspring production

At three months of age, F1 animals were mated in all combinations (Veh female x Veh male, Dex female x Dex male, Dex female x Veh male and Veh female x Dex male) to give four groups of F2 litters (VV, DD, DV and VD) (Figure 2.1). Pregnant females were group housed with animals from the same group at a maximum of five animals per cage until last week of pregnancy after which females were singly housed. Females were killed and tissues collected at E20. The animals were sacrificed using the same method as described above for F1 animals. Tissues were collected and stored as described above.

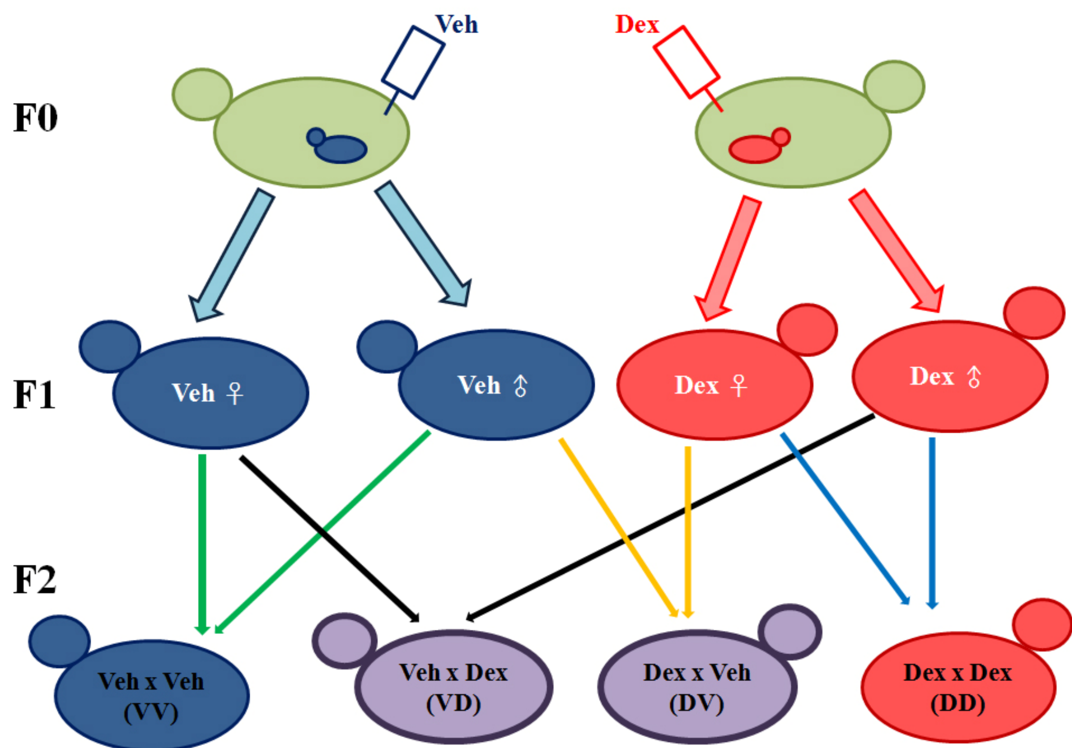


Figure 2.1 Schematic diagram of the glucocorticoid programmed rat experimental protocol

2.5 Mice

2.5.1 Production of F1 offspring

In house C57Bl/6 wild type mice were reared by their biological mothers on standard laboratory chow (RM1) and were weaned at three weeks of age onto standard laboratory chow. At five weeks of age, these F0 females were randomly assigned to either CON or DIO diets for 12 weeks. At 17 weeks of age, F0 CON and DIO females were singly housed with a stud male in the afternoon, remaining on their assigned diet. Vaginal plugs were checked in the morning, and when found, the male was removed from cage and the day was designated as E0 of pregnancy. Pregnant females were singly housed until delivery which occurred between E19 and E21 and were weighed at E10 and E18. Females were maintained on their designated diets through pregnancy and lactation. At delivery, F1 CON and F1 DIO pups were weighed, sexed, and culled back to five per litter with similar numbers of male and female kept in each litter. F1 offspring were weaned onto standard laboratory chow at three weeks of age.

2.5.2 Production of F2 offspring

F1 males and females were allowed to grow until three months of age, and then mated in all combinations (F1 CON female x F1 CON male, F1 DIO female x F1 DIO male, F1 DIO female x F1 CON male and F1 CON female x F1 DIO male) Pregnant females were singly housed until delivery which occurred between E19 and E21 and were weighed at E10 and E18. At delivery, F2 pups (designated CC, DD, DC and CD) were weighed, sexed, and culled back to five per litter with two males and three females or vice versa kept in each litter.

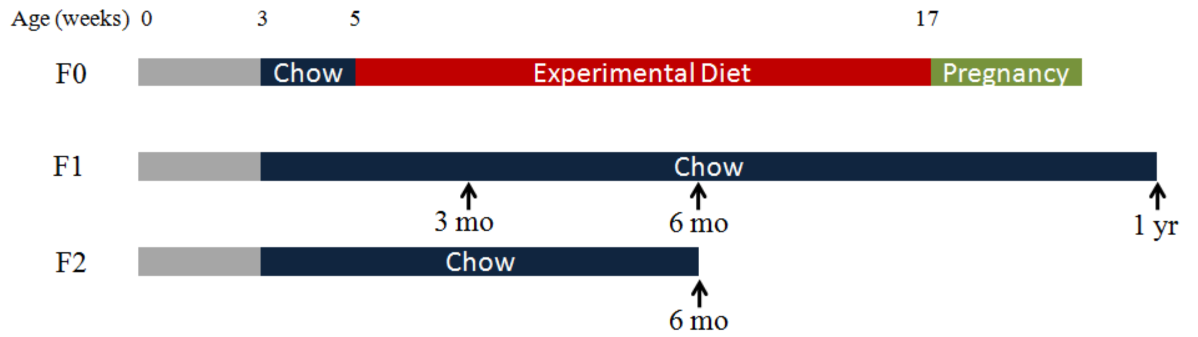


Figure 2.2 Schematic diagram showing the duration of exposure to experimental diets in F0 mice and time points of experimental procedures in F1 and F2 offspring

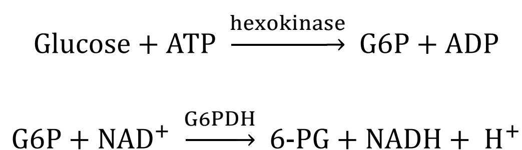
2.6 Biochemical assays

2.6.1 Measurement of plasma insulin concentrations

Plasma insulin concentrations were measured using the ultra sensitive rat insulin ELISA kit for rat and ultra sensitive mouse insulin ELISA kit for mice (Crystal Chem Inc., Illinois, USA). The manufacturer reports 100% reactivity to rat and mouse insulin as well as an intra- and inter-assay coefficient of variation (CV) of ≤10%. The manufacturer's instructions for the low range assay (able to measure insulin levels between 0.1 – 6.4 ng/ml) were followed accordingly for both rats and mice. Samples were loaded as 5µl per well in singlicate and standard in duplicates along with 95µl of sample diluent. Each well was pre-coated with primary guinea pig anti-insulin antibody. For the blank negative, sample diluent was used. The plate was then incubated at 4°C for 2 hours followed by five washes with supplied wash buffer to remove excess insulin not bound to the plate. This was then followed by the addition of 100µl of horse-radish peroxidase conjugated anti-insulin antibody and incubated at RT° for 30 minutes. The plate was washed again seven times with wash buffer to remove excess anti-insulin antibody. 100µl of enzyme substrate containing tetramethylbenzidine was then added to each well and incubated in the dark for 40 minutes at RT°. This was then followed by the addition of 100µl of reaction enzyme stop solution. The plate was then read using a plate reader (Molecular Devices, Berkshire, UK) and the difference between the absorbances at 630nm and 450nm was measured. The assay was deemed satisfactory if the standard curve had an R^2 value >0.98.

2.6.2 Measurement of plasma glucose

The measurement of plasma glucose level was achieved by using Infinity™ glucose hexokinase reagent. Hexokinase acts to catalyse the phosphorylation of glucose by removing a phosphate from adeno-triphosphate (ATP) to form glucose-6-phosphate (G6P) and adeno-diphosphate (ADP). Glucose-6-phosphate then oxidises to become 6-phosphogluconate (6-PG) while reducing nicotinamide adenine dinucleotide from its oxidised form NAD^+ to NADH with the help of glucose-6-phosphate dehydrogenase (G6PDH) (Carroll et al. 1970). The amount of NADH formed is thus in direct proportion to the concentration of glucose in the plasma.



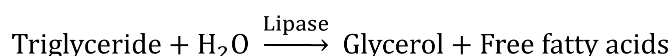
$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{G6P} + \text{ADP}$$

$$\text{G6P} + \text{NAD}^+ \xrightarrow{\text{G6PDH}} \text{6-PG} + \text{NADH} + \text{H}^+$$

Therefore, by measuring the absorbance of NADH at 340nm, it is possible to calculate the concentration of glucose. The concentration of glucose in each individual sample was calculated by setting a glucose standard (Thermo Fisher Scientific) of 0-400mg/dl in duplicate on each 96-well plate. 2µl of each plasma sample was used for the experiment. After the standard and sample had been loaded on the plate, 200µl of glucose hexokinase reagent were added to each well and incubated for 10 minutes at room temperature. Thereafter, by reading the UV absorbance of the standard glucose and sample, the concentration of each sample was calculated. The manufacturer reported an intra- and inter-assay CV of 1.6%.

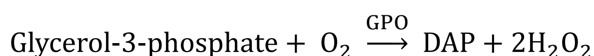
2.6.3 Measurement of plasma triglyceride

Plasma triglyceride concentrations were measured by using the Infinity™ triglyceride liquid stable reagent. The mechanisms of the reaction are as follow: The hydrolysis of triglyceride molecules by specific lipases results in glycerol and free fatty acid production. Glycerol is then phosphorylated by ATP into glycerol-3-phosphate and ADP by glycerol kinase (GK). Glycerol-3-phosphate is then oxidised to form dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2) by glycerolphosphate (GPO). The reaction of H_2O_2 with 4-aminoantipyrine (4-AAP) and 3,5-dichloro-2-hydroxybenzene sulfonate (3,5 DHBS) with peroxidase (POD) as catalyst produces a red coloured dye (Fossati and Prencipe 1982, McGowan et al. 1983). The absorbance of this dye correlates to the triglyceride level in the sample.

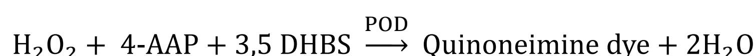


$$\text{Triglyceride} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$



$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{DAP} + 2H_2O_2$$

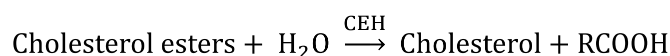


$$H_2O_2 + \text{4-AAP} + \text{3,5 DHBS} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 2H_2O$$

Standard curves were generated by serial dilution (0.565 – 7.91mmol/l) of triglyceride standard solution and Milli-Q water. Fasted plasma samples were loaded in singlicate at 2µl per well and standards in duplicate on a 96 well plate. 200µl of triglyceride reagent was added to each well and the plate incubated in dark at 37°C for 5 minutes. The difference between absorbance at 500nm and 600nm were calculated. A standard curve $R^2 > 0.98$ was deemed satisfactory. The manufacturer reported an intra- and inter-assay CV were reported of 2.1%.

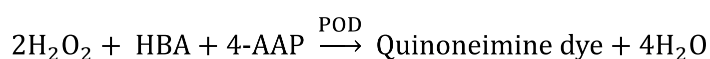
2.6.4 Measurement of plasma cholesterol

Plasma cholesterol concentration was measured by using Infinity™ total cholesterol reagent. The mechanism of reactions is similar to that of the measurement of plasma triglyceride. Cholesterol esters were hydrolyzed to cholesterol and fatty acids by the action of cholesterol ester hydrolase (CEH). Free cholesterols were then oxidised by cholesterol oxidase (CO) to cholest-4-en-3-one and hydrogen peroxide (H_2O_2). Hydrogen peroxide then combines with hydroxybenzonic acid (HBA) and 4-aminoantipyrine (4-AAP) to form a red coloured dye by the catalyst action of peroxidase (POD) (Allain et al. 1974).



$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{CEH}} \text{Cholesterol} + \text{RCOOH}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CO}} \text{Cholest-4-en-3-one} + H_2O_2$$



$$2H_2O_2 + \text{HBA} + \text{4-AAP} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4H_2O$$

Standard curves were generated by serial dilution (0.97 – 27.16mmol/l) of cholesterol standard solution and Milli-Q water. Samples were loaded in singlicate at 2µl per well and standards in duplicate on a 96 well plate. 200µl of cholesterol reagent was added to each well and the plate incubated in dark at 37°C for 5 minutes. The difference between absorbance at 500nm and 600nm were calculated. A standard curve $R^2 > 0.98$ was deemed satisfactory. The manufacturer reported an intra- and inter-assay CV of 2.8%.

2.6.5 Measurement of glycogen in tissue

The measurement of tissue glycogen level was performed using Infinity™ glucose hexokinase reagent. Tissues were cut and weighed (40 – 50mg for placental labyrinth; 20 – 35mg for the placental junctional layer). Tissues were placed in a glass bijou and 500µl of 2N HCl were added. Glass bijous were then put in a 50ml BD Falcon™ tube with 5ml of 95°C water. The Falcon™ tubes were incubated in a water bath at 95°C for 2 hours with gentle shaking. After the incubation, 500µl of sodium hydroxide (NaOH) was added to each sample. Each sample was adjusted with a pH 4.5 – 10 strip (Sigma-Aldrich, Dorset, UK) with either 2N HCl or 2N NaOH until the pH was close to 7.0; the volume added and removed for each sample was recorded for later calculation of glycogen levels. The samples were left on ice after pH adjustment and were transferred into a new 2ml Eppendorf™ tube. Samples were then centrifuged at 2000rpm at 4°C for 4 minutes. The supernatant was then removed into a new 2ml Eppendorf™ tube and analysed using the Infinity™ glucose hexokinase reagent (section 2.6.2). Samples were loaded on a 96 well plate in duplicate. A protein assay was performed on each sample for accurate calculation of glycogen levels. The quantification of protein in samples is described in 2.7.7.1. The level of glycogen was then calculated using the equation below:

$$\frac{\text{glucose concentration}}{2} \times \frac{\text{volume after pH}}{\mu\text{g of protein}}$$

2.7 Molecular procedures

2.7.1 Extraction of total RNA from tissues

Snap frozen liver and placental tissues were used. Total RNA was extracted by using the RNeasy® mini kit (Qiagen, West Sussex, UK) and the manufacturer's instructions were followed. Approximately 30-50mg of tissue was used for the extraction of RNA. An on-column DNase treatment step was performed on liver and placental RNA according to the manufacturer's instructions.

2.7.2 Extraction of total RNA from adipose tissue

The same procedures were followed for the extraction of RNA from adipose tissue except for the homogenisation step. About 60mg of snap frozen adipose tissue was added to 1ml of QIAzol® for homogenisation. 300µl of chloroform was added and mixed by shaking vigorously; the mixture was then left at RT° for five minutes. Samples were centrifuged at 13,000rpm for 15 minutes at 4°C. The clear aqueous supernatant was removed into a new Eppendorf™ and mixed with equal volume of 70% ethanol. The samples were then transferred to the RNeasy® spin columns and the remainder of the steps were followed as described in section 2.7.1.

2.7.3 RNA quantification and agarose gel electrophoresis

Approximately 2µl of RNA extracted was used undiluted for quantification using the NanoDrop ND-1000 quantification system. The RNA concentration was recorded and the ultraviolet absorbance at 260nm/280nm and 260nm/230nm was also recorded as an indication of purity of the RNA extracted. A value between 1.8 – 2.1 was considered satisfactory.

To ensure extracted RNA was intact, all RNA samples underwent an agarose gel electrophoresis step on a 1% TBE agarose gel (section 2.2) containing 1:10 GelRed™ (for nucleic acid fluorescent under ultraviolet light exposure). Approximately 0.8 – 1.2µg of RNA was mixed with 2µl of loading buffer (Orange G). A 1kb DNA ladder was loaded along with samples to estimate size and to act as control. The gel was run at 100 volts for 45 – 60 minutes and viewed using a Gel-doc system at 240nm. The presence of a thick 28S and a thinner 18S ribosomal RNA bands (at about 2:1 ratio) indicates intact RNA and was considered satisfactory.

2.7.4 Reverse transcription of RNA

The synthesis of cDNA from RNA (~1µg for liver and placenta, ~0.5µg for adipose) was performed by using the reverse transcription system from Promega®. After normalisation of all samples to a similar concentration and volume (10.7µl), a final quantification step was performed using the NanoDrop system (as described in 2.7.3). The samples were then denatured in 70°C for 10 minutes, then immediately placed on ice. A master mix was made consisting of: $MgCl_2$ (4µl), 10x reverse transcription buffer (2µl), dNTP (2µl), RNasin inhibitor (0.5µl), AMV reverse transciptase (0.6µl), Oligo-dT (0.2µl) per sample. After the addition of this master mix, samples were reverse transcribed at 42°C for 15 minutes followed by 95°C for 5 minutes then gradually cooled down to 4°C in a G-Storm thermal cycler. A –RT (no reverse transcriptase) sample was made; the sample underwent all the procedures mentioned above except that nuclease free water was added in place of AMV reverse transcriptase. This acts as a negative control to ensure no genomic DNA contamination occurred. Samples were stored at -20°C.

2.7.5 Real-time polymerase chain reaction

The quantity of mRNA was measured using a LightCycler® 480. Either a Taqman® primer-probe mix or self designed primers were used for specific gene amplification. Self designed intron spanning assays were designed using the Universal ProbeLibrary™ (UPL) system (Roche Diagnostics Ltd., West Sussex, UK). A standard curve was made by pooling cDNA from all the samples for the experiment and serially diluted in nuclease free water (1:4, 1:8, 1:16, 1:32, 1:64, 1:128). Individual samples of cDNA were diluted to 1:20 in nuclease free water. 2μl of samples and standard curve were loaded into the bottom of a 384 well plate in triplicate, followed by a master mix containing primers, probe and Roche® probes master (Table 2.1).

Plates were centrifuged for 2 minutes at 2000rpm and then loaded into the LightCycler® 480. The reaction conditions comprised rapid heating to 95°C for 5 minutes for denaturation followed by 50 cycles of a denaturation step at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 1 second. The samples were then cooled to 40°C for 30 seconds for completion. Acceptable assays had a reaction efficiency value between 1.7 – 2.1 and slope between -3.1 - -3.6. Data were analysed with the Roche LightCycler® software (ver. 1.5.0 SP1). All genes of interest were normalised to housekeeping genes.

	Taqman® primer-probe	**Self designed primer**
cDNA (1:20)	2µl	2µl
Roche Probes Master	5µl	5µl
Taqman® probe	0.5µl	-
Forward primer	-	0.02µl (100µM)
Reverse primer	-	0.02µl (100µM)
Corresponding UPL probe	-	0.1µl (10µM)
Nuclease free water	2.5µl	2.7µl

Table 2.1 Recipe for real time PCR using either Taqman® primer-probe mix or self designed UPL primers with corresponding UPL probe

2.7.5.1 Rat self designed primers (UPL)

Primer	Accession Number	Forward Primer	Reverse Primer	UPL Probe No.	Amplicon (nt)
Akt1	ENSRNOT00000031164.2	aacgacgtagccattgtgaa	ccatcattcttgaggaggaagt	71	95
IRS1	NM_012969.1	tggacgtcacaggcagaat	gggatgcatcgtaccatctac	69	142
Igf1	NM_178866.4	tgacatgcccaagactcaga	cgtggcattttctgttcctc	63	114
Igf1r	NM_052807.1	gcctccaactttgtctttgc	cactgggccaggaatgtc	68	66
Phlda2	XM_001065824.1	gcgctgatcgactaccaga	ctactcccgtgggtctctga	25	69
GAPDH	M17701.1	agctggtcatcaatgggaaa	atttgatgttagcgggatcg	9	83
11β-HSD1	NM_017080.2	tctacaaatgaagagttcagaccag	gccccagtgacaatcacttt	1	62
Grb10	NM_00110903.1	caaccaagaagccaaccag	tccacggatgagttaatatcgtt	117	74
IgfBP2	NM_013122.2	gcgggtacctgtgaaaagag	tccctccgagtggtcatc	112	78
IgfBP3	NM_012588.1	cacactgaatcatctgaagttcct	tttggaagggcgacactg	95	109
Igf2r	ENSRNOT00000021840.3	gcaacaaaaccgcaggtc	tgcagtccacctcacctgt	55	63
TATA box binding protein (TBP)	NM_001004198.1	cccaccagcagttcagtagc	caattctgggtttgatcattctg	129	75
H19	NR_027324.1	tcctttgcccaaagagctaa	gctgggtagcaccatttctt	84	76

2.7.5.2 Rat Taqman® primer-probe set

Primer	Accession Number	Reference Sequence	Amplicon (nt)
Cyclophilin-A (PPiA)	RN00690933_m1	NM_017101.1	149
Actin β (ActB)	RN00667869_m1	NM_031144.2	91
Igf2	RN01454518_m1		74
Igf2r	RN01636937_m1	NM_012756.1	82
GR (Nr3c1)	RN01405584_m1	NM_012576.2	75
Mest	RN01500324_m1	NM_001009617.1	101
CDKn1c	RN00711097_m1		70
11βHSD2	RN00492539_m1	NM_017081.1	100
Slc2a1 (Glut1)	RN01417099_m1	NM_138827.1	73
Slc2a3 (Glut3)	RN00567331_m1	NM_017102.2	99
Slc38a1	RN00593696_m1	NM_138832.1	95
Slc38a2	RN00710421_m1	NM_181090.2	57
Slc38a4	RN00590667_m1	NM_130748.1	101

2.7.5.3 Mouse self designed primers (UPL)

Primer	Accession Number	Forward Primer	Reverse Primer	UPL Probe No.	Amplicon (nt)
11β-HSD1		tctacaaatgaagagttcagaccag	gccccagtgacaatcacttt	1	62
Lipoprotein lipase (LPL)	NM_008509.2	ctcgctctcagatgccctac	aggcctggttgtgttgctt	95	90
Hormone sensitive lipase (HSL)	ENSMUST00000054301.6	gcgctggaggagtgttttt	ccgctctccagttgaacc	3	75
Fatty acid synthase (FAS)	NM_007988.3	ccaaatccaacatgggaca	tgctccagggataacagca	34	76
PPAR α	ENSMUST00000109423.1	ccttccctgtgaactgacg	caccatgttggatggatgtg	5	107
PPAR γ	ENSMUST00000000450.3	gctgttatgggtgaaactctgg	tctgtgtcaaccatggtaatttct	2	110
5α Reductase	NM_175283.3	gggaaactggatacaaaataccc	ccacgagctccccaaaata	41	78
5β Reductase	NM_145364.2	gaaaagatagcagaagggaaggt	gggacatgctctgtattccataa	103	77
PEPCK	ENSMUST00000029017.4	gatgacattgcctggatgaa	cgttttctgggttgatagcc	105	64
GR	ENSMUST00000115567.1	tgacgtgtggaagctgtaaagt	catttcttccagcacaaaggt	56	78
FTO	NM_011936.2	cacagcctcggtttagttcc	aatccaaggtgcctgttgag	53	60
TATA box binding protein	NM_013684.3	gggagaatcatggaccagaa	gatgggaattccaggagtca	97	90

2.7.5.4 Mouse Taqman® primer-probe set

Primer	Accession Number	Reference Sequence	Amplicon (nt)
Cyclophilin-A (PPiA)	Mm02342430_g1	NM_008907.1	148
ActB (Actin β)	Mm01205647_g1	NM_007393.3	72

2.7.6 Detection and quantification of Igf2 promoter transcripts

2.7.6.1 SYBR® Green quantification of Igf2 promoter transcripts

SYBR® green is a cyanine dye that is widely used in various type of nucleic acid analysis (Zipper et al. 2004). Intron-spanning primers were designed by Dr. Amanda J. Drake (Table 2.3); with the reverse primers situated in exon 4 (code: 4R in this experiment) of the rat Igf2 gene. cDNA was diluted 1:20 with a standard curve generated as previously described in section 2.7.5.

Samples were loaded in triplicate along with primers and SYBR® Green (Table 2.2) on to a 384 well real-time plate which was centrifuged for 2 minutes at 2000rpm. Using a LightCycler® 480 real time machine, the samples were rapidly heated to 95°C for 5 minutes for denaturation then underwent 50 cycles of amplification process. Each cycle of amplification consists of a denaturation step at 95°C for 10 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 30 second. After the amplification, the samples underwent one cycle of melting curve formation at 95°C for 5 seconds, 65°C for 1 minute, a rise of temperature to 97°C and rapid cooling to 40°C for 10 seconds for completion. The presence of a single peak on the melt curve indicated a single product was formed. This was confirmed by gel electrophoresis. Data were analysed with the Roche LightCycler® software (ver. 1.5.0 SP1). All genes of interest were normalised back to housekeeping genes.

	Volume per well
cDNA (1:20)	2.5µl
Roche SYBR® Green I	5µl
Forward primer	0.05µl (100µM)
Reverse primer	0.05µl (100µM)
Nuclease free water	2.4µl

Table 2.2 Recipe for SYBR® Green quantification of Igf2 promoter transcripts

Rat Igf2 promoter	Primer Sequence	Amplicon (nt)
P0 (Forward primer)	cctgaggcaaaaccagagaa	151
P1 (Forward primer)	acttcagcagctcccacttc	123
P2 (Forward primer)	ccgctgttcggtttgcatac	102
P3 (Forward primer)	cggcctccttatccaacttc	64
4R (Reverse primer)	agcacaacatcgacttccc	

Table 2.3 List of different rat Igf2 transcript primers (designed by Dr. Amanda J. Drake)

2.7.6.2 Standard polymerase chain reaction for rat Igf2 promoters

A standard PCR was done to confirm the presence or absence of transcripts from the different Igf2 promoters in rat fetal liver and placental labyrinth. Primers used in this experiment were same as for the SYBR® green experiment listed in table 2.3. Two random cDNA sample from each treatment group: Veh and Dex, were chosen and mixed together for this experiment. PCR mix was added to the cDNA mix along with nuclease free water, forward and reverse primers (Table 2.4) and the PCR reaction was performed in a G-Storm thermal cycler. Samples were rapidly heated to 95°C for 5 minutes for denaturation then underwent 30 cycles of amplification process. Each cycle consists of a denaturation step at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 second. The samples were then left at 72°C for an extra five minutes then cooled to 4°C for completion.

	Volume per well
cDNA (1:20)	0.5µl
Promega Master PCR mix	5µl
Forward primer	0.5µl (20µM)
Reverse primer	0.5µl (20µM)
Nuclease free water	3.5µl

Table 2.4 Recipe for standard PCR reaction

2.7.7 Western blotting

2.7.7.1 Protein extraction and quantification

Approximately 50mg of fetal liver and placental labyrinth were used for protein extraction. Tissues were homogenised in 500µl protein lysis buffer (section 2.2) with protease inhibited water and $NaVO_4$ for phosphorylated protein examination. Tissue homogenates were centrifuged at 14,000rpm for 15 minutes at 4°C. The supernatant was then transferred into a new Eppendorf™ tube and a protein quantification assay was performed.

Protein concentration assays were performed using the Bio-Rad® protein assay kit using 5µl of samples. BSA standards were used at a serial dilution (10, 8, 6, 4, 2, 1, 0.5, 0 mg/ml) to determine the concentration of individual samples. 20µl of Reagent S was added to each millilitre of Reagent A before use. 25µl of Reagent A and 200µl of Reagent B was added to each sample in duplicate in a 96-well plate. The plate was covered and incubated at room temperature for 15 minutes. The plate was then measured with a plate reader at an absorbance of 750nm. Individual sample concentrations were recorded. A standard curve R^2>0.98 was considered satisfactory.

2.7.7.2 Protein gel electrophoresis

Protein samples (50µg) were reconstituted with 1x NuPAGE® LDS sample loading buffer and 1x NuPAGE® sample reducing agent to equal volume not exceeding a total of 20µl. Samples were mixed gently, centrifuged briefly and incubated at 70°C for 10 minutes to denature protein. Samples were then loaded on to a NuPAGE® Novex 4 – 12 % Bis-Tris gel along with a Kaleidoscope™ protein ladder for

separation. A XCell Surelock™ Mini-Cell was used for this process. The central chamber of the XCell tank was filled with 200ml of 1x running buffer (section 2.2) and 1ml antioxidant solution, added immediately before use. The outer chamber was filled with 1x running buffer (section 2.2). The gel was run at 200V for 1 hour at RT° (or longer if required).

2.7.7.3 Gel transfer

Hybond™-C extra nitrocellulose membrane with 0.45µm pores were used for the transfer of protein. XCell Surelock™ tank were used for this procedure. A gel sandwich was prepared within the cassette provided for XCell, depending on the amount of gel to be transferred a different gel sandwich configuration was used (Figure 2.3). The gel cassette was placed in the XCell tank and was filled with 1x transfer buffer (section 2.2) with 500µl of antioxidant solution. The outer chamber was filled with deionised water. The transfer procedure was run at 30V for 1 hour at RT°.

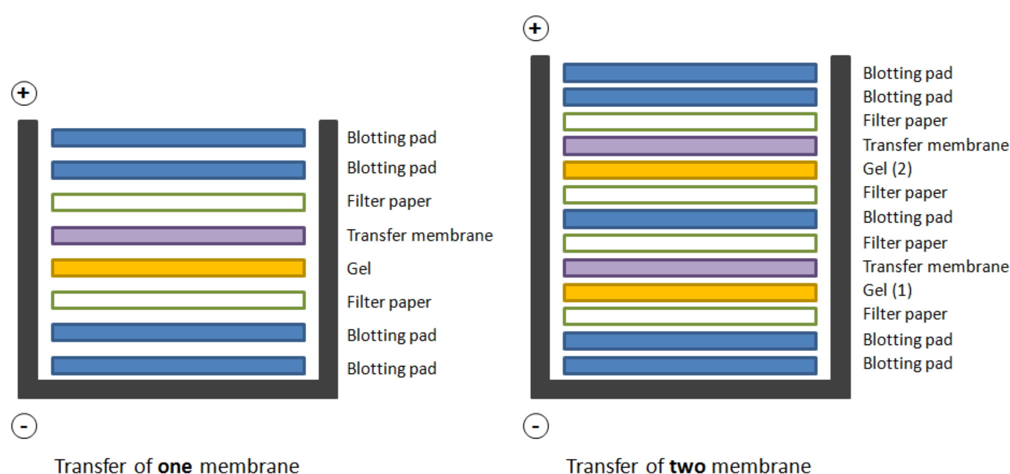


Figure 2.3 Gel sandwich configuration for one or two gel transfer

2.7.7.4 Protein immunoblotting

After the transfer of protein to the nitrocellulose membrane, the membrane was stained quickly with Ponceau-S solution to confirm successful transfer. The membrane was then washed with Milli-Q water to remove excess Ponceau-S and blocked with 5% milk (section 2.2) for 1hour. The membrane was then washed three times (5 minutes each) with TBST (section 2.2), then incubated with primary antibody (Table 2.5) at a concentration of 1:1,000 in 5% BSA solution (section 2.2) overnight at 4°C on a rocker. The membrane was then washed three times (5 minutes each) in TBST prior to secondary antibody incubation. Secondary antibody was made in 5% milk at a concentration of 1:10,000. The membrane was incubated on a rocker for 1 hour at RT°. Lastly the membrane was washed again with TBST twice (5 minutes each) and was kept in 1x TBS (section 2.2) solution.

2.7.7.5 Blot reading

Membranes were visualised using the Odyssey® Infrared Imaging System. A scanned image of the membrane was taken using the infrared imaging system and the density of the band measured using the Odyssey® imaging system software.

Primary antibody	Phosphorylation site	Size (kDa)	Company
IRS1	-	165	Millipore
Phospho-IRS1	Tyr^{612}	165	Invitrogen
Pi3k	-	85	Cell Signaling
Phospho-Pi3k	Tyr^{458}	85	Cell Signaling
AKT1	-	60	Cell Signaling
Phospho-AKT1	Ser^{473}	60	Cell Signaling
GSK3β	-	46	Cell Signaling
Phospho-GSK3β	Ser^{9}	46	Cell Signaling
mTOR	-	289	Cell Signaling
Phospho-mTOR	Ser^{2448}	289	Cell Signaling
β-tubulin	-	55	Invitrogen

Table 2.5 Primary antibodies for immunoblotting of protein

IRS1 = Insulin receptor substrate 1; Pi3k = Phosphoinositide 3-kinase; GSK3β = Glycogen synthase kinase 3β; mTOR = Mammalian target of rapamycin; Tyr = Tyrosine; Ser = Serine

2.8 Histological procedures:

2.8.1 Tissue collection:

Rat placentas were collected at embryonic day 20 (E20), fixed in 10% neutral buffered formalin and kept at 4°C overnight, then transferred into 70% ethanol and kept at RT° until embedding in paraffin. Before embedding, placentas were weighed and halved mid-sagittally. The placentas were orientated with the cut surface facing up and embedded in paraffin.

2.8.2 Tissue sectioning

Embedded samples were sectioned at 7-μm thickness with a rotary microtome machine and MB35 blades (Thermo Fisher Scientific, Leicestershire, UK). The tissue were cut into a 10 section ribbon each time and laid out on a platform in sequential series. After the tissue has been exhaustively sectioned, a systematic random sampling (Gundersen and Jensen 1987) approach was used to select sections without bias. A total of seven sections were chosen from each sample. The selected sections were carefully dissected from the ribbon and placed in a water bath heated to 45°C then transferred to a glass slide. Slides were dried and incubated overnight at 37°C; the slides were then kept at RT° until use.

2.8.3 Histological staining:

For examination of the sections, a standard haematoxylin and eosin stain approach was used. Haematoxylin stains the nuclei of the cells blue and eosin counter-stains most other tissues and displays as pink.

The slides were first de-waxed by immersing in xylene and rehydrated through a series of diluted ethanol. The slides were then rinsed in distilled water to remove excess ethanol before staining in Harris' haematoxylin. The slides were then washed under running tap water and checked under a microscope for sufficient staining. The slides were then neutralised in alkaline tap water (section 2.2) followed by washing under running tap water. The slides were stain with Eosin-Y, washed under running tap water and dehydrated using a series of concentrated ethanol solutions. Lastly, the sections were then cleared with xylene. The complete procedures were outlined in table 2.6. Glass coverslips (VWR International, Leicestershire, UK) were mounted with dystyrene plasticiser and xylene (DPX) (Thermo Fisher Scientific, Leicestershire, UK).

Solution	Duration
Xylene	10 minutes (x2)
100% Ethanol	2 minutes (x2)
95% Ethanol	2 minutes
70% Ethanol	2 minutes
50% Ethanol	2 minutes
30% Ethanol	2 minutes
Distilled water	5 minutes
Harris' haematoxylin	1 – 2 minutes
Running tap water	3 minutes
Alkaline tap water	1 minute
Running tap water	3 minutes
Eosin-Y (Alcoholic)	30 seconds
30% Ethanol	10 seconds
50% Ethanol	10 seconds
70% Ethanol	10 seconds
95% Ethanol	10 seconds
100% Ethanol	2 minutes
Xylene	5 minutes (2x)

Table 2.6 Haematoxylin and Eosin staining protocol

2.8.4 Slides analysis:

In order to analyse the sections, the histologically stained tissues were visualised under a photographic CoolSNAP CCD camera and microscope system with liquid crystal RGB filter at 1.25x magnification for a full view of the placenta. Areas of the different zones of the placenta were measured using the software MCID Basic 7.0 software (InterFocus Imaging Ltd., Cambridge, UK). The absolute volume was calculated by applying the Cavalieri principle and an estimated volume was established by using the following equations. The measurements and calculations were performed blinded as to prenatal treatment groups.

Equation 1:

$$V_i = A_x \times T$$

Equation 2:

$$V_{est} = \sum_{i=1}^{6} (V_i + V_{i+1}) * \frac{t_i}{2}$$

Where in *equation 1*: V_i is the volume of the section; A_x is the area of the section; T is the thickness of the section (in this case, T = 7, as a constant number as all sections were cut at 7µm).

Where in *equation 2*: $V_{(est)}$ is the estimated placental volume; V_i is the volume of the section (calculated by *equation 1*); V_{i+1} being the volume of next section and so forth; t_i is the thickness between the section V_i and V_{i+1} and so forth.

2.9 Experimental diet constituents:

Diets were purchased from Research Diets Inc., New Brunswick, New Jersey, USA

	Control diet (CON) D12328		Obesogenic diet (DIO) D12331	
	gm%	kcal%	gm%	kcal%
Protein	16.8	16.4	23.0	16.4
Carbohydrate	***74.3***	***73.1***	***35.5***	***25.5***
Fat	***4.8***	***10.5***	***35.8***	***58.0***
Total kcal/gm	***4.07***		***5.56***	
Casein, 80 Mesh	228	912	228	912
DL-Methionine	2	0	2	0
Maltodextrin 10	170	680	170	680
Corn starch	***835***	***3340***	***0***	***0***
Sucrose	***0***	***0***	***175***	***700***
Soybean oil	25	225	25	225
Coconut oil (hydrogenated)	***40***	***360***	***333.5***	***3001.5***
Mineral mix (S10001)	40	0	40	0
Sodium bicarbonate	10.5	0	10.5	0
Potassium citrate, 1 H_2O	4	0	4	0
Vitamin mix (V10001)	10	40	10	40
Choline bitartrate	2	0	2	0
Colour dye	0.1	0	0.1	0
Total	***1366.6***	***5557***	***1000.1***	***5558.5***

These two diets were chosen because the micro- and macronutrient constituents of the two diets were identical; the only difference being the fat, carbohydrate content, and sugar content.

CHAPTER 3

Programming effects on placenta and liver in the glucocorticoid programmed rat

3.1 Introduction

3.1.1 Introduction

Numerous studies in humans (Barker et al. 1989, Rich-Edwards et al. 1997) and animals have suggested that exposure of the fetus to an insult during a specific period of development may lead to programming effects on many organ systems including the cardiovascular system (Koenen et al. 2002, O'Regan et al. 2004, Hadoke et al. 2006), liver and pancreas (Nyirenda et al. 1998, Dahlgren et al. 2001, Moss et al. 2001, Drake et al. 2005), and the brain (Brabham et al. 2000, Welberg et al. 2001, Emgård et al. 2007). One mechanism leading to these effects may be prenatal exposure to excess glucocorticoid.

The placenta plays a central role in modulating the transfer of nutrients, waste and hormones between the maternal and fetal circulation and may be a key target for programming effects (Langley-Evans et al. 1996, Ain et al. 2005, Fowden et al. 2006b). Studies have suggested that nutrient transport across the placenta correlates with fetal growth (Fowden and Forhead 2004) supporting a potential role for this organ in mediating programming effects. Exposure to excess glucocorticoid during pregnancy has been suggested to have effects on the placenta by changing hormone secretion (Ain et al. 2005), nutrient transport (Hahn et al. 1999, Langdown and Sugden 2001, Audette et al. 2011), placental growth (Benediktsson et al. 1993, Ozmen et al. 2011) and placental vascular development (Hewitt et al. 2006). Interestingly, studies have shown that whilst administration of synthetic glucocorticoids to a pregnant mother causes growth restriction in the offspring, direct glucocorticoid administration to the fetus does not necessarily decrease birth weight

(Jobe et al. 1998a), suggesting an important role for the placenta in glucocorticoid-induced programming effects.

As discussed in chapter one, Igf1 and Igf2 are two potent growth factors that are important in promoting fetal and placental growth and may have a role in programming effects. During fetal life, Igf2 has a more important role than Igf1 (DeChiara et al. 1991). Both of these growth factors exhibit their effect by binding to the Igf1r and thereafter signalling a cascade of proteins downstream in the Igf pathway (Figure 1.6). Glucocorticoids may have direct effect on Igf2 expression suggesting a role for Igf2 in the glucocorticoid programmed rat (Li et al. 1998). In addition, it has been suggested that Igf2 may act as a 'call for help' signalling from the fetus to the placenta to increase placental nutrient transport capacity (Langdown and Sugden 2001, Constância et al. 2005). Therefore the Igf system may play an important role in programming.

3.1.2 Hypothesis

1. Overexposure to glucocorticoids in utero during the last week of gestation induces programming effects in the developing offspring; these programming effects may in part be modulated by the placenta.
2. Changes in the Igf-signalling pathway may be important in programming effects.

3.1.3 Aims

The aim of this study is to explore the possible underlying mechanisms of glucocorticoid programming. I will study the effects of prenatal glucocorticoid overexposure on gene expression in placenta and fetal liver, placental volume and glycogen storage, and the level of proteins in the Igf pathway.

3.2 Methods

3.2.1 Animal maintenance, treatment and tissue collection

Female Wistar rats were time-mated at a weight of around 250g. Cages were checked for the presence of a vaginal plug daily each morning. Females were singly housed after a vaginal plug was found (denoted as E0) and were left undisturbed with *ad libitum* access to food and water. Pregnant female rats were randomly assigned to dexamethasone (Dex) or vehicle (Veh) treatment. Between E15 and E20 inclusive, pregnant females were given a subcutaneous injection each morning of either Dex in 0.9% saline and 4% ethanol (100µg/kg body weight) or the equivalent volume of Veh (4% ethanol saline) (n= Veh: 9; Dex: 8).

At E20, at least one hour after the final injection, pregnant females were killed by carbon dioxide asphyxiation. Maternal blood was collected by cardiac puncture into a 0.5M EDTA rinsed 2ml syringe. Fetal blood was collected into Microvette® CB-300 tubes after decapitation. Blood was placed on wet ice until centrifugation (5000 rpm, 10 mins, 4°C) and plasma removed and frozen at -20°C. Fetuses and placentas were dissected and weighed. Placentas were either fixed in 10% neutral buffered formalin, stored overnight in 4°C and transferred into 70% ethanol and kept at RT° until embedding in paraffin or dissected into labyrinth and junctional layers. Fetal liver was dissected. Dissected tissues were snap frozen on dry ice and stored at -80°C.

3.2.2 Metabolic experiments

Fetal plasma was pooled from all fetuses within a litter. Non-fasting plasma glucose was measured using an enzymatic (hexokinase) method (Thermo Fisher Scientific); plasma insulin was measured using an ELISA kit (Crystal Chem Inc.).

3.2.3 Molecular experiments

Total mRNA was extracted from fetal liver and placental labyrinth using RNeasy® mini kit (Qiagen) and reverse transcribed (Promega) to produce cDNA. Quantitative real-time PCR was used to examine gene expression by TaqMan™ (Applied Biosystems) or self designed UPL (Roche) primers with the addition of MasterMix (Roche). The expression levels of different Igf2 transcripts was examined by quantitative real-time PCR using SYBR® green and primers designed by Dr. Amanda J. Drake. The presence of different Igf2 transcripts in fetal liver and placenta was assessed by gel electrophoresis (2% TBE agarose gel with 1kb DNA ladder at 100V for 1 hour) following PCR. Expression of genes was normalised to the average of two reference genes Cyclophilin-A and Tata box binding protein (TBP) in placental labyrinth and to Cyclophilin-A in the fetal liver.

Fetal liver and placental labyrinth were both weighed and protein extracted for Western Blot analysis of protein levels. In particular, proteins involved in the Igf pathway were explored. Total protein is expressed relative to the control protein: β-tubulin. Analysis of phosphorylated protein is expressed relative to total protein.

3.2.4 Placental stereology procedures

Paraffin embedded placentas were thoroughly sectioned at 7µm per section. Sections were then fixed on glass slides, stained with haematoxylin and counter-stained with eosin. Stained sections were examined under a microscope with a CCD camera at 1.25x magnification. Areas of the two different zones, labyrinth and junctional, were measured by MCID Basic 7.0 software (InterFocus Imaging Ltd., Cambridge, UK), and volumes calculated. Ratio of volumes were in relation to total placental volume measured. Part of this work was performed by Ms. Fiona E. Graham, as part of an Honours project for the University of Edinburgh. This experiment was with the assessor blinded as to prenatal exposure.

3.2.5 Glycogen measurement in the placenta

Placental labyrinth and junctional zones were weighed and glycogen levels measured using the colourimetric method used for measuring plasma glucose. Glycogen levels are expressed relative to the amount of protein per gram of placenta.

3.2.6 Statistical analysis

Data were analysed using SPSS® and GraphPad®. Statistical significance was calculated using Student's *t*-test or Mann-Whitney *u*-test when the distribution of data was non-parametric. Data are expressed as mean ± SEM unless otherwise stated. Results were considered significant if $p<0.05$.

3.3 Results

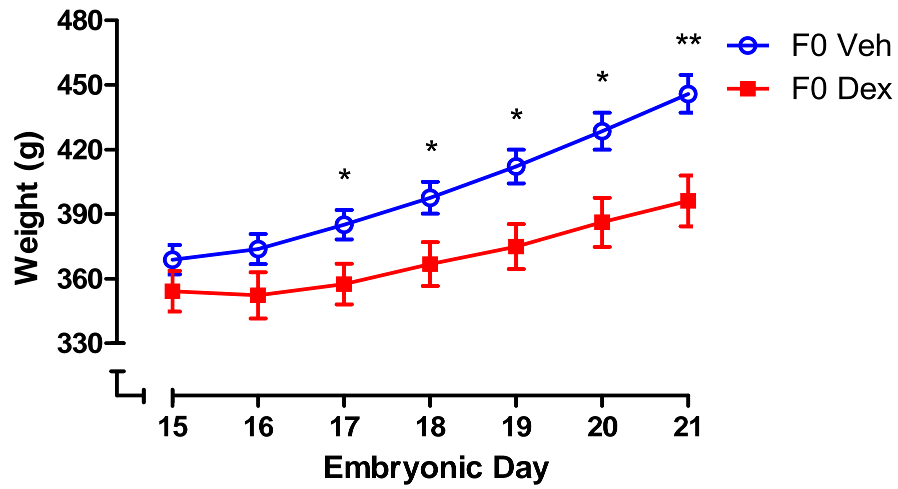
3.3.1 Mother (F0) and F1 offspring

Pregnant females exposed to Dex gained less weight compared to Veh exposed females; at E20, F0 Dex mothers were lighter than F0 Veh mothers (Figure 3.1), this is in line with previous findings (Simpson et al. 1974, LaBorde et al. 1992).

Offspring of mothers treated with Dex (F1 Dex, n=113 from 8 litters) were significantly lighter than mothers treated with Veh (F1 Veh, n=119 from 9 litters) at E20 (Figure 3.2). The placentas of F1 Dex offspring (n=112) were also significantly lighter compared to F1 Veh offspring (n=119) (Figure 3.2). These results are in agreement with previous studies of prenatal glucocorticoid over-exposure (Langdown and Sugden 2001, Ain et al. 2005, Ozmen et al. 2011). There was no difference in the number of pups per litter between the two treatment groups (Figure 3.2).

There was no difference in maternal non-fasted plasma glucose levels, however, Dex increased maternal non-fasted plasma insulin levels (Figure 3.3a). In contrast, Dex exposure was associated with decreased fetal insulin levels in the absence of any alteration in plasma glucose (Figure 3.3b).

A)



B)

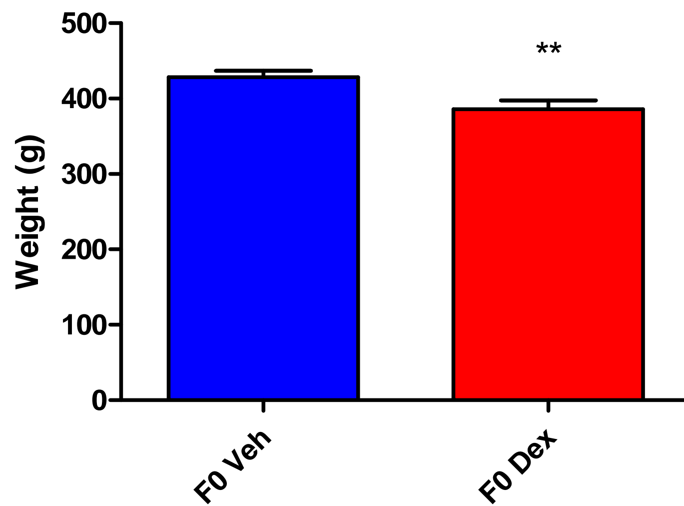


Figure 3.1 Maternal weight gain during pregnancy and maternal weights at E20
Dex exposure decreased **A)** maternal weight gain during the last week of gestation so that **B)** Dex treated F0 females were lighter at E20
Data are mean ± SEM, analysed by Student's *t*-test
n = Veh: 8, Dex: 9
* = p<0.05, **= p<0.01

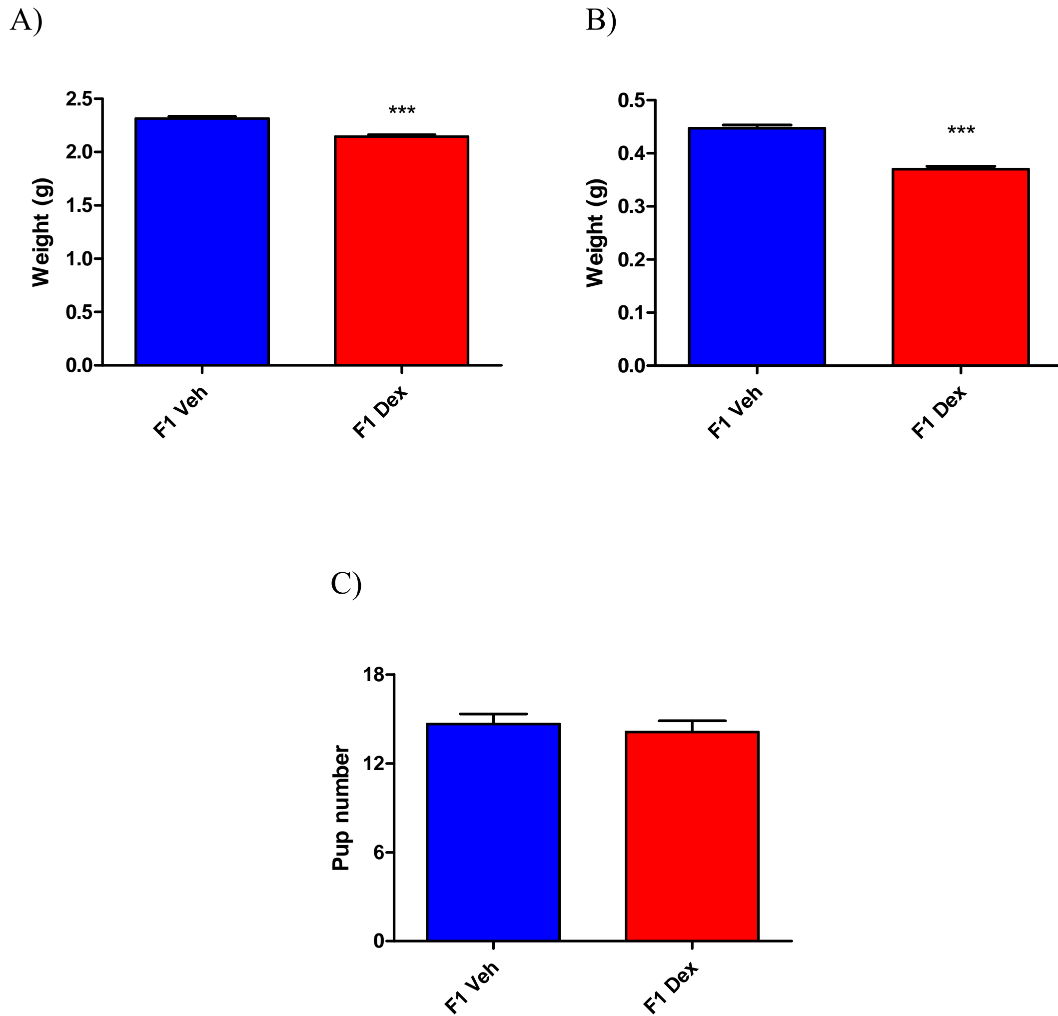


Figure 3.2 F1 offspring fetal and placental weights and litter size

Prenatal Dex exposure decreased **A)** pup weight and **B)** placental weight at E20. **C)** There was no difference in litter size

Data are mean ± SEM, analysed by Student's *t*-test

n =	Fetus	Veh: 79, Dex: 74
	Placenta	Veh: 79, Dex: 73
	Litter	Veh: 9, Dex: 8

*** = $p<0.001$

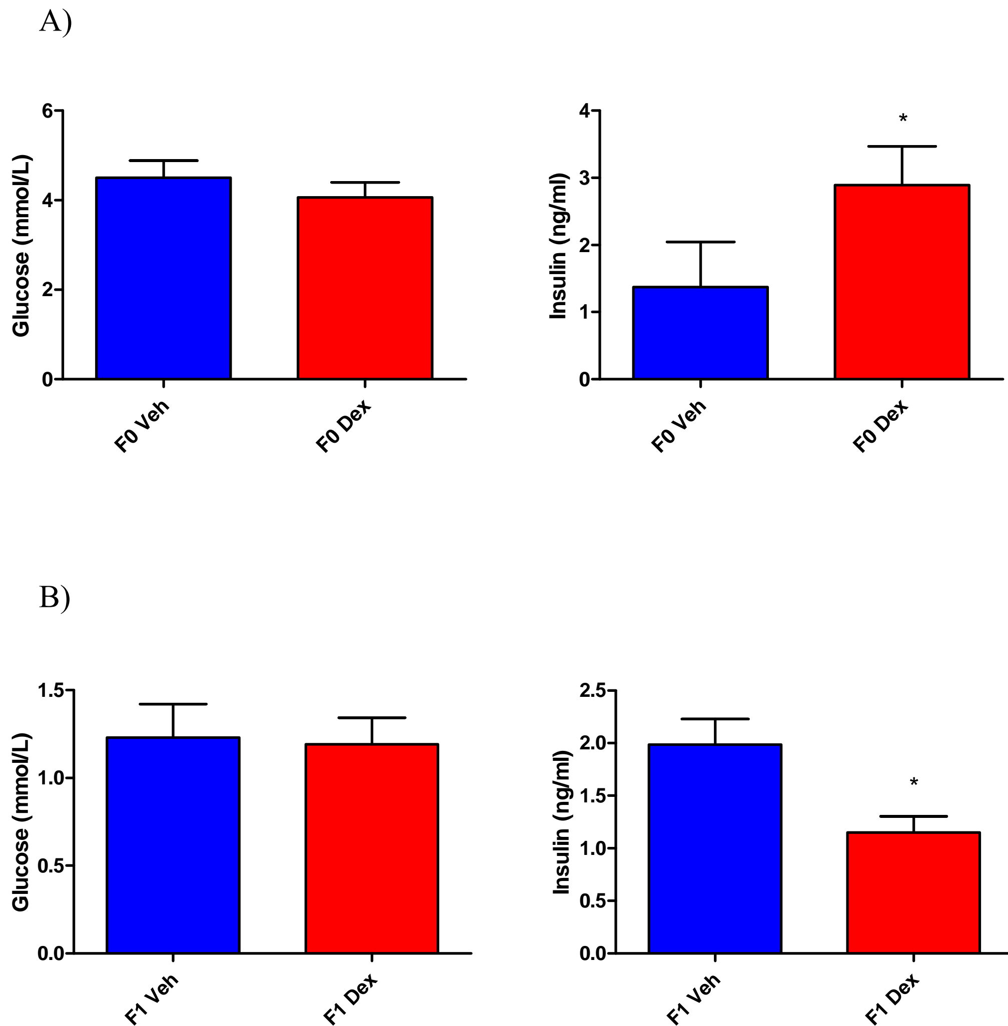


Figure 3.3 Maternal and fetal glucose and insulin levels at E20

There were **A)** no differences in F0 maternal plasma glucose but increased maternal insulin levels in Dex exposed animals at E20. **B)** In the fetuses, there were no differences in fetal glucose but decreased fetal insulin levels in Dex exposed animals at E20

Data are mean ± SEM, glucose analysed by Students *t* test and insulin by Mann-Whitney *u*-test as it was not normally distributed

n =	Maternal	Veh: 9, Dex: 8
	Fetal	Veh: 9, Dex: 8

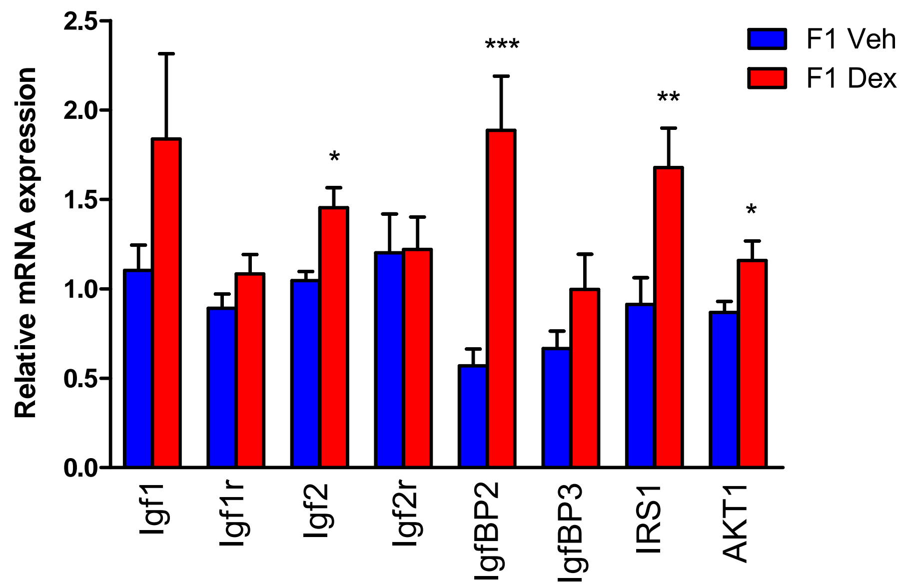
* = $p<0.05$

3.3.2 F1 fetal liver gene expression

Dex exposure resulted in increased Igf2 gene expression in fetal liver (Figure 3.4a). This reflected an increase in the expression of mRNA transcripts from the three Igf2 promoters: P1, P2, and P3 (Figure 3.5). There appeared to be no transcripts from the P0 promoter in fetal liver (Figure 3.8). Dex also resulted in increased mRNA expression of IRS1 and AKT1 (Figure 3.4a).

Expression of IgfBP2 was also increased in Dex exposed offspring (Figure 3.4a) although there were no differences in the expression of Igf1, Igf1r, Igf2r and IgfBP3 (Figure 3.4a). The expression of the potent growth inhibitors CDKn1c and Grb10 were both increased in liver of offspring exposed to Dex. No difference was seen in the expression of another growth inhibitor, Phlda2 (Figure 3.4b).

A)



B)

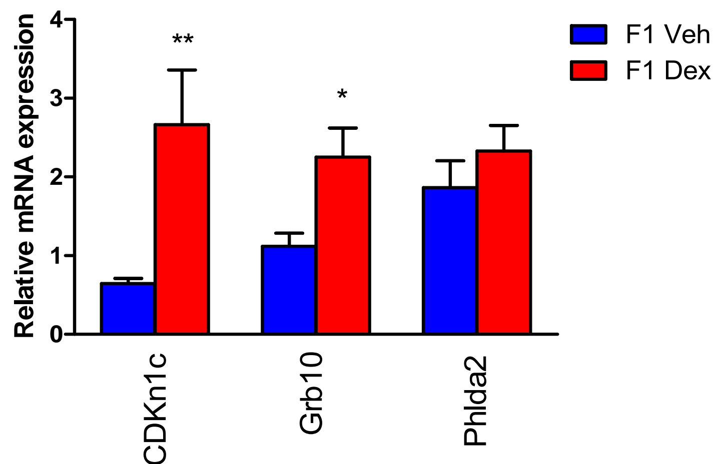


Figure 3.4 Gene expression in F1 fetal liver at E20

Dex exposure was associated with **A)** increased expression of genes in the Igf pathway: Igf2, IgfBP2, IRS1, AKT1 and with **B)** increased expression of the imprinted genes CDKn1c, and Grb10 in fetal liver at E20

Data are mean ± SEM, all data analysed by Student's *t*-test except Igf1 and CDKn1c which were analysed by Mann-Whitney *u*-test

n = Veh: 12, Dex: 12

* = p<0.05, ** = p<0.01, *** = p<0.001

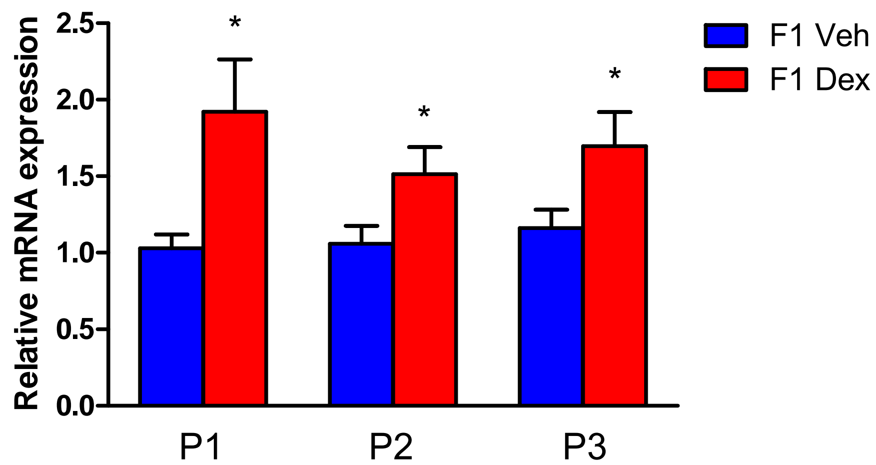


Figure 3.5 Expression of transcripts from Igf2 promoters in fetal liver

Dex exposure increased expression of transcripts from Igf2 promoters P1, P2, and P3 in fetal liver at E20

Data are mean ± SEM, analysed by Student's *t*-test

n = Veh: 12, Dex: 12

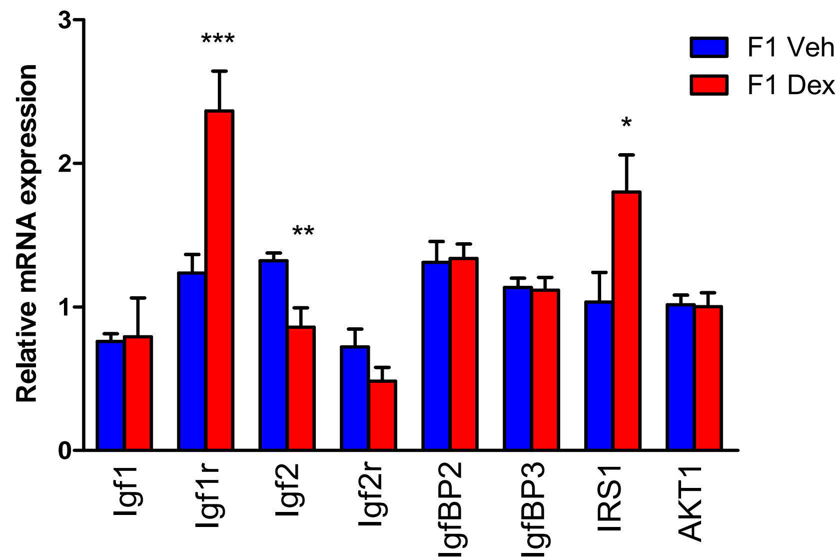
* = $p<0.05$

3.3.3 F1 placental labyrinth gene expression

In contrast to the pattern of gene expression in fetal liver, the expression of Igf2 was decreased in Dex exposed offspring placenta (Figure 3.6a); this reflected a decrease in expression of transcripts from the P0 (exclusively expressed in placenta) (Nyirenda et al. 1998, Langdown and Sugden 2001, Constância et al. 2002, Drake et al. 2005), P1, and P2 promoters (Figure 3.8). No difference was seen in transcript levels from the P3 promoter (Figure 3.8). The presence of transcripts from the P0 promoter in placental labyrinth was confirmed on gel electrophoresis (Figure 3.9). Dex exposure was associated with an increase in the expression of IRS1 but not in the expression of AKT1 (Figure 3.6a).

Despite the reduction in the size of the placenta, Dex exposure was associated with an increase in the mRNA expression of the system A amino acid transporters: Slc38a2 and Slc38a4 as well as increased expression of the glucose transporters: Glut1 and Glut3 (Figure 3.7). There was a trend to an increase in the expression of the amino acid transporter Slc38a1 (p=0.057) in Dex exposed placentas (Figure 3.7). There were no differences in the mRNA expression of the organic cation transporters: Slc22a1 and Slc22a3 (Figure 3.7) or in the expression of the imprinted genes Igf2r, CDKn1c, Grb10, Phlda2, or Mest (Figure 3.6a, b).

A)



B)

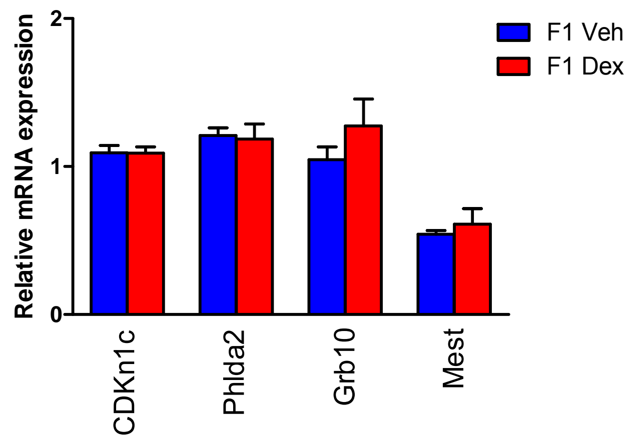


Figure 3.6 Expression of genes in E20 placental labyrinth

Dex exposure was associated with **A)** increased expression of genes in the Igf signalling pathway: Igf1r and IRS1 and with decreased expression of Igf2 in placental labyrinth at E20. **B)** No differences were found in the expression of a number of imprinted genes important in growth

Data are mean ± SEM, analysed by Student's *t*-test

n = Veh: 12, Dex: 12

* = p<0.05, ** = p<0.01, *** = p<0.001

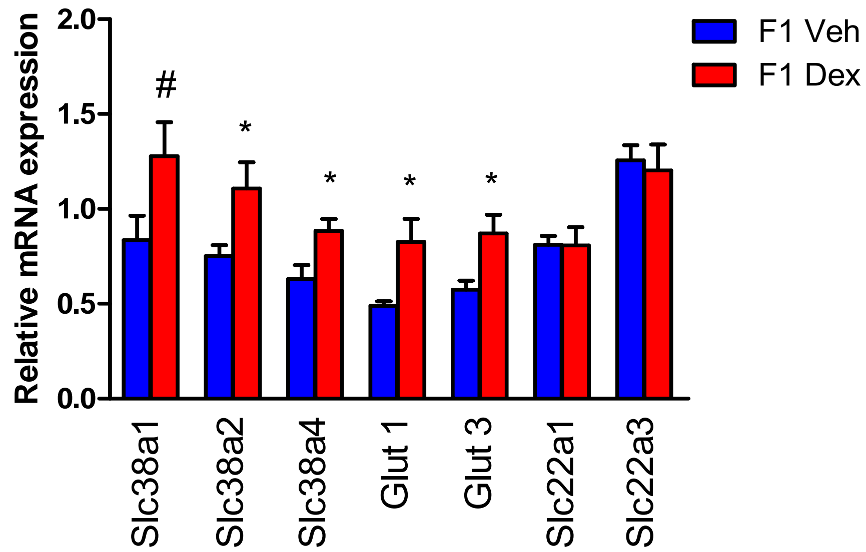


Figure 3.7 Expression of nutrient transporters in E20 placental labyrinth

Dex exposure increased expression in Slc38a2, Slc38a4, Glut1, Glut3 and was associated with a trend for an increased expression of Slc38a1 in placental labyrinth at E20

Data are mean ± SEM, analysed by Student's *t*-test

n = Veh: 12, Dex: 12

= p=0.057, * = p<0.05

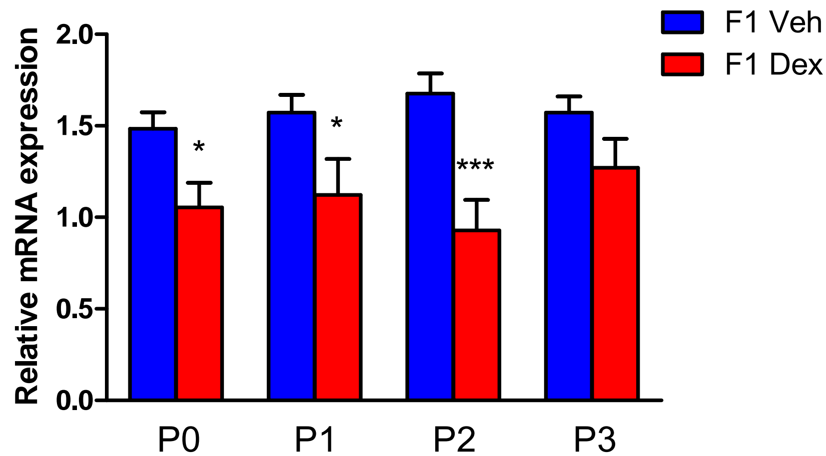


Figure 3.8 Expression of transcripts from Igf2 promoters in placental labyrinth

Dex exposure decreased expression of transcripts from Igf2 promoters P0, P1, and P2 in placental labyrinth at E20

Data are mean ± SEM, analysed by Student's *t*-test

n = Veh: 12, Dex: 12

* = $p<0.05$, *** = $p<0.001$

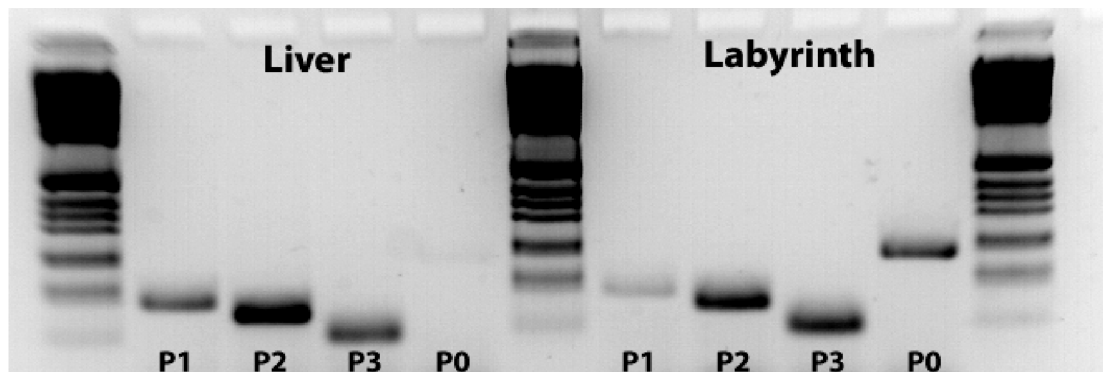


Figure 3.9 Gel electrophoresis of Igf2 transcripts from P1, P2, P3, and P0 promoters in E20 fetal liver and placental labyrinth

This shows that the P0 promoter transcript is only detected in placental labyrinth

3.3.4 F1-E20 placental stereology

Despite the Dex-induced reduction in placental size (Figure 3.2b), the area, and thus the volume of the placental labyrinth (Figure 3.10a) and junctional zone (Figure 3.10b) were reduced equally. Data presented are in relation to total placental volume.

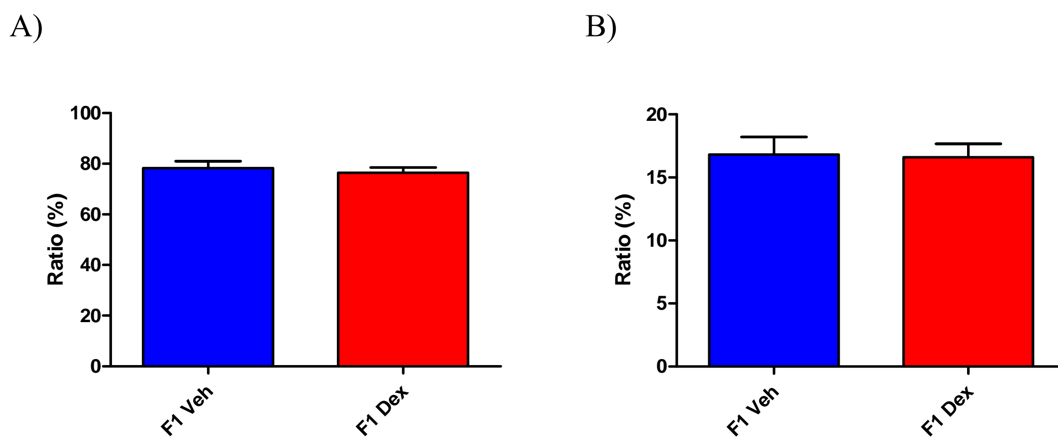


Figure 3.10 Ratio of the volume of labyrinth and junctional zones in placenta at E20
There was no difference in the ratio of volume of **A)** labyrinth and **B)** junctional zones between groups in relation to total placental volume
Data are mean ± SEM, analysed by Student's *t*-test
n = Veh: 11, Dex: 16
Part of this work was performed by Ms. Fiona E. Graham

3.3.5 F1 placental glycogen

No differences were seen in the amount of glycogen in placental labyrinth or junctional zones of the placenta (Figure 3.11a, b).

A) B)

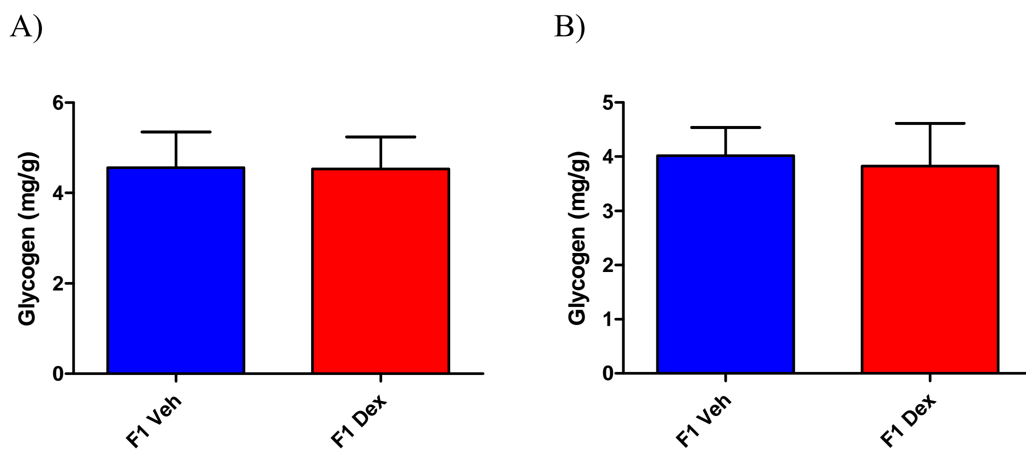


Figure 3.11 Glycogen levels in placental labyrinth and junctional zones at E20

There was no difference in glycogen level in **A)** labyrinth and **B)** junctional zones between groups

Data are mean ± SEM, analysed by Student's *t*-test

n = Labyrinth Veh: 7, Dex: 8

Junctional zone Veh: 8, Dex: 8

3.3.6 Western blot analysis of proteins involved in the Igf2 signalling pathway in placental labyrinth

Protein levels of pIRS1 and pmTOR were both elevated in placental labyrinth with Dex exposure. In contrast, prenatal Dex exposure was associated with a reduction in pAKT1 and pGSK3β. No difference was seen in pPi3k (Figure 3.12, Figure 3.13).

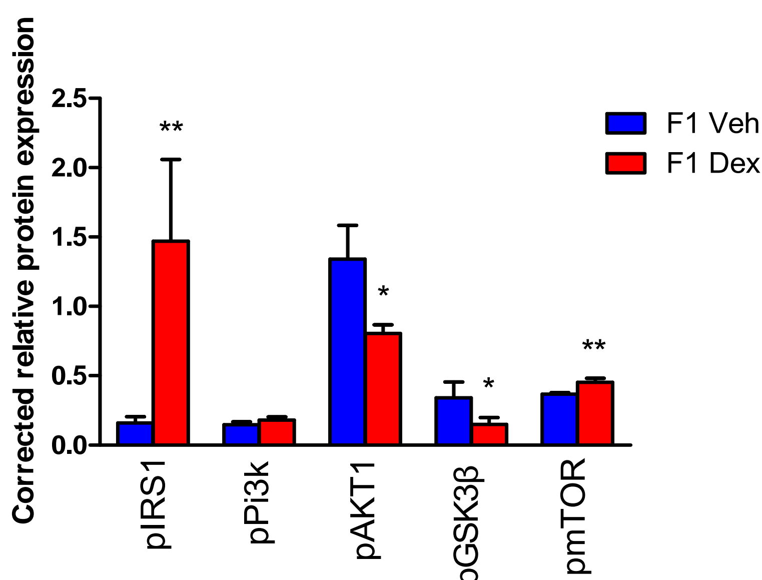


Figure 3.12 Placental labyrinth Igf2 pathway proteins

Increased protein level of pIRS1, pmTOR and decreased level of pAKT1 and pGSK3β were found in placental labyrinth of Dex exposed animals at E20

Data are mean ± SEM, all genes analysed by Student's *t*-test except pIRS1 which was analysed by Mann-Whitney *u*-test

n = Veh: 7, Dex: 7

* = $p<0.05$, ** = $p<0.01$

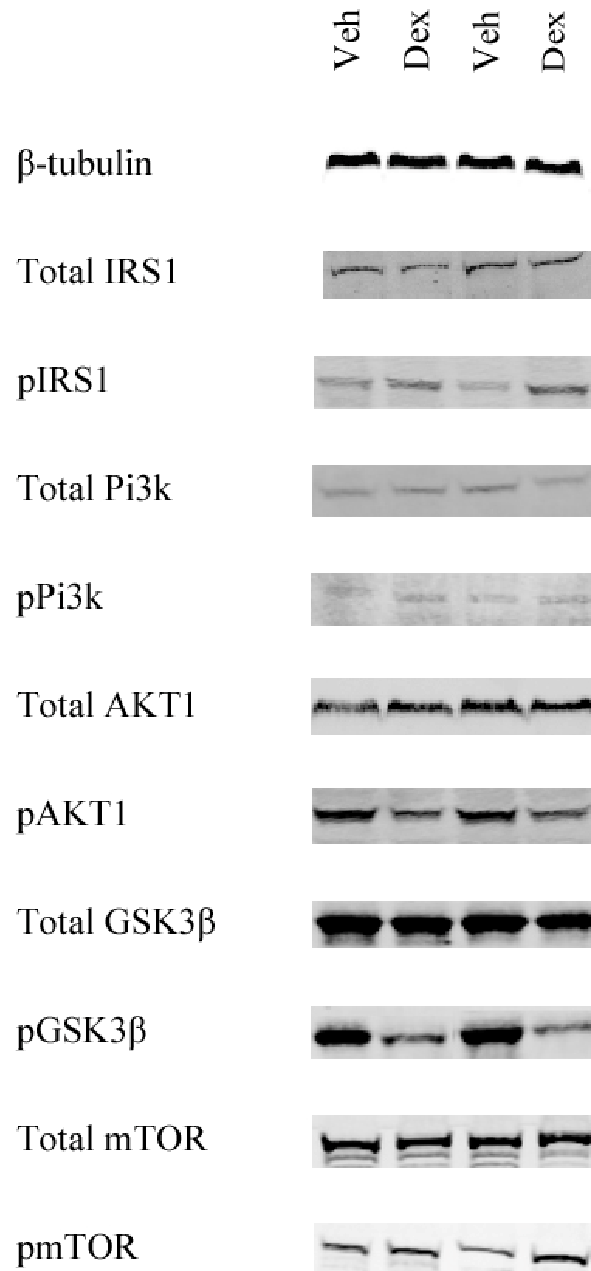


Figure 3.13 Representative blots of placental labyrinth Igf2 pathway proteins

IRS1 = Insulin receptor substrate 1; pIRS1 = Phosphorylated IRS1 (Tyr^{612}); Pi3k = Phosphoinositide 3-kinase; pPi3k = Phosphorylated Pi3k (Tyr^{458}); pAKT1 = Phosphorylated AKT1 (Ser^{473}); GSK3β = Glycogen synthase kinase 3β; pGSK3β = Phosphorylated GSK3β (Ser^{9}); mTOR = Mammalian target of rapamycin; pmTOR = Phosphorylated mTOR (Ser^{2448})

3.3.7 Western blot analysis of proteins involved in the Igf2 signalling pathway in fetal liver

Phosphorylated GSK3β protein levels were increased with Dex exposure. No difference was seen in phosphorylated AKT1 (Figure 3.14 and 3.15). Protein levels of pIRS1, pPi3k, and pmTOR were extremely low and could not be measured.

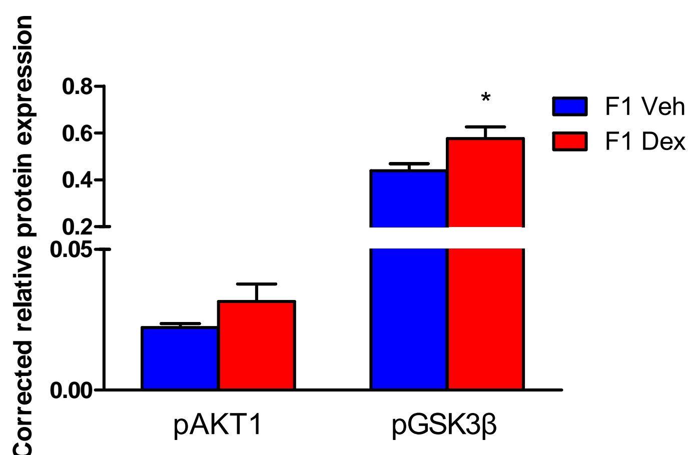


Figure 3.14 Fetal liver Igf2 pathway proteins

Dex exposure increased protein levels of pGSK3β in fetal liver at E20

Data are mean ± SEM, analysed by Student's *t*-test

n = Labyrinth Veh: 7, Dex: 7

* = $p<0.05$

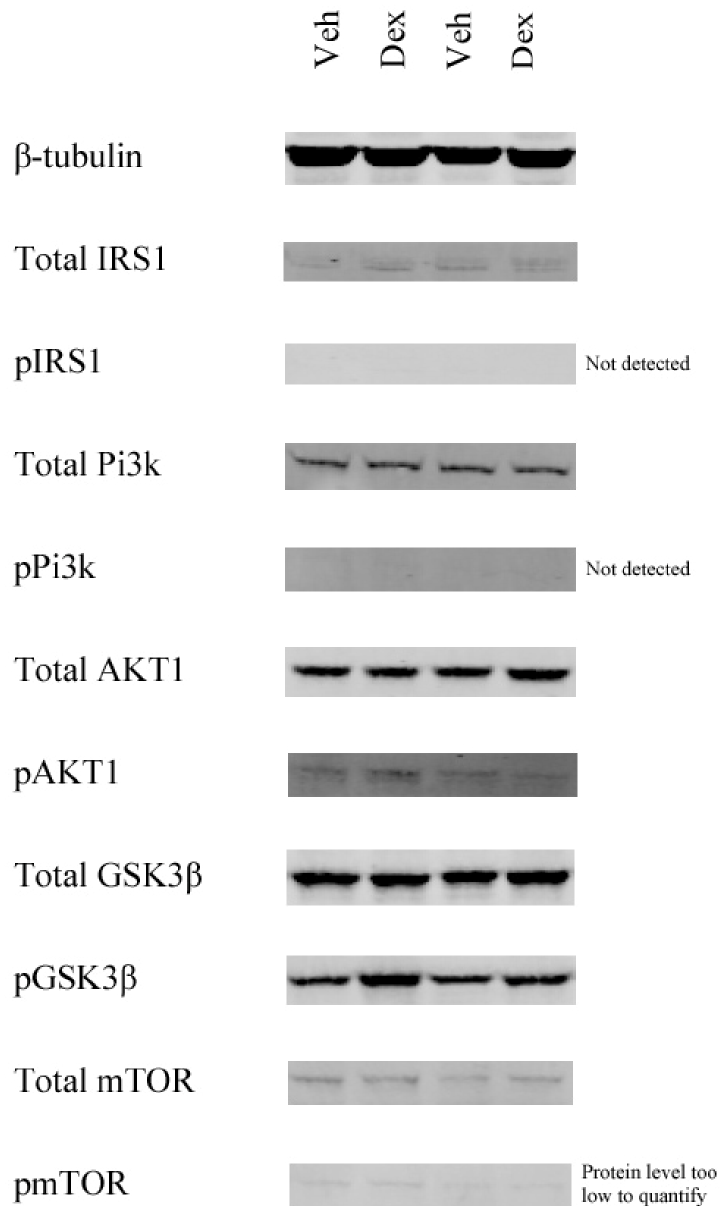


Figure 3.15 Representative blots of fetal liver Igf2 pathway proteins

IRS1 = Insulin receptor substrate 1; pIRS1 = Phosphorylated IRS1 (Tyr^{612}); Pi3k = Phosphoinositide 3-kinase; pPi3k = Phosphorylated Pi3k (Tyr^{458}); pAKT1 = Phosphorylated AKT1 (Ser^{473}); GSK3β = Glycogen synthase kinase 3β; pGSK3β = Phosphorylated GSK3β (Ser^{9}); mTOR = Mammalian target of rapamycin; pmTOR = Phosphorylated mTOR (Ser^{2448})

3.4 Discussion

These results demonstrate that prenatal Dex exposure is associated with effects on both fetus and placenta. The reduction in fetal and placental weights with Dex exposure are consistent with previous reports (Langdown and Sugden 2001, Ain et al. 2005). These results suggest that the programming effects of prenatal glucocorticoid overexposure may, at least in part, be due to alterations in placental growth and function.

Decreased maternal weight gain during the last week of pregnancy was also observed and is consistent with previous reports (Ain et al. 2005). This reduction in maternal weight gain is not due to a reduction in litter size as no difference was seen in pup number. One possible explanation for this could be the direct effect of Dex on eating behaviour. It has been shown previously that a single dose of Dex treatment in rats can result in anorexic behaviour within 24 hours and reduce weight gain (Simpson et al. 1974). This is an important finding as lower gestational weight gain has been linked to an increased risk of giving birth to small for gestational age babies (Nohr et al. 2008, Margerison Zilko et al. 2010).

An increase in plasma insulin levels was noted in non-fasted plasma of Dex exposed mothers. An increase in insulin levels is a known effect of chronic glucocorticoid treatment and has been observed in both animals and humans (Lenzen and Bailey 1984). In contrast, a reduction in fetal insulin levels was seen with Dex exposure. Previous studies have suggested that fetal overexposure to glucocorticoid may impair pancreatic β-cell function and result in a decrease in insulin secretion from the fetal

pancreas (Blondeau et al. 2001). Insulin has long been linked to fetal growth as pregnant women with diabetes are at risk of giving birth to babies that are larger in size (Ehrenberg et al. 2004). This increase in fetal growth appears to be related to the level of maternal blood glucose which stimulates fetal insulin production (Tallarigo et al. 1986, Farmer et al. 1988). In this study, the finding of reduced fetal insulin in Dex exposed animals may contribute to the decreased weight in offspring exposed to Dex in utero.

Igf2 has been suggested to be one of the most important growth factors during fetal life (Randhawa and Cohen 2005). In the Igf2 knockout mouse, fetal growth was reduced by 40 – 50% and placental growth by about 60% (DeChiara et al. 1990, Coan et al. 2008b). In this study, I have shown that despite a reduced weight at E20 in offspring exposed to Dex, there was an increase in Igf2 mRNA expression in the fetal liver. However, there was also an increase in the expression of IgfBP2, which primarily acts to inhibit the actions of Igfs particularly Igf2 (Jones and Clemmons 1995, Firth and Baxter 2002). Studies in pigs have shown that despite an increase in hepatic Igf2 mRNA expression in IUGR piglets in utero, IgfBP2 mRNA expression is also increased (Kampman et al. 1993) and previous studies in rodents have reported increased Igf2 and IgfBP2 gene expression in Dex exposed fetal liver (Price et al. 1992). Along with the increase in IgfBP2 which may counteract the actions of Igf2, I also found an increase in the expression of the potent growth inhibitors CDKn1c and Grb10 in Dex exposed fetal liver. CDKn1c has been suggested to be the major regulator of fetal growth within its domain which contains other imprinted genes such as Phlda2 (Andrews et al. 2007). It acts by negatively regulating cell

proliferation and when over-expressed, has been linked to a reduction in growth (Andrews et al. 2007). Interestingly, CDKn1c has a glucocorticoid response element 5kb upstream of its promoter site, suggesting that glucocorticoids may regulate the expression of this gene directly (Alheim et al. 2003). Grb10 is able to interact with receptor tyrosine kinases and may also play an important role in fetal and placental growth. Loss of function of Grb10 results in fetal and placental overgrowth, suggesting a role for this gene as a growth suppressor (Charalambous et al. 2003). Thus, the effects of Dex exposure on fetal growth may reflect a complex interaction between the expression of genes promoting and repressing fetal growth. The interaction between the imprinted genes in the fetal liver also mirrors the parental conflict hypothesis, which suggests that maternally expressed genes act to restrict growth, whereas paternally expressed genes act to enhance growth (Moore and Haig 1991). In this study, there was an increase in the expression of the maternally expressed genes, CDKn1c and Grb10 which may act to suppress growth and the paternally expressed gene, Igf2 which would be predicted to enhance growth. The increased expression of Igf2 may reflect an attempt to compensate for the growth retardation caused by CDKn1c and Grb10 (Caspary et al. 1999, Grandjean et al. 2000).

In the placental labyrinth, the expression of mRNA encoding several nutrient and glucose transporters important in growth was increased with Dex exposure, including Slc38a2, Slc38a4, Glut1, and Glut3. This is consistent with previous reports of the effects of glucocorticoid overexposure on the expression of nutrient transporters (Langdown and Sugden 2001, Wyrwoll et al. 2009). A further study showed no effect

of Dex exposure on the mRNA expression of these nutrient transporters after Dex exposure, but found increased nutrient transport (Audette et al. 2011). The discrepancy reported between this last study and the others could be a consequence of the time-point of glucocorticoid exposure, since studies that showed a difference in mRNA expression involved exposure of the mother to glucocorticoids during the last week of gestation, whereas Audette et al. performed the study at mid-gestation.

It has been proposed that smaller placentas may be more efficient in providing nutrients to the fetus (Coan et al. 2008a) and may be able to adapt to the increasing fetal demands by up-regulating the nutrient transfer capability (Constância et al. 2005). Indeed, it has also been proposed that an increase in fetal Igf2 expression may be a “call for help” from the fetus, signalling to the placenta to increase nutrient supply from the mother (Constância et al. 2005, Coan et al. 2008a). Thus, the increased expression of the nutrient and glucose transporters Slc38a2, Slc38a4, Glut1 and Glut3 in the Dex exposed placentas may reflect this increased demand from the fetus in Dex exposed animals mediated by Igf2. In contrast to the findings in fetal liver, the expression of Igf2 was decreased in the placental labyrinth. The levels of transcripts from three of the four promoters: P0, P1, and P2 of Igf2 was also decreased in placental labyrinth. The P0 promoter of the Igf2 gene has been shown to be placenta specific and acts as a major modulator of placental and fetal growth (Constância et al. 2002).

The exact function of glycogen stored in the placenta remains to be determined. Some studies have suggested that glycogen may act as a source of energy for the

placenta's own needs (Barash and Shafrir 1990), whilst others have suggested that it may be used for fetal growth and survival (Selye and McKeown 1935, Bouillot et al. 2006, Leonce et al. 2006, Esquiliano et al. 2009). The majority of the glycogen in the placenta is stored in the cytoplasm of the glycogen cells, which migrate to the junctional zone close to the end of pregnancy (Tunster et al. 2010). In the current study, no difference was seen in glycogen levels between Dex and Veh in either the placental labyrinth or the junctional zone. It has been suggested recently that Phlda2 may be a major regulator of glycogen storage in the placenta (Tunster et al. 2010) so that the lack of change in glycogen levels are consistent with the lack of change in Phlda2 gene expression.

Due to the important role of Igf2 in fetal and placental growth, I measured the protein levels of important components of the Igf2 signalling pathway which play an important role in cellular growth and protein synthesis. In the placental labyrinth, pIRS1 and pmTOR were both increased with Dex exposure, whereas pAKT1 and pGSK3β were decreased and no difference was seen in pPi3k. Upon insulin stimulation, IRS1 is phosphorylated and activates downstream Pi3k/AKT pathways (Esposito et al. 2001, Gual et al. 2005). Thus, the rise in pIRS1 level seen in placental labyrinth with Dex exposure may be due to a direct effect of increased maternal circulating insulin. Although activation of IRS1 through phosphorylation would be expected to increase placental growth, Dex exposed placentas were smaller. One mechanism for this may be the decrease in the level of activated phosphorylated AKT1 and the lack of change in activation of Pi3k through phosphorylation. GSK3β is constitutively active and thus a decrease in phosphorylation of GSK3β would be

associated with activation of this protein (Welsh and Proud 1993, Welsh et al. 1996). The activation of GSK3β would result in deactivation of glycogen synthase and therefore reduced glycogen synthesis. The increased phosphorylation of mTOR may be a consequence of the increase in nutrient transport which would be predicted to occur in Dex exposed placentas, since it has been suggested that mTOR can be regulated by the presence of nutrients, especially amino acids (Roos et al. 2009). Therefore, the observed change in mTOR could be independent of Igf signalling. In the fetal liver, no difference was seen in pAKT1 but an increase was seen in pGSK3β in offspring exposed to Dex. IRS1 and mTOR protein was present at very low levels. The phosphorylation, and therefore activation, of these two proteins was also seen at very low levels and phosphorylation of Pi3k was not seen. Previous work has demonstrated that these proteins are indeed present at extremely low levels in fetal liver (Anand et al. 2002, Khamzina et al. 2003).

From this study, it is clear that exposure to Dex during the last week of gestation in rats is associated with tissue-specific effects on both fetus and placenta. One explanation for these tissue-specific effects is that during the last week of gestation, the growth of the fetus and the fetal liver is at its maximal rate; whereas the lifespan of the placenta is coming to an 'end', therefore, it is likely that the fetus and the placenta were 'insulted' by Dex at different points in the lifespan of the organ leading to different outcomes. Additionally, glucocorticoids may act to induce epigenetic changes directly by modifying DNA methylation patterns (Thomassin et al. 2001) and changing chromatin conformation (Fryer and Archer 1998). The patterns of DNA methylation are known to be tissue-specific (Fowden et al. 2006a), so that

glucocorticoid induced effects on DNA methylation may result in tissue-specific effects.

In conclusion, this study has demonstrated that glucocorticoid overexposure during the final week of pregnancy in the rat results in effects on fetal weight, placental weight, placental gene expression and function, and fetal liver gene expression. The precise mechanisms underlying these effects remain to be determined but may include direct effects of glucocorticoids on growth and gene expression and/or epigenetic changes.

CHAPTER 4

Intergenerational programming in the glucocorticoid programmed rat

4.1 Introduction

4.1.1 Introduction

Previous studies from our group and others have provided evidence that programming effects may be transmissible to a second generation (F2) in the absence of a direct exposure to an insult. As discussed in chapter 1, this phenomenon has been reported in both humans and animals. Epidemiological studies in humans have provided evidence for intergenerational effects on birth weight (Klebanoff et al. 1984, Emanuel et al. 1992, Collins et al. 2003) and obesity (Labayen et al. 2010), cardiovascular (Davey Smith et al. 1997, Walker et al. 1998, Kuznetsova et al. 2003, Bunt et al. 2005, Labayen et al. 2010) and type 2 diabetes risk (Dabelea et al. 2000, Hyppönen et al. 2003, Dabelea et al. 2008). Similar results have been reported in animal studies with a number of studies showing evidence for multigenerational effects occurring as a consequence of prenatal exposure to a low protein diet, including effects on birth weight (Snoeck et al. 1990, Benyshek et al. 2008), the pancreas (Snoeck et al. 1990), brain development (Stewart et al. 1975, Hoet and Hanson 1999), nephrogenesis and renal function (Langley-Evans et al. 1999, Watkins et al. 2010), blood pressure (Langley Evans and Jackson 1994, Harrison and Langley-Evans 2009), and glucose homeostasis (Benyshek et al. 2006). Early postnatal exposure to dietary manipulations has also been shown to have multigenerational effects on pancreatic function, insulin secretion, and insulin sensitivity (Laychock et al. 1995, Srinivasan et al. 2003, Benyshek et al. 2004). In most of these studies, similar phenotypes were observed in both F1 and F2 generations even though the F2 offspring were not directly exposed to the 'insult'. Additionally, in a number of these 'across generation' studies, effects have been

demonstrated to be passed through either the maternal (Drake et al. 2005, Zambrano et al. 2005, Burdge et al. 2007, Skinner et al. 2008) or paternal (Anway et al. 2005, Drake et al. 2005, Kaati et al. 2007) lines.

We have previously shown multigenerational effects on birth weight and glucose homeostasis in the Dex programmed rat (Drake et al. 2005). In the previous chapter, I showed effects in the placenta and fetus in F1 offspring directly exposed to Dex treatment during the last week of gestation. Here I have explored effects on the placenta and fetal liver in a second (F2) generation.

4.1.2 Hypothesis

I hypothesise that overexposure to glucocorticoids during the last week of gestation may induce programming effects not only in the developing offspring but also in the grand offspring. I also hypothesise that the underlying mechanisms of programming between the two generations are different, as the exposure to glucocorticoids occurs at different stages of development.

4.1.3 Aims

The aim of this study was to explore whether the effects of prenatal glucocorticoid overexposure differ in the F1 and F2 generations. This will be achieved by studying fetal and placental size and gene expression in the F2 generation at the same time point as in the F1.

4.2 Methods

4.2.1 Animal maintenance, treatment, tissue collection, and offspring production

Female Wistar rats were time mated with a stud Wistar male when they weighed around 250g. The presence of a vaginal plug was checked daily in the morning. The females were singly housed after a vaginal plug was found (denoted as E0) and were left undisturbed with *ad libitum* access to food and water. Pregnant female rats were randomly assigned to Dex or Veh treatment. On E15, pregnant females were given a subcutaneous injection of either Dex in 0.9% saline and 4% ethanol (100μg/kg body weight) or an equivalent volume of Veh (1ml/kg). The injection was given every day in the morning from E15 to E21 inclusive after weighing the animals.

Litters (F1 Veh and F1 Dex) were weighed at birth and culled back to eight per group. Pups were reared by their biological mother until three weeks of age and were then weaned and housed with animals from the same treatment group. When F1 females weighed around 250g, they were housed singly with an F1 male from a different litter of either the same or a different treatment group. This lead to the production of four different groups: F1 Veh female x F1 Veh male (VV), F1 Veh female x F1 Dex male (VD), F1 Dex female x F1 Veh male (DV), and F1 Dex female x F1 Dex male (DD) (Figure 2.1).

At E20, F1 pregnant females were killed by carbon dioxide asphyxiation. Maternal blood was collected by cardiac puncture into a 0.5M EDTA rinsed 2ml syringe. Fetal blood was collected into Microvette® CB-300 tubes after decapitation. Blood was

placed on wet ice until centrifugation (5000 rpm, 10 mins, 4°C) and plasma removed and frozen at -20°C. Fetuses and placentas were dissected and weighed. Placentas were either fixed in 10% neutral buffered formalin, stored overnight in 4°C and transferred into 70% ethanol and kept at RT° until embedding in paraffin or dissected into labyrinth and junctional layers. Fetal liver was dissected. Dissected tissues were snap frozen on dry ice and stored at -80°C.

4.2.2 Metabolic experiments

Fetal plasma was pooled from all fetuses within a litter. Non-fasting plasma glucose was measured using an enzymatic (hexokinase) method (Thermo Fisher Scientific); plasma insulin was measured using an ELISA kit (Crystal Chem Inc.).

4.2.3 Molecular experiments

Total mRNA was extracted from fetal liver and placental labyrinth using the RNeasy® mini kit (Qiagen) and reverse transcribed (Promega) to produce cDNA. Quantitative real-time PCR was used to examine gene expression by TaqMan™ (Applied Biosystems) or self designed UPL (Roche) primers with the addition of MasterMix (Roche). The expression levels of different Igf2 transcripts was examined by quantitative real-time PCR using SYBR® green and primers designed by Dr. Amanda J. Drake. The presence of different Igf2 transcripts in fetal liver and placenta was assessed by gel electrophoresis (2% TBE agarose gel with 1kb DNA ladder at 100V for 1 hour) following PCR. Expression of all genes examined in placental labyrinth were normalised to the average of two reference genes

Cyclophilin-A and Tata box binding protein (TBP) and to Cyclophilin-A in the fetal liver.

4.2.4 Placental stereology procedures

Paraffin embedded placentas were thoroughly sectioned at 7μm per section. Sections were then fixed on glass slides, stained with haematoxylin and counter-stained with eosin. Stained sections were examined under a microscope with a CCD camera at 1.25x magnification. The areas of the two different zones, labyrinth and junctional, were measured by MCID Basic 7.0 software (InterFocus Imaging Ltd., Cambridge, UK), and volumes calculated. Ratio of volumes were in relation to total placental volume were measured. This experiment was performed with the assessor blinded to experimental groups.

4.2.5 Statistical analysis

Data were analysed by using SPSS® and GraphPad®. Statistical significance was calculated using Student's *t*-test, two-way ANOVA, Chi-Square test, or Pearson's correlation and is expressed as mean ± SEM unless otherwise stated. Results were considered significant if $p<0.05$.

4.3 Results

4.3.1 F1 cohort

No difference was seen in length of gestation or litter size between females receiving Dex (F0 Dex) or Veh (F0 Veh) (Table 4.1). Offspring of Dex treated mothers (F1 Dex) were lighter than Veh (F1 Veh). At weaning, both F1 Dex males (Table 4.2) and F1 Dex females (Table 4.2) were lighter than Veh males and females respectively.

4.3.2 F2 cohort

Difficulties were encountered when setting up F1 Dex females to plug with F1 Veh males (DV) as well as F1 Veh females to plug with F1 Dex males (VD). Both these groups took longer to mate and had higher rate of failed pregnancy after confirmation of the presence of vaginal plug (Table 4.3). Possible reasons for this are discussed below in section 4.4. At E20, analysis by two-way ANOVA showed an effect of maternal prenatal Dex exposure to increase fetal weight ($p<0.001$; $F=16.67$) and paternal prenatal Dex exposure to decrease fetal weight ($p<0.05$; $F=4.09$) (Figure 4.1a). For the placenta, maternal prenatal Dex exposure was associated with increased placental weight ($p<0.001$; $F=11.25$), and paternal prenatal Dex exposure with decreased placental weight ($p<0.001$; $F=13.55$) (Figure 4.1b). At birth, weight was decreased in DD, DV and VD offspring, with the VD group having the lowest weight (Figure 4.1c). No difference was seen in the number of pups per litter across the four groups (Table 4.3). There were no differences in glucose and insulin levels in non-fasted plasma from the F1 mothers at E20 (Figure 4.2a). There were also no differences in fetal glucose or insulin levels in pooled plasma at E20 (Figure 4.2b).

	F0 Veh	F0 Dex	p value
Gestation length (days)	22.50 ± 0.18	22.67 ± 0.16	0.51
Litter size	12.50 ± 1.15	13.13 ± 0.39	0.61

Table 4.1 F0 maternal cohort
No difference in gestation length and litter size
Data are mean ± SEM, analysed by Student's *t*-test
n = Gestation length Veh: 8, Dex: 9
Litter number Veh: 8, Dex: 8 (one Dex litter was cannibalised)

	F1 Veh	F1 Dex	p value
Birth weight (g)			
Male	6.761 ± 0.09	5.897 ± 0.11	< 0.0001
Female	6.434 ± 0.09	5.541 ± 0.08	< 0.0001
Wean weight (g)			
Male	68.08 ± 1.56	61.42 ± 1.21	< 0.01
Female	64.30 ± 1.44	60.37 ± 1.08	< 0.05

Table 4.2 F1 cohort for 8 litters per group
Offspring exposed to Dex were significantly lighter at birth and at weaning
Data are mean ± SEM, analysed by Student's *t*-test

	VV	DD	DV	VD
Setup to plug	14	13	21	22
Successful plug	9	10	10	12
Plug percentage	64.28%	76.92%	47.61%	54.54%
Successful pregnancy	9	8	8	8
Pregnancy percentage	100.00%	80.00%	80.00%	66.66%
Litters with reabsorption sites	0	0	1	3
Litter size	14.1 ± 0.9	13.1 ± 1.5	11.7 ± 0.8	13.4 ± 2.0

Table 4.3 F2 cohort – pregnancy setup

Chi-Square analysis of observed versus expected plugs: Chi-square 12.79; $p<0.05$ and Chi-square analysis of observed versus expected pregnancies following successful plugging: Chi-square 2.13; $p=0.5$

Data for litter size were mean ± SEM, analysed by two-way ANOVA

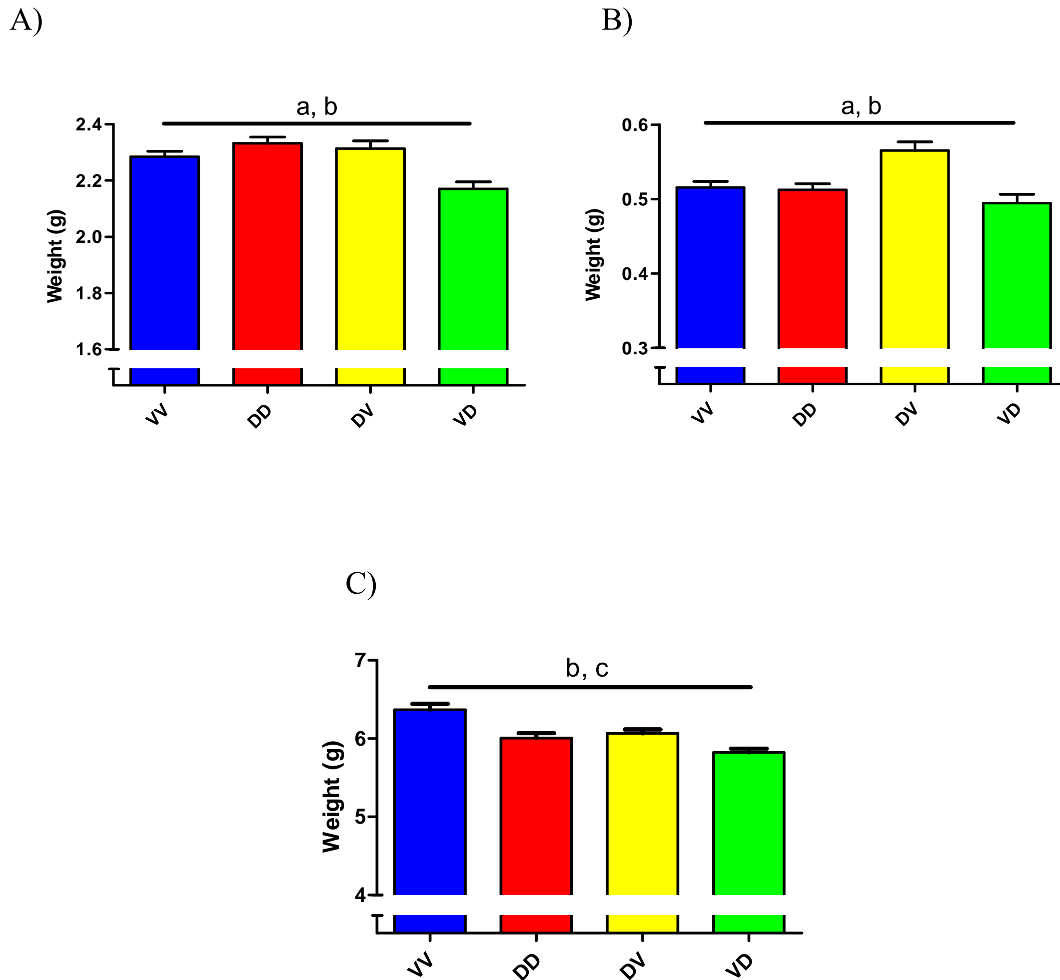


Figure 4.1 F2 weights of **A)** fetus, **B)** placenta, and **C)** at birth

There was an effect of maternal prenatal Dex exposure to increase fetal and placental weight at E20; an effect of paternal prenatal Dex exposure effect to reduce fetal and placental weight at E20 and weight at birth; and an interaction between parental prenatal Dex exposure to reduce weight at birth

Data are mean ± SEM, analysed by two-way ANOVA

n =		
	Fetuses	VV: 127, DD: 91, DV: 94, VD: 106
	Placentas	VV: 126, DD: 91, DV: 94, VD: 106
	Pups	VV: 109, DD: 110, DV: 109, VD: 84

a = Maternal prenatal Dex exposure effect to increase, b = Paternal prenatal Dex exposure effect to decrease, c = Interaction between parental prenatal Dex exposure status to decrease birth weight

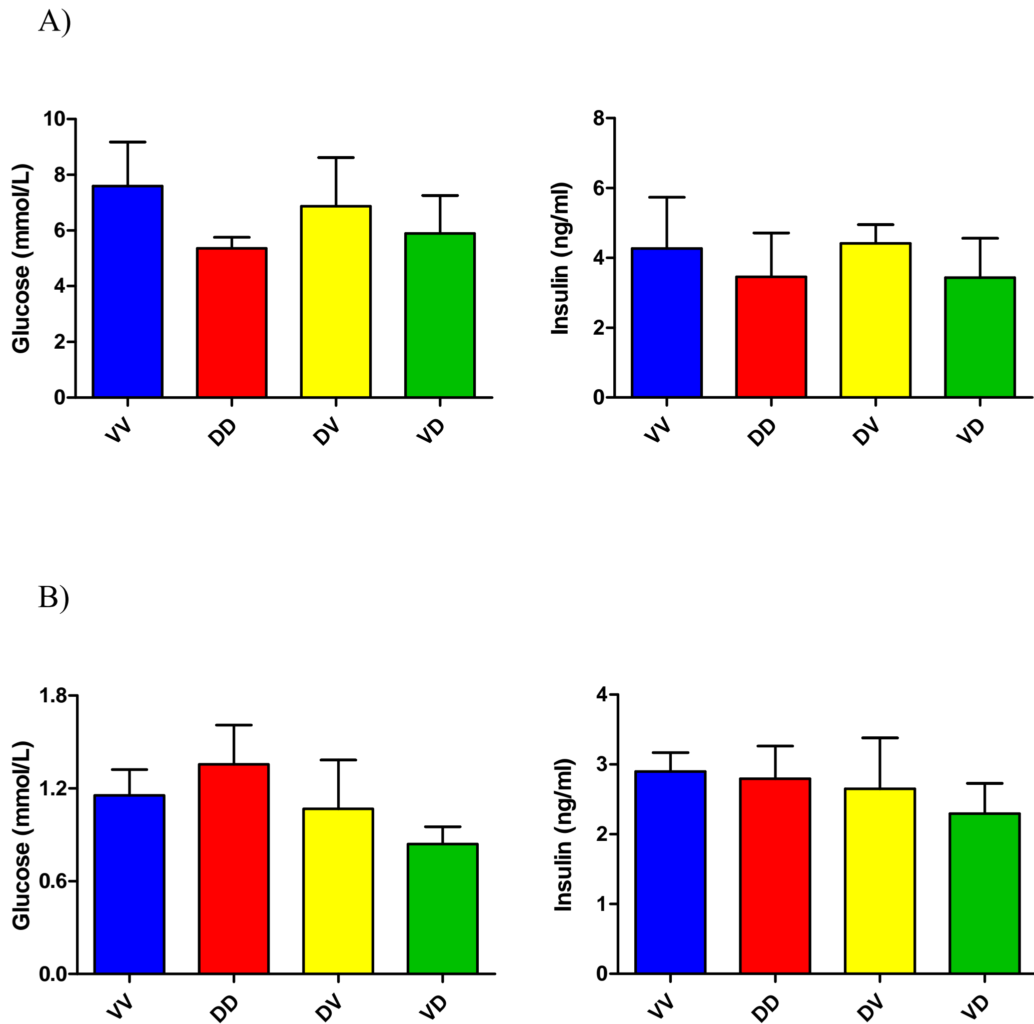


Figure 4.2 Maternal and fetal glucose and insulin at E20

There was **A)** no difference in F1 maternal plasma glucose and insulin levels in all four groups at E20 and **B)** no difference in F2 fetal plasma glucose and insulin levels in all four groups at E20

Data are mean ± SEM, analysed by two-way ANOVA

n =	Maternal and fetal glucose	6 per group
	Maternal and fetal insulin	VV: 3, DD: 4, DV: 3, VD: 4

4.3.3 F2-E20 fetal liver gene expression

At E20, there was an interaction between maternal and paternal prenatal Dex exposure to decrease the mRNA expression of Igf2 ($p<0.05$; $F=5.66$) and Phlda2 ($p<0.05$; $F=6.62$); an interaction between maternal and paternal prenatal Dex exposure to increase IgfBP2 ($p<0.05$; $F=6.15$) mRNA expression; and an effect of paternal prenatal Dex exposure to decrease IgfBP3 ($p<0.05$; $F=4.62$) mRNA expression (Figure 4.3). No difference was seen in the expression of Igf1, CDKn1c, Grb10 (Figure 4.3), or in the mRNA expression levels of transcripts from the three promoters of Igf2 (P1, P2, P3) (Figure 4.4a). There was a significant correlation between the expression of the three Igf2 promoters and total Igf2 (Figure 4.4b). No expression of a transcript from the P0 promoter of Igf2 was seen in fetal liver as expected.

4.3.4 F2-E20 placental labyrinth gene expression

In the placental labyrinth, there was an effect of prenatal maternal Dex exposure to increase the expression of IgfBP2 ($p<0.01$; $F=6.85$), Mest ($p<0.05$; $F=6.33$), Slc38a4 ($p<0.05$; $F=5.33$), Glut1 ($p<0.01$; $F=10.11$), and Slc22a3 ($p<0.01$; $F=7.78$); and a trend for an increase in the expression of Slc38a1 ($p=0.058$; $F=3.89$) (Figure 4.5a, b and 4.6). There was an effect of paternal prenatal Dex exposure effect to increase mRNA expression of CDKn1c ($p<0.05$; $F=6.41$), Phlda2 ($p<0.05$; $F=6.08$), and Slc22a1 ($p<0.05$; $F=6.28$) (Figure 4.5b and 4.6). No differences were seen in the expression of Slc38a2, Igf2, Igf2r, IgfBP3, Grb10 (Figure 4.5a, b and 4.6), or in the transcripts from all four Igf2 promoters (P0, P1, P2, P3) (Figure 4.7a). There was a significant correlation between the expression of all four Igf2 promoters and total Igf2 (Figure 4.7b).

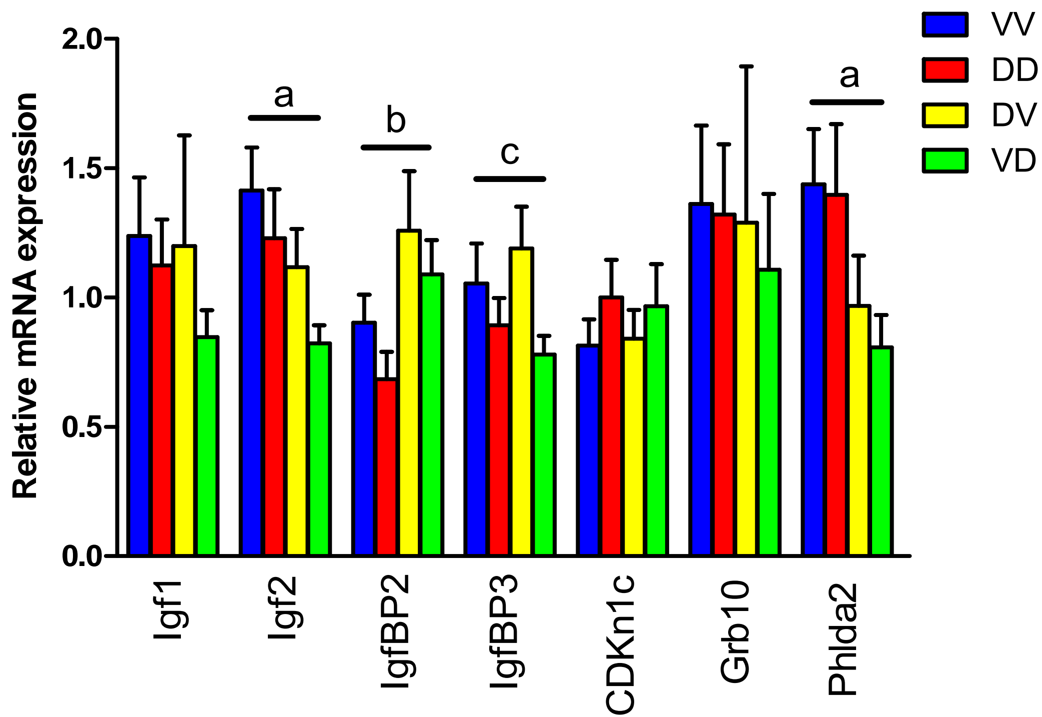


Figure 4.3 F2-E20 fetal liver gene expression

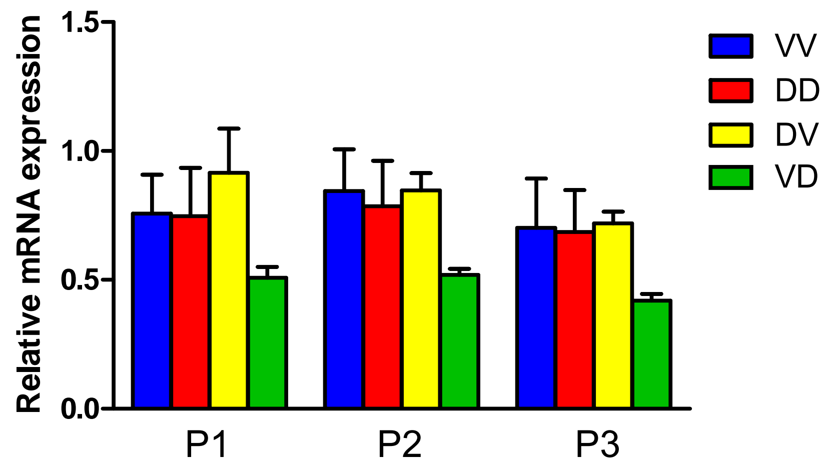
There was an interaction between parental prenatal Dex exposure effect to reduce expression of Igf2 and Phlda2; an interaction between parental prenatal Dex exposure effect to increase expression of IgfBP2; and a paternal prenatal Dex exposure effect to decrease expression of IgfBP3

Data are mean ± SEM, analysed by two-way ANOVA

n = VV: 9, DD: 7, DV: 8, VD: 8

a = Parental interaction of prenatal Dex exposure to decrease, b = Parental interaction of prenatal Dex exposure to increase, c = Paternal prenatal Dex exposure effect to decrease

A)



B)

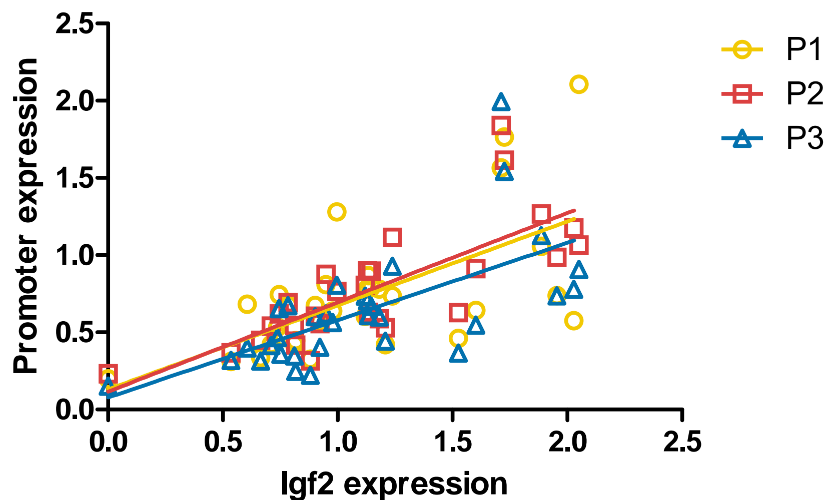


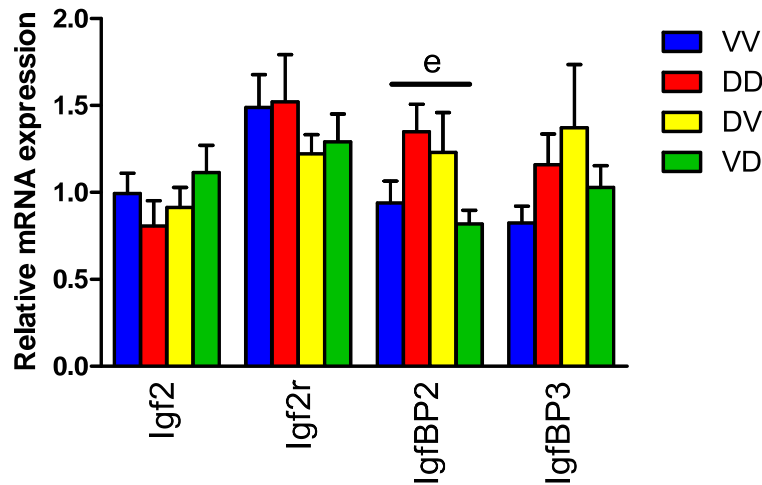
Figure 4.4 F2 fetal liver Igf2 promoter transcript levels and correlation

There was **A)** no difference in the level of mRNA transcripts of all three promoters in fetal liver at E20 but **B)** there is a positive correlation between total Igf2 expression and all three promoter transcripts. The significance of the correlation between total Igf2 expression and all three promoter transcripts (P1, P2, P3) was $p<0.001$

Data are mean ± SEM, analysed by two-way ANOVA and Pearson's correlation

n = VV: 9, DD: 7, DV: 8, VD: 8

A)



B)

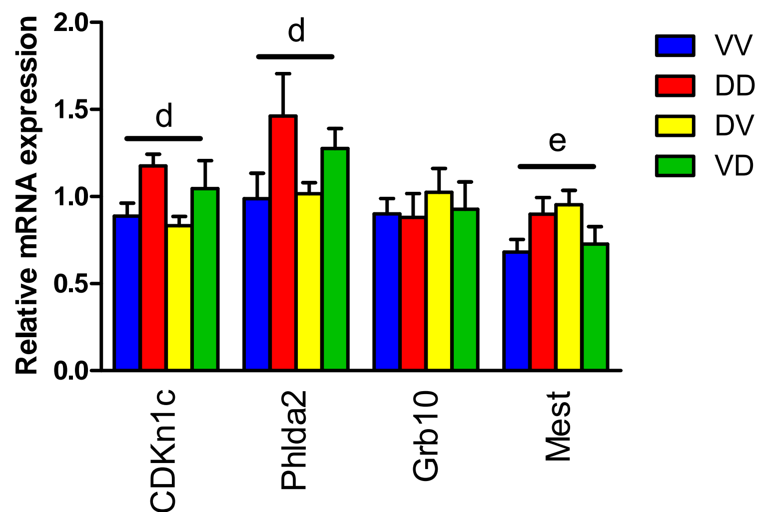


Figure 4.5 F2 placental labyrinth Igf pathway and imprinted gene expression

There was a paternal prenatal Dex exposure effect to increase expression of CDKn1c and Phlda2; and a maternal prenatal Dex exposure effect to increase expression of Mest and IgfBP2

Data are mean ± SEM, analysed by two-way ANOVA

n = VV: 9, DD: 7, DV: 8, VD: 8

d = Paternal prenatal Dex exposure effect to increase, e = Maternal prenatal Dex exposure effect to increase

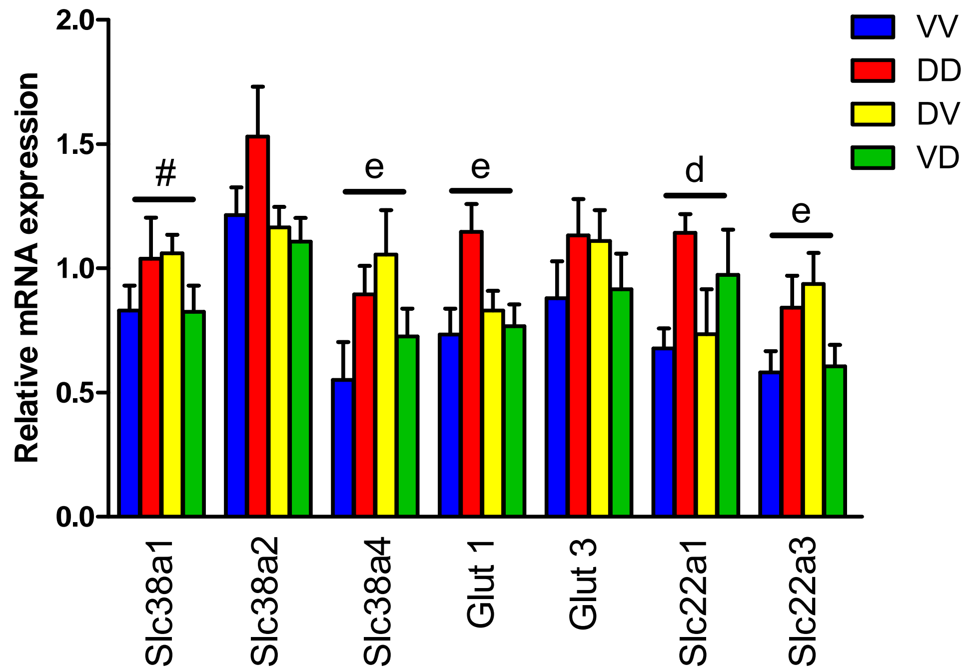


Figure 4.6 F2-E20 placental labyrinth nutrient transporter gene expression

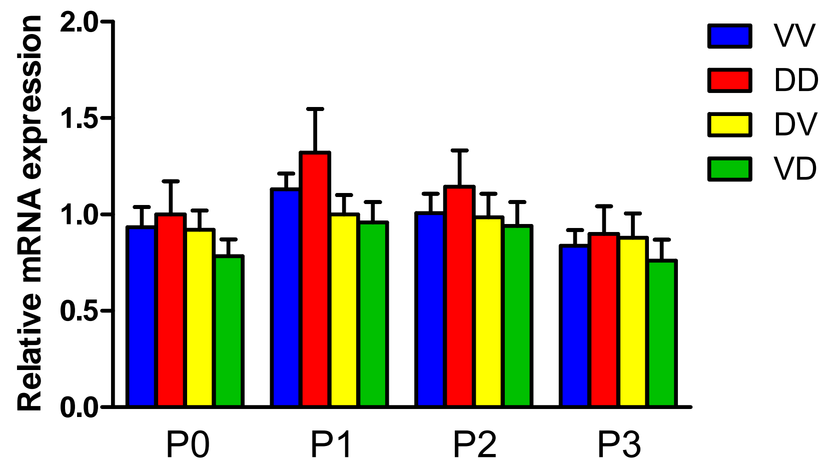
There was a paternal prenatal Dex exposure effect to increase expression of Slc22a1; and a maternal prenatal Dex exposure effect to increase expression of Slc38a4, Glut1, and Slc22a3

Data are mean ± SEM, analysed by two-way ANOVA

n = VV: 9, DD: 7, DV: 8, VD: 8

d = Paternal Dex exposure effect to increase, e = Maternal Dex exposure effect to increase, # = trend (p=0.058) to significance for maternal Dex exposure effect to increase

A)



B)

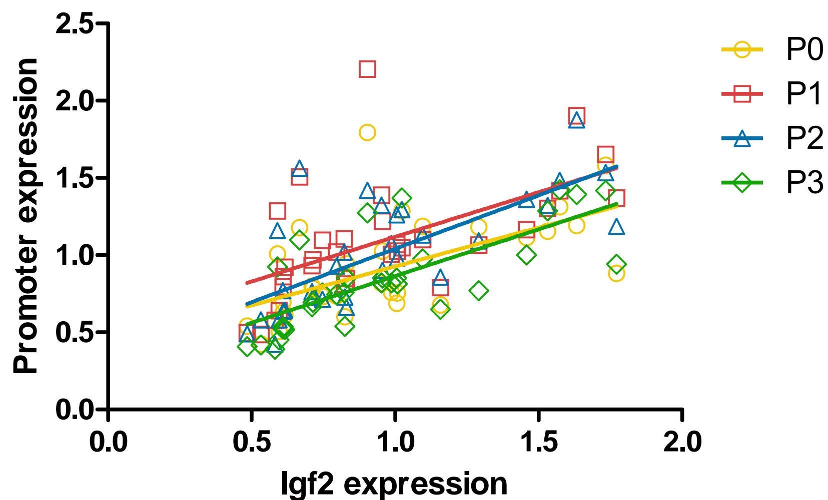


Figure 4.7 F2 placental labyrinth Igf2 promoters' transcription level and correlation

There was **A)** no difference in the level of mRNA transcripts of all four promoters in labyrinth at E20 but **B)** there is a positive correlation between total Igf2 expression and all four promoter transcripts. The significance of the correlation between total Igf2 expression and all four promoter transcripts (P0, P1, P2, P3) was $p<0.001$

Data are mean ± SEM, analysed by two-way ANOVA and Pearson's correlation

n = VV: 9, DD: 7, DV: 8, VD: 8

4.3.5 F2-E20 placental stereology

There were no differences in the volume of placental labyrinth between the four groups (Figure 4.8a); however there was an effect of paternal prenatal Dex exposure to decrease the volume of the junctional zone (Figure 4.8b). Data presented are in relation to total placental volume.

A) B)

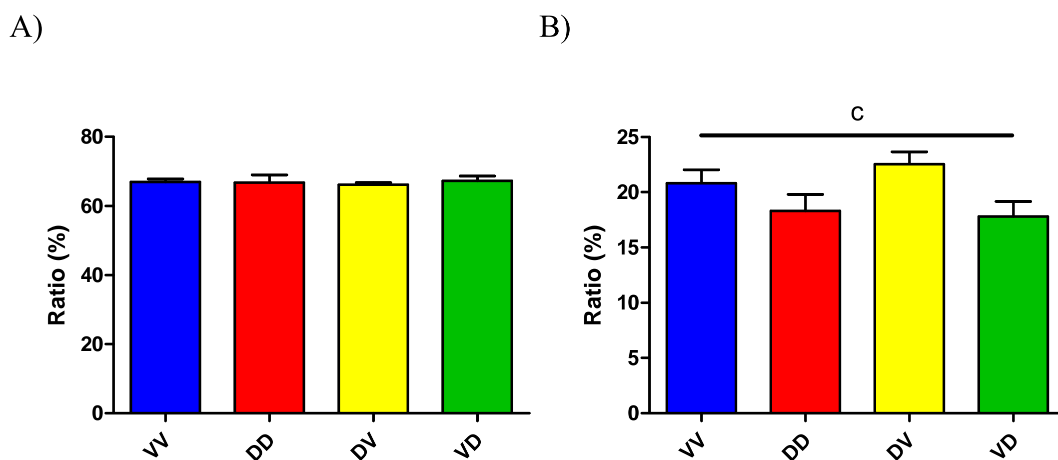


Figure 4.8 Ratio of the volume of F2 placental labyrinth and junctional zones
There was **A)** no difference between the volume of placental labyrinth in all four groups but **B)** there is a paternal prenatal Dex exposure effect to decrease the volume of the junctional zone in relation to total placental volume
Data are mean ± SEM, analysed by two-way ANOVA
n = 6 per group
c = Paternal effect to decrease

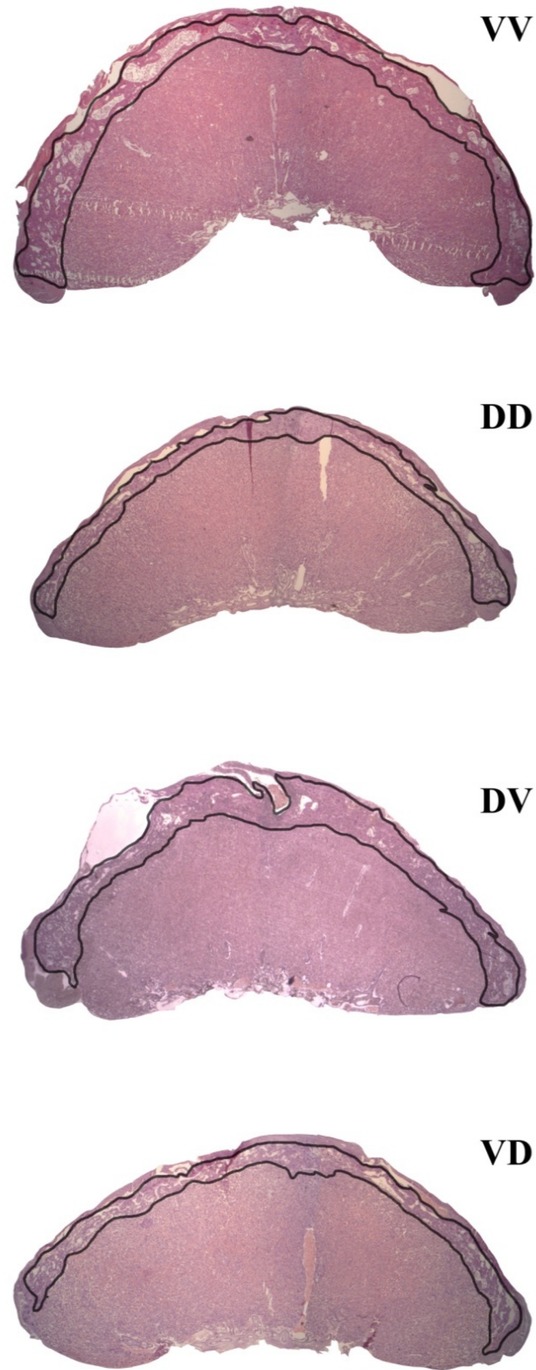


Figure 4.9 Representative pictures of H&E stained placentas from each group
Picture shows the decreased junctional zone in DD and VD groups
Area encircled by the black marker represents the junctional zone

4.4 Discussion

These results demonstrate programming effects of parental prenatal Dex exposure on placental and fetal development in the F2 generation. Previous reports have suggested that the transmission of programming effects across generations results in similar phenotypes being reproduced in the subsequent generations (Drake et al. 2005, Harrison and Langley-Evans 2009, Jimenez-Chillaron et al. 2009). These results confirm that prenatal Dex exposure is associated with effects on fetal and placental growth and gene expression in the F2 generation but suggests that despite similar phenotypes in both F1 and F2 offspring, the mechanisms underlying the programming effects on growth in the two generations may be different.

In this study, difficulties were encountered when mating DV and VD groups. These two groups took longer to mate and there were a number of resorption sites in the litters of the VD group. It is not clear at what point in gestation this resorption of embryos occurred and whether this was a result of failed implantation or as a consequence of maternal physiological error. Although the cause of the resorption sites in the VD group is not clear, but one explanation could be that this occurred as a consequence of altered gene expression, since many of the genes that showed changes in expression in this study, in particular imprinted genes, are important in growth and fetal survival (Walsh et al. 1994, Barton et al. 2005). Alterations in the expression of these genes may lead to unfavourable pregnancy outcome (Walsh et al. 1994, Dean et al. 1998). Indeed, some studies have previously shown that disruption in the expression of some imprinted genes leads to post-implantation embryo loss (Walsh et al. 1994, Pathak et al. 2010) and others studies using a chemotherapeutic

agent, cyclophosphamide, have shown increased resorption sites associated with epigenetic changes (Hales et al. 1992, Barton et al. 2005). Possible explanations for the increase in the length of time to plug may be the altered sexual behaviour in F1 Dex males. Previous studies have suggested that prenatal exposure to glucocorticoids can alter sexual preference and behaviour in male rats (Holson et al. 1995, Piffer et al. 2009). Additionally, studies have shown that prenatal overexposure to stress or glucocorticoids may impact on females; altering the oestrous cycle, increasing avoidance behaviour during mating, increasing spontaneous abortions during pregnancy, and altering fertility in female offspring (Herrenkohl 1979, Frye and Orecki 2002, Piffer and Pereira 2004).

In the second generation, I found parent-of-origin effects on placental and fetal weight at E20 and on birth weight. The parental conflict hypothesis suggests that the influence of the father promotes growth enhancement, whereas the influence of the mother is to restrict the growth of the developing fetus (Moore and Haig 1991). My results showing a maternal effect to increase and a paternal effect to reduce fetal and placental growth in combination with maternal and paternal effects on placental gene expression appear to be in opposition to this hypothesis. However, an alternative way to look at this would be that the normal mechanisms by which the mother acts to restrain growth and by which the father acts to promote growth have failed as a consequence of prenatal Dex exposure, thus agreeing with the parental conflict hypothesis.

In the F2 fetal liver, gene expression studies showed an interaction between parental

prenatal Dex exposure effects to decrease the expression of Igf2. There was also an interaction between parental prenatal Dex exposure to decrease the expression of the imprinted gene Phlda2. In opposition to the findings in F1, but in agreement with the observed effects on fetal growth, the lowest expression of Igf2 and Phlda2 was found in the smallest offspring, VD. The reduction in Igf2 may be the reason for the small offspring in VD group since Phlda2 has been shown to have its primary function in the placenta (Tunster et al. 2010). In contrast, there was an interaction between parental prenatal Dex exposure to increase the expression of IgfBP2. As discussed in chapter 3, IgfBP2 acts primarily to inhibit Igf2 action (Jones and Clemmons 1995). The reduction in Igf2 expression together with an increase in IgfBP2 expression, would be predicted to reduce fetal growth. Although a marked change in hepatic CDKn1c expression was observed in the F1 Dex offspring, CDKn1c expression was unchanged in the F2 generation. This gives additional support to the possibility that mechanisms underlying programming effects are different between the F1 and F2 generations.

In the placental labyrinth, we found a paternal effect to increase the expression of the maternally expressed genes CDKn1c and Phlda2. In transgenic mouse models, knockout of CDKn1c results in placentomegaly with abnormalities of the labyrinth (Takahashi et al. 2000); and deletion of Phlda2 gene leads to placentomegaly with expansion of both labyrinth and junctional layers (Frank et al. 2002, Salas et al. 2004). In contrast, over-expression of Phlda2 leads to a reduction in the growth of the placenta (especially the junctional zone), in association with a significant reduction in glycogen storage (Tunster et al. 2010). In this study, there was an effect of

paternal prenatal Dex exposure to decrease the area of the placental junctional zone This is consistent with the pattern of Phlda2 gene expression, since we found an effect of paternal prenatal Dex exposure to increase its expression. The expression of Mest, a paternally expressed imprinted gene known to be related to placental and fetal growth (Lefebvre et al. 1998, McMinn et al. 2006) is increased in placental labyrinth by maternal prenatal Dex exposure. The increased expression of Mest would be expected to result in increased fetal and placental growth; and indeed analysis by two-way ANOVA showed an effect of maternal Dex to increase fetal growth, although placental growth was only increased in the DV group. One reason for the differences seen between this model and the models using knockouts/overexpression of single genes might be that the effects of the original glucocorticoid overexposure result in the altered expression of multiple genes that play a role in fetal and placental growth and it is the balance of changes which determines the patterns of growth that were observed in this study.

Although the mechanisms that underlie all of the programming effects in the glucocorticoid programmed rat remain undefined, epigenetic modifications have been suggested as a likely mechanism. Previous reports have shown multigenerational epigenetic changes as a consequence of maternal dietary manipulation (Morgan et al. 1999) as well as exposure to an endocrine disruptor which induced epigenetic changes in multiple generations that were not exposed (Anway et al. 2005, Stouder and Paoloni-Giacobino 2010). A recent report has shown that the multigenerational epigenetic effects induced by endocrine disruptors can specifically affect imprinted genes in the germline (Stouder and Paoloni-Giacobino

2011), therefore suggesting the potential for these epigenetic marks to be transmitted to subsequent generations. In our recent report, fetal overexposure to glucocorticoids led to changes in methylation pattern of Igf2 in F1 and F2 fetal liver (Drake et al. 2011), thus giving further support to the concept that epigenetic modifications are a possible mechanism in multigenerational programming.

In this study, I have shown that the gene expression changes in F2 placental labyrinth and fetal liver are dependent on parental prenatal Dex exposure. This is best exemplified by the expression of a number of imprinted genes which are important in both fetal and placental growth. This suggests that in the glucocorticoid overexposure model, the transmission of programming effects to the second generation may occur as a consequence of epigenetic changes, since imprinted genes are epigenetically regulated. The erasing of imprinted marks in the developing germ line occurs at around embryonic day 12 – 13 (Reik and Walter 2001, Hajkova et al. 2002) and there is re-establishment of DNA methylation at imprinted genes later in fetal development, at least in the male germ line (Kafri et al. 1992, Tada et al. 1998, Reik and Walter 2001) (Figure 4.10). Thus, the germ cells which will go on to form the F2 generation are exposed to Dex during the period in which DNA methylation is re-established. If this process was disrupted and the effects preserved following fertilisation, this could result in the transmission of effects to the F2 generation. Indeed, previous reports using endocrine disruptors have shown epigenetic programming effects in the male germ line (Anway et al. 2006b, Stouder and Paoloni-Giacobino 2010, Stouder and Paoloni-Giacobino 2011) which appear to escape the epigenetic reprogramming that occurs following fertilisation (Anway et al.

2005, Stouder et al. 2009). Although the exact mechanism of such resistance to resetting of epigenetic marks remains to be explored (Youngson and Whitelaw 2008), it has been proposed that under the effect of these ‘external agents’, alterations in DNA methylation or the induction of new differentially methylated regions can persist through generations (Anway et al. 2005, Guerrero-Bosagna et al. 2005, Skinner et al. 2010). Other than DNA methylation, emerging evidence suggests that changes in histone marks (Fu et al. 2004, Park et al. 2008, Fu et al. 2009, Hammoud et al. 2011) and non-coding RNAs (Rassoulzadegan et al. 2006, Lalancette et al. 2008) may play a role in the transmission of effects across generations through sperm.

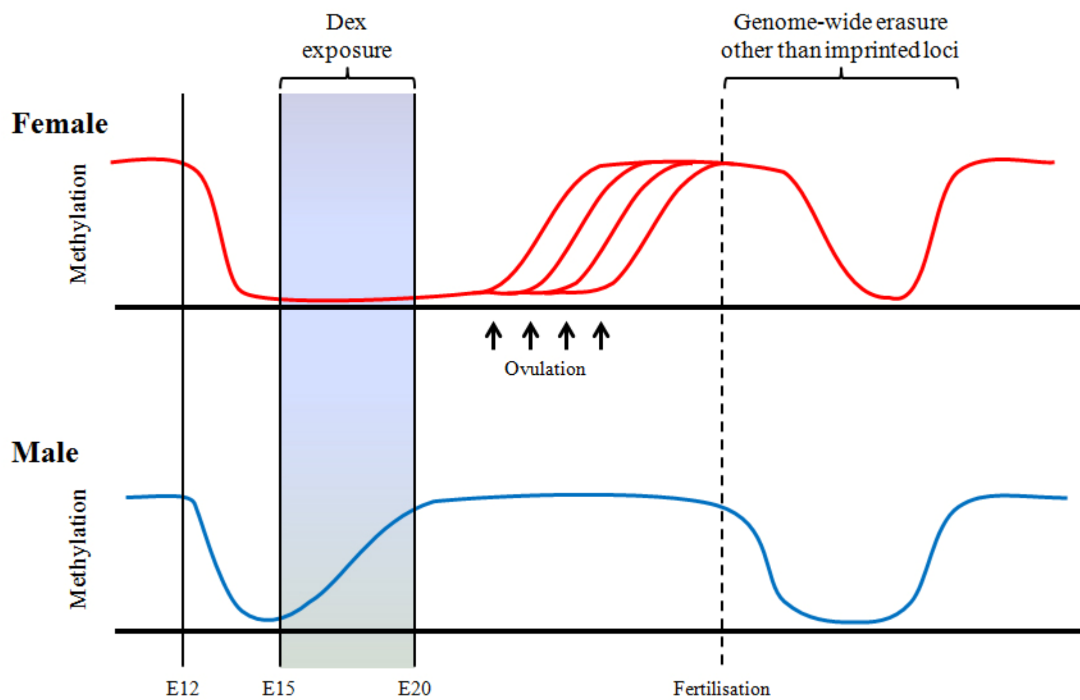


Figure 4.10 DNA methylation in germ cells of females and males
Diagrammatic representation of methylation levels in germ cells showing the erasure of methylation marks during fetal life and following fertilisation

The timing of exposure to excess glucocorticoids may also lead to generation specific programming effects. In the F1, the programming effects may be due to direct exposure to glucocorticoids during late gestation; whereas in the F2, the exposure to excess glucocorticoids may occur at the level of the germ cell in the developing F1 gonad so that the changes seen in F1 and F2 may be different due to the effects of Dex at different time points of development.

Another possible factor that may play a role in the transmission of programming effects specifically through the maternal line could be alterations in maternal physiology and behaviour. As previously discussed, maternal size has been linked to birth weight in horses (Walton and Hammond 1938), pigs (Gama and Johnson 1993), mice (Cowley et al. 1989), and humans (Ibanez et al. 2000). In this study, F1 female exposed to Dex were lighter, and previous data has shown that despite slight catch up growth in Dex exposed females, this reduced weight at birth can continue till 6 months of age (Cleasby et al. 2003, Drake 2004). Smaller F1 Dex females may give birth to offspring that are lighter at birth as was seen in DD and DV groups. Also, Dex exposure has been reported to cause altered behaviour in animals which are directly exposed (here the F0 mothers) as well as behavioural changes in offspring (here the F1 offspring) (Muneoka et al. 1997, Brown 2009). In rats, maternal behaviour has been shown to affect the behaviour of female offspring, potentially through alterations in DNA methylation (Meaney 2001, Weaver et al. 2004). Thus, it is possible that the F0 mothers treated with Dex had altered maternal behaviour toward their F1 offspring postnatally, leading to a similarly altered maternal behaviour in F1 females which could then impact on the F2 generation. However a

previous study using a cross fostering paradigm has shown that in the glucocorticoid programmed rat, Dex exposed fetuses do not escape the fate of developing metabolic phenotypes despite postnatal rearing by an unexposed mother. Similarly, pups exposed to Veh in utero do not develop metabolic phenotypes even if reared by a Dex exposed mother postnatally (Nyirenda et al. 2001), suggesting that the programming effects are determined in utero rather than by maternal behaviour postnatally.

In conclusion, prenatal Dex overexposure during the last week of gestation in rats is associated with programming effects not only in the F1 offspring, but also in the next generation.

CHAPTER 5

Programming effects in first generation offspring of obese female mice

5.1 Introduction

5.1.1 Introduction

Epidemiological studies in humans have shown that an increased maternal body mass index during pregnancy is linked to offspring obesity and metabolic illnesses (Eriksson et al. 2003, Whitaker 2004, Gale et al. 2007, Catalano et al. 2009). In animal studies, maintenance of females on a high fat and/or high sugar diet during and after pregnancy has also been associated with effects on hypertension, metabolic syndrome, muscle development, reproduction, and obesity in their offspring (Khan et al. 2005, Samuelsson et al. 2008, Bayol et al. 2009, Jungheim et al. 2010).

Various approaches have been developed to determine the underlying mechanisms linking maternal obesity with programming effects in the offspring. These studies have included varying the content of the maternal diet, for example using lard to induce maternal obesity (Khan et al. 2003, Chechi et al. 2010); using 'junk food' including chocolate biscuits, marshmallow and cheese (Bayol et al. 2007, Akyol et al. 2009, Bayol et al. 2009) or using commercially manufactured high fat diets with or without additional ingredients such as vegetable oil and sweetened condensed milk (Guo and Jen 1995, Samuelsson et al. 2008, Elahi et al. 2009). In addition to the dietary differences between studies, the age of introduction of dietary interventions and the duration of exposure prior to pregnancy differs between studies. In some studies dietary changes were introduced shortly after weaning (Jungheim et al. 2010, Massiera et al. 2010), whilst in others the experimental diets were introduced 55 weeks before mating (Sumiyoshi et al. 2006), 4 weeks before mating (Samuelsson et al. 2008, Nivoit et al. 2009), a week before mating (Guo and Jen 1995, Taylor et al.

2005), or at day of conception (Bayol et al. 2007). In this study, we set out to characterise a model of maternal obesity and the effects on offspring by using a high fat, high sugar, balanced protein diet. There were two primary intentions for this study. Firstly, we wished to generate a model of maternal obesity in pregnancy which mimics the human state by inducing obesity prior to and during pregnancy and then to examine the programming effects on offspring. Second, we used a diet matched in terms of protein, micro and macronutrient content in order to exclude an altered protein content as a cause of programming effects in the offspring.

5.1.2 Hypothesis

I hypothesised that maternal obesity induced by exposure to a high fat and high sugar diet would lead to programming effects on metabolism in the offspring.

5.1.3 Aims

The aim of this study was to generate a mouse model of maternal obesity and to characterise the phenotype of the offspring by studying effects on glucose/insulin homeostasis and gene expression in offspring liver and adipose tissue at different postnatal ages. This study was part of a larger study performed with another PhD student Ms. Rachel S. Dakin, and a post-doctoral researcher Dr. Vicky King. I focused mainly on the F1 female offspring at 3 months and 6 months of age but also undertook studies in both genders at 12 months of age. I also collected body weights in both genders at all time points of development. Here I show only my own experimentally derived data unless otherwise stated.

5.2 Methods

5.2.1 Animal maintenance and diet

Female C57bl/6 mice were bred in-house and mated with a stud C57bl/6 male to produce an F0 generation. The presence of vaginal plug was checked daily in the morning and females were singly housed after a vaginal plug was found (denoted as E0) and were left undisturbed with *ad libitum* access to food and water. Pregnant female mice were allowed to litter and pups (F0) were sexed, culled back to five pups per litter and weaned at three weeks of age. The pups were then randomly assigned to house with pups from different litters at a maximum of five per cage and were assigned a standard laboratory chow (RM1) (Special diet services, Witham, UK) for two weeks.

At five weeks of age, F0 females were either assigned to a diet-induced obesity (DIO) diet or a control (CON) diet that was matched in all macro- and micronutrients except fat and carbohydrate. After 12 weeks of exposure to the experimental diet, females were singly housed with a stud male for mating, remaining on their assigned experimental diet. Cages were checked for the presence of a vaginal plug daily and when found, males were removed from the cage. Throughout pregnancy, intake of diet was weighed and recorded although to avoid stress in the pregnant mother this procedure was not performed in metabolic cages. At birth, F1 pups were weighed, sexed and culled back to five per litter. F1 pups were reared by their biological mother (which remained on their respective experimental diets) until weaning at three weeks of age. At weaning, the pups were then randomly assigned to be group housed with other same sexed littermates from the same treatment group and were

put on to standard laboratory chow. Dr. Vicky King (Centre for Reproductive Health, University of Edinburgh) helped with the production of F1 offspring in two of the cohorts in this study.

Groups of F1 offspring underwent intra-peritoneal glucose tolerance testing (IPGTT), and diurnal tail tip blood collections at 3 months, 6 months or 1 year of age and were killed after the experimental procedures by carbon dioxide asphyxiation at each of the three time points. Organs were dissected, weighed and snap frozen on dry ice and stored at -80°C. Organ weights were normalised to body weight before statistical analysis.

5.2.2 Metabolic experiments

Plasma from IPGTT was measured by using an enzymatic (hexokinase) method (Thermo Fisher Scientific) for glucose; enzymatic colourimetric tests were used for triglyceride (Thermo Fisher Scientific) and cholesterol (Thermo Fisher Scientific) measurements; plasma insulin was measured by using an ELISA kit (Crystal Chem Inc.).

5.2.3 Molecular experiments

Liver and adipose total RNA was extracted using the RNeasy® mini kit (Qiagen) and reverse transcribed (Promega) to produce cDNA. Quantitative real-time PCR was used to examine gene expression using TaqMan™ (Applied Biosystems) or self designed UPL (Roche) primers with the addition of MasterMix (Roche). Expression of all genes examined were normalised to the reference gene: Cyclophilin-A.

5.2.4 Statistical analysis

Data were analysed by using SPSS® and GraphPad®. Statistical significance was calculated by Student's *t*-tests, Mann-Whitney *u*-tests when the distribution of data was non-parametric or area under curve (AUC) analysis. Data are expressed as mean ± SEM unless otherwise stated. Results were considered significant if $p<0.05$.

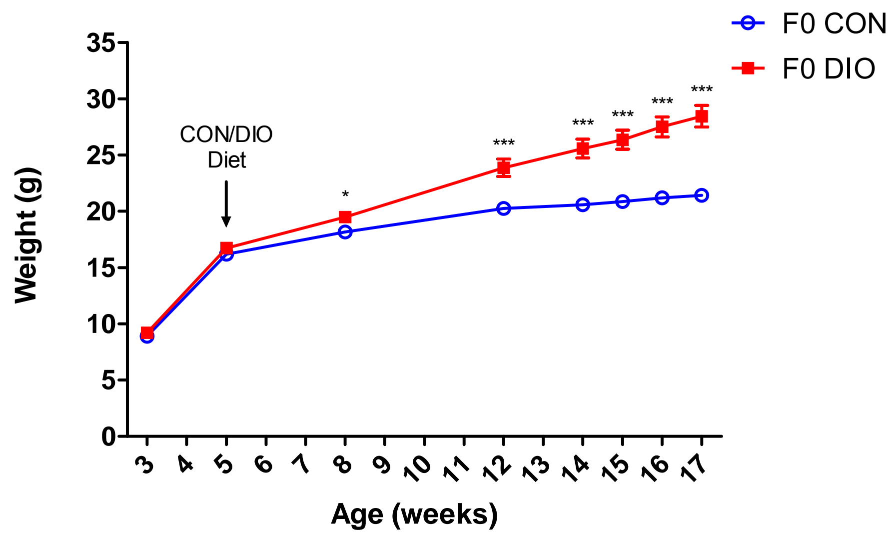
5.3 Results

5.3.1 F0 maternal physiology and F1 offspring

F0 females assigned to DIO diet were heavier than controls at 12 weeks of age (Figure 5.1a). There was no difference between the groups in terms of time taken to plug (CON = 3.00±0.43, DIO = 3.05±0.34 days; p=0.92), however F0 DIO females lost more weight between the time of introduction of a male and the identification of a vaginal plug (Figure 5.2). During pregnancy, F0 DIO females gained less weight (Figure 5.1b) and consumed less diet (Table 5.1) compared to F0 CON females. Although less diet in grams was consumed by the F0 DIO females, they consumed more fat than controls. There was no difference in either total calorie or protein intake between groups (Table 5.1).

At birth, both male and female F1 DIO offspring were lighter than F1 CON offspring (Table 5.2). There was no difference in litter size between groups (Table 5.2). At weaning, both F1 DIO males and females were heavier than F1 CON offspring; this difference between weights disappeared by 2 months of age (Table 5.2) and no weight difference was seen at three months (Table 5.2). At six months of age, F1 DIO females were lighter than F1 CON females although no difference was seen in the males (Table 5.2). At one year of age, F1 DIO females were lighter than F1 CON females, but no difference was seen in males (Table 5.2).

A)



B)

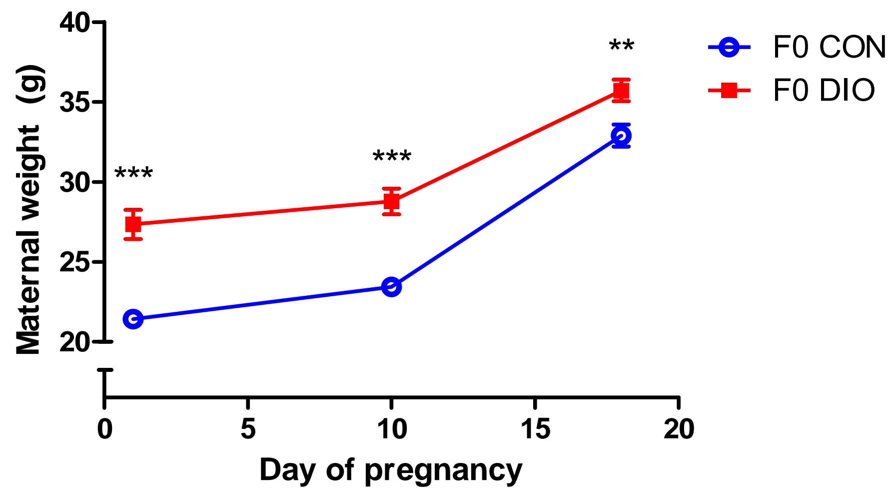


Figure 5.1 Weight of F0 pregnant females before and during pregnancy

A) F0 females on DIO had increased weight gain and were heavier before mating, however, **B)** F0 DIO females gained less weight throughout pregnancy compared to F0 CON females

Data are mean ± SEM, analysed by Student's *t*-test

n = F0 CON: 22, F0 DIO: 23 litters

* = p<0.05, ** = p<0.01, *** = p<0.001

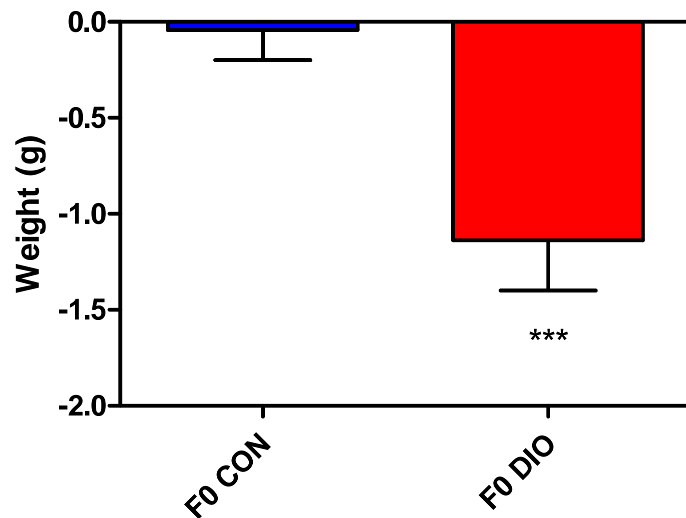


Figure 5.2 Maternal weight change following introduction of male to cage
Females were mated after 12 weeks on experimental diet. F0 DIO females lost more weight than controls following the introduction of a male to the cage
Data are mean ± SEM, analysed by Student's *t*-test
n = F0 CON: 19, F0 DIO: 19 litters
*** = p<0.001

	F0 CON	F0 DIO	p value
Diet consumed (g)	51.01 ± 0.88	38.45 ± 2.19	< 0.0001
Fat intake (%gm)	2.45 ± 0.04	13.77 ± 0.78	< 0.0001
Protein intake (%gm)	8.57 ± 0.14	8.84 ± 0.50	0.60
Total energy (kcal)	207.6 ± 3.59	213.8 ± 12.16	0.63

Table 5.1 F0 maternal diet consumption during pregnancy from E1 – E18
n = F0 CON: 22, F0 DIO: 22 litters

	F1 CON	F1 DIO	p value
Birth weight (g)			
Male	1.33 ± 0.02 (67)	1.22 ± 0.02 (48)	< 0.0001
Female	1.27 ± 0.02 (58)	1.19 ± 0.02 (52)	< 0.001
Wean weight (g)			
Male	8.14 ± 0.15 (48)	10.39 ± 0.2 (39)	< 0.0001
Female	7.87 ± 0.15 (41)	10.01 ± 0.17 (42)	< 0.0001
Weight at 2 months (g)			
Male	25.39 ± 0.25 (47)	25.16 ± 0.32 (30)	0.57
Female	19.29 ± 0.25 (31)	18.79 ± 0.21 (39)	0.13
Weight at 3 months (g)			
Male	30.39 ± 0.67 (12)	28.69 ± 0.73 (9)	0.11
Female	20.80 ± 0.45 (15)	21.55 ± 0.63 (16)	0.34
Weight at 6 months (g)			
Male	38.33 ± 0.99 (12)	36.48 ± 1.02 (9)	0.21
Female	26.06 ± 0.58 (14)	24.11 ± 0.57 (16)	< 0.05
Weight at 12 months (g)			
Male	44.35 ± 1.36 (12)	44.76 ± 0.83 (9)	0.82
Female	28.10 ± 1.17 (8)	25.67 ± 0.54 (11)	= 0.05

Table 5.2 F1 offspring weights at birth, weaning, 2, 3, 6 and 12 months
Numbers in brackets indicates number of animals at each time point
n = F0 CON: 22, F0 DIO: 22 litters

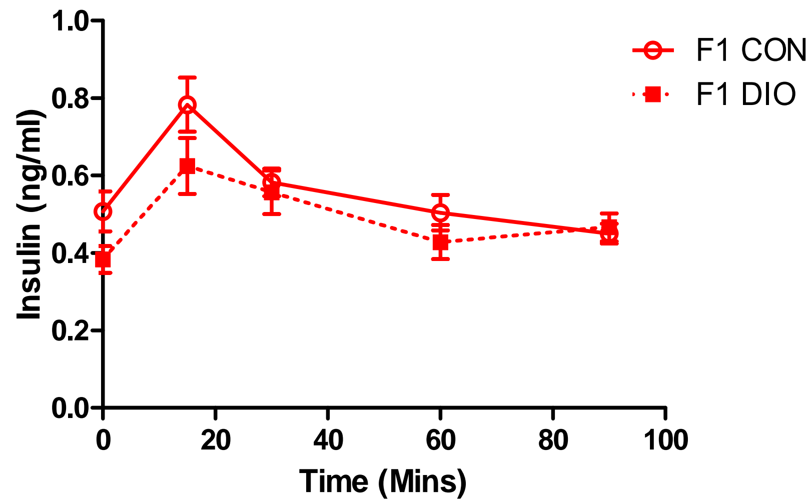
5.3.2 F1 offspring plasma analysis and hepatic triglyceride

At three months of age, there were no differences in plasma glucose or insulin levels during IPGTT between F1 CON and DIO female offspring (Figure 5.3). There was no difference in plasma triglyceride levels between the groups, but a trend to increased plasma cholesterol level was seen in F1 DIO females (Table 5.3).

At six months of age, there was a trend for F1 DIO females to have lower plasma glucose levels than F1 CON females during the IPGTT (Figure 5.4). No differences were seen in plasma triglyceride and cholesterol levels (Table 5.3).

At one year of age, no differences were seen in glucose or insulin levels in F1 females (Figure 5.5) however there was a trend for F1 DIO males to have higher glucose levels during IPGTT (Figure 5.6). No differences were seen in plasma triglyceride and cholesterol levels between F1 control and F1 DIO females however both triglyceride and cholesterol were increased in F1 DIO males (Table 5.3).

A)



B)

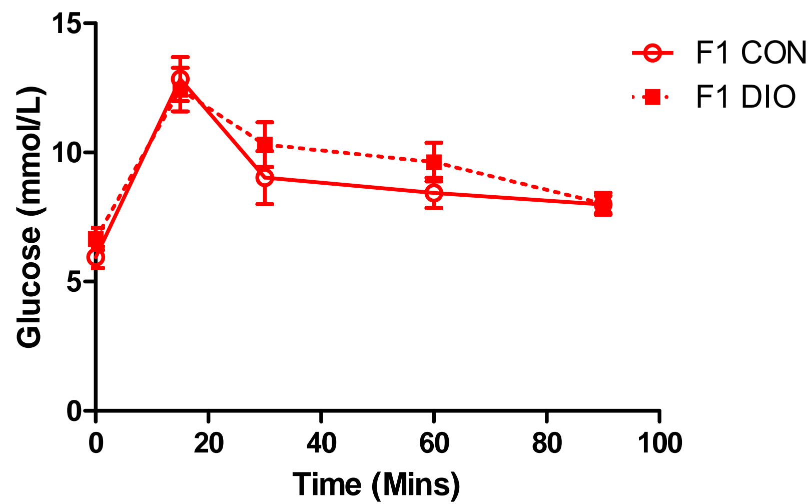
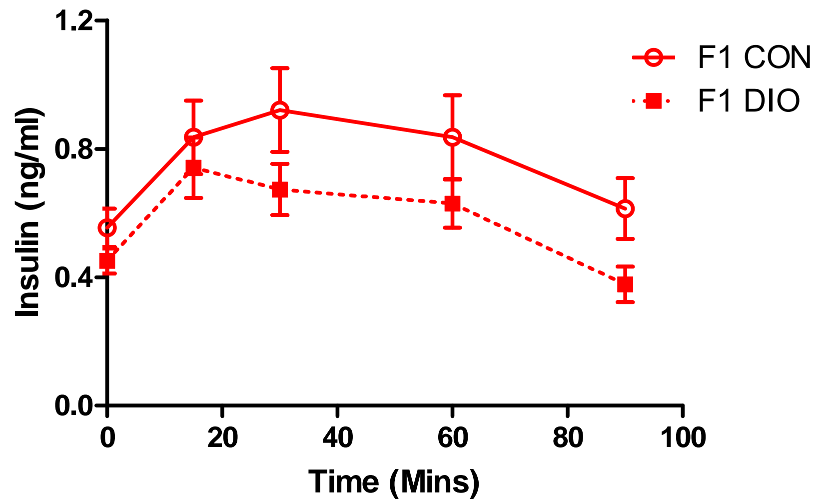


Figure 5.3 Plasma insulin and glucose levels at 3 months of age in F1 females
There were no differences in plasma **A)** insulin or **B)** glucose between the groups at 3 months of age
Data are mean ± SEM, analysed by AUC
n = F1 CON: 8, F1 DIO: 8

A)



B)

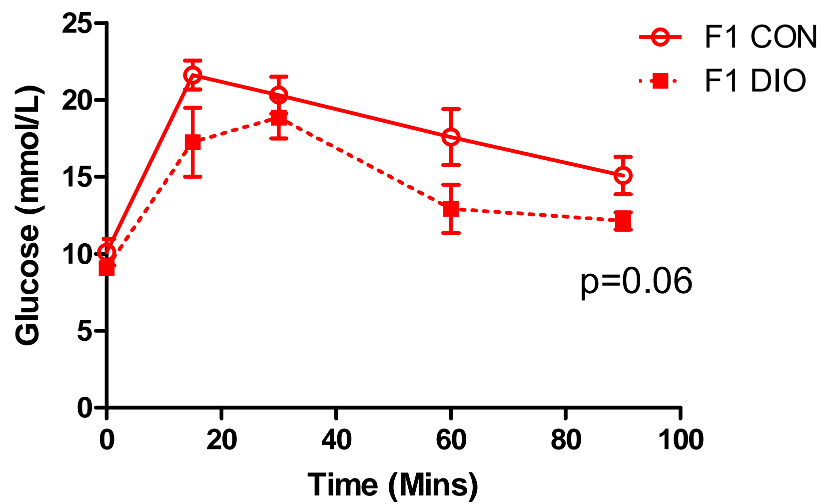


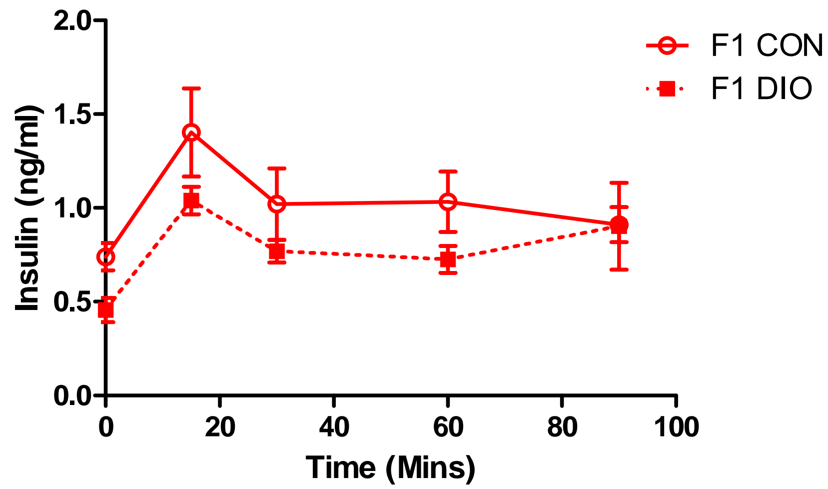
Figure 5.4 Plasma insulin and glucose levels at 6 months of age in F1 females

There were no differences in **A)** plasma insulin levels following an intraperitoneal glucose load; however F1 DIO females had a trend towards a decrease in **B)** plasma glucose levels at 6 months of age

Data are mean ± SEM, analysed by AUC

n = F1 CON: 6, F1 DIO: 5

A)



B)

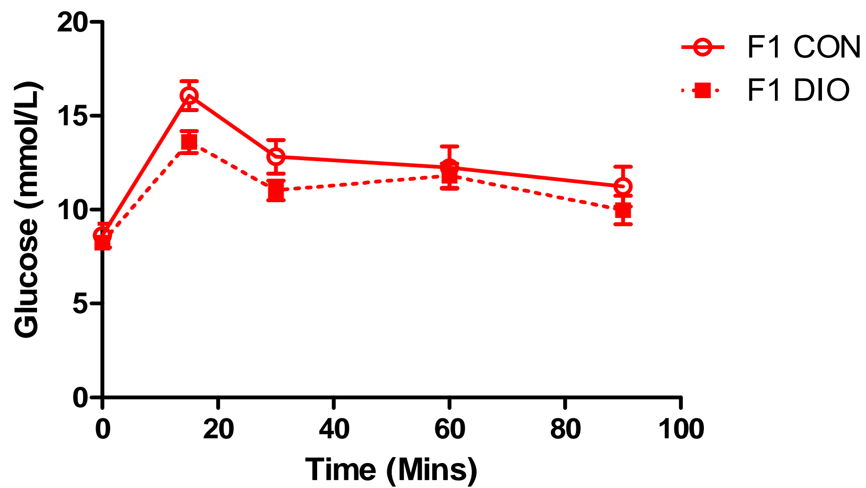


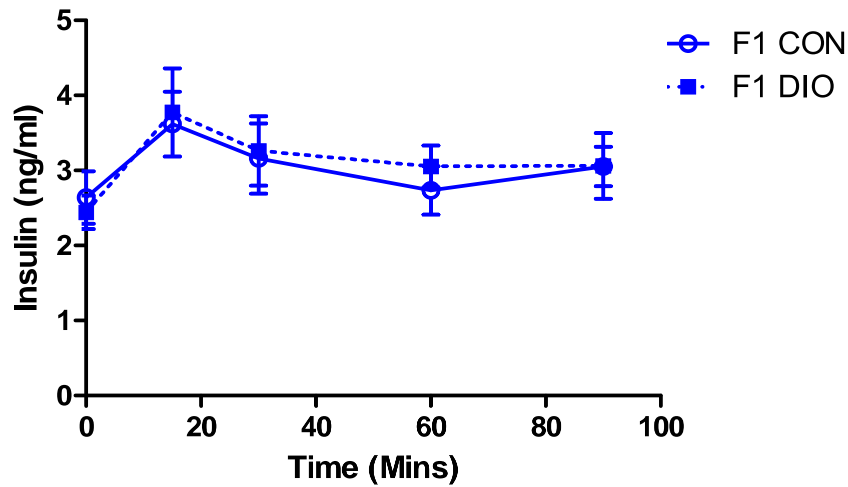
Figure 5.5 Plasma insulin and glucose levels at one year of age in F1 females

There were no differences in **A)** plasma insulin and **B)** glucose levels following an intraperitoneal glucose load in F1 females at one year of age

Data are mean ± SEM, analysed by AUC

n = F1 CON: 8, F1 DIO: 11

A)



B)

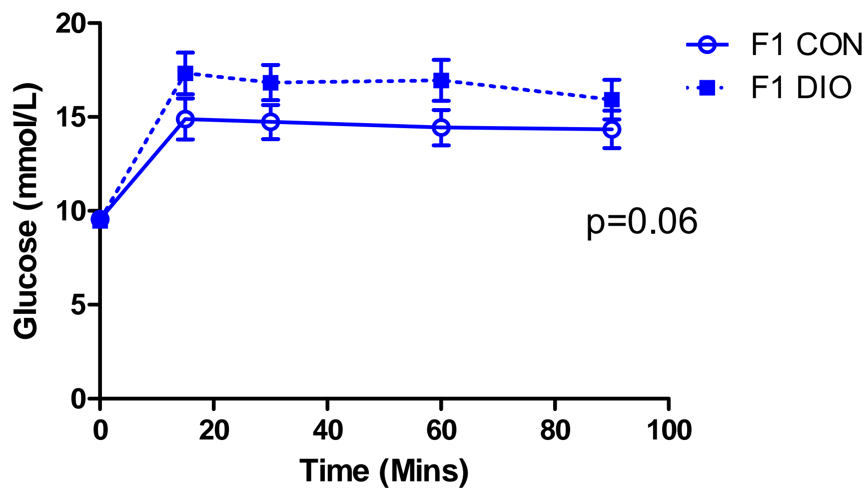


Figure 5.6 Plasma insulin and glucose levels at one year of age in F1 males

There were **A)** no differences in plasma insulin levels following an intraperitoneal glucose load; however **B)** F1 DIO males had a trend towards an increase in plasma glucose levels at one year of age

Data are mean ± SEM, analysed by AUC

n = F1 CON: 12, F1 DIO: 10

	F1 CON	F1 DIO	p value
3 month female			
Plasma TG (mmol/L)	0.65 ± 0.02	0.72 ± 0.05	0.18
Plasma chol (mmol/L)	0.87 ± 0.15	1.47 ± 0.26	0.06
6 month female			
Plasma TG (mmol/L)	0.61 ± 0.09	0.38 ± 0.06	0.07
Plasma chol (mmol/L)	2.16 ± 0.27	2.03 ± 0.16	0.70
1 year female			
Plasma TG (mmol/L)	0.72 ± 0.03	0.67 ± 0.02	0.12
Plasma chol (mmol/L)	0.95 ± 0.20	1.21 ± 0.08	0.19
1 year male			
Plasma TG (mmol/L)	0.64 ± 0.02	0.71 ± 0.03	< 0.05
Plasma chol (mmol/L)	2.13 ± 0.25	2.80 ± 0.23	< 0.05

Table 5.3 F1 plasma triglyceride, plasma cholesterol, and hepatic triglyceride levels
Data are mean ± SEM, analysed by Student's *t*-test and Mann-Whitney *u*-test
n = 3 month F1 CON: 5, F1 DIO: 4
6 month F1 CON: 6, F1 DIO: 5
1 year female F1 CON: 8, F1 DIO: 11
1 year male F1 CON: 12, F1 DIO: 10
TG = triglyceride, chol = cholesterol

5.3.3 F1 offspring organ weight

In F1 females, no difference was seen between groups in the weight of liver, kidney, mesenteric adipose, subcutaneous adipose, and retroperitoneal adipose tissue weight at 3 months, 6 months or one year of age (Table 5.4).

There was increased weight of mesenteric and gonadal adipose tissue in F1 DIO male offspring at one year of age; although no differences were seen in liver, kidney, subcutaneous and retroperitoneal adipose tissue weights (Table 5.5).

	F1 CON	F1 DIO	p value
3 month female organ weights (mg/g)			
Liver	4.64 ± 0.09	4.92 ± 0.17	0.15
Kidney	0.61 ± 0.02	0.61 ± 0.01	0.89
Mesenteric ad.	0.66 ± 0.14	0.58 ± 0.24	0.78
Subcutaneous ad.	0.95 ± 0.13	1.18 ± 0.14	0.25
Retroperitoneal ad.	0.49 ± 0.12	0.41 ± 0.17	0.70
6 month female organ weights (mg/g)			
Liver	4.32 ± 0.18	4.51 ± 0.06	0.39
Kidney	0.50 ± 0.10	0.59 ± 0.04	0.08
Mesenteric ad.	1.24 ± 0.38	1.02 ± 0.33	0.33
Subcutaneous ad.	1.04 ± 0.15	1.02 ± 0.10	0.92
Retroperitoneal ad.	1.15 ± 0.17	0.73 ± 0.09	0.07
1 year female organ weights (mg/g)			
Liver	3.91 ± 0.21	4.16 ± 0.16	0.35
Kidney	0.54 ± 0.02	0.55 ± 0.02	0.74
Mesenteric ad.	0.88 ± 0.12	0.81 ± 0.04	0.53
Subcutaneous ad.	1.47 ± 0.15	1.30 ± 0.08	0.31
Retroperitoneal ad.	0.22 ± 0.04	0.18 ± 0.01	0.35

Table 5.4 F1 female offspring organ weights
Data are normalised to the body weight of animal
Data are mean ± SEM, analysed by Student's *t*-test
n = 3 month F1 CON: 5, F1 DIO: 4
6 month F1 CON: 6, F1 DIO: 5
1 year F1 CON: 8, F1 DIO: 11
ad. = adipose

	F1 CON	F1 DIO	p value
1 year male organ weights (mg/g)			
Liver	4.11 ± 0.15	4.33 ± 0.20	0.37
Kidney	0.47 ± 0.01	0.44 ± 0.01	0.07
Mesenteric ad.	1.53 ± 0.09	1.88 ± 0.10	< 0.05
Subcutaneous ad.	2.19 ± 0.13	2.24 ± 0.15	0.79
Retroperitoneal ad.	0.36 ± 0.03	0.42 ± 0.02	0.15
Gonadal ad.	1.63 ± 0.08	1.98 ± 0.11	< 0.05

Table 5.5 F1 male offspring organ weights at one year of age
Data are normalised to the body weight of animal
Data are mean ± SEM, analysed by Student's *t*-test
n = CON: 12, DIO: 9
ad. = adipose

5.3.4 F1 female offspring hepatic gene expression

In the female liver, there was an increase in the expression of fatty acid synthase (Fas) and a trend towards an increase in lipoprotein lipase (LPL) expression ($p=0.058$) at three months of age (Figure 5.7). There were no differences in the expression of hormone sensitive lipase (HSL), peroxisome proliferator-activated receptor α (PPARα), PPARγ, phosphoenolpyruvate carboxykinase (PEPCK), glucocorticoid receptor (GR), 11βHSD1, 5-α reductase (5αR), or 5-β reductase (5βR) (Figure 5.7). At six months of age, no differences were seen in the expression of: LPL, PPARα, PEPCK, GR, 11βHSD1, 5αR, or 5βR in female offspring (Figure 5.8).

5.3.5 F1 female offspring adipose tissue gene expression

At six months of age, no differences were seen in the expression of LPL, HSL, FTO, GR or 11βHSD1 in mesenteric adipose tissue (Figure 5.9a). In the subcutaneous adipose tissue, no differences were seen in the expression of LPL, Fas, HSL, PEPCK, FTO, GR, or 11βHSD1 (Figure 5.9b).

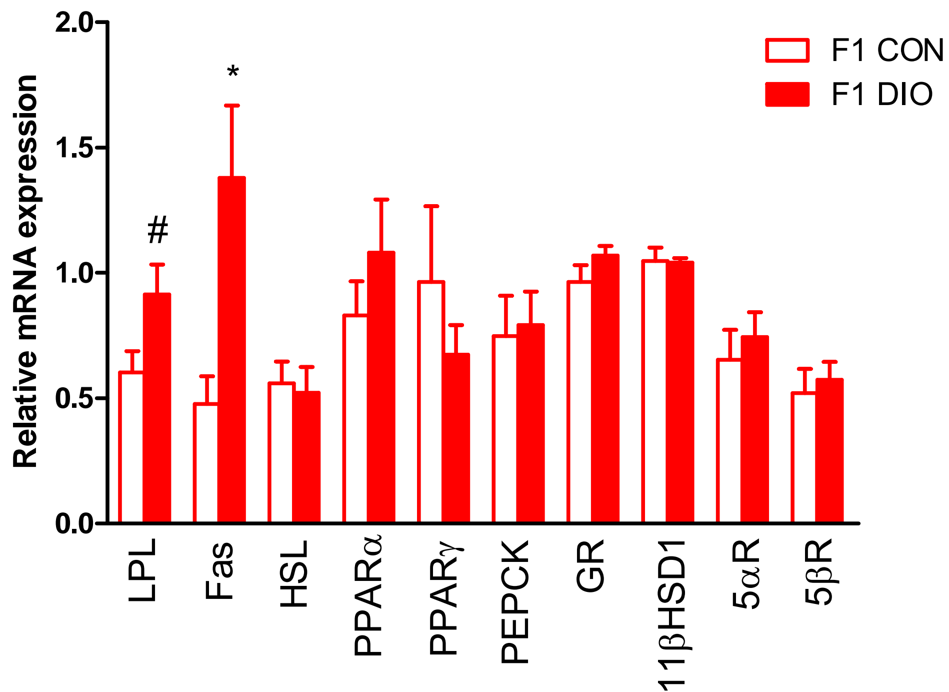


Figure 5.7 Hepatic gene expression in three months old F1 females

At three months of age there was increased expression of Fas and a trend towards an increase in the expression of LPL in F1 DIO liver but no differences were seen in the expression of a number of other genes important in glucose, lipid and glucocorticoid metabolism.

Data are mean ± SEM, analysed by Student's *t*-test

n = F1 CON: 6, F1 DIO: 5

* = $p<0.05$, # = $p=0.058$

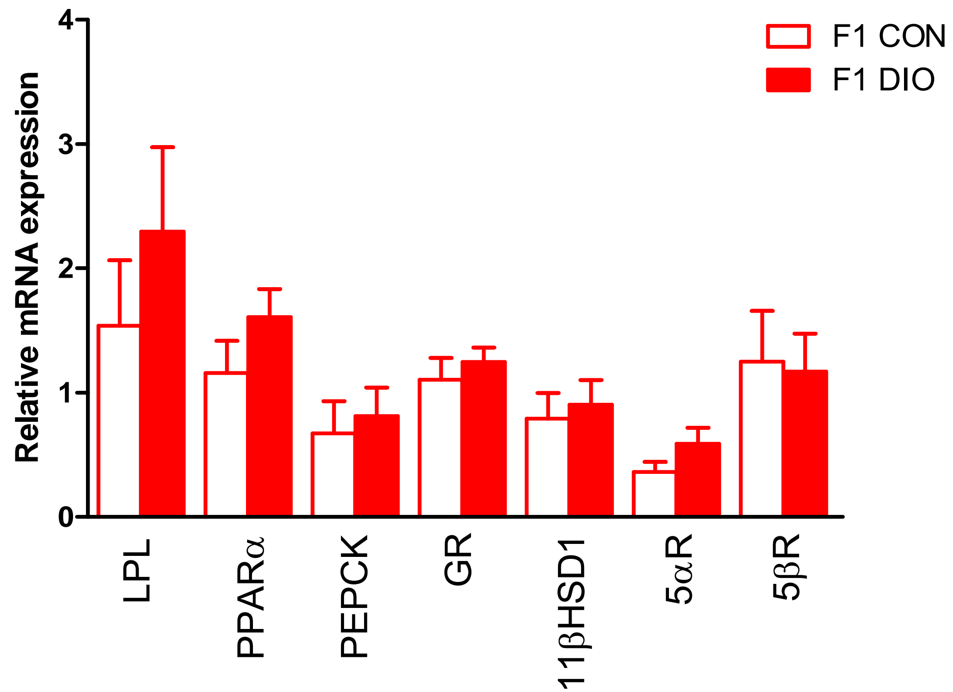


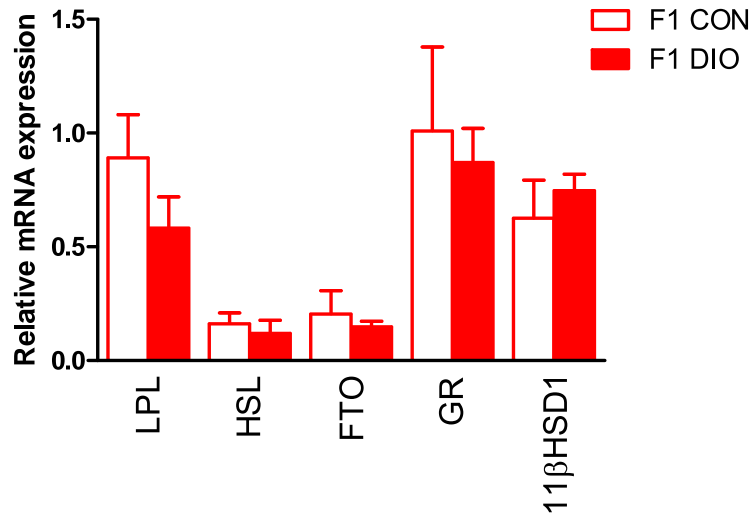
Figure 5.8 Hepatic gene expression in six months old F1 females

There were no differences in gene expression in liver from F1 females at six months of age

Data are mean ± SEM, analysed by Student's *t*-test

n = F1 CON: 6, F1 DIO: 5

A)



B)

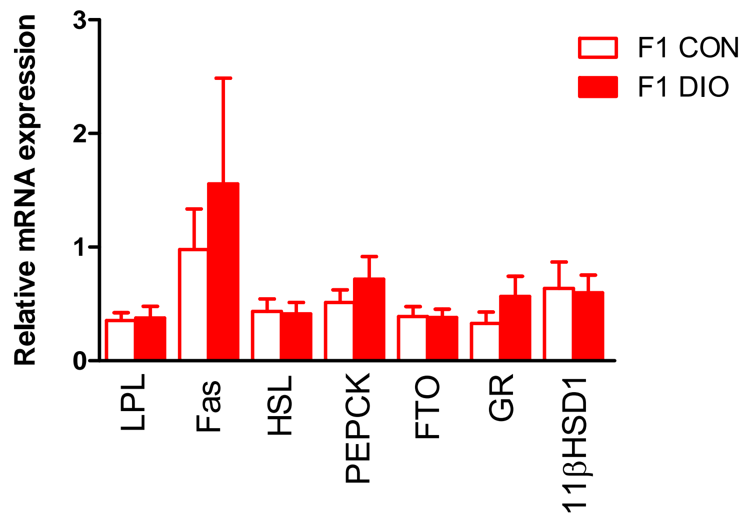


Figure 5.9 Gene expression in adipose depots from six month old F1 females

There were no differences in the expression of genes important in glucose, lipid and glucocorticoid metabolism in A) mesenteric or B) subcutaneous adipose at 6 months of age

Data are mean ± SEM, analysed by Student's *t*-test

n = F1 CON: 6, F1 DIO: 5

5.4 Discussion

These results demonstrate that in this model, maternal obesity does not result in major effects on female offspring health at least until one year of age. This finding is in disagreement with a number of published studies which reported programming effects in both genders (Guo and Jen 1995, Khan et al. 2003, Bayol et al. 2008, Samuelsson et al. 2008). In male offspring of obese mothers, there was a trend to decreased insulin sensitivity at one year of age. This suggests possible effects of aging in male animals exposed to maternal obesity prenatally. Females that were assigned DIO diets gained more weight and were heavier than animals on CON at the time of mating. Throughout pregnancy, DIO females gained less weight compared to CON. This reduced weight gain was not due to alterations in litter size. This inverse relationship between maternal size and weight gain during pregnancy has also been shown in humans (CMACE 2010, Park et al. 2011). At birth, both male and female F1 offspring of DIO females were lighter in weight, but by weaning, both male and female F1 DIO offspring were heavier than F1 CON offspring. A recent study which analysed the contents of the milk from mothers maintained on a high fat diet showed a higher fat content in milk (Purcell et al. 2011), so that the 'catch-up' growth observed in the F1 DIO offspring in this study may be due to the ingestion of milk that is higher in fat content from their mothers, as the mothers remained on their assigned experimental diet during lactation. At two months of age, this difference in weight between the two groups was no longer present.

No differences were seen in plasma triglyceride and cholesterol levels in F1 females at 3, 6, and 12 months of age. This is in contrast to some reports showing an increase

in plasma triglyceride levels at 3 months of age and increased cholesterol at 6 months of age (Samuelsson et al. 2008) in offspring of obese mothers, but in agreement with other reports showing no difference in plasma lipid levels in female offspring of high fat and high protein fed mothers (Zhang et al. 2005). In males, there was an increase in plasma triglyceride and cholesterol levels in DIO offspring at 12 months of age. There were no differences in glucose homeostasis in F1 female offspring at 3, 6 and 12 months of age although in DIO F1 males, we found a trend towards insulin insensitivity at 12 months of age. Gene expression was not analysed in the adipose tissue of three months old females because of the relatively minor changes seen in six month old females.

One possible explanation for our finding of minimal effects of maternal obesity on F1 female offspring is the diets used in this model in comparison to others. In this study, the DIO diet was completely matched with CON diet in terms of the micro- and macronutrients and protein and only differed in fat and carbohydrate content. This is important as low protein intake during pregnancy has been associated with programming effects in the offspring, including programming of hypertension (Langley-Evans 1997) and glucose intolerance and homeostasis (Langley et al. 1994, Ozanne et al. 1996, Ozanne and Hales 2002). In some of the other studies of maternal obesity, dietary constituents were not matched between the experimental and control groups; diets included lard (Khan et al. 2003, Taylor et al. 2005), cheese, biscuits, marshmallow, pork pie and other human foods (Bayol et al. 2005, Bayol et al. 2007, Bayol et al. 2008, Akyol et al. 2009), or a supplementation of sweet condensed milk (Samuelsson et al. 2008). Other studies have used commercially

manufactured DIO diets in the experimental group, but used a standard laboratory chow as control (Zhang et al. 2005, Liang et al. 2010), therefore also potentially not matching protein intake. Other than a protein imbalance leading to programming effects, differences in micronutrient levels, such as folate may also induce programming effects (Ashworth and Antipatis 2001). In my study, the F0 DIO females consumed less diet during gestation but the actual protein (per gram) consumed was the same between the two groups. This recording of dietary intake was not performed in metabolic cages, since although this is the ideal way to accurately measure dietary intake, solitary housing and novel cage environments cause a significant amount of stress to the pregnant animals which has its own programming effects. A second explanation for the differences between studies may be due to differences in the species and strains of animals used (Surwit et al. 1988). Some studies have used rats: either Wistar (Guo and Jen 1995, Bayol et al. 2005, Akyol et al. 2009, Nivoit et al. 2009) or Sprague-Dawley (Levin and Govek 1998, Khan et al. 2003, Shankar et al. 2008); while others have used mice: C57bl/6 (Sumiyoshi et al. 2006, Samuelsson et al. 2008, Bruce et al. 2009, Elahi et al. 2009, Liang et al. 2009, Chechi et al. 2010, Gregorio et al. 2010) or BALB/c (Zhang et al. 2005). It is known that some strains of mice are resistant to the development of obesity on a high fat diet (Surwit et al. 1988, Surwit et al. 1995b) so that the effects induced in females and their offspring may differ between animal strains.

The duration and timing of exposure to the experimental diets may be of importance in inducing programming effects. Some studies have introduced experimental diets a week prior to pregnancy (Guo and Jen 1995, Taylor et al. 2005) whereas others have

introduced experimental diets for as long as a year before mating (Sumiyoshi et al. 2006). This difference in the duration of exposure to diets may induce variable maternal phenotypes; as one week of high fat feeding may not be sufficient to generate 'maternal obesity' in comparison to months of high fat feeding. In this study, we introduced the experimental diets two weeks after weaning and maintained the animals on their assigned diets for a duration of 12 weeks. This was sufficient to induce obesity in the animals prior to mating, thus allowing us to look more specifically at the programming effects of maternal obesity.

Catch-up growth has been shown to be important in a number of studies. In humans, catch-up growth has been associated with increased risk of coronary heart diseases (Eriksson et al. 1999), increased insulin secretion (Soto et al. 2003b) and childhood obesity (Ong et al. 2000). In animals, it has also been associated with a shorter lifespan (Ozanne and Hales 2004). In this study, F1 DIO offspring had a lighter birth weight compared to F1 CON offspring. This is in agreement with a previous study in cafeteria diet fed rats which showed a reduction in fetal weight at E20 of pregnancy (Akyol et al. 2009), however F1 DIO offspring were heavier than F1 CON at weaning. Despite this 'catch up growth' in F1 DIO female offspring, very minor programming effects were seen in F1 DIO females, although in F1 DIO males there was a trend to decreased insulin sensitivity at one year of age suggesting that programming effects may become more apparent with ageing. This could be due to the time point at which the catch up growth occurred, as studies in humans suggest that catch up growth at different time points and for different durations is important in determining disease risk (Ong et al. 2000, Eriksson et al. 2001). Furthermore, most

reports on catch up growth have examined offspring that were exposed to an environment of undernutrition in utero, whereas in this study the lower birth weight was seen in the over- nourished group.

From an evolutionary point of view, the minimal programming effects seen in DIO offspring may reflect a protective effect of maternal obesity. Since nutrition was plentiful for the developing fetus(es) in this model, there may have been no need for them to ‘adapt’ to a potentially difficult environment in the postnatal period. The consequences of early life programming have been considered to reflect the adaptation of the fetus(es) to an expected poor or altered environment postnatally, resulting in some organs that are less important in survival to be less developed than other organs which may be critical for survival (Hales and Barker 1992). Thus in the setting of maternal obesity and relative fetal overnutrition, perhaps minimal programming effects are necessary in the next generation. Whether a programmed phenotype in the DIO offspring would be more apparent with exposure to the obesogenic diet after weaning is the subject of ongoing studies.

This finding of minimal programming effects from maternal obesity may have public health implications. Many previous studies in humans have suggested that maternal obesity is linked to childhood obesity (Whitaker 2004, Gale et al. 2007). However, it is uncertain to what extent this link is due to programming effects of maternal obesity, shared genes or the effects of exposure to a poor diet / lack of exercises postnatally. In this study, I have shown that programming effects are not an inevitable consequence of maternal obesity in utero.

CHAPTER 6

Programming effects in second generation offspring of obese female mice

6.1 Introduction

6.1.1 Introduction

As discussed in chapter four, programming effects may be transmissible to a second generation although the mechanisms by which effects are transmitted remains unclear. In humans the offspring of obese mothers are at increased risk of developing childhood obesity (Whitaker 2004, Gale et al. 2007), so that maternal obesity in pregnancy may lead to the establishment of a cycle of obesity which continues across a number of generations. Maternal effects may be one mechanism underlying the transmission of programmed effects across generations. Additionally, studies in other programmed models of overnutrition suggest that programmed effects may also be transmitted through the paternal line (Pentinat et al. 2010) so that an alternative mechanism for the transmission of programming effects as a consequence of overnutrition may be through effects which are transmissible through the germline.

Thus, although minimal programming effects were seen in F1 DIO female offspring in our mouse model as described in chapter five, we hypothesised that there may still be programming effects in the second generation.

6.1.2 Hypothesis

We hypothesise that the programming effects may be found in the second generation in a mouse model of maternal obesity and that these effects may differ from that in the first generation. Secondly, we hypothesise that the programming effects in the F2 generation will be both parental exposure status specific and gender specific.

6.1.3 Aims

The aim of this study was to determine whether the programming effects of maternal obesity differ in the F1 and F2 generations. In this initial study, this will involve studying the F2 generation in adulthood at 6 months of age.

6.2 Methods

6.2.1 Animal maintenance, diet induction, and offspring production

Female C57bl/6 mice were bred in-house and mated with a stud C57bl/6 male to produce an F0 generation. The presence of a vaginal plug was checked for daily in the morning. The females were singly housed when vaginal plug was found (denoted as E0) and were left undisturbed with *ad libitum* access to food and water. Pregnant female mice were allowed to litter and pups (F0) were sexed, culled back to five pups per litter and weaned at three weeks of age. The pups were then randomly assigned to house with pups from different litters at a maximum of five per cage and were assigned a standard laboratory chow (RM1) (Special diet services, Witham, UK) for two weeks.

At five weeks of age, F0 females were either assigned to a diet-induced obesity (DIO) diet or a control (CON) diet that was matched in all macro- and micronutrients except fat and carbohydrate. After 12 weeks of exposure to the experimental diet, females were singly housed with a stud male for mating, remaining on their assigned experimental diet. Cages were checked for the presence of a vaginal plug daily and when found, males were removed from the cage.

Litters (F1 CON and F1 DIO) were weighed at birth and culled back to five per group. Pups were reared by their biological mother until three weeks of age and were then weaned and housed with animals from same treatment group. At three months of age, F1 females were housed singly with an F1 male from a different litter of either the same or a different treatment group. This lead to the production of four

different groups: F1 CON female x F1 CON male (CC), F1 CON female x F1 DIO male (CD), F1 DIO female x F1 CON male (DC), and F1 DIO female x F1 DIO male (DD).

At six months of age, all four groups of F2 animals underwent IPGTT and were killed after the experimental procedures by carbon dioxide asphyxiation. Organs were dissected, weighed, snap frozen on dry ice and stored at -80°C. Organ weights were normalised to body weight before statistical analysis.

6.2.2 Metabolic experiments

Plasma from IPGTT was measured by using an enzymatic (hexokinase) method (Thermo Fisher Scientific) for glucose; plasma insulin was measured using an ELISA kit (Crystal Chem Inc.).

6.2.3 Statistical analysis

Data were analysed using SPSS® and GraphPad®. Statistical significance was calculated by two-way ANOVA or area under curve (AUC) analysis and is expressed as mean ± SEM unless otherwise stated. Results were considered significant if $p<0.05$.

6.3 Results

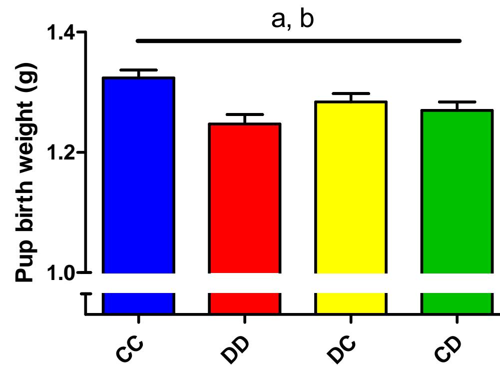
6.3.1 F2 weights

At birth, analysis by two-way ANOVA showed an effect of both paternal ($p<0.001$, $F=10.3$) and maternal ($p<0.05$, $F=4.9$) prenatal DIO exposure to decrease birth weight (Figure 6.1a). In F2 male pups, there was an effect of maternal prenatal DIO exposure ($p<0.05$, $F=4.30$) to decrease birth weight (Figure 6.1b) whereas in F2 female pups, there was an effect of paternal prenatal DIO exposure ($p<0.01$, $F=8.77$) to decrease birth weight (Figure 6.1c). There were no differences between litter sizes in all four groups (Figure 6.2). At weaning, the differences between weights had disappeared in both male (Figure 6.3a) and female (Figure 6.3b) offspring and by six months of age, no differences in weights were seen in F2 males (Figure 6.4a) or females (Figure 6.4b).

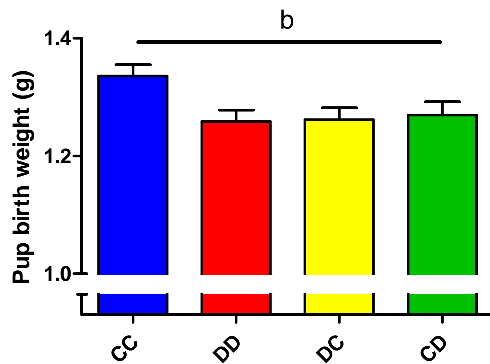
In F2 males, at six months, there was an effect of maternal prenatal DIO exposure to increase the weight of mesenteric adipose ($p<0.05$, $F=4.93$), subcutaneous adipose ($p<0.01$, $F=9.87$) and retroperitoneal adipose tissue ($p<0.05$, $F=6.73$); as well as a trend to increase gonadal adipose tissue weight ($p=0.07$, $F=3.35$) (Figure 6.5). No differences were seen between groups in the weight of liver and kidney (Figure 6.5).

In F2 females at six months, there was a trend for maternal prenatal DIO exposure to increase the weight of liver ($p=0.06$, $F=3.97$) (Figure 6.6). No differences were seen between groups in the weight of kidney, mesenteric adipose, subcutaneous adipose and retroperitoneal adipose tissue (Figure 6.6).

A)



B)



C)

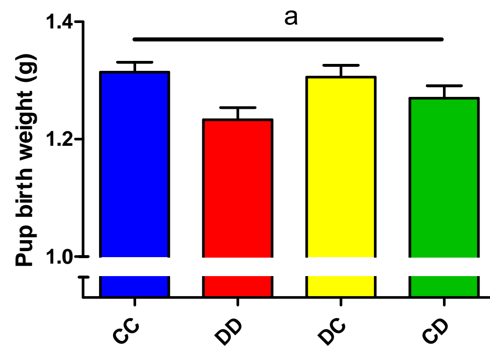


Figure 6.1 F2 birth weights

There was **A)** an effect of both paternal and maternal prenatal DIO exposure to decrease birth weight with both offspring sexes combined. **B)** In males, there was an effect of maternal prenatal DIO diet exposure to decrease birth weight and **C)** in females there was an effect of paternal prenatal DIO exposure to decrease birth weight

Data are mean ± SEM, analysed by two-way ANOVA

n = CC: 11, DD: 9, DC: 9, CD: 8 litters per group

a = Effect of paternal prenatal DIO exposure to decrease, b = effect of maternal prenatal DIO exposure to decrease

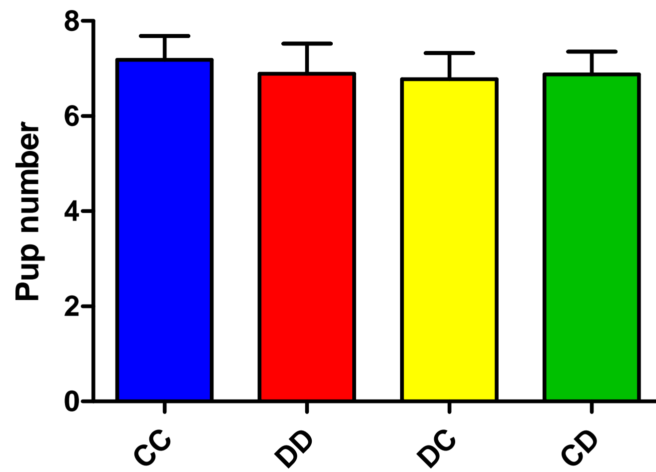


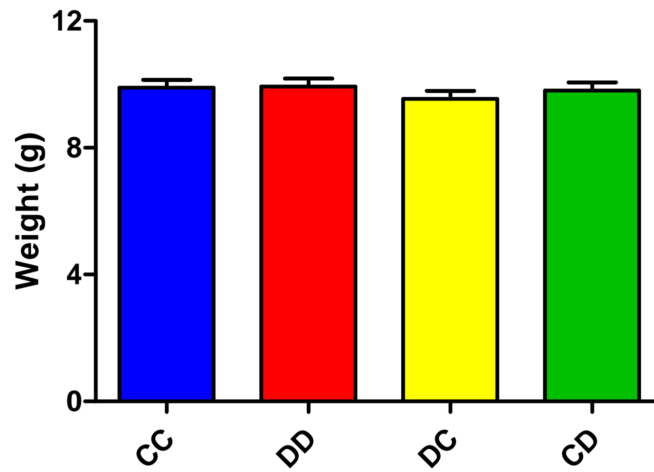
Figure 6.2 F2 litter size

There were no differences between the litter size in all four groups

Data are mean ± SEM, analysed by two-way ANOVA

n = CC: 11, DD: 9, DC: 9, CD: 8 litters per group

A)



B)

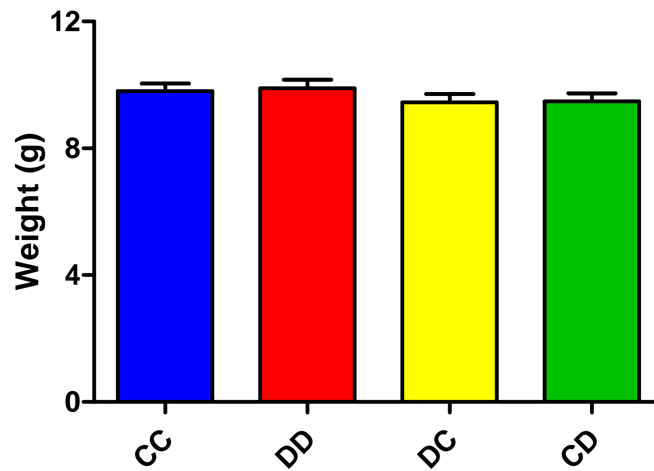


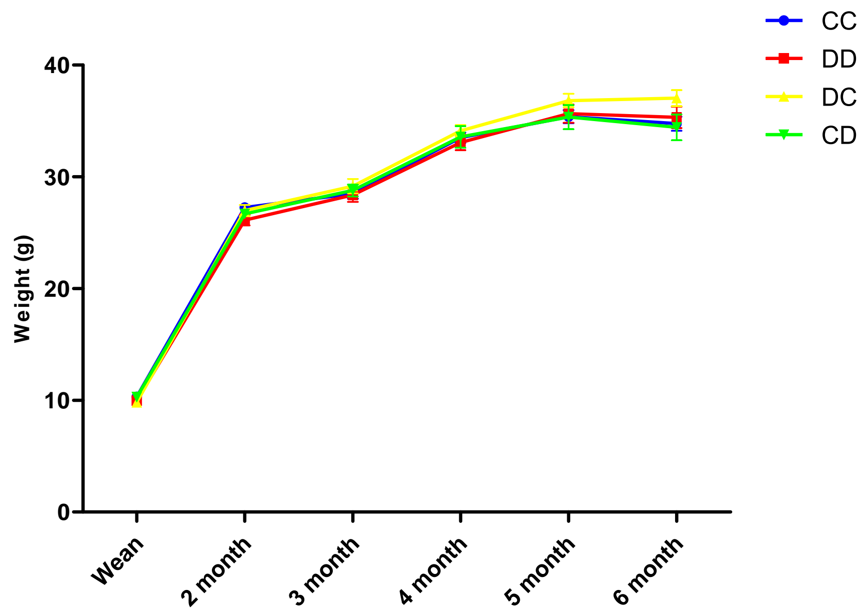
Figure 6.3 F2 weaning weights

There were no differences in the weights of **A)** male and **B)** female offspring at weaning in all four groups

Data are mean ± SEM, analysed by two-way ANOVA

n =	Male	CC: 25, DD: 22, DC: 23, CD: 20
	Female	CC: 26, DD: 20, DC: 21, CD: 22

A)



B)

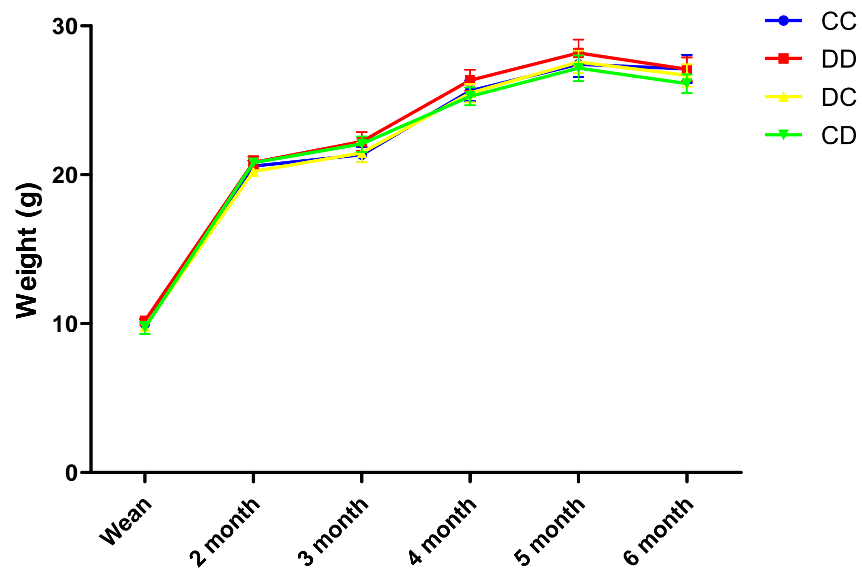


Figure 6.4 F2 male and female offspring growth trajectory

There were no differences in weight gain in all four groups in **A)** male or **B)** female offspring till six months of age

Data are mean ± SEM, analysed by AUC

n = Males CC: 15, DD: 11, DC: 14, CD: 10

Females CC: 11, DD: 10, DC: 11, CD: 12

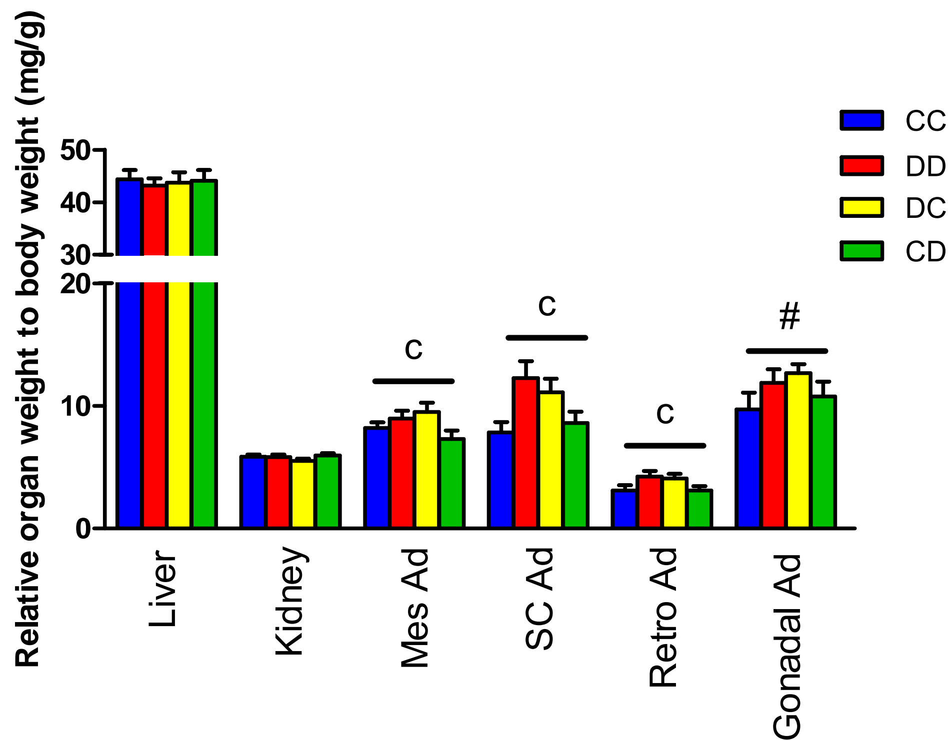


Figure 6.5 Organ weights of F2 male offspring at six months of age

There was an effect of maternal prenatal DIO exposure to increase the weight of mesenteric adipose, subcutaneous adipose and retroperitoneal adipose tissue. There was also a trend for maternal prenatal DIO exposure to increase the weight of gonadal adipose tissue

Data are mean ± SEM, analysed by two-way ANOVA

n = CC: 7, DD: 8, DC: 9, CD: 9 litters per group

c = Maternal prenatal DIO exposure effect to increase, # = trend (p=0.07) to significance for maternal prenatal DIO exposure effect to increase

Mes Ad = Mesenteric adipose, SC Ad = Subcutaneous adipose, Retro Ad = Retroperitoneal adipose

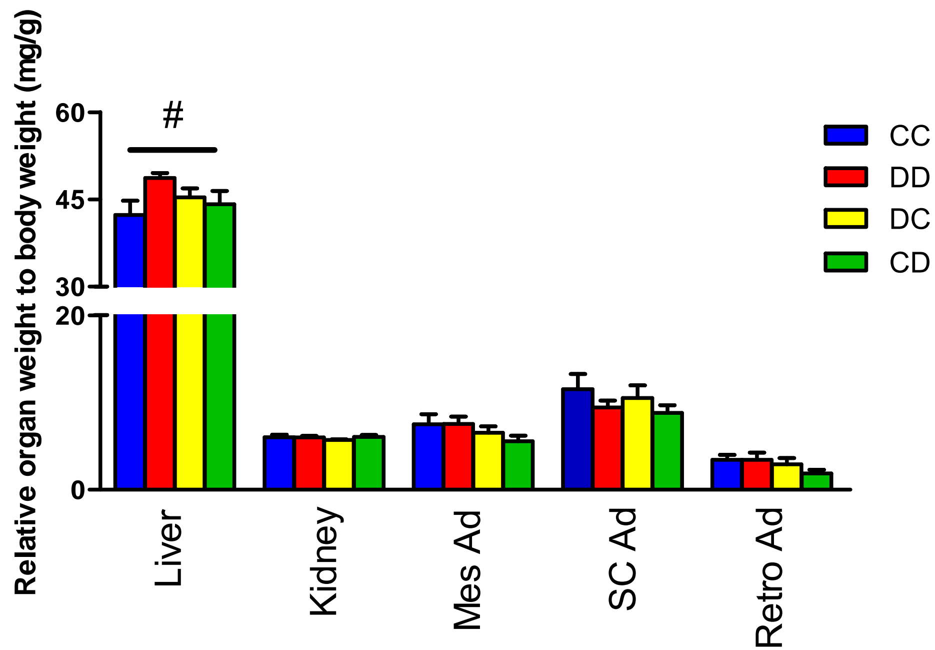


Figure 6.6 Organ weights of F2 female offspring at six months of age

There was a trend for maternal prenatal DIO exposure to increase the weight of liver

Data are mean ± SEM, analysed by two-way ANOVA

n = 8 per group

= trend (p=0.06) to significance for maternal prenatal DIO diet exposure effect to increase

Mes Ad = Mesenteric adipose, SC Ad = Subcutaneous adipose, Retro Ad = Retroperitoneal adipose

6.3.2 F2 offspring plasma analysis

At six months of age, there was an effect of maternal prenatal DIO exposure ($p<0.01$, $F=9.36$) to increase insulin levels during IPGTT in F2 male offspring (Figure 6.7a). There were no differences in glucose levels (Figure 6.7b).

In F2 female offspring, there was an interaction between maternal and paternal prenatal diet exposure ($p<0.05$, $F=5.47$) to reduce insulin levels during IPGTT (Figure 6.8a). There were no differences in glucose levels (Figure 6.8b).

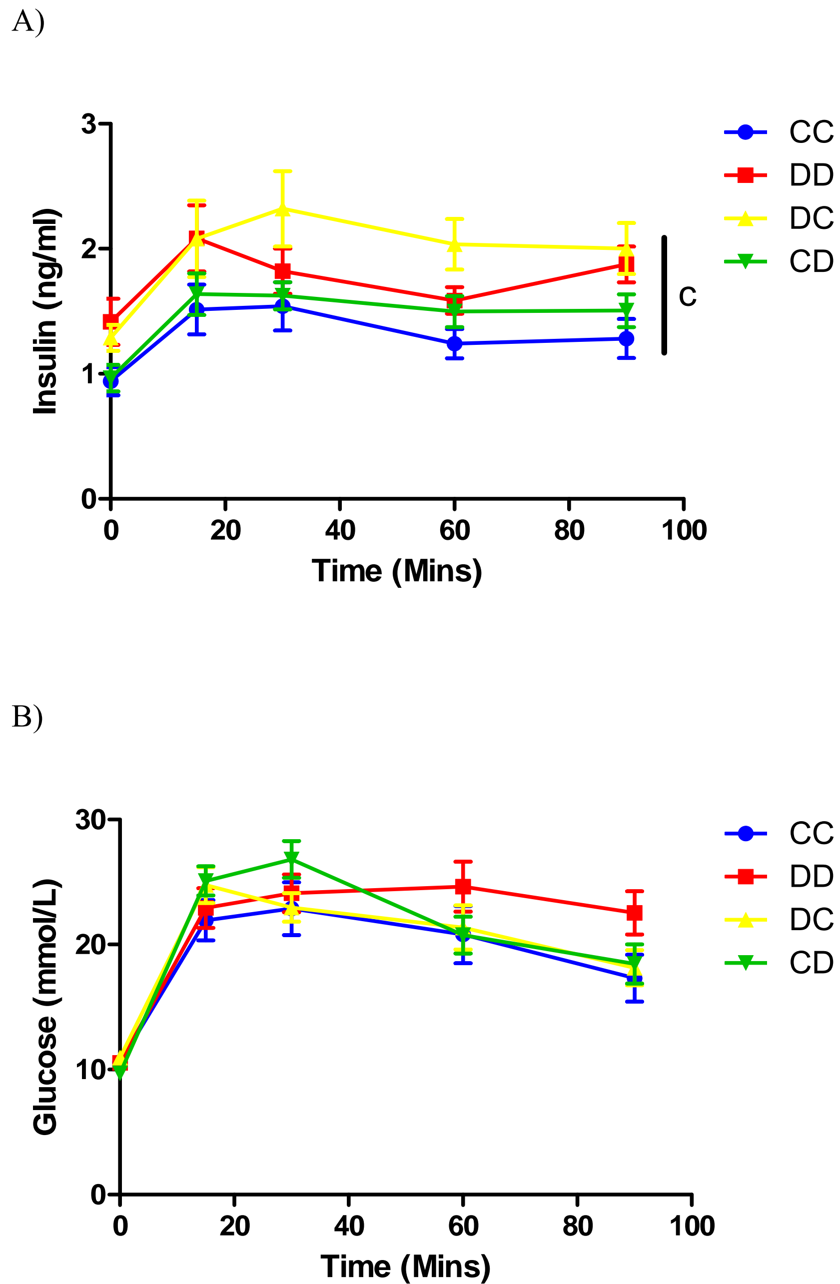


Figure 6.7 Plasma insulin and glucose levels at six months of age in F2 males

There was A) an effect of maternal prenatal DIO exposure effect to increase insulin secretion, but B) no difference in glucose levels following an intraperitoneal glucose load

Data are mean ± SEM, analysed by AUC

n = CC: 8, DD: 9, DC: 9, CD: 10

c = Maternal prenatal DIO diet exposure effect to increase

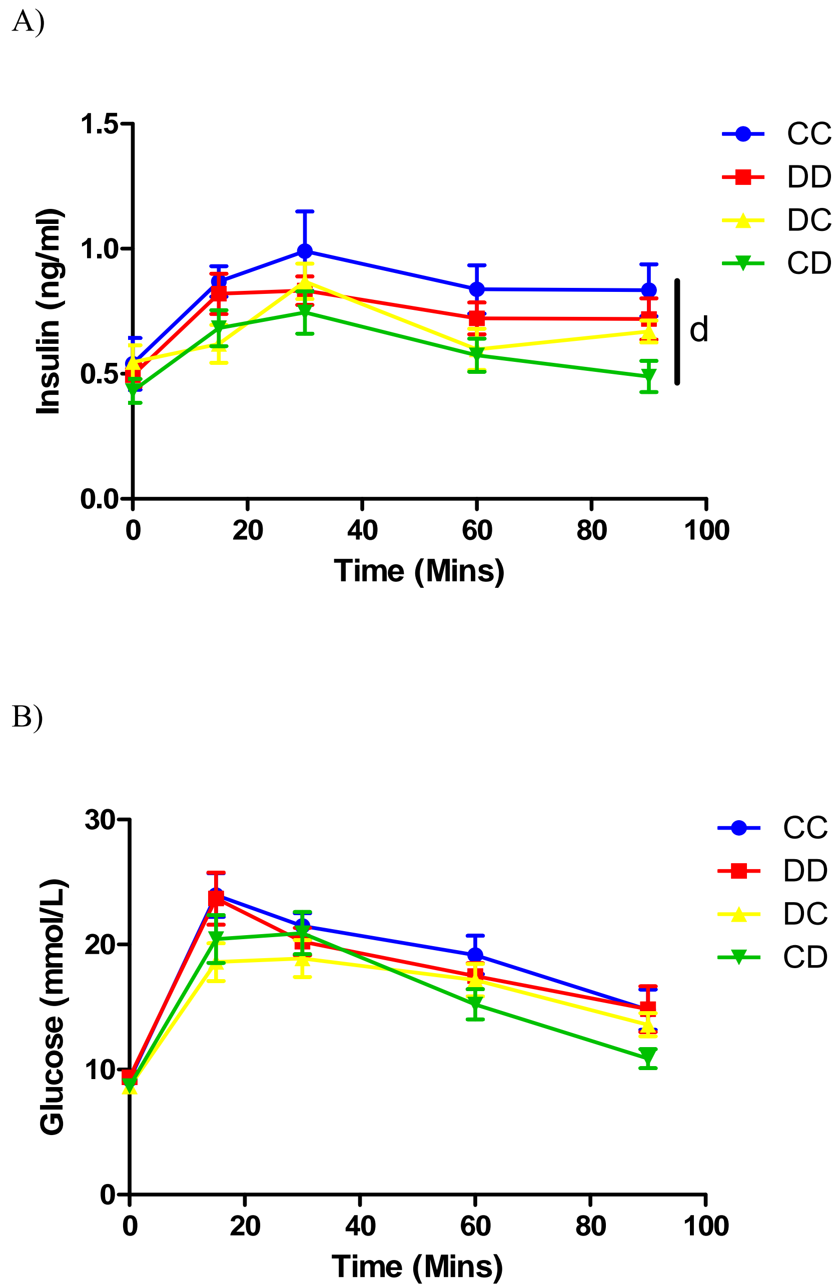


Figure 6.8 Plasma insulin and glucose levels at six months of age in F2 females

There was **A)** an interaction between maternal and paternal prenatal DIO exposure to decrease insulin levels, but **B)** no differences in glucose levels following an intraperitoneal glucose load

Data are mean ± SEM, analysed by AUC

n = CC: 8, DD: 9, DC: 9, CD: 8

d = Interaction between maternal and paternal prenatal DIO exposure effect to decrease

6.4 Discussion

Although minimal programming effects were seen in the F1 generation directly exposed to maternal obesity, there were both gender- and parent-of-origin specific programming effects on birth weight, fat distribution and glucose homeostasis in the second generation. At birth, there were parent-of-origin effects on male and female pup birth weights. There was an effect of maternal prenatal DIO exposure to decrease birth weight in male pups and an effect of paternal prenatal DIO exposure to decrease birth weight in female pups. At six months of age, no differences were seen in the body weights of F2 males, but there was an effect of maternal prenatal DIO exposure to increase the weight of adipose tissue. Interestingly, the character of this fat distribution suggests central fat deposition. In human males, central obesity has been linked to metabolic illnesses such as diabetes and dyslipidaemia as well as an increased risk of cardiovascular disease (Björntorp 1991, Bujalska et al. 1997). In this study, mice with an increase in adipose tissue, groups DD and DC, also displayed insulin insensitivity.

In F2 females at six months of age, there were no differences in body weight or in the weight or distribution of adipose depots. However, there was an interaction between maternal and paternal prenatal DIO exposure effect to decrease the insulin response to an intraperitoneal glucose load, suggesting increased insulin sensitivity in these animals.

Previous studies using high fat diets (Ng et al. 2010, Dunn and Bale 2011) and other programming models (Dahlgren et al. 2001, Sugden and Holness 2002, O'Regan et al.

2004, Zambrano et al. 2006, Kaati et al. 2007) have shown gender specific programming effects. A study of the effects of paternal obesity showed effects mainly in daughters (Ng et al. 2010) and using a similar model of maternal obesity, Dunn et al. showed programming effects of increased body size and reduced insulin sensitivity in both F1 and F2 offspring (Dunn and Bale 2009) and an increased body size in F3 only in females (Dunn and Bale 2011). Notably, these effects in F3 appear to be passed down through the paternal lineage to the daughters only (Dunn and Bale 2011). These findings are similar to the findings in my study showing gender specific programming effects of mother to son or father to daughter transmission of programmed effects (Figure 6.9).

Although the mechanisms of such gender specific transmission are unknown, there are a number of possibilities. Firstly, maternal investment in the offspring may play a role in such gender specific programming (Haig 1990). F1 DIO mothers could have 'invested' more in their male offspring postnatally, either through preferential feeding or increased maternal caring behaviour to increase the chances of survival; thus leading to a mother to son transmission pattern. This explanation is supported by previous reports showing maternal retrieval of pups being influenced by pup gender so that mothers retrieve male pups before females (Deviterne and Desor 1990) and additionally retrieve pups that are less developed and perhaps less likely to survive (Bolivar and Brown 1995). Secondly, these gender specific changes could be due to in utero hormonal exposure leading to different programming effects in male and female offspring. For example, maternal obesity leads to increased oestrogen exposure of the fetus (Pettigrew and Hamilton-Fairley 1997), such effects might

plausibly affect one sex but not the other. One previous report in mice has shown that male fetuses positioned in between two female fetuses are exposed to higher level of oestrogen than those position between male fetus(es), as the amniotic fluid of female fetuses contains higher oestrogen levels. This increased in utero exposure of oestrogen in the male fetus(es) was associated with less aggressive behaviour in adulthood and an increased sexual activity (Vom Saal et al. 1983). Lastly, the placenta may have played a role in gender specific programming effects. Previous studies have shown that gene expression, protein levels, hormone levels and immunological function in the placenta differ according to the gender of the developing fetus (Mueller and Bale 2008b, Clifton 2010). However, none of these mechanisms explain the transmission of effects from father to daughter. As the father was removed from the cage after mating and only contributes sperm to the next generation, this suggests an epigenetic mode of transmission leading to the transmission of effects. How this might affect daughters but not sons is unclear although it has been proposed that effects on the X chromosome might mediate father-to-daughter transmission (Pembrey et al. 2006). However, I am not aware of any reports providing clear evidence of such transmission.

As mentioned previously, in addition to the gender specific programming effects in the F2, there were also generation specific programming effects in this model of maternal obesity. There are a few possibilities which might explain such generation specific programming effects. Firstly, as discussed in chapter four, epigenetic effects in the germline as a consequence of disrupted epigenetic reprogramming may impact on the next generation. In the germline of both sexes, epigenetic marks are erased

during early development. In males, the re-establishment of epigenetic marks in the developing germline occurs during late fetal development whereas in females, this occurs mainly after birth (Reik et al. 2001, Sasaki and Matsui 2008). Although the developing F1 generation offspring were exposed to the abnormal maternal environment throughout gestation and lactation, they were also potentially exposed at the level of the germ cell. We maintained F0 mothers on obesogenic diets from 5 weeks of age, so that the germ cells which would form the F1 generation were also potentially exposed to, and may have been influenced by this environment both during the period of re-establishment of methylation marks which occurs at ovulation and during the period of epigenetic reprogramming post-fertilisation. In contrast, the F2 were first exposed at the level of the developing germ cell (Figure 6.10). These germ cells were exposed throughout development, during the time at which epigenetic marks are both erased and re-established (at least in the male germline). Thus, the difference in timing and extent to which the F1 and F2 germ cells and offspring were exposed to the insult may lead to different programming effects in the two generations.

Alterations in F1 maternal or paternal physiology may also play a role. In other programming models, altered maternal physiology such as a smaller maternal size has been shown to have programming effects (Walton and Hammond 1938, Cowley et al. 1989). In my model, although there were minimal programming effects seen in the directly exposed F1 females, however this was assessed in the non-pregnant state. Normal pregnancy is a state of physiological insulin resistance (Spellacy and Goetz 1963), and it is possible that there was decompensation during pregnancy in F1 DIO

females, leading to programming effects in the F2 generation. This is supported by previous studies using a model of undernutrition in rats in which a reduction in β cell mass was associated with alterations in glucose homeostasis during pregnancy (Blondeau et al. 1999). Additionally, alterations in the F1 males, such as changes in sperm may be of importance. Studies in both humans (Fejes et al. 2005, Hammoud et al. 2008) and animals (Ghanayem et al. 2010, Bakos et al. 2011) have shown effects of obesity on sperm count, motility, sperm function and DNA damage, suggesting that sperm may be a target for programming effects. Emerging evidence suggests that effects on histones (Hammoud et al. 2009) and siRNA (Rassoulzadegan et al. 2006, Lalancette et al. 2008) in sperm may be associated with the transmission of effects to subsequent generations. Although I am not aware of reports suggesting epigenetic changes in histones and siRNA in sperm in models of paternal or maternal obesity, this may be an important direction in future work.

In summary, maternal obesity induced by a high fat and high sugar diet in mice is associated with minimal programming effects in the F1 offspring, but leads to gender specific and parent-of-origin specific programming effects in the F2.

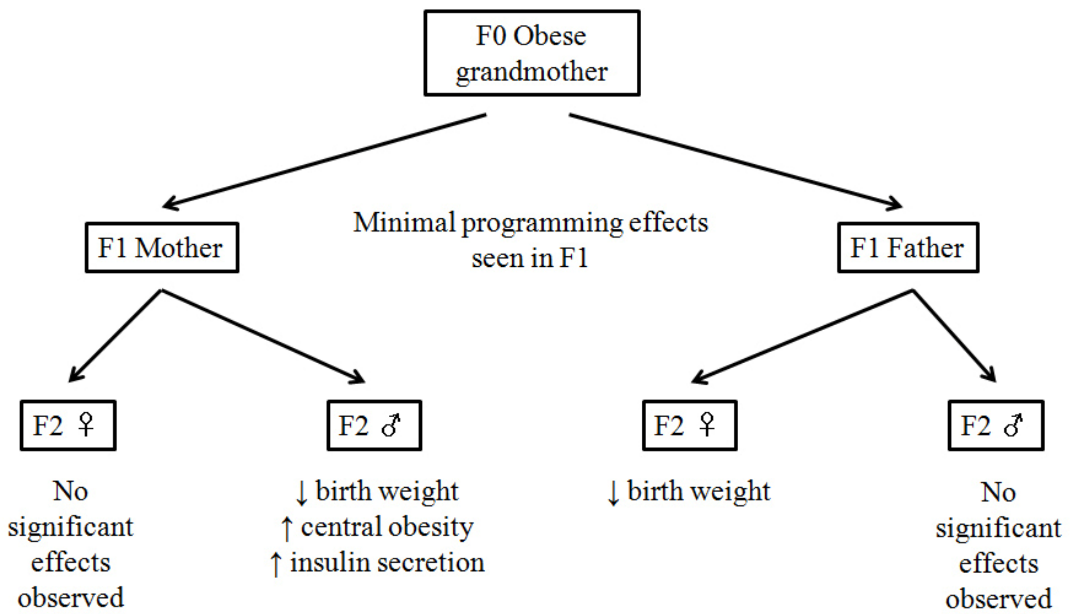


Figure 6.9 Summary of findings in F2 mice
This shows the mother to son and father to daughter effects

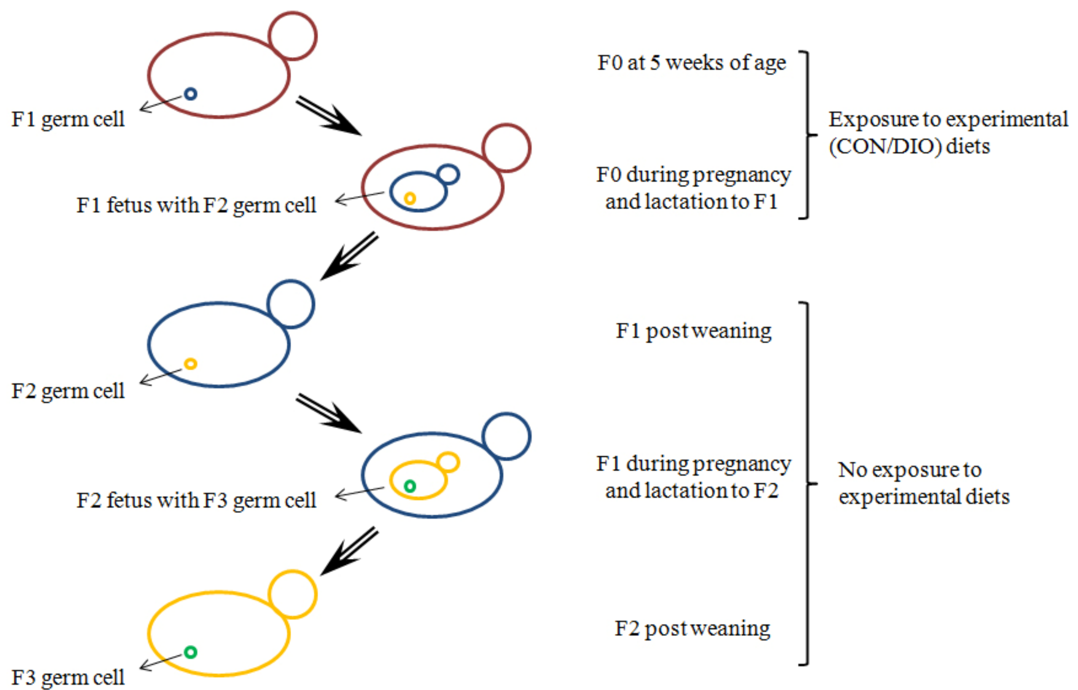


Figure 6.10 Schematic of F1 and F2 exposure to experimental diets
The F1 generation was initially exposed to the experimental diets as a germ cell in the gonad of the F0 mother as diets were commenced at 5 weeks of age. This exposure was also present during fertilisation, throughout the period of in utero development and also postnatally during lactation. In contrast, the F2 was only exposed to the experimental diets as a germ cell in the developing gonad of the F1 generation in utero

CHAPTER 7

Concluding discussion and future work

7.1 Introduction

The aim of this thesis was to explore the possible mechanisms of multigenerational programming in two different animal models: i) the glucocorticoid programmed rat model, and ii) a mouse model of maternal obesity. In the rat model of glucocorticoid overexposure, I have explored changes in placental and fetal hepatic gene expression in both generations and protein levels in the first generation. In the mouse model of maternal obesity, I studied body weight, metabolic changes and gene expression changes in the first generation and body weight and glucose homeostasis in the second generation. A summary of the main findings in the glucocorticoid programmed rat and the maternal obesity programmed mice is presented in figure 7.1 and 7.2 respectively.

7.2 Multigenerational programming in the glucocorticoid programmed rat

Previous reports from our laboratory have shown that programming effects on glucose homeostasis and hypertension may be induced by administering a synthetic glucocorticoid, dexamethasone, during the last week of gestation in rats (Benediktsson et al. 1993, Nyirenda et al. 1998, Drake et al. 2005). Such programming effects have also been shown to be passed down to the 'not directly exposed' second generation (Drake et al. 2005). I have continued to use this model to explore the underlying mechanisms of multigenerational programming. In this study, I have found that Dex lowers fetal and placental weight at E20 and increases the expression of several placental nutrient transporters important in fetal growth. This enhancement of the nutrient transporters may be a result of a 'call for help' signal from the fetus mediated through increased expression of Igf2 (Constância et al. 2005).

However, despite the increased expression of Igf2 in the fetal liver and the increased expression of important nutrient transporters in the placenta, Dex exposed fetuses remained small at E20 and at birth. This may be due to a variety of reasons such as: i) a smaller maternal weight gain during the period of Dex administration, ii) reduced fetal insulin secretion and / or signalling, and iii) the increased expression of several other growth restricting genes (e.g. CDKn1c, Grb10) in the fetal liver. Our recent study has also shown DNA methylation changes in the offspring that were exposed to Dex prenatally (Drake et al. 2011).

Previous reports have shown that Dex exposure in rats during the last week of gestation alters the expression of vascular endothelial growth factor and placental vascularity (Hewitt et al. 2006) as well as changes in the secretion of hormones by the placenta that are important in maintaining pregnancy in rats (Ain et al. 2005). These changes, although not explored in my study, may potentially lead to an altered in utero environment for the developing fetus leading to programming effects.

In addition to the molecular changes in the placenta and fetal liver, maternal physiology and behaviour may also play a role in programming of the fetus. Previous reports have shown that Dex exposure can lead to an anorexic behaviour in rats (Simpson et al. 1974) resulting in decreased weight gain. In my study, at E20, F0 Dex mothers are lighter in comparison to F0 Veh mothers. Reduced maternal weight and size has been shown to be linked to reduced birth weight in a variety of species such as horses (Allen et al. 2002), sheep (Hunter 1956), pigs (Gama and Johnson 1993), mice (Cowley et al. 1989), and humans (Ibanez et al. 2000). Thus, the

underlying mechanism(s) that lead to the final phenotype in the F1 offspring of glucocorticoid programmed rat may reflect an interaction between gene expression, placental morphology, altered hormone secretion, and altered maternal behaviour.

Although previous reports have suggested that the programmed phenotype in F2 offspring is similar to that in the F1 (Drake et al. 2005, Harrison and Langley-Evans 2009, Jimenez-Chillaron et al. 2009), the mechanisms by which programming effects are transmitted across generations remain to be determined. In my study, despite the similarity in metabolic phenotypes in the two generations, the underlying mechanisms appear to be different. In the F2 fetuses, I found parent-of-origin effects on both placental and fetal weight at E20 and on birth weight. These results suggest that the growth of the F2 fetuses and placentas are closely related to their parental Dex exposure status and that the programming effects may be passed down through either the maternal or paternal lineage. One of the explanations for the differences in results seen as a consequence of Dex exposure in either the mother or the father is the parental conflict hypothesis. This hypothesis suggests that the influence of the father promotes growth enhancement, whereas the influence of the mother is to restrict the growth of the developing fetus (Moore and Haig 1991).

The generation specific programming effects seen in this model may be explained by the timing of exposure to the Dex insult at different time points of development. F1 offspring were directly exposed to Dex during the last week of in utero development. In the F2, the exposure to Dex was at the level of the germ cell within the gonads of the developing F1 during a crucial time of epigenetic reprogramming. The fact that

epigenetic reprogramming differs in timing between male and female germ cells - occurring predominantly during late fetal development in males (Kafri et al. 1992, Tada et al. 1998, Reik and Walter 2001) but predominantly after birth in females (Tada et al. 1998, Reik et al. 2001) may underlie some of the parent-of-origin specific effects seen in the F2.

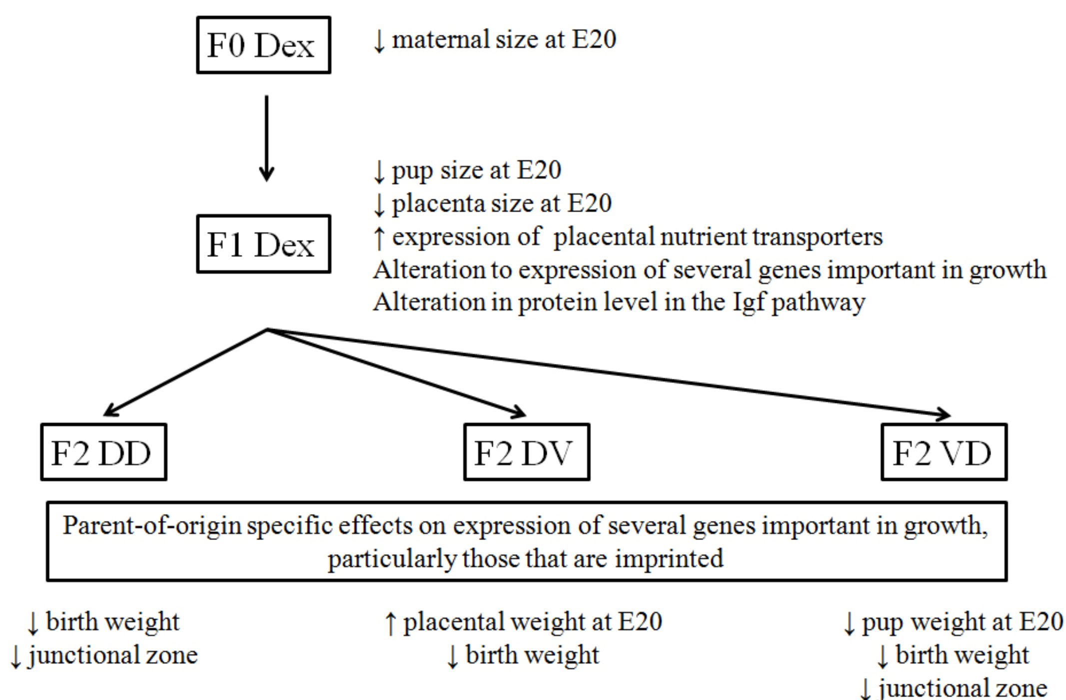


Figure 7.1 Summary of main findings in the glucocorticoid programmed rat model

7.3 Multigenerational programming as a consequence of maternal obesity

Obesity has become an increasing issue worldwide (WHO 2011). In the second part of this thesis, I described a model of maternal obesity by feeding a high fat and high sugar diet to mice after weaning to induce a phenotype of obesity prior to pregnancy; mimicking the human state of maternal dietary obesity. In this study, we found relatively minimal programming effects in our F1 offspring. This finding is in contrast to previous reports showing programming effects by maternal obesity on offspring glucose homeostasis, adipose deposition, metabolic disorders, and cardiovascular disease (Guo and Jen 1995, Bayol et al. 2005, Samuelsson et al. 2008, Elahi et al. 2009). There are several possible reasons for this difference in findings. As discussed in chapter five, the differences in diets used to induce obesity in our model compared to others may be an important factor. In our model, we used a diet that is high fat and high sugar but identical in other micro- and macronutrient contents including protein. Secondly, the duration of exposure to the experimental diets may be an important factor. In our model, we aimed to induce maternal obesity prior to pregnancy thus mimicking human maternal obesity. In other models, the duration of experimental diets ranges from a week (Guo and Jen 1995) to 55 weeks (Sumiyoshi et al. 2006) prior to pregnancy, potentially leading to different programming results.

Despite the lack of changes in the F1 offspring, I found changes in the F2 offspring. The findings are similar to those in the glucocorticoid programmed rat which also showed generation specific and parent-of-origin specific programming effects. Additionally, there were gender specific effects in the F2 offspring with mother to

son and father to daughter transmission effects. Possible mechanisms of such specific parental transmission effects include: i) epigenetic effects on the germline which may be transmitted to subsequent generations, ii) maternal investment on the offspring in response to the environment the mother was exposed to, iii) in utero exposure to hormones which may affect one gender but not the other. The mechanisms which explain the father to daughter transmission remain unclear.

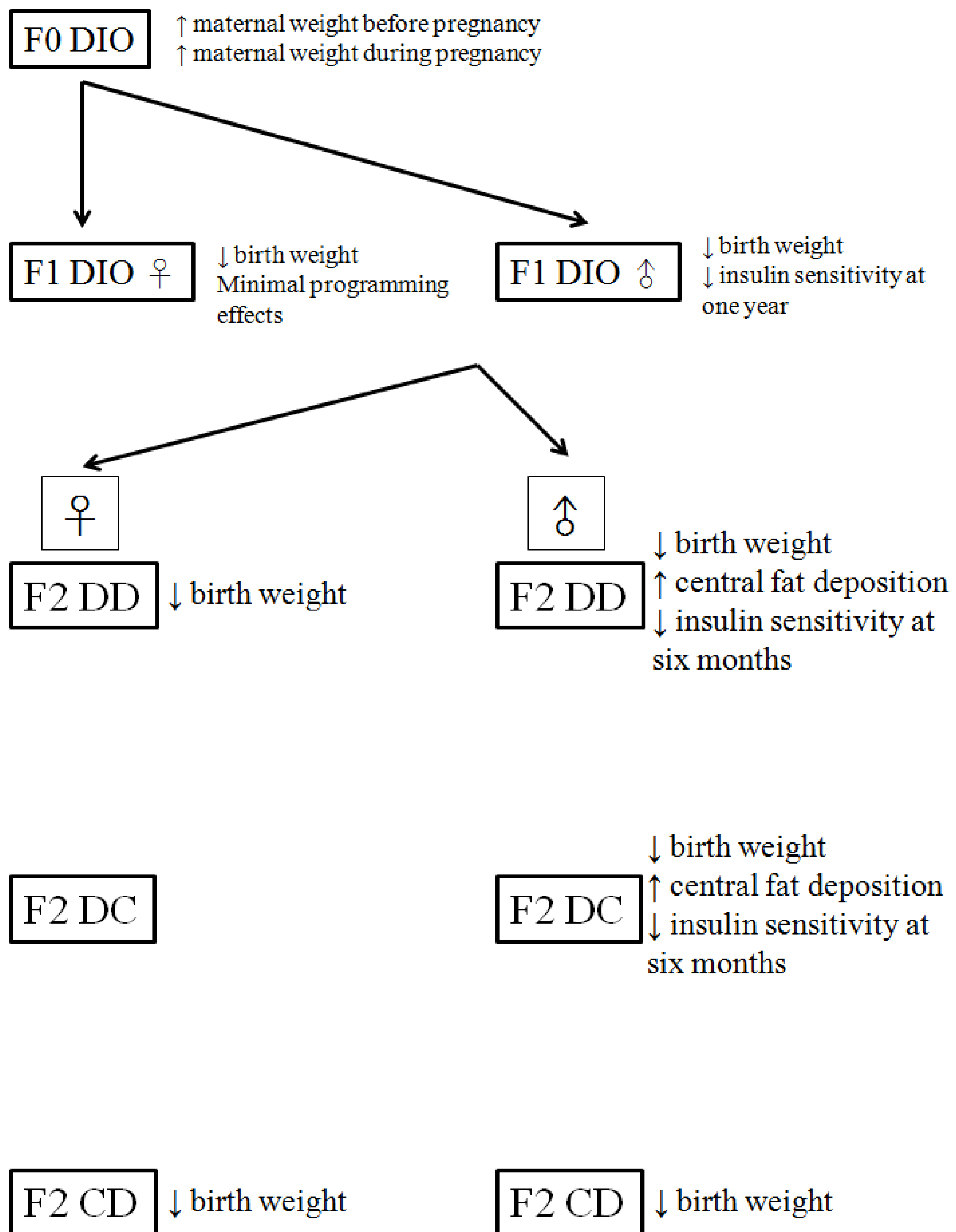


Figure 7.2 Summary of findings in the mouse model of maternal obesity

7.4 Public health implications

In the past two decades, numerous large scale human epidemiological studies from different geographical areas and in different ethnicities have suggested that exposure to an adverse environment during specific time points in development is associated with adult disease risk. Such adverse environments may include direct physiological alterations such as starvation, malnutrition, or over nutrition as well as psychological stress such as the 9/11 event. Interestingly, it has been shown that some of these programming effects are reproduced in subsequent generations. Programming of the offspring has been viewed as a consequence of fetal adaptation to a poor environment, resulting in lower investment in organs that are less important in survival compared with organs which may be critical for survival (Hales and Barker 1992). The exact underlying mechanisms of such programming and multigenerational transmission effects remain largely unknown and animal models have been developed in which to explore potential mechanisms. The advantages of using animal models for programming studies are the i) controlled environment and ii) genetic background to minimise bias as well as iii) the shorter life cycle which allows rapid reproduction of subsequent generations.

In the glucocorticoid programmed rat model, I have shown programming effects in the placenta including changes in placental size, structure, and gene expression. If such findings can be translated into human studies, they may be useful in the early detection and prevention of future health risks in the offspring.

Previous studies in humans have suggested that maternal obesity is linked to

childhood obesity (Whitaker 2004, Gale et al. 2007). However, it is uncertain to what extent this link is due to programming effects of maternal obesity, shared genes or the effects of exposure to a poor diet / lack of exercise postnatally. In this study, I have shown that programming effects are perhaps not an inevitable consequence of exposure to maternal obesity in utero. However, despite this, there are programming effects in the F2. This model will allow future exploration of how maternal overnutrition may lead to programming effects in the grand-offspring and potentially in subsequent generations.

In conclusion, the two animal models presented in this thesis have confirmed that an adverse environment during early life may lead to programming effects in the offspring and grand-offspring. Along with the numerous large scale human epidemiological studies, this indicates a link between early life exposure to 'insults' and future disease risks.

7.5 Future work

As presented in this thesis, and from reports in the literature, epigenetic modifications may play an important role in early life programming and in the transmission of effects to subsequent generations. Future studies should be directed at exploring epigenetic changes, such as DNA methylation changes and histone modifications, in both models as mediators of programming effects. Reports have suggested possible transmission of programming effects via sperm (Anway et al. 2005, Stouder and Paoloni-Giacobino 2010) and further studies aimed at exploring whether epigenetic effects may be transmissible through the germline will be important in increasing our understanding of the mechanisms by which effects are transmitted across generations. In order to explore the full effect of obesity in programming, I would also like to generate a model of paternal obesity and determine its effect on offspring and grand-offspring. Additionally, as previously discussed, dietary constituents may be important in different programming effects. Future studies aimed at teasing out programming effects of different micro- and macronutrients will be important. Lastly, although minimal programming effects were seen in F1 females of the maternal obese mice, this does not exclude possible decompensation during pregnancy therefore studies should also be aimed at exploring possible maternal effects in this regard.

References

Aerts, L, Holemans, K & Van Assche, F A 1990. Maternal diabetes during pregnancy: Consequences for the offspring. *Diabetes/Metabolism Reviews,* **6,** 147-67.

Ain, R, Canham, L N & Soares, M J 2005. Dexamethasone-induced intrauterine growth restriction impacts the placental prolactin family, insulin-like growth factor-II and the Akt signaling pathway. *Journal of Endocrinology,* **185,** 253-63.

Ain, R, Konno, T, Canham, L N & Soares, M J 2006. Phenotypic analysis of the rat placenta. *Methods in Molecular Medicine,* **121,** 295-313.

Akyol, A, Langley-Evans, S C & McMullen, S 2009. Obesity induced by cafeteria feeding and pregnancy outcome in the rat. *British Journal of Nutrition,* **102,** 1601-10.

Alessi, D R, Andjelkovic, M, Caudwell, B, Cron, P, Morrice, N, Cohen, P & Hemmings, B A 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. *The EMBO Journal,* **15,** 6541-51.

Alheim, K, Corness, J, Samuelsson, M, Bladh, L, Murata, T, Nilsson, T & Okret, S 2003. Identification of a functional glucocorticoid response element in the promoter of the cyclin-dependent kinase inhibitor p57Kip2. *Journal of Molecular Endocrinology,* **30,** 359-68.

Allain, C C, Poon, L S, Chan, C S G, Richmond, W & Fu, P C 1974. Enzymatic Determination of Total Serum Cholesterol. *Clinical Chemistry,* **20,** 470-75.

Allen, W, Wilsher, S, Turnbull, C, Stewart, F, Ousey, J, Rossdale, P & Fowden, A 2002. Influence of maternal size on placental, fetal and postnatal growth in the horse. I. Development in utero. *Reproduction,* **123,** 445-53.

Anand, P, Boylan, J M, Ou, Y & Gruppuso, P A 2002. Insulin signaling during perinatal liver development in the rat. *American Journal of Physiology - Endocrinology and Metabolism,* **283,** E844-E52.

Andjelković, M, Jakubowicz, T, Cron, P, Ming, X F, Han, J W & Hemmings, B A 1996. Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC-PK/PKB) promoted by serum and protein phosphatase inhibitors. *Proceedings of the National Academy of Sciences of the United States of America,* **93,** 5699-704.

Andrews, S C, Wood, M D, Tunster, S J, Barton, S C, Surani, M A & John, R M 2007. Cdkn1c (p57(Kip2)) is the major regulator of embryonic growth within its imprinted domain on mouse distal chromosome 7. *BMC Developmental*

Biology, **7**.

Antonov, A N 1947. Children born during The Siege of Leningrad in 1942. *The Journal of Pediatrics,* **30,** 250-59.

Antonow-Schlorke, I, Schwab, M, Li, C & Nathanielsz, P W 2003. Glucocorticoid exposure at the dose used clinically alters cytoskeletal proteins and presynaptic terminals in the fetal baboon brain. *The Journal of Physiology,* **547,** 117-23.

Anway, M D, Cupp, A S, Uzumcu, M & Skinner, M K 2005. Epigenetic transgenerational actions of endocrine disruptors and mate fertility. *Science,* **308,** 1466-69.

Anway, M D, Leathers, C & Skinner, M K 2006a. Endocrine disruptor vinclozolin induced epigenetic transgenerational adult-onset disease. *Endocrinology,* **147,** 5515-23.

Anway, M D, Memon, M A, Uzumcu, M & Skinner, M K 2006b. Transgenerational Effect of the Endocrine Disruptor Vinclozolin on Male Spermatogenesis. *Journal of Andrology,* **27,** 868-79.

Archer, J E & Blackman, D E 1971. Prenatal psychological stress and offspring behavior in rats and mice. *Developmental Psychobiology,* **4,** 193-248.

Ashworth, C & Antipatis, C 2001. Micronutrient programming of development throughout gestation. *Reproduction,* **122,** 527-35.

Audette, M C, Challis, J R G, Jones, R L, Sibley, C P & Matthews, S G 2011. Antenatal Dexamethasone Treatment in Midgestation Reduces System A-Mediated Transport in the Late-Gestation Murine Placenta. *Endocrinology*.

Audette, M C, Greenwood, S L, Sibley, C P, Jones, C J P, Challis, J R G, Matthews, S G & Jones, R L 2010. Dexamethasone stimulates placental system A transport and trophoblast differentiation in term villous explants. *Placenta,* **31,** 97-105.

Backer, J M, Myers, M G, Jr., Shoelson, S E, Chin, D J, Sun, X J, Miralpeix, M, Hu, P, Margolis, B, Skolnik, E Y, Schlessinger, J & et al. 1992. Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *The EMBO Journal,* **11,** 3469-79.

Baker, J, Liu, J-P, Robertson, E J & Efstratiadis, A 1993. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell,* **75,** 73-82.

Bakos, H W, Mitchell, M, Setchell, B P & Lane, M 2011. The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model. *International Journal of Andrology,* **34,** 402-10.

Barash, V & Shafrir, E 1990. Mobilization of placental glycogen in diabetic rats. *Placenta,* **11,** 515-21.

Barbazanges, A, Piazza, P V, Le Moal, M & Maccari, S 1996. Maternal Glucocorticoid Secretion Mediates Long-Term Effects of Prenatal Stress. *The Journal of Neuroscience,* **16,** 3943-49.

Barker, D J 1990. The fetal and infant origins of adult disease. *British Medical Journal,* **301,** 1111.

Barker, D J P, Godfrey, K M, Gluckman, P D, Harding, J E, Owens, J A & Robinson, J S 1993. Fetal nutrition and cardiovascular disease in adult life. *The Lancet,* **341,** 938-41.

Barker, D J P, Osmond, C, Winter, P D, Margetts, B & Simmonds, S J 1989. Weight in infancy and death from ischaemic heart disease. *The Lancet,* **334,** 577-80.

Barton, T S, Robaire, B & Hales, B F 2005. Epigenetic programming in the preimplantation rat embryo is disrupted by chronic paternal cyclophosphamide exposure. *Proceedings of the National Academy of Sciences of the United States of America,* **102,** 7865.

Battaglia, F C & Meschia, G 1978. Principal substrates of fetal metabolism. *Physiological Reviews,* **58,** 499-527.

Bayol, S, Macharia, R, Farrington, S, Simbi, B & Stickland, N 2009. Evidence that a maternal “junk food” diet during pregnancy and lactation can reduce muscle force in offspring. *European Journal of Nutrition,* **48,** 62-65.

Bayol, S A, Farrington, S J & Stickland, N C 2007. A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. *British Journal of Nutrition,* **98,** 843-51.

Bayol, S A, Simbi, B H, Bertrand, J A & Stickland, N C 2008. Offspring from mothers fed a 'junk food' diet in pregnancy and lactation exhibit exacerbated adiposity that is more pronounced in females. *The Journal of Physiology,* **586,** 3219-30.

Bayol, S A, Simbi, B H & Stickland, N C 2005. A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning. *The Journal of Physiology,* **567,** 951-61.

Beatty, L, Weksberg, R & Sadowski, P D 2006. Detailed analysis of the methylation patterns of the KvDMR1 imprinting control region of human chromosome 11. *Genomics,* **87,** 46-56.

Benediktsson, R, Calder, A A, Edwards, C R W & Seckl, J R 1997. Placental 11β-hydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure. *Clinical Endocrinology,* **46,** 161-66.

Benediktsson, R, Lindsay, R S, Noble, J, Seckl, J R & Edwards, C R 1993. Glucocorticoid exposure in utero: new model for adult hypertension. *The Lancet,* **341,** 339-41.

Benyshek, D C, Johnston, C S & Martin, J F 2004. Post-natal diet determines insulin resistance in fetally malnourished, low birthweight rats (F1) but diet does not modify the insulin resistance of their offspring (F2). *Life Sciences,* **74,** 3033-41.

Benyshek, D C, Johnston, C S & Martin, J F 2006. Glucose metabolism is altered in the adequately-nourished grand-offspring (F-3 generation) of rats malnourished during gestation and perinatal life. *Diabetologia,* **49,** 1117-19.

Benyshek, D C, Johnston, C S, Martin, J F & Ross, W D 2008. Insulin sensitivity is normalized in the third generation (F3) offspring of developmentally programmed insulin resistant (F2) rats fed an energy-restricted diet. *Nutrition and Metabolism,* **5**.

Bernstein, E & Allis, C D 2005. RNA meets chromatin. *Genes & Development,* **19,** 1635-55.

Bestor, T H 2000. The DNA methyltransferases of mammals. *Human Molecular Genetics,* **9,** 2395-402.

Bieswal, F, Ahn, M-T, Reusens, B, Holvoet, P, Raes, M, Rees, W D & Remacle, C 2006. The Importance of Catch-up Growth after Early Malnutrition for the Programming of Obesity in Male Rat. *Obesity,* **14,** 1330-43.

Bifulco, A, Brown, G W & Adler, Z 1991. Early sexual abuse and clinical depression in adult life. *The British Journal of Psychiatry,* **159,** 115-22.

Björntorp, P 1991. Metabolic Implications of Body Fat Distribution. *Diabetes Care,* **14,** 1132-43.

Blondeau, B, Garofano, A, Czernichow, P & Breant, B 1999. Age-dependent inability of the endocrine pancreas to adapt to pregnancy: a long-term consequence of perinatal malnutrition in the rat. *Endocrinology,* **140,** 4208.

Blondeau, B, Lesage, J, Czernichow, P, Dupouy, J P & Bréant, B 2001. Glucocorticoids impair fetal β-cell development in rats. *American Journal of Physiology - Endocrinology and Metabolism,* **281,** E592-E99.

Bloom, S L, Sheffield, J S, Mcintire, D D & Leveno, K J 2001. Antenatal dexamethasone and decreased birth weight. *Obstetrics & Gynecology,* **97,** 485-90.

Bolivar, V J & Brown, R E 1995. Selective retrieval of jimpy mutant pups over normal male littermates by lactating female B6CBACa-Aw-J/A-Ta jp mice. *Behavior Genetics,* **25,** 75-80.

Bouillot, S, Rampon, C, Tillet, E & Huber, P 2006. Tracing the Glycogen Cells with Protocadherin 12 During Mouse Placenta Development. *Placenta,* **27,** 882-88.

Brabham, T, Phelka, A, Zimmer, C, Nash, A, López, J F & Vázquez, D M 2000. Effects of prenatal dexamethasone on spatial learning and response to stress is influenced by maternal factors. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **279,** R1899-R909.

Brooks, A A, Johnson, M R, Steer, P J, Pawson, M E & Abdalla, H I 1995. Birth weight: nature or nurture? *Early Human Development,* **42,** 29-35.

Brown, E S 2009. Effects of Glucocorticoids on Mood, Memory, and the Hippocampus. *Annals of the New York Academy of Sciences,* **1179,** 41-55.

Brown, R W, Diaz, R, Robson, A C, Kotelevtsev, Y V, Mullins, J J, Kaufman, M H & Seckl, J R 1996. The ontogeny of 11 beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. *Endocrinology,* **137,** 794-7.

Bruce, K D, Cagampang, F R, Argenton, M, Zhang, J, Ethirajan, P L, Burdge, G C, Bateman, A C, Clough, G F, Poston, L, Hanson, M A, McConnell, J M & Byrne, C D 2009. Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. *Hepatology,* **50,** 1796-808.

Bujalska, I J, Kumar, S & Stewart, P M 1997. Does central obesity reflect “Cushing's disease of the omentum”? *The Lancet,* **349,** 1210-13.

Bunt, J C, Tataranni, P A & Salbe, A D 2005. Intrauterine Exposure to Diabetes Is a Determinant of Hemoglobin A1c and Systolic Blood Pressure in Pima Indian Children. *The Journal of Clinical Endocrinology & Metabolism,* **90,** 3225-29.

Burdge, G C, Slater-Jefferies, J, Torrens, C, Phillips, E S, Hanson, M A & Lillycrop, K A 2007. Dietary protein restriction of pregnant rats in the F-0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F-1 and F-2 generations. *British Journal of Nutrition,* **97,** 435-39.

Cantley, L C 2002. The Phosphoinositide 3-Kinase Pathway. *Science,* **296,** 1655-57.

Carmichael, S L & Shaw, G M 2000. Maternal Life Event Stress and Congenital Anomalies. *Epidemiology,* **11,** 30-35.

Carpenter, C L, Duckworth, B C, Auger, K R, Cohen, B, Schaffhausen, B S & Cantley, L C 1990. Purification and characterization of phosphoinositide 3-kinase from rat liver. *Journal of Biological Chemistry,* **265,** 19704-11.

Carroll, J J, Smith, N & Babson, A L 1970. A colorimetric serum glucose

determination using hexokinase and glucose-6-phosphate dehydrogenase. *Biochemical Medicine,* **4,** 171-80.

Caspary, T, Cleary, M A, Perlman, E J, Zhang, P, Elledge, S J & Tilghman, S M 1999. Oppositely imprinted genes p57Kip2 and Igf2 interact in a mouse model for Beckwith–Wiedemann syndrome. *Genes & Development,* **13,** 3115-24.

Catalano, P M, Presley, L, Minium, J & Hauguel-de Mouzon, S 2009. Fetuses of Obese Mothers Develop Insulin Resistance in Utero. *Diabetes Care,* **32,** 1076-80.

Charalambous, M, Smith, F M, Bennett, W R, Crew, T E, Mackenzie, F & Ward, A 2003. Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism. *Proceedings of the National Academy of Sciences of the United States of America,* **100,** 8292-97.

Chechi, K, R. Herzberg, G & K. Cheema, S 2010. Maternal dietary fat intake during gestation and lactation alters tissue fatty acid composition in the adult offspring of C57Bl/6 mice. *Prostaglandins, leukotrienes, and essential fatty acids,* **83,** 97-104.

Christy, C, Hadoke, P W F, Paterson, J M, Mullins, J J, Seckl, J R & Walker, B R 2003. 11beta-hydroxysteroid dehydrogenase type 2 in mouse aorta: localization and influence on response to glucocorticoids. *Hypertension,* **42,** 580-7.

Chu, C Y & Rana, T M 2007. Small RNAs: Regulators and guardians of the genome. *Journal of Cellular Physiology,* **213,** 412-19.

Chu, S, Kim, S & Bish, C 2009. Prepregnancy Obesity Prevalence in the United States, 2004–2005. *Maternal and Child Health Journal,* **13,** 614-20.

Clarke, A S, Wittwer, D J, Abbott, D H & Schneider, M L 1994. Long-term effects of prenatal stress on HPA axis activity in juvenile rhesus monkeys. *Developmental Psychobiology,* **27,** 257-69.

Clarke, K, Ward, J, Forhead, A, Giussani, D & Fowden, A 2002. Regulation of 11 beta-hydroxysteroid dehydrogenase type 2 activity in ovine placenta by fetal cortisol. *Journal of Endocrinology,* **172,** 527-34.

Cleasby, M E, Kelly, P A T, Walker, B R & Seckl, J R 2003. Programming of rat muscle and fat metabolism by in utero overexposure to glucocorticoids. *Endocrinology,* **144,** 999.

Clifton, V L 2010. Review: Sex and the Human Placenta: Mediating Differential Strategies of Fetal Growth and Survival. *Placenta,* **31, Supplement,** S33-S39.

CMACE 2010. *Maternal obesity in the UK: Findings from a national project*,

London
Coan, P M, Angiolini, E, Sandovici, I, Burton, G J, Constância, M & Fowden, A L 2008a. Adaptations in placental nutrient transfer capacity to meet fetal growth demands depend on placental size in mice. *The Journal of Physiology,* **586,** 4567-76.
Coan, P M, Conroy, N, Burton, G J & Ferguson-Smith, A C 2006. Origin and characteristics of glycogen cells in the developing murine placenta. *Developmental Dynamics,* **235,** 3280-94.
Coan, P M, Ferguson-Smith, A C & Burton, G J 2005. Ultrastructural changes in the interhaemal membrane and junctional zone of the murine chorioallantoic placenta across gestation. *Journal of Anatomy,* **207,** 783-96.
Coan, P M, Fowden, A L, Constancia, M, Ferguson-Smith, A C, Burton, G J & Sibley, C P 2008b. Disproportional effects of Igf2 knockout on placental morphology and diffusional exchange characteristics in the mouse. *The Journal of Physiology,* **586,** 5023-32.
Cogswell, M E & Yip, R 1995. The influence of fetal and maternal factors on the distribution of birthweight. *Seminars in Perinatology,* **19,** 222-40.
Collins, J W, David, R J, Prachand, N G & Pierce, M L 2003. Low Birth Weight Across Generations. *Maternal and Child Health Journal,* **7,** 229-37.
Constância, M, Angiolini, E, Sandovici, I, Smith, P, Smith, R, Kelsey, G, Dean, W, Ferguson-Smith, A, Sibley, C P, Reik, W & Fowden, A 2005. Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems. *Proceedings of the National Academy of Sciences of the United States of America,* **102,** 19219-24.
Constância, M, Hemberger, M, Hughes, J, Dean, W, Ferguson-Smith, A, Fundele, R, Stewart, F, Kelsey, G, Fowden, A, Sibley, C & Reik, W 2002. Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature,* **417,** 945-48.
Cowley, D E, Pomp, D, Atchley, W R, Eisen, E J & Hawkins-Brown, D 1989. The Impact of Maternal Uterine Genotype on Postnatal Growth and Adult Body Size in Mice. *Genetics,* **122,** 193-203.
Cropley, J E, Suter, C M, Beckman, K B & Martin, D I K 2006. Germ-line epigenetic modification of the murine Avy allele by nutritional supplementation. *Proceedings of the National Academy of Sciences,* **103,** 17308-12.
Cross, D A E, Alessi, D R, Cohen, P, Andjelkovich, M & Hemmings, B A 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature,* **378,** 785-89.

Crowley, P, Chalmers, I & Ke1Rse, M J N C 1990. The effects of corticosteroid administration before preterm delivery: an overview of the evidence from controlled trials. *BJOG: An International Journal of Obstetrics & Gynaecology,* **97,** 11-25.

Curhan, G C, Willett, W C, Rimm, E B, Spiegelman, D, Ascherio, A L & Stampfer, M J 1996. Birth Weight and Adult Hypertension, Diabetes Mellitus, and Obesity in US Men. *Circulation,* **94,** 3246-50.

Dabelea, D, Hanson, R L, Lindsay, R S, Pettitt, D J, Imperatore, G, Gabir, M M, Roumain, J, Bennett, P H & Knowler, W C 2000. Intrauterine exposure to diabetes conveys risks for type 2 diabetes and obesity: a study of discordant sibships. *Diabetes,* **49,** 2208-11.

Dabelea, D, Mayer-Davis, E J, Lamichhane, A P, D'Agostino, R B, Liese, A D, Vehik, K S, Narayan, K M V, Zeitler, P & Hamman, R F 2008. Association of Intrauterine Exposure to Maternal Diabetes and Obesity With Type 2 Diabetes in Youth. *Diabetes Care,* **31,** 1422-26.

Dahl, C, Grønbæk, K & Guldberg, P 2011. Advances in DNA methylation: 5-hydroxymethylcytosine revisited. *Clinica Chimica Acta,* **412,** 831-36.

Dahlgren, J, Nilsson, C, Jennische, E, Ho, H-P, Eriksson, E, Niklasson, A, Björntorp, P, Wikland, K A & Holmäng, A 2001. Prenatal cytokine exposure results in obesity and gender-specific programming. *American Journal of Physiology - Endocrinology and Metabolism,* **281,** E326-E34.

Davenport, M L, Clemmons, D R, Miles, M V, Camacho-Hubner, C, Joseph d'Ercole, A & Underwood, L E 1990. Regulation of Serum Insulin-Like Growth Factor-I (IGF I) and IGF Binding Proteins during Rat Pregnancy. *Endocrinology,* **127,** 1278-86.

Davey Smith, G, Hart, C, Ferrell, C, Upton, M, Hole, D, Hawthorne, V & Watt, G 1997. Birth weight of offspring and mortality in the Renfrew and Paisley study: Prospective observational study. *British Medical Journal,* **315,** 1189-93.

de Vries, A, Holmes, M C, Heijnis, A, Seier, J V, Heerden, J, Louw, J, Wolfe-Coote, S, Meaney, M J, Levitt, N S & Seckl, J R 2007. Prenatal dexamethasone exposure induces changes in nonhuman primate offspring cardiometabolic and hypothalamic-pituitary-adrenal axis function. *The Journal of Clinical Investigation,* **117,** 1058-67.

Dean, W, Bowden, L, Aitchison, A, Klose, J, Moore, T, Meneses, J J, Reik, W & Feil, R 1998. Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes.

Development, **125,** 2273.

DeChiara, T M, Efstratiadis, A & Robertsen, E J 1990. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature,* **345,** 78-80.

DeChiara, T M, Robertson, E J & Efstratiadis, A 1991. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell,* **64,** 849-59.

Delcuve, G P, Rastegar, M & Davie, J R 2009. Epigenetic Control. *Journal of Cellular Physiology,* **219,** 243-50.

Dennis, P B, Fumagalli, S & Thomas, G 1999. Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. *Current Opinion in Genetics & Development,* **9,** 49-54.

Dennis, P B, Jaeschke, A, Saitoh, M, Fowler, B, Kozma, S C & Thomas, G 2001. Mammalian TOR: A Homeostatic ATP Sensor. *Science,* **294,** 1102-05.

Desforges, M & Sibley, C P 2010. Placental nutrient supply and fetal growth. *International Journal of Developmental Biology,* **54,** 377-90.

Deuchar, G A, McLean, D, Hadoke, P W F, Brownstein, D G, Webb, D J, Mullins, J J, Chapman, K, Seckl, J R & Kotelevtsev, Y V 2011. 11β-Hydroxysteroid Dehydrogenase Type 2 Deficiency Accelerates Atherogenesis and Causes Proinflammatory Changes in the Endothelium in Apoe−/− Mice. *Endocrinology,* **152,** 236-46.

Deviterne, D & Desor, D 1990. Selective pup retrieving by mother rats: Sex and early development characteristics as discrimination factors. *Developmental Psychobiology,* **23,** 361-68.

Dickinson, H, Walker, D W, Wintour, E M & Moritz, K 2007. Maternal dexamethasone treatment at midgestation reduces nephron number and alters renal gene expression in the fetal spiny mouse. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **292,** R453-R61.

Dobson, P D & Kell, D B 2008. Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? *Nature Reviews Drug Discovery,* **7,** 205-20.

Dolinoy, D C, Weidman, J R, Waterland, R A & Jirtle, R L 2006. Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environmental Health Perspectives,* **114,** 567-72.

Doumas, D, Margolin, G & John, R S 1994. The intergenerational transmission of aggression across 3 generations. *Journal of Family Violence,* **9,** 157-75.

Drake, A J. 2004. *'The Intergenerational Consequences of Fetal Programming'.* Doctor of Philosophy Thesis, The University of Edinburgh.

Drake, A J & Liu, L 2010. Intergenerational transmission of programmed effects: public health consequences. *Trends in Endocrinology & Metabolism,* **21,** 206-13.

Drake, A J, Liu, L, Kerrigan, D, Meehan, R R & Seckl, J R 2011. Multigenerational programming in the glucocorticoid programmed rat is associated with generation-specific and parent of origin effects. *Epigenetics : official journal of the DNA Methylation Society,* **6,** 1334-43.

Drake, A J & Reynolds, R M 2010. Impact of maternal obesity on offspring obesity and cardiometabolic disease risk. *Reproduction,* **140,** 387-98.

Drake, A J, Walker, B R & Seckl, J R 2005. Intergenerational consequences of fetal programming by in utero exposure to glucocorticoids in rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **288,** R34-R38.

Dunn, G A & Bale, T L 2009. Maternal High-Fat Diet Promotes Body Length Increases and Insulin Insensitivity in Second-Generation Mice. *Endocrinology*.

Dunn, G A & Bale, T L 2011. Maternal high-fat diet effects on third-generation female body size via the paternal lineage. *Endocrinology,* **152,** 2228.

Edinger, A L 2007. Controlling cell growth and survival through regulated nutrient transporter expression. *Biochemical Journal,* **406,** 1-12.

Edwards, C A & Ferguson-Smith, A C 2007. Mechanisms regulating imprinted genes in clusters. *Current Opinion in Cell Biology,* **19,** 281-89.

Edwards, C R W, Benediktsson, R, Lindsay, R S & Seckl, J R 1996. 11[beta]-Hydroxysteroid dehydrogenases: Key enzymes in determining tissue-specific glucocorticoid effects. *Steroids,* **61,** 263-69.

Ehrenberg, H M, Mercer, B M & Catalano, P M 2004. The influence of obesity and diabetes on the prevalence of macrosomia. *American Journal of Obstetrics and Gynecology,* **191,** 964-68.

Elahi, M M, Cagampang, F R, Mukhtar, D, Anthony, F W, Ohri, S K & Hanson, M A 2009. Long-term maternal high-fat feeding from weaning through pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice. *British Journal of Nutrition,* **102,** 514-19.

Emanuel, I, Filakti, H, Alberman, E & Evans, S J W 1992. Intergenerational studies of human birthweight from the 1958 birth cohort. 1. Evidence for a multigenerational effect. *BJOG: An International Journal of Obstetrics & Gynaecology,* **99,** 67-74.

Emgård, M, Paradisi, M, Pirondi, S, Fernandez, M, Giardino, L & Calzà, L 2007.

Prenatal glucocorticoid exposure affects learning and vulnerability of cholinergic neurons. *Neurobiology of Aging,* **28,** 112-21.

Eriksson, J, Forsen, T, Tuomilehto, J, Osmond, C & Barker, D 2000. Fetal and Childhood Growth and Hypertension in Adult Life. *Hypertension,* **36,** 790-94.

Eriksson, J G, Forsén, T, Tuomilehto, J, Osmond, C & Barker, D J P 2001. Early growth and coronary heart disease in later life: longitudinal study. *British Medical Journal,* **322,** 949-53.

Eriksson, J G, Forsén, T, Tuomilehto, J, Osmond, C & Barker, D J P 2003. Early adiposity rebound in childhood and risk of Type 2 diabetes in adult life. *Diabetologia,* **46,** 190-94.

Eriksson, J G, Forsen, T, Tuomilehto, J, Winter, P D, Osmond, C & Barker, D J P 1999. Catch-up growth in childhood and death from coronary heart disease: longitudinal study. *British Medical Journal,* **318,** 427-31.

Esposito, D L, Li, Y, Cama, A & Quon, M J 2001. Tyr612 and Tyr632 in Human Insulin Receptor Substrate-1 Are Important for Full Activation of Insulin-Stimulated Phosphatidylinositol 3-Kinase Activity and Translocation of GLUT4 in Adipose Cells. *Endocrinology,* **142,** 2833-40.

Esquiliano, D R, Guo, W, Liang, L, Dikkes, P & Lopez, M F 2009. Placental Glycogen Stores are Increased in Mice with H19 Null Mutations but not in those with Insulin or IGF Type 1 Receptor Mutations. *Placenta,* **30,** 693-99.

Fang, J, Madhavan, S & Alderman, M 1999. The influence of maternal hypertension on low birth weight: differences among ethnic populations. *Ethnicity & disease,* **9,** 369.

Farmer, G, Russell, G, Hamilton-Nicol, D, Ogenbede, H, Ross, I, Pearson, D, Thom, H, Kerridge, D & Sutherland, H 1988. The influence of maternal glucose metabolism on fetal growth, development and morbidity in 917 singleton pregnancies in nondiabetic women. *Diabetologia,* **31,** 134-41.

Fejes, I, Koloszár, S, Szöllo˝si, J, Závaczki, Z & Pál, A 2005. Is semen quality affected by male body fat distribution? *Andrologia,* **37,** 155-59.

Felitti, V J, Anda, R F, Nordenberg, D, Williamson, D F, Spitz, A M, Edwards, V, Koss, M P & Marks, J S 1998. Relationship of Childhood Abuse and Household Dysfunction to Many of the Leading Causes of Death in Adults: The Adverse Childhood Experiences (ACE) Study. *American Journal of Preventive Medicine,* **14,** 245-58.

Firth, S M & Baxter, R C 2002. Cellular Actions of the Insulin-Like Growth Factor Binding Proteins. *Endocrine Reviews,* **23,** 824-54.

Fitzpatrick, G V, Pugacheva, E M, Shin, J-Y, Abdullaev, Z, Yang, Y, Khatod, K,

Lobanenkov, V V & Higgins, M J 2007. Allele-Specific Binding of CTCF to the Multipartite Imprinting Control Region KvDMR1. *Molecular and Cellular Biology,* **27,** 2636-47.

Fitzpatrick, G V, Soloway, P D & Higgins, M J 2002. Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. *Nature Genetics,* **32,** 426-31.

Forbes, K & Westwood, M 2008. The IGF Axis and Placental Function. *Hormone Research in Paediatrics,* **69,** 129-37.

Fossati, P & Prencipe, L 1982. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clinical Chemistry,* **28,** 2077-80.

Fowden, A L 2003. The Insulin-like Growth Factors and feto-placental Growth. *Placenta,* **24,** 803-12.

Fowden, A L & Forhead, A J 2004. Endocrine mechanisms of intrauterine programming. *Reproduction,* **127,** 515-26.

Fowden, A L, Sibley, C, Reik, W & Constancia, M 2006a. Imprinted genes, placental development and fetal growth. *Hormone Research in Paediatrics,* **65 Suppl 3,** 50-8.

Fowden, A L, Ward, J W, Wooding, F P B, Forhead, A J & Constancia, M 2006b. Programming placental nutrient transport capacity. *The Journal of Physiology,* **572,** 5-15.

Francis, D, Diorio, J, Liu, D & Meaney, M J 1999. Nongenomic Transmission Across Generations of Maternal Behavior and Stress Responses in the Rat. *Science,* **286,** 1155-58.

Frank, D, Fortino, W, Clark, L, Musalo, R, Wang, W, Saxena, A, Li, C-M, Reik, W, Ludwig, T & Tycko, B 2002. Placental overgrowth in mice lacking the imprinted gene Ipl. *Proceedings of the National Academy of Sciences of the United States of America,* **99,** 7490-95.

Franke, T F, Kaplan, D R & Cantley, L C 1997. PI3K: Downstream AKTion Blocks Apoptosis. *Cell,* **88,** 435-37.

Franke, T F, Yang, S-I, Chan, T O, Datta, K, Kazlauskas, A, Morrison, D K, Kaplan, D R & Tsichlis, P N 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell,* **81,** 727-36.

Franklin, T B, Russig, H, Weiss, I C, Gräff, J, Linder, N, Michalon, A, Vizi, S & Mansuy, I M 2010. Epigenetic Transmission of the Impact of Early Stress Across Generations. *Biological Psychiatry,* **68,** 408-15.

French, N P, Hagan, R, Evans, S F, Godfrey, M & Newnham, J P 1999. Repeated antenatal corticosteroids: Size at birth and subsequent development. *American Journal of Obstetrics and Gynecology,* **180,** 114-21.

Frye, C A & Orecki, Z A 2002. Prenatal Stress Alters Reproductive Responses of Rats in Behavioral Estrus and Paced Mating of Hormone-Primed Rats. *Hormones and Behavior,* **42,** 472-83.

Fryer, C J & Archer, T K 1998. Chromatin remodelling by the glucocorticoid receptor requires the BRG1 complex. *Nature,* **393,** 88-91.

Fu, Q, McKnight, R A, Yu, X, Wang, L, Callaway, C W & Lane, R H 2004. Uteroplacental insufficiency induces site-specific changes in histone H3 covalent modifications and affects DNA-histone H3 positioning in day 0 IUGR rat liver. *Physiological Genomics,* **20,** 108-16.

Fu, Q, Yu, X, Callaway, C W, Lane, R H & McKnight, R A 2009. Epigenetics: intrauterine growth retardation (IUGR) modifies the histone code along the rat hepatic IGF-1 gene. *The FASEB Journal,* **23,** 2438-49.

Funder, J 1993. Mineralocorticoids, glucocorticoids, receptors and response elements. *Science,* **259,** 1132-33.

Gale, C R, Javaid, M K, Robinson, S M, Law, C M, Godfrey, K M & Cooper, C 2007. Maternal Size in Pregnancy and Body Composition in Children. *The Journal of Clinical Endocrinology & Metabolism,* **92,** 3904-11.

Galtier-Dereure, F, Boegner, C & Bringer, J 2000. Obesity and pregnancy: complications and cost. *The American Journal of Clinical Nutrition,* **71,** 1242S-48.

Gama, L L & Johnson, R K 1993. Changes in ovulation rate, uterine capacity, uterine dimensions, and parity effects with selection for litter size in swine. *Journal of Animal Science,* **71,** 608-17.

Gardner, D S, Tingey, K, Van Bon, B W M, Ozanne, S E, Wilson, V, Dandrea, J, Keisler, D H, Stephenson, T & Symonds, M E 2005. Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **289,** R947-R54.

Gauguier, D, Bihoreau, M T, Ktorza, A, Berthault, M F & Picon, L 1990. Inheritance of diabetes mellitus as consequence of gestational hyperglycemia in rats. *Diabetes,* **39,** 734-39.

Ghanayem, B I, Bai, R, Kissling, G E, Travlos, G & Hoffler, U 2010. Diet-Induced Obesity in Male Mice Is Associated with Reduced Fertility and Potentiation of Acrylamide-Induced Reproductive Toxicity. *Biology of Reproduction,* **82,**

96-104.

Gilbert, S F 2005. Mechanisms for the environmental regulation of gene expression: ecological aspects of animal development. *Journal of Biosciences,* **30,** 65-74.

Globisch, D, Münzel, M, Müller, M, Michalakis, S, Wagner, M, Koch, S, Brückl, T, Biel, M & Carell, T 2010. Tissue Distribution of 5-Hydroxymethylcytosine and Search for Active Demethylation Intermediates. *PLoS ONE,* **5,** e15367.

Gluckman, P D, Morel, P C H, Ambler, G R, Breier, B H, Blair, H T & McCutcheon, S N 1992. Elevating Maternal Insulin-Like Growth Factor-I In Mice and Rats Alters the Pattern of Fetal Growth By Removing Maternal Constraint. *Journal of Endocrinology,* **134,** R1-3.

Godfrey, K M, Lillycrop, K A, Burdge, G C, Gluckman, P D & Hanson, M A 2007. Epigenetic Mechanisms and the Mismatch Concept of the Developmental Origins of Health and Disease. *Pediatric Research,* **61,** 5R-10R 10.1203/pdr.0b013e318045bedb.

Gould, G W & Holman, G D 1993. The glucose transporter family: structure, function and tissue-specific expression. *Biochemical Journal,* **295 (Pt 2),** 329-41.

Grandjean, V, Smith, J, Schofield, P N & Ferguson-Smith, A C 2000. Increased IGF-II protein affects p57kip2 expression in vivo and in vitro: Implications for Beckwith–Wiedemann syndrome. *Proceedings of the National Academy of Sciences,* **97,** 5279-84.

Gregorio, B M, Souza-Mello, V, Carvalho, J J, Mandarim-de-Lacerda, C A & Aguila, M B 2010. Maternal high-fat intake predisposes nonalcoholic fatty liver disease in C57BL/6 offspring. *American Journal of Obstetrics and Gynecology,* **203,** 495.e1-95.e8.

Group, H S C R 2010. Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) Study: associations with maternal body mass index. *BJOG: An International Journal of Obstetrics & Gynaecology,* **117,** 575-84.

Gual, P, Le Marchand-Brustel, Y & Tanti, J-F 2005. Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. *Biochimie,* **87,** 99-109.

Guerrero-Bosagna, C, Sabat, P & Valladares, L 2005. Environmental signaling and evolutionary change: can exposure of pregnant mammals to environmental estrogens lead to epigenetically induced evolutionary changes in embryos? *Evolution & Development,* **7,** 341-50.

Gundersen, H J & Jensen, E B 1987. The efficiency of systematic sampling in stereology and its prediction. *Journal of Microscopy,* **147,** 229-63.

Guo, F & Jen, K L C 1995. High-fat feeding during pregnancy and lactation affects

offspring metabolism in rats. *Physiology & Behavior,* **57,** 681-86.

Hadoke, P W F, Lindsay, R S, Seckl, J R, Walker, B R & Kenyon, C J 2006. Altered vascular contractility in adult female rats with hypertension programmed by prenatal glucocorticoid exposure. *Journal of Endocrinology,* **188,** 435-42.

Hahn, T, Barth, S, Graf, R, Engelmann, M, Beslagic, D, Reul, J M H M, Holsboer, F, Dohr, G & Desoye, G 1999. Placental Glucose Transporter Expression Is Regulated by Glucocorticoids. *The Journal of Clinical Endocrinology & Metabolism,* **84,** 1445-52.

Haig, D 1990. Brood Reduction and Optimal Parental Investment when Offspring Differ in Quality. *The American Naturalist,* **136,** 550-56.

Haig, D 1993. Genetic Conflicts in Human Pregnancy. *The Quarterly Review of Biology,* **68,** 495-532.

Haig, D 2000. The kinship theory of genomic imprinting. *Annual Review of Ecology and Systematics,* **31,** 9-32.

Hajkova, P, Erhardt, S, Lane, N, Haaf, T, El-Maarri, O, Reik, W, Walter, J & Surani, M A 2002. Epigenetic reprogramming in mouse primordial germ cells. *Mechanisms of Development,* **117,** 15-23.

Hales, B F, Crosman, K & Robaire, B 1992. Increased postimplantation loss and malformations among the F2 progeny of male rats chronically treated with cyclophosphamide. *Teratology,* **45,** 671-78.

Hales, C N, Barker, D J, Clark, P M, Cox, L J, Fall, C, Osmond, C & Winter, P D 1991. Fetal and infant growth and impaired glucose tolerance at age 64. *British Medical Journal,* **303,** 1019-22.

Hales, C N & Barker, D J P 1992. Type-2 (on-insulin-dependent) diabetes-mellitus - The thrifty phenotype hypothesis. *Diabetologia,* **35,** 595-601.

Hall, W 1975. Weaning and growth of artificially reared rats. *Science,* **190,** 1313-15.

Hammoud, A O, Wilde, N, Gibson, M, Parks, A, Carrell, D T & Meikle, A W 2008. Male obesity and alteration in sperm parameters. *Fertility and Sterility,* **90,** 2222-25.

Hammoud, S S, Nix, D A, Hammoud, A O, Gibson, M, Cairns, B R & Carrell, D T 2011. Genome-wide analysis identifies changes in histone retention and epigenetic modifications at developmental and imprinted gene loci in the sperm of infertile men. *Human Reproduction*.

Hammoud, S S, Nix, D A, Zhang, H, Purwar, J, Carrell, D T & Cairns, B R 2009. Distinctive chromatin in human sperm packages genes for embryo development. *Nature,* **460,** 473-78.

Han, J, Xu, J, Epstein, P & Liu, Y 2005. Long-term effect of maternal obesity on

pancreatic beta cells of offspring: reduced beta cell adaptation to high glucose and high-fat diet challenges in adult female mouse offspring. *Diabetologia,* **48,** 1810-18.

Harding, J 2001. The nutritional basis of the fetal origins of adult disease. *International Journal of Epidemiololgy,* **30,** 15-23.

Harrison, M & Langley-Evans, S C 2009. Intergenerational programming of impaired nephrogenesis and hypertension in rats following maternal protein restriction during pregnancy. *British Journal of Nutrition,* **101,** 1020-30.

Hatada, I, Ohashi, H, Fukushima, Y, Kaneko, Y, Inoue, M, Komoto, Y, Okada, A, Ohishi, S, Nabetani, A, Morisaki, H, Nakayama, M, Niikawa, N & Mukai, T 1996. An imprinted gene p57(KIP2) is mutated in Beckwith-Wiedemann syndrome. *Nature Genetics,* **14,** 171-73.

Hay, N & Sonenberg, N 2004. Upstream and downstream of mTOR. *Genes & Development,* **18,** 1926-45.

Haydak, M H 1970. Honey Bee Nutrition. *Annual Review of Entomology,* **15,** 143-56.

Hernández-Martínez, C, Arija, V, Balaguer, A, Cavallé, P & Canals, J 2008. Do the emotional states of pregnant women affect neonatal behaviour? *Early human development,* **84,** 745-50.

Herr, F, Liang, O D, Herrero, J, Lang, U, Preissner, K T, Han, V K M & Zygmunt, M 2003. Possible Angiogenic Roles of Insulin-Like Growth Factor II and Its Receptors in Uterine Vascular Adaptation to Pregnancy. *The Journal of Clinical Endocrinology & Metabolism,* **88,** 4811-17.

Herrenkohl, L 1979. Prenatal stress reduces fertility and fecundity in female offspring. *Science,* **206,** 1097-99.

Herrick, K, Phillips, D I W, Haselden, S, Shiell, A W, Campbell-Brown, M & Godfrey, K M 2003. Maternal Consumption of a High-Meat, Low-Carbohydrate Diet in Late Pregnancy: Relation to Adult Cortisol Concentrations in the Offspring. *The Journal of Clinical Endocrinology & Metabolism,* **88,** 3554-60.

Heslehurst, N, Ells, L J, Simpson, H, Batterham, A, Wilkinson, J & Summerbell, C D 2007. Trends in maternal obesity incidence rates, demographic predictors, and health inequalities in 36 821 women over a 15-year period. *BJOG: An International Journal of Obstetrics & Gynaecology,* **114,** 187-94.

Hewitt, D P, Mark, P J & Waddell, B J 2006. Glucocorticoids prevent the normal increase in placental vascular endothelial growth factor expression and placental vascularity during late pregnancy in the rat. *Endocrinology,* **147,**

5568-74.

Hoet, J J & Hanson, M A 1999. Intrauterine nutrition: its importance during critical periods for cardiovascular and endocrine development. *The Journal of Physiology,* **514,** 617-27.

Holson, R R, Gough, B, Sullivan, P, Badger, T & Sheehan, D M 1995. Prenatal dexamethasone or stress but not ACTH or corticosterone alter sexual behavior in male rats. *Neurotoxicology and Teratology,* **17,** 393-401.

Huang, W L, Beazley, L D, Quinlivan, J A, Evans, S F, Newnham, J P & Dunlop, S A 1999. Effect of Corticosteroids on Brain Growth in Fetal Sheep. *Obstetrics & Gynecology,* **94,** 213-18.

Humbel, R E 1990. Insulin-like growth factors I and II. *European Journal of Biochemistry,* **190,** 445-62.

Hundertmark, S, Buhler, H, Fromm, M, Kruner-Gareis, B, Kruner, M, Ragosch, V, Kuhlmann, K & Seckl, J R 2001. Ontogeny of 11beta-hydroxysteroid dehydrogenase: activity in the placenta, kidney, colon of fetal rats and rabbits. *Hormone and Metabolic Research,* **33,** 78-83.

Hunter, G L 1956. The maternal influence on size in sheep. *The Journal of Agricultural Science,* **48,** 36-60.

Hwa, V, Oh, Y & Rosenfeld, R G 1999. The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocrine Reviews,* **20,** 761-87.

Hyppönen, E, Smith, G D & Power, C 2003. Parental diabetes and birth weight of offspring: intergenerational cohort study. *British Medical Journal,* **326,** 19-20.

Ibanez, L, Potau, N, Enriquez, G & De Zegher, F 2000. Reduced Uterine and Ovarian Size in Adolescent Girls Born Small for Gestational Age. *Pediatric Research,* **47,** 575-77.

Inawaka, K, Kawabe, M, Takahashi, S, Doi, Y, Tomigahara, Y, Tarui, H, Abe, J, Kawamura, S & Shirai, T 2009. Maternal exposure to anti-androgenic compounds, vinclozolin, flutamide and procymidone, has no effects on spermatogenesis and DNA methylation in male rats of subsequent generations. *Toxicology and Applied Pharmacology,* **237,** 178-87.

Jansson, T 2001. Amino Acid Transporters in the Human Placenta. *Pediatric Research,* **49,** 141-47.

Jansson, T, Cowley, E & Illsley, N 1995. Cellular localization of glucose transporter messenger RNA in human placenta. *Reproduction, Fertility and Development,* **7,** 1425-30.

Jansson, T, Wennergren, M & Illsley, N P 1993. Glucose transporter protein

expression in human placenta throughout gestation and in intrauterine growth retardation. *The Journal of Clinical Endocrinology & Metabolism,* **77,** 1554-62.

Jansson, T, Ylvén, K, Wennergren, M & Powell, T L 2002. Glucose Transport and System A Activity in Syncytiotrophoblast Microvillous and Basal Plasma Membranes in Intrauterine Growth Restriction. *Placenta,* **23,** 392-99.

Jennings, B J, Ozanne, S E, Dorling, M W & Hales, C N 1999. Early growth determines longevity in male rats and may be related to telomere shortening in the kidney. *FEBS Letters,* **448,** 4-8.

Jimenez-Chillaron, J C, Isganaitis, E, Charalambous, M, Gesta, S, Pentinat-Pelegrin, T, Faucette, R R, Otis, J P, Chow, A, Diaz, R, Ferguson-Smith, A & Patti, M-E 2009. Intergenerational Transmission of Glucose Intolerance and Obesity by In Utero Undernutrition in Mice. *Diabetes,* **58,** 460-68.

Jobe, A H, Newnham, J, Willet, K, Sly, P & Ikegami, M 1998a. Fetal Versus Maternal and Gestational Age Effects of Repetitive Antenatal Glucocorticoids. *Pediatrics,* **102,** 1116-25.

Jobe, A H, Wada, N, Berry, L M, Ikegami, M & Ervin, M G 1998b. Single and repetitive maternal glucocorticoid exposures reduce fetal growth in sheep. *American Journal of Obstetrics and Gynecology,* **178,** 880-85.

Jones, H N, Powell, T L & Jansson, T 2007. Regulation of Placental Nutrient Transport – A Review. *Placenta,* **28,** 763-74.

Jones, H N, Woollett, L A, Barbour, N, Prasad, P D, Powell, T L & Jansson, T 2009. High-fat diet before and during pregnancy causes marked up-regulation of placental nutrient transport and fetal overgrowth in C57/BL6 mice. *The FASEB Journal,* **23,** 271-78.

Jones, J I & Clemmons, D R 1995. Insulin-Like Growth Factors and Their Binding Proteins: Biological Actions. *Endocrine Reviews,* **16,** 3-34.

Jungheim, E S, Schoeller, E L, Marquard, K L, Louden, E D, Schaffer, J E & Moley, K H 2010. Diet-Induced Obesity Model: Abnormal Oocytes and Persistent Growth Abnormalities in the Offspring. *Endocrinology,* **151,** 4039-46.

Kaati, G, Bygren, L O & Edvinsson, S 2002. Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. *European Journal of Human Genetics,* **10,** 682-88.

Kaati, G, Bygren, L O, Pembrey, M & Sjostrom, M 2007. Transgenerational response to nutrition, early life circumstances and longevity. *European Journal of Human Genetics,* **15,** 784-90.

Kafri, T, Ariel, M, Brandeis, M, Shemer, R, Urven, L, McCarrey, J, Cedar, H &

Razin, A 1992. Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. *Genes & Development,* **6,** 705-14.

Kalaria, R N, Gravina, S A, Schmidley, J W, Perry, G & Harik, S I 1988. The glucose transporter of the human brain and blood-brain barrier. *Annals of Neurology,* **24,** 757-64.

Kampman, K A, Ramsay, T G & White, M E 1993. Developmental changes in hepatic IGF-2 and IGFBP-2 mRNA levels in intrauterine growth-retarded and control swine. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* **104,** 415-21.

Kaneko-Ishino, T, Kuroiwa, Y, Miyoshi, N, Kohda, T, Suzuki, R, Yokoyama, M, Viville, S, Barton, S C, Ishino, F & Surani, M A 1995. Peg1/MEST imprinted gene on chromosome-6 identified by cDNA subtraction hybridization. *Nature Genetics,* **11,** 52-59.

Kannisto, V, Christensen, K & Vaupel, J W 1997. No Increased Mortality in Later Life for Cohorts Bom during Famine. *American Journal of Epidemiology,* **145,** 987-94.

Kauffman, K S, Seidler, F J & Slotkin, T A 1994. Prenatal dexamethasone exposure causes loss of neonatal hypoxia tolerance: cellular mechanisms. *Pediatric Research,* **35,** 515-22.

Kaufmann, P, Black, S & Huppertz, B 2003. Endovascular Trophoblast Invasion: Implications for the Pathogenesis of Intrauterine Growth Retardation and Preeclampsia. *Biology of Reproduction,* **69,** 1-7.

Khamzina, L, Gruppuso, P A & Wands, J R 2003. Insulin signaling through insulin receptor substrate 1 and 2 in normal liver development. *Gastroenterology,* **125,** 572-85.

Khan, I Y, Dekou, V, Douglas, G, Jensen, R, Hanson, M A, Poston, L & Taylor, P D 2005. A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **288,** R127-33.

Khan, I Y, Taylor, P D, Dekou, V, Seed, P T, Lakasing, L, Graham, D, Dominiczak, A F, Hanson, M A & Poston, L 2003. Gender-Linked Hypertension in Offspring of Lard-Fed Pregnant Rats. *Hypertension,* **41,** 168-75.

Klebanoff, M A, Graubard, B I, Kessel, S S & Berendes, H W 1984. Low Birth Weight Across Generations. *JAMA: The Journal of the American Medical Association,* **252,** 2423-27.

Koenen, S V, Mecenas, C A, Smith, G S, Jenkins, S & Nathanielsz, P W 2002. Effects of maternal betamethasone administration on fetal and maternal blood

pressure and heart rate in the baboon at 0.7 of gestation. *American Journal of Obstetrics and Gynecology,* **186,** 812-17.

Kouzarides, T 2002. Histone methylation in transcriptional control. *Current Opinion in Genetics & Development,* **12,** 198-209.

Kriaucionis, S & Heintz, N 2009. The Nuclear DNA Base 5-Hydroxymethylcytosine Is Present in Purkinje Neurons and the Brain. *Science,* **324,** 929-30.

Krozowski, Z, MaGuire, J A, Stein-Oakley, A N, Dowling, J, Smith, R E & Andrews, R K 1995. Immunohistochemical localization of the 11 beta-hydroxysteroid dehydrogenase type II enzyme in human kidney and placenta. *The Journal of Clinical Endocrinology & Metabolism,* **80,** 2203-9.

Kuznetsova, T, Staessen, J A, Olszanecka, A, Ryabikov, A, Stolarz, K, Malyutina, S, Fagard, R, Kawecka-Jaszcz, K, Nikitin, Y & Investigators, E 2003. Maternal and paternal influences on left ventricular mass of offspring. *Hypertension,* **41,** 69-74.

Labayen, I, Ruiz, J R, Ortega, F B, Loit, H-M, Harro, J, Veidebaum, T & Sjöström, M 2010. Intergenerational Cardiovascular Disease Risk Factors Involve Both Maternal and Paternal BMI. *Diabetes Care,* **33,** 894-900.

LaBorde, J B, Hansen, D K, Young, J F, Sheehan, D M & Holson, R R 1992. Prenatal dexamethasone exposure in rats: Effects of dose, age at exposure, and drug-induced hypophagia on malformations and fetal organ weights. *Fundamental and Applied Toxicology,* **19,** 545-54.

Lalancette, C, Miller, D, Li, Y & Krawetz, S A 2008. Paternal contributions: new functional insights for spermatozoal RNA. *Journal of Cellular Biochemistry,* **104,** 1570-79.

Langdown, M L & Sugden, M C 2001. Enhanced placental GLUT1 and GLUT3 expression in dexamethasone-induced fetal growth retardation. *Molecular and Cellular Endocrinology,* **185,** 109-17.

Langley-Evans, S C 1997. Hypertension induced by foetal exposure to a maternal low-protein diet, in the rat, is prevented by pharmacological blockade of maternal glucocorticoid synthesis. *Journal of Hypertension,* **15,** 537-44.

Langley-Evans, S C, Phillips, G J, Benediktsson, R, Gardner, D S, Edwards, C R W, Jackson, A A & Seckl, J R 1996. Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat. *Placenta,* **17,** 169-72.

Langley-Evans, S C, Welham, S J M & Jackson, A A 1999. Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. *Life Sciences,* **64,** 965-74.

Langley Evans, S F & Jackson, A A 1994. Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. *Clinical Science,* **86,** 217-22.

Langley, S C, Browne, R F & Jackson, A A 1994. Altered glucose tolerance in rats exposed to maternal low protein diets in utero. *Comparative Biochemistry and Physiology Part A: Physiology,* **109,** 223-29.

Laplante, D P, Barr, R G, Brunet, A, Fort, G G D, Meaney, M L, Saucier, J-F, Zelazo, P R & King, S 2004. Stress During Pregnancy Affects General Intellectual and Language Functioning in Human Toddlers. *Pediatric Research,* **56,** 400-10.

Laplante, D P, Brunet, A, Schmitz, N, Ciampi, A & King, S 2008. Project Ice Storm: Prenatal Maternal Stress Affects Cognitive and Linguistic Functioning in 5½-Year-Old Children. *Journal of the American Academy of Child and Adolescent Psychiatry,* **47,** 1063-72.

Lau, M M, Stewart, C E, Liu, Z, Bhatt, H, Rotwein, P & Stewart, C L 1994. Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes & Development,* **8,** 2953-63.

Launer, L J, Hofman, A & Grobbee, D E 1993. Relation between birth weight and blood pressure: longitudinal study of infants and children. *British Medical Journal,* **307,** 1451-54.

Laychock, S G, Vadlamudi, S & Patel, M S 1995. Neonatal rat dietary carbohydrate affects pancreatic islet insulin secretion in adults and progeny. *American Journal of Physiology - Endocrinology and Metabolism,* **269,** E739-E44.

Lee, J, Inoue, K, Ono, R, Ogonuki, N, Kohda, T, Kaneko-Ishino, T, Ogura, A & Ishino, F 2002. Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. *Development,* **129,** 1807-17.

Lefebvre, L, Viville, S, Barton, S C, Ishino, F, Keverne, E B & Surani, M A 1998. Abnormal maternal behaviour and growth retardation associated with loss of the imprinted gene Mest. *Nature Genetics,* **20,** 163-9.

Lenzen, S & Bailey, C J 1984. Thyroid Hormones, Gonadal and Adrenocortical Steroids and the Function of the Islets of Langerhans. *Endocrine Reviews,* **5,** 411-34.

Leonce, J, Brockton, N, Robinson, S, Venkatesan, S, Bannister, P, Raman, V, Murphy, K, Parker, K, Pavitt, D, Teoh, T G, Regan, L, Burchell, A, Steer, P & Johnston, D G 2006. Glucose Production in the Human Placenta. *Placenta,* **27,** 103-08.

Leung, K & Munck, A 1975. Peripheral Actions of Glucocorticoids. *Annual Review of Physiology,* **37,** 245-72.

Levin, B E & Govek, E 1998. Gestational obesity accentuates obesity in obesity-prone progeny. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **275,** R1374-79.

Levine, S, Haltmeyer, G C, Karas, G G & Denenberg, V H 1967. Physiological and behavioral effects of infantile stimulation. *Physiology & Behavior,* **2,** 55-59.

Levitt, N S, Lindsay, R S, Holmes, M C & Seckl, J R 1996. Dexamethasone in the Last Week of Pregnancy Attenuates Hippocampal Glucocorticoid Receptor Gene Expression and Elevates Blood Pressure in the Adult Offspring in the Rat. *Neuroendocrinology,* **64,** 412-18.

Li, J, Saunders, J C, Fowden, A L, Dauncey, M J & Gilmour, R S 1998. Transcriptional Regulation of Insulin-like Growth Factor-II Gene Expression by Cortisol in Fetal Sheep during Late Gestation. *Journal of Biological Chemistry,* **273,** 10586-93.

Liang, C, DeCourcy, K & Prater, M R 2010. High-saturated-fat diet induces gestational diabetes and placental vasculopathy in C57BL/6 mice. *Metabolism,* **59,** 943-50.

Liang, C, Oest, M E & Prater, M R 2009. Intrauterine exposure to high saturated fat diet elevates risk of adult-onset chronic diseases in C57BL/6 mice. *Birth Defects Research Part B: Developmental and Reproductive Toxicology,* **86,** 377-84.

Lillycrop, K A, Phillips, E S, Jackson, A A, Hanson, M A & Burdge, G C 2005. Dietary Protein Restriction of Pregnant Rats Induces and Folic Acid Supplementation Prevents Epigenetic Modification of Hepatic Gene Expression in the Offspring. *The Journal of Nutrition,* **135,** 1382-86.

Lillycrop, K A, Phillips, E S, Torrens, C, Hanson, M A, Jackson, A A & Burdge, G C 2008. Feeding pregnant rats a protein-restricted diet persistently alters the methylation of specific cytosines in the hepatic PPAR alpha promoter of the offspring. *British Journal of Nutrition,* **100,** 278-82.

Lillycrop, K A, Slater-Jefferies, J L, Hanson, M A, Godfrey, K M, Jackson, A A & Burdge, G C 2007. Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and changes in histone modifications. *British Journal of Nutrition,* **97,** 1064-73.

Lindsay, R S, Lindsay, R M, Waddell, B J & Seckl, J R 1996. Prenatal glucocorticoid exposure leads to offspring hyperglycaemia in the rat: studies with the 11 b -hydroxysteroid dehydrogenase inhibitor carbenoxolone. *Diabetologia,* **39,**

1299-305.

Lissau, I & Sorensen, T I A 1994. Parental neglect during childhood and increased risk of obesity in young adulthood. *The Lancet,* **343,** 324-27.

Louvi, A, Accili, D & Efstratiadis, A 1997. Growth-Promoting Interaction of IGF-II with the Insulin Receptor during Mouse Embryonic Development. *Developmental Biology,* **189,** 33-48.

Lu, N Z & Cidlowski, J A 2004. The Origin and Functions of Multiple Human Glucocorticoid Receptor Isoforms. *Annals of the New York Academy of Sciences,* **1024,** 102-23.

Lumey, L H 1992. Decreased birthweights in infants after maternal in utero exposure to the Dutch famine of 1944-1945. *Paediatric Perinatal Epidemiology,* **6,** 240-53.

Malandro, M S, Beveridge, M J, Kilberg, M S & Novak, D A 1996. Effect of low-protein diet-induced intrauterine growth retardation on rat placental amino acid transport. *American Journal of Physiology - Cell Physiology,* **271,** C295-303.

Margerison Zilko, C E, Rehkopf, D & Abrams, B 2010. Association of maternal gestational weight gain with short- and long-term maternal and child health outcomes. *American Journal of Obstetrics and Gynecology,* **202,** 574.e1-74.e8.

Massiera, F, Barbry, P, Guesnet, P, Joly, A, Luquet, S, Moreilhon-Brest, C, Mohsen-Kanson, T, Amri, E-Z & Ailhaud, G 2010. A Western-like fat diet is sufficient to induce a gradual enhancement in fat mass over generations. *Journal of Lipid Research,* **51,** 2352-61.

Mattick, J S & Makunin, I V 2006. Non-coding RNA. *Human Molecular Genetics,* **15,** R17-R29.

Mayer, W, Hemberger, M, Frank, H-G, Gr mer, R, Winterhager, E, Kaufmann, P & Fundele, R 2000a. Expression of the imprinted genes MEST/Mest in human and murine placenta suggests a role in angiogenesis. *Developmental Dynamics,* **217,** 1-10.

Mayer, W, Niveleau, A, Walter, J, Fundele, R & Haaf, T 2000b. Embryogenesis: Demethylation of the zygotic paternal genome. *Nature,* **403,** 501-02.

McCauley, J, Kern, D E, Kolodner, K, Dill, L, Schroeder, A F, DeChant, H K, Ryden, J, Derogatis, L R & Bass, E B 1997. Clinical Characteristics of Women With a History of Childhood Abuse. *JAMA: The Journal of the American Medical Association,* **277,** 1362-68.

McCowan, L M E, Buist, R G, North, R A & Gamble, G 1996. Perinatal morbidity in

chronic hypertension. *BJOG: An International Journal of Obstetrics & Gynaecology,* **103,** 123-29.

McDonald, T J, Franko, K L, Brown, J M, Jenkins, S L, Nathanielsz, P W & Nijland, M J 2003. Betamethasone in the Last Week of Pregnancy Causes Fetal Growth Rtardation but Not Adult Hypertension in Rats. *Journal of the Society for Gynecologic Investigation,* **10,** 469-73.

McEwen, B S, De Kloet, E R & Rostene, W 1986. Adrenal steroid receptors and actions in the nervous system. *Physiological Reviews,* **66,** 1121-88.

McGowan, M, Artiss, J, Strandbergh, D & Zak, B 1983. A peroxidase-coupled method for the colorimetric determination of serum triglycerides. *Clinical Chemistry,* **29,** 538-42.

McKinnon, T, Chakraborty, C, Gleeson, L M, Chidiac, P & Lala, P K 2001. Stimulation of Human Extravillous Trophoblast Migration by IGF-II Is Mediated by IGF Type 2 Receptor Involving Inhibitory G Protein(s) and Phosphorylation of MAPK. *The Journal of Clinical Endocrinology & Metabolism,* **86,** 3665-74.

McMillen, I C, Adam, C L & Mühlhäusler, B S 2005. Early origins of obesity: programming the appetite regulatory system. *The Journal of Physiology,* **565,** 9-17.

McMinn, J, Wei, M, Sadovsky, Y, Thaker, H M & Tycko, B 2006. Imprinting of PEG1/MEST Isoform 2 in Human Placenta. *Placenta,* **27,** 119-26.

Meaney, M J 2001. Maternal care, gene expression, and the transmission of individual differences in stress reactivity accross generations. *Annual Review of Neuroscience,* **24,** 1161-92.

Monk, D, Arnaud, P, Frost, J, Hills, F A, Stanier, P, Feil, R & Moore, G E 2009. Reciprocal imprinting of human GRB10 in placental trophoblast and brain: evolutionary conservation of reversed allelic expression. *Human Molecular Genetics,* **18,** 3066-74.

Moore, T, Constancia, M, Zubair, M, Bailleul, B, Feil, R, Sasaki, H & Reik, W 1997. Multiple imprinted sense and antisense transcripts, differential methylation and tandem repeats in a putative imprinting control region upstream of mouse Igf2. *Proceedings of the National Academy of Sciences of the United States of America,* **94,** 12509-14.

Moore, T & Haig, D 1991. Genomic imprinting in mammalian development: a parental tug-of-war. *Trends in Genetics,* **7,** 45-49.

Morgan, H D, Sutherland, H G E, Martin, D I K & Whitelaw, E 1999. Epigenetic inheritance at the agouti locus in the mouse. *Nature Genetics,* **23,** 314-18.

Moss, T J M, Sloboda, D M, Gurrin, L C, Harding, R, Challis, J R G & Newnham, J P 2001. Programming effects in sheep of prenatal growth restriction and glucocorticoid exposure. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **281,** R960-R70.

Mueller, B R & Bale, T L 2008a. Sex-specific programming of offspring emotionality after stress early in pregnancy. *Journal of Neuroscience,* **28,** 9055-65.

Mueller, B R & Bale, T L 2008b. Sex-Specific Programming of Offspring Emotionality after Stress Early in Pregnancy. *The Journal of Neuroscience,* **28,** 9055-65.

Muneoka, K, Mikuni, M, Ogawa, T, Kitera, K, Kamei, K, Takigawa, M & Takahashi, K 1997. Prenatal dexamethasone exposure alters brain monoamine metabolism and adrenocortical response in rat offspring. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **273,** R1669-R75.

Murthy, K K & Moya, F R 1994. Effect of betamethasone on maternal, fetal and neonatal rat cellular immunity. *Early Human Development,* **36,** 1-11.

Navé, B T, Ouwens, M, Withers, D J, Alessi, D R & Shepherd, P R 1999. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochemical Journal,* **344,** 427-31.

Neria, Y, Gross, R, Olfson, M, Gameroff, M J, Wickramaratne, P, Das, A, Pilowsky, D, Feder, A, Blanco, C, Marshall, R D, Lantigua, R, Shea, S & Weissman, M M 2006. Posttraumatic stress disorder in primary care one year after the 9/11 attacks. *General hospital psychiatry,* **28,** 213-22.

Ng, S-F, Lin, R C Y, Laybutt, D R, Barres, R, Owens, J A & Morris, M J 2010. Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. *Nature,* **467,** 963-66.

Nilsson, E E, Anway, M D, Stanfield, J & Skinner, M K 2008. Transgenerational epigenetic effects of the endocrine disruptor vinclozolin on pregnancies and female adult onset disease. *Reproduction,* **135,** 713-21.

Nivoit, P, Morens, C, Van Assche, F, Jansen, E, Poston, L, Remacle, C & Reusens, B 2009. Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. *Diabetologia,* **52,** 1133-42.

Nohr, E A, Vaeth, M, Baker, J L, Sørensen, T I, Olsen, J & Rasmussen, K M 2008. Combined associations of prepregnancy body mass index and gestational

weight gain with the outcome of pregnancy. *The American Journal of Clinical Nutrition,* **87,** 1750-59.

Noma, K-i, Allis, C D & Grewal, S I S 2001. Transitions in Distinct Histone H3 Methylation Patterns at the Heterochromatin Domain Boundaries. *Science,* **293,** 1150-55.

Novak, D, Lehman, M, Bernstein, H, Beveridge, M & Cramer, S 2006. SNAT Expression in Rat Placenta. *Placenta,* **27,** 510-16.

Nyirenda, M J, Lindsay, R S, Kenyon, C J, Burchell, A & Seckl, J R 1998. Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. *The Journal of Clinical Investigation,* **101,** 2174-81.

Nyirenda, M J, Welberg, L A & Seckl, J R 2001. Programming hyperglycaemia in the rat through prenatal exposure to glucocorticoids-fetal effect or maternal influence? *Journal of Endocrinology,* **170,** 653-60.

O'Regan, D, Kenyon, C J, Seckl, J R & Holmes, M C 2004. Glucocorticoid exposure in late gestation in the rat permanently programs gender-specific differences in adult cardiovascular and metabolic physiology. *American Journal of Physiology - Endocrinology and Metabolism,* **287,** E863-E70.

O'Regan, D, Kenyon, C J, Seckl, J R & Holmes, M C 2008. Prenatal dexamethasone 'programmes' hypotension, but stress-induced hypertension in adult offspring. *Journal of Endocrinology,* **196,** 343-52.

Odell, C D, Kotelchuck, M, Chetty, V K, Fowler, J, Stubblefield, P G, Orejuela, M & Jack, B W 2006. Maternal Hypertension as a Risk Factor for Low Birth Weight Infants: Comparison of Haitian and African-American Women. *Maternal and Child Health Journal,* **10,** 39-46.

Ogden, C L, Carroll, M D, Curtin, L R, McDowell, M A, Tabak, C J & Flegal, K M 2006. Prevalence of Overweight and Obesity in the United States, 1999-2004. *JAMA: The Journal of the American Medical Association,* **295,** 1549-55.

Oliva, R & Dixon, G H 1991. Vertebrate protamine genes and the histone-to-protamine replacement reaction. *Progress in nucleic acid research and molecular biology,* **40,** 25-94.

Oliva, R & Mateo, S 2011. Medical Implications of Sperm Nuclear Quality. In: Rousseaux, S & Khochbin, S (eds.) *Epigenetics and Human Reproduction.* Springer Berlin Heidelberg.

Ong, K K L, Ahmed, M L, Emmett, P M, Preece, M A & Dunger, D B 2000. Association between postnatal catch-up growth and obesity in childhood:

prospective cohort study. *British Medical Journal,* **320,** 967-71.

Osmond, C, Barker, D J, Winter, P D, Fall, C H & Simmonds, S J 1993. Early growth and death from cardiovascular disease in women. *British Medical Journal,* **307,** 1519-24.

Ozanne, S E & Hales, C N 2002. Early programming of glucose-insulin metabolism. *Trends in Endocrinology and Metabolism,* **13,** 368-73.

Ozanne, S E & Hales, C N 2004. Lifespan: Catch-up growth and obesity in male mice. *Nature,* **427,** 411-12.

Ozanne, S E, Smith, G D, Tikerpae, J & Hales, C N 1996. Altered regulation of hepatic glucose output in the male offspring of protein-malnourished rat dams. *American Journal of Physiology - Endocrinology and Metabolism,* **270,** E559-E64.

Ozmen, A, Unek, G, Kipmen-Korgun, D & Korgun, E 2011. The expression of Akt and ERK1/2 proteins decreased in dexamethasone-induced intrauterine growth restricted rat placental development. *Journal of Molecular Histology,* **42,** 237-49.

Painter, R C, Roseboom, T J & Bleker, O P 2005. Prenatal exposure to the Dutch famine and disease in later life: An overview. *Reproductive Toxicology,* **20,** 345-52.

Park, J H, Stoffers, D A, Nicholls, R D & Simmons, R A 2008. Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1. *The Journal of Clinical Investigation,* **118,** 2316-24.

Park, S, Sappenfield, W, Bish, C, Salihu, H, Goodman, D & Bensyl, D 2011. Assessment of the Institute of Medicine Recommendations for Weight Gain During Pregnancy: Florida, 2004–2007. *Maternal and Child Health Journal,* **15,** 289-301.

Parker, P J, Caudwell, F B & Cohen, P 1983. Glycogen Synthase from Rabbit Skeletal Muscle; Effect of Insulin on the State of phosphorylation of the Seven Phosphoserine Residues in vivo. *European Journal of Biochemistry,* **130,** 227-34.

Patel, M S & Srinivasan, M 2002. Metabolic Programming: Causes and Consequences. *Journal of Biological Chemistry,* **277,** 1629-32.

Pathak, S, Saxena, M, D'Souza, R & Balasinor, N 2010. Disrupted imprinting status at the H19 differentially methylated region is associated with the resorbed embryo phenotype in rats. *Reproduction, Fertility and Development,* **22,** 939-48.

Peel, S & Bulmer, D 1977. Proliferation and differentiation of trophoblast in the establishment of the rat chorio-allantoic placenta. *Journal of Anatomy,* **124,** 675-87.

Peixoto-Silva, N, Frantz, E D C, Mandarim-de-Lacerda, C A & Pinheiro-Mulder, A 2011. Maternal protein restriction in mice causes adverse metabolic and hypothalamic effects in the F1 and F2 generations. *British Journal of Nutrition,* **FirstView,** 1-10.

Pembrey, M E, Bygren, L O, Kaati, G, Edvinsson, S, Northstone, K, Sjostrom, M & Golding, J 2006. Sex-specific, male-line transgenerational responses in humans. *European Journal of Human Genetics,* **14,** 159-66.

Penn, N W, Suwalski, R, O'Riley, C, Bojanowski, K & Yura, R 1972. The presence of 5-hydroxymethylcytosine in animal deoxyribonucleic acid. *Biochemical Journal,* **126,** 781-90.

Pentinat, T, Ramon-Krauel, M, Cebria, J, Diaz, R & Jimenez-Chillaron, J C 2010. Transgenerational Inheritance of Glucose Intolerance in a Mouse Model of Neonatal Overnutrition. *Endocrinology,* **151,** 5617-23.

Pepe, G J, Burch, M G & Albrecht, E D 1999. Expression of the 11[beta]-Hydroxysteroid Dehydrogenase Types 1 and 2 Proteins in Human and Baboon Placental Syncytiotrophoblast. *Placenta,* **20,** 575-82.

Persson, E & Jansson, T 1992. Low birth weight is associated with elevated adult blood pressure in the chronically catheterized guinea-pig. *Acta Physiologica Scandinavica,* **145,** 195-96.

Pettigrew, R & Hamilton-Fairley, D 1997. Obsesity and female reproductive function. *British Medical Bulletin,* **53,** 341.

Piffer, R C, Garcia, P C & Pereira, O C M 2009. Adult partner preference and sexual behavior of male rats exposed prenatally to betamethasone. *Physiology & Behavior,* **98,** 163-67.

Piffer, R C & Pereira, O C M 2004. Reproductive aspects in female rats exposed prenatally to hydrocortisone. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* **139,** 11-16.

Pladys, P, Sennlaub, F, Brault, S, Checchin, D, Lahaie, I, Le, N L O, Bibeau, K, Cambonie, G, Abran, D, Brochu, M, Thibault, G, Hardy, P, Chemtob, S & Nuyt, A M 2005. Microvascular rarefaction and decreased angiogenesis in rats with fetal programming of hypertension associated with exposure to a low-protein diet in utero. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **289,** R1580-88.

Price, W A, Stiles, A D, Moats-Staats, B M & D'Ercole, A J 1992. Gene expression of

insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. *Endocrinology,* **130,** 1424-32.

Purcell, R H, Sun, B, Pass, L L, Power, M L, Moran, T H & Tamashiro, K L K 2011. Maternal stress and high-fat diet effect on maternal behavior, milk composition, and pup ingestive behavior. *Physiology & Behavior,* **104,** 474-79.

Rana, T M 2007. Illuminating the silence: understanding the structure and function of small RNAs. *Nature Reviews Molecular Cell Biology,* **8,** 23-36.

Randhawa, R & Cohen, P 2005. The role of the insulin-like growth factor system in prenatal growth. *Molecular Genetics and Metabolism,* **86,** 84-90.

Rassoulzadegan, M, Grandjean, V, Gounon, P, Vincent, S, Gillot, I & Cuzin, F 2006. RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. *Nature,* **441,** 469-74.

Ravelli, A C J, Van der Meulen, J H P, Michels, R P J, Osmond, C, Barker, D J P, Hales, C N & Bleker, O P 1998. Glucose tolerance in adults after prenatal exposure to famine. *The Lancet,* **351,** 173.

Ravelli, G-P, Stein, Z A & Susser, M W 1976. Obesity in Young Men after Famine Exposure in Utero and Early Infancy. *New England Journal of Medicine,* **295,** 349-53.

Reik, W, Constancia, M, Fowden, A, Anderson, N, Dean, W, Ferguson-Smith, A, Tycko, B & Sibley, C. Year. Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. *In:* 49th Annual Meeting of the Society-for-Gynecologic-Investigation, Mar 20-23 2002 Los Angeles, California. Cambridge Univ Press, 35-44.

Reik, W, Dean, W & Walter, J 2001. Epigenetic Reprogramming in Mammalian Development. *Science,* **293,** 1089-93.

Reik, W & Walter, J 2001. Genomic imprinting: Parental influence on the genome. *Nature Reviews Genetics,* **2,** 21-32.

Reinisch, J, Simon, N, Karow, W & Gandelman, R 1978. Prenatal exposure to prednisone in humans and animals retards intrauterine growth. *Science,* **202,** 436-38.

Rennie, K L & Jebb, S A 2005. Prevalence of obesity in Great Britain. *Obesity Reviews,* **6,** 11-12.

Reynolds, R M, Godfrey, K M, Barker, M, Osmond, C & Phillips, D I W 2007. Stress Responsiveness in Adult Life: Influence of Mother's Diet in Late Pregnancy. *The Journal of Clinical Endocrinology & Metabolism,* **92,**

2208-10.

Reynolds, R M, Osmond, C, Phillips, D I W & Godfrey, K M 2010. Maternal BMI, Parity, and Pregnancy Weight Gain: Influences on Offspring Adiposity in Young Adulthood. *The Journal of Clinical Endocrinology & Metabolism,* **95,** 5365-69.

Rich-Edwards, J W, Colditz, G A, Stampfer, M J, Willett, W C, Gillman, M W, Hennekens, C H, Speizer, F E & Manson, J E 1999. Birthweight and the Risk for Type 2 Diabetes Mellitus in Adult Women. *Annals of Internal Medicine,* **130,** 278-84.

Rich-Edwards, J W, Stampfer, M J, Manson, J E, Rosner, B, Hankinson, S E, Colditz, G A, Hennekens, C H & Willet, W C 1997. Birth weight and risk of cardiovascular disease in a cohort of women followed up since 1976. *British Medical Journal,* **315,** 396-400.

Richards, E J 2006. Inherited epigenetic variation - revisiting soft inheritance. *Nature Reviews Genetics,* **7,** 395-401.

Rignano, E 1911, *Upon the inheritance of acquired characters: a hypothesis of heredity, development, and assimilation,* Chicago, The Open Court Publication Company.

Rinkenberger, J & Werb, Z 2000. The labyrinthine placenta. *Nature Genetics,* **25,** 248-50.

Roland, B L & Funder, J W 1996. Localization of 11beta-hydroxysteroid dehydrogenase type 2 in rat tissues: in situ studies. *Endocrinology,* **137,** 1123-8.

Roos, S, Jansson, N, Palmberg, I, Säljö, K, Powell, T L & Jansson, T 2007. Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth. *The Journal of Physiology,* **582,** 449-59.

Roos, S, Powell, T L & Jansson, T 2009. Placental mTOR links maternal nutrient availability to fetal growth. *Biochemical Society Transactions,* **37,** 295-8.

Rosario, F J, Jansson, N, Kanai, Y, Prasad, P D, Powell, T L & Jansson, T 2011. Maternal Protein Restriction in the Rat Inhibits Placental Insulin, mTOR, and STAT3 Signaling and Down-Regulates Placental Amino Acid Transporters. *Endocrinology,* **152,** 1119-29.

Roseboom, T J, van der Meulen, J H P, Osmond, C, Barker, D J P, Ravelli, A C J, Schroeder-Tanka, J M, van Montfrans, G A, Michels, R P J & Bleker, O P 2000. Coronary heart disease after prenatal exposure to the Dutch famine, 1944-45. *Heart,* **84,** 595-98.

Roseboom, T J, van der Meulen, J H P, Ravelli, A C J, Osmond, C, Barker, D J P & Bleker, O P 2001. Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. *Molecular and Cellular Endocrinology,* **185,** 93-98.

Roseboom, T J, van der Meulen, J H P, Ravelli, A C J, van Montfrans, G A, Osmond, C, Barker, D J P & Bleker, O P 1999. Blood pressure in adults after prenatal exposure to famine. *Journal of Hypertension,* **17,** 325-30.

Rossant, J & Cross, J C 2001. Placental development: Lessons from mouse mutants. *Nature Reviews Genetics,* **2,** 538-48.

Saad, M J, Araki, E, Miralpeix, M, Rothenberg, P L, White, M F & Kahn, C R 1992. Regulation of insulin receptor substrate-1 in liver and muscle of animal models of insulin resistance. *The Journal of Clinical Investigation,* **90,** 1839-49.

Salas, M, John, R, Saxena, A, Barton, S, Frank, D, Fitzpatrick, G, Higgins, M J & Tycko, B 2004. Placental growth retardation due to loss of imprinting of Phlda2. *Mechanisms of Development,* **121,** 1199-210.

Samuelsson, A M, Matthews, P A, Argenton, M, Christie, M R, McConnell, J M, Jansen, E H, Piersma, A H, Ozanne, S E, Twinn, D F, Remacle, C, Rowlerson, A, Poston, L & Taylor, P D 2008. Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. *Hypertension,* **51,** 383-92.

Sandovici, I, Smith, N H, Nitert, M D, Ackers-Johnson, M, Uribe-Lewis, S, Ito, Y, Jones, R H, Marquez, V E, Cairns, W, Tadayyon, M, O'Neill, L P, Murrell, A, Ling, C, Constância, M & Ozanne, S E 2011. Maternal diet and aging alter the epigenetic control of a promoter–enhancer interaction at the Hnf4a gene in rat pancreatic islets. *Proceedings of the National Academy of Sciences,* **108,** 5449-54.

Sands, J & Dobbing, J 1985. Continuing growth and development of the third-trimester human placenta. *Placenta,* **6,** 13-21.

Sarbassov, D D, Guertin, D A, Ali, S M & Sabatini, D M 2005. Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex. *Science,* **307,** 1098-101.

Sasaki, H & Matsui, Y 2008. Epigenetic events in mammalian germ-cell development: reprogramming and beyond. *Nature Reviews Genetics,* **9,** 129-40.

Scheepens, A, van de Waarenburg, M, van den Hove, D & Blanco, C E 2003. A

Single Course of Prenatal Betamethasone in the Rat Alters Postnatal Brain Cell Proliferation but not Apoptosis. *The Journal of Physiology,* **552,** 163-75.

Scheid, M P, Marignani, P A & Woodgett, J R 2002. Multiple Phosphoinositide 3-Kinase-Dependent Steps in Activation of Protein Kinase B. *Molecular and Cellular Biology,* **22,** 6247-60.

Seckl, J R & Chapman, K E 1997. The 11β-Hydroxysteroid Dehydrogenase System, A Determinant of Glucocorticoid and Mineralocorticoid Action. *European Journal of Biochemistry,* **249,** 361-64.

Seckl, J R & Meaney, M J 2004. Glucocorticoid Programming. *Annals of the New York Academy of Sciences,* **1032,** 63-84.

Selye, H & McKeown, T 1935. Studies on the Physiology of the Maternal Placenta in the Rat. *Proceedings of the Royal Society of London. Series B, Biological Sciences,* **119,** 1-31.

Sferruzzi-Perri, A N, Owens, J A, Pringle, K G, Robinson, J S & Roberts, C T 2006. Maternal Insulin-Like Growth Factors-I and -II Act via Different Pathways to Promote Fetal Growth. *Endocrinology,* **147,** 3344-55.

Shams, M, Kilby, M D, Somerset, D A, Howie, A J, Gupta, A, Wood, P J, Afnan, M & Stewart, P M 1998. 11Beta-hydroxysteroid dehydrogenase type 2 in human pregnancy and reduced expression in intrauterine growth restriction. *Human Reproduction,* **13,** 799-804.

Shankar, K, Harrell, A, Liu, X, Gilchrist, J M, Ronis, M J J & Badger, T M 2008. Maternal obesity at conception programs obesity in the offspring. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **294,** R528-38.

Shankar, K, Kang, P, Harrell, A, Zhong, Y, Marecki, J C, Ronis, M J J & Badger, T M 2010. Maternal Overweight Programs Insulin and Adiponectin Signaling in the Offspring. *Endocrinology***,** en.2010-0017.

Shibata, E, Hubel, C A, Powers, R W, von Versen-Hoeynck, F, Gammill, H, Rajakumar, A & Roberts, J M 2008. Placental System A Amino Acid Transport is Reduced in Pregnancies With Small For Gestational Age (SGA) Infants but Not in Preeclampsia with SGA Infants. *Placenta,* **29,** 879-82.

Shiell, A W, Campbell-Brown, M, Haselden, S, Robinson, S, Godfrey, K M & Barker, D J P 2001. High-Meat, Low-Carbohydrate Diet in Pregnancy: Relation to Adult Blood Pressure in the Offspring. *Hypertension,* **38,** 1282-88.

Shin, B-C, Fujikura, K, Suzuki, T, Tanaka, S & Takata, K 1997. Glucose Transporter GLUT3 in the Rat Placental Barrier: A Possible Machinery for the Transplacental Transfer of Glucose. *Endocrinology,* **138,** 3997-4004.

Shin, J-Y, Fitzpatrick, G V & Higgins, M J 2008. Two distinct mechanisms of silencing by the KvDMR1 imprinting control region. *EMBO J,* **27,** 168-78.

Shoener, J A, Baig, R & Page, K C 2006. Prenatal exposure to dexamethasone alters hippocampal drive on hypothalamic-pituitary-adrenal axis activity in adult male rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* **290,** R1366.

Simpson, C W, Dicara, L V & Wolf, G 1974. Glucocorticoid anorexia in rats. *Pharmacology Biochemistry and Behavior,* **2,** 19-25.

Singh, R R, Cullen-McEwen, L A, Kett, M M, Boon, W-M, Dowling, J, Bertram, J F & Moritz, K M 2007. Prenatal corticosterone exposure results in altered AT1/AT2, nephron deficit and hypertension in the rat offspring. *The Journal of Physiology,* **579,** 503-13.

Skinner, M K 2008. What is an epigenetic transgenerational phenotype?: F3 or F2. *Reproductive Toxicology,* **25,** 2-6.

Skinner, M K, Anway, M D, Savenkova, M I, Gore, A C & Crews, D 2008. Transgenerational epigenetic programming of the brain transcriptome and anxiety behavior. *PLoS ONE,* **3,** e3745.

Skinner, M K, Manikkam, M & Guerrero-Bosagna, C 2010. Epigenetic transgenerational actions of environmental factors in disease etiology. *Trends in Endocrinology & Metabolism,* **21,** 214-22.

Snoeck, A, Remacle, C, Reusens, B & Hoet, J J 1990. Effect of a Low Protein Diet during Pregnancy on the Fetal Rat Endocrine Pancreas. *Neonatology,* **57,** 107-18.

Soares, M J, Chapman, B M, Rasmussen, C A, Dai, G, Kamei, T & Orwig, K E 1996. Differentiation of trophoblast endocrine cells. *Placenta,* **17,** 277-89.

Soto, N, Bazaes, R A, Peña, V, Salazar, T, Ávila, A, Iñiguez, G, Ong, K K, Dunger, D B & Mericq, M V 2003a. Insulin Sensitivity and Secretion Are Related to Catch-Up Growth in Small-for-Gestational-Age Infants at Age 1 Year: Results from a Prospective Cohort. *The Journal of Clinical Endocrinology & Metabolism,* **88,** 3645-50.

Soto, N, Bazaes, R A, Peña, V, Salazar, T, Ávila, A, Iñiguez, G, Ong, K K, Dunger, D B & Mericq, M V 2003b. Insulin Sensitivity and Secretion Are Related to Catch-Up Growth in Small-for-Gestational-Age Infants at Age 1 Year: Results from a Prospective Cohort. *Journal of Clinical Endocrinology & Metabolism,* **88,** 3645-50.

Spellacy, W N & Goetz, F C 1963. Plasma insulin in normal late pregnancy. *New England Journal of Medicine,* **268,** 988-91.

Srinivasan, M, Aalinkeel, R, Song, F & Patel, M S 2003. Programming of Islet Functions in the Progeny of Hyperinsulinemic/Obese Rats. *Diabetes,* **52,** 984-90.

St Clair, D, Xu, M, Wang, P, Yu, Y, Fang, Y, Zhang, F, Zheng, X, Gu, N, Feng, G, Sham, P & He, L 2005. Rates of Adult Schizophrenia Following Prenatal Exposure to the Chinese Famine of 1959-1961. *JAMA: The Journal of the American Medical Association,* **294,** 557-62.

Stanner, S A, Bulmer, K, Andres, C, Lantseva, O E, Borodina, V, Poteen, V V & Yudkin, J S 1997. Does malnutrition in utero determine diabetes and coronary heart disease in adulthood? Results from the Leningrad siege study, a cross sectional study. *British Medical Journal,* **315,** 1342-48.

Staud, F, Mazancová, K, Mikšík, I, Pávek, P, Fendrich, Z & Pácha, J 2006. Corticosterone Transfer and Metabolism in the Dually Perfused Rat Placenta: Effect of 11β-hydroxysteroid Dehydrogenase Type 2. *Placenta,* **27,** 171-80.

Stewart, P M, Corrie, J E, Shackleton, C H & Edwards, C R 1988. Syndrome of apparent mineralocorticoid excess. A defect in the cortisol-cortisone shuttle. *The Journal of Clinical Investigation,* **82,** 340-49.

Stewart, P M, Rogerson, F M & Mason, J I 1995. Type 2 11 beta-hydroxysteroid dehydrogenase messenger ribonucleic acid and activity in human placenta and fetal membranes: its relationship to birth weight and putative role in fetal adrenal steroidogenesis. *The Journal of Clinical Endocrinology & Metabolism,* **80,** 885-90.

Stewart, R J, Preece, R F & Sheppard, H G 1975. Twelve generations of marginal protein deficiency. *British Journal of Nutrition,* **33,** 233-53.

Stouder, C, Deutsch, S & Paoloni-Giacobino, A 2009. Superovulation in mice alters the methylation pattern of imprinted genes in the sperm of the offspring. *Reproductive Toxicology,* **28,** 536-41.

Stouder, C & Paoloni-Giacobino, A 2010. Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. *Reproduction,* **139,** 373-79.

Stouder, C & Paoloni-Giacobino, A 2011. Specific transgenerational imprinting effects of the endocrine disruptor methoxychlor on male gametes. *Reproduction,* **141,** 207-16.

Sugden, M & Holness, M 2002. Gender-specific programming of insulin secretion and action. *Journal of Endocrinology,* **175,** 757-67.

Sumiyoshi, M, Sakanaka, M & Kimura, Y 2006. Chronic Intake of High-Fat and High-Sucrose Diets Differentially Affects Glucose Intolerance in Mice. *The*

Journal of Nutrition, **136,** 582-87.

Sun, X J, Rothenberg, P, Kahn, C R, Backer, J M, Araki, E, Wilden, P A, Cahill, D A, Goldstein, B J & White, M F 1991. Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature,* **352,** 73-77.

Surve, V V 2008. *High fructose/surcose diets for inducing hypertriglyceridemia and insulin resistance in rodents*, New Brunswick, NJ

Surwit, R S, Feinglos, M N, Rodin, J, Sutherland, A, Petro, A E, Opara, E C, Kuhn, C M & Rebuffe-Scrive, M 1995a. Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. *Metabolism: Clinical and Experimental,* **44,** 645-51.

Surwit, R S, Feinglos, M N, Rodin, J, Sutherland, A, Petro, A E, Opara, E C, Kuhn, C M & Rebuffe-Scrive, M 1995b. Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. *Metabolism,* **44,** 645-51.

Surwit, R S, Kuhn, C M, Cochrane, C, McCubbin, J A & Feinglos, M N 1988. Diet-induced type II diabetes in C57BL/6J mice. *Diabetes,* **37,** 1163-67.

Susser, E S & Lin, S P 1992. Schizophrenia After Prenatal Exposure to the Dutch Hunger Winter of 1944-1945. *Archives of General Psychiatry,* **49,** 983-88.

Szabó, P E & Mann, J R 1995. Biallelic expression of imprinted genes in the mouse germ line: implications for erasure, establishment, and mechanisms of genomic imprinting. *Genes & Development,* **9,** 1857-68.

Szabo, P E, Tang, S-H E, Silva, F J, Tsark, W M K & Mann, J R 2004. Role of CTCF Binding Sites in the Igf2/H19 Imprinting Control Region. *Molecular and Cellular Biology,* **24,** 4791-800.

Tachibana, M, Matsumura, Y, Fukuda, M, Kimura, H & Shinkai, Y 2008. G9a/GLP complexes independently mediate H3K9 and DNA methylation to silence transcription. *The EMBO Journal,* **27,** 2681-90.

Tada, T, Tada, M, Hilton, K, Barton, S C, Sado, T, Takagi, N & Surani, M A 1998. Epigenotype switching of imprintable loci in embryonic germ cells. *Development Genes and Evolution,* **207,** 551-61.

Tahiliani, M, Koh, K P, Shen, Y, Pastor, W A, Bandukwala, H, Brudno, Y, Agarwal, S, Iyer, L M, Liu, D R, Aravind, L & Rao, A 2009. Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1. *Science,* **324,** 930-35.

Takahashi, K, Kobayashi, T & Kanayama, N 2000. p57Kip2 regulates the proper development of labyrinthine and spongiotrophoblasts. *Molecular Human Reproduction,* **6,** 1019-25.

Takata, K, Kasahara, T, Kasahara, M, Ezaki, O & Hirano, H 1992. Localization of erythrocyte/HepG2-type glucose transporter (GLUT1) in human placental villi. *Cell and Tissue Research,* **267,** 407-12.

Takata, K, Kasahara, T, Kasahara, M, Ezaki, O & Hirano, H 1994. Immunolocalization of glucose transporter GLUT1 in the rat placental barrier: possible role of GLUT1 and the gap junction in the transport of glucose across the placental barrier. *Cell and Tissue Research,* **276,** 411-18.

Tallarigo, L, Giampietro, O, Penno, G, Miccoli, R, Gregori, G & Navalesi, R 1986. Relation of Glucose Tolerance to Complications of Pregnancy in Nondiabetic Women. *New England Journal of Medicine,* **315,** 989-92.

Tang, J I, Seckl, J R & Nyirenda, M J 2011. Prenatal Glucocorticoid Overexposure Causes Permanent Increases in Renal Erythropoietin Expression and Red Blood Cell Mass in the Rat Offspring. *Endocrinology,* **152,** 2716-21.

Tangalakis, K, Lumbers, E, Moritz, K, Towstoless, M & Wintour, E 1992. Effect of cortisol on blood pressure and vascular reactivity in the ovine fetus. *Experimental Physiology,* **77,** 709-17.

Taylor, P D, McConnell, J, Khan, I Y, Holemans, K, Lawrence, K M, Asare-Anane, H, Persaud, S J, Jones, P M, Petrie, L, Hanson, M A & Poston, L 2005. Impaired glucose homeostasis and mitochondrial abnormalities in offspring of rats fed a fat-rich diet in pregnancy. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **288,** R134-R39.

Taylor, P D & Poston, L 2007. Developmental programming of obesity in mammals. *Experimental Physiology,* **92,** 287-98.

Thomassin, H, Flavin, M, Espinas, M-L & Grange, T 2001. Glucocorticoid-induced DNA demethylation and gene memory during development. *The EMBO Journal,* **20,** 1974-83.

Tunster, S J, Tycko, B & John, R M 2010. The Imprinted Phlda2 Gene Regulates Extraembryonic Energy Stores. *Molecular and Cellular Biology,* **30,** 295-306.

Tycko, B & Morison, I M 2002. Physiological functions of imprinted genes. *Journal of Cellular Physiology,* **192,** 245-58.

Valdez, R, Athens, M, Thompson, G, Bradshaw, B & Stern, M 1994. Birthweight and adult health outcomes in a biethnic population in the USA. *Diabetologia,* **37,** 624-31.

Vikse, B E, Irgens, L M, Leivestad, T, Hallan, S & Iversen, B M 2008. Low birth weight increases risk for end-stage renal disease. *Journal of the American Society of Nephrology,* **19,** 151-57.

Vom Saal, F, Grant, W, McMullen, C & Laves, K 1983. High fetal estrogen

concentrations: correlation with increased adult sexual activity and decreased aggression in male mice. *Science,* **220,** 1306-09.

Waddell, B J, Benediktsson, R, Brown, R W & Seckl, J R 1998. Tissue-Specific Messenger Ribonucleic Acid Expression of 11β-Hydroxysteroid Dehydrogenase Types 1 and 2 and the Glucocorticoid Receptor within Rat Placenta Suggests Exquisite Local Control of Glucocorticoid Action. *Endocrinology,* **139,** 1517-23.

Waddington, C H 1957, *The Strategy of the Genes,* London, George Allen & Unwin.

Walker, B R, McConnachie, A, Noon, J P, Webb, D J & Watt, G C M 1998. Contribution of parental blood pressures to association between low birth weight and adult high blood pressure: cross sectional study. *British Medical Journal,* **316,** 834-37.

Walsh, C, Glaser, A, Fundele, R, Ferguson-Smith, A, Barton, S, Azim Surani, M & Ohlsson, R 1994. The non-viability of uniparental mouse conceptuses correlates with the loss of the products of imprinted genes. *Mechanisms of Development,* **46,** 55-62.

Walton, A & Hammond, J 1938. The Maternal Effects on Growth and Conformation in Shire Horse-Shetland Pony Crosses. *Proceedings of the Royal Society of London. Series B, Biological Sciences,* **125,** 311-35.

Wang, X, He, C, Moore, S C & Ausió, J 2001. Effects of histone acetylation on the solubility and folding of the chromatin fiber. *Journal of Biological Chemistry,* **276,** 12764.

Waterland, R A & Jirtle, R L 2003. Transposable Elements: Targets for Early Nutritional Effects on Epigenetic Gene Regulation. *Molecular and Cellular Biology,* **23,** 5293-300.

Watkins, A J, Lucas, E S, Torrens, C, Cleal, J K, Green, L, Osmond, C, Eckert, J J, Gray, W P, Hanson, M A & Fleming, T P 2010. Maternal low-protein diet during mouse pre-implantation development induces vascular dysfunction and altered renin-angiotensin-system homeostasis in the offspring. *British Journal of Nutrition,* **103,** 1762-70.

Watkins, M L, Rasmussen, S A, Honein, M A, Botto, L D & Moore, C A 2003. Maternal Obesity and Risk for Birth Defects. *Pediatrics,* **111,** 1152-58.

Weaver, I C G, Cervoni, N, Champagne, F A, D'Alessio, A C, Sharma, S, Seckl, J R, Dymov, S, Szyf, M & Meaney, M J 2004. Epigenetic programming by maternal behavior. *Nature Neuroscience,* **7,** 847-54.

Weaver, N 1955. Rearing of Honeybee Larvae on Royal Jelly in the Laboratory. *Science,* **121,** 509-10.

Weismann, A 1875. Ueber den Saison-Dimorphismus der Schmetterlinge. *Studien zur Descendez-Theorie.*

Welberg, L A M, Seckl, J R & Holmes, M C 2001. Prenatal glucocorticoid programming of brain corticosteroid receptors and corticotrophin-releasing hormone: possible implications for behaviour. *Neuroscience,* **104,** 71-79.

Welsh, G I & Proud, C G 1993. Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. *Biochemical Journal,* **294,** 625-29.

Welsh, G I, Wilson, C & Proud, C G 1996. GSK3: a SHAGGY frog story. *Trends in Cell Biology,* **6,** 274-79.

Wermann, H, Stoop, H, Gillis, A J M, Honecker, F, van Gurp, R J, Ammerpohl, O, Richter, J, Oosterhuis, J W, Bokemeyer, C & Looijenga, L H J 2010. Global DNA methylation in fetal human germ cells and germ cell tumours: association with differentiation and cisplatin resistance. *The Journal of Pathology,* **221,** 433-42.

Whitaker, R C 2004. Predicting Preschooler Obesity at Birth: The Role of Maternal Obesity in Early Pregnancy. *Pediatrics,* **114,** e29-e36.

WHO 2011. WHO Obesity and overweight factsheet. *World Health Organization*

Whorwood, C B, Ricketts, M L & Stewart, P M 1994. Epithelial cell localization of type 2 11 beta-hydroxysteroid dehydrogenase in rat and human colon. *Endocrinology,* **135,** 2533-41.

Wichi, R B, Souza, S B, Casarini, D E, Morris, M, Barreto-Chaves, M L & Irigoyen, M C 2005. Increased blood pressure in the offspring of diabetic mothers. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **288,** R1129-R33.

Williams, S, St George, I M & Silva, P A 1992. Intrauterine growth retardation and blood pressure at age seven and eighteen. *Journal of Clinical Epidemiology,* **45,** 1257-63.

Wood, C E, Cheung, C Y & Brace, R A 1987. Fetal heart rate, arterial pressure, and blood volume responses to cortisol infusion. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **253,** R904-R09.

Wykes, S M & Krawetz, S A 2003. The structural organization of sperm chromatin. *Journal of Biological Chemistry,* **278,** 29471.

Wyrwoll, C S, Seckl, J R & Holmes, M C 2009. Altered Placental Function of 11{beta}-Hydroxysteroid Dehydrogenase 2 Knockout Mice. *Endocrinology,* **150,** 1287-93.

Yang, K 1995. Co-expression of two distinct isoforms of 11[beta]-hydroxysteroid

dehydrogenase in the ovine placenta. *The Journal of Steroid Biochemistry and Molecular Biology,* **52,** 337-43.

Yang, Z, Zhao, W, Zhang, X, Mu, R, Zhai, Y, Kong, L & Chen, C 2008. Impact of famine during pregnancy and infancy on health in adulthood. *Obesity Reviews,* **9,** 95-99.

Yehuda, R & Bierer, L M 2007. Transgenerational transmission of cortisol and PTSD risk. In: E. Ronald De Kloet, M S O & Eric, V (eds.) *Progress in Brain Research.* Elsevier.

Yehuda, R, Bierer, L M, Schmeidler, J, Aferiat, D H, Breslau, I & Dolan, S 2000. Low Cortisol and Risk for PTSD in Adult Offspring of Holocaust Survivors. *The American Journal of Psychiatry,* **157,** 1252-59.

Yehuda, R, Engel, S M, Brand, S R, Seckl, J, Marcus, S M & Berkowitz, G S 2005. Transgenerational Effects of Posttraumatic Stress Disorder in Babies of Mothers Exposed to the World Trade Center Attacks during Pregnancy. *The Journal of Clinical Endocrinology & Metabolism,* **90,** 4115-18.

Youngson, N A & Whitelaw, E 2008. Transgenerational epigenetic effects. *Annual Review of Genomics and Human Genetics,* **9,** 233-57.

Yung, H-w, Calabrese, S, Hynx, D, Hemmings, B A, Cetin, I, Charnock-Jones, D S & Burton, G J 2008. Evidence of Placental Translation Inhibition and Endoplasmic Reticulum Stress in the Etiology of Human Intrauterine Growth Restriction. *The American Journal of Pathology,* **173,** 451-62.

Zalensky, A O, Siino, J S, Gineitis, A A, Zalenskaya, I A, Tomilin, N V, Yau, P & Bradbury, E M 2002. Human testis/sperm-specific histone H2B (hTSH2B). *Journal of Biological Chemistry,* **277,** 43474.

Zambrano, E, Bautista, C J, Deás, M, Martínez-Samayoa, P M, González-Zamorano, M, Ledesma, H, Morales, J, Larrea, F & Nathanielsz, P W 2006. A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. *The Journal of Physiology,* **571,** 221-30.

Zambrano, E, Martínez-Samayoa, P M, Bautista, C J, Deás, M, Guillén, L, Rodríguez-González, G L, Guzmán, C, Larrea, F & Nathanielsz, P W 2005. Sex differences in transgenerational alterations of growth and metabolism in progeny (F2) of female offspring (F1) of rats fed a low protein diet during pregnancy and lactation. *The Journal of Physiology,* **566,** 225-36.

Zhang, J, Wang, C, Terroni, P L, Cagampang, F R A, Hanson, M & Byrne, C D 2005. High-unsaturated-fat, high-protein, and low-carbohydrate diet during

pregnancy and lactation modulates hepatic lipid metabolism in female adult offspring. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,* **288,** R112-R18.

Zhou, J & Bondy, C A 1993. Placental glucose transporter gene expression and metabolism in the rat. *The Journal of Clinical Investigation,* **91,** 845-52.

Zipper, H, Brunner, H, Bernhagen, J & Vitzthum, F 2004. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Research,* **32,** e103.

Zohar, A H, Giladi, L & Givati, T 2007. Holocaust exposure and disordered eating: a study of multi-generational transmission. *European Eating Disorders Review,* **15,** 50-57.

Zwart, R, Sleutels, F, Wutz, A, Schinkel, A H & Barlow, D P 2001. Bidirectional action of the Igf2r imprint control element on upstream and downstream imprinted genes. *Genes & Development,* **15,** 2361-66.