Exposure of Mouse Embryos to Ethanol During Preimplantation Development: Effect on DNA-Methylation in the *H19* Imprinting Control Region

Philip Charles Haycock

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg, September 2007

DECLARATION

I, Philip Charles Haycock, declare that this dissertation is my own work, unless otherwise stated. It is being submitted for the degree of Master of Science in Medicine in the branch of Human Genetics, in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

……...….

…….…...day of…….…., 2007.

ABSTRACT

Ethanol is a classic teratogen capable of inducing a wide range of developmental abnormalities that vary in severity, from the barely perceptible to spontaneous abortion. These defects are collectively referred to as foetal alcohol spectrum disorders (FASD). Foetal alcohol syndrome (FAS) lies at the extreme end of this spectrum and is associated with three broad domains: prenatal and/or postnatal growth retardation, distinctive facial features and brain damage. Epidemiological and animal studies clearly indicate that the clinical variability of FASD is related to four distinct window periods: preconception, preimplantation, gastrulation and postorganogenesis. These developmental windows are correlated with peak periods of epigenetic reprogramming, suggesting a common mechanism of ethanol teratogenesis. Together with experimental evidence that ethanol inhibits DNA-methyltransferase, as well as folate metabolism, this suggests an 'epigenetic model of FASD'.

The aim of the present study was to explore the validity of this model by investigating the relationship between ethanol-induced growth retardation and imprinting, following ethanol exposure during the preimplantation period. Employing an experimental study design, together with a hybrid mouse model, embryos and placentae were harvested at 10.5 days post coitus (dpc). The weights of embryos and placentae, as well as methylation profiles at the *H19* imprinting control region (ICR) – an important regulator of growth - were measured.

It was found that ethanol-treated embryos and placentae were severely growth retarded in comparison to controls: r=-0.760 ($p<0.01$, one-tailed) and r=-0.816 ($p<0.05$,

two-tailed), respectively. Bisulphite genomic sequencing revealed that the methylation profile at the *H19* ICR was unaffected in ethanol-treated embryos, in comparison to saline-treated controls. Conversely, methylation at the paternal and maternal alleles in placentae was found to be reduced and increased, respectively, in comparison to embryos. These results imply that mechanisms for the maintenance of imprinting in the embryo are more robust than in the placenta. This is consistent with the relatively long-lived nature of the embryo, which must maintain imprinting for a considerably longer period of time than the placenta.

Bisulphite sequencing also revealed that the paternal allele of the *H19* ICR had significantly decreased levels of methylation, while the maternal allele had increased levels of methylation, in ethanol treated-placentae, in comparison to saline controls. The changes observed at the paternal allele were localized to the CTCF1 DNA-binding site, while a trend for increased methylation at the maternal allele was observed at the CTCF2 site. A partial correlation further revealed that demethylation at the paternal allele in placentae partly mediated the effect of ethanol on placental weight. An 'epigenetic switch model', whereby paternal *Igf2* is downregulated by the epigenetic switching of the paternal allele to the maternal epigenotype, is proposed to explain this relationship. However, partial correlations also indicated that demethylation at the paternal allele of the *H19* ICR, as well as placental growth retardation, did not mediate the effect of ethanol on embryo growth.

Collectively, these data suggest that imprinting at the *H19* ICR is not a mechanism of embryo growth retardation prior to 10.5 dpc. In explaining these results, it is proposed that the growth retarded placenta was able to meet the nutritional demands of

the similarly growth retarded embryo up until 10.5 dpc. However, an important question for future research would be to examine the relationship between ethanol-induced growth retardation and imprinting during late gestation. During the final growth spurt (>14.5 dpc) the growth retarded placenta may become unable to meet the increased demands for nutrition, which would exacerbate foetal growth restriction.

In sum, the present study revealed a novel mechanism of ethanol-induced growth retardation in the placenta but indicated that imprinting at the *H19* ICR does not mediate the effect of ethanol on the early embryo. Further research is required to resolve the relationship between imprinting and ethanol-induced growth retardation.

ACKNOWLEDGEMENTS

I would like to acknowledge the following people for their guidance and support during the course of this project:

- My supervisor, Prof Michele Ramsay, for her constant encouragement, support and advice over the last three years.
- Dr Stephanie Dawes and Dr Digby Warner of the Mycobacterial Research Unit (University of the Witwatersrand) for their advice regarding the cloning experiments.
- Prof. Beverly Kramer (Department of Anatomy, University of the Witwatersrand) for her advice regarding the animal dissections.
- Mr Hilton Ngwenya for his technical assistance.
- Dr Lillian Ouko for her advice regarding various aspects of this project.
- Everyone at Central Animal Services (University of the Witwatersrand)
- Everyone from the FAS research group for their valuable insights and ideas.

I would also like to acknowledge the following sources of funding:

- University of the Witwatersrand, the German Academic Exchange Service (DAAD) and the National Research Foundation (NRF), who provided financial support for my personal maintenance.
- The March of Dimes who provided financial support for the present study.

On a personal note, I would like to thank the following people:

- Prof. Himla Soodyall and Prof. Trefor Jenkins for their mentorship, support and guidance over the last four years.
- Ms Fahmida Essop, Mrs Claire ('stationary-thief') Mitchell, Mrs Carol ('friendly friend') Robinson, Ms Silke Arndt, Mr Thejane Matladile (Mr 'T'), Mrs Vanessa Pillay ('V'), Mrs Akashnie Maharaj, Prof. Arnold Christianson, Dr Tony Lane ('The Tonester'), Kathpaham Shantikumar ('Wacko Kato'), Mr Jaysen Knezovich, Ms Jacqueline Frost, Ms Maria Mudau, Ms Punita Pitamber, Ms Pareen ('Pesty') Patel, Ms Candice de Carlvalho, Ms Zane Lombard, Mrs Karen Kuhn, Mr Heeran ('Hero') Makkan and Mr Thejissen Naidoo ('TJ') for keeping things interesting.
- My parents, Kenneth and Elvira Haycock, my granddad, Kenneth Haycock, my brother, Andrew Haycock and my sister, Sarah Haycock, for their love and support. And most importantly I would like to thank my wife, Ralitsa Stoyanova, for her love and support, without which I would not be where I am today.

TABLE OF CONTENTS

DECLARATION ii
ABSTRACT iii
ACKNOWLEDGEMENTS vi
TABLE OF CONTENTS viii
LIST OF FIGURES xiv
LIST OF TABLES xv

CHAPTER 1: INTRODUCTION 1

1.1 What are Foetal Alcohol Spectrum Disorders? 1
1.2 What causes Foetal Alcohol Spectrum Disorders? 3
1.3 Outline of literature review 5
1.4 The importance of timing in the aetiology of FASD 7
1.4.1 Taxonomic heterogeneity 7
1.4.2 Variability in timing may underlie taxonomic heterogeneity 8
1.4.2.1 The preconception period: early investigations 9
1.4.2.2 The preconception period: recent findings 11
1.4.2.2.1 Preconceptional effects mediated by paternal consumption 12
1.4.2.2.2 Preconceptional effects mediated by maternal consumption 13
1.4.2.2 The preimplantation period 15
1.4.2.2.1 In vivo administration of ethanol during the preimplantation period 16
1.4.2.2.2 In vitro administration of ethanol during the preimplantation period 18
1.4.2.3 Gastrulation 21
1.4.2.4 Post-gastrulation 22

1.4.3 DNA-methylation as a mechanism of teratogenesis 24
1.5 Epigenetics 26
1.5.1 What is Epigenetics? 26
1.5.1.2 Effect of epigenetic modifications on chromatin structure and function 27
1.5.1.3 Heritability of epigenetic modifications through mitosis 28
1.5.1.4 Environmental origins of epigenetic modifications 28
1.5.2 Epigenetic reprogramming 29
1.5.3 Genomic imprinting 32
1.5.3.1 Epigenetic reprogramming at imprinted loci 35
1.5.3.2 Preimplantation as a sensitive window period of imprinting regulation 35
1.5.3.2.1 Growth abnormalities following in vitro culture of the early mammalian embryo 36
1.5.3.2.2 Imprinting as a mechanism of growth abnormalities 37
1.5.4 An epigenetic model of FASD 39
1.6 Aims and rationale 41

CHAPTER 2: MATERIALS AND METHODS 43

2.1 Materials 44
2.1.1 Mouse studies 44
2.2 Methods 45
2.2.1 Mouse treatments 45
2.2.2 DNA extractions 46
2.2.3 Restriction enzyme digests 46
2.2.4 Bisulphite modification 47
2.2.5 Amplification of the *H19* ICR by the polymerase chain reaction 47
2.2.6 Cloning of PCR products 51

2.2.6.1 Ligation of PCR products into pGEM T-easy vector 52
2.2.6.2 Transformation of plasmids into DH5α cells 52
2.2.6.3 Isolation and amplification of inserts 52
2.2.7 Dideoxy chain-termination based sequencing of inserts 53
2.2.8 Data cleanup and storage 54
2.2.8.1 Preparation of sequence data for computational analysis 54
2.2.8.2 Generation of methylation data, scoring of strain specific polymorphisms and calculation of bisulphite conversion error rate 54
2.3 Statistical analyses 55
2.3.1 Descriptive statistics 55
2.3.2 Hypothesis testing 56
2.3.2.1 Hierarchical linear modelling 57
2.3.2.2 Mann-Whitney U-tests 61
2.3.2.3 Correlations between weight, treatment and DNA-methylation 62

CHAPTER 3: RESULTS 64

3.1 Mouse experiments 64
3.1.1 Weight 65
3.1.1.1 Hierarchical linear modelling of the relationship between weight and treatment 66
3.2 Molecular genetic analyses 69
3.2.1 Confirmation of strain-specific DNA polymorphisms 69
3.2.2 Analyses of DNA-methylation 70
3.2.2.1 Visual inspection of methylation profiles 72
3.2.2.2 Distribution of % DNA-methylation across 17/16 CpG sites 76
3.2.2.3 Distribution of % DNA-methylation at the CTCF1 and

CTCF2 sites 79
3.2.2.4 Hierarchical linear modelling of DNA-methylation 80
3.2.2.5 Analyses of the aggregated DNA-methylation data 84
3.2.2.6 The relationship between weight and methylation at the paternal allele of the *H19* ICR in placentae 86
3.2.2.7 Partial correlations of the relationship between treatment, weight and methylation at the paternal allele of the *H19* ICR in placentae 88
3.2.2.7.1 Partial correlations of the relationship between placental weight and treatment, controlling for the effect of methylation at the paternal allele 89
3.2.2.7.2 Partial correlations of the relationship between embryo weight and treatment, controlling for the effect of placental weight or methylation at the paternal allele 90
3.3. Summary of results 91

CHAPTER 4: DISCUSSION 93

4.1 Outline of discussion 94
4.2 Effect of ethanol on embryo and placental weight 95
4.3 Relaxation of imprinting in placental tissue 97
4.3.1 Asymmetry between the upstream and downstream portions of the *H19* ICR 97
4.3.2 Relaxation of imprinting in placentae: evidence from previous studies 98
4.3.3 Evolutionary models predict robust maintenance of imprinting in the embryo but not the placenta 99
4.3.4 Protection against loss of imprinting: role of delayed activation 101
4.3.5 Dynamic imprinting in the placenta: role of chromatin assembly factors 102

4.3.6 Summary of findings regarding relaxation of imprinting in the placenta 104
4.4 Preferential loss of imprinting in ethanol-treated trophectoderm 105
4.4.1 Mechanisms of preferential loss of imprinting in ethanol-treated trophectoderm 106
4.4.2 Preferential loss of imprinting in trophectoderm induced by *in vitro* culture 108
4.4.3 Summary of findings regarding preferential loss of imprinting in ethanol-treated trophectoderm 111
4.5 Ethanol-induced demethylation and imprinting at the *H19/Igf2* domain 112
4.5.1 Mechanisms of imprinted gene expression in the *H19*/*Igf2* domain 112
4.5.2 Ethanol induced epigenetic switching of the paternal to the maternal epigenotype 114
4.5.3 In *cis* and in *trans* effects of demethylation at the paternal allele of the *H19* ICR 115
4.5.4 Summary of findings regarding the effect of demethylation at the paternal allele of the *H19* ICR on imprinting in the *H19/Igf2* domain 116
4.6 Ethanol-induced epigenetic switching as a mechanism of growth retardation 117
4.6.1 Clinical and experimental consequences of epimutations and mutations at the *H19/Igf2* domain 118
4.6.2 Mechanisms of *Igf2*-mediated growth control 120
4.6.3 Summary of findings regarding mechanisms of ethanol-induced growth retardation 122
4.7 Study limitations and strengths 123
4.7.1 Limitations 123
4.7.2 Strengths 126
4.8 Future studies 126
4.9 Conclusion 128

REFERENCES 129

APPENDIX A: Ethics clearance certificate 143

APPENDIX B: Protocols 144

APPENDIX C: Reagents 147

APPENDIX D: Weight data 152

LIST OF FIGURES

Figure 1.1. Stages of preimplantation development 16
Figure 1.2. Epigenetic reprogramming during development 30
Figure 1.3. The *H19* ICR in the context of the *H19/Igf2* imprinted domain 34
Figure 2.1. Flow diagram of methodology 43
Figure 2.2. The genomic context of the *H19* imprinting control region in mouse 49
Figure 2.3. Sequence of the region amplified by the primers of the present study 51
Figure 2.4. Partial correlations of the relationship between treatment and weight, controlling for the effect of DNA-methylation on the latter 63
Figure 3.1. Mean embryo weights and standard deviations across ten mothers 65
Figure 3.2. Mean placenta weights and standard deviations across nine mothers 66
Figure 3.3. Confirmation of four strain-specific DNA polymorphisms 70
Figure 3.4. Number of clones distributed by sample origin, treatment type and parental origin 71
Figure 3.5. Analysis of DNA-methylation at 17 CpG sites in the H19 Imprinting Control Region in ten embryonic and ten placental samples from five ethanol treated mothers 73
Figure 3.6. Analysis of DNA-methylation at 17 CpG sites in the H19 Imprinting Control Region in ten embryonic and ten placental samples from five control mothers 74
Figure 3.7. Analysis of DNA-methylation at CpG sites in the H19 imprinting control region in the CAST/Ei and C57BL/6 mouse strains 75
Figure 3.8. Distribution of DNA-methylation in the H19 imprinting control region 78
Figure 3.9. Distribution of DNA-methylation at two CTCF DNA-binding regions within the H19 Imprinting Control Region 80

LIST OF TABLES

Table 2.1. The primers used in the present study 48
Table 3.1. Hierarchical Linear Modelling of the Relationship Between Ethanol and Weight in Embryos and Placentae 67
Table 3.2. Median Weights of Aggregated Embryos and Placentae as well as Median Number of Resorptions and Implantations in Ethanol-Exposed Mothers and Controls 69
Table 3.3. Strain-specific polymorphisms 70
Table 3.4. Hierarchical linear modelling of the relationship between ethanol and DNA-methylation in the *H19* imprinting control region 82
Table 3.5. Multilevel logistic modelling of the relationship between DNA-methylation and ethanol at individual CpG Sites within the *H19* imprinting control region in placentae in non-aggregated data 83
Table 3.6. Median Levels of Paternal DNA-Methylation in the *H19* Imprinting Control Region in Ethanol-Exposed Placentae and Controls 84
Table 3.7. Median levels of DNA-methylation at individual CpG sites in the *H19* imprinting control region in ethanol-exposed placentae and controls using the aggregated dataset at the level of mothers 86
Table 3.8. Correlations between Weight and Paternal DNA-methylation in the *H19* Imprinting Control Region in Placenta 88
Table 3.9. Partial Correlations Between Treatment and Placental Weight Controlling for the Effect of Paternal DNA-Methylation in Placentae on Placental Weight 90
Table 3.10. Partial Correlations Between Treatment and Embryo Weight Controlling for the Effect of Paternal DNA-Methylation in Placentae or Placental Weight on Embryo Weight 91

CHAPTER 1: INTRODUCTION

1.1 WHAT ARE FOETAL ALCOHOL SPECTRUM DISORDERS?

Alcohol is the generic label for a large family of organic compounds in which a hydroxyl group (-*OH*) is bound to a carbon atom of an alkyl or substituted alkyl group. Ethyl alcohol (ethanol), produced from the fermentation of fruits or grains with yeast, is by far the most common variety, being a key ingredient in many human beverages. Unfortunately, ethanol is also a classic teratogen capable of inducing a wide range of developmental abnormalities that vary in severity, from the barely perceptible to spontaneous abortion, and which are collectively referred to as foetal alcohol spectrum disorders (FASD; Astley 2004). It is important to emphasize that FASD is an umbrella term for all pathologies resulting from the exposure of the developing foetus to alcohol but does not in itself constitute a clinical diagnosis. In other words, an individual suffering from a congenital abnormality, as a result of *in utero* ethanol exposure, would not receive a diagnosis of FASD but rather a diagnosis falling within the FASD continuum. According to the Institute of Medicine's revised classification system (Hoyme et al. 2005) there are currently six recognized diagnoses: foetal alcohol syndrome (FAS) with and without confirmed maternal alcohol exposure; partial FAS with and without confirmed maternal alcohol exposure; alcohol related birth defects (ARBDs); and alcohol related neurodevelopmental disorder (ARND). After spontaneous abortion, FAS is considered the most adverse clinical outcome resulting from prenatal alcohol exposure.

Although knowledge of the teratogenic properties of ethanol may extend as far back as antiquity, the first epidemiological and scientific investigations of the effects of ethanol on the human organism, and lower animals, are comparatively recent (Sullivan, 1899; Whitney 1912; Stockard 1913; Pearl 1916; Nice 1917; MacDowell 1922; Hanson and Handy 1923; Hanson and Florence 1927a; Hanson and Florence 1927b). Moreover, the formal recognition of an ethanol-induced birth defect syndrome was not made prior to the modern era.

FAS was first delineated in 1973 (Jones and Smith 1973; Jones et al. 1973) and encompasses three broad domains, or categories: prenatal and/or postnatal growth retardation; distinctive facial features (short palpebral fissures, smooth philtrum, thin vermillion border of the upper lip) and brain damage (May et al. 2004). Soon after its recognition, it was realized that the manifestation of classic FAS features could be highly variable, and a number of related diagnoses soon evolved to reflect this fact. These included ARBDs (Jones and Smith, 1973) and foetal alcohol effects (FAE; Clarren and Smith, 1978). The latter was defined as a partial expression of FAS but the diagnostic guidelines were so non-specific that a child with attention deficit disorder (ADD), whose mother had consumed a few glasses of wine during pregnancy, would meet the criteria for FAE (Astley 2004). Following the call of Aase et al. (1995) for its abandonment, FAE has not been included in recent diagnostic guides.

One of the main problems faced by FAE, and indeed all the diagnoses within the FASD continuum, is the high degree of overlap between FAS features and other genetic and teratogenic birth defect syndromes. By including the word ‘alcohol’ in the name of the diagnosis, a strong claim is made as to the cause of the birth defect syndrome and

consequently great care must be taken to exclude other known causes of FAS-like features. Some authors have suggested that the word alcohol should be removed or that the phrase 'alcohol associated' should be emphasized instead, since the aetiology may often involve a number of interacting factors (Abel, 2006). The labelling of these syndromes as alcohol-induced is all the more complicated by the fact that not all women who consume alcohol during pregnancy have children with FAS, suggesting that alcohol is a necessary but insufficient causal factor (Abel, 1984; 2006). This begs the question as to the aetiological heterogeneity of FASD and whether it makes sense to ascribe to it a single cause. Unsurprisingly, the origination of the FAS label is strongly rooted in didactic, and not academic, considerations, being intended to help facilitate the education of women about the teratogenic nature of alcohol (Jones 1973). Recognising the difficulties faced by a classification system that underscores the aetiological importance of alcohol, diagnostic guides emphasize the importance of differential diagnoses and multidisciplinary teams, so as to rule out other known syndromes.

1.2 WHAT CAUSES FOETAL ALCOHOL SPECTRUM DISORDERS?

Soon after its recognition, research turned towards the mechanistic bases of FASD. As alluded to above, the clinical consequences of *in utero* ethanol exposure are highly variable and one of the early research questions focused on whether this variability could be related to variability in dosage and timing. Unsurprisingly, the FASD research community has relied heavily on animal models in addressing such key questions.

Other questions have focused on the aetiological basis of FASD. Generally speaking, researchers attempt to explain the mechanisms of alcohol teratogenesis from one or more of the following perspectives: genetic, biochemical, cellular and morphological. For example, research has shown that ethanol is associated with reduced growth factor levels (Resnicoff et al. 1994; Goodlett and Horn, 2001); inhibition of such factors is likely to result in reduced cellular proliferation (Armant and Saunders, 1996; Wozniak et al 2004) which may, in turn, result in reduced brain mass (Wozniak et al 2004); and it is reasonable to propose that genetic variation in enzymes that regulate alcohol metabolism (e.g. alcohol dehydrogenases) influence an individual's susceptibility to FASD (Warren and Li, 2005). The key challenge facing the FASD research field is the integration of this wide, and oft-times seemingly disparate, body of research into a coherent whole such that a more complete explanation of alcohol-induced birth defect syndromes may be attained.

This is a monumental task because FASD cannot be understood as if it were a single localized insult on an otherwise normal whole. Instead, it must be approached as an emergent property of deregulated developmental pathways and interactions, the primary origins of which will be a great number of steps removed from the ultimate end result, such as altered brain function. The wide range of morphological and physiological abnormalities that have been associated with *in utero* alcohol exposure suggest that there is a high degree of 'causal fan out' from the primary insults at the molecular and cellular levels to the defects observed at the clinical level. This, in turn, suggests that the mechanistic bases of FASD involve a potentially bewildering array of heterogeneity, at least in terms of the cascade of events linking the primary insult with the end clinical

outcome. On the other hand, understanding the primary mechanisms, lying at the root of alcohol teratogenesis, is an imminently more tractable problem, and is the focus of much research, as is the present study.

1.3 OUTLINE OF LITERATURE REVIEW

In the literature review to follow, an overview will be given of what is known about FASD aetiology, with particular emphasis on the importance of timing and critical window periods of development. It will be shown that alcohol consumption at any time in an organism's life-cycle – including the preconception period (i.e. before pregnancy), the preimplantation period, gastrulation and postgastrulation - has variable and deleterious consequences for foetal growth and development. Despite this seemingly broad window period, alcohol teratogenesis is correlated with non-overlapping 'peaks' of vulnerability. One of the key challenges facing the FASD-research field is determining whether any common mechanisms lie at the root of alcohol teratogenesis at these different time points and, if so, reconciling this with the wide variability in phenotypic outcomes, as exemplified by the FASD continuum and studies in animal models. In other words, does variability arise from common mechanisms operating at different time points, or different mechanisms operating at different time points? The answers to these questions will contribute to a greater understanding of FASD and may have important consequences for the treatment and prevention of FASD in the future.

Following a summary of the importance of timing and critical window periods of development, an 'epigenetic model of FASD' is presented that attempts to provide an

integration of these research findings into a coherent whole. Moreover, the model presents a common mechanism of alcohol teratogenesis that is proposed to operate throughout these periods - namely, epigenetic reprogramming – and also implies a number of experimentally testable hypotheses. The model is based on the correlation between critical window periods of teratogenesis with known peak periods of epigenetic reprogramming, as well as the available evidence that alcohol directly interferes with epigenetic mechanisms. Finally, special attention is drawn to the phenomenon of genomic imprinting and epigenetic reprogramming during the preimplantation period. It is proposed that an epigenetic model is particularly well poised to explain the teratogenic consequences of alcohol exposure during the preimplantation period.

Thus, the aim of the present study is to partially validate the epigenetic model presented by testing the hypothesis that ethanol exposure during the preimplantation period is associated with deregulation of imprinting and that this is, in turn, is a mechanism of foetal growth retardation in the postimplantation period.

1.4 THE IMPORTANCE OF TIMING IN THE AETIOLOGY OF FASD

1.4.1 Taxonomic heterogeneity

Taxonomically, FASD is a highly heterogeneous group of developmental disorders, as exemplified by the wide spectrum of birth defects associated with prenatal alcohol exposure. Clinically, FASD is associated with the following recognized diagnoses: FAS with and without confirmed maternal alcohol exposure; partial FAS with and without confirmed maternal alcohol exposure; ARBDs; and ARND (Hoyme et al. 2005). All six diagnoses represent variations of the following theme: prenatal and/or postnatal growth retardation; distinctive facial features (short palpebral fissures, smooth philtrum, thin vermillion border of the upper lip) and brain damage (May et al. 2004). However, each of these categories is broadly defined and much variation exists in what constitutes satisfaction of the criteria for diagnosis. For example, microcephaly or Attention Deficit Disorder (ADD) could both constitute evidence for brain damage. Moreover, FASD has also been associated with a number of other morphological and physiological defects, some of which are included with the ARBD rubric. The more common features include cardiac septal defects and minor joint abnormalities, while less common presentations include various skeletal anomalies, as well as ocular, vestibular, urinary, hepatic, skin and immune defects (Chaudhuri 2000).

1.4.2 Variability in timing may underlie taxonomic heterogeneity

Research in animal models strongly suggests that the above variability in clinical outcomes is related to variations in timing of alcohol exposure, as well as dosage. By far the most popular animal used in the FASD research field has been the mouse, particularly with regards to studies of morphological damage, followed by the rat, and other animal species including, fish, chickens, guinea pigs, dogs, ferrets, non-human primates and pigs (Becker et al. 1996). Virtually all FAS related features have been replicated in the mouse, using a wide range of dosage regimens, as well as variations in developmental timing (Becker et al. 1996).

The following section covers the teratogenic consequences of ethanol exposure during the following developmental periods: preconception; preimplantation; gastrulation; and post-gastrulation. Most animal studies typically employ one of two dosage paradigms: acute dosage regimens, which typically involve 2.9-6.0 g/kg ethanol administered intraperitoneally or intragastrically, on one or two occasions within the same day, or chronic dosage regimens, which typically involve smaller ($\leq$3g/kg) doses of ethanol administered intraperitoneally, intragastrically, or as part of their liquid diet, throughout the period of development of interest.

1.4.2.1 The preconception period: early investigations

A preconceptional effect can be said to occur when the consumption of alcohol prior to conception (in either the male or female parent) is associated with birth abnormalities in the offspring, despite the latter not being directly exposed to the teratogen *in utero*.

The first preconceptional studies of ethanol extend back to the early 1900s when Lamarckian ideas of inheritance were still in-vogue and the subject of much investigation (Whitney 1912; Pearl 1916; Nice 1917; MacDowell 1922; Hanson and Handy 1923; Hanson and Florence 1927a; Hanson and Florence 1927b). Ethanol was a favourite experimental system because of its myriad effects on the human organism – considered both deleterious and beneficial at the time - and the known fact that ethanol distributed to the male and female genitalia quite readily. Thus, ethanol seemed well suited to addressing questions pertaining to the inheritance of acquired characters i.e., Lamarckian inheritance.

The results of this research are quite mixed, many studies purportedly finding evidence for, and against, alcohol-induced modifications of the parental germlines. With regards to this literature review, the most salient and consistent research findings, particularly those that would probably stand up to modern day statistical standards, have been selected.

The favourite method of ethanol administration was by inhalation: placed in a copper tank, with a screen floor or wire mesh, animals would be forced to breathe in the fumes of 95% ethanol, 30 minutes to several hours every day for months to a year,

depending on the nature of the particular experiment. During the course of the study various mating conditions would be setup to test a number of questions, such as the effect of chronic alcoholism in the male or female on fecundity or future offspring vitality. Often such experiments would be continued for several generations, to test whether any effects detected in the F1 generation persisted into future descendents, without further alcohol treatment.

In one extensive series of experiments by Stockard (1913) guinea pigs were treated by the inhalation method to the point of intoxication every day, except Sundays, for approximately three years. “From time to time” treated animals (males and females) were mated with untreated controls. Various experimental conditions were tested, such as ‘alcoholised females’ x ‘normal males’, ‘alcoholised females’ x ‘alcoholised males’ and ‘alcoholised males’ x ‘normal females’. However, alcoholised females were treated both before and after conception and are therefore irrelevant here because they do not constitute effective tests of preconceptional exposure.

It was found that, following 34 successful crossings between alcoholised males and normal females, 24% of litters were stillborn. The remaining litters produced 54 offspring, 39% of which died soon after birth. In comparison, a ‘normal male’ x ‘normal female’ crossing resulted in 33 litters, 1 of which (3%) was stillborn, and of the 60 live offspring, 4 (7%) died soon after birth. In addition, crossings amongst the untreated offspring (males and females), i.e. offspring of parents from the ‘alcoholised conditions’ who were not themselves subjected to the inhalation method, tended to have fewer surviving offspring than controls (54% versus 93%; Stockard, 1918).

In sum, these results suggest that alcohol administered to males during the preconceptional period resulted in high rates of perinatal mortality in offspring and that these effects persisted into the F2 generation.

Utilizing a similar experimental design but with white mice and over a shorter period of time (7 months), Nice (1917) found that the crossing of one 'alcoholised male' with two 'normal females' resulted in 10 litters and 66 offspring, 6% of which died soon after birth. In comparison, 9 litters from control matings resulted in 47 mice, all of which were viable and survived the duration of the experiment. In addition, it was reported that the same crossings resulted in, on average, larger litter sizes (6.6 versus 5.1 in controls), more litters (1.66 versus 1.3 in controls) and a greater number of live offspring, although these were less viable, (11 versus 6.5 in controls) in the experimental group. Whether or not these differences are significant is difficult to determine because the raw data is unavailable. However, given the small sample size, it is likely that only the average difference in number of live offspring, which is reasonably large, could be considered significant by today's standards. If taken at face value, the results suggest that 'alcoholised males' produced more, but less viable, offspring than the control group.

1.4.2.2 The preconception period: recent findings

The findings described above, although arguably deficient in various aspects of study design, are consistent with more recent investigations of preconceptional effects. These effects have been uncovered following both paternal and maternal preconceptional consumption of ethanol. The findings regarding the former are particularly convincing

because they are unaffected by the confounding factors usually associated with maternal alcoholism.

1.4.2.2.1 Preconceptional effects mediated by paternal consumption

For example, relatively recent epidemiological studies indicate an association between lowered birth weight in offspring and paternal alcoholism (Little et al. 1987). In addition, adoption studies suggest an increased association between hyperactivity and lowered cognitive abilities in offspring and alcoholism in the biological father but not the adoptive father (Hegedus et al. 1984; Tarter et al. 1984). These effects may be mediated by social facilitation i.e. paternal drinking may encourage maternal drinking, but animal studies, described below, strongly suggest a more direct relationship between paternal alcohol exposure and offspring health and behaviour.

For example, studies employing both acute and chronic dosage regimens provide evidence for a relationship between paternal alcohol exposure in rats and 'large' reductions (defined as two or more standard deviations below the mean of *ad libitum* controls) in birth weight and an increase in physical and organ abnormalities (Anderson et al 1981; Mankes et al. 1982; Abel and Tan, 1988; Cicero et al. 1990; Cicero et al. 1994; Abel, 1995; Bielawski and Abel, 1997).

The latter include decreased spleen weights at weaning, increased adrenal weights at birth (Abel, 1993) and decreased testosterone levels at sexual maturity (Abel and Tan, 1988; Cicero et al. 1990). With regards to birth weight, some studies report effects in the opposite direction i.e. increases in birth weight in offspring of alcohol-treated fathers

(Randall et al. 1982; Leichter, 1986; Abel and Lee, 1988; Abel and Tan, 1988; Abel EL, 1993; Bielawski and Abel, 1997).

Alcoholic male rats also produce offspring with various behavioural abnormalities. For example, a strain-dependent effect exists on hyperactivity in Sprague-Dawley rats but not Long-Evans rats (Abel and Lee, 1988). Other behavioural effects include associations with difficulties in passive avoidance learning tasks (Abel, 1994) and exaggerated stress responses (Abel and Bilitzke, 1990).

1.4.2.2.2 Preconceptional effects mediated by maternal consumption

Preconceptional effects may also be mediated by the female but, in practice, these effects are more difficult to disentangle from possible confounding factors, such as malnutrition and generally reduced vitality in alcoholic mothers.

In one study, Livy et al. (2004) investigated the effect of preconceptional alcohol exposure using the following treatment paradigm: 3.0g/kg of ethanol administered intragastrically (IG), every day for 60 days, to C57BL/6J mice, prior to conception. Following this chronic dosage regimen, various mating conditions were setup: 'alcoholised males' x 'alcoholised females', 'alcoholised males' x 'control females', and 'control males' x 'control females'. Alcoholic and control treatments were continued until conception, at which point they were halted. Harvested on the 14th day of gestation, Livy et al. (2004) found that foetuses from alcoholic females were significantly growth retarded in comparison to controls. Moreover, the male treatment paradigm did not seem to affect embryo weight.

It should be emphasized that female body weight, food consumption and fecundity was not significantly affected by alcohol treatment, suggesting that these results are not necessarily the result of an altered physiology or anatomy in the mother and that the reduced weights of the foetuses, who were not themselves directly exposed to alcohol, were not the result of altered maternal nutrition.

These findings parallel those of Becker and Randall (1987), who reported growth retardation in untreated offspring of prenatally exposed F1 individuals. Similar results were also reported by Little et al. (1980), who observed a relationship between alcoholism in women, who abstained during pregnancy, and reduced birth weights in their offspring (Little et al. 1980).

In sum, a wide range of birth defects and foetal abnormalities have been reported in animal models and human studies following preconceptional alcohol exposure. These findings suggest that offspring not directly exposed to alcohol *in utero* may nevertheless be born with developmental abnormalities if their father or mother consumed alcohol prior to conception. In addition, the existence of preconceptional effects in both males and females suggests that the latter are not wholly due to the confounding effects of maternal malnutrition. These mechanisms, particularly in males, are likely to involve alcohol-induced changes in the gametes or, alternatively, selection effects within the germline (Abel, 2004).

1.4.2.2 The preimplantation period

The preimplantation period corresponds to the first 4-6 days of mouse development, which roughly corresponds to the first 2 weeks of human pregnancy. It begins with fertilization and subsequent formation of the zygote (Figure 1.1). This is followed by a rapid period of mitotic cell divisions which, by 2.5 days post coitus (dpc), gives rise to a solid spherical mass of blastomeres, also referred to as the morula (Figure 1.1). By 3.5 dpc the ball of cells has developed into a blastocyst: an asymmetric and hollow spherical body with an outer layer of cells (the blastoderm) enveloping a fluid-filled cavity (Figure 1.1). The outer layer will give rise to the trophoblast, which is involved in the implantation of the embryo into the uterine wall, and eventually develops into the chorion, while the inner cell mass (the epiblast) eventually gives rise to the embryo (Figure 1.1). The preimplantation period ends with the onset of implantation, which begins around day 4.5, and is completed by day 6. The implantation of the embryo into the uterine wall also corresponds to the onset of gastrulation, during which time the three primary germ layers – the mesoderm, ectoderm and endoderm – are formed.

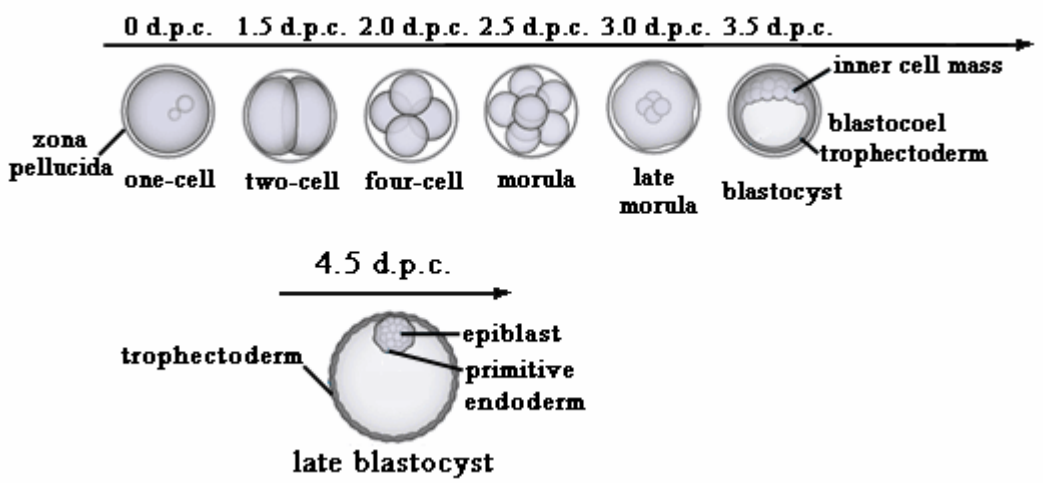

Figure 1.1. Stages of preimplantation development. Preimplantation begins with fertilization and subsequent formation of the zygote and ends with formation of the blastocyst and onset of implantation into the uterine wall. Epiblast – future embryo; trophectoderm – future placenta; primitive endoderm – future yolk sac; modified from http://www.sickkids.ca/rossant/custom/people_amy.asp

1.4.2.2.1 In vivo administration of ethanol during the preimplantation period

The teratogenic consequences of ethanol exposure during this period have received surprisingly little attention in comparison to other developmental periods. The reason for this is perhaps rooted in the traditional belief that the mammalian conceptus is refractory to teratogenic stimuli prior to implantation – an idea that traces its origins to early X-ray and irradiation experiments (Padmanabhan and Hameed, 1988). These early experiments led to the general assumption that the preimplantation mammalian conceptus responds to teratogenic stimuli in an 'all-or-none' fashion, either failing to develop at all, or surviving with no malformations (Padmanabhan and Hameed, 1988).

Despite this general belief, research in mice suggests that *in utero* ethanol exposure during the preimplantation period manifests in adverse outcomes towards the

extreme end of the FASD continuum. For example, in one study undertaken in MF1 mice, it was found that intraperitoneal (IP) administration of 5.8 g/kg ethanol, on any day during the preimplantation period (days 1-4) resulted in severe malformations as well as embryo growth retardation in 80-100% of viable embryos, as assessed on day 15 in gestation. In the same study, administration of a reduced alcohol dosage, 3.9 g/kg, did not significantly reduce embryo weights. Interestingly, even though embryo resorption rates were 2-3 times greater in the ethanol than in the saline and untreated control groups, the number of successful implantations was unaffected (Padmanabhan and Hameed, 1988). In the same study, Padmanabhan and Hameed (1988) observed variable effects of preimplantation ethanol exposure on placental weight. Depending on the precise timing of administration, as well as the day of dissection, placentae were sometimes growth reduced, growth enhanced or unaffected.

Consistent with the idea that *in vivo* treatment does not deleteriously affect implantation rates, Mitchell et al. (1994) found that administration of 4 g/kg ethanol during the first 4 days of gestation promoted pregnancy by inducing earlier onset of implantation in rats, while the postimplantation period was characterized by increased rates of abortion. Similar findings were reported by Checiu and Sandor (1986) in mice and Clarren and Astley (1992) in primates. In the latter, it was found that administration of 1.8g/kg Ethanol during the first 3 weeks of gestation in *Macaca nemistrina* was associated with increased rates of abortion in late, but not early, gestation.

Thus, following treatment across a number of animal species (mouse, rat and primates), *in vivo* administration of ethanol does not seem to deleteriously affect preimplantation development *per se*. However, following implantation, and the

concomitant onset of gastrulation and cellular differentiation, embryos clearly start to abort in large numbers or survive with gross physical abnormalities, such as growth retardation (Padmanabhan and Hameed, 1988).

1.4.2.2.2 In vitro administration of ethanol during the preimplantation period

In stark contrast, studies that expose the preimplantation embryo to ethanol in culture i.e., *in vitro*, generally report findings in the opposite direction of those described above. However, these findings are also quite varied, the effect depending on the precise timing of administration as well as the *in vitro* concentration of ethanol. For example, Leach et al. (1993) reported that development was inhibited following exposure of 1-cell embryos to 0.4% or 1.6% (w/v) ethanol. Two-cell embryos were similarly affected but only at 1.6% (w/v) ethanol. Some of the defects observed included reduced blastocyst formation and hatching. These deleterious effects are usually limited to high concentrations (≥220mM or 1%) that generally exceed the range observed *in vivo* in more traditional animal studies (Leach et al 1993). For example, the peak blood-alcohol concentration attained by acute administration, intraperitoneally and orally, of 2.9 g/kg ethanol in a mouse model did not exceed 0.4% (Webster et al. 1983).

At lower, and physiologically more realistic, concentrations (0.1%), treatment of 1-cell and 2-cell embryos encouraged blastocyst formation. Moreover, the onset of differentiation of trophoblast cells occurred earlier in embryos treated at the 1-cell stage. In contrast, 4-cell embryos seemed refractory to treatment over the entire range of levels tested (0.1-1.6%), while treatment of 8-cell morula with 0.1% ethanol solution was found

to increase the rate of blastulation (Stachecki et al. 1994; Armant and Saunders, 1996). Similarly, Wiebold and Becker (1987) found that exposure of 2-cell embryos to 0.1% ethanol enhanced blastulation and, following transfer to pseudopregnant dams, an increased rate of implantation. Enhanced implantation rates of *in vitro* treated mouse blastocysts, at similar ethanol concentrations, were also reported by Stachecki et al. (1994).

These generally enhancing effects of *in vitro* treatment were not associated with differences in long-term pregnancy outcome. For example, *in vitro* treatment of blastocysts did not affect their resorption rate once transferred to foster mothers, nor was it associated with morphological abnormalities in live offspring (Stachecki et al. 1994; Armant and Saunders, 1996). In addition, in the study by Wiebold and Becker (1987) it was found that, whilst implantation development seemed encouraged by *in vitro* treatment, parturition rates were not different between the controls and experimental groups.

Thus, *in vitro* treatment of preimplantation embryos appears capable of enhancing or inhibiting cell growth and differentiation, depending on the dosage and precise timing of administration. In addition, the postimplantation development of embryos does not appear to be deleteriously affected by *in vitro* culture in the presence of ethanol. These findings are in stark contrast to the *in vivo* studies, described above, which generally report abnormalities towards the extreme end of the FASD continuum i.e., high perinatal death, growth retardation and physical malformations. In other words, the teratogenic consequences of alcohol exposure seem to be abolished when ethanol treatment occurs *in*

vitro, suggesting that this effect is not mediated by ethanol directly, and requires some interaction with the maternal system.

One possibility is that toxic metabolites arising from the maternal system mediate the teratogenic effects of alcohol (when administered *in vivo*). For example, much evidence suggests that the teratogenic effects of ethanol are mediated, at least in part, by its primary metabolite acetaldehyde (Campbell and Fantel, 1983; Guerri and Sanchis, 1985; Lau et al. 1991). The preimplantation embryo is unable to metabolize ethanol to acetaldehyde (Rout and Armant, 2002), suggesting a possible explanation for the discrepancy between *in vivo* and *in vitro* studies i.e., that acetaldehyde produced by the maternal system mediates the teratogenic consequences of *in vivo* ethanol exposure during the preimplantation period.

In sum, the available evidence suggests that alcohol, administered either *in vivo* or *in vitro,* is not deleterious *per se* to the preimplantation embryo. However, following implantation, embryos exposed to ethanol *in vivo,* but not *in vitro,* start to abort in large numbers or survive with gross abnormalities. This suggests that toxic metabolites of the maternal system, such as acetaldehyde, mediate an unknown effect on the preimplantation embryo: subtle enough not to deregulate the preimplantation period but sufficient to result in foetal abortion and gross physical abnormalities during the periods of gastrulation and organ differentiation.

1.4.2.3 Gastrulation

Following implantation of the blastocyst into the uterine wall, which is completed by day 6, gastrulation continues with the onset of the organogenic period, corresponding to days 7-14 in mouse development and weeks 3-8 in humans. During this time there is progressive subdivision of the germinal layers and rudimentary organ formation. It is this period of development that is generally considered the most sensitive to teratogenic insult Armant and Saunders, 1996).

Administration of acute doses of alcohol during the gastrulation phase of mouse development results in a myriad of morphological abnormalities, including skeletal and organ malformations and increased rates of embryo resorption. Interestingly, distinct malformation profiles correspond to distinct timings of ethanol insult, with the most striking anomalies arising during gestational days 7-10. In addition, particular organ abnormalities seem to follow alcohol insult during distinct organ differentiation periods (reviewed in Becker et al. 1996). For example, craniofacial abnormalities, many of which are strikingly reminiscent of FAS facial features, result primarily from acute dosages on gestational days 7, 8 and 9; brain abnormalities seem to arise following acute treatments on gestational days 7 and 8; ocular abnormalities correspond to insult on gestational days 7, 8, 9 and 10; urogenital anomalies arise following treatment on days 9 and 10; and skeletal and limb anomalies correspond to days 9, 10 and 11 (reviewed in Becker et al 1996).

Although acute dosage regimens during the gastrulation phase of mouse development are capable of producing a myriad of abnormalities, encompassing most of

the known spectrum of clinical abnormalities seen in humans, acute exposure on any single day in this period is not enough by itself to produce the full spectrum of birth defects associated with FAS. A more realistic model seems to involve chronic alcohol dosage paradigms that occur throughout the gastrulation phase. For example, chronic exposure regimens that last throughout the gastrulation phase (days 4-12) result in the following abnormalities: brain defects, ocular defects, cardiovascular defects, urogenital defects and skeletal (limb) defects (reviewed in Becker et al 1996). Interestingly, chronic treatments during this period do not seem to result in growth retardation (Becker et al. 1996). These abnormalities seem localized to ethanol insults during either the postgastrulation period, when differentiated systems enter a period of growth (Becker et al. 1996) or preimplantation development (Padmanabhan and Hameed 1988), described above.

1.4.2.4 Post-gastrulation

The postgastrulation phase of mouse development begins around day 15 and lasts until after birth, which occurs approximately around days 17-19, and corresponds to weeks 9-26 in human pregnancy (Becker et al. 1996). During this stage the primordial organ formations enter a period of intense growth, increasing in both size and volume, and continue to differentiate, becoming more mature with regards to function (Becker et al. 1996).

Acute and chronic ethanol insults during this period are associated with growth retardation and abnormalities of the brain but do not usually result in gross morphological abnormalities of other organ systems.

Taken together, the full spectrum of birth defects arising from *in utero* alcohol exposure in humans has been reproduced in animal studies, employing acute and chronic dosage regimen paradigms, during the preconception, preimplantation and gastrulation stages of mouse development. Despite this, no single paradigm is capable of producing the full spectrum in isolation. In mouse models it seems that exposure throughout pregnancy is required for the full FAS profile (Becker et al. 1996). Growth retardation is particularly interesting because it seems to arise following ethanol administration during all the periods described above, with the exception of gastrulation, which is characterized predominantly by intense cellular and organ differentiation and is also a peak period of teratogenesis. These findings suggest that differentiating cells are particularly vulnerable to the teratogenic effects of alcohol.

Consistent with this idea, *in vitro* studies of the cellular response to ethanol suggest that cells far away from terminal end points, which are typically proliferating cells and pluripotent, respond to ethanol by differentiating, while fully differentiated cells may be refractory to ethanol treatment (reviewed in Armant and Saunders, 1996). In contrast, cells in between these end points respond to ethanol by delaying their onset of differentiation.

For example, ethanol enhances differentiation of rat pheochromocytoma (PC12) cells (reviewed in Armant and Saunders 1996). PC12 is a pluripotent cell line that is widely used in studies of growth-factor mediated differentiation (Bai et al. 2005). Ethanol

treatment was found to enhance neural growth factor (NGF) induced differentiation in this cell line i.e., greater neurite outgrowth (Bai et al. 2005; and reviewed in Armant and Sauders 1996).

In contrast to the above effects, less pluripotent cells tend to respond to ethanol by delaying differentiation. For example, ethanol inhibits NGF-induced neural differentiation when the treated cells are dorsal root ganglion sensory neurons, which are far more differentiated than PC12 cells (Luo et al. 1996), while rat cerebellar granule cells, which are terminally differentiated, appear to be refractory to ethanol treatment (Armant and Saunders, 1996).

Thus, the teratogenesis of alcohol at the cellular level seems to correlate with the differentiated state of the cell at the time of insult. This may partly explain why ethanol exposure is not teratogenic to the preimplantation embryo *per se*, which is relatively pluripotent, but directly teratogenic to the gastrulating embryo. Moreover, it may also explain why the latter is particularly sensitive to ethanol.

1.4.3 DNA-methylation as a mechanism of teratogenesis

The above literature review summarized what is generally known about FASD from the perspective of timing and what the pattern of teratogenesis says about the underlying mechanisms.

What is of particular interest is the mechanism of alcohol teratogenesis at the molecular and biochemical levels. Research from this perspective will help unravel the primary nature of alcohol teratogenesis, since this is the first level at which alcohol is

likely to operate. The fan out from the initial alcohol insult at this level is clearly quite broad. Although timing is probably a key factor in this variability, the mechanistic basis of alcohol teratogenesis across these different periods is poorly understood. In particular, it is not known whether variability arises from the same mechanisms operating at different time points or whether different mechanisms operate throughout.

A possible candidate mechanism of alcohol teratogenesis throughout the developmental period – from preconception to parturition - is DNA-methylation. This hypothesis is partly based on the findings that ethanol causes genome-wide hypomethylation in midgestation mouse foetuses (Garro et al. 1991); that acetaldehyde inhibits DNA-methyltransferase (Garro et al. 1991); that alcohol exposure causes alterations in DNA-methyltransferase mRNA in rat sperm (Bielawski et al. 2002); and that alcohol deregulates folate metabolism – a primary source of the methyl cofactors in the methylation pathway (Halsted et al. 2002).

Despite this, and as far as this author is aware, the relationship between alcohol teratogenesis and DNA-methylation has not been subject to any systematic investigation in the alcohol research field. This is surprising considering that DNA-methylation is an important mechanism in a number of epigenetic phenomena, including genomic imprinting and cellular differentiation, which could plausibly be involved in the teratogenic pathway. Moreover, the major periods of epigenetic reprogramming in development correspond to, and potentially explain, the peak periods of alcohol teratogenesis i.e., preconception, preimplantation and gastrulation.

In the next section an ‘epigenetic model of FASD’ will be outlined that is capable of integrating the wide body of findings described above into a coherent whole. By

generating a number of experimentally testable hypotheses, it is hoped that this model will contribute to a greater understanding of FASD. Particular emphasis will be given to epigenetic reprogramming in the preimplantation embryo since this seems especially well correlated with the nature of alcohol teratogenesis.

1.5 EPIGENETICS

1.5.1 What is Epigenetics?

The cells of a multicellular organism are genetically identical (with the exception of cells residing within the immune system) but are functionally heterogeneous. It is generally believed that functional heterogeneity results from the differential expression of genes. Put in other words, one way to generate functional diversity is to use the genetic code in different ways, in different cell types, during different times of development. Thus, understanding how functional diversity is generated requires an understanding of how differences in gene expression arise during development. Epigenetic modifications of chromatin are an important mechanism of this process because they cause heritable alterations in gene expression, amongst different cell-types, that are not mediated by DNA-sequence (Holliday, 1987; Arney and Fisher, 2004 Cheung and Lau, 2005).

As a mechanism of cellular differentiation, epigenetic modifications have three important properties: (1) they affect cell function because they affect chromatin structure and gene expression (2) they are heritable across cell division because they can be replicated and transmitted to daughter cells through mitosis; and (3) their origin is under

the control of the cellular environment (Arney and Fisher, 2004). The last property is particularly important because *different* cellular environments are a source of *different* epigenetic modifications – i.e. *epigenetic variation*.

1.5.1.2 Effect of epigenetic modifications on chromatin structure and function

The link between epigenetic modifications and chromatin structure is mediated chiefly by the ability of the former to recruit chromatin remodelling enzymes and other non-histone proteins. For example, methylation at the paternal allele of the *H19* imprinting control region (ICR) blocks the binding of the boundary element CCCTC-binding factor (CTCF). This allows the promoter of the *Igf2* gene to physically interact with an enhancer located >80kb downstream (Lopes et al. 2004; Kurukuti et al. 2006) which, in-turn, partitions the *Igf2* and *H19* genes into 'silent' and 'active' chromatin domains (Murrell et al. 2004; Kurukuti et al. 2006). In contrast, binding of CTCF to the unmethylated maternal allele of the *H19* ICR partitions the *Igf2* gene into a silent chromatin domain (Murrell et al. 2004; Kurukuti et al. 2006). Thus, DNA-methylation is able to alter higher-order chromatin structure through its ability to recruit, or block, DNA-binding proteins

Other chromatin proteins are also known to preferentially bind methylated DNA. These include methyl CpG-binding protein 2 (MECP2), which has been functionally linked to Rett's syndrome (Guy et al. 2001; Li, 2002), methyl CpG binding domain protein (MBD) 1, MBD2, MBD4 and Kaiso (Hendrich et al. 2001; Li, 2002).

These methylation-binding proteins have important effects on gene expression by, for example, recruiting chromatin remodelling enzymes, such as histone deacetylase, that increase the 'openness' of the chromosomal region to be transcribed and, thereby, a gene's access to the transcriptional machinery (Lopes et al. 2004).

1.5.1.3 Heritability of epigenetic modifications through mitosis

These effects on gene expression are also heritable across cell division because DNA-methylation at CpG dinucleotides can be stably replicated during DNA-synthesis and transmitted through mitosis (Li, 2002). The copying of 'old' DNA-methylation profiles onto newly synthesized DNA strands is mediated by DNA methyltransferase 1 (*DNMT1*), which preferentially methylates hemimethylated DNA (Li, 2002). In this way, the methylation profile of the 'old half' serves as a template for synthesis of the 'new half'. It has been suggested that histone modifications may be replicated in a similar fashion, although this is not the only proposed model (Cheung and Lau (2005).

1.5.1.4 Environmental origins of epigenetic modifications

During development, a myriad number of signal transduction pathways, under the control of growth factors, hormones and other signalling molecules, mediate their effects on cellular function through their influence on epigenetic modifications and chromatin remodelling (Arney and Fisher, 2004; Cheung and Lau 2005). In this way, the genome of

a cell monitors the cellular environment for important developmental information, in order to determine its functional destiny (Arney and Fisher, 2004).

The sensitivity of the epigenetic state of the genome to its environment has also been demonstrated by experimental studies. For example, the 'epigenome' can be altered by dietary supplements, (e.g. folic acid, vitamin B_{12}, choline, and betaine), ethanol, endocrine disruptors, *in vitro* culture techniques, 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and maternal care (Cooney et al. 2002; Waterland and Jirtle, 2003; Garro et al. 1991; Anway et al. 2005. Newbold et al. 2006; Thompson et al. 2001; Wu et al. 2004; Weaver et al. 2004).

Environmentally induced epigenetic modifications are likely to originate during important periods of epigenetic reprogramming.

1.5.2 Epigenetic reprogramming

Developmentally, the establishment or erasure of chromatin modifications is known as epigenetic reprogramming (Figure 1.2). Significantly, the prenatal period is characterized by dynamic epigenetic rearrangements: during the preimplantation period genome-wide DNA-methylation is almost entirely erased; this is followed by genome-wide *de novo* methylation during gastrulation (Reik and Walter, 2001) (Figure 1.2). Within the germline, epigenetic changes are no less dynamic: similar to the case in somatic cells, primordial germ cells (PGCs) also acquire genome-wide *de novo* methylation but following their entry into the genital ridge there is rapid erasure of DNA-methylation at both imprinted and non-imprinted loci, with the exception of repetitive

elements, which seem to partially escape methylation erasure (Hajkova et al. 2002) (Figure 1.2). Later periods of development, during the onset of terminal differentiation events, are correlated with localized chromatin remodelling, such as the NGF-induced neuronal differentiation pathway (Bai et al. 2005), the JAK-STAT-induced astroglial differentiation pathway (Fan et al. 2005) and the differentiation of neural stem cells (Hsieh and Gage, 2004) (Figure 1.2). Ethanol-induced insults during the prenatal period could mediate their effects through disruption of these epigenetic reprogramming events (Figure 1.2).

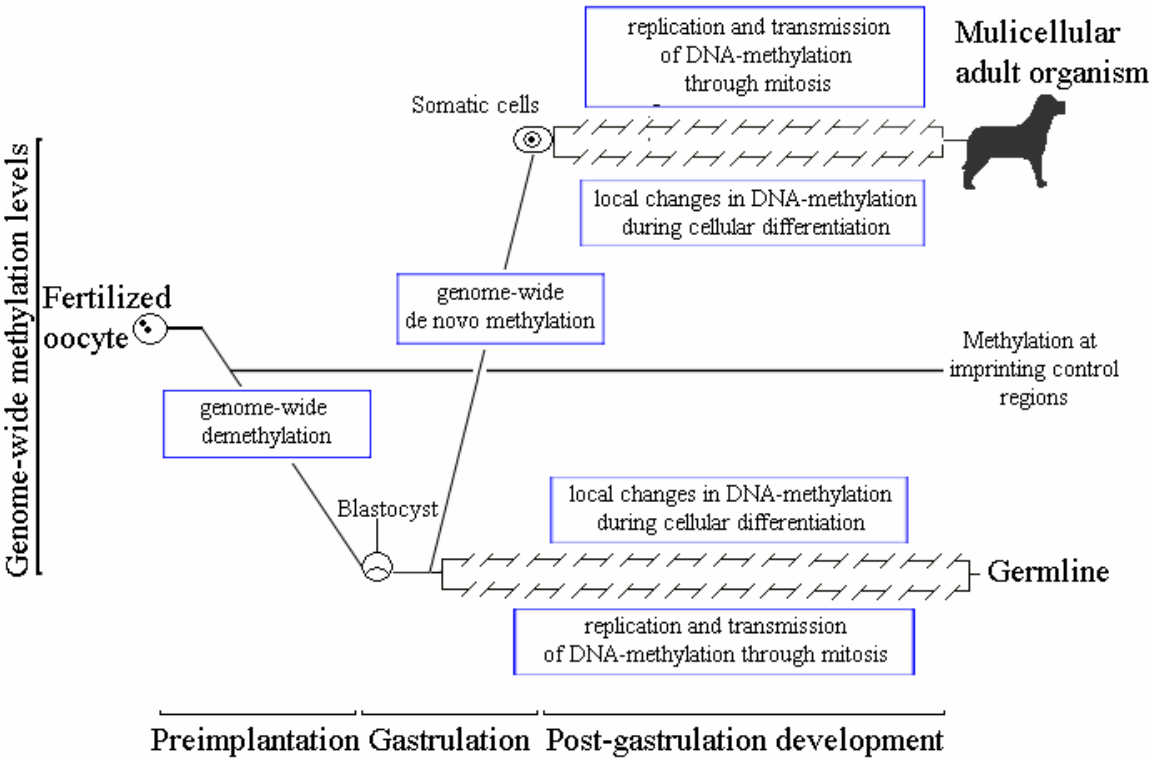

Figure 1.2. Epigenetic reprogramming during development. Preimplantation is characterised by genome-wide demethylation; gastrulation is characterised by genome-wide *de novo* methylation (Reik and Walter, 2001). The germline is characterised by dynamic epigenetic changes, including genome-wide *de novo* methylation and demethylation at both imprinted and non-imprinted loci during later stages (Hajkova et al. 2002). Localised epigenetic changes are associated with cellular differentiation. These periods of epigenetic rearrangement correlate with peak periods of ethanol teratogenesis, suggesting an 'epigenetic model of FASD'; broken lines indicate dynamic changes in methylation.

For example, the most dynamic period of epigenetic reprogramming, gastrulation (Reik and Walter, 2001) is also the peak window period of ethanol teratogenesis (Armant and Saunders, 1996; Becker et al. 1996). In addition, cells at different stages of differentiation are epigenetically distinct (Arney and Fisher, 2004), which may explain the correlation between ethanol teratogenesis and cellular differentiation (Armant and Saunders, 1996). The proposal that ethanol teratogenesis may be linked to inappropriate epigenetic reprogramming is consistent with the foetal programming hypothesis, which proposes that environmental insults during the prenatal period predispose the fetus to a adult onset diseases, such as coronary heart disease and hypertension (Fleming et al., 2004; Gluckman et al., 2005; Dolinoy et al. 2007). It has been proposed that this 'programming' is mediated, in part, by epigenetic mechanisms (Fleming et al., 2004; Jablonka, 2004; Gluckman et al., 2005).

When the teratogenic insult occurs in the germline, the result may be the transmission of FASD, or FASD-like phenotypes, to the next generation. This latter idea is not without precedent. For example, exposure of gestating female rats during the period of gonad sex differentiation to endocrine disruptors vinclozolin (an antiandrogenic compound) or methoxychlor (an estrogenic compound) resulted in decreased spermatogenic capacity (cell number and viability) and increased incidence of male infertility and adult onset diseases in the F1 generation (Anway et al. 2005; Anway and Skinner, 2006). This phenotype was heritable up to the F4 generation and was associated with alterations in global DNA-methylation in the germline.

As a model of alcohol teratogenesis, the preimplantation period is particularly interesting. First, from an epigenetic stand-point, preimplantation is a period of genome

wide *de*methylation, a process for removal of the epigenetic marks of the previous generation and thus a means for restoring pluripotency to the cells of the early embryo (Reik and Walter, 2001). However, the exception to this rule is genomic imprinting.

1.5.3 Genomic imprinting

Imprinting is an epigenetic marking mechanism that results in the preferential expression of either the paternal or maternal allele of certain genes (Thomson et al. 2001) and is 'epigenetic' because it is heritable across cellular and organismal generations and mediated by chromatin marks, such as DNA-methylation and histone modifications (Tycko and Morison, 2002). In addition, imprinted genes are regulated by DNA-methylation at differentially methylated regions (DMRs), of which there are two types: those that are differentially methylated in all tissues, throughout development; and those that acquire differential methylation during somatic development, in a tissue-specific manner (Reik and Walter, 2001). The former are often referred to as 'imprinting control regions' (ICRs) because they are the primary regulators of imprinting in their respective chromosomal domains (Reik and Walter, 2001). Another general feature of imprinted genes is their tendency to appear in clusters (Reik and Walter, 2001). Important mediators of foetal growth and development (Tycko and Morison, 2002), imprinted genes have been found to underlie a number of birth defect syndromes in humans (Reik and Walter, 2001; Bliek et al. 2006) and animal species, including large offspring syndrome (LOS) in ruminant species and culture-induced growth abnormalities in mice (Walker et al. 1996; Young et al. 1998; Sinclair et al. 2000; Khosla et al. 2001; McLaren, 2000;

Sinclair et al. 2000; Nagy et al. 1993; Dean et al. 1998). Functionally haploid (being predominantly expressed from either the maternal or paternal allele), imprinted genes are more sensitive to physiological conditions than their diploid counterparts (Jirtle et al. 2000).

For example, the imprinted cluster on distal chromosome 7 in mouse (syntenic to chromosome 11p15.5 in human) contains at least 2 imprinted domains and eight imprinted genes (Reik and Walter, 2001). One of these domains contains the best characterized imprinted gene cluster in the mammalian genome: the *Igf2/H19* domain, containing the paternally expressed *Igf2* and maternally expressed *H19* genes (Figure 1.3), as well as three DMRs associated with *Igf2* and a single DMR associated with *H19* (Lopes et al. 2003). The *H19* DMR is considered an ICR because hypermethylation of the paternal allele is established during spermatogenesis, and maintained during pre- and post-implantation development (Tremblay et al. 1995; 1997; Warnecke et al. 1998; Weber et al. 2001). In contrast, the other three DMRs, which are associated with the *Igf2* gene, show considerable methylation changes during development – losing their paternal methylation during preimplantation - and acquiring tissue specific methylation patterns during somatic development (*i.e.,* postimplantation; Lopes et al. 2003). The latter, as well as expression of *Igf2*, is regulated by the influence of the *H19* ICR on higher order chromatin structure in the region. The mechanism involves binding of the CTCF zinc-finger protein to the *H19* ICR, which prevents the physical interaction of *Igf2* with enhancer elements located downstream of *H19*, partitioning the two genes into 'silent' and 'active' chromatin domains (Kurukuti et al. 2006; Figure 1.3). Maternal *Igf2* is silenced because CTCF preferentially binds to the unmethylated maternal allele, while

methylation on the paternal allele prevents binding, thus allowing *Igf2* promoter-enhancer interaction and expression of paternal *Igf2* (Delaval and Feil, 2004; Figure 1.3). Perturbation of this process has deleterious effects on foetal growth because *Igf2* is the precursor peptide for a mitogen factor known as 'insulin-like growth factor II', which is active in foetal and placental tissues (Tycko and Morison, 2002). For example, hypomethylation of the *H19* ICR has been associated with the Silver-Russell birth defect syndrome, which is characterized by intrauterine growth retardation (IUGR), poor postnatal health, classic facial features and asymmetry (Price et al. 1999). Moreover, together with its receptors, and associated signal-transduction pathways, *Igf2* signaling is one of two known systems of growth control in the mammalian genome – the other being the insulin-mediated growth pathway (Reik and Walter, 2001).

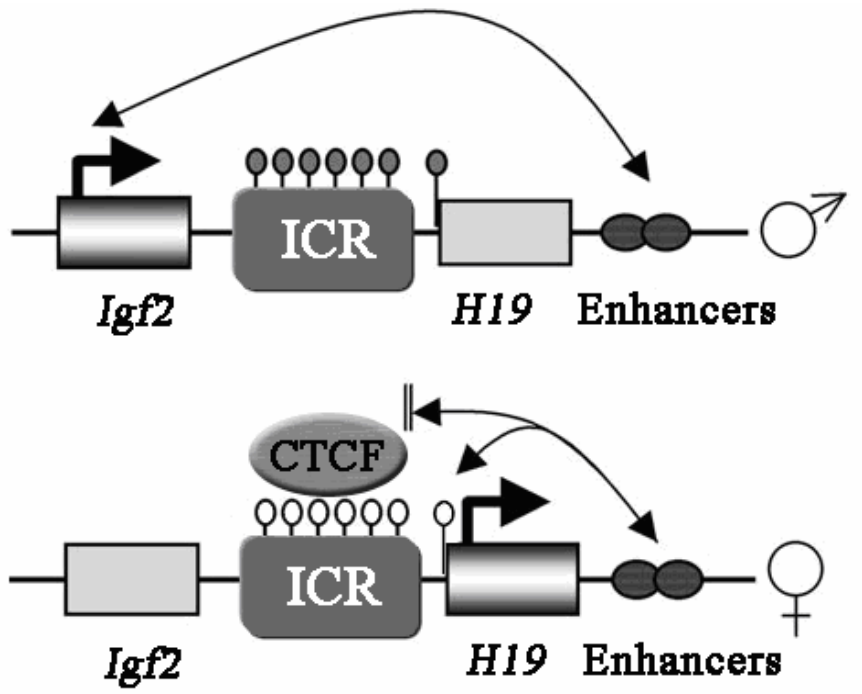

Figure 1.3. The *H19* ICR in the context of the *H19/Igf2* imprinted domain. Filled and unfilled lollipops represent methylated and unmethylated CpG dinucleotides, respectively. Arrows represent transcription start sites of the *Igf2* and *H19* genes; when CTCF binds to the unmethylated maternal allele it blocks access of maternal *Igf2* to enhancer sequences located downstream of *H19;* conversely the insulator function of the *H19* ICR is abrogated by methylation on the paternal allele, blocking CTCF and allowing paternal *Igf2* access to the enhancers (Engel et al. 2006). Thus, paternal *Igf2* is expressed and maternal *Igf2* is silenced. Figured derived from Engel et al. (2006).

1.5.3.1 Epigenetic reprogramming at imprinted loci

There are three important periods in the life of an imprint: (1) gametogenesis, when imprints must be reset according to the sex of the transmitting parent, (2) preimplantation and (3) gastrulation, when secondary imprints arise with important roles in cellular differentiation and proliferation in late gestation (Weinstein, 2001). Thus, there are major parallels between genome-wide epigenetic reprogramming and reprogramming at imprinted loci, making genomic imprinting an excellent model of epigenetic regulation in general (Reik and Walter, 2001).

As a model of alcohol-teratogenesis, genomic imprinting during the preimplantation period is particularly interesting. For example, epigenetic information at imprinted loci must be maintained in the face of dramatic genome-wide epigenetic change i.e., the complete erasure of almost all epigenetic marks from the previous generation (Reik and Walter, 2001). This suggests that imprints might be particularly vulnerable to environmental perturbations during the preimplantation period.

1.5.3.2 Preimplantation as a sensitive window period of imprinting regulation

In support of this idea, it has been known for many years that manipulation of the early embryo in culture, whether for animal cloning or *in vitro* fertility treatments, is associated with high embryo and/or foetal resorption rates post-implantation, as well as gross physical abnormalities and aberrant growth in the offspring surviving parturition (Thomson et al. 2001). Although the number of studies is large, and markedly different in

their aims, all involve the removal of embryos from, or their conception outside of, their natural environment i.e., the reproductive tract, and subsequent maintenance in artificial culture until ready for transferral to a foster mother (Thomson et al. 2001). A growing body of evidence implicates deregulated genomic imprinting as a mechanism of the abnormal foetal growth and development associated with these methods.

1.5.3.2.1 Growth abnormalities following in vitro culture of the early mammalian embryo

The techniques to come under the spotlight most recently are Human IVF and intra-cytoplasmic sperm injection (ICSI). Typically, fertilized oocytes are maintained by *in vitro* culture up until the 2-4 cell stage before being transferred to a foster mother. Although the number of cases is still too small to draw firm conclusions, several studies have found increased risks of lowered birth weights and birth defect syndromes associated with fertility treatment by these techniques (te Velde et al. 1998; van Steirteghem, 1998; Khosla et al. 2001; Niemitz and Feinberg, 2004; Gicquel et al. 2003). On the other hand, the population seeking fertility treatment is reproductively 'abnormal' to begin with and the increased incidence of birth defects may be a consequence of the same factors associated with the underlying infertility.

Most of the evidence for adverse effects of *in vitro* culture comes from animal cloning experiments. During these procedures the nucleus of a somatic cell is transferred to an enucleated oocyte and the resulting clone is maintained for an extended period of time in culture (McLaren, 2000). Such experiments in sheep and cattle are associated with increased foetal resorption rates, enhanced foetal growth, high birth weights and

malformations of the skeletal and organ systems, collectively referred to as the large offspring syndrome (LOS; Walker et al. 1996; Young et al. 1998; Sinclair et al. 2000). *In vitro* culture of preimplantation mouse embryos, derived from embryonic stem cells as well as somatic cells, is associated with similar abnormalities (Khosla et al. 2001; McLaren, 2000; Sinclair et al. 2000; Nagy et al. 1993; Dean et al. 1998).

1.5.3.2.2 Imprinting as a mechanism of growth abnormalities

In sum, across a wide spectrum of animal species, a stressful environmental condition, *in vitro* culture, during the preimplantation period, or the equivalent in ruminants, is associated with high perinatal death and physical abnormalities in surviving offspring. With regards to the mechanisms of these defects, an extensive body of research has accumulated implicating culture-induced epigenetic defects at imprinted loci.

For example Dean et al. (1998) observed changes in DNA-methylation at the *Igf2, H19, Igf2r* and *U2af1-rs1* imprinted genes in cultured embryonic stem cells. Similar changes, together with aberrant imprinted gene expression, were observed in 13-14 day old foetuses derived from these cells, suggesting that imprinting defects arising in the early embryo persisted into the postimplantation period (Dean et al. 1998). In addition, these epigenetic defects were associated with abnormally large foetuses and various physical abnormalities, including interstitial bleeding, poor mandible development, polyhydramnios and endematous skin (Nagy et al. 1993; Wang et al. 1997; Dean et al. 1998).

A similar study investigated the influence of different culture media on imprinting and subsequent mouse development (Khosla et al. 2001). Two-thirds of the blastocysts cultured in M16 medium supplemented with foetal calf serum (FCS), failed to develop to day 14 foetuses after transfer to foster mothers. These foetuses also displayed reduced growth size in comparison to controls and embryos cultured in the absence of FCS. In addition, the foetuses derived from embryos cultured in the M16+FCS medium showed decreased expression of the *H19* and *Igf2* genes, as well as increased DNA-methylation at the ICR of the former (Khosla et al. 2001). The expression of the imprinted genes G*rb10* and *Grb7* was also affected (Khosla et al. 2001).

Consistent with the idea that specific components of the culture medium may interfere with development and imprinting, Wu et al. (2004) found that *in vitro* exposure of preimplantation mouse embryos to TCDD, a toxic environmental contaminant, was associated with altered DNA-methylation at the *H19* and *Igf2* imprinted genes as well as significant growth retardation in 14 day foetuses, in comparison to controls.

In sum, these studies demonstrate that culture media, and particular components within these media, can deregulate imprinting at multiple growth-related genes and that this deregulation may be a mechanism of cultured-induced abnormalities in foetal growth and development. These findings suggest that imprinting is sensitive to the preimplantation environment and, therefore, could be a mediator of the teratogenic consequences of ethanol exposure during the preimplantation period. This hypothesis could explain a number of features associated with *in vivo* alcohol exposure of preimplantation mouse embryos. First, epigenetic information is not required *per se* during preimplantation, since this is a period characterized by the removal of epigenetic

information and the establishment of pluripotency (Reik et al. 2001). This may explain why alcohol is not directly teratogenic to the preimplantation embryo. Second, disruption of imprinting during the preimplantation period, as a result of alcohol-induced changes in DNA-methylation, would be expected to manifest in growth and developmental abnormalities during the postimplantation period.

1.5.4 An epigenetic model of FASD

In sum, alcohol-induced defects in epigenetic reprogramming are a plausible mechanism of alcohol teratogenesis and potentially reconcile a broad body of findings from the alcohol research field, i.e., the consequences of alcohol exposure during the preconception, preimplantation and gastrulation phases of development (Figure 1.2). In support of this model, alcohol is known to affect methyl metabolism (Halsted et al. 2002) as well as DNA-methyltransferases (Garro et al. 1991; Bielawski et al. 2002).

For example, Garro et al. (1991) found that acute administration of ethanol to pregnant mice during midgestation resulted in genome wide hypomethylation in 11 day old foetuses. Pregnant MF1 mice were dosed with either 50% ethanol (3g/kg) or a caloric equivalent of glucose-saline by gavage on the 9^{th}, 10^{th} and 11^{th} days of pregnancy. Employing a methyl accepting assay, the authors measured the ability of harvested DNA to act as substrate for *Hpa*II methylase. Under saturating conditions of S-Adenosyl-L-methionine (SAM) – a methyl donor – it was found that DNA of foetuses extracted from ethanol fed dams was a significantly better ($p<0.01$) substrate as compared to the control group, suggesting a reduced level of methylation in the former (Garro et al 1991). The

authors also showed that nuclei extracted from the ethanol group had significantly reduced (p<0.001) methylase activity as compared to the control group, suggesting lower levels of DNA-methyltransferase in foetuses harvested from ethanol-fed dams (Garro et al 1991). The mechanism of this effect may be mediated by acetaldehyde, which was found to inhibit DNA-methyltransferase activity by 20% to 90% over a wide concentration range (3 μm to 100 μm) *in vitro* (Garro et al. 1991). In contrast, ethanol did not inhibit DNA-methyltransferase activity *in vitro*, even at very high concentrations (100mM; Garro et al 1991).

The reduction in genome-wide DNA-methylation could be due to inhibition of either *Dnmt1* (a maintenance DNA-methyltransferase) or the *de novo* DNA-methyltransferases, *Dnmt3a* and *Dnmt3b*. Complete inactivation of either group of enzymes results in the abortion of gastrulating embryos (reviewed in Li, 2002).

In another study it was found that absorption of folic acid in the intestine was inhibited in a model of chronic alcoholism (Halsted et al. 2002). Folate is an important source of the 5-methyl group in 5-methyldeoxycytidine observed at CpG dinucleotides, and reductions in this cofactor during critical stages of pregnancy could lead to altered epigenetic reprogramming (Figure 1.2).

Finally, reductions in DNA-methyltransferase RNA in sperm and reduction of offspring weight were observed following chronic alcohol treatment of male rats (Bielawski et al. 2002), supporting the hypothesis that ethanol-induced deregulation of epigenetic programming during gametogenesis may partly explain the preconception effects of ethanol (Figure 1.2).

1.6 AIMS AND RATIONALE

As the above literature review highlighted, the effect of *in utero* alcohol exposure is highly variable. Studies in animal models suggest that differences in timing and dosage may underlie this variability. Three time points are particularly important - preconception, preimplantation and gastrulation – and ethanol treatment has been shown to have deleterious consequences across all three developmental periods. Moreover, the peak periods of teratogenesis correlate with peak periods of epigenetic reprogramming, which is highly suggestive of an epigenetic model of FASD.

Finally, it was argued that the preimplantation period is particularly interesting because (1) genomic imprinting seems particularly vulnerable at this stage and (2) an epigenetic model accounts for all the salient features associated with *in vivo* exposure of preimplantation embryos to ethanol.

Thus, the aim of the present study was to explore the validity of an 'epigenetic model of FASD' by testing the hypothesis that deregulation of imprinting mediates the effect of ethanol on foetal growth, following *in vivo* administration of ethanol during the preimplantation period. As a prelude to a wider analysis of imprinting more generally, it was decided to focus on the *H19/Igf2* domain, for the following reasons: this domain has an established role in foetal growth – a key aspect of alcohol teratogenesis; it is the most intensively studied imprinted domain in the mammalian genome, with unparralled levels of information regarding methylation dynamics over time and between tissues; and the role of this region in the aetiology of Silver-Russell syndrome in humans – a disorder

with superficial similarities to FAS, including growth retardation and distinctive facial features (Bliek et al. 2006). However, it should be emphasized that, insofar as deregulation of epigenetic reprogramming is a mechanism of FASD, other imprinted genes are likely to be involved.

Since previous studies have shown that hypomethylation of the *H19* ICR is associated with growth retardation in humans (e.g. Silver-Russell Syndrome, Bliek et al. 2006) and that methylation occurs preferentially at the paternal allele of the *H19* ICR in mouse (Tremblay et al. 1995; 1997; Warnecke et al. 1998; Weber et al. 2001) it was predicted that alcohol exposure during the preimplantation period would be associated with (1) embryo and placental growth retardation; (2) hypomethylation at the paternal allele of the *H19* ICR; and that (3) the effect of ethanol on embryonic and placental growth would be indirect i.e., would be mediated through its effect on methylation at the paternal allele of the *H19* ICR.

In sum, the specific aims of the study were to expose mouse embryos to ethanol during the preimplantation period and assess the effect on:

1. Foetal and placental weights at 10.5 dpc, as well as the implantation rate.
2. Methylation patterns at the paternal and maternal alleles of the *H19* ICR.
3. To examine whether methylation at the paternal allele of the *H19* ICR mediates the effect of ethanol on embryo and/or placenta weight.

CHAPTER 2: MATERIALS AND METHODS

An experimental study design was employed to test the hypotheses described above, a summarized version of which is presented in Figure 2.1.

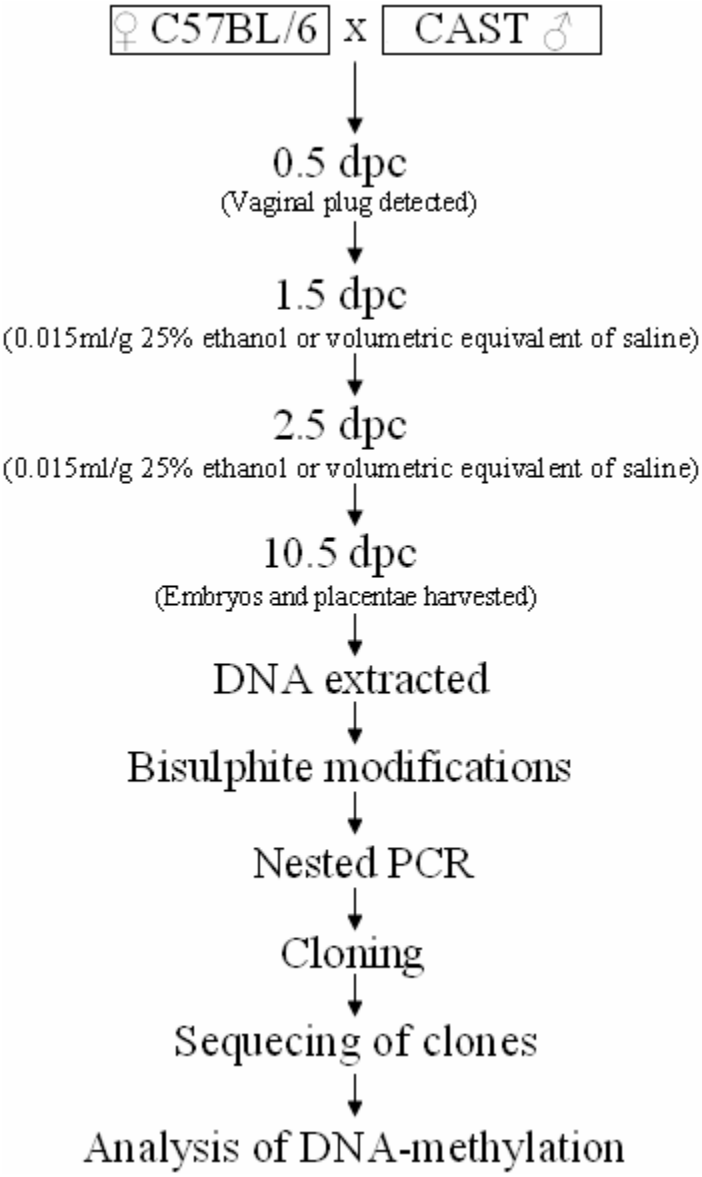

Figure 2.1. Flow diagram of methodology. The C57BL/6 strain served as the maternal genetic source, while the CAST/Ei strain served as the paternal genetic source. The day of vaginal plug detection was scored as 0.5 dpc. Ethanol (0.015ml/g of 25%) or saline (volumetric equivalent) treatment occurred at 1.5 and 2.5 d.p.c; placentae and embryos were harvested at 10.5 d.p.c; extracted DNA was subjected to bisulphite mutagenesis, which was followed by nested PCR, cloning, sequencing and analysis of DNA-methylation; see text for details.

2.1 MATERIALS

2.1.1 Mouse studies

In order to distinguish parental genetic contributions it was necessary to use two different mouse strains. The C57BL/6 strain, purchased from the National Health Laboratory Service (NHLS), served as the maternal genetic source, while the *Mus musculus castaneus* (CAST/Ei) strain, purchased from the Jackson Laboratory, served as the paternal genetic source.

Ten female C57BL/6 mice were randomly assigned to two groups: 'ethanol-treated' and control. It was originally planned that these females would be mated by a single male ('CAST male 1') to keep sources of variation to a bare minimum and because every additional male would have required a doubling of the sample size in order to control for between male differences. However, only a single pregnancy was derived from 'CAST male 1' before he became sterile, necessitating a replacement male. Thus, 'CAST male 2', the male offspring of 'CAST male 1', was used to generate the nine remaining pregnancies.

Ethics approval was granted by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand (AESC clearance number: 2004-86-3; Appendix A). Animals were handled in accordance with the animal care procedures of the Animal Ethics Control Committee (AECC) and the AESC.

2.2 METHODS

The protocols and reagent recipes used in the present study are described in Appendices B and C, respectively, except where these were commercially obtained. It should also be noted that all experimental steps were done at separate locations. In addition, all molecular genetic experimental steps were done using dedicated sets of pipettes. The bisulphite modifications, DNA extractions and DNA digests were done in a separate laboratory, in a separate building, from the cloning and PCR steps. The preparation of PCR reaction mixes was done in a separate room from post-PCR steps.

2.2.1 Mouse treatments

The generation of mouse pregnancies involved the following procedure. A single C57BL/6 female would be placed with a CAST/Ei male in the evening. The presence of a vaginal plug the following morning would be taken as evidence of copulation and that day would subsequently be scored as 0.5 dpc (Figure 2.1). The experimental treatments followed an acute dosage regimen paradigm. Thus, at 1.5 and 2.5 dpc, 0.015ml/g of 25% ethanol (2.9g/kg) was administered to putatively pregnant females (Figure 2.1). A volumetric equivalent of phosphate buffered saline (PBS) was administered to control females (Figure 2.1). At 10.5 dpc, pregnant females were euthenased and their placentae and embryos harvested (Figure 2.1). The amniotic and chorionic sacs were completely dissected from the embryonic and placental tissues and discarded. Great care was taken to exclude maternally derived tissue from the placenta.

Placentae and embryos were subsequently weighed on a regularly calibrated Sartorius scale with 0.001g readability. Three readings was taken per sample to ensure the accuracy of the measurement. The weights reported in the results section are averages of these three weight measurements. Plug-positive females were also monitored for weight gain to ensure that only pregnant females were dissected at 10.5 dpc.

2.2.2 DNA extractions

DNA was extracted from the embryos and placentae using the High Pure PCR Template Preparation Kit (Roche Applied Science) according to the manufacturer's instructions, with the exception that EDTA was added to the elution buffer to a final concentration of 10mM. DNA-concentrations were determined on a Nanodrop ND-1000 spectrophotometer. Given the high costs of the bisulphite mutagenesis and cloning procedures (described below), it was necessary to pool embryonic and placental DNA samples that came from the same mother. Thus, there were 20 pooled DNA samples in total: 10 embryonic and 10 placental DNA samples from 10 mothers.

2.2.3 Restriction enzyme digests

To improve the efficiency of the bisulphite modification, the pooled DNA samples were digested using the *HindIII* restriction endonuclease (New England Biolabs), which cuts outside of the region of interest. Briefly, 5 µg of DNA was digested using 10 units of restriction enzyme at 37^{o}C overnight (12-16 hours) according to the

manufacturer's instructions. Digested DNA was subsequently purified using a phenol:chloroform:isoamyl alcohol (25:24:1) protocol (Appendix B). To increase the amount of precipitated DNA, glycogen was added to the aqueous phase to a final concentration of 0.5-1 μg/μl (Appendix B). DNA was precipitated using a salting out procedure and stored in Tris buffer (pH 8.0) (Appendix B).

2.2.4 Bisulphite modification

Digested DNA samples were subjected to bisulphite mutagenesis using the CpGenome modification kit from Chemicon. The procedure was done according to the manufacturer's instructions, with the exception that the first denaturation step was done in 10mM NaOH and incubated at 96^{o}C for 5 minutes instead of 50^{o}C for 10 minutes. Following addition of the first modification reagent, samples were incubated at 55^{o}C, instead of 50^{o}C, overnight (12-14 hours) in the dark. The method relies on the ability of sodium bisulphite to convert cytosine residues into uracil under conditions whereby methylated cytosine remains unreactive (Clark et al. 1994). The full protocol is described in Appendix B.

2.2.5 Amplification of the *H19* ICR by the polymerase chain reaction

Bisulphite modified DNA was subsequently amplified by two rounds of the polymerase chain reaction (PCR) using a nested set of primers specific for nucleotides 1278 to 1706 in the U19619 genomic contig (GenBank;

http://www.ncbi.nlm.nih.gov/Genbank; Figures 2.2 and 2.3). Nested PCR was employed because of the highly degraded nature of the bisulphite modified genomic DNA.

The region amplified corresponds to the upstream portion of the *H19* ICR and includes 16 and 17 CpG dinucleotides in the C57BL/6 and CAST/Ei strains, respectively (Tremblay et al. 1995; Figures 2.2 and 2.3). The entire *H19* ICR includes four GC-rich DNA-binding sites for CTCF - a zinc finger protein that preferentially binds unmethylated DNA and which is required for the insulator function of the *H19* ICR (Bell et al. 2000; Hark et al. 2000; Kaffer et al. 2000; Kanduri et al. 2000; Szabo et al. 2000; Holmgren et al. 2001; Thorvaldsen et al. 2002; Szabo et al. 2004). The region amplified by the PCR primer set of the present study includes two of these CTCF DNA-binding sites: CTCF1 and CTCF2, located at positions 1359-1402 and 1603-1648 in the U19619 genomic contig, respectively (Tremblay et al. 1995) (Figures 2.2 and 2.3). Methylation occurs at six CpG dinucleotides in the former and five CpG dinucleotides in the latter (Figures 2.2 and 2.3). The genomic context of the region under study, as well as the *H19* ICR as a whole, is illustrated in Figure 2.2.

Table 2.1. The primers used in the present study

Primer ID	Primer sequence	Position in the U19619 genomic contig*
BS_2t1	5'-GAGTATTTAGGAGGTATAAGAATT-3'	1278-1302
BS_1t3	5'-ATCAAAAACTAACATAAACCCCT-3'	1728-1751
BS_2t2	5'-GTAAGGAGATTATGTTTATTTTTGG-3'	1304-1328
BS_1t4	5'-CCTCATAAAACCCATAACTAT-3'	1705-1726
WT_2t1	5'-GAGCATCCAGGAGGCATAAGAATT-3'	1728-1751
WT_1t3	5'-ATCAAGGACTAGCATGAACCCCT-3'	1728-1751

'BS' primers were used for the amplification of bisulphite modified DNA; 'WT' primers were used for the amplification of non-bisulphite modified DNA; the primer sequences were obtained from Tremblay et al. (1997) with modification of position 1711-1714 in BS_1t4, which was mistakenly reported as TAAT instead of AAAA; *http://www.ncbi.nlm.nih.gov/Genbank

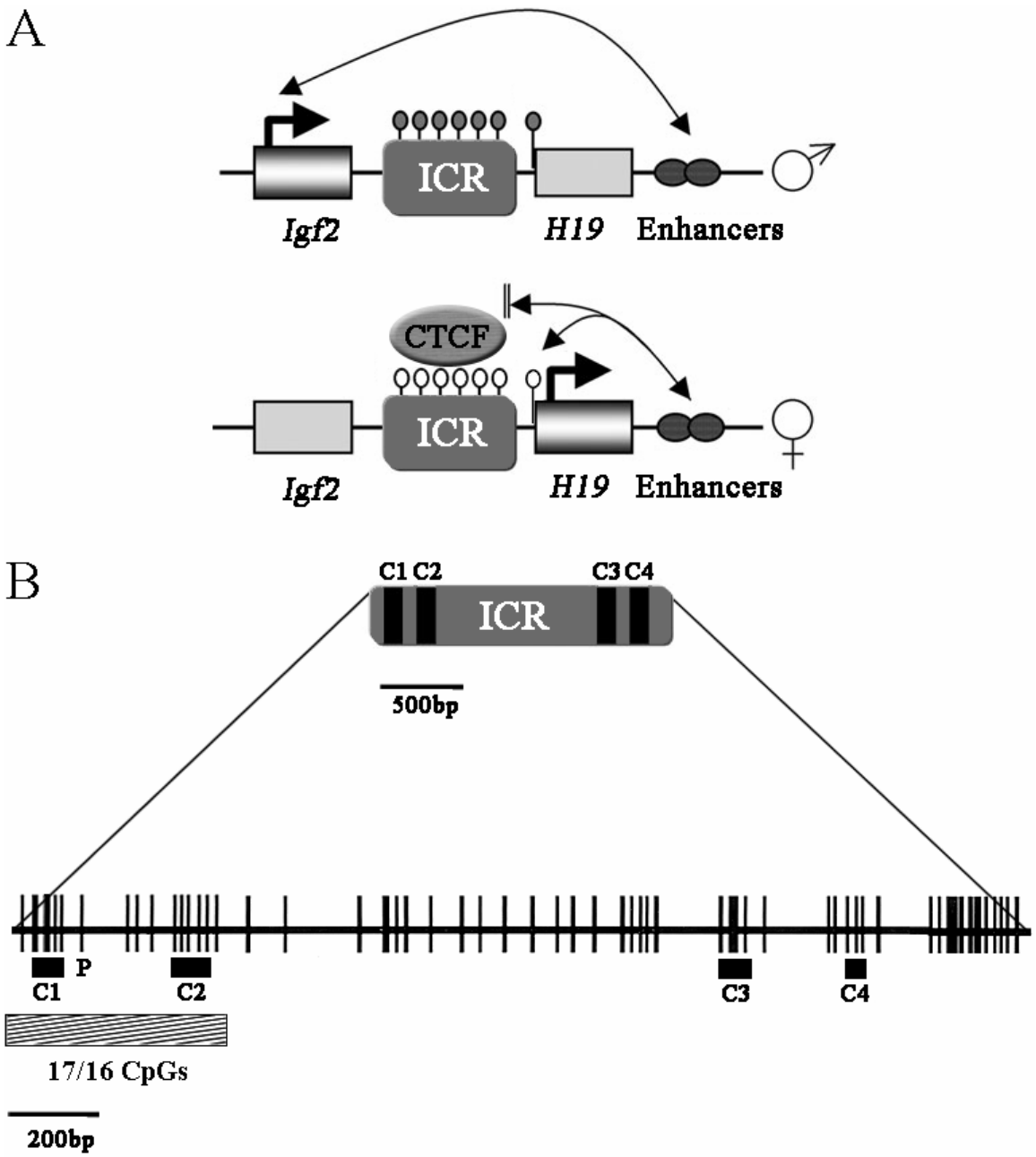

Figure 2.2. The genomic context of the *H19* imprinting control region in mouse. A – the *H19* ICR in the context of the *H19/Igf2* imprinted domain. Filled and unfilled lollipops represent methylated and unmethylated CpG dinucleotides, respectively. Arrows represent transcription start sites of the *Igf2* and *H19* genes; when CTCF binds to the unmethylated maternal allele it blocks access of maternal *Igf2* to enhancer sequences located downstream of *H19;* conversely the insulator function of the *H19* ICR is abrogated by methylation on the paternal allele, blocking CTCF and allowing paternal *Igf2* access to the enhancers (Engel et al. 2006). Thus, paternal *Igf2* is expressed and maternal *Igf2* is silenced. B – Location of CpG dinucleotides (vertical lines) within the *H19* ICR at nucleotides 1330-3147 in the U19619 genomic contig in GenBank (Tremblay et al. 1995). The region contains four CTCF DNA-binding sites (Hark et al. 2000), represented by black boxes: CTCF1, CTCF2, CTCF3 and CTCF4.The hatched box corresponds to nucleotides 1278 to 1706, which is amplified by the primers of the present study, and includes CTCF sites '1' and '2' . This region contains 17 and 16 CpG dinucleotides in the CAST/Ei and C57BL/6 mouse strains, respectively. The CpG site that is specific to the former is labelled 'P'. The figure was derived, with some modifications, from Tremblay et al. (1997) and Engel (2006); C - CTCF.

The first round of PCR included the following primer set: BS_2t1 (forward) and BS_1t3 (reverse) (Table 2.1). The final volume of each first round PCR reaction mix was made up to 50µl in deionised water and included 5-10 µl of bisulphite modified DNA, as well as the following reagents (final concentration indicated in brackets): primers (5 µM each; Inqaba Biotechnical Industries); deoxyribonucleotide triphosphates (128µM each; Bioline); $MgCl_2$ (2mM; Applied Biosystems); 1 unit of AmpliTaq Gold DNA polymerase (0.02 units/µl; Applied Biosystems). First round PCR was carried out in a GeneAmp 2720 thermal cycler (Applied Biosystems) for two cycles at 94°C (4 minutes), 55°C (2 minutes), and 72°C (2 minutes), followed by 35 cycles at 94°C (1 minute), 55°C (2 minutes) and 72°C (2 minutes). One µl of the first round PCR reaction was used as template in the second round, using the following set of nested primers: BS_2t2 (forward) and BS_1t4 (reverse) (Table 2.1). The PCR mix was identical to the first round, with the exception that the final volume was made up to 25µl in deionised water. The thermocycler conditions were also identical, with the exception that the first two cycles were omitted. It should be noted that, given the nature of the bisulphite modification, which induces non-complementarity in DNA, the nested set of PCR primers is specific for the top strand i.e., only a single strand serves as template during PCR.

Figure 2.3. Sequence of the region amplified by the primers of the present study. The sequence shown is the C57BL/6 reference sequence at nucleotides 1278-1751 in the U19619 genomic contig (http://www.ncbi.nlm.nih.gov/Genbank). CpG dinucleotides are bold and highlighted in grey. The positions of the CTCF1 and CTCF2 binding sites are underlined. CTCF1 is closest to the 5' end. A – the sequence before bisulphite modification; B – the sequence following bisulphite modification and PCR amplification; note that the only cytosines present in this sequence are those in the context of CpG dinucleotides. These sites are all methylated in this hypothetical sequence and, as a result, are unaffected by bisulphite mutagenesis. §T-C transition mutation in CAST/Ei; *G-A transition mutation in CAST/Ei.

2.2.6 Cloning of PCR products

The resulting PCR products were cloned using a pGEM T-easy vector system and transformed into competent DH5α cells.

2.2.6.1 Ligation of PCR products into pGEM T-easy vector

Following inspection on 2% agarose gels for successful sample amplification, as well as the absence of contamination in the negative control, PCR products were precipitated using a salting out procedure (Appendix B). Precipitated products were stored in 10 µl of deionised water for a short period of time (never longer than a day) before being ligated into pGEM T-easy vector according to the manufacturer's instructions (Promega). All ligation steps were carried out overnight at 4^{o}C.

2.2.6.2 Transformation of plasmids into DH5α cells

Competent DH5α cells, with transformation efficiencies $\geq 1x10^{8}$ cfu/µg, were prepared according to the rubidium chloride method, described in Appendix B. Fifty µl aliquots of competent DH5α cells were subsequently transformed using the ligation products of pGEM T-easy vector cloning. The transformation procedure is described in more detail in Appendix B. In brief, transformed cells, together with 50 µl of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), were plated on LA plates, containing 100 µg/ml ampicillin, and incubated overnight at 37^{o}C.

2.2.6.3 Isolation and amplification of inserts

Colonies containing the insert of interest were initially identified by blue/white colony screening, with white colonies reflecting the presence of an insert. Presence of the

insert was confirmed by PCR, using the BS_2t2/BS_1t4 set of primers (Table 2.1) and the same protocol used in second round nested PCR (described above). Clones were prepared for amplification according to the following procedure: white colonies were picked using sterile pipette tips and immersed in 50 µl of deionised water in 96-well PCR plates. The plate was subsequently incubated at 96^{o}C for 2-5 minutes. Three µl of the resulting mixture was used as a template for PCR.

2.2.7 Dideoxy chain-termination based sequencing of inserts

Following inspection on 2% agarose gels for successful amplification, as well as the absence of contamination in negative controls, PCR products were filter purified using MultiScreen PCR$_{\mu 96}$ plates, in accordance with the manufacturer's instructions (Millipore). Purified products were resuspended in 20µl of deionised water, 5-6.5µl of which was used as template for dideoxy chain-termination based sequencing. In brief, a 1/8th reaction of BigDye Terminator v3.1 reaction mix (Applied Biosystems), together with reverse primer (BS_1t4, Table 2.1; 5µM), was made up to 10 µl in deionised water and incubated in a GeneAmp 2720 thermal cycler (Applied Biosystems) for 25 cycles at 96^{o}C (30 seconds), 50^{0}C (15 seconds) and 60^{o}C (4 minutes). The resulting cycle sequencing products were filter purified on Montage SEQ$_{96}$ cleanup plates, in accordance with the manufacturer's instructions (Millipore).

Purified cycle sequencing products were resolved by capillary electrophoresis on a 3130*xl* Genetic Analyzer (Applied Biosystems). Sequence data was generated by Ultra-Rapid sequencing using the 36cm capillary array and 3130 POP-7 polymer (Applied

Biosystems). The raw sequence data was subsequently analyzed using Sequencing Analysis v5.2 to generate sequence files in .abi format (Applied Biosystems).

2.2.8 Data cleanup and storage

During the next stage, the relevant sequence information was extracted. This included whether the sequence contained C57BL/6 or CAST/Ei polymorphisms, the amount of 'non-CpG Cs', and the status of each CpG site i.e., whether it was a 'TpG' or a 'CpG'. This information was extracted using a computational approach.

2.2.8.1 Preparation of sequence data for computational analysis

The ABI sequence files were imported into SeqMan (DNASTAR) for multiple sequence alignment so that the following post-sequencing analysis steps could be conducted: sequences were trimmed so that they had the same read length; sequences with poor or low quality reads were discarded; and all CpG dinucleotide sites were checked for correct base calls.

2.2.8.2 Generation of methylation data, scoring of strain specific polymorphisms and calculation of bisulphite conversion error rate

Given the large amount of clone sequence data, e.g. processing 20 clones per sample would generate 400 sequence reads, it was not practical to score clone

methylation and parental origin manually. Thus, a computational approach was taken whereby the status of each CpG dinucleotide, the status of each polymorphic site and the amount of 'non-CpG Cs' was scored automatically using a Python script (written by Andries Oelofse, Bioinformatics Consultant) and subsequently exported into an excel file format.

2.3 STATISTICAL ANALYSES

2.3.1 Descriptive statistics

Since unmethylated cytosine is converted to uracil during the bisulphite mutagenesis procedure, which is subsequently converted to thymine during PCR, the presence of thymine or cytosine can be used to determine the status of methylation at a CpG dinucleotide. Thus, the presence of thymine at a CpG site is scored as 'unmethylated', while the presence of cytosine is scored as 'methylated'. In this way, the status of methylation can be determined for each CpG site for each clone. In addition, by scoring the presence and absence of methylation as '1' and '0', respectively, the total level of methylation in a clone can be determined. For example, the percentage level of methylation for each clone can be calculated by multiplying the average level of methylation (sum of '1s' divided by number of CpG sites) by 100. This procedure was used to calculate the total percentage level of methylation (17 sites in 'paternal clones' and 16 sites in 'maternal clones'), as well as the percentage level of methylation at CTCF sites '1' and '2'. In addition to scoring the level of methylation, the parental origins of

each clone can be determined by the presence or absence of strain specific polymorphisms. Finally, since these sites are always unmethylated, the presence of non-CpG 'Cs' can be taken as an estimate of the bisulphite conversion error rate. Clones with non-CpG 'C' contents >5% were discarded. The raw DNA-methylation data is graphically represented in the results section. Median and mean levels of methylation were calculated and are reported in the results section.

The weight data is reported in mg and, as was described above, is based on averages of three measurements per sample. The average measurements for each sample are reported in Appendix D. Medians, means and standard deviations of weights were calculated and are reported in the results section.

2.3.2 Hypothesis testing

The predictions of the present study were the following: that ethanol administration during the preimplantation period would be associated with (1) growth retardation in embryos and placentae; (2) reduced methylation at the paternal allele of the *H19* ICR; and that (3) the effect of ethanol on embryonic and placental growth would be indirect i.e., would be mediated through its effect on methylation at the paternal allele of the *H19* ICR. Hierarchical linear modelling, loglikelihood ratio tests, Wald-chi-square tests, t-tests and Mann-Whitney U tests were used to test predictions (1) and (2). Partial correlations were used to test prediction (3).

Mann-Whitney U and t-tests of weight and 'paternal methylation' differences, between ethanol-treated and control embryos and placentae, were one-sided because of

the prediction that ethanol would cause retardation and hypomethylation, respectively. Tests of methylation differences on the maternal allele, between the two treatment groups, were two-sided because no effect was predicted.

2.3.2.1 Hierarchical linear modelling

The data of the present study is hierarchically structured i.e., measurements of level-1 units are nested within level-2 units. For example, weight measurements are taken at the level of embryos and their placentae, which are nested within mothers. Similarly, methylation measurements are taken at the level of clones, which are also nested within mothers. As a consequence of this hierarchical data structure, not all observations are independent i.e., measurements that come from the same mother are related. Traditional approaches to this type of dataset include: (1) analyzing at the level of the higher unit e.g., averaging over the weight and methylation measurements that come from the same mother; (2) analyzing at the level of the lower unit e.g., at the level of embryos and placentae or at the level of clones; (3) treating the higher level as an independent variable, with the number of categories equivalent to the number of units e.g., treating 'mother' as a grouping variable with the number of categories equivalent to the number of mothers (Osborne, 2000; Rasbash et al. 2005).

However, all three approaches have their disadvantages: (1) analyzing at the level of aggregated data involves potentially large losses in information, leading to inflated standard errors, reduced effect sizes and a higher type-II error rate; (2) conversely, analysis of non-aggregated data, without taking into account the non-independence of observations, leads to underestimated standard errors, inflated effect sizes and an elevated

type-I error rate; and (3) treating the level-2 unit as a grouping variable is inefficient when the number of level-2 units is large and when there is no natural reference category (Osborne, 2000; Rasbash et al. 2005).

With these limitations in mind, it was decided to employ hierarchical linear modelling (Bryk & Raudenbush, 1992; Goldstein, 1995), which explicitly takes into account the hierarchical structure of such datasets. All analyses were done in MLwiN v2.02 downloaded from the Centre for Multilevel Modelling website (http://www.cmm.bristol.ac.uk/).

Hierarchical linear modelling is an extension of simple linear regression, in which the relationship between two variables is expressed as:

$$\text{(i) } y_i = a + bx_i + e_i$$

where y is the dependent variable; a is the intercept; b is the coefficient or slope of x, the predictor variable; e is the residual variation not explained by the model; and i takes on values '1' to the number of observations. What makes a model hierarchical is the nature of the residual variation. In a variance components model (a restricted form of the hierarchical linear model), the regression equation is rewritten as:

$$\text{(ii) } y_{ij} = a + bx_{ij} + u_j + e_{ij}$$

where a and b are the fixed parts of the model and u_j represents the deviation of the *jth* level-2 unit from the predicted value and e_{ij} represents the deviation of the *ith* level-1 unit, nested within the *jth* level-2 unit, from the predicted value.

The above hierarchical linear model (equation two) was adapted to the present study to test the relationship between treatment (ethanol versus control) and weight, as well as between treatment and DNA-methylation. Thus, in equation two, y corresponds to either weight or DNA-methylation and x corresponds to treatment, a binary predictor variable with ethanol coded '1' and control coded '0'. Moreover, u_j represents the deviation of the *jth* mother from the predicted value and e_{ij} represents the deviation of the level-1 unit, nested within the *jth* mother, from the predicted value. With regards to the weight data, e_{ij} corresponds to the deviation of the *ith* embryo or placenta, nested within the *jth* mother, from the predicted value. For the DNA-methylation data, e_{ij} corresponds to the deviation of the *ith* clone, nested within the *jth* mother, from the predicted value.

The same general strategy was employed to test the hypotheses that treatment predicts (1) embryo weight; (2) placental weight; and (3) DNA-methylation. First, loglikelihood ratio tests compared the full model (equation two) with the reduced model:

$$\text{(iii) } y_{ij} = a + u_j + e_{ij}$$

where only the intercept (a) is taken as a predictor of the dependent variable. A significant test statistic indicates that the full model (equation two) is a significantly better predictor of the dependent variable than the reduced model (equation 3). In the second step, the significance of the coefficient (b) of the treatment predictor term is

assessed by t-tests. These were one-sided with regards to the effect of treatment on embryo weight because of the *a priori* prediction that ethanol would induce embryo-growth retardation. Similarly, the t-tests of the relationship between ethanol and DNA-methylation were also one-sided given the *a priori* prediction that ethanol would induce hypomethylation. However, it should be noted that, given the skew in methylation data, which tends to be either 'high' or 'low', the assumptions of the t-test may be violated and, consequently, invalid. Thus, the loglikelihood ratio test is probably a more reliable indicator of the relationship between treatment and DNA-methylation. For the placental weight data, no *a priori* prediction was made about the direction of ethanol's effect and, consequently, the t-tests are two sided.

As alluded to above, the relationship between treatment and clone methylation can be assessed in a number of ways: (1) at the level of individual CpG sites, (2) at the level of overall methylation within the *H19* ICR, i.e. averaged over the 17/16 CpG sites, and (3) at the level of overall methylation within the individual CTCF DNA-binding regions. With regards to (1), the analysis strategy must be modified to take into account the binary nature (methylated or unmethylated) of the response variable. Thus, a multilevel logistic regression model was utilized to determine whether treatment predicted the status of methylation at individual CpG sites, the significance of which was assessed by Wald chi-square tests. Separate models were constructed for each CpG site.

In addition to the multiple ways in which the methylation data could be analyzed, there were also multiple sets of methylation data: (a) paternal clones in embryos; (b) paternal clones in placentae; (c) maternal clones in embryos; and (d) maternal clones in placentae. It was decided that the relationship between ethanol and the overall level of

methylation (across the 17/16 CpG dinucleotides), as well as between ethanol and the level of methylation at individual CTCF DNA-binding sites, would be assessed in all four datasets (a, b, c and d), using the hierarchical linear modelling strategy described above. Furthermore, it was decided that the relationship between treatment and methylation at individual CpG sites would only be assessed in the datasets in which the level of methylation, at either CTCF '1' or '2', was significantly predicted by treatment. The purpose of this was to determine which CpG sites contributed to the overall relationship between treatment and DNA-methylation.

2.3.2.2 Mann-Whitney U-tests

The statistical analyses described below were conducted in SPSS for Windows v13.0 (SPSS Inc., Chicago IL).

As described above, it was not financially feasible to carry out the bisulphite mutagenesis and cloning procedures for each embryo and each placenta. As a consequence, the weight and methylation data were not analyzed at the same level. The latter was measured in pooled embryonic and placental samples, while the former was measured for each embryo and each placenta separately. Thus, in order to assess the three way relationship between treatment, weight and DNA-methylation, it was necessary to aggregate both datasets to the level of mothers. Thus, average weights were calculated for embryos and placentae from the same mother, resulting in two sets of weight data: 10 embryonic and 10 placental weight estimates, corresponding to 10 mothers. Similarly, averages were taken of the clone-level methylation data to obtain aggregated estimates

for each mother, resulting in four sets of data, corresponding to 10 mothers: 10 estimates of paternal methylation in embryos; 10 estimates of paternal methylation in placentae; 10 estimates of maternal methylation in embryos; and 10 estimates of maternal methylation in placentae. The resulting datasets were initially analyzed separately, to determine whether any substantial loss in information or bias was incurred by aggregating the data.

The aggregated data is continuous and distributed in two groups (ethanol and control). This type of data is traditionally analyzed using t-tests, which assume that the dependent variables i.e., weight and DNA-methylation, are normally distributed in both treatment groups. However, given the small sample (N=5 per treatment group), a normal distribution cannot be assumed. Moreover, the skew in the methylation data, alluded to above, is a further violation of the t-test assumptions. Thus, it was decided to employ Mann-Whitney U exact tests, which are the non-parametric equivalents of the t-test.

2.3.2.3 Correlations between weight, treatment and DNA-methylation

The two-way relationships between weight, methylation and treatment were subsequently assessed, non-parametrically, by calculating Spearman's correlation coefficients, except in cases of tied ranks, in which case Kendall's tau-b was used instead. The two way correlations were followed up by partial correlations of the relationship between weight and treatment, controlling for the effect of methylation on the former (Figure 2.4). By comparing the resulting reduction in the correlation coefficient between treatment and weight, it is possible to assess whether this relationship is wholly, or in part, dependent on methylation (Figure 2.4). For example, if the correlation coefficient is

reduced to zero, this would indicate that the relationship between ethanol and weight is entirely dependent on methylation (Figure 2.4). This would be consistent with the hypothesis that ethanol indirectly affects weight, i.e. affects weight via its effect on methylation (Figure 2.4). Alternatively, if the correlation coefficient remained the same, this would indicate that the relationship is entirely independent of methylation (Figure 2.4).

To determine whether the reduction in the correlation between treatment and weight i.e., the indirect effect, was significant, 95% confidence intervals were calculated using a nonparametric bootstrapping procedure described by Preacher and Hayes (2004).

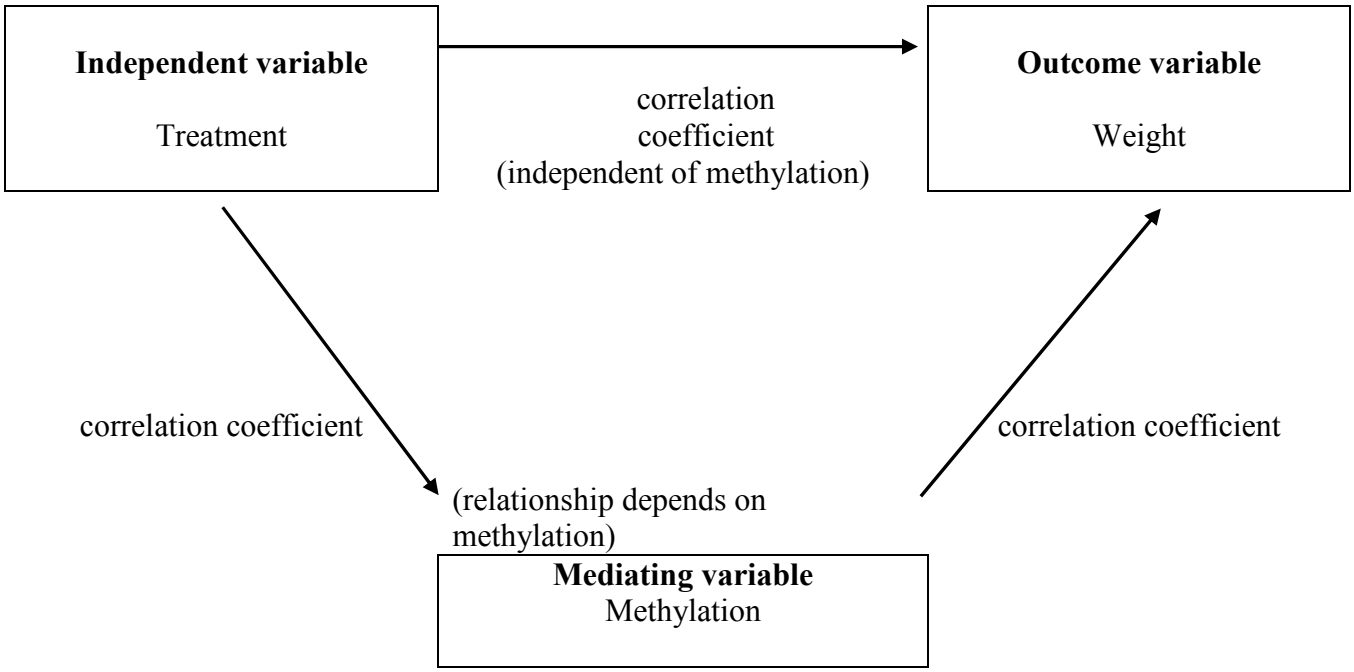

Figure 2.4. Partial correlations of the relationship between treatment and weight, controlling for the effect of DNA-methylation on the latter. The size of the reduction in the correlation coefficient between treatment and weight is an indication of the importance of methylation in mediating the relationship. There are three possibilities: (1) ethanol entirely depends on methylation for its effects on weight; (2) ethanol is entirely independent of methylation for its effect on weight; and (3) ethanol is partly dependent on methylation for its effect on weight.

CHAPTER 3: RESULTS

The aim of the present study was to test the predictions that ethanol administration during the preimplantation period would be associated with: (1) growth retardation in postimplantation embryos and placentae; (2) reduced methylation at the paternal allele of the *H19* ICR in postimplantation embryos and placentae; and that (3) the effect of ethanol on embryonic and placental growth would be indirect i.e., would be mediated through its effect on methylation at the paternal allele of the *H19* ICR. To test the predictions, the weight data were analyzed at the level of both individual embryos/placentae and mothers. The methylation data were also analyzed at two different levels: the level of individual clones and mothers. As described in the methods section, the analyses at the level of mothers were required in order to test the relationship between weight and methylation and, thereby test prediction (3).

3.1 MOUSE EXPERIMENTS

In total, dissections of ten mothers (five controls and five ethanol-treated) at 10.5 dpc yielded 81 embryos and their placentae (42 controls and 39 ethanol-treated); nine resorptions (two controls and seven ethanol-treated). The total number of successful implantations was 90 (44 controls and 46 ethanol-treated).

3.1.1 Weight

The mean embryo weights for the control and ethanol treatment groups were 48.4mg (SD=17.2mg) and 19.8mg (SD=6.5mg), respectively. The average embryo weights were similar across mothers within the same treatment group, with the exception of mother C5 who was a clear outlier (Figure 3.1).

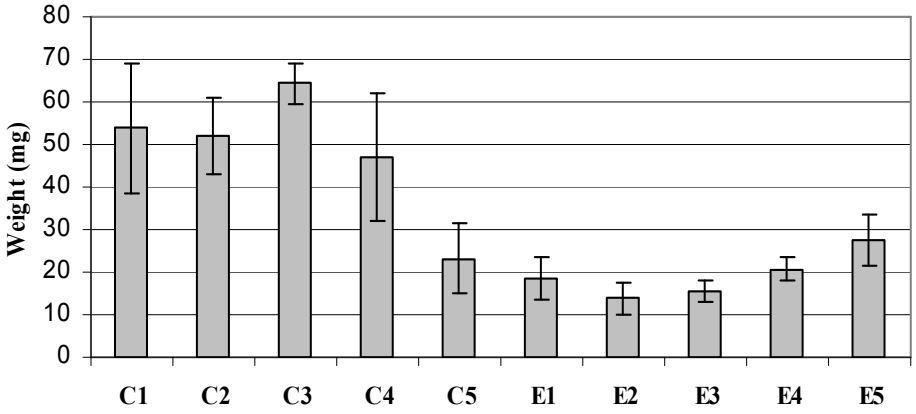

Figure 3.1. Mean embryo weights and standard deviations across ten mothers. C = control mother, E = ethanol-treated mother

The mean placentae weights for the control and ethanol treatment groups were 50.5mg (SD=16.5mg) and 33.4mg (SD=12.2), respectively. The mean weights of placentae for the control and ethanol-treated mothers were consistently >40mg and <40mg, respectively (Figure 3.2). Due to incomplete dissection of the amniotic and chorionic sacs, the weights of the E1 placental samples were not analyzed.

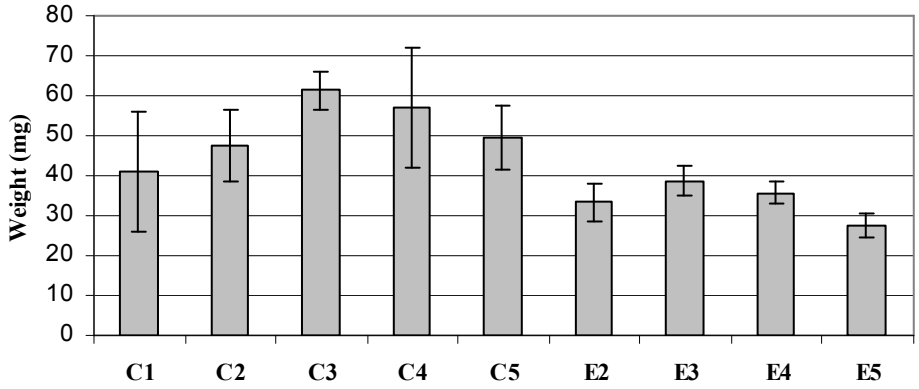

Figure 3.2. Mean placenta weights and standard deviations across nine mothers. C = control mother, E = ethanol-treated mother; E1 was excluded from the weight analyses because of incomplete dissection of the amniotic and chorionic sacs.

3.1.1.1 Hierarchical linear modelling of the relationship between weight and treatment

Hierarchical linear modelling was used to explore the relationship between individual weights and treatment (Table 3.1). Loglikelihood ratio tests, as well as t-tests, indicate that treatment type (ethanol versus control) is a highly significant predictor of the differences in weight between ethanol-treated and control placentae and embryos (Table 3.1). In addition, hierarchical linear modelling indicates that 29.2% and 79.7% of the variation in placenta and embryo weights, respectively, is structured between mothers and that most of this variation is explained by treatment type (Table 3.1).

Table 3.1. Hierarchical Linear Modelling of the Relationship Between Ethanol and Weight in Embryos and Placentae

Dependent Variable	ΔLoglikelihood[b]	Beta	SE	t	% Variation Structured Between Mothers	% Variation Between Mothers Explained by Ethanol
Placenta Weight	9.691**	-17.159	4.223	4.063***	29.2	83.5
Embryo Weight	10.923***	-28.799	6.481	4.444***	79.7	68.5

[a]$p<0.10$, *$p<0.05$, **$p<0.01$, ***$p<0.001$; [b]change in the loglikelihood statistic upon addition of the ethanol predictor term to the null model; beta represents average difference in weight (mg) between ethanol-exposed and control embryos

As explained in the methods section, to analyze the relationship between weight and DNA-methylation it was necessary to aggregate the embryos and placentae that came from the same mother. The reason for this was that methylation was analyzed in pooled DNA samples, while weights were analyzed for individual embryos and placentae separately. Thus, in order to compare the two sets of data, they had to be aggregated to the same level of analysis i.e., the level of mothers.

Thus, average embryo and placenta weights were calculated for each mother. As indicated by hierarchical linear modelling, most of the variation in embryo weights was structured between mothers (79.7%) and thus very little information is lost by aggregating over embryos (Table 3.1). In contrast, most of the variation in placenta weights was not structured between mothers (29.2%), indicating a substantial loss in information. Nevertheless, comparisons of the aggregated weights between control and ethanol-treated groups revealed similar differences, indicating that much of the within mother variation is redundant for both embryos and placentae (Table 3.2). For example,

the median difference in weight for ethanol and control embryos and placentae was 33.75mg and 14.87mg, respectively, which is similar to the average differences reported for the non-aggregated data: 28.8mg and 17.2mg for embryos and placentae, respectively (Tables 3.1 and 3.2).

Mann-Whitney U tests confirmed that the differences between ethanol-treated and control embryos and placentae remained significant after aggregating the weight data (Table 3.2). As would be expected, given the small sample size (N=10), these significant differences were associated with large effect sizes (r = 0.760 and 0.816 for embryos and placentae, respectively; Table 3.2). Similarly to the analysis of the non-aggregated data, most of the variation in weight between mothers is explained by treatment type (both R^2s > 0.55; Table 3.2).

Despite the significant differences in embryo and placenta weights, between the control and ethanol-treated mothers, the number of successful implantations per treatment group was very similar (p>0.60, two-tailed; Table 3.2). In addition, the resorption rate seemed unaffected by treatment-type, although this was numerically greater in the ethanol-exposed mothers (p>0.10, one-tailed; Table 3.2). The absence of a significant difference in the resorption rate could be due to a lack of power because the estimated effect size is reasonably large (r=0.493) and 24.3% of the variation in embryo resorptions is potentially explained by treatment type (Table 3.2).

Table 3.2. Median Weights of Aggregated Embryos and Placentae as well as Median Number of Resorptions and Implantations in Ethanol-Exposed Mothers and Controls

Dependent Variable	Median Ethanol	Median Control	U	Z	r	R^2
Embryo Weight	18.06	51.81	1**	-2.402	-0.760	0.577
Placenta Weight	34.51	49.38	0**	-2.449	-0.816	0.667
Number of Resorptions	1.5	0	5.5	-1.560	-0.493	0.243
Number of Implantations	9	9	8.5	-0.949	-0.300	0.090

[a] $p<0.10$, *$p<0.05$, **$p<0.01$

3.2 MOLECULAR GENETIC ANALYSES

3.2.1 Confirmation of strain-specific DNA polymorphisms

As was expected, the T-C and G-A transitions described in previous studies (Figure 2.3; Tremblay et al. 1997) were observed in the C57BL/6 and CAST/Ei strains. However, sequencing of non-bisulphite modified DNA from a CAST/Ei male and C57BL/6 female, as well as the pooled embryonic and placental samples, revealed the presence of two additional strain-specific polymorphisms (Figure 3.3; Table 3.3). These were, with the position in the U19619 genomic contig indicated in brackets: a G indel in a string of six Gs (1501-1506) (deletion absent in the C57BL/6 strain) and an A-G transition (1654) (A in C57BL/6) (Figure 3.3).

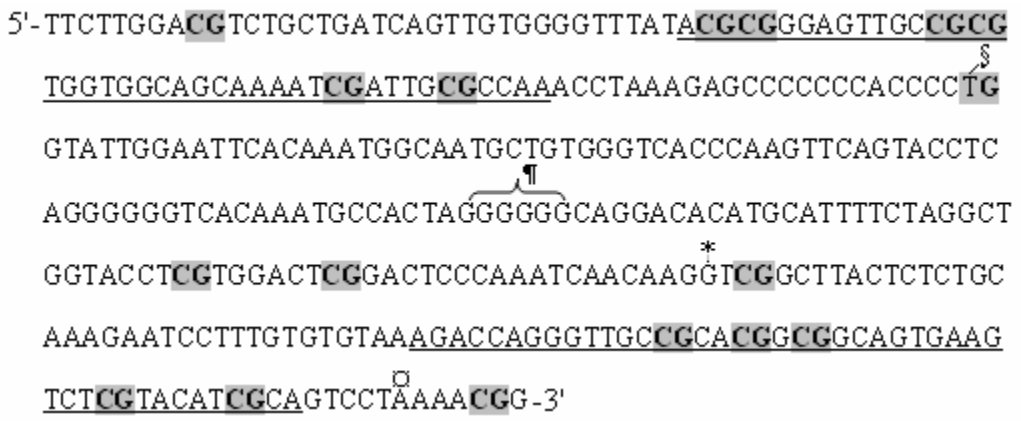

Figure 3.3. Confirmation of four strain-specific DNA polymorphisms, two reported here for the first time. The sequence shown corresponds to the C57Bl/6 strain sequenced in the present study. The new mutations are indicated by symbols: ¤A-G transition; ¶G indel. The previously identified variants were §T-C transition; *G-A transition.

Table 3.3. Strain-specific polymorphisms

Polymorphism	C57BL/6	CAST/Ei	Position*	Reported previously
T-C	T	C	1506	yes
G indel	six Gs	five Gs	1501-1506	no
G-A	G	A	1566	yes
A-G	A	G	1654	no

*in the U19619 genomic contig (http://www.ncbi.nlm.nih.gov/Genbank)

3.2.2 Analyses of DNA-methylation

Of 594 clones sequenced during the course of this study, 257 (43%) were discarded, leaving 337 clones in total for the analyses. Criteria for excluding a clone included the following: a non-conversion error rate of unmethylated cytosine to thymine >5%, which indicated inefficient bisulphite mutagenesis, and whether any ambiguities existed in the parental origin of a clone i.e., the presence of a paternal and maternal sequence variant on the same clone. The latter criterion accounted for about 25% of the discarded clones. These ‘mosaic clones’ are likely to have arisen as a result of ‘template

switching' – a PCR artefact (e.g. Ford et al. 1994; Yu et al. 2003) - during nested PCR of bisulphite modified DNA. Since this phenomenon is only evident following PCR of non-clonal DNA templates, the mosaic clones could also be an indicator of contamination of clone DNA with spurious ligation products.

The number of clones representing each treatment group was evenly split, with 144 and 148 coming from ethanol-exposed and control samples, respectively. In total, 165 and 127 clones were inherited paternally and maternally, respectively, indicating a slight bias in favour of the former. The clones not accounted for by treatment group came from two parental controls – a C57Bl/6 female (23 clones) and a CAST male (22 clones). Figure 3.4 describes the number of clones according to sample origin (placenta, embryo, or internal control), treatment type (ethanol or control), and parental origin (maternal or paternal).

There was major variability in terms of the number of clones representing the embryonic and placental samples for each mother. The average embryonic sample was represented by 8.6 clones but ranged from 1 to 27, while the average placental sample was represented by 6.9 clones with a range of 3 to 20.

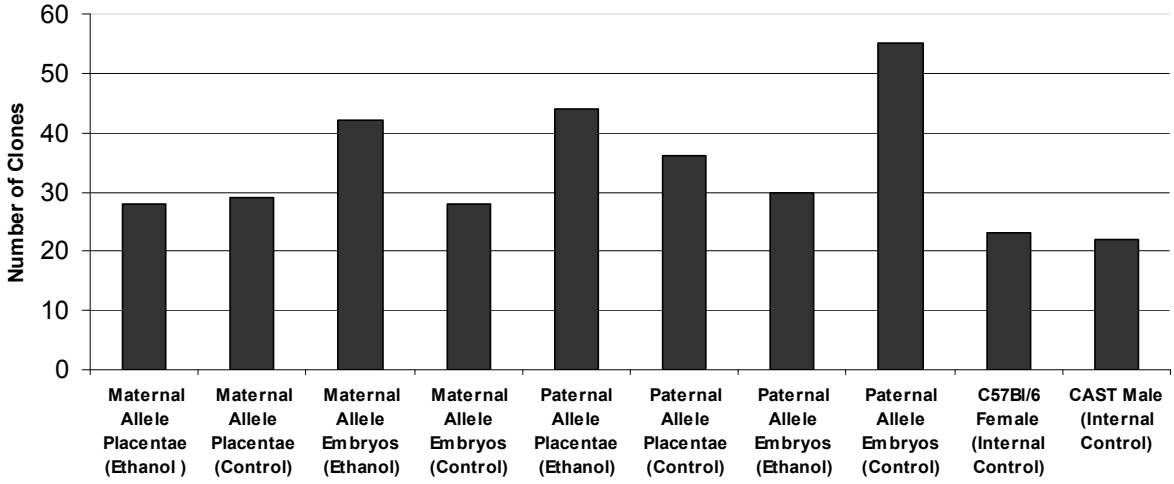

Figure 3.4 Number of clones distributed by sample origin, treatment type and parental origin

3.2.2.1 Visual inspection of methylation profiles

Figures 3.5 and 3.6 describe the DNA-methylation profiles for ten embryonic and ten placental samples from five control and five ethanol-treated mothers, constructed from 292 clone sequences. Figure 3.7 describes the DNA-methylation profiles of a C57BL/6 female and CAST/Ei male, constructed using 23 and 22 clones, respectively. For one control mother and one ethanol-treated mother, no clones were available for analysis of maternal DNA-methylation in embryos.

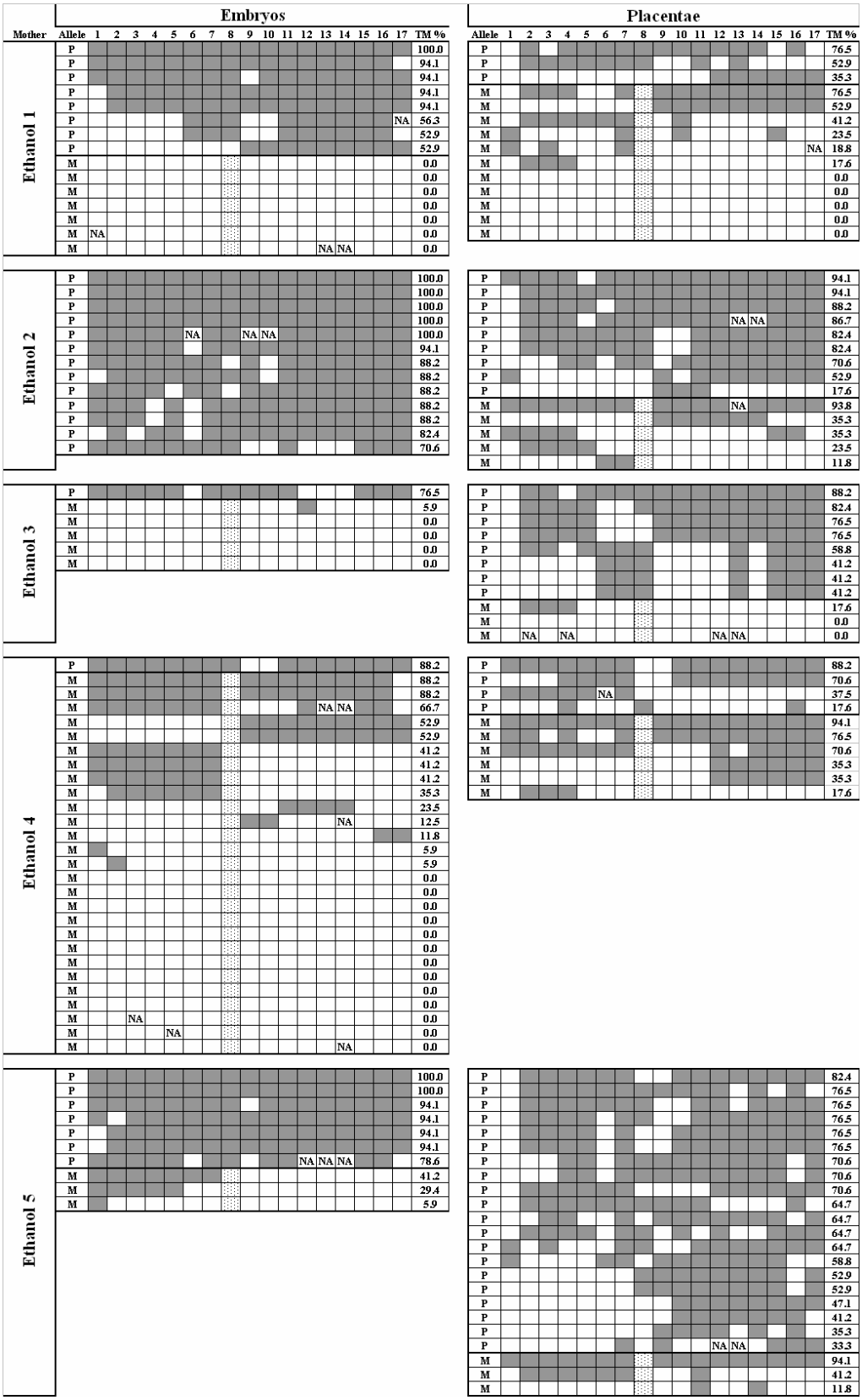

Figure 3.5. Analysis of DNA-methylation at 17 CpG sites in the H19 Imprinting Control Region in ten embryonic and ten placental samples from five ethanol treated mothers. Grey and white blocks represent methylated and unmethylated CpG sites, resepectively. Site 8 is not a CpG dinucleotide in the C57Bl/6 strain and, consequently, is not analyzed on the maternal allele; TM - total methylation, M - maternal allele, P - paternal allele; NA indicates CpG sites that could not be analyzed due to poor sequence data; each row preceded by an M or P represents a single clone.

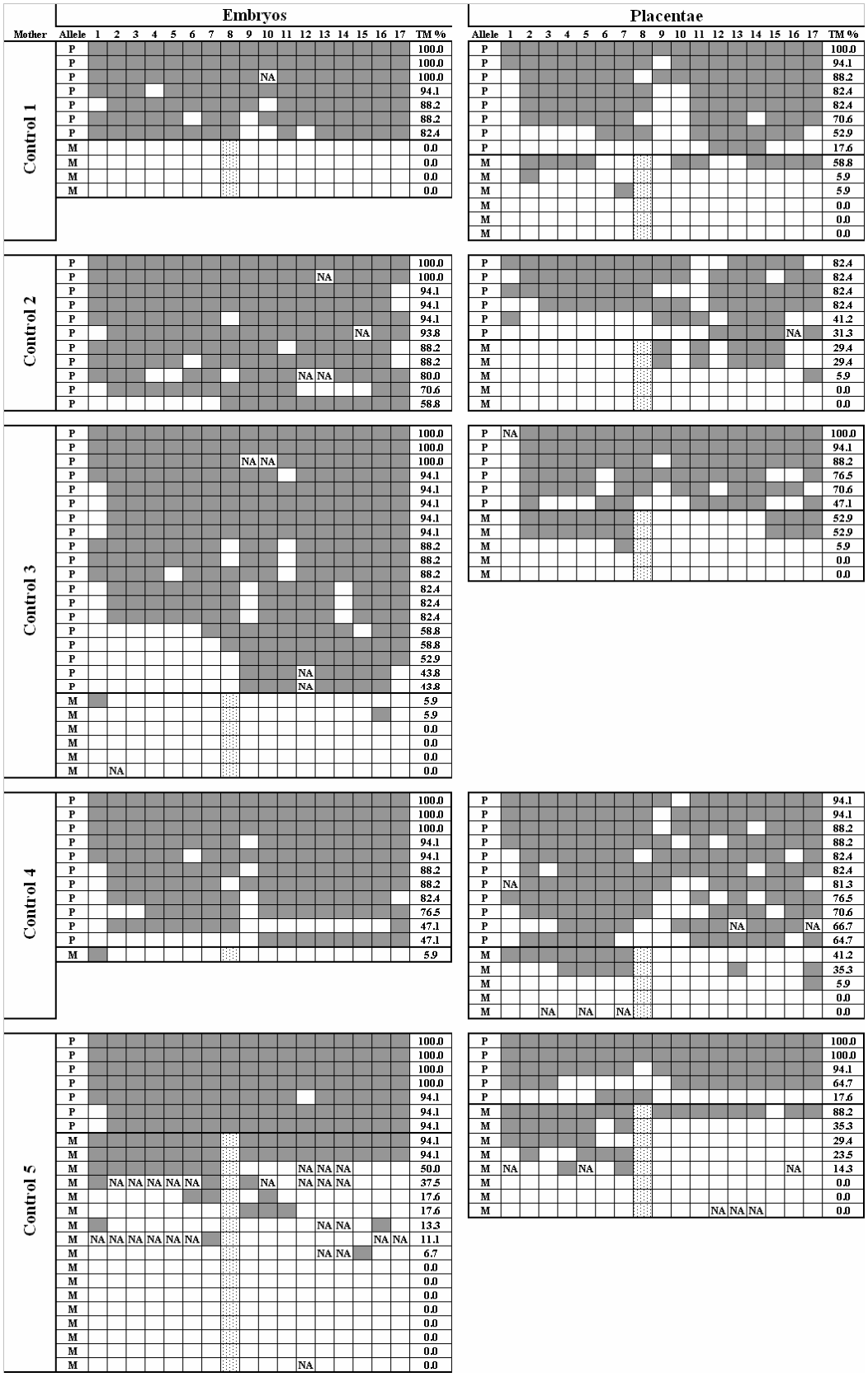

Figure 3.6. Analysis of DNA-methylation at 17 CpG sites in the H19 Imprinting Control Region in ten embryonic and ten placental samples from five control mothers. Grey and white blocks represent methylated and unmethylated CpG sites, resepectively. Site 8 is not a CpG dinucleotide in the C57Bl/6 strain and, consequently, is not analyzed on the maternal allele; TM - total methylation, M - maternal allele, P - paternal allele; NA indicates CpG sites that could not be analyzed due to poor sequence data; each row preceded by an M or P represents a single clone.

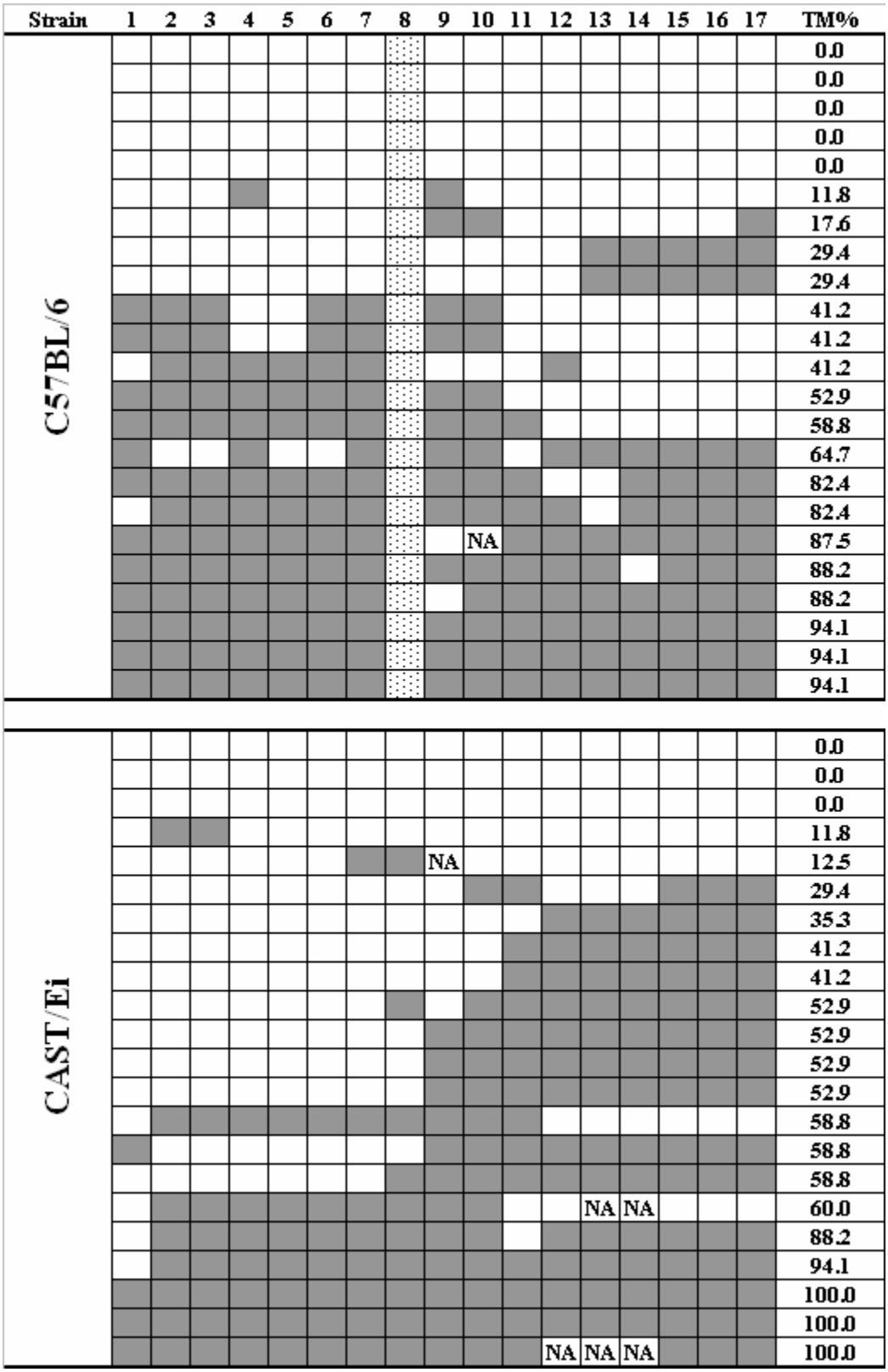

Figure 3.7. Analysis of DNA-methylation at CpG sites in the H19 imprinting control region in the CAST/Ei and C57BL/6 mouse strains. 17 sites are present in the CAST/Ei strain, while 16 sites are present in the C57BL/6 strain. Grey and white blocks represent methylated and unmethylated CpG sites, resepectively. TM - total methylation; NA indicates CpG sites that could not be analyzed due to poor sequence data; each row represents a single clone.

Visual inspection of the DNA-methylation profiles of clones from control and ethanol-treated embryos revealed high correlations with parental origin i.e., maternal and paternal alleles are clearly hypomethylated and hypermethylated, respectively (Figures 3.5 and 3.6).

The relationship between parental origin and DNA-methylation is less striking in the placentae of both control and ethanol-treated mothers (Figures 3.5 and 3.6). Although the pattern is similar to embryos, paternal and maternal alleles being predominantly hyper- and hypomethylated, respectively, several maternal and paternal clones are characterized by blocks of hyper- and hypomethylation, respectively (Figures 3.5 and 3.6). These 'relaxed' methylation profiles are particularly striking in the placentae from 'ethanol 5' and 'ethanol 3', in which large blocks of demethylation are apparent on the paternal allele (Figure 3.5).

Another striking pattern is the almost complete absence of DNA-methylation at CpG site '1' in paternal clones from ethanol-treated placentae but not embryos (Figure 3.5). Smaller blocks of demethylation are also apparent in placentae from 'Ethanol 4', 'Ethanol 2', 'control 1' and 'control 2' (Figures 3.5 and 3.6). Contrary to expectation, a number of maternal clones show blocks of hypermethylation (Figures 3.5 and 3.6).

3.2.2.2 Distribution of % DNA-methylation across 17/16 CpG sites

Overall, the methylation profiles of placentae seem 'patchier' and less 'block-like', in comparison to embryos, and this pattern seems particularly evident in placentae from ethanol-treated mothers relative to controls (Figures 3.5 and 3.6).

Analysis of the distribution of % DNA-methylation revealed a 'bell-shaped' profile, with a long lagging tail, for paternal alleles from placentae, which is in stark contrast to the strongly right skewed profile of paternal alleles from embryos (Figures 3.8a and 3.8b). The distribution of paternal DNA-methylation in ethanol-exposed placentae is shifted to the left, relative to controls, as evidenced by the placement of the 'ethanol-treated' mode at 76.5% (versus 82.4% in the controls) and the absence of 100% methylated paternal clones from the ethanol-treatment group (Figure 3.8a).

On the maternal allele, the distribution of % DNA-methylation was clearly hypomethylated in both placentae and embryos (3.8c and 3.8d). This pattern was particularly striking in embryos, in which >50% of clones were fully unmethylated in both ethanol-treated and control groups (Figure 3.8d). In placentae, although the mode was also positioned at 0% methylation, the overall profile was 'shallower', and less 'left-skewed'. This 'relaxed' methylation profile was more pronounced in placentae from ethanol-exposed mothers (Figure 3.8c).

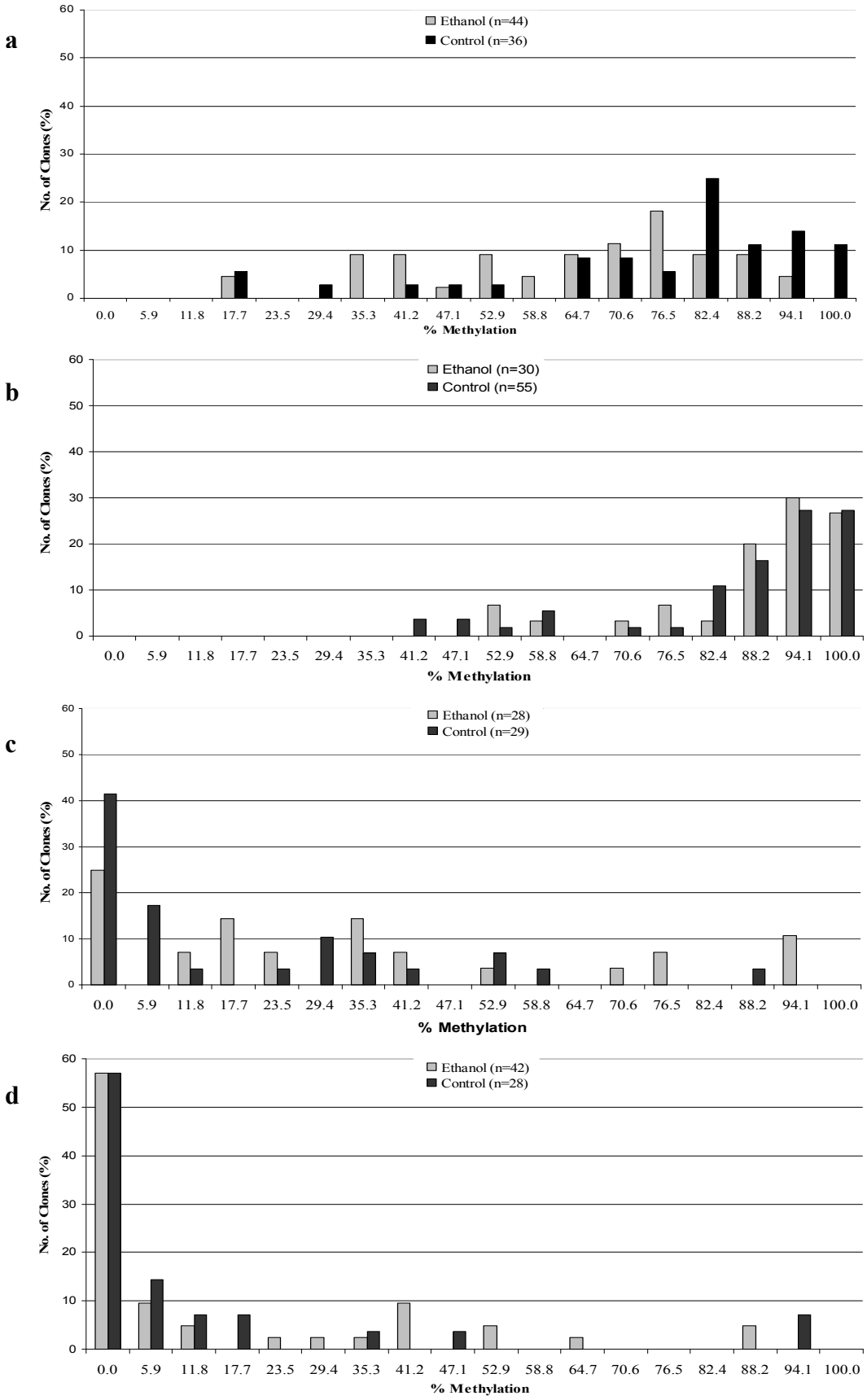

Figure 3.8. Distribution of DNA-methylation in the H19 imprinting control region. Percentage methylation based on 17 CpG sites for paternal alleles and 16 CpG sites for maternal alleles; n = number of clones; a - paternal alleles from placentae; b- paternal alleles from embryos; c - maternal alleles from placentae; d - maternal alleles from embryos; n = number of clones.

3.2.2.3 Distribution of % DNA-methylation at the CTCF1 and CTCF2 sites

When the *H19* ICR is broken down into CTCF sites '1' and '2' (CpG dinucleotides 2-7 and 12-16, respectively) some striking asymmetries within the region become apparent (Figure 3.9). For example, >50% of paternal clones are fully (100%) methylated at the CTCF2 site in both ethanol-treated and control placentae, making this distribution 'right skewed' (Figure 3.9a). Similarly, >50% of paternal clones from control placentae are fully methylated at the CTCF1 site (Figure 3.9a). However, in stark contrast to these distributions, paternal methylation at the CTCF1 site in ethanol-treated placentae is 'plateau-like' and almost U-shaped (Figure 3.9a).

In embryos, paternal methylation is highly skewed to the right, with >50% of clones carrying full methylation profiles at CTCF sites '1' and '2' (Figure 3.9b). In contrast, these sites are completely unmethylated in approximately 80% of maternal clones in both ethanol-treated and control embryos (Figure 3.9d). Similarly, almost 80% of maternal clones from control placentae are completely unmethylated at the CTCF2 site, while slightly less than 60% of clones from ethanol exposed placentae show complete demethylation at this site (Figure 3.9c). The distribution of the latter is slightly 'U-shaped', with approximately 25% of maternal clones being completely methylated (Figure 3.9c). There were fewer maternal clones from ethanol-treated and control placentae that were completely unmethylated at the CTCF1 site (both <60%; Figure 3.9c).

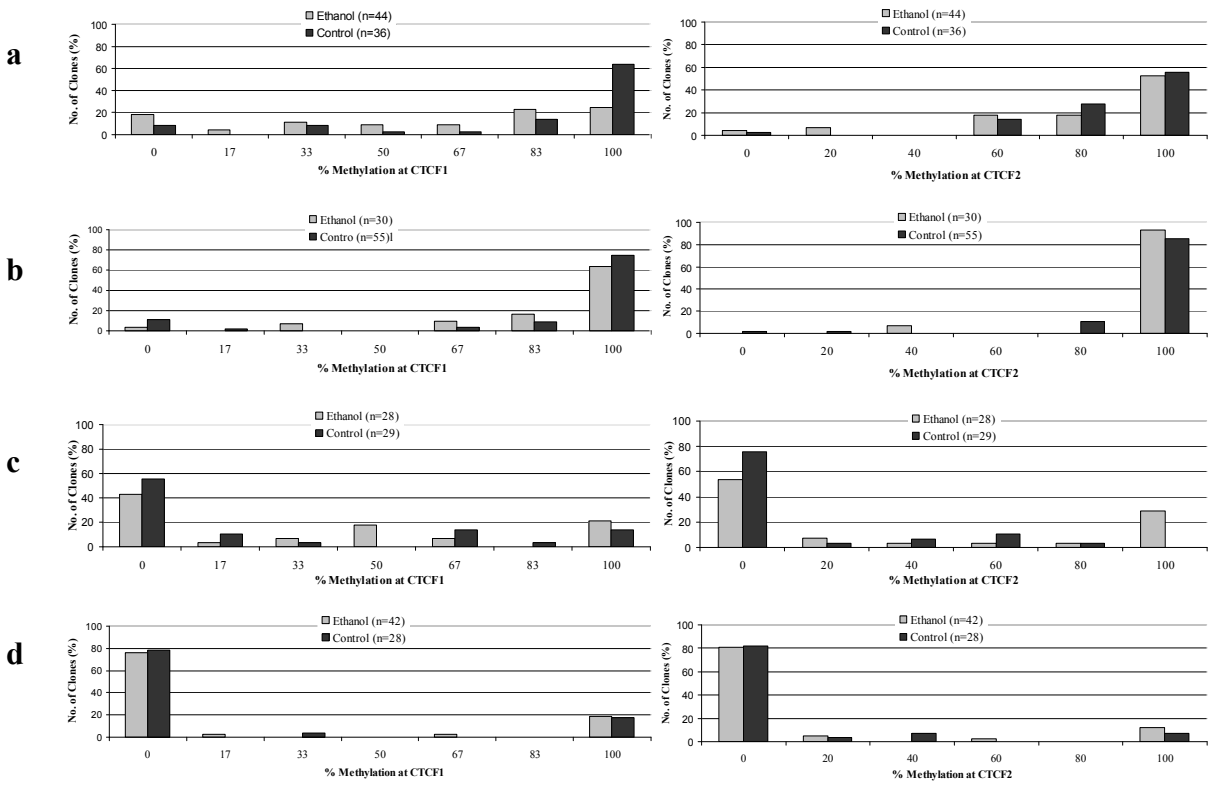

Figure 3.9. Distribution of DNA-methylation at two CTCF DNA-binding regions within the H19 Imprinting Control Region. Figures on the left correspond to CTCF1 (CpG sites 2-7); figures on the right correspond to CTCF2 (CpG sites 12-16); a - paternal alleles from placentae; b - paternal alleles from embryos; c - maternal alleles from placentae; d - maternal alleles from embryos; n = number of clones.

3.2.2.4 Hierarchical linear modelling of DNA-methylation

Hierarchical linear modelling was used to test the relationship between DNA-methylation at the level of clones and treatment (Table 3.4). Loglikelihood ratio tests indicated that the treatment predictor term (ethanol versus control) significantly improved a hierarchical linear model of the relationship between the latter and total paternal DNA-methylation in placentae ($p<0.05$; Table 3.4). Moreover, paternal clones from ethanol exposed placentae were significantly less methylated (12% on average, $p<0.05$) than paternal clones from control placentae (Table 3.4). Loglikelihood ratio tests also revealed

a significant improvement in a model of paternal DNA-methylation at the CTCF1 site in placentae, upon addition of a treatment predictor term ($p<0.01$; Table 3.4). A highly significant difference in paternal DNA-methylation at this site, between ethanol-exposed and control clones, was also observed (Table 3.4). The former carried, on average, 22% less methylation than the latter ($p<0.01$; Table 3.4). Hierarchical linear modelling suggests that the treatment predictor term explains 100% of the between-mother variation in paternal DNA-methylation in placentae (Table 3.4).

The treatment predictor term was not found to significantly improve any other models of DNA-methylation, although trends for an improvement were observed for total maternal methylation, as well as methylation at the CTCF2 site, in placentae (Table 3.4). Moreover, t-tests indicated significant relationships between treatment and DNA-methylation in both of these models (in each case $p<0.05$; Table 3.4). Total methylation and methylation at the CTCF2 site were elevated (+16% and +25%, respectively) on the maternal allele in ethanol-exposed placentae relative to controls (Table 3.4). However, as stated in the methods section, the results of the t-test may be unreliable due to the skewed nature of methylation data.

The treatment predictor term was found to explain 69% and 49% of between-mother variation in total and CTCF2 DNA-methylation, respectively (Table 3.4). However, hierarchical linear modelling revealed that the vast majority of variation in DNA-methylation was not structured between mothers. Instead, it was almost entirely structured between clones within mothers (Table 3.4). This is perhaps unsurprising, considering the highly variable number of clones available for each mother (Figures 3.5 and 3.6). Despite this, there was evidence for the existence of a small amount of structure

at the level of mothers (Table 3.4). This was particularly striking at the maternal allele of the CTCF2 site in placentae, in which 21.8% of the variation was structured between mothers (Table 3.4).

Table 3.4. Hierarchical linear modelling of the relationship between ethanol and DNA-methylation in the *H19* imprinting control region

Dependent Variable	ΔLoglikelihood ratio statistic[b]	Beta	SE	t	% Variation Structured Between Mothers	% Variation Between Mothers Explained by Treatment
Paternal Allele Placentae						
TM	6.141*	-12.018	4.641	2.59*	2.8	100.0
CTCF1	6.492**	-22.391	7.772	2.881**	5.4	100.0
CTCF2	1.457	-6.868	5.664	1.213	0.0	0.0
Paternal Allele Embryos						
TM	0.031	0.76	4.111	0.185	4.8	7.8
CTCF1	0.015	0.907	7.039	0.129	1.0	16.3
CTCF2	0.146	1.455	3.805	0.382	0.0	0.0
Maternal Allele Placentae						
TM	3.649[a]	15.684	7.752	2.02*	9.4	69.3
CTCF1	0.796	9.529	10.588	0.9	1.0	0.0
CTCF2	3.493[a]	24.65	11.936	2.07*	21.8	48.7
Maternal Allele Embryos						
TM	0.095	2.42	7.865	0.308	5.8	2.4
CTCF1	0.077	3.349	11.947	0.28	4.2	0.0
CTCF2	0.235	4.05	7.464	0.543	1.2	100.0

Note: beta = change in % DNA-methylation in ethanol group relative to control group; TM - % DNA-methylation measured across 17 CpG sites on the paternal allele or 16 CpG Sites on the maternal allele; [b]change in the loglikelihood statistic upon addition of the treatment predictor term to the null model; [a]$p<0.10$, *$p<0.05$, **$p<0.01$

To determine the origins of the significant differences described above, the relationship between treatment and DNA-methylation at individual CpG dinucleotides

was explored using 'two-level random intercepts logistic modelling', the results of which are shown in Table 3.5.

Table 3.5. Multilevel logistic modelling of the relationship between DNA-methylation and ethanol at individual CpG Sites within the *H19* imprinting control region in placentae in non-aggregated data

CpG Site	beta	SE	Wald Chi-square	beta	SE	Wald Chi-square
	Paternal Allele			Maternal Allele		
CpG1	-1.448	0.627	5.333*	0.896	0.713	0.209
CpG2	-1.239	0.518	5.716*	0.724	0.556	1.696
CpG3	-0.978	0.503	3.779[a]	0.956	0.578	2.734[a]
CpG4	-0.862	0.525	2.695	0.726	0.558	1.697
CpG5	-1.054	0.521	4.094*	-0.358	0.64	0.313
CpG6	-1.665	0.574	8.415**	0.264	0.65	0.165
CpG7	-0.844	0.589	2.052	0.152	0.553	0.076
CpG8	-0.453	0.474	0.914	NA	NA	NA
CpG9	0.02	0.45	0.002	1.061	0.749	2.006
CpG10	0.223	0.461	0.234	1.855	0.837	4.913*
CpG11	-0.028	0.793	0.001	1.123	0.708	2.514
CpG12	-0.432	0.67	0.415	2.823	1.196	5.575*
CpG13	-2.079	1.085	3.670[a]	0.989	0.715	1.911
CpG14	-0.811	0.624	1.693	1.433	0.794	3.252[a]
CpG15	-0.105	0.594	0.031	1.098	0.735	2.230
CpG16	-0.028	0.564	0.002	1.239	0.79	2.461
CpG17	-0.163	0.555	0.086	0.437	0.666	0.431

Note: beta = log-of-the-odds of a CpG Site being methylated in the ethanol group relative to the saline group; [a]$p<0.10$, *$p<0.05$, **$p<0.01$

Wald tests indicated that ethanol significantly increased the log-of-the-odds of a site being unmethylated on the paternal allele in placentae at the following CpG dinucleotides: '1', '2', '5' and '6' (in each case $p<0.05$; Table 3.5). There were also trends for an increased risk of demethylation at CpG sites '3' and '13' in the ethanol-treatment group (in each case $p<0.10$; Table 3.5). Four out of six of the affected sites lie within the CTCF1 region.

On the maternal allele in placentae, it was found that ethanol significantly increased the log-of-the-odds of methylation at CpG sites 10 and 12 (in each case

$p<0.05$), with trends for similar effects at sites '3' and '14' (in each case $p<0.10$; Table 3.5). Two out of four of these affected sites lie within the CTCF2 region.

3.2.2.5 Analyses of the aggregated DNA-methylation data

After aggregating the data to the level of mothers i.e., by averaging over clones, paternal methylation in placentae remained significantly different between ethanol-treated and control mothers, in terms of both total and CTCF1 DNA-methylation (Table 3.6). Mann-Whitney U tests revealed no other significant differences in DNA-methylation (in each case $p>.10$; Table 3.6).

Table 3.6. Median Levels of Paternal DNA-Methylation in the *H19* Imprinting Control Region in Ethanol-Exposed Placentae and Controls

Methylation variable	Ethanol Median	Control Median	Mann-Whitney U	Z	r	R^2
Paternal Allele Placentae						
Total Methylation	62.5%	75.3%	2*	2.193	-0.693	0.481
CTCF 1	61.1%	79.2%	2*	2.193	-0.693	0.481
CTCF 2	80.0%	85.5%	5.5	1.467	-0.464	0.215
Paternal Allele Embryos						
Total Methylation	88.2%	87.5%	10	0.522	-0.165	0.027
CTCF 1	89.7%	86.4%	12	0.105	-0.033	0.001
CTCF 2	100.0%	95.8%	8	0.955	-0.302	0.091
Maternal Allele Placentae						
Total Methylation	42.5%	17.5%	7	1.149	-0.363	0.132
CTCF 1	50.0%	33.3%	6.5	1.261	-0.399	0.159
CTCF 2	40.0%	10.0%	6	1.366	-0.432	0.187
Maternal Allele Embryos						
Total Methylation	11.8%	4.2%	6.5	0.436	-0.154	0.024
CTCF 1	13.3%	0.0%	6	0.661	-0.234	0.055
CTCF 2	2.0%	1.7%	7	0.308	-0.109	0.012

[a] $p<0.10$, *$p<0.05$, **$p<0.01$

Carrying out a similar analysis at individual CpG dinucleotides on the paternal allele in ethanol-treated and control placentae, it was found that sites ‘2’, ‘3’, 6 and ‘7’ were significantly less methylated in the former (Table 3.7). In addition, trends for less methylation in the ethanol-treatment group were observed at sites ‘5’ and ‘14’ (Table 3.7). These results are similar to the analyses carried out on the non-aggregated clone data (Table 3.5). The only major exceptions are at CpG sites ‘1’ and ‘7’, which were significant and non-significant, respectively, in the logistic models described above (Table 3.5).

On the maternal allele, CpG site 12 was significantly more methylated in ethanol treated placentae than controls (Table 3.7), while trends for similar differences were observed at CpG sites ‘2’, ‘3’, ‘4’ and ‘10’. These effects were also similar to the results of logistic modelling, with the exception of sites ‘2’ and ‘3’, which were not significantly different between ethanol-treated and control placentae in the non-aggregated clone data (Table 3.5).

Thus, the analyses of DNA-methylation performed on the aggregated and non-aggregated clone data are generally quite similar. This suggests that, by averaging over clones to produce aggregated estimates of DNA-methylation for each mother, no major biases were introduced by the highly variable number of clones. However, some biases seem to have been introduced, as evidenced by a few inconsistent results, but these were minor and do not affect the overall conclusions that (1) treatment is a predictor of total and CTCF1 paternal DNA-methylation, as well as methylation at a number of CpG sites within the CTCF1 region, in placentae; (2) that treatment predicts maternal DNA-

methylation at CpG sites 10 and 14 (in the CTCF2 region) in placentae; and (3) that treatment does not significantly affect maternal or paternal DNA-methylation in embryos.

Table 3.7. Median levels of DNA-methylation at individual CpG sites in the *H19* imprinting control region in ethanol-exposed placentae and controls using the aggregated dataset at the level of mothers

	Paternal Allele			Maternal Allele		
CpG Site	Ethanol Median	Control Median	Mann-Whitney U	Ethanol Median	Control Median	Mann-Whitney U
CpG Site 1	10.0%	50.0%	6	33.3%	0.0%	7.5
CpG Site 2	62.5%	80.0%	3.5*	60.0%	33.3%	3.5[a]
CpG Site 3	55.0%	80.0%	0.5**	50.0%	25.0%	4[a]
CpG Site 4	66.7%	75.0%	9.5	60.0%	40.0%	3.5[a]
CpG Site 5	62.5%	75.0%	5[a]	33.3%	40.0%	11
CpG Site 6	62.5%	80.0%	2*	33.3%	25.0%	10
CpG Site 7	75.0%	87.5%	3.5*	40.0%	50.0%	12.5
CpG Site 8	66.7%	66.7%	10.5	NA	NA	NA
CpG Site 9	50.0%	50.0%	10.5	33.3%	0.0%	7
CpG Site 10	50.0%	66.7%	9.5	33.3%	0.0%	3.5[a]
CpG Site 11	66.7%	81.8%	10.5	33.3%	12.5%	6.5
CpG Site 12	66.7%	81.8%	8.5	33.3%	0.0%	3*
CpG Site 13	87.5%	100.0%	7	25.0%	14.3%	8
CpG Site 14	66.7%	87.5%	4.5[a]	40.0%	14.3%	5.5
CpG Site 15	85.0%	83.3%	12	33.3%	16.7%	9
CpG Site 16	75.0%	80.0%	12.5	33.3%	14.3%	6.5
CpG Site 17	75.0%	80.0%	10.5	20.0%	20.0%	12

[a]$p<0.10$, *$p<0.05$, **$p<0.01$

3.2.2.6 The relationship between weight and methylation at the paternal allele of the H19 ICR in placentae

The similar sets of findings between the two datasets suggest that it is valid to analyze at the level of mothers. Thus, the relationship between weight and DNA-methylation at the paternal allele in placentae was analyzed, using the aggregated dataset described above.

Spearman's correlations revealed highly significant relationships between placental weight and total and CTCF1 DNA-methylation (Table 3.8). Significant relationships between placental weight and DNA-methylation at CpG sites 2, 3, 5, 7 were also observed (Table 3.8). In addition, there was a trend for a relationship between placental weight and paternal DNA-methylation at CpG site 13 (Table 3.8). These sites all fall within a CTCF DNA-binding region.

With regards to embryo weight, a number of significant relationships with paternal DNA-methylation at CpG sites 3, 5, 7 and 14 in placentae occurred (Table 3.8). Several trend level relationships were also observed, including correlations between embryo weight and paternal DNA-methylation at CTCF sites 1 and 2, as well as CpG sites 6 and 12 (Table 3.8). Similar to the relationship between paternal DNA-methylation and placental weight, these sites all fall within a CTCF DNA-binding region.

Table 3.8. Correlations between Weight and Paternal DNA-methylation in the *H19* Imprinting Control Region in Placenta

Independent Variable	Embryo Weight		Placenta Weight	
	r	R^2	r	R^2
Total Methylation	0.418	0.175	0.767**	0.588
CTCF 1	0.539[a]	0.291	0.767**	0.588
CTCF 2[b]	0.360[a]	0.130	0.028	0.001
CpG Site 1[b]	0.072	0.005	0.236	0.056
CpG Site 2[b]	0.250	0.063	0.704**	0.496
CpG Site 3	0.608*	0.370	0.870**	0.757
CpG Site 4[b]	0.230	0.053	0.171	0.029
CpG Site 5	0.567*	0.322	0.720*	0.518
CpG Site 6[b]	0.406[a]	0.165	0.609	0.371
CpG Site 7[b]	0.477*	0.228	0.479*	0.229
CpG Site 8	-0.012	0.000	0.400	0.160
CpG Site 9[b]	-0.116	0.013	0.000	0.000
CpG Site 10[b]	0.114	0.013	0.057	0.003
CpG Site 11[b]	0.250	0.063	0.057	0.003
CpG Site 12[b]	0.341[a]	0.116	0.028	0.001
CpG Site 13[b]	0.272	0.074	0.458[a]	0.209
CpG Site 14[b]	0.506*	0.256	0.203	0.041
CpG Site 15	-0.139	0.019	-0.167	0.028
CpG Site 16	-0.335	0.112	-0.192	0.037
CpG Site 17	-0.109	0.012	0.234	0.055

Note: [b]Kendall's tau_b used to estimate the correlation coefficient instead of Spearman's rho because of tied ranks; [a]$p<0.10$, *$p<0.05$, **$p<0.01$

3.2.2.7 Partial correlations of the relationship between treatment, weight and methylation at the paternal allele of the H19 ICR in placentae

Two sets of partial correlations were carried out. The first investigated the relationship between placental weight and treatment, controlling for the effect of 'methylation at the paternal allele in placentae' on the former. The second investigated the relationship between embryo weight and treatment, controlling for the effect of placental weight or 'methylation at the paternal allele in placentae' on the former.

3.2.2.7.1 Partial correlations of the relationship between placental weight and treatment, controlling for the effect of methylation at the paternal allele

Partial correlations were carried out to determine the extent to which these relationships account for the effects of treatment on DNA-methylation, placenta weight and embryo weight, the results of which are reported in Tables 3.8 and 3.9. Partial correlations were also carried out on the three way relationship between placenta weight, embryo weight and treatment (Table 3.10).

In the first set of analyses, partial correlations were used to test the hypothesis that paternal DNA-methylation in placentae mediates the effect of treatment on placental weight. After controlling for the effect of paternal DNA-methylation, partial correlations revealed small to large reductions in the correlation coefficients between treatment and placental weight (Table 3.9). The most striking effect occurred at CpG site 3, which was found to mediate >50% of the relationship between treatment and placental weight (Table 3.9). Medium size effects were also observed for the CTCF1 site and CpG site 2, which both mediate approximately 40% of the relationship between treatment and placental weight (Table 3.9). These reductions suggest that the effect of treatment on placental weight partly depends on paternal DNA-methylation at CTCF1, CpG site 2 and CpG site 3 (Table 3.9). However, these indirect effects were just short of significance, as evidenced by the borderline 95% confidence intervals, which included '0' (Table 3.9). Given the small sample size in the present study and the reasonably large size of the indirect effects, it cannot be concluded that mediation is not occurring.

Table 3.9. Partial Correlations Between Treatment and Placental Weight Controlling for the Effect of Paternal DNA-Methylation in Placentae on Placental Weight

			95% C.I.for indirect effect (mg)	
Mediator Variable	Treatment and placental weight partial correlations[b]	Standardised indirect effect[c]	Lower	Upper
Total Methylation	-0.662*	-0.204	-15.9	1.2
CTCF1	-0.521*	-0.345	-11.2	0
CpG Site 2	-0.508*	-0.358	-16.0	0
CpG Site 3	-0.408	-0.458	-18.8	0
CpG Site 5	-0.616*	-0.250	-3.3	0
CpG Site 7	-0.692*	-0.174	-10.2	3.6

[b]Correlation between treatment and placental weight controlling for the effect of the mediator variable on the latter; [c]the effect of treatment on placental weight mediated by the mediator variable; C.I. - confidence interval; [a]$p<0.10$, *$p<0.05$, **$p<0.01$

3.2.2.7.2 Partial correlations of the relationship between embryo weight and treatment, controlling for the effect of placental weight or methylation at the paternal allele

Partial correlations were used to test the hypothesis that paternal DNA-methylation in placentae mediates the effect of treatment on embryo weight (Table 3.10). Small reductions in the correlation coefficients between treatment and embryo weights were observed after controlling for the effect of paternal DNA-methylation at CpG sites 3, 5, 7 and 14. This suggests that the effect of treatment on embryo weight is independent of paternal DNA-methylation in placentae (Table 3.10).

A related hypothesis tested the effect of placental weight on the relationship between treatment and embryo weight (Table 3.10). As suggested by the small reduction in the correlation coefficient, the effect of treatment on embryo weight seems to be largely independent of placental weight (Table 3.10).

Table 3.10. Partial Correlations Between Treatment and Embryo Weight Controlling for the Effect of Paternal DNA-Methylation in Placentae or Placental Weight on Embryo Weight

Mediator variable	Treatment and embryo weight partial correlation[b]	Standardised indirect effect[c]	95% C.I. for indirect effect (mg) Lower	Upper
CpG3	-0.903**	0.102	-5.6	11.4
CpG5	-0.674*	-0.126	0	0
CpG7	-0.648*	-0.153	-17.3	0
CpG14	-0.657*	-0.144	-14.7	0
Placental Weight	-0.699[a]	-0.102	-20.8	7.8

[b]Correlation between treatment and embryo weight controlling for the effect of the mediator variable on the latter; [c]the effect of treatment on embryo weight mediated by the mediator variable; C.I. - confidence interval; [a]$p<0.10$, *$p<0.05$, **$p<0.01$

3.3. SUMMARY OF RESULTS

In sum, ethanol exposure was associated with significant growth retardation in embryos and placentae. With regards to the methylation data, imprinting seemed more relaxed in placentae relative to embryos, i.e. methylation was less parent-of-origin specific in the former. In addition, reduced and increased levels of methylation at the paternal and maternal alleles of the *H19* ICR, respectively, were observed in ethanol-treated placentae, relative to controls. The ethanol-related decreases in methylation at the paternal allele were associated with the CTCF1 DNA-binding site, while increases at the maternal allele were associated with the CTCF2 DNA-binding site. However, the statistical evidence for the latter was weaker and could not be replicated in the aggregated dataset and, therefore, could not be statistically associated with growth retardation in either placental or embryonic tissue. The loss in methylation at the paternal CTCF1 allele, in ethanol treated placentae, was primarily associated with CpG sites ‘2’, ‘3’, ‘5’

and ‘6’, in both the aggregated and non-aggregated datasets. Partial correlations suggest that CpG sites 2 and 3 mediate 41% and 53%, respectively, of ethanol-induced growth retardation in placentae. Finally, the mediation effect of placental weight, as well as methylation at the paternal allele in placentae, on the relationship between ethanol and embryo growth retardation was small and highly non-significant. This suggests that the effect of ethanol on embryo weight is direct i.e. is independent of placenta weight and methylation at the paternal allele in placentae.

The implications of these findings are discussed in the next section.

CHAPTER 4: DISCUSSION

In the present study, it is proposed that the nature of alcohol teratogenesis is consistent with an epigenetic model of FASD. A collection of ethanol-induced birth defect syndromes, FASD is characterized by variable manifestations of brain damage, distinctive facial features and pre- and/or postnatal growth retardation (Manning and Hoyme, 2007). Intriguingly, these three domains are not perfectly correlated i.e., they do not always co-occur in affected individuals, which may suggest distinctive aetiological profiles for each. Of these, growth retardation is particularly interesting.

Required for diagnosis of FAS but absent from the ARBD and ARND clinical profiles (Manning and Hoyme, 2007), pre- and postnatal growth retardation is associated with two non-overlapping peaks of teratogenesis: the preimplantation and late gestational periods, in animal models (Checiu and Sandor, 1986; Padmanabhan and Hameed, 1988; Mitchell et al. 1994; Clarren and Astley 1992; Becker et al. 1996) as well as in human epidemiological studies (Rosett et al. 1980; Yang et al. 2001).

It was subsequently proposed that genomic imprinting may underlie the above phenomena. Consistent with this prediction, imprinted genes are important regulators of growth in the mammalian foetus, and their deregulation has previously been associated with physical abnormalities in a wide range of mammalian species, including humans (Thomson et al. 2001). During preimplantation development, imprinted genes must maintain their epigenetic state in the face of genome-wide demethylation, suggesting that this may be a particularly sensitive window period for environmentally induced deregulation.

Thus, the aim of the present study was to test the relationship between ethanol-induced growth retardation and imprinting following acute ethanol treatment during the preimplantation period, in a mouse model. Focusing on the most highly studied imprinted chromosomal domain in the mammalian genome, the present study investigated the effect of ethanol on DNA-methylation at the *H19* ICR. Based on previous research, it was predicted that ethanol would induce hypomethylation at the paternal allele and this would, in turn, be associated with growth-retardation.

4.1 OUTLINE OF DISCUSSION

The results of the present study are partly consistent with the above prediction. Although clearly associated with growth retardation in both embryos and placentae, ethanol was only associated with altered DNA-methylation at the *H19* ICR in the latter. Moreover, despite being unrelated to embryo weight, partial correlations suggest that demethylation of the paternal allele partly mediates the effect of ethanol on placental growth. This, in turn, implies a functional effect of reduced methylation at the paternal allele in placentae, perhaps as a result of disrupted imprinting control in the *H19/Igf2* domain. The discussion that follows explores the implications of each of these findings in turn. The first section deals with the relationship between ethanol and growth retardation. In the second section, the more relaxed state of imprinting in placental, relative to embryonic, tissue is thoroughly explored and is discussed in the context of the asymmetry in the life-span of the two tissues. The relaxed state of placental imprinting may underlie the finding that ethanol is unrelated to methylation at the *H19* ICR in embryos but is

associated with decreased and increased methylation at the paternal and maternal alleles, respectively, in placentae. Thus, in the third section, it is proposed that the relaxed state of imprinting in placental tissue may predispose it to further relaxation as a result of inappropriate environmental cues, such as ethanol. The reduced levels of methylation at the paternal *H19* ICR suggest a functional effect, possibly as a result of altered imprinting control in the *H19/Igf2* domain. In the fourth section, it is proposed that ethanol induces an epigenetic switch from the paternal to the maternal epigenotype and that this, in turn, underlies ethanol-induced growth retardation in the placenta, the possible mechanisms of which are also discussed. The discussion ends with a consideration of the limitations of the present study.

4.2 EFFECT OF ETHANOL ON EMBRYO AND PLACENTAL WEIGHT

In the present study it was found that *in vivo* administration of 0.015ml/g of 25% ethanol on days 1.5 and 2.5 dpc resulted in severe growth retardation of embryos ($r= -0.760$, $p<0.01$) and placentae ($r= -0.816$, $p<0.05$). Interestingly, implantation rates were clearly unaffected between the two treatment groups ($p>0.60$), suggesting that embryo development was not deleteriously altered during the preimplantation period. These findings are in very close agreement with previous studies employing *in vivo* administration of ethanol (Padmanabhan and Hameed, 1988; Clarren and Astley, 1992; Mitchell, 1994). This is in stark contrast to the findings of studies that employed *in vitro* routes of administration, which generally reported enhanced preimplantation but unaffected postimplantation embryo development (Wiebold and Becker 1987).

The relationship between ethanol exposure during the preimplantation period and placental growth has received comparatively little attention. In a study by Padmanabhan and Hameed (1988) the effect on placental weight was found to be highly variable. Administration of 0.02ml/g or 0.03ml/g of 25% ethanol, at either 1.5 or 2.5 dpc, resulted in growth enhancement of placentae at 15.5 d.p.c, relative to saline-treated but not untreated controls. However, placentae harvested at either 14.5 dpc or 18.5 dpc were unaffected following administration of 0.03ml/g of 25% ethanol at 2.5 dpc. When the day of administration was shifted to 6.5 dpc the placental weights were reduced in comparison to controls at both 14.5 dpc and 18.5 dpc (Padmanabhan and Hameed, 1988). Their results suggest that the effect of ethanol, administered during the pre- and peri-implantation periods, depends on dosage, as well as timing. Direct comparison with the present findings is difficult because Padmanabhan and Hameed (1988) treated each placenta as an independent observation i.e., they did not take into account the relatedness of placentae from the same mother. Given the large litter sizes, which averaged 10 offspring per mother, their standard errors were grossly underestimated. Moreover, the two studies were different with regards to the mouse strains employed, dosage regimen, and the day at which placentae were harvested. Future studies should more firmly establish the relationship between ethanol administration during the preimplantation period and placental growth.

4.3 RELAXATION OF IMPRINTING IN PLACENTAL TISSUE

The results of the present study indicate that imprinting is more relaxed in placental than embryonic tissue, as evidenced by reduced and increased levels of methylation at the paternal and maternal alleles of the *H19* ICR, respectively. However, it should be noted that the increased levels of methylation at the maternal allele in placentae, relative to embryos, may be partly due to contamination with maternal tissue, which would have contained both paternal and maternal C57Bl/6 alleles. On the other hand, when hypermethylated maternally derived clones were observed they tended to fall into an intermediate category i.e., they were never fully methylated and tended to show low to medium levels of methylation (10% to 50%), which is consistent with a gain in methylation as a result of relaxed imprinting in placental tissue. Moreover, the gains in methylation tended to be greater on the maternal allele in ethanol-treated placentae, consistent with a model whereby relaxation of placental imprinting is enhanced by ethanol treatment.

4.3.1 Asymmetry between the upstream and downstream portions of the *H19* ICR

Interestingly, the apparent gains and losses in methylation tended to occur in blocks in either the upstream (CpG sites 1-7) or downstream (CpG sites 9-17) portions of the *H19* ICR and rarely extended through the whole region, although many instances of non-block like changes were also observed. These changes also seemed associated with particular CTCF DNA-binding regions on the paternal allele in embryos and placentae.

For example, blocks of demethylation in the upstream (CpG sites 1-7) portion of the ICR were observed on the paternal allele in embryonic and placental tissue, while the downstream region (CpG sites 9-17) was rarely demethylated. Significantly, bisulphite sequencing of 4kb of DNA, encompassing 68 CpG dinucleotides, narrowed the *H19* ICR to a 2.2kb region 2kb upstream of the *H19* transcription start site (Tremblay et al. 1997). The border of the ICR appears to be 52 base pairs upstream of CpG site 1330 in the U19619 genomic contig (Tremblay et al. 1997; Warnecke, 1998), which is equivalent to CpG site 1 in the present study. Thus, CpG sites 1-7 lie directly adjacent to the border of the ICR, which may partly explain their greater sensitivity to demethylation. It can only be speculated as to the precise mechanisms underlying this greater sensitivity, but Murrell et al. (2004) proposed a model of epigenetic switching in which putative protein factors bind to the paternal *H19* ICR, facilitating an interaction with DMR2 of *Igf2,* and the subsequent switching of the latter into an active chromatin domain. It is plausible that CpG sites located within the interior of this protein-DNA binding complex i.e., sites located downstream of CpG dinucleotides 1-7, would be less accessible to, and thus relatively protected from, ethanol. One way in which to test this proposal would be to sequence the downstream region, including the 3' border, to determine whether demethylation correlates with proximity to either the 5' or 3' ends.

4.3.2 Relaxation of imprinting in placentae: evidence from previous studies

The finding of relaxed imprinting in placentae, in comparison to embryos is not without precedent. For example Mann et al. (2004) studied DNA-methylation in the *H19*

and *Snrpn* ICRs in 9.5 dpc embryos derived from blastocysts cultured in Whitten's media. In addition to observing various changes resulting from the media itself, Mann et al. (2004) reported sporadic losses of methylation on the normally hypermethylated paternal and maternal alleles of the *H19* and *Snrpn* ICRs, respectively, in control placentae, relative to control embryos. However, the statistical significance of this finding is difficult to ascertain because the controls were derived from a single mother (Mann et al. 2004).

Consistent with the more relaxed state of imprinting in the placenta, sporadic activation of the normally silent paternal *H19* gene is sometimes observed in tissue of trophectoderm origin but not the inner cell mass and the postimplantation embryo (Svensson et al. 1998).

4.3.3 Evolutionary models predict robust maintenance of imprinting in the embryo but not the placenta

The above findings may be related to the relatively short lived existence of the placenta. From an evolutionary perspective, the consequences of relaxed imprinting are not identical for the embryo and the placenta and are probably far more serious for the former, which must maintain imprinted expression for a considerably longer period of time. Thus, it is reasonable to propose selective pressure for more stringently regulated imprinting in the embryo. This proposal is consistent with a growing body of evidence for the placental origins of imprinting as well as an ancestral imprinting mechanism that originally depended on histone modifications – a relatively unstable epigenetic mark

(Lewis et al. 2004). For example, a second imprinted domain on distal chromosome 7 associated with the Kcnq1ot1/ KvDMR1 imprinting center (IC2), contains nine imprinted genes, eight of which are maternally expressed and one paternally expressed (Lewis et al. 2004). Five of the maternally expressed (paternally repressed) genes are imprinted exclusively in the placenta. Notably, when methylation is genetically ablated (in a *Dnmt* -/- mouse model) genes imprinted in both the embryo and the placenta become biallelically expressed, while genes imprinted exclusively in the placenta are unaffected (Lewis et al. 2004). Moreover, the latter are associated with repressive histone modifications on the paternally silent allele, the recruitment of which depends on IC2 (Lewis et al. 2004). Interestingly, considerable expression was observed from the 'silent' allele when the imprinting depended on histone modifications (Lewis et al. 2004). This suggests that histone modifications are a relatively less stable mechanism of imprinting in comparison to DNA-methylation (Lewis et al. 2004).

Lewis et al. (2004) subsequently propose an evolutionary model of genomic imprinting, in which the ancestral mechanism, initially limited to the placenta, originally depended on histone modifications, and was later transferred to the embryo. The relatively unstable nature of histone modifications created a selective pressure for a more stable silencing mechanism in the embryo i.e. DNA-methylation. Being short-lived, it is presumed that relaxed imprinting is better tolerated in the placenta and thus a selective pressure for more stringent imprinting would be weaker. This would explain why all genes depending exclusively on histone modifications for their imprinting have so far only been observed in the placenta (Lewis et al. (2004).

4.3.4 Protection against loss of imprinting: role of delayed activation

Although DNA-methylation is crucial, it is likely that additional mechanisms exist for the stabilization and maintenance of imprinting in the embryo. Delayed activation of imprinted gene expression until after the major periods of epigenetic rearrangement in the early embryo may represent one such mechanism. For example, expression of *H19* is not detected in the postimplantation embryo until 8.5 dpc (Poirier et al. 1991). In contrast, *H19* RNA is detected in the trophectoderm of late stage blastocysts (4.5 dpc) and persists in extraembryonic tissues through the remainder of gestation (Poirier et al. 1991).

The silencing of maternal *H19* until comparatively late in development (8.5 dpc) may be part of an overall control strategy, designed to protect the locus from loss of imprinting during dynamic periods of epigenetic change. For example, an open question concerns the mechanism by which imprinted loci are protected from genome-wide demethylation during the preimplantation period. It has been proposed that specialized chromatin structures protect imprints from active demethylation in the zygote (Oswald et al. 2000). During the cleavage stages, when demethylation occurs by passive mechanisms, it is proposed that the *de novo* DNA-methyltransferases, *Dnmt3b* and *Dnmt3a,* protect methylated imprints from demethylation, while the unmethylated imprints are protected from *Dnmt3b* and *Dnmt3a* by assuming inaccessible higher-order chromatin structures (Reik et al. 2001). Thus, the complete absence of *H19* expression prior to 4.5 dpc (Poirier et al. 1991) may be a consequence of maintaining the maternal allele in a repressive state in order to protect it from *de novo* methylation. These silencing

factors may persist in the inner cell mass but not the trophectoderm, perhaps to protect the unmethylated maternal copy from the wave of genome wide *de novo* methylation that is associated with gastrulation but which is absent in the trophectoderm (Reik et al. 2001). The presence of *Dnmt3b* and *Dnmt3a* in the gastrulating embryo, but their absence in trophectoderm, may also explain the greater levels of methylation at the paternal *H19* ICR in embryos, relative to placentae (present study; Mann et al. 2004).

4.3.5 Dynamic imprinting in the placenta: role of chromatin assembly factors

Consistent with the more relaxed state of imprinting in the placenta, variegated expression of paternal *H19* occurs in trophectoderm and extraembryonic tissues but not the gastrulating embryo (Svensson et al. 1998). Moreover, trichostatin-A, an inhibitor of histone deacetylase, is able to activate paternal *H19* in the placenta, but not the embryo. These findings suggest that placental imprinting is more dependent on a dynamic interplay between silencer and enhancer chromatin assembly factors (Svensson et al. 1998). This, in turn, may lead to mosaic patterns of expression from the paternal *H19* allele. For example, biallelic expression may occur when the ratio of silencers to enhancers randomly fluctuates below a certain threshold level in particular cells (Svensson et al. 1998). The absence of sporadic activation of the paternal *H19* allele may be due to a much greater abundance of repressive chromatin factors. As discussed above, two of these factors may be *Dnmt3a* and *Dnmt3b,* which are active in the early embryo but not the placenta (Reik et al. 2001).

CTCF, a DNA-binding protein with an affinity for unmethylated DNA, is presumably another one of these factors (Engel et al. 2006; Kurukuti et al. 2006). The ability of this protein to partition the *Igf2/H19* domain into specialized chromatin structures (Kurukuti et al. 2006) probably underlies its ability to protect the maternal *H19* ICR from *de novo* methylation (Engel et al. 2006). Since CTCF is present in finite amounts, fluctuations below a certain threshold may lead to sporadic gains in methylation on the maternal allele. This effect would be exacerbated by the presence of extra maternal copies of *H19*, which would place demands on CTCF beyond the available supply. As a consequence, some maternal copies of *H19* would go unprotected, leading to sporadic gains of methylation and, thereby, silencing of maternal *H19*. In support of this scenario, deletion of CTCF DNA-binding sites results in *de novo* methylation of the maternal *H19* ICR and concomitant gene silencing (Engel et al. 2006).

Conversely, the presence of extra copies of the paternal allele should be associated with the opposite effect i.e., sporadic losses in methylation. Consistent with this prediction, 9.5 dpc PatDup.d7 placentae, which are bipaternal with regards to the distal region of chromosome 7, display variegated patterns of paternal *H19* expression (Svensson et al. 1998). This suggests that the presence of two paternal copies of the *H19* gene places excessive demands on a finite pool of chromatin repressive factors which, in turn, leads to sporadic activation of paternal *H19* (Svensson et al. 1998). *Dnmt1* may be one of these factors, since it is required for the maintenance of methylation at imprinted loci through mitosis (Reik et al. 2001).

An open question in the imprinting research field is whether the silencing of maternal *Igf2* and paternal *H19* is absolute or whether it depends on a dynamic

equilibrium between silencers and enhancers (Svensson et al. 1998; Fourel et al. 2004; Kurkuti et al. 2006). The results of the present study suggest that imprinting control is indeed more stringent in the embryo but, conversely, is dynamically regulated in the placenta. Presumably, the differences in imprinting control between the embryo and placenta are related to the relatively long-lived status of the former and relatively transient nature of the latter. An absolute system of epigenetic control is particularly important in the gastrulating embryo, when long-term developmental potentials are initially laid down.

4.3.6 Summary of findings regarding relaxation of imprinting in the placenta

In sum, mechanisms for the maintenance of imprinting are more robust in the early and postimplantation embryos than in the placenta. The transfer of imprinting to the embryo from the placenta, in early mammalian history, may have lead to selection for more robust mechanisms of imprinting in the former but not the latter. The reasons for this are probably related to the more long-lived nature of the embryo, for which loss of imprinting is presumably more harmful than in the placenta. As a consequence of this asymmetry, imprinting in the placenta seems to depend on a more dynamic interplay between silencing and activating factors.

Robust imprinting mechanisms include DNA-methylation, specialized chromatin structures, and chromatin assembly factors. For example, relaxation of imprinting in the placenta may be the result of sporadic fluctuations in silencers and enhancers (Svensson et al. 1998). The embryo is presumably protected from the consequences of such

fluctuations because of specialized chromatin structures at the unmethylated maternal allele, which protect it from *de novo* methylation, and the presence of *Dnmt3a* and *Dnmt3b,* which confer protection against demethylation to the paternal *H19* ICR (Reik et al. 2001). The absence of these repressive chromatin states in trophectoderm leads to early expression of maternal *H19* (4.5 dpc), while the occasional fluctuation of silencer and enhancer chromatin assembly factors occasionally leads to activation of paternal *H19* in the placenta (Svensson et al. 1998).

The less stringently maintained state of imprinting in the placenta may underlie its greater sensitivity to environmental disruption, such as ethanol treatment, as discussed below.

4.4 PREFERENTIAL LOSS OF IMPRINTING IN ETHANOL-TREATED TROPHECTODERM

As described above, it was predicted that ethanol-treatment of mouse embryos during the preimplantation period would result in reduced methylation at the paternal allele of the *H19* ICR in midgestation mouse embryos and placentae. This prediction was partially validated: methylation was unaffected in embryos, while reduced and increased methylation of the paternal and maternal alleles, respectively, was observed in ethanol-treated placentae.

4.4.1 Mechanisms of preferential loss of imprinting in ethanol-treated trophectoderm

Since treatment occurred prior to gastrulation, these results suggest two possible scenarios: (1) DNA-methylation at the paternal allele was reduced in both the inner cell mass and the trophectoderm but recovered in the descendents of the former (the postimplantation embryo) but not the latter (the placenta); (2) ethanol-induced demethylation occurred preferentially in the outer layer of the early stage blastocyst/morula, as a result of its closer proximity to the maternal environment. It is also possible that the preferential loss of imprinting observed in ethanol-treated trophectoderm partly arose post-implantation, as a result of lingering by-products of ethanol metabolism, such as acetaldehyde, in the maternal blood stream.

With regards to the first hypothesis, i.e. preferential recovery of imprinting in the postimplantation embryo, two possible mechanisms suggest themselves: (a) preferential selection in the inner cell mass of unaffected cells and/or (b) the presence of recovery mechanisms in the gastrulating embryo but not the trophectoderm. An explanation by a selection-based mechanism is unlikely because *H19* is not expressed in the embryo until 8.5. dpc (Poirier et al. 1991), suggesting that the putative reductions in paternal *H19* methylation in the inner cell mass did not deleteriously affect the growth of the early embryo. Moreover, implantation rates were virtually identical across the two treatment groups, suggesting that preimplantation embryos were equally viable at the implantation stage (4.5 dpc).

The second proposed mechanism of recovery (b) is consistent with the wave of genome wide *de novo* methylation associated with the onset of gastrulation in the inner

cell mass around E4.5 to E7.0 (Monk et al. 1987; Santos et al. 2002). The *de novo* DNA-methyltransferases *Dnmt3a* and *Dnmt3b,* which have an established role in the maintenance and acquisition of methylation at imprinted loci (Chen et al. 2003; Lei et al. 1996; Okano et al. 1998) are particularly interesting in this regard because they preferentially locate to the inner cells mass during the onset of gastrulation. Thus, the putative recovery of the demethylated paternal allele in the inner cell mass and postimplantation embryo may be mediated by *de novo* methylation, catalyzed by *Dnmt3a* and *Dnmt3b*, while the absence of these enzymes in trophectoderm may explain the persistence of demethylation in 10.5 dpc placentae (Watanabe et al. 2002).

Increased methylation on the maternal allele was also observed in ethanol-treated placentae relative to controls. This is consistent with the role of a dynamic interplay between silencers and enhancers in the regulation of imprinting in the placenta, discussed above, as well as the DNA-methyltransferase inhibition model proposed by Garro et al. (1991). For example, in one scenario, ethanol, or its metabolite acetaldehyde, inhibits DNA-methyltransferase activity at the gene transcription or protein level, causing a partial loss of methylation at the paternal allele. Given the finite nature of the cellular pool of CTCF, the partial acquisition of this 'maternal-like' epigenotype at the paternal allele would sequester CTCF proteins away from maternal copies of the *H19* ICR causing sporadic gains in methylation at the maternal allele, discussed in more detail below.

Significantly, the apparent gains in methylation were preferentially associated with CpG sites in the downstream region (CpG sites 9-17), which contrasts with the losses in methylation on the paternal allele, which preferentially affected the upstream portion (CpG sites 1-7). The latter may be related to its location at the 5' border, which,

as discussed above, may confer greater susceptibility to demethylation. Conversely, the gains in methylation in the downstream region suggest that the losses in methylation initially occurred 3' of CpG sites 9-17 of the maternal allele and spread upstream, preferentially affecting the CTCF2 region because of its closer proximity to the 3' end. This is consistent with targeted deletion studies of the region, which seem to indicate that acquisition of methylation on the paternal allele is initiated in the 3' region and spreads out in proximal and distal directions (Engel et al. 2006).

4.4.2 Preferential loss of imprinting in trophectoderm induced by *in vitro* culture

Interestingly, the above findings are in close agreement with Mann et al. (2004) who reported loss of imprinted expression of *H19, Aascl2, Snrpn, Peg3 and Xist* in mouse conceptuses, following *in vitro* culture in Whitten's medium. These changes were associated with reduced methylation at the normally hypermethylated alleles of the *H19* and *Snrpn* ICRs in preimplantation mouse blastocysts, which persisted into postimplantation mouse placentae but not embryos harvested at 9.5 dpc. Moreover, the authors also observed sporadic gains in methylation on the normally unmethylated maternal and paternal alleles of the *H19* and *Snrpn* ICRs, respectively, in placentae derived from blastocysts cultured in Whitten's media.

Thus, similarly to the results of the present study, losses and gains in methylation at the paternal and maternal allele of the *H19* ICR, respectively, occurred preferentially in placental tissue following exposure to a stressful environmental agent (Whitten's medium) during the preimplantation period. The authors made similar predictions about

the origins of the preferential loss of imprinting in placental tissue, namely that (1) trophectoderm cells are more sensitive to disruption or (2) imprinting is disrupted randomly in the cells of the blastocyst, affecting both the inner cell mass and the trophectoderm, but mechanisms for the recovery and/or maintenance of imprinting in the former are more robust. It was argued that disruption of imprinting occurs in the precursor cells of the foetus but recovers during postimplantation development (Mann et al. 2004). Consistent with this prediction, preliminary findings from the same research group revealed loss of methylation in the inner cell mass similar to the loss of methylation observed in blastocysts as a whole (unpublished findings by Mann et al. 2004). However, the authors could not rule out the possibility that the loss of methylation in the inner cell mass occurred preferentially in cells destined to give rise to extra-embryonic tissue, such as precursor primitive endoderm. Consistent with the 'recovery' hypothesis, the authors also reported occasional biallelic expression in the embryo, suggesting incomplete recovery in embryos (Mann et al. 2004).

Mann et al. (2004) investigated the same region of the *H19* ICR as in the present study and thus it is noteworthy that preferential demethylation of the upstream region (CpG sites 1-7) was observed in a few clones but was not a general observation. Rather, paternal demethylation tended to be complete, extending throughout the region under study i.e. CpG sites 1-17 (Mann et al. 2004). This is consistent with the idea, proposed above, that demethylation initiates in the upstream region i.e. at the 5' border of the ICR, and gradually spreads downstream to the 3' end. However, contrary to this hypothesis, some clones were observed with preferential demethylation in the downstream region

(Mann et al. 2004), suggesting that culture-induced loss of methylation may be more sporadic, in comparison to ethanol treatment.

Nevertheless, their results are commensurate with the findings of the present study that ethanol induces a relaxation of imprinting in placentae but not embryos. Given the greater extent of paternal demethylation, relative to maternal gains in methylation, it is likely that the former occurred first. This begs the question as to how demethylation on the paternal allele may lead to gains in methylation on the maternal allele. As discussed above, imprinting at the *H19* gene in the placenta involves a stochastic interplay between silencer and enhancer chromatin assembly factors (Svensson et al. 1998). One of these factors is CTCF DNA-binding protein. In the present case, ethanol-induced demethylation of the paternal allele would be expected to result in increased demands for CTCF DNA-binding factor, which would have reduced the pool of factors available for the normally unmethylated maternal alleles. Since CTCF DNA-binding protein is required for the maintenance of a hypomethylated state, this reduced availability would be expected to result in *de novo* methylation of maternal copies of the *H19* ICR (Engel et al. 2006), which would explain the gains in maternal methylation observed in the present study. Sporadic gains in methylation on the maternal allele of the *H19* ICR, in conjunction with gains in methylation on the paternal allele of the *Snrpn* ICR, were also reported by Mann et al. (2004), consistent with this proposal.

4.4.3 Summary of findings regarding preferential loss of imprinting in ethanol-treated trophectoderm

In sum, the present findings echo those of previous studies (Svensson et al. 1998; Mann et al, 2004; Lewis et al. 2004), suggesting that (1) imprinting is inherently more relaxed in placental tissue, relative to embryos and (2) that imprinting in the trophectoderm lineage is generally more sensitive to the environment than the inner cell mass, either as a result of its closer proximity to the maternal environment or the more robust state of imprinting in the gastrulating embryo. The former may underlie the latter i.e., the relatively relaxed state of imprinting control in placentae may make it more vulnerable to environmental insult. On the other hand, this also implies that the placenta normally tolerates a certain degree of imprinting relaxation. In fact, there is much evidence to suggest that the placenta is able to compensate for major disturbances in function by, for example, increasing the efficiency of nutrient supply to the foetus (Constancia et al. 2002; 2005). Indeed, Mann et al. (2004) did not observe a relationship between imprinting defects in midgestation mouse embryos and gross embryo abnormalities, although subtle effects could not be ruled out. Similarly, in the present study, both paternal demethylation and growth retardation in the placenta were unrelated to embryo weight, suggesting the operation of compensatory mechanisms that protect the embryo from these defects. Alternatively, since both embryos and placentae were similarly growth retarded, there may have been no net change in nutrient supply and demand. In other words, the reduced supply capabilities of the growth retarded placenta were matched by the reduced demands of the growth retarded embryo.

4.5 ETHANOL-INDUCED DEMETHYLATION AND IMPRINTING AT THE *H19/IGF2* DOMAIN

In the present thesis, it was hypothesized that deregulated genomic imprinting underlies the physical growth component of FASD. More specifically, it was predicted that ethanol exposure during the preimplantation period would cause hypomethylation of the *H19* ICR and this would, in turn, result in growth retarded in midgestation (10.5 dpc) mouse embryos and placentae. The results of the present study are partly consistent with this prediction. Ethanol exposure was not associated with altered DNA-methylation in the *H19* ICR in embryos but was associated with paternal demethylation in placentae. However, hypomethylation at the paternal allele in placentae was unrelated to embryo growth retardation. This suggests that loss of imprinting, at least at the *H19* ICR, is not a mechanism of ethanol-induced growth retardation in midgestation mouse embryos.

Despite being unrelated to embryo weight, analysis of partial correlations suggested that paternal demethylation partly mediates the effect of ethanol on placental weight. This, in turn, implies a functional effect of paternal demethylation in placentae, perhaps as a result of disrupted imprinting control in the *H19/Igf2* domain.

4.5.1 Mechanisms of imprinted gene expression in the *H19/Igf2* domain

The ability of the *H19* ICR to regulate imprinting over 100kb of DNA-sequence implies the existence of long-range chromatin interactions, involving enhancers, silencers, insulators and boundary elements (Lopes et al. 2003; Fourel et al. 2004). For

example the *H19* ICR contains a chromatin boundary and silencer element which, when unmethylated, is able to bind the CTCF zinc finger protein (Lopes et al. 2003). The insulator function of the *H19* ICR depends principally on its ability to bind CTCF which is, in turn, dependent on four GC rich 21bp repeats (Hark et al. 2000; Pant et al. 2003; Schoenherr et al. 2003; Engel et al. 2004; Pant et al. 2004; Szabo et al. 2004).

The formation of the boundary element prevents the interaction of the *Igf2* promoter with enhancers downstream of *H19.* The mechanism depends on the ability of the *H19* ICR-CTCF protein complex to physically interact with *Igf2* DMR1 (Lopes et al. 2003; Kurukuti et al. 2006). This interaction partitions maternal *Igf2* into a silent chromosomal domain, thereby abrogating its contact with the enhancer sequence elements downstream of *H19* (Kurukuti et al. 2006). Since the maternal *H19* ICR is hypomethylated, maternal *Igf2* is repressed, while maternal *H19* is expressed. Binding of CTCF protects the *H19* ICR from *de novo* methylation and also initiates the transcription of *H19 (*Pant et al. 2003; Schoenherr et al. 2003; Engel et al. 2004; Pant et al. 2004; Szabo et al. 2004). Conversely, paternal *Igf2* is expressed because methylation of the paternal *H19* ICR blocks CTCF from binding which, in turn, facilitates the access of the *Igf2* promoter to the enhancer sequence element downstream of *H19* (Kurukuti et al. 2006). In addition, paternal *H19* is silenced because of hypermethylation in its promoter region, which seems to originate from the *H19* ICR (Engel et al. 2004).

4.5.2 Ethanol induced epigenetic switching of the paternal to the maternal epigenotype

Since abrogation of CTCF binding sites on the paternal allele results in the epigenetic switching of the paternal to the maternal epigenotype (Engel et al. 2004), it is proposed that ethanol-induced demethylation at the paternal *H19* ICR results in a similar switching in placentae. Switching to the maternal epigenotype would result in the partitioning of the paternal *Igf2* gene into a silent chromatin state. This mechanism is predicted to involve binding of CTCF to the hypomethylated paternal allele.

However, ethanol-induced demethylation was principally associated with CTCF1 and not CTCF2. Moreover, the epigenetic status of the other two CTCF binding sites, which were not investigated in the present study, is unknown. Thus, it is difficult to ascertain whether the reduced methylation detected in the present study had a functional effect in placentae. On the other hand, significant correlations with placental weight were only detected at CpG sites specifically located within CTCF1, which supports a functional effect. In addition, small changes at these CTCF binding sites are able to switch the *Igf2/H19* domain from the paternal to the maternal epigenotype (Engel et al. 2004). For example, targeted mutations at just 9 CpG dinucleotides across the four CTCF DNA-binding sites eliminated the ability of the hypermethylated paternal allele to block CTCF binding which was, in turn, associated with biallelic expression of *H19,* silencing of normally active paternal *Igf2* and a 40% reduction in mouse birth weight (Engel et al. 2004). In addition, targeted deletion of a single CTCF DNA-binding site (CTCF site 4) was enough to switch the maternal to the paternal epigenotype (Pant et al. 2004). This

deletion was associated with *de novo* methylation at CTCF sites 1, 2 and 3, as well as activation of normally silent maternal *Igf2* (Pant et al. 2004).

4.5.3 In *cis* and in *trans* effects of demethylation at the paternal allele of the *H19* ICR

The reduction of *Igf2* protein levels, following the epigenetic switch of the paternal to the maternal epigenotype, may occur either in *cis* or in *trans*. For example, loss of methylation at the paternal allele of the *H19* ICR may lead to partitioning of paternal *Igf2* into a silent chromatin state. In *trans* effects may be mediated by negative interactions between paternal *H19* and maternal *Igf2*. Although normally silent, the gains in methylation observed on the maternal allele in placentae may have lead to activation of maternal *Igf2*. Consistent with in *trans* effects, *Igf2* mRNA levels, as well as translatability, are negatively correlated with *H19* mRNA levels in the cytoplasm (Li et al. 1998). Moreover, *H19* RNA has been shown to inhibit tumorigenicity in some cancer cell types, which may be mediated by a negative regulatory effect on *Igf2* (Hao et al., 1993; Casola et al. 1997; Isfort et al., 1997; Fukuzawa et al. 1999). It has been proposed that these in *trans* effects are mediated by RNA interference (Lewis and Redrup, 2005). Indeed, a similarly imprinted non-coding RNA, *antiPeg11*, has been shown to inhibit translation of *Peg11* mRNA by such a mechanism (Davis et al. 2005).

4.5.4 Summary of findings regarding the effect of demethylation at the paternal allele on imprinting in the *H19/Igf2* domain

In sum, the *H19* ICR regulates the imprinted expression of both *H19* and *Igf2*. The latter depends on the ability of the ICR to function as a boundary element when unmethylated which is principally related to its ability to bind CTCF protein. This function partitions *Igf2* into a silent chromatin state, which abrogates its access to enhancers downstream of *H19*. Genetic studies indicate that abrogation of this function results in an epigenetic switch of the paternal to the maternal epigenotype. Thus, it is proposed that ethanol induced demethylation at the paternal *H19* ICR results in a similar epigenetic switch. This proposal is supported by mutation targeting studies, which suggest that demethylation of the entire ICR is not required for epigenetic switching, while deletion of a single CTCF binding site is enough to switch the maternal to the paternal epigenotype. Since the relationship between methylation and CTCF binding is qualitative, demethylation at a small number of sites may be enough for the acquisition of enhancer blocking activity at the paternal *H19* ICR. This epigenetic switch may, in turn, result in placental growth retardation as a result of reduced *Igf2* protein levels, which may occur either in *cis* or in *trans*.

4.6 ETHANOL-INDUCED EPIGENETIC SWITCHING AS A MECHANISM OF GROWTH RETARDATION

The ethanol-induced epigenetic switch proposed above is predicted to reduce *Igf2* protein levels either in *trans* or in *cis*. Such an effect would be expected to inhibit placental growth because *Igf2* is the precursor peptide for a mitogen factor known as 'insulin-like growth factor II', which is active in foetal and placental tissues (Tycko and Morison, 2002; Fowden et al. 2006). Moreover, together with its receptors, and associated signal-transduction pathways, *Igf2* signaling is one of two known systems of growth control in the mammalian genome – the other being the insulin-mediated growth pathway (Efstratiadis, 1998).

Reduced levels of *Igf2* may explain the observed relationship between ethanol induced hypomethylation at the paternal *H19* ICR in placentae and placental growth retardation. This proposal is consistent with the nature of *Igf2* expression and translation. For example, *Igf2* mRNA is first detected by *in situ* hybridization in the trophoblast at 5.5 d.p.c and continues throughout the remainder of placental development (Lee et al. 1990). IGF-II protein is also detected in the trophoblast at 5.5 dpc and has a similar pattern of expression to *Igf2* thereafter (Pringle and Roberts, 2007). These findings suggest that *Igf2* and its protein product support placental growth throughout the postimplantation period (Pringle and Roberts, 2007).

On the other hand, a more direct link to growth control for *H19* cannot be ruled out. The reason for this is that deletion of a silencer element, located midway between the ICR and the *H19* promoter, did not disrupt the insulator function of the upstream region,

nor did it affect imprinted expression of *Igf2*. However, it did result in expression of paternal *H19* which was also associated with growth retardation in mouse foetuses, suggesting a role in growth control independent of *Igf2* (Drewell et al. 2000).

Thus, there are three possible mechanisms by which ethanol-induced epigenetic switching may result in placental growth retardation: *H19* induced repression of *Igf2,* either (1) in *trans* or (2) in *cis* or, more controversially, by (3) direct inhibition of growth via an unknown mechanism.

4.6.1 Clinical and experimental consequences of epimutations and mutations at the *H19/Igf2* domain

All three possibilities are consistent with the clinical and experimental manifestations of mutations and epimutations in the *H19/Igf2* domain. For example, demethylation of the *H19* ICR has been associated with the Silver-Russell birth defect syndrome, which is characterized by intrauterine growth retardation (IUGR), poor postnatal health, classic facial features and asymmetry (Price et al. 1999). Conversely, hypermethylation, or deletion, of the maternal *H19* ICR and/or activation of paternal *Igf2* is associated with overgrowth phenotypes, such as Wilm's tumour and some cases of Beckwith-Wiedemann syndrome (BWS) (Moulton et al. 1994; Catchpoole et al. 1997).

Deletion studies in animal models have also drawn attention to the importance of the *Igf2/H19* domain in foetal and placental growth. For example, deletion of the entire *Igf2* gene results in severely growth retarded but viable offspring when inherited on the paternal allele (DeChiara et al. 1991; Baker et al. 1993; Burns and Hassan, 2001).

Intriguingly, growth restriction is not detected until E9.5, becoming significant by E11 (Burns and Hassan, 2001). As was described above, deletion of CTCF binding sites on the paternal allele results in demethylation of the *H19* ICR and, consequently, epigenetic switching of the paternal to the maternal epigenotype. This, in turn, results in biallelic expression of *H19,* as well as reductions in *Igf2* mRNA and growth retardation in mouse offspring (Engel et al. 2004). Conversely, deletion of the *H19* gene region is associated with somatic overgrowth when inherited on the maternal allele (Leighton et al. 1995). This effect is presumably due to loss of in *cis* control of *Igf2* by *H19* (Leighton et al. 1995).

Thus, the reductions and increases in *Igf2* expression are associated with growth retardation and overgrowth, respectively, consistent with the proposed epigenetic switch mechanism of ethanol-induced growth retardation in placentae. However, the effect of ethanol on embryo growth is independent of its effect on methylation at the paternal allele of the *H19* ICR in placentae. This suggests that imprinting, at least at the *H19* ICR, is not a mechanism of ethanol-induced growth retardation in midgestation mouse embryos. However, functional and genetic studies support the existence of a mismatch between onset of imprinting-mediated growth retardation in the placenta and the embryo, with the former preceding the latter (Constancia et al. 2002; 2005). Thus, the absence of a relationship between loss of imprinting at the *H19* ICR in placentae and embryo growth retardation may be due to the early day of dissection in the present study (10.5 dpc). It is possible that placental loss of imprinting becomes a mechanism of embryo growth retardation only during later stages of pregnancy. In other words, the placentae from the present study may have been able to cope with the nutrient demands of the severely

growth retarded embryos prior to 10.5 dpc. However, the ability of the placenta to maintain adequate nutrient supply may begin to wane during late gestation when a larger foetus exerts concomitantly greater demands on the placenta for nutrition. The nature of *Igf2*-mediated growth control is consistent with this prediction.

4.6.2 Mechanisms of *Igf2*-mediated growth control

In one study, deletion of the *Igf2* P0 transcript, which is specifically expressed in the labyrinthe trophoblast of the placenta, resulted in reduced passive permeability of the placenta for nutrients but increased efficiency of glucose and amino acid active transport (Constancia et al. 2002; 2005). These increases were mediated, at least in part, by upregulation of the *Slc2a3* and *Slc38a4* amino acid transporter genes (Constancia et al. 2002; 2005). Intriguingly, this mechanism seems to depend on foetal *Igf2* because its deletion in the foetus abolishes the expression of *Slc2a3* and *Slc38a4* and reduces active amino acid transport (Constancia et al. 2002; 2005). Moreover, there is a mismatch between the onset of placental and embryonic growth retardation. The former is observed from embryonic day (E) 12 onwards, while foetal growth retardation follows several days later (from E16 onwards; Constancia et al. 2002; 2005). This suggests that the increased efficiency of nutrient transport initially compensates for the growth retarded placenta, resulting from the *Igf2* P0 deletion (Constancia et al. 2002; 2005). However, by E16 the placenta is unable to maintain increased transporter activity which, together with the reduced surface area and permeability of the placenta, leads to foetal growth restriction (Constancia et al. 2002; 2005).

Thus, *Igf2* is characterized by two distinct mechanisms of growth control. First, it directly controls the growth of the placenta – as exemplified by the *Igf2* P0 deletion – which, in turn, affects nutrient supply of the foetus. Moreover, *Igf2* regulates foetal demand for nutrients from the placenta – as exemplified by the upregulation in placental transport systems. However, by E16, foetal demand exceeds the supply capability of the mutant placenta, resulting in foetal growth retardation.

Interestingly, both *Igf2* mRNA and its protein product are detected in the trophoblast from 5.5 dpc onwards, suggesting that *Igf2* supports placental growth throughout the postimplantation period (Lee et al. 1990; Pringle and Roberts, 2007). This implies that reductions in *Igf2* in placenta should inhibit placental growth in the early postimplantation embryo. However, deletion of the entire *Igf2* gene does not affect embryo growth until 9.5 dpc, suggesting an offset between the onset of placental and embryonic growth restriction, with the former preceding the latter (Burns and Hassan, 2001).

Partial correlations suggested that paternal demethylation at the *H19* ICR in placentae was unrelated to the effect of ethanol on embryo weight. The results of Constancia et al. (2002, 2005) suggest that the reason for this may be increased placental transport efficiency. In other words, embryonic *Igf2* may have triggered the upregulation of transporter systems in the placenta in order to compensate for the reduction in placental size. This line of reasoning is supported by the observation that methylation at the *H19* ICR in embryos was completely unaffected by ethanol, suggesting that expression of *Igf2* in the embryo was also normal. Alternatively, increased transporter efficiency may not have been required because the embryos were similarly growth

retarded. Thus, the reduced supply capabilities of the smaller placentae may have been matched by the reduced demands of the smaller embryos. However, the findings of Constancia et al. (2002, 2005) imply that the supply sufficiency of the growth retarded placenta may begin to wane as the embryo increases in size during late gestation. Thus, it is possible that ethanol-induced foetal growth retardation may become partly dependent on loss of placental imprinting during late gestation (>14.5 dpc).

4.6.3 Summary of findings regarding mechanisms of ethanol-induced growth retardation

In sum, it is proposed that ethanol-induced demethylation at the paternal *H19* ICR in placentae lead to an epigenetic switch of the paternal to the maternal epigenotype and, as a consequence, reductions of *Igf2* and placental growth retardation. In addition, it is proposed that ethanol-induced epigenetic switching in the placenta did not mediate the effect of ethanol on embryo weight for two reasons: (1) increased functional efficiency in the placenta or (2) relatively modest nutritional demands of the smaller embryos, which would have been within the supply capabilities of their placentae. Thus, prior to 10.5 dpc embryo growth retardation does not depend on loss of imprinting at the *H19* ICR in placentae. However, it is possible that the nutritional demands of the foetus could exceed the supply capabilities of the growth retarded placenta during the later stages of gestation, exacerbating foetal growth retardation.

4.7 STUDY LIMITATIONS AND STRENGTHS

4.7.1 Limitations

By focusing on a single imprinted locus – the *H19* ICR – the conclusions that can be drawn from the present findings are severely limited. Although this region was unrelated to embryo growth retardation, the possibility that other imprinted loci may be involved cannot be ruled out. Moreover, this region is preferentially methylated on the paternal allele and demethylation is expected to result in reduced growth, consistent with the nature of ethanol teratogenesis. However, other ICRs, such as the one associated with the *Snrpn* imprinted gene, are preferentially methylated on the maternal allele, and hypomethylation is expected to result in growth enhancement. Thus, insofar as ethanol-induced growth retardation depends on imprinting, this would imply that paternally methylated ICRs are preferentially affected. However, if ethanol-induced demethylation occurred indiscriminately, this would preclude imprinting as a general mechanism of ethanol teratogenesis. For example, it is possible that ethanol-induced demethylation at repetitive elements, which normally escape demethylation during the preimplantation period (Hajkova et al. 2002), may underlie placental growth retardation. Thus, by focusing on a single locus in the genome, the present study cannot conclude that imprinting is not involved in ethanol-induced embryonic growth retardation prior to 10.5 dpc, nor conclude that deregulation of imprinting is the only epigenetic mechanism of ethanol-induced placental growth retardation.

The relationship between demethylation at the paternal allele of the *H19* ICR and reduced placental growth implies that gene expression from the *H19* and *Igf2* genes was similarly altered. However, without directly determining the expression levels from the paternal and maternal alleles, it can only be speculated as to whether imprinted gene expression was altered. Thus, although methylation was altered in placentae, it is possible that imprinted expression remained unchanged. Similarly, although methylation was unaffected in embryos it is possible that imprinted gene expression was altered. Although the relationship between methylation at the *H19* ICR and imprinted gene expression in the *H19/Igf2* domain is firmly established, direct functional assays of the imprinted gene expression would have made the present findings more convincing. This point is underscored by the observation of increased methylation at the maternal allele of the *H19* ICR in ethanol-treated placentae. Although demethylation at the paternal allele was clearly more severe, this implies that the net change in imprinted gene expression may have been close to zero, undermining any conclusions regarding the relationship between relaxation of imprinting and placental growth retardation.

Generalising the present findings depends on the assumption that paternal alleles inherited from the C57BL/6 strain would have responded similarly to ethanol treatment. Thus, another limitation was the use of the CAST/Ei strain as the exclusive source of the paternal allele.

Likewise, generalising the present findings is further undermined by the utilization of two CAST/Ei males throughout the study. Since a single male was used to derive all control pregnancies and four of the five ethanol-exposed pregnancies, the findings of the present study may not extend to a wider population of CAST/Ei males.

For example, different CAST/Ei paternal alleles may display differential sensitivities to ethanol treatment.

Finally, it should be noted that an acute dosage regimen paradigm, such as the one employed in the present study, may not be well-suited to the detection of ethanol-induced epigenetic defects. Genomic imprinting depends on multiple layers of epigenetic information, including histone modifications, asynchronous replication timing and DNA-methylation (Paulsen and Ferguson-Smith, 2001). Thus, there is much redundancy in epigenetic regulation and DNA-methylation seems to be principally tailored for long-term gene silencing i.e., it follows, rather than precedes, gene silencing (Bird, 2002). Moreover, genomic imprinting may depend on a dynamic interplay between various silencing and enhancing factors as well as between different epigenetic modifications (Svensson et al. 1998; Jaenisch and Bird, 2003; Kurukuti et al. 2006). For example, methylation at lysine 9 on histone H3 promotes *de novo* DNA-methylation, while methylation at CpG dinucleotides encourages histone deacetylation and methylation at lysine 9 on histone H3 (Jaenisch and Bird, 2003). Thus, insofar as imprinting depends on a dynamic balance between silencing and activating factors, acute dosages of ethanol may not be enough to permanently shift the balance. Consistent with this view, the *in vitro* culture of preimplantation embryos for hours to days is associated with severe imprinting defects (Thomson et al. 2001). However, in the present study it was found that two ‘once-off’ doses of ethanol produced relatively modest imprinting defects. This suggests that a chronic dosage regimen paradigm may be more appropriate to the detection of ethanol-induced epigenetic defects at imprinted loci.

4.7.2 Strengths

The ability to distinguish between paternal and maternal genetic contributions was clearly a major strength of the present study. Without this capability, it may have been concluded that methylation is unaffected in both embryos and placentae i.e., the net effect of decreased and increased methylation at the paternal and maternal alleles, respectively, may have been close to zero. Moreover, the losses and gains in methylation at the paternal and maternal alleles, respectively, suggest that imprinting control is dynamic in the placenta, consistent with previously proposed models that placental imprinting depends on a stochastic interplay between silencer and enhancer chromatin assembly factors (Svensson et al. 1998). This finding depended on the ability to distinguish between maternal and paternal genetic contributions.

4.8 FUTURE STUDIES

As the above discussion of the limitations suggests, future studies should include an expanded list of imprinted genes, to more conclusively determine the role of imprinting as a mechanism of ethanol-induced growth retardation in the early embryo. For example, imprinted ICRs should be included that are preferentially methylated on the maternal allele, such as the *Snrpn* ICR, to determine whether demethylation similarly occurs at these loci. Insofar as ethanol-induced growth retardation depends on loss of imprinting, it is predicted that ethanol preferentially affects paternally expressed genes. Consistent with this prediction, the placenta is particularly sensitive to environmentally

induced imprinting defects and paternally expressed imprinted genes function predominantly in the regulation of placental development. This line of reasoning suggests that growth enhancement is not observed following ethanol treatment during the preimplantation period because maternally expressed genes function predominantly in the regulation of embryo growth and, as discussed above, imprinting mechanisms are particularly robust in the embryo. Alternatively, ethanol may affect the epigenetic state of the genome indiscriminately, implying that genomic imprinting is not a direct mechanism of ethanol teratogenesis.

In addition to more imprinted loci, future studies should employ functional assays of imprinted gene expression, such as RT-PCR, in order to directly determine the effect of ethanol on imprinted gene expression. The importance of such assays is underscored by the existence of a number of imprinted genes in placental tissue that do not depend on DNA-methylation as well as the possibility that such loci may be particularly sensitive to ethanol induced deregulation.

Finally, as suggested above, a chronic dosage regimen paradigm may be more conducive to the detection of epigenetic defects at imprinted loci. A future study could dose pregnant females with a reduced dose every day from conception, perhaps up until the end of gastrulation, when the dramatic epigenetic rearrangements of the early embryo are completed. Moreover, the day of harvesting should be shifted to a later period of development. Insofar as the growth retarded placenta is able to meet the nutritional needs of the growth retarded embryo, imprinting defects may be unrelated to embryo growth retardation until late gestation.

4.9 CONCLUSION

In conclusion, the findings of the present study were more complex than originally anticipated. Although paternal demethylation was detected in ethanol-treated placentae, and even though this may mediate the effect of ethanol on placental growth, an unanticipated finding was a partial gain in methylation at the maternal allele. Moreover, *H19* methylation in midgestation mouse embryos (10.5 dpc) was clearly unaffected by ethanol treatment on days 1.5 and 2.5 dpc. Focusing on the relationship between paternal demethylation in the placenta and embryonic growth, the present findings suggest that the *H19* ICR is not a mechanism of ethanol-induced growth retardation prior to 10.5 dpc. However, the *H19* ICR cannot be conclusively ruled out because the functional relationship between the placenta and the embryo depends on the ability of the former to supply the latter with sufficient nutrition. Since both the embryo and the placenta were growth retarded there may have been no net change in nutrient turnover i.e., no net change in 'supply and demand'. Following this line of reasoning, it is unsurprising that demethylation of the paternal *H19* ICR in placenta was unrelated to embryo growth retardation. However, during later gestational periods, when nutritional demands are expected to increase dramatically during the final growth spurt of the foetus, it is to be expected that the supply capabilities of the growth retarded placenta would be unable to maintain sufficient supply. Thus, more research is required before any firm conclusions can be drawn regarding the relationship between ethanol-induced growth retardation and the *H19/Igf2* chromosomal domain.

REFERENCES

Aase JM, Jones KL, Clarren SK (1995) Do we need the term "FAE"? Pediatrics 95:428-430

Astley SJ (2004) Diagnostic guide for fetal alcohol spectrum disorders: The 4-Digit Diagnostic Code. Seattle: University of Washington

Abel E (2004) Paternal contribution to fetal alcohol syndrome. Addict Biol 9:127-133; discussion 135-126

Abel EL (1984) Prenatal effects of alcohol. Drug Alcohol Depend 14:1-10

Abel EL (2006) Fetal alcohol syndrome: a cautionary note. Curr Pharm Des 12:1521-1529

Anway MD, Cupp AS, Uzumcu M, Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308:1466-1469

Anway MD, Skinner MK (2006) Epigenetic transgenerational actions of endocrine disruptors. Endocrinology 147:S43-49

Armant DR, Saunders DE (1996) Exposure of embryonic cells to alcohol: contrasting effects during preimplantation and postimplantation development. Semin Perinatol 20:127-139

Arney KL, Fisher AG (2004) Epigenetic aspects of differentiation. J Cell Sci 117:4355-4363

Baker J, Liu JP, Robertson EJ, Efstratiadis A (1993) Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75:73-82

Becker HC, Diaz-Granados JL, Randall CL (1996) Teratogenic actions of ethanol in the mouse: a minireview. Pharmacol Biochem Behav 55:501-513

Becker HC, Randall CL (1987) Two generations of maternal alcohol consumption in mice: effect on pregnancy outcome. Alcohol Clin Exp Res 11:240-242

Bell AC, Felsenfeld G (2000) Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature 405:482-485

Bielawski DM, Zaher FM, Svinarich DM, Abel EL (2002) Paternal alcohol exposure affects sperm cytosine methyltransferase messenger RNA levels. Alcohol Clin Exp Res 26:347-351

Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16:6-21

Bliek J, Terhal P, van den Bogaard MJ, Maas S, Hamel B, Salieb-Beugelaar G, Simon M, Letteboer T, van der Smagt J, Kroes H, Mannens M (2006) Hypomethylation of the H19 gene causes not only Silver-Russell syndrome (SRS) but also isolated asymmetry or an SRS-like phenotype. Am J Hum Genet 78:604-614

Burns JL, Hassan AB (2001) Cell survival and proliferation are modified by insulin-like growth factor 2 between days 9 and 10 of mouse gestation. Development 128:3819-3830

Bryk AS, Raudenbush SW (1992) Hierarchical linear models: Applications and data analysis methods. Newbury Park, CA: Sage Publications

Campbell MA, Fantel AG (1983) Teratogenicity of acetaldehyde in vitro: relevance to the fetal alcohol syndrome. Life Sci 32:2641-2647

Casola S, Pedone PV, Cavazzana AO, Basso G, Luksch R, d'Amore ES, Carli M, Bruni CB, Riccio A (1997) Expression and parental imprinting of the H19 gene in human rhabdomyosarcoma. Oncogene 14:1503-1510

Catchpoole D, Lam WW, Valler D, Temple IK, Joyce JA, Reik W, Schofield PN, Maher ER (1997) Epigenetic modification and uniparental inheritance of H19 in Beckwith-Wiedemann syndrome. J Med Genet 34:353-359

Chaudhuri JD (2000) Alcohol and the developing fetus--a review. Med Sci Monit 6:1031-1041

Checiu M, Sandor S (1986) The effect of ethanol upon early development in mice and rats. IX. Late effect of acute preimplantation intoxication in mice. Morphol Embryol (Bucur) 32:5-11

Chen RZ, Akbarian S, Tudor M, Jaenisch R (2001) Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. Nat Genet 27:327-331

Chen T, Ueda Y, Dodge JE, Wang Z, Li E (2003) Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. Mol Cell Biol 23:5594-5605

Cheung P, Lau P (2005) Epigenetic regulation by histone methylation and histone variants. Mol Endocrinol 19:563-573

Clark SJ, Harrison J, Paul CL, Frommer M (1994) High sensitivity mapping of methylated cytosines. Nucleic Acids Res 22:2990-2997

Clarren SK, Astley SJ (1992) Pregnancy outcomes after weekly oral administration of ethanol during gestation in the pig-tailed macaque: comparing early gestational exposure to full gestational exposure. Teratology 45:1-9

Clarren SK, Smith DW (1978) The fetal alcohol syndrome. Lamp 35:4-7

Constancia M, Angiolini E, Sandovici I, Smith P, Smith R, Kelsey G, Dean W, Ferguson-Smith A, Sibley CP, 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. Proc Natl Acad Sci U S A 102:19219-19224

Constancia 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 417:945-948

Cooney CA, Dave AA, Wolff GL (2002) Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring. J Nutr 132:2393S-2400S

Davis E, Caiment F, Tordoir X, Cavaille J, Ferguson-Smith A, Cockett N, Georges M, Charlier C (2005) RNAi-mediated allelic trans-interaction at the imprinted Rtl1/Peg11 locus. Curr Biol 15:743-749

Dean W, Bowden L, Aitchison A, Klose J, Moore T, Meneses JJ, Reik W, Feil R (1998) Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. Development 125:2273-2282

DeChiara TM, Robertson EJ, Efstratiadis A (1991) Parental imprinting of the mouse insulin-like growth factor II gene. Cell 64:849-859

Dolinoy DC, Weidman JR, Jirtle RL (2007) Epigenetic gene regulation: linking early developmental environment to adult disease. Reprod Toxicol 23:297-307

Drewell RA, Brenton JD, Ainscough JF, Barton SC, Hilton KJ, Arney KL, Dandolo L, Surani MA (2000) Deletion of a silencer element disrupts H19 imprinting independently of a DNA methylation epigenetic switch. Development 127:3419-3428

Efstratiadis A (1998) Genetics of mouse growth. Int J Dev Biol 42:955-976

Engel N, Thorvaldsen JL, Bartolomei MS (2006) CTCF binding sites promote transcription initiation and prevent DNA methylation on the maternal allele at the imprinted H19/Igf2 locus. Hum Mol Genet 15:2945-2954

Engel N, West AG, Felsenfeld G, Bartolomei MS (2004) Antagonism between DNA hypermethylation and enhancer-blocking activity at the H19 DMD is uncovered by CpG mutations. Nat Genet 36:883-888

Fan G, Martinowich K, Chin MH, He F, Fouse SD, Hutnick L, Hattori D, Ge W, Shen Y, Wu H, ten Hoeve J, Shuai K, Sun YE (2005) DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. Development 132:3345-3356

Fleming TP, Kwong WY, Porter R, Ursell E, Fesenko I, Wilkins A, Miller DJ, Watkins AJ, Eckert JJ (2004) The embryo and its future. Biol Reprod 71:1046-1054

Ford JE, McHeyzer-Williams MG, Lieber MR (1994) Chimeric molecules created by gene amplification interfere with the analysis of somatic hypermutation of murine immunoglobulin genes. Gene 142:279-283

Fourel G, Magdinier F, Gilson E (2004) Insulator dynamics and the setting of chromatin domains. Bioessays 26:523-532

Fowden AL, Sibley C, Reik W, Constancia M (2006) Imprinted genes, placental development and fetal growth. Horm Res 65 Suppl 3:50-58

Fukuzawa R, Umezawa A, Ochi K, Urano F, Ikeda H, Hata J (1999) High frequency of inactivation of the imprinted H19 gene in "sporadic" hepatoblastoma. Int J Cancer 82:490-497

Garro AJ, McBeth DL, Lima V, Lieber CS (1991) Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. Alcohol Clin Exp Res 15:395-398

Gicquel C, Gaston V, Mandelbaum J, Siffroi JP, Flahault A, Le Bouc Y (2003) In vitro fertilization may increase the risk of Beckwith-Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene. Am J Hum Genet 72:1338-1341

Gluckman PD, Hanson MA, Spencer HG, Bateson P (2005) Environmental influences during development and their later consequences for health and disease: implications for the interpretation of empirical studies. Proc Biol Sci 272:671-677

Goldstein H (1995) Multilevel statistical models. London: Arnold.

Goodlett CR, Horn KH (2001) Mechanisms of alcohol-induced damage to the developing nervous system. Alcohol Res Health 25:175-184

Guerri C, Sanchis R (1985) Acetaldehyde and alcohol levels in pregnant rats and their fetuses. Alcohol 2:267-270

Guy J, Hendrich B, Holmes M, Martin JE, Bird A (2001) A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nat Genet 27:322-326

Hajkova P, Erhardt S, Lane N, Haaf T, El-Maarri O, Reik W, Walter J, Surani MA (2002) Epigenetic reprogramming in mouse primordial germ cells. Mech Dev 117:15-23

Halsted CH, Villanueva JA, Devlin AM, Niemela O, Parkkila S, Garrow TA, Wallock LM, Shigenaga MK, Melnyk S, James SJ (2002) Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig. Proc Natl Acad Sci U S A 99:10072-10077

Hanson FB, Florence H (1927a) Do albino rats having ten generations of alcoholic ancestry inherit resistance to alcohol fumes? The American Naturalist 61:43-53

Hanson FB, Florence H (1927b) The effects of alcohol on birth weight and litter size in the albino rat. The American Naturalist 61:503-519

Hanson FB, Handy V (1923) The effects of alcohol fumes on the albino rat: introduction and sterility data for the first treated generation. The American Naturalist 57:532-544

Hao Y, Crenshaw T, Moulton T, Newcomb E, Tycko B (1993) Tumour-suppressor activity of H19 RNA. Nature 365:764-767

Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM (2000) CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature 405:486-489

Hegedus AM, Tarter RE, Hill SY, Jacob T, Winsten NE (1984) Static ataxia: a possible marker for alcoholism. Alcohol Clin Exp Res 8:580-582

Hendrich B, Guy J, Ramsahoye B, Wilson VA, Bird A (2001) Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. Genes Dev 15:710-723

Holliday R (1987) The inheritance of epigenetic defects. Science 238:163-170

Holmgren C, Kanduri C, Dell G, Ward A, Mukhopadhya R, Kanduri M, Lobanenkov V, Ohlsson R (2001) CpG methylation regulates the Igf2/H19 insulator. Curr Biol 11:1128-1130

Hoyme HE, May PA, Kalberg WO, Kodituwakku P, Gossage JP, Trujillo PM, Buckley DG, Miller JH, Aragon AS, Khaole N, Viljoen DL, Jones KL, Robinson LK (2005) A practical clinical approach to diagnosis of fetal alcohol spectrum disorders: clarification of the 1996 institute of medicine criteria. Pediatrics 115:39-47

Hsieh J, Gage FH (2004) Epigenetic control of neural stem cell fate. Curr Opin Genet Dev 14:461-469

Isfort RJ, Cody DB, Kerckaert GA, Tycko B, LeBoeuf RA (1997) Role of the H19 gene in Syrian hamster embryo cell tumorigenicity. Mol Carcinog 20:189-193

Jablonka E, Lamb MJ (1995). *Epigenetic Inheritance and Evolution: The Lamarckian Dimension.* Oxford: Oxford University Press

Jablonka E (2004) Epigenetic epidemiology. Int J Epidemiol 33:929-935

Jablonka E, Lamb MJ (2002) The changing concept of epigenetics. Ann N Y Acad Sci 981:82-96

Jablonka E, Lamb MJ. (2005). *Evolution in Four Dimensions*. Cambridge, MA: MIT Press

Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 33 Suppl:245-254

Jirtle RL, Sander M, Barrett JC (2000) Genomic imprinting and environmental disease susceptibility. Environ Health Perspect 108:271-278

Jones KL, Smith DW (1973) Recognition of the fetal alcohol syndrome in early infancy. Lancet 2:999-1001

Jones KL, Smith DW, Ulleland CN, Streissguth P (1973) Pattern of malformation in offspring of chronic alcoholic mothers. Lancet 1:1267-1271

Kaffer CR, Srivastava M, Park KY, Ives E, Hsieh S, Batlle J, Grinberg A, Huang SP, Pfeifer K (2000) A transcriptional insulator at the imprinted H19/Igf2 locus. Genes Dev 14:1908-1919

Kanduri C, Pant V, Loukinov D, Pugacheva E, Qi CF, Wolffe A, Ohlsson R, Lobanenkov VV (2000) Functional association of CTCF with the insulator upstream of the H19 gene is parent of origin-specific and methylation-sensitive. Curr Biol 10:853-856

Khosla S, Dean W, Brown D, Reik W, Feil R (2001) Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. Biol Reprod 64:918-926

Kurukuti S, Tiwari VK, Tavoosidana G, Pugacheva E, Murrell A, Zhao Z, Lobanenkov V, Reik W, Ohlsson R (2006) CTCF binding at the H19 imprinting control region mediates maternally inherited higher-order chromatin conformation to restrict enhancer access to Igf2. Proc Natl Acad Sci U S A 103:10684-10689

Lau CF, Vogel R, Obe G, Spielmann H (1991) Embryologic and cytogenetic effects of ethanol on preimplantation mouse embryos in vitro. Reprod Toxicol 5:405-410

Lau MM, Stewart CE, Liu Z, Bhatt H, Rotwein P, Stewart CL (1994) Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. Genes Dev 8:2953-2963

Leach RE, Stachecki JJ, Armant DR (1993) Development of in vitro fertilized mouse embryos exposed to ethanol during the preimplantation period: accelerated embryogenesis at subtoxic levels. Teratology 47:57-64

Lee JE, Pintar J, Efstratiadis A (1990) Pattern of the insulin-like growth factor II gene expression during early mouse embryogenesis. Development 110:151-159

Lei H, Oh SP, Okano M, Juttermann R, Goss KA, Jaenisch R, Li E (1996) De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development 122:3195-3205

Leighton PA, Ingram RS, Eggenschwiler J, Efstratiadis A, Tilghman SM (1995) Disruption of imprinting caused by deletion of the H19 gene region in mice. Nature 375:34-39

Lewis A, Mitsuya K, Umlauf D, Smith P, Dean W, Walter J, Higgins M, Feil R, Reik W (2004) Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. Nat Genet 36:1291-1295

Lewis A, Redrup L (2005) Genetic imprinting: conflict at the Callipyge locus. Curr Biol

15:R291-294

Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3:662-673

Li YM, Franklin G, Cui HM, Svensson K, He XB, Adam G, Ohlsson R, Pfeifer S (1998) The H19 transcript is associated with polysomes and may regulate IGF2 expression in trans. J Biol Chem 273:28247-28252

Little RE, Sing CF (1987) Father's drinking and infant birth weight: report of an association. Teratology 36:59-65

Little RE, Streissguth AP, Barr HM, Herman CS (1980) Decreased birth weight in infants of alcoholic women who abstained during pregnancy. J Pediatr 96:974-977

Liu J, Kahri AI, Heikkila P, Ilvesmaki V, Voutilainen R (1995) H19 and insulin-like growth factor-II gene expression in adrenal tumors and cultured adrenal cells. J Clin Endocrinol Metab 80:492-496

Livy DJ, Maier SE, West JR (2004) Long-term alcohol exposure prior to conception results in lower fetal body weights. Birth Defects Res B Dev Reprod Toxicol 71:135-141

Lopes S, Lewis A, Hajkova P, Dean W, Oswald J, Forne T, Murrell A, Constancia M, Bartolomei M, Walter J, Reik W (2003) Epigenetic modifications in an imprinting cluster are controlled by a hierarchy of DMRs suggesting long-range chromatin interactions. Hum Mol Genet 12:295-305

MacDowell EC (1922) Experiments with alcohol and white rats. The American Naturalist 56:289-311

Mann MR, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM, Bartolomei MS (2004) Selective loss of imprinting in the placenta following preimplantation development in culture. Development 131:3727-3735

Manning MA, Hoyme HE (2007) Fetal ethanol spectrum disorders: A practical clinical approach to diagnosis. Neuroscience and Biobehavioural Reviews 31:230-238

May PA, Gossage JP, White-Country M, Goodhart K, Decoteau S, Trujillo PM, Kalberg WO, Viljoen DL, Hoyme HE (2004) Alcohol consumption and other maternal risk factors for fetal alcohol syndrome among three distinct samples of women before, during, and after pregnancy: the risk is relative. Am J Med Genet C Semin

Med Genet 127:10-20

McLaren A (2000) Cloning: pathways to a pluripotent future. Science 288:1775-1780

Mitchell JA (1994) Effects of alcohol on blastocyst implantation and fecundity in the rat. Alcohol Clin Exp Res 18:29-34

Monk M, Boubelik M, Lehnert S (1987) Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development 99:371-382

Moulton T, Crenshaw T, Hao Y, Moosikasuwan J, Lin N, Dembitzer F, Hensle T, Weiss L, McMorrow L, Loew T, et al. (1994) Epigenetic lesions at the H19 locus in Wilms' tumour patients. Nat Genet 7:440-447

Murrell A, Heeson S, Reik W (2004) Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. Nat Genet 36:889-893

Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A 90:8424-8428

Newbold RR, Padilla-Banks E, Jefferson WN (2006) Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. Endocrinology 147:S11-17

Nice L (1917) Further observations on the effects of alcohol on white mice. The American Naturalist 51:596-607

Niemitz EL, Feinberg AP (2004) Epigenetics and assisted reproductive technology: a call for investigation. Am J Hum Genet 74:599-609

Pearl R (1916) Some Effects of the Continued Administration of Alcohol to the Domestic Fowl, with Special Reference to the Progeny. Proceedings of the National Academy of Sciences of the United States of America 2:675-683

Okano M, Xie S, Li E (1998) Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. Nucleic Acids Res 26:2536-2540

Osborne JW (2000) Advantages of hierarchical linear modeling. Practical Assessment, Research & Evaluation 7:1 Retrieved September 16, 2007 from

http://PAREonline.net/getvn.asp?v=7&n=1

Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W, Walter J (2000) Active demethylation of the paternal genome in the mouse zygote. Curr Biol 10:475-478

Padmanabhan R, Hameed MS (1988) Effects of acute doses of ethanol administered at pre-implantation stages on fetal development in the mouse. Drug Alcohol Depend 22:91-100

Pant V, Kurukuti S, Pugacheva E, Shamsuddin S, Mariano P, Renkawitz R, Klenova E, Lobanenkov V, Ohlsson R (2004) Mutation of a single CTCF target site within the H19 imprinting control region leads to loss of Igf2 imprinting and complex patterns of de novo methylation upon maternal inheritance. Mol Cell Biol 24:3497-3504

Pant V, Mariano P, Kanduri C, Mattsson A, Lobanenkov V, Heuchel R, Ohlsson R (2003) The nucleotides responsible for the direct physical contact between the chromatin insulator protein CTCF and the H19 imprinting control region manifest parent of origin-specific long-distance insulation and methylation-free domains. Genes Dev 17:586-590

Paulsen M, Ferguson-Smith AC (2001) DNA methylation in genomic imprinting, development, and disease. J Pathol 195:97-110

Pearl R (1916) Some Effects of the Continued Administration of Alcohol to the Domestic Fowl, with Special Reference to the Progeny. Proc Natl Acad Sci U S A 2:675-683

Poirier F, Chan CT, Timmons PM, Robertson EJ, Evans MJ, Rigby PW (1991) The murine H19 gene is activated during embryonic stem cell differentiation in vitro and at the time of implantation in the developing embryo. Development 113:1105-1114

Preacher KJ, Hayes AF (2004) SPSS and SAS procedures for estimating indirect effects in simple mediation models. Behav Res Methods Instrum Comput 36:717-731

Price SM, Stanhope R, Garrett C, Preece MA, Trembath RC (1999) The spectrum of Silver-Russell syndrome: a clinical and molecular genetic study and new diagnostic criteria. J Med Genet 36:837-842

Pringle KG, Roberts CT (2007) New light on early post-implantation pregnancy in the mouse: roles for insulin-like growth factor-II (IGF-II)? Placenta 28:286-297

Rasbash J, Steele F, Browne W, Prosser B (2004) A User's Guide to *MLwiN* http://www.cmm.bristol.ac.uk/index.shtml

Reik W, Dean W, Walter J (2001) Epigenetic reprogramming in mammalian development. Science 293:1089-1093

Reik W, Walter J (2001) Genomic imprinting: parental influence on the genome. Nat Rev Genet 2:21-32

Resnicoff M, Rubini M, Baserga R, Rubin R (1994) Ethanol inhibits insulin-like growth factor-1-mediated signalling and proliferation of C6 rat glioblastoma cells. Lab Invest 71:657-662

Rosett HL, Weiner L, Zuckerman B, McKinlay S, Edelin KC (1980) Reduction of alcohol consumption during pregnancy with benefits to the newborn. Alcohol Clin Exp Res 4:178-184

Santos F, Hendrich B, Reik W, Dean W (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 241:172-182

Schoenherr CJ, Levorse JM, Tilghman SM (2003) CTCF maintains differential methylation at the Igf2/H19 locus. Nat Genet 33:66-69

Shahbazian M, Young J, Yuva-Paylor L, Spencer C, Antalffy B, Noebels J, Armstrong D, Paylor R, Zoghbi H (2002) Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3. Neuron 35:243-254

Sinclair KD, Young LE, Wilmut I, McEvoy TG (2000) In-utero overgrowth in ruminants following embryo culture: lessons from mice and a warning to men. Hum Reprod 15 Suppl 5:68-86

Stachecki JJ, Yelian FD, Leach RE, Armant DR (1994) Mouse blastocyst outgrowth and implantation rates following exposure to ethanol or A23187 during culture in vitro. J Reprod Fertil 101:611-617

Steenman MJ, Rainier S, Dobry CJ, Grundy P, Horon IL, Feinberg AP (1994) Loss of imprinting of IGF2 is linked to reduced expression and abnormal methylation of H19 in Wilms' tumour. Nat Genet 7:433-439

Stockard CR (1913) The effect on the offspring of intoxicating the male parent and the transmission of the defects to subsequent generations. The American Naturalist 47:641-682

Sullivan WC (1899) A note on the influence of maternal inebriety on the offspring. Journal of Mental Science 45: 489–503

Sun FL, Dean WL, Kelsey G, Allen ND, Reik W (1997) Transactivation of Igf2 in a mouse model of Beckwith-Wiedemann syndrome. Nature 389:809-815

Svensson K, Mattsson R, James TC, Wentzel P, Pilartz M, MacLaughlin J, Miller SJ, Olsson T, Eriksson UJ, Ohlsson R (1998) The paternal allele of the H19 gene is progressively silenced during early mouse development: the acetylation status of histones may be involved in the generation of variegated expression patterns. Development 125:61-69

Szabo P, Tang SH, Rentsendorj A, Pfeifer GP, Mann JR (2000) Maternal-specific footprints at putative CTCF sites in the H19 imprinting control region give evidence for insulator function. Curr Biol 10:607-610

Szabo PE, Tang SH, Silva FJ, Tsark WM, Mann JR (2004) Role of CTCF binding sites in the Igf2/H19 imprinting control region. Mol Cell Biol 24:4791-4800

Tarter RE, Hegedus AM, Goldstein G, Shelly C, Alterman AI (1984) Adolescent sons of alcoholics: neuropsychological and personality characteristics. Alcohol Clin Exp Res 8:216-222

Thompson SL, Konfortova G, Gregory RI, Reik W, Dean W, Feil R (2001) Environmental effects on genomic imprinting in mammals. Toxicol Lett 120:143-150

Thorvaldsen JL, Mann MR, Nwoko O, Duran KL, Bartolomei MS (2002) Analysis of sequence upstream of the endogenous H19 gene reveals elements both essential and dispensable for imprinting. Mol Cell Biol 22:2450-2462

Tremblay KD, Duran KL, Bartolomei MS (1997) A 5' 2-kilobase-pair region of the imprinted mouse H19 gene exhibits exclusive paternal methylation throughout development. Mol Cell Biol 17:4322-4329

Tremblay KD, Saam JR, Ingram RS, Tilghman SM, Bartolomei MS (1995) A paternal-specific methylation imprint marks the alleles of the mouse H19 gene. Nat Genet

9:407-413

Tycko B, Morison IM (2002) Physiological functions of imprinted genes. J Cell Physiol 192:245-258

Walker SK, Hartwich KM ,Seamark RF (1996) The production of unusually large offspring following embryo manipulation: Concepts and challenges. Theriogenology 45:111–120

Warnecke PM, Biniszkiewicz D, Jaenisch R, Frommer M, Clark SJ (1998) Sequence-specific methylation of the mouse H19 gene in embryonic cells deficient in the Dnmt-1 gene. Dev Genet 22:111-121

Warren KR, Li TK (2005) Genetic polymorphisms: impact on the risk of fetal alcohol spectrum disorders. Birth Defects Res A Clin Mol Teratol 73:195-203

Watanabe D, Suetake I, Tada T, Tajima S (2002) Stage- and cell-specific expression of Dnmt3a and Dnmt3b during embryogenesis. Mech Dev 118:187-190

Waterland RA, Jirtle RL (2003) Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Mol Cell Biol 23:5293-5300

Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ (2004) Epigenetic programming by maternal behavior. Nat Neurosci 7:847-854

Weber M, Milligan L, Delalbre A, Antoine E, Brunel C, Cathala G, Forne T (2001) Extensive tissue-specific variation of allelic methylation in the Igf2 gene during mouse fetal development: relation to expression and imprinting. Mech Dev 101:133-141

Webster WS, Walsh DA, McEwen SE, Lipson AH (1983) Some teratogenic properties of ethanol and acetaldehyde in C57BL/6J mice: implications for the study of the fetal alcohol syndrome. Teratology 27:231-243

Weinstein LS (2001) The role of tissue-specific imprinting as a source of phenotypic heterogeneity in human disease. Biol Psychiatry 50:927-931

Whitney DD (1912) The effects of alcohol not inherited in hydatina senta. The American Naturalist 46:41-56

Wiebold JL, Becker WC (1987) In-vivo and in-vitro effects of ethanol on mouse preimplantation embryos. J Reprod Fertil 80:49-57

Wilkins JF, Haig D (2003) What good is genomic imprinting: the function of parent-specific gene expression. Nat Rev Genet 4:359-368

Wozniak DF, Hartman RE, Boyle MP, Vogt SK, Brooks AR, Tenkova T, Young C, Olney JW, Muglia LJ (2004) Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults. Neurobiol Dis 17:403-414

Wu Q, Ohsako S, Ishimura R, Suzuki JS, Tohyama C (2004) Exposure of mouse preimplantation embryos to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters the methylation status of imprinted genes H19 and Igf2. Biol Reprod 70:1790-1797

Yang Q, Witkiewicz BB, Olney RS, Liu Y, Davis M, Khoury MJ, Correa A, Erickson JD (2001) A case-control study of maternal alcohol consumption and intrauterine growth retardation. Ann Epidemiol 11:497-503

Young LE, Sinclair KD, Wilmut I (1998) Large offspring syndrome in cattle and sheep. Rev Reprod 3:155-163

Yu K, Chedin F, Hsieh CL, Wilson TE, Lieber MR (2003) R-loops at immunoglobulin class switch regions in the chromosomes of stimulated B cells. Nat Immunol 4:442-451

Zou J, Rabin RA, Pentney RJ (1993) Ethanol enhances neurite outgrowth in primary cultures of rat cerebellar macroneurons. Brain Res Dev Brain Res 72:75-84

APPENDIX A:

ETHICS CLEARANCE CERTIFICATE

AESC 4

STRICTLY CONFIDENTIAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:

2004	86	3

APPLICANT: Philip C Haycock

DEPARTMENT: Human Genetics

PROJECT TITLE. **Exposure of Mouse Embryos to Alcohol During Preimplantation Development: Effect on Methylation in the HI9 Imprinting Control Region**

Species	Number	Expiry Date
Mus Musculus	4 (M)	September 2006
Mus Musculus	8 (F)	September 2006

i) Approval is hereby given for the experiment described in the above application.
The use of these animals is subject to AESC Guidelines for the use and care of animals, is limited to the procedures specified in the application form, and to:

APPROVED subject to supplying
- list of references for the dose of alcohol given to the mice
- clarifying the method of euthanasia to be used
- discussing the role of CAS in the this study with the CAS Director

SIGNED D A. Grey DATE: 3 September 2004
(Chairman: ~~Animal~~ Ethics Screening Committee)

ii) I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23(1)(c) of the Veterinary and Para-veterinary Professions Act (19 of 1982)

SIGNED _____ DATE: 3 September 2004
(Registered Veterinarian)

NOTE:
First-time users of the CAS should contact the Director of the CAS in order to familiarise themselves with the facilities available, and the procedures required by the CAS for the carrying out of experiments.

APPENDIX B

PROTOCOLS

Post restriction digest cleanup

1. Adjust the volume of the digest to 100 µl
2. Add glycogen to a final concentration of 0.5-1 µg/µl
3. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1)
4. Leave on ice for 5 minutes
5. Spin for 5 minutes at 11 000g and recover top aqueous phase
6. Add 1/10 volume of 3M sodium acetate pH 5.2 and an equal volume of 100% ethanol
7. Spin for 10 minutes at 11 000g
8. Wash pellot with 70% ethanol and air dry
9. Resuspend in approximately 50 µl of deionised water

Precipitation of PCR products

1. Adjust volume of PCR product to 45 µl in deionised water
2. Add 5 µl of 3M sodium acetate pH 5.2
3. Add 125 µl 100% ethanol
4. Spin for 5 minutes at approximately 12 000g
5. Wash pellot with 70% ethanol and air dry
6. Resuspend in approximately 50 µl of deionised water

Rubidium chloride method for preparation of competent cells

1. Inoculate 1ml from overnight culture into 100 ml Psi broth and incubate at 37^{o}C with aeration to A550=0.48
2. Put on ice for 15 minutes
3. Spin cells at 3-5000g for 5 minutes
4. Discard supernatant and resuspend in 0.4 volume (of original volume) of TfbI and place one ice for 15 minutes
5. Repeat step 3
6. Discard supernatant and resuspend in 0.04 volume TfbII and place on ice for 15 minutes
7. Freeze in liquid nitrogen and store at -70^{o}C

Transformations

1. Defrost 50 µl of competent cells (per sample) on ice for approximately 5 minutes
2. Add 5µl of ligation product to 50 µl of competent cells and mix by gently flicking the tube
3. Heat shock for 45-50 seconds at 42^{o}C
4. Return the tubes to ice for 2 minutes
5. Add 950 µl of SOC medium to the tubes
6. Incubate for 1.5 hours at 37^{o}C in a shaking incubator (100-150rpm)
7. Plate 50-200 µl of each transformation culture onto LB/ampicillin/X-Gal plates

8. Incubate the plates overnight (16-24 hours) at 37^{o}C

APPENDIX C

REAGENTS

0.5 M EDTA (pH 8.0)

93.06 g EDTA dihydrate

100 ml ddH_20

pH to 8 with 5 M NaOH pellets

1M Tris-HCl pH (8.0)

12.11 g Tris

Adjust pH to 8.0 and make up to 100 ml with distilled water

Autoclave before use

1 x TE buffer (pH 8.0)

1 ml 1 M Tris-HCl

200 µl 0.5 M EDTA

Make up to 100 ml with distilled water

Autoclave before use

Primer dilutions

All primers were resuspended in Tris buffer (pH 8.0) to a final concentration of 100 µM. Working solutions were diluted to 5 µM in deionised water. All primers were stored at -20^0C.

dNTP mix (Bioline)

A 10mM working solution was prepared from 100mM stock dNTPs.

Take 10 µl of each dNTP and make up to 100µl in deionised water.

2% Agarose gel

8 g Agarose

400 ml 1x TBE buffer

Heat in the microwave until fully dissolved.

Once slightly cooled, add 12 µl of a 10 mg/µl ethidium bromide.

Pour the gel mix into a gel tray.

10 x TBE buffer

432 g Tris

220 g Boric acid

29.7 g EDTA dihydrate

Add distilled water to a final volume of 4 *l.*

Autoclave before use.

3M Sodium Acetate (pH 5.2)

204.5g sodium acetate

Add 400ml deionised water

Adjust pH to 5.2 using glacial acetic acid

SOB medium

40ml of deionised water

0.8g tryptone

0.2g yeast extract

0.02g NaCl

Adjust pH to 7.0 using 5M NaOH

Adjust volume to 50ml using deionised water

Autoclave the broth before use

1M $MgCl_2$/1M $MgSO_4$ solution

10ml deionised water

2.03g of 1M $MgCl_2$

2.47g of 1M $MgSO_4$

Sterilize using a 0.45µm filter

2M glucose solution

3.6g

10ml deionised water

Sterilize using a 0.45µm filter

SOC medium

890µl of SOB medium

100µl of 1M $MgCl_2$/1$MgSO_4$ solution

10µl of 2M glucose solution

LA agar plates

100ml distilled water

1g NaCl2

1g tryptone

0.5g yeast extract

2g agar

Adjust the pH to 7.0 using 5M NaOH

Autoclave the broth

Add 200µl ampicillin (50mg/ml)

Ampicillin solution (Roche)

Resuspended to a final concentration of 50mg/ml and sterilised using a 0.45µm filter

Psi brother

1000ml distilled water

5g Bacto yeast extract

20g Bacto tryptone

5g magnesium sulphate

Adjust pH to 7.6 with potassium hydroxide

TfbI

200ml distilled water

0.588g potassium acetate

2.42g rubidium chloride

0294g calcium chloride

2.0g manganese chloride

30ml glycerol

Adjust pH to 5.8 with dilute acetic acid

TfbII

100ml distilled water

0.21g MOPS

1.1g calcium chloride

APPENDIX D:

WEIGHT DATA

Table B1. Weights (mg) of embryonic and placental samples of five control and five ethanol-treated mothers

Embryo ID	Mother ID	Mother Code	Treatment	Date of dissection	Embryo Weight (mg)	Placenta Weight (mg)
E1	NoholesB6.2	C1	saline	11/9/2006	56	41
E2	NoholesB6.2	C1	saline	11/9/2006	60	47
E3	NoholesB6.2	C1	saline	11/9/2006	14	37
E4	NoholesB6.2	C1	saline	11/9/2006	58	50
E5	NoholesB6.2	C1	saline	11/9/2006	54	36
E6	NoholesB6.2	C1	saline	11/9/2006	60	43
E7	NoholesB6.2	C1	saline	11/9/2006	60	37
E8	NoholesB6.2	C1	saline	11/9/2006	46	34
E9	NoholesB6.2	C1	saline	11/9/2006	68	42
E10	NoholesB6.2	C1	saline	11/9/2006	63	45
E1	2LB6.4	C2	saline	2/10/2006	55	99
E2	2LB6.4	C2	saline	2/10/2006	60	63
E3	2LB6.4	C2	saline	2/10/2006	33	25
E4	2LB6.4	C2	saline	2/10/2006	54	50
E5	2LB6.4	C2	saline	2/10/2006	63	49
E6	2LB6.4	C2	saline	2/10/2006	47	43
E7	2LB6.4	C2	saline	2/10/2006	46	31
E8	2LB6.4	C2	saline	2/10/2006	54	40
E9	2LB6.4	C2	saline	2/10/2006	57	32
E1	2L2RB6.3	C3	saline	15/9/2006	71	63
E2	2L2RB6.3	C3	saline	15/9/2006	62	78
E3	2L2RB6.3	C3	saline	15/9/2006	69	56
E4	2L2RB6.3	C3	saline	15/9/2006	56	41
E5	2L2RB6.3	C3	saline	15/9/2006	66	85
E6	2L2RB6.3	C3	saline	15/9/2006	61	60
E7	2L2RB6.3	C3	saline	15/9/2006	66	51
E8	2L2RB6.3	C3	saline	15/9/2006	64	57
E1	1R2LB3	C4	saline	25/5/2005	21	79
E2	1R2LB3	C4	saline	25/5/2005	37	56
E4	1R2LB3	C4	saline	25/5/2005	55	45
E5	1R2LB3	C4	saline	25/5/2005	66	94
E6	1R2LB3	C4	saline	25/5/2005	57	49
E7	1R2LB3	C4	saline	25/5/2005	54	39
E8	1R2LB3	C4	saline	25/5/2005	41	37
E1	1L2RB3	C5	saline	16/5/2005	24	56
E2	1L2RB3	C5	saline	16/5/2005	25	60
E3	1L2RB3	C5	saline	16/5/2005	20	43
E5	1L2RB3	C5	saline	16/5/2005	9	46
E6	1L2RB3	C5	saline	16/5/2005	21	52
E7	1L2RB3	C5	saline	16/5/2005	20	44

E8	1L2RB3	C5	saline	16/5/2005	31	35
E9	1L2RB3	C5	saline	16/5/2005	36	60
E1	C572010	E1	ethanol	31/10/2004	15	19
E2	C572010	E1	ethanol	31/10/2004	15	
E3	C572010	E1	ethanol	31/10/2004	24	59
E4	C572010	E1	ethanol	31/10/2004	26	72
E6	C572010	E1	ethanol	31/10/2004	16	64
E7	C572010	E1	ethanol	31/10/2004	19	49
E8	C572010	E1	ethanol	31/10/2004	13	64
E9	C572010	E1	ethanol	31/10/2004	24	62
E10	C572010	E1	ethanol	31/10/2004	15	68
E1	2RB6	E2	ethanol	6/9/2006	8	44
E2	2RB6	E2	ethanol	6/9/2006	13	13
E4	2RB6	E2	ethanol	6/9/2006	13	47
E5	2RB6	E2	ethanol	6/9/2006	19	21
E6	2RB6	E2	ethanol	6/9/2006		39
E8	2RB6	E2	ethanol	6/9/2006	16	49
E9	2RB6	E2	ethanol	6/9/2006	15	22
E1	1RB5	E3	ethanol	7/7/2006	19	37
E2	1RB5	E3	ethanol	7/7/2006	13	52
E3	1RB5	E3	ethanol	7/7/2006	15	43
E4	1RB5	E3	ethanol	7/7/2006	15	43
E6	1RB5	E3	ethanol	7/7/2006		17
E7	1RB5	E3	ethanol	7/7/2006	19	22
E8	1RB5	E3	ethanol	7/7/2006	13	60
E9	1RB5	E3	ethanol	7/7/2006	15	37
E2	1R1LB6.4	E4	ethanol	28/9/2006	18	32
E3	1R1LB6.4	E4	ethanol	28/9/2006	25	26
E4	1R1LB6.4	E4	ethanol	28/9/2006	21	20
E6	1R1LB6.4	E4	ethanol	28/9/2006	21	41
E8	1R1LB6.4	E4	ethanol	28/9/2006	18	60
E9	1R1LB6.4	E4	ethanol	28/9/2006	23	37
E1	1LB6.4	E5	ethanol	12/10/2006	25	26
E2	1LB6.4	E5	ethanol	12/10/2006	31	24
E3	1LB6.4	E5	ethanol	12/10/2006	41	32
E4	1LB6.4	E5	ethanol	12/10/2006	30	32
E5	1LB6.4	E5	ethanol	12/10/2006	21	26
E6	1LB6.4	E5	ethanol	12/10/2006	25	27
E7	1LB6.4	E5	ethanol	12/10/2006	21	20
E8	1LB6.4	E5	ethanol	12/10/2006	28	28
E9	1LB6.4	E5	ethanol	12/10/2006	29	34

Embryo IDs are sorted according to order of dissection; missing data correspond to samples that were not measured, either by mistake or because they were lost during the dissection; each weight is an average of three measurements.