BOR Papers in Press. Published on May 27, 2009 as DOI:10.1095/biolreprod.108.074690

Fetal Alcohol Spectrum Disorders: The Epigenetic Perspective[1]

Short title: Epigenetics and ethanol teratogenesis

Summary sentence: Important periods of ethanol teratogenesis – preconception, preimplantation and gastrulation – correspond to peak periods of epigenetic reprogramming, suggesting an epigenetic model of fetal alcohol spectrum disorders.

Keywords: epigenetics, fetal alcohol spectrum disorders, ethanol teratogenesis

Philip C Haycock[2,3]

[2]Division of Human Genetics, University of the Witwatersrand and National Health Laboratory Service, Johannesburg, South Africa

[1]Supported by a March of Dimes Grant 6-FY0470

[3]Present address and reprint requests:
Mr Philip Haycock
Department of Public Health and Primary Care
Strangeways Research Laboratory
University of Cambridge
CB1 8RN
E-mail: pch43@medschl.cam.ac.uk

ABSTRACT

Ethanol is a classic teratogen capable of inducing a wide range of developmental abnormalities. Studies in animal models suggest that differences in timing and dosage underlie this variability, with three particularly important developmental periods: preconception, preimplantation and gastrulation. These periods of teratogenesis correlate with peak periods of epigenetic reprogramming which, together with the evidence that ethanol interferes with one carbon metabolism, DNA-methylation, histone modifications and noncoding RNA (ncRNA), suggests an important role for epigenetic mechanisms in the aetiology of fetal alcohol spectrum disorders (FASD). In addition to a number of testable hypotheses, an epigenetic model suggests that the concept of a 'fetal alcohol spectrum' should be expanded to include 'preconceptional effects'. This proposal has important public health implications, highlighting the urgency of research into the epigenetic basis of FASD.

Copyright 2009 by The Society for the Study of Reproduction.

INTRODUCTION

In utero alcohol exposure is associated with a wide range of neurobehavioural and physical abnormalities. These vary in severity, from the barely perceptible to spontaneous abortion, and are collectively referred to as fetal alcohol spectrum disorders (FASD) [1]. According to the Institute of Medicine's revised classification system [2] there are currently six recognized diagnoses: fetal 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 the most adverse clinical outcome resulting from prenatal alcohol exposure.

First delineated in 1973 [3, 4] FAS encompasses three broad domains: prenatal and/or postnatal growth retardation; distinctive facial features (short palpebral fissures, smooth philtrum, thin vermillion border of the upper lip) and brain damage. 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 [5].

What causes fetal alcohol spectrum disorders?

Soon after its recognition, research turned towards the mechanistic basis 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 accounted for by differences 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 have approached alcohol teratogenesis from one or more of the following perspectives: genetic, biochemical, cellular and morphological. For example, research has associated ethanol with reduced growth factor levels [6, 7]; inhibition of such factors is likely to result in reduced cellular proliferation [8, 9] which may, in turn, result in reduced brain mass [9]; and it is reasonable to propose that genetic variation in enzymes that regulate alcohol metabolism (e.g. alcohol dehydrogenases) influences an individual's susceptibility to FASD [10]. The key challenge facing the FASD research field is the integration of this wide and disparate body of research into a coherent whole.

This is a monumental task because FASD cannot be understood as if it were a single localized insult on an otherwise normal organism. Instead, it must be approached as an emergent property of deregulated developmental pathways and interactions. 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 aetiology of FASD involves a potentially bewildering array of heterogeneity.

In this review, special attention is drawn to the possible role of epigenetic factors and epigenetic reprogramming as mechanisms of ethanol teratogenesis (Figure 1). The relationship has received little attention in the alcohol research field. This is surprising, considering that epigenetic factors

are important mechanisms of developmental events, such as genomic imprinting and cellular differentiation, which could plausibly be involved in the teratogenic pathway. Moreover, it is argued that an epigenetic perspective is able to explain a large number of phenomena, including the behavioural and physical abnormalities arising from preconceptional and *in utero* ethanol exposure. Evidential support for this proposal comes from epidemiological and experimental studies linking ethanol to alterations in one carbon metabolism, DNA-methyltransferase, DNA-methylation, histone modifications and noncoding RNAs (ncRNAs), as well as the important role of epigenetic mechanisms in CNS development and dysfunction (a key feature of FASD). Before proceeding, it should be noted that alcohol refers exclusively to ethyl alcohol (i.e. ethanol) in this review.

EPIGENETICS

What is epigenetics?

The cells of a multicellular organism are genetically identical (with the exception of germ cells and cells residing within the immune system) but are functionally heterogeneous. Understanding how functional diversity is generated requires an understanding of how heritable differences in gene expression arise during development amongst different cell types. The mechanisms governing these processes are *epi*genetic, since they cannot solely reside within the DNA-sequence, and are sometimes described as epigenetic inheritance systems (EISs) [11]. Some of the most widely recognized mechanisms of epigenetic inheritance include covalent modifications of DNA and histones (collectively referred to as chromatin marks), as well as small (~20-30 nucleotides) ncRNAs [11].

As mechanisms of cellular differentiation, chromatin marks and small ncRNAs have three important properties: (1) they affect cell function because they affect chromatin structure and/or 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 [12].

Effect of epigenetic factors on chromatin structure and gene expression

The basic building block of chromatin is the nucleosome, a structure formed by the association of DNA with histone proteins in a bead-on-a-string-like configuration. The core of the nucleosome is made up of histones H2A, H2B, H3 and H4, with histone H1 acting as a linker between the nucleosomes [13]. Epigenetic modifications of chromatin include methylation at carbon five of cytosine (one of the four nitrogenous bases in DNA) and covalent modifications of histone proteins. DNA-methylation occurs almost entirely in the context of CpG dinucleotides, which are generally methylated in vertebrate genomes, with the exception of CpG islands which tend to escape methylation. Histone modifications occur in the N-terminal tails of histones H3 and H4 as well as the core of histones H2A and H3. Amino acid residues within these histones can be covalently modified by acetylation, methylation, phosphorylation, ubiquitinylation, ADP-ribosylation and sumoylation [13]. These covalent modifications cause chromatin to assume various states of compaction and folding, which affects the accessibility of gene promoter regions to the transcriptional machinery of the cell. For example, heterochromatin is generally condensed and silent, while euchromatin is open and transcriptionally active. Further distinctions are possible between constitutive and facultative heterochromatin [12]. The former are important maintainers of genome stability, being associated with highly repetitive

centromeric and telomeric DNA. In contrast, facultative heterochromatin is generally associated with gene silencing, the inactive X chromosome in mammalian females being the most obvious example of this type [12].

The two main types of chromatin tend to be associated with distinct classes of epigenetic modifications. For example, heterochromatin is associated with methylation of CpG dinucleotides, hypoacetylation of histones H3 and H4 and di/tri-methylation of lysine 9 on histone H3 (H3K9Me) [12]. Euchromatin, on the other hand, is associated with hypomethylation of CpG dinucleotides, acetylation of histones H3 and H4 and di/tri-methylation of lysine 4 on histone H3 (H3K4Me) [12].

These modifications are regulated by a range of chromatin modifying enzymes, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), which catalyze the reversible acetylation of histones [14]. Other important enzymes include histone methyltransferases (HMTs), the maintenance DNA methyltransferase DNMT1, and the *de novo* methyltransferases DNMT3A and DNMT3B [15, 16].

The mechanism of the association between these epigenetic modifications and chromatin structure is partly mediated by the ability of chromatin marks to recruit chromatin remodelling enzymes and other non-histone proteins. For example, methylation at the paternal allele of the *H19* imprinting control region (ICR) in mouse 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 [17, 18], which, in-turn, partitions the *Igf2* and *H19* genes into 'silent' and 'active' chromatin domains [17, 19]. In contrast, binding of CTCF to the unmethylated maternal allele of the *H19* ICR partitions the *Igf2* gene into a silent chromatin domain [17, 19]. Thus, DNA-methylation is able to alter higher-order chromatin structure through its ability to block DNA-binding proteins.

In contrast to the above mechanism, which is based on the ability of DNA-methylation to abolish protein binding, other chromatin proteins are known to preferentially bind methylated DNA. These include methyl CpG-binding protein 2 (MECP2), which has been functionally linked to Rett's syndrome [20, 21], methyl CpG binding domain protein (MBD) 1, MBD2, MBD4 and kaiso [21, 22]. Through chromatin silencing mechanisms that involve the recruitment of HDACs, these proteins are able to repress gene promoter access to the transcriptional machinery [18].

DNA methylation and histone modifications regulate gene expression at the level of chromatin, specifying silencing/expression prior to transcription through their effects on chromatin structure. In contrast, small (~20-30 nucleotides) ncRNAs regulate gene expression through RNA silencing pathways that operate at both the level of chromatin, as well as post-transcriptionally [23]. The two most widely recognised RNA silencing pathways are RNA interference (RNAi), regulated by short interfering RNAs (siRNAs), and developmentally programmed silencing pathways regulated by micro RNAs (miRNAs). Both RNAs are produced from longer double stranded RNA (dsRNA) precursors by the enzyme Dicer and function as specificity factors for protein effector complexes that silence/degrade homologous sequences [23]. Although there is considerable mechanistic overlap amongst RNA silencing pathways there are some notable differences. For example, the dsRNA precursors of siRNAs generally have

exogenous origins, e.g. transgenes or viruses, while miRNAs are generally produced from dsRNAs with endogenous origins, e.g. miRNA genes [23]. Moreover, siRNAs generally target their own precursors for degradation, while miRNAs generally inhibit translation of target mRNAs originating from other genes [23].

In addition to post-transcriptional gene silencing, RNA silencing pathways may also operate at the level of chromatin through RNA-directed modulation of DNA-methylation and histone modifications [23]. For example, siRNAs in plants can direct the silencing of transgenes via DNA methylation of homologous sequences through a process known as RNA-directed DNA methylation (RdDM) [24, 25].

Heritability of epigenetic factors through mitosis

Epigenetic factors must, by definition, be capable of transmitting functional information through mitosis, a criterion that is generally satisfied by DNA-methylation, many (but not all) histone modifications and small RNAs. For example, the copying of 'old' DNA-methylation profiles onto newly synthesized DNA strands is mediated by DNA methyltransferase 1 (*DNMT1*), which preferentially methylates hemimethylated DNA [21]. In this way, the methylation profile of the 'old half' serves as a template for synthesis of the 'new half'. The processes by which histone modifications are replicated and transmitted through mitosis are poorly understood, although it is known that 'old' histones are randomly distributed to newly synthesized DNA strands. It has been suggested that histone modifications might be replicated in a similar fashion to DNA-methylation, although this is not the only proposed model [15]. In the 'nascent transcript model' it is proposed that the inheritance of heterochromatin depends on a positive feedback loop between siRNAs and H3K9 methylation [23].

Small RNAs can also be transmitted through mitosis, suggesting that RNA, like chromatin modifications, might also mediate the inheritance of cell-specific gene expression programs through mitosis. Moreover, small RNAs can be stably replicated via RNA-dependent RNA polymerase, suggesting that small RNAs could, in principle, maintain patterns of gene silencing for relatively long periods of time, over several cell divisions. This is consistent with the observation that RNA-induced gene silencing can persist for several generations in *Caenorhabditis elegans* [26]

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 chromatin and gene expression [12, 15]. The ability of the genome to respond to developmental signals through heritable alterations in chromatin structure is a key aspect of cellular differentiation and is clearly important to functional variation between cells within the same individual.

The ability of epigenetic factors to respond to developmental signals probably underlies their sensitivity to exogenous environmental stimuli. For example, the 'epigenome' can be altered by dietary supplements, (e.g. folic acid, vitamin B12, choline, and betaine) [27, 28], ethanol [29], endocrine disruptors [30, 31], *in vitro* culture techniques [32], 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [33] and maternal care [34]. The epigenetic changes associated with these

environmental manipulations have also been associated with various behavioural and physical abnormalities, raising the possibility that the epigenetic processes underlying variability between cells may also contribute to phenotypic variation between individuals. This variation is likely to arise during important periods of epigenetic reprogramming.

Epigenetic reprogramming

Developmentally, the establishment or erasure of chromatin modifications is known as epigenetic reprogramming (Figure 1). Significantly, the prenatal period is characterized by dramatic epigenetic changes: during the preimplantation period genome-wide DNA-methylation is almost entirely erased; this is followed by genome-wide *de novo* methylation during gastrulation [16]. 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 [35]. Later periods of development, during the onset of terminal differentiation events, are correlated with localized chromatin remodelling, such as the nerve growth factor (NGF) induced neuronal differentiation pathway [36], the JAK-STAT-induced astroglial differentiation pathway [37] and the differentiation of neural stem cells [14]. Research from the Human Epigenome Project (HEP) supports the view that DNA-methylation correlates with tissue differentiation [38]. For example, bisulphite sequencing of chromosomes 6, 20 and 22 in 12 tissues revealed small differences in methylation between functionally similar cells, such as CD4+ lymphocytes and CD8+ lymphocytes (difference ~6%), and relatively larger differences in methylation between functionally dissimilar tissues, such as sperm and melanocytes (difference ~20%) [38].

Epigenetic reprogramming as a mechanism of ethanol teratogenesis

Ethanol-induced abnormalities could arise through disruption of these epigenetic reprogramming events (Figure 1). In support of this epigenetic perspective, alcohol is known to affect one carbon metabolism, the primary source of methyl donors in DNA transmethylation reactions [39], DNA-methyltransferase [29, 40], DNA-methylation [29], histone modifications [13] and small ncRNAs [41].

Ethanol and DNA-methylation

For example, Garro et al. [29] found that acute administration of ethanol to pregnant mice during midgestation resulted in genome wide hypomethylation in 11 day old fetuses. Pregnant MF1 mice were dosed with either 50% ethanol (3g/kg) or a caloric equivalent of glucose-saline by gavage on the 9th, 10th and 11th 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 fetuses from ethanol-fed dams was a significantly better substrate as compared to the control group, suggesting a reduced level of methylation in the former [29]. The authors also showed that nuclei extracted from the ethanol group had significantly reduced methylase activity as compared to the control group, suggesting lower levels of DNA-methyltransferase in fetuses harvested from ethanol-fed dams [29]. 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* [29]. In contrast, ethanol did not inhibit DNA-methyltransferase activity *in vitro*, even at very high concentrations (100mM) [29].

In a separate study, reductions in DNA-methyltransferase RNA in sperm, as well as reductions in offspring weight, were observed following chronic alcohol treatment of male rats, suggesting that alterations in DNA methylation in the germline may lead to physical abnormalities in offspring [40].

Ethanol and one-carbon metabolism
It has been known for many years that alcohol interacts with various components of one-carbon metabolism, including folate and homocysteine, suggesting a potential link between ethanol and DNA transmethylation reactions. In a case-control study, reduced blood folate and elevated plasma homocysteine was reported in a sample of 32 chronic alcoholics in comparison to non-drinking controls [42]. In an animal model of chronic alcoholism, it was found that ethanol, together with a folate restricted diet, resulted in elevated homocysteine and global reductions in DNA methylation in liver [39]. Inhibition of one carbon metabolism could lead to alterations in epigenetic reprogramming (Figure 1).

Ethanol and histone modifications
Prior research in the alcoholism research field has also highlighted the likely importance of histone modifications, including acetylation, methylation and phosphorylation, in the pathophysiology of chronic alcohol abuse [13]. For example, Park et al [43] observed increased acetylation of histone H3 at lysine 9 (H3K9) but not Lys14, -18, and -23 following primary culture of rat hepatocytes in ethanol [43]. Liver cells obtained from rats exposed to ethanol (6 g/kg) intragastrically produced similar results. Increased H3K9 acetylation at the alcohol dehydrogenase (*ADHI*) gene was also observed [43], which might account for the ethanol-induced increases in *ADH1* gene expression observed in a separate study [44].

Employing a dosage regimen paradigm meant to mimic binge drinking behaviour, Kim and Shukla [45] investigated the tissue-specificity of ethanol-induced acetylation and methylation at H3K9 in rat. Increases in acetylation were observed in liver, lung, spleen and testis but not kidney, brain, heart, stomach, colorectum, pancreas or vessels, with no significant effects on methylation in any tissues.

In a study by Pal-Bhadra et al. [46] it was observed that ethanol caused an increase in acetylation and a decrease in methylation of H3K9, as well as an increase in methylation of H3K4, in rat hepatocytes. The latter changes were associated with the upregulation of a number of genes, including *ADH* and *GSTA3*, while the methylation changes at H3K9 were associated with a downregulation of genes, including *Lsdh* and *Cyp2c11*.

A role for histone modifications in the mediation of ethanol-induced alterations in mitogen-activated protein (MAP) kinase signalling pathways has recently been demonstrated. Lee and Shukla [47] observed increased phosphorylation of histone H3 serine 10 (H3S10) and H3S28 following exposure of rat hepatocytes to either ethanol or acetaldehyde *in vitro* – an effect mediated by MAPK14 (p38 MAPK) but not MAPK3 (p42⁄44 MAP kinase) or JNK kinases.

Ethanol and small noncoding RNAs
Research has recently established a role for RNA directed gene silencing in mediating the

relationship between alcohol and the large–conductance calcium- and voltage-activated potassium (BK) channel, a well known molecular mechanism of alcohol addiction [41]. Increased levels of MIR9 (miR-9) miRNA were observed following exposure of rat neurons to 20 mM alcohol *in vitro* which, in turn, resulted in considerable reorganization of the BK RNA landscape. More specifically, it was found that MIR9 downregulated BK RNA splice variants carrying MIR9 recognition elements (MRE) in their 3' ends, leading to the enrichment of splice variants for alcohol-resistant BK isoforms.

In sum, ethanol is a known inhibitor of one carbon metabolism and DNA-methyltransferase; and interferes with various epigenetic factors, including DNA-methylation, histone modifications and small ncRNAs. Despite this, little is known about the relationship between epigenetic factors and ethanol teratogenesis. As argued below, the correlation between major periods of epigenetic reprogramming and critical periods of teratogenesis, suggests that an epigenetic perspective is able to explain a large number of phenomena associated with ethanol exposure.

THE IMPORTANCE OF TIMING AND DOSAGE IN THE AETIOLOGY OF FASD

Research in animal models strongly suggests that the variability observed within the FASD continuum 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 [48]. 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 of exposure [48].

The following section covers the teratogenic consequences of ethanol exposure during specific developmental periods: preconception; preimplantation; and 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 (≤3g/kg) doses of ethanol administered intraperitoneally, intragastrically, or as part of their liquid diet, throughout the developmental period of interest.

These dosage levels generally model the 'binge drinking' end of the behavioural spectrum in humans. For example, in an epidemiological study of FAS in the Western Cape province of South Africa, case mothers who admitted drinking, self reported consumption of 13.6 drinks (SD=8.9) over the weekend. Assuming a weight of ~60kg (the average weight of case mothers), 13.6 drinks would be equivalent to ~3-4.3 g/kg ethanol. However, these estimates could vary quite considerably depending on the weight of the case mothers (SD = 14.2 kg) and the number of self reported drinks (SD = 8.9 drinks) [49].

Perhaps more important than the number of drinks consumed is the actual blood alcohol concentration (BAC) achieved, which will vary according to a person's weight and alcohol metabolism, and which is more comparable between species. Pharmacologically relevant BACs, i.e. doses that would realistically be expected in humans, probably rarely exceed 200mM. In a casualty ward at a German hospital, unusually high BACs of 109mM – 170 mM were reported at an incidence of 3 per 1000 patients over a three month period [50]. In an animal model, delivery

of two doses of 2.9g/kg four hours apart resulted in BACs of ~65mM-109mM (the authors do not state whether this was by intraperitoneal injection or by gavage). It has also been reported that 3-5 drinks produces an average BAC of ~33mM in humans [51]. According to Pantazis et al [52], pharmacologically relevant BACs should lie in the range of ~20mM to ~170mM, which is a realistic estimate of the levels likely to be attained by moderate to very heavy drinkers. Clearly, interpretation of findings in the alcohol research field should be tempered by whether they are reflective of the human condition, and whether they model "low to moderate" or binge drinking behaviour.

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 date to the early 1900s [53-60] when Lamarckian ideas of inheritance were still in-vogue and the subject of much investigation. Ethanol was a popular experimental system because of its myriad effects on the human organism 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 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 guinea pigs were treated by the inhalation method to the point of intoxication every day, except Sundays, for approximately three years [60]. "From time to time" treated animals (males and females) were mated with untreated controls [60]. Various experimental conditions were tested, such as 'alcoholised females' x 'normal males', 'alcoholised females' x 'alcoholised males' and 'alcoholised males' x 'normal females'.

It was found that, following 34 successful crossings between alcoholised males and normal females, 24% of litters were stillborn [60]. 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, the untreated offspring of parents from 'alcoholised conditions' tended to have fewer surviving offspring than controls (54% versus 93%) [60]. 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.

The preconception period: recent findings
The findings described above 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.

Preconceptional effects mediated by paternal consumption
Relatively recent epidemiological studies indicate an association between lowered birth weight in offspring and paternal alcoholism [61]. In addition, adoption studies suggest positive associations between hyperactivity and lowered cognitive abilities in offspring and alcoholism in the biological father but not the adoptive father [62, 63]. These effects may be mediated by social facilitation i.e. paternal drinking may encourage maternal drinking, but animal studies, described below, suggest a more direct relationship between paternal alcohol exposure and offspring health and behaviour [64].

For example, significantly fewer matings were produced by male mice maintained on a liquid diet for 28 days, in which alcohol comprised 32% of the caloric content [65]. In addition, significantly reduced birth weights and crown-rump-lengths were reported for offspring of alcohol treated males harvested at 18 days of gestation. However, the frequency of congenital defects did not differ between the alcohol treatment group and controls [65].

In another study, 25 day old male rats were maintained on a liquid diet, in which 35% of the calories were derived from alcohol, until adulthood (~60 days old) [66]. Following a two week alcohol free period, the alcohol treated males were mated to non-alcohol treated females. Although litter size was significantly reduced in the alcohol treatment group, the number of matings, offspring weight, sex ratios, mortality and gross abnormalities did not differ from the control group. However, serum testosterone, seminal vesicle weights and beta-endorphin levels in the hypothalamus were significantly reduced in adult offspring derived from alcohol treated males [66].

Another study found that male rats maintained on a 20% v/v ethanol liquid diet for 60 days sired fewer offspring [67]. This outcome may have arisen from an increased number of resorptions, higher preimplantation loss and fewer implantations. Paternal alcohol treatment was also associated with an increase in the number of malformations, including microcephalus, microphthalmia, cranial fissure, and hydronephrosis, and a reduction in fetal weights in offspring harvested after 20 days of gestation [67].

Male fertility and mean birth weights were not affected in a study in which male rats were intubated with 2 or 3 g/kg ethanol twice daily for nine weeks [68]. However, alcohol treated males in the 3g/kg exposure group produced a greater number of "runts" (<5.5g at birth). Alcohol treated males also sired relatively fewer males, and their offspring showed increases in adrenal weights at birth and a decrease in spleen weights at 21 days of age [68].

It should be noted that not all studies have reported effects of preconceptional alcohol exposure. For example, no effects on the number of implantations, prenatal mortality, sex ratio, soft tissue malformations, or fetal weights, were observed on day 19 of gestation, following chronic

treatment of male mice for a month [69]. In this study, fathers were maintained on a liquid diet, in which alcohol comprised 20% or 30% of the dietary calories, for four weeks before being mated.

In contrast to the above studies, in which fetal weights were either unaffected or reduced, daily administration of 2.5 g/kg or 5g/kg ethanol to male rats by gavage, over three or nine weeks, resulted in higher fetal weights in offspring harvested just prior to term [70]. In the "three week" exposure group, a significant increase in the number of fetuses was also observed, while a significant increase in placental weight and decreased placental index was reported in the "nine week" exposure group [70].

Studies employing chronic dosage regimens have also uncovered behavioural effects of paternal alcohol exposure. For example, in one study, 18 day old offspring of male rats intubated with 3 or 2g/kg ethanol twice a day for seven months were more active in the open field compared to controls and took a greater number of trials to complete a passive avoidance learning task [71].

In another study, male mice were maintained on a liquid diet, in which alcohol provided 0%, 10% or 20% of their calories, for 56-61 days [72]. Organ weights (except for thymus), litter size, and weight at birth, 21 days and 55 days of age were not affected. However, a dose-dependent decrease in physical activity at 20 and 24 days of age, as well as decreases in serum testosterone at 55 days of age, were observed in offspring derived from the alcohol treatment groups. In addition, offspring of fathers receiving 20% of their calories from alcohol performed better on a passive avoidance task but more poorly in a T maze task [72].

In a study of both mice and rats, males received 0%, 10% or 25% of the calories from ethanol over 7-14 weeks [73]. It was observed that mice from the alcohol exposed group were relatively more immobile in a swimming behavioural task. In contrast, rats from the alcohol exposed group displayed an opposite effect, being less immobile in the same task [73].

In a separate study, male rats receiving 17.5% or 35% of their dietary calories from alcohol sired female offspring who performed worse in a "two-way shock avoidance learning" task [74]. However, no effects on birth weight, spontaneous alternation or passive avoidance learning were observed [74].

Studies employing acute dosage regimens have also reported effects of paternal alcohol exposure on offspring development. In one study, in which male rats were intubated with a once off dose of either 6, 4, 2 or 0 g/kg ethanol, there was a significant dose response effect on the frequency of "runts" (<5.5g at birth) and the number of malformations [75]. However, no differences in mating, fecundity or litter size were reported. In a separate study, a once off dose of 5g/kg administered by intraperitoneal injection to male rats, 24 hours prior to mating, resulted in fewer matings, smaller litter sizes and higher fetal mortality [76].

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. [77] 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. [77] found that fetuses from alcoholic females were significantly growth retarded in comparison to controls. However, the male treatment paradigm did not seem to affect embryo weight.

These findings parallel those of Becker and Randall [78], who reported growth retardation in mouse offspring of F1 females exposed to ethanol prior to conception. Similar results were reported by Little et al. [79], who observed a relationship between alcoholism in women, who abstained during pregnancy, and reduced birth weight in their offspring [79].

Epigenetic mechanisms of preconceptional effects

In sum, a wide range of birth defects and fetal 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. The mechanisms, particularly in males, are likely to involve alcohol-induced epigenetic changes in the gametes or, alternatively, selection effects within the germline [64], resulting in the ontogenesis of "FASD-like" phenotypes in unexposed generations.

This is consistent with an accumulating body of evidence for the transmissibility of environmentally acquired epigenetic states between generations in animal models and humans. For example, exposure of gestating female rats during the period of gonad sex differentiation to the endocrine disruptors vinclozolin (an antiandrogenic compound) or methoxychlor (an estrogenic compound) resulted in decreased spermatogenic capacity and an increased incidence of adult onset diseases and hypercholesterolemia in the males of the F1-F4 generations [30, 80]. The heritability of these phenotypes up until the F4 generation may have been mediated by DNA methylation, as suggested by the presence of global DNA-methylation changes in the germline of F1 individuals [30].

More recently, direct evidence for transgenerational inheritance of an environmentally induced epigenetic state was uncovered in a study of the viable yellow allele of *agouti* (A^{vy}) – a gene that controls coat colour in mice [81]. Nutritional supplementation of pregnant mice shifts the coat colour of their offspring towards the pseudoagouti phenotype through changes in DNA methylation at the A^{vy} allele [27]. Expanding upon prior research, Cropley et al. [81] were able to show that epigenetic and phenotypic effects of nutritional supplementation persist into unexposed generations.

The possibility of transgenerational responses to historical environments in humans has recently been uncovered by studies of food availability in northern Sweden [82]. In this study, a surfeit of food during the paternal grandfather's slow growth period (SGP, 9-12 years), was associated with reduced longevity, as well as increased diabetes mortality, in their grandchildren [82]. In the same cohort, poor food availability in the paternal grandparental generation was associated with decreased mortality risk ratios (RR) in grandchildren [83]. Epigenetic changes in the grandparental germline are a candidate mechanism of these transgenerational effects of diet, although confounding by socioeconomic variables cannot be entirely ruled out.

In sum, the preconceptional effects of ethanol described above are consistent with a growing body of evidence for the existence of transgenerational response phenomena, i.e. the transmission of environmentally induced states from exposed generations to unexposed generations. Although selection within the germline cannot be ruled out, epigenetic reprogramming could be a mechanism of these phenomena (Figure 1).

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. 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. By 3.5 dpc the cell aggregates have developed into a blastocyst: an asymmetric and hollow spherical body with an outer layer of cells (the blastoderm) enveloping a fluid-filled cavity. 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. The preimplantation period in mouse 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.

In vivo administration of ethanol during the preimplantation period

The teratogenic consequences of ethanol exposure during this period have received surprisingly little attention. 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 [84]. 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, or surviving with no malformations [84].

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 [84]. Variable effects on placental weight were also observed [84]. Depending on the precise timing of administration, as well as the day of dissection, placentae were sometimes growth reduced, growth enhanced or unaffected.

In a separate study, Mitchell et al. [85] 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 [86] in mice and Clarren and Astley [87] 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.

In stark contrast, studies that expose the preimplantation embryo to ethanol *in vitro*, generally report findings in the opposite direction of those described above. For example, the *in vitro* culture of 4-cell embryos in ethanol (22mM – 348mM) did not affect their resorption rate once transferred to foster mothers, nor was it associated with morphological abnormalities in live offspring [8, 88]. These findings suggest that the teratogenic consequences of ethanol exposure during the preimplantation period partly depends on an interaction with the maternal system, e.g. through production of toxic metabolites such as acetaldehyde.

These findings have similar parallels in the animal cloning research field, in which it has been known for many years that exposure of the early embryo to abnormal environmental conditions results in severe physical and growth abnormalities in clones surviving transfer to foster mothers [32]. The abnormalities include increased fetal resorption rates, enhanced fetal growth, high birth weights and malformations of the skeletal and organ systems, collectively referred to as the large offspring syndrome (LOS) in sheep and cattle [89-91]. The *in vitro* culture of preimplantation mouse embryos, derived from embryonic stem cells or somatic cells, is associated with similar abnormalities [89, 92-96].

A growing body of evidence implicates culture-induced imprinting defects as mechanisms of these abnormalities. For example, Dean et al. [92] observed changes in DNA-methylation at the *Igf2, H19, Igf*2*r* and *Zrsr1* (*U2af1-rs1)* imprinted genes in cultured embryonic stem cells. Similar changes, together with aberrant imprinted gene expression, were observed in 13-14 day old fetuses derived from these cells, suggesting that imprinting defects arising in the early embryo persisted into the postimplantation period [92]. In addition, these epigenetic defects were associated with abnormally large fetuses and various physical abnormalities, including interstitial bleeding, poor mandible development, polyhydramnios and endematous skin [92, 95].

Consistent with the idea that specific components of the culture medium may interfere with development and imprinting, Wu et al. [33] 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 fetuses, in comparison to controls.

The above findings indicate that imprinting is sensitive to the preimplantation environment, raising the possibility that ethanol-induced imprinting defects might be a mechanism of ethanol teratogenesis (Figure 1). Since imprinted gene expression does not normally occur prior to gastrulation, these defects would not be expected to affect preimplantation development but would be expected to result in severe physical and growth abnormalities in late gestation – as is generally reported by '*in vivo* studies' of alcohol. In support of the above 'imprinting' hypothesis, a recent study by Haycock and Ramsay [97] reported an effect of preimplantation ethanol exposure (2.9g/kg/day over two days) on DNA methylation at the paternal allele of the *H19* imprinting control region (ICR) in placentae derived from midgestation mouse embryos. Alternatively, defects in genome wide epigenetic reprogramming could also potentially account for the teratogenic effects of ethanol exposure during the preimplantation period (Figure 1).

Gastrulation and cellular differentiation

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 intense cellular differentiation and 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, suggesting that differentiating cells might be particularly vulnerable to the teratogenic effects of alcohol [8].

Both acute and chronic dosage regimens have been utilized by researchers investigating the teratogenic properties of alcohol during the postimplantation period in mouse [reviewed in 48]. Administration of acute doses (2.9g/kg-5.8g/kg ethanol) results in a wide range of morphological abnormalities, including skeletal and organ malformations and increased rates of embryo resorption [48]. Interestingly, distinct malformation profiles correspond to distinct timings of ethanol insult: craniofacial abnormalities, many of which are strikingly reminiscent of FAS facial features, result primarily from acute doses 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 [48].

Studies employing chronic dosage regimens have reported phenotypes characteristic of the entire FAS spectrum in mouse. These studies have generally relied on liquid diets in which alcohol provided 15-30% of dietary calories, although some studies have also employed intragastric and intraperitoneal routes of administration [48]. Similar to the result of studies employing acute dosage regimens, associations between developmental timing and particular malformations have also been observed: ocular, cardiovascular and skeletal systems seem particularly vulnerable on gestational days 4-12, while the urogenital system is particularly vulnerable on gestational days 4-10; and growth retardation seems strongly associated with chronic alcohol exposure during late gestation (gestational days 12-17 in mouse) [48].

The major exception to this pattern is the central nervous system (CNS), which is sensitive to chronic alcohol exposure throughout gastrulation - unsurprising, considering that CNS development coincides with the entire postimplantation period [48]. In animal models,

abnormalities arising from chronic alcohol exposure have included exencephaly, hydrocephaly, microcephaly, dilated ventricles and various structural defects [48]. Neuroimaging studies in humans have confirmed the existence of structural abnormalities as well as reduced overall brain volume in FASD and FAS cases [98]. These physical brain abnormalities may underlie the various behavioural and cognitive characteristics of individuals prenatally exposed to alcohol, such as deficits in learning, memory, attention, behavioural inhibition and cognitive control [98].

Ultimately, such behavioural and morphological deficits in the CNS are likely to arise as a result of insults at the cellular level, through alterations in proliferation and differentiation. In one study, acute (5 g/kg) and chronic alcohol (9.3 g/kg/day over four days) exposure was shown to reduce proliferation of neural progenitors in the hippocampus *in vivo* [99]. In addition, alcohol delivered as part of a liquid diet (7% alcohol w/v), with an average consumption of 14–15 g/kg/day and an average weekly BAC of 44mM-52mM, was associated with inhibited proliferation of neural progenitors, inhibited dendritic differentiation and greater cell death in the hippocampus [100]. In a study of neural stem cells, treatment inhibited differentiation into neurons at a concentration of 25-100mM but promoted differentiation into astrocytes and oligodendrocytes at a concentration of 100mM [101].

Given the major epigenetic rearrangements that occur during gastrulation (Figure 1), as well as the role of ncRNA and chromatin modifications in the origins and maintenance of cellular identity, it is likely that epigenetic factors will be found to play a role in the cellular response to alcohol. Moreover, given the importance of epigenetic mechanisms in normal CNS development and dysfunction, it is likely that epigenetic research will provide valuable insights into the mechanistic basis of alcohol-induced brain damage.

Epigenetics and the developing brain

The RE1 silencing transcription factor (REST) has emerged as one of the key early players in neurogenesis [102]. This zinc finger transcription factor coordinates silencing of neuron-specific genes in embryonic stem cells and neural progenitors, through the recruitment of a chromatin repressive complex to RE1 binding sites [102]. Maintained in a silent state, neuron-specific genes are nonetheless poised for activation through permissive chromatin modifications that include DNA hypomethylation, the absence of H3K9 methylation and the presence of H3K4 methylation [102]. Activation of neuron specific genes occurs during the transition of progenitor cells into post-mitotic neurons as a result of transcriptional silencing of *REST*, a process regulated by the recruitment of a chromatin enzyme complex to a retinoic acid receptor element (RARE) in the REST promoter region [102]. In addition to post-translational and transcriptional silencing mechanisms, the transition of neural progenitors might also be facilitated by conversion of REST into an activator of neuronal gene expression by a small noncoding dsRNA [103].

Epigenetic mechanisms have also been shown to play a role in gliogenesis. For example, glial fibrillary acidic protein (GFAP) contains a binding site for signal transducer and activation of transcription 3 (STAT3) that when methylated abrogates the binding of STAT3, and the accessibility of the GFAP promoter to the transcriptional machinery [104]. A CpG site within the STAT3 recognition sequence is demethylated in astrocytes, where GFAP is usually expressed, but highly methylated in cells that do not normally express GFAP [104]. In a separate study it was found that ciliary neurotrophic factor (CNTF) and fibroblast growth factor 2 (FGF2)

regulated differential patterns of H3 lysine methylation in progenitor and differentiated glial cells, with increased levels of H3K9 methylation (a marker of repressed chromatin) in cortical progenitors and increased levels of H3K4 methylation (a marker of activated chromatin) in astrocytes [105].

The importance of epigenetic mechanisms in normal CNS development suggests that defects in these factors should contribute to abnormal CNS function, as demonstrated by a growing number of neurological disorders with established epigenetic components. Perhaps the most famous example is Rett syndrome, an autism spectrum disorder that primarily affects females, develops from ~6-18 months of age and which is characterized by major cognitive impairments, including loss of voluntary speech and hand skills [106]. The syndrome results from mutations in the methyl DNA binding protein MeCP2 [107].

With regards to deficits of the CNS more broadly, Petronis and colleagues have argued extensively for the importance of epigenetic factors in the origins of a wide range of psychiatric disorders, arguing that the various non-Mendelian features of these conditions, such as late-age of onset and monozygotic twin discordance, are more consistent with an 'epigenetic perspective' than the classic 'gene plus environment' paradigm [108-112]. Experimental verification of their claim is beginning to emerge [113].

There is also evidence for epigenetic mechanisms in the mediation of gene environment interactions in behavioural phenotypes. For example, it is known that rat offspring receiving relatively greater levels of "maternal care", defined as "pup licking and grooming" and "arched-back nursing", have more modest hypothalamic-pituitary-adrenal (HPA) stress responses, a phenomenon involving epigenetic rearrangements at the glucocorticoid receptor gene [34, 114].

Thus, although clearly a wide and diverse range of developmental factors are essential to brain development, a complete understanding of the teratogenic relationship between alcohol, gastrulation and the CNS is unlikely to be attained without an understanding of epigenetic factors.

CONCLUSION

In sum, alterations in epigenetic programming may underlie the teratogenic consequences of ethanol exposure prior to conception, as well as post-conception, during preimplantation and gastrulation. One of the main implications of an epigenetic perspective is that the FASD spectrum is not limited to clinical defects arising from *in utero* ethanol exposure, suggesting that the concept of a 'fetal alcohol spectrum' should be expanded to include preconceptional effects. The disentanglement of the latter from socioeconomic factors, maternal malnutrition, and other potential confounders in humans may prove an intractable problem. Nevertheless, the possibility that ethanol consumption prior to conception may induce epigenetic abnormalities in the germline has major public health implications and requires urgent attention. In particular, research needs to establish whether preconceptional effects are limited to chronic alcohol abuse, or include low-to--moderate and "once-off" binge drinking episodes. This will require some modification to current dosage regimen paradigms, which generally test the effect of alcohol levels towards the "binge drinking" end of the spectrum. Questions pertaining to the temporality

of preconceptional effects also need to be addressed. For example, if transgenerational responses to ethanol are real, for how many generations do they occur?

Finally, an epigenetic perspective suggests that alcohol exposure outside of the organogenic period, e.g. during preimplantation or prior to conception, might have teratogenic consequences for the CNS. Indeed, the association of paternal alcohol consumption with behavioural and cognitive abnormalities in offspring in some animal and human studies (discussed above) supports this view. Since such cases are unlikely to receive a diagnosis within the FASD spectrum (gestational alcohol exposure being a requirement for diagnosis), this raises the possibility that transgenerational responses to alcohol might account for a significant proportion of idiopathic neurodevelopmental disorders (e.g. idiopathic autism) in humans.

Acknowledgements

The author gratefully acknowledges the constructive input of Dr Michele Ramsay and Dr Lillian Ouko. The author also acknowledges the financial support of the German Academic Exchange Service (DAAD), the University of the Witwatersrand and the South African National Research Foundation (NRF).

REFERENCES

1. Astley SJ. Fetal alcohol syndrome prevention in Washington State: evidence of success. Paediatr Perinat Epidemiol 2004; 18: 344-351.

2. 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. A practical clinical approach to diagnosis of fetal alcohol spectrum disorders: clarification of the 1996 institute of medicine criteria. Pediatrics 2005; 115: 39-47.

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

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

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

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

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

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

9. Wozniak DF, Hartman RE, Boyle MP, Vogt SK, Brooks AR, Tenkova T, Young C, Olney JW, Muglia LJ. 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 2004; 17: 403-414.

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

11. Jablonka E, Lamb MJ. Evolution in four dimensions : genetic, epigenetic, behavioral, and symbolic variation in the history of life. Cambridge, Mass.: MIT Press; 2005.

12. Arney KL, Fisher AG. Epigenetic aspects of differentiation. J Cell Sci 2004; 117: 4355-4363.
13. Shukla SD, Velazquez J, French SW, Lu SC, Ticku MK, Zakhari S. Emerging Role of Epigenetics in the Actions of Alcohol. Alcohol Clin Exp Res 2008.
14. Hsieh J, Gage FH. Epigenetic control of neural stem cell fate. Curr Opin Genet Dev 2004; 14: 461-469.
15. Cheung P, Lau P. Epigenetic regulation by histone methylation and histone variants. Mol Endocrinol 2005; 19: 563-573.
16. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. Science 2001; 293: 1089-1093.
17. Kurukuti S, Tiwari VK, Tavoosidana G, Pugacheva E, Murrell A, Zhao Z, Lobanenkov V, Reik W, Ohlsson R. 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 2006; 103: 10684-10689.
18. Lopes S, Lewis A, Hajkova P, Dean W, Oswald J, Forne T, Murrell A, Constancia M, Bartolomei M, Walter J, Reik W. Epigenetic modifications in an imprinting cluster are controlled by a hierarchy of DMRs suggesting long-range chromatin interactions. Hum Mol Genet 2003; 12: 295-305.
19. Murrell A, Heeson S, Reik W. Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. Nat Genet 2004; 36: 889-893.
20. Guy J, Hendrich B, Holmes M, Martin JE, Bird A. A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nat Genet 2001; 27: 322-326.
21. Li E. Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 2002; 3: 662-673.
22. Hendrich B, Guy J, Ramsahoye B, Wilson VA, Bird A. Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. Genes Dev 2001; 15: 710-723.
23. Moazed D. Small RNAs in transcriptional gene silencing and genome defence. Nature 2009; 457: 413-420.
24. Pelissier T, Wassenegger M. A DNA target of 30 bp is sufficient for RNA-directed DNA methylation. Rna 2000; 6: 55-65.
25. Wassenegger M, Heimes S, Riedel L, Sanger HL. RNA-directed de novo methylation of genomic sequences in plants. Cell 1994; 76: 567-576.
26. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998; 391: 806-811.
27. Cooney CA, Dave AA, Wolff GL. Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring. J Nutr 2002; 132: 2393S-2400S.
28. Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Mol Cell Biol 2003; 23: 5293-5300.
29. Garro AJ, McBeth DL, Lima V, Lieber CS. Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. Alcohol Clin Exp Res 1991; 15: 395-398.
30. Anway MD, Cupp AS, Uzumcu M, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 2005; 308: 1466-1469.

31. Newbold RR, Padilla-Banks E, Jefferson WN. Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. Endocrinology 2006; 147: S11-17.
32. Thompson SL, Konfortova G, Gregory RI, Reik W, Dean W, Feil R. Environmental effects on genomic imprinting in mammals. Toxicol Lett 2001; 120: 143-150.
33. Wu Q, Ohsako S, Ishimura R, Suzuki JS, Tohyama C. 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 2004; 70: 1790-1797.
34. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ. Epigenetic programming by maternal behavior. Nat Neurosci 2004; 7: 847-854.
35. Hajkova P, Erhardt S, Lane N, Haaf T, El-Maarri O, Reik W, Walter J, Surani MA. Epigenetic reprogramming in mouse primordial germ cells. Mech Dev 2002; 117: 15-23.
36. Bai S, Ghoshal K, Datta J, Majumder S, Yoon SO, Jacob ST. DNA methyltransferase 3b regulates nerve growth factor-induced differentiation of PC12 cells by recruiting histone deacetylase 2. Mol Cell Biol 2005; 25: 751-766.
37. 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. DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. Development 2005; 132: 3345-3356.
38. Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M, Burton J, Cox TV, Davies R, Down TA, Haefliger C, Horton R, Howe K, Jackson DK, Kunde J, Koenig C, Liddle J, Niblett D, Otto T, Pettett R, Seemann S, Thompson C, West T, Rogers J, Olek A, Berlin K, Beck S. DNA methylation profiling of human chromosomes 6, 20 and 22. Nat Genet 2006; 38: 1378-1385.
39. Halsted CH, Villanueva JA, Devlin AM, Niemela O, Parkkila S, Garrow TA, Wallock LM, Shigenaga MK, Melnyk S, James SJ. Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig. Proc Natl Acad Sci U S A 2002; 99: 10072-10077.
40. Bielawski DM, Zaher FM, Svinarich DM, Abel EL. Paternal alcohol exposure affects sperm cytosine methyltransferase messenger RNA levels. Alcohol Clin Exp Res 2002; 26: 347-351.
41. Pietrzykowski AZ, Friesen RM, Martin GE, Puig SI, Nowak CL, Wynne PM, Siegelmann HT, Treistman SN. Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. Neuron 2008; 59: 274-287.
42. Cravo ML, Gloria LM, Selhub J, Nadeau MR, Camilo ME, Resende MP, Cardoso JN, Leitao CN, Mira FC. Hyperhomocysteinemia in chronic alcoholism: correlation with folate, vitamin B-12, and vitamin B-6 status. Am J Clin Nutr 1996; 63: 220-224.
43. Park PH, Lim RW, Shukla SD. Involvement of histone acetyltransferase (HAT) in ethanol-induced acetylation of histone H3 in hepatocytes: potential mechanism for gene expression. Am J Physiol Gastrointest Liver Physiol 2005; 289: G1124-1136.
44. Mistilis SP, Birchall A. Induction of alcohol dehydrogenase in the rat. Nature 1969; 223: 199-200.
45. Kim JS, Shukla SD. Acute in vivo effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. Alcohol Alcohol 2006; 41: 126-132.

46. Pal-Bhadra M, Bhadra U, Jackson DE, Mamatha L, Park PH, Shukla SD. Distinct methylation patterns in histone H3 at Lys-4 and Lys-9 correlate with up- & down-regulation of genes by ethanol in hepatocytes. Life Sci 2007; 81: 979-987.
47. Lee YJ, Shukla SD. Histone H3 phosphorylation at serine 10 and serine 28 is mediated by p38 MAPK in rat hepatocytes exposed to ethanol and acetaldehyde. Eur J Pharmacol 2007; 573: 29-38.
48. Becker HC, Diaz-Granados JL, Randall CL. Teratogenic actions of ethanol in the mouse: a minireview. Pharmacol Biochem Behav 1996; 55: 501-513.
49. May PA, Gossage JP, Brooke LE, Snell CL, Marais AS, Hendricks LS, Croxford JA, Viljoen DL. Maternal risk factors for fetal alcohol syndrome in the Western cape province of South Africa: a population-based study. Am J Public Health 2005; 95: 1190-1199.
50. Lindblad B, Olsson R. Unusually high levels of blood alcohol? Jama 1976; 236: 1600-1602.
51. Gohlke JM, Griffith WC, Faustman EM. A systems-based computational model for dose-response comparisons of two mode of action hypotheses for ethanol-induced neurodevelopmental toxicity. Toxicol Sci 2005; 86: 470-484.
52. Pantazis NJ, Dohrman DP, Luo J, Goodlett CR, West JR. Alcohol reduces the number of pheochromocytoma (PC12) cells in culture. Alcohol 1992; 9: 171-180.
53. Hanson F, Florence H. The effects of alcohol on birth weight and litter size in the albino rat. The American Naturalist 1927; 61: 503-519.
54. Hanson F, Florence H. Do albino rats having ten generations of alcoholic ancestry inherit resistance to alcohol fumes? The American Naturalist 1927 61: 43-53.
55. Hanson F, Handy V. The effects of alcohol fumes on the albino rat: introduction and sterility data for the first treated generation. The American Naturalist 1923; 57: 532-544.
56. MacDowell E. Experiments with alcohol and white rats. The American Naturalist 1922; 56: 289-311.
57. Nice L. Further observations on the effects of alcohol on white mice. The American Naturalist 1917; 51: 596-607.
58. Pearl R. 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 1916; 2: 675-683.
59. Whitney D. The effects of alcohol not inherited in hydatina senta. The American Naturalist 1912; 46: 41-56.
60. Stockard C. The effect on the offspring of intoxicating the male parent and the transmission of the defects to subsequent generations. The American Naturalist 1913; 47: 641-682.
61. Little RE, Sing CF. Father's drinking and infant birth weight: report of an association. Teratology 1987; 36: 59-65.
62. Hegedus AM, Tarter RE, Hill SY, Jacob T, Winsten NE. Static ataxia: a possible marker for alcoholism. Alcohol Clin Exp Res 1984; 8: 580-582.
63. Tarter RE, Hegedus AM, Goldstein G, Shelly C, Alterman AI. Adolescent sons of alcoholics: neuropsychological and personality characteristics. Alcohol Clin Exp Res 1984; 8: 216-222.
64. Abel E. Paternal contribution to fetal alcohol syndrome. Addict Biol 2004; 9: 127-133; discussion 135-126.
65. Anderson RA, Jr., Furby JE, Oswald C, Zaneveld LJ. Tetratological evaluation of mouse fetuses after paternal alcohol ingestion. Neurobehav Toxicol Teratol 1981; 3: 117-120.

66. Cicero TJ, Adams ML, O'Connor L, Nock B, Meyer ER, Wozniak D. Influence of chronic alcohol administration on representative indices of puberty and sexual maturation in male rats and the development of their progeny. J Pharmacol Exp Ther 1990; 255: 707-715.
67. Mankes RF, LeFevre R, Benitz KF, Rosenblum I, Bates H, Walker AI, Abraham R, Rockwood W. Paternal effects of ethanol in the long-evans rat. J Toxicol Environ Health 1982; 10: 871-878.
68. Abel EL. Rat offspring sired by males treated with alcohol. Alcohol 1993; 10: 237-242.
69. Randall CL, Burling TA, Lochry EA, Sutker PB. The effect of paternal alcohol consumption on fetal development in mice. Drug Alcohol Depend 1982; 9: 89-95.
70. Abel EL. A surprising effect of paternal alcohol treatment on rat fetuses. Alcohol 1995; 12: 1-6.
71. Abel EL. Effects of physostigmine on male offspring sired by alcohol-treated fathers. Alcohol Clin Exp Res 1994; 18: 648-652.
72. Abel EL, Lee JA. Paternal alcohol exposure affects offspring behavior but not body or organ weights in mice. Alcohol Clin Exp Res 1988; 12: 349-355.
73. Abel EL, Bilitzke P. Paternal alcohol exposure: paradoxical effect in mice and rats. Psychopharmacology (Berl) 1990; 100: 159-164.
74. Abel EL, Tan SE. Effects of paternal alcohol consumption on pregnancy outcome in rats. Neurotoxicol Teratol 1988; 10: 187-192.
75. Bielawski DM, Abel EL. Acute treatment of paternal alcohol exposure produces malformations in offspring. Alcohol 1997; 14: 397-401.
76. Cicero TJ, Nock B, O'Connor LH, Sewing BN, Adams ML, Meyer ER. Acute paternal alcohol exposure impairs fertility and fetal outcome. Life Sci 1994; 55: PL33-36.
77. Livy DJ, Maier SE, West JR. Long-term alcohol exposure prior to conception results in lower fetal body weights. Birth Defects Res B Dev Reprod Toxicol 2004; 71: 135-141.
78. Becker HC, Randall CL. Two generations of maternal alcohol consumption in mice: effect on pregnancy outcome. Alcohol Clin Exp Res 1987; 11: 240-242.
79. Little RE, Streissguth AP, Barr HM, Herman CS. Decreased birth weight in infants of alcoholic women who abstained during pregnancy. J Pediatr 1980; 96: 974-977.
80. Anway MD, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors. Endocrinology 2006; 147: S43-49.
81. Cropley JE, Suter CM, Beckman KB, Martin DI. Germ-line epigenetic modification of the murine A vy allele by nutritional supplementation. Proc Natl Acad Sci U S A 2006; 103: 17308-17312.
82. Bygren LO, Kaati G, Edvinsson S. Longevity determined by paternal ancestors' nutrition during their slow growth period. Acta Biotheor 2001; 49: 53-59.
83. Pembrey ME, Bygren LO, Kaati G, Edvinsson S, Northstone K, Sjostrom M, Golding J. Sex-specific, male-line transgenerational responses in humans. Eur J Hum Genet 2006; 14: 159-166.
84. Padmanabhan R, Hameed MS. Effects of acute doses of ethanol administered at pre-implantation stages on fetal development in the mouse. Drug Alcohol Depend 1988; 22: 91-100.
85. Mitchell JA. Effects of alcohol on blastocyst implantation and fecundity in the rat. Alcohol Clin Exp Res 1994; 18: 29-34.
86. Checiu M, Sandor S. The effect of ethanol upon early development in mice and rats. IX. Late effect of acute preimplantation intoxication in mice. Morphol Embryol (Bucur) 1986; 32: 5-11.

87. Clarren SK, Astley SJ. Pregnancy outcomes after weekly oral administration of ethanol during gestation in the pig-tailed macaque: comparing early gestational exposure to full gestational exposure. Teratology 1992; 45: 1-9.
88. Stachecki JJ, Yelian FD, Leach RE, Armant DR. Mouse blastocyst outgrowth and implantation rates following exposure to ethanol or A23187 during culture in vitro. J Reprod Fertil 1994; 101: 611-617.
89. Sinclair K, Young L, Wilmut I, McEvoy T. In-utero overgrowth in ruminants following embryo culture: lessons from mice and a warning to men. Hum Reprod 2000; 15 Suppl 5: 68-86.
90. Walker S, Hartwich K, Seamark R. The production of unusually large offspring following embryo manipulation: Concepts and challenges. Theriogenology 1996; 45: 111–120.
91. Young LE, Sinclair KD, Wilmut I. Large offspring syndrome in cattle and sheep. Rev Reprod 1998; 3: 155-163.
92. Dean W, Bowden L, Aitchison A, Klose J, Moore T, Meneses JJ, Reik W, Feil R. Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. Development 1998; 125: 2273-2282.
93. Khosla S, Dean W, Brown D, Reik W, Feil R. Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. Biol Reprod 2001; 64: 918-926.
94. McLaren A. Cloning: pathways to a pluripotent future. Science 2000; 288: 1775-1780.
95. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A 1993; 90: 8424-8428.
96. Khosla S, Dean W, Reik W, Feil R. Culture of preimplantation embryos and its long-term effects on gene expression and phenotype. Hum Reprod Update 2001; 7: 419-427.
97. Haycock PC, Ramsay M. Exposure of Mouse Embryos to Ethanol During Preimplantation Development: Effect on DNA Methylation in the H19 Imprinting Control Region. Biol Reprod 2009.
98. Spadoni AD, McGee CL, Fryer SL, Riley EP. Neuroimaging and fetal alcohol spectrum disorders. Neurosci Biobehav Rev 2007; 31: 239-245.
99. Nixon K, Crews FT. Binge ethanol exposure decreases neurogenesis in adult rat hippocampus. J Neurochem 2002; 83: 1087-1093.
100. He J, Nixon K, Shetty AK, Crews FT. Chronic alcohol exposure reduces hippocampal neurogenesis and dendritic growth of newborn neurons. Eur J Neurosci 2005; 21: 2711-2720.
101. Tateno M, Ukai W, Yamamoto M, Hashimoto E, Ikeda H, Saito T. The effect of ethanol on cell fate determination of neural stem cells. Alcohol Clin Exp Res 2005; 29: 225S-229S.
102. Ballas N, Grunseich C, Lu DD, Speh JC, Mandel G. REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. Cell 2005; 121: 645-657.
103. Kuwabara T, Hsieh J, Nakashima K, Taira K, Gage FH. A small modulatory dsRNA specifies the fate of adult neural stem cells. Cell 2004; 116: 779-793.
104. Takizawa T, Nakashima K, Namihira M, Ochiai W, Uemura A, Yanagisawa M, Fujita N, Nakao M, Taga T. DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev Cell 2001; 1: 749-758.
105. Song MR, Ghosh A. FGF2-induced chromatin remodeling regulates CNTF-mediated gene expression and astrocyte differentiation. Nat Neurosci 2004; 7: 229-235.
106. Hagberg B, Aicardi J, Dias K, Ramos O. A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls: Rett's syndrome: report of 35 cases. Ann Neurol 1983; 14: 471-479.

107. Jung BP, Jugloff DG, Zhang G, Logan R, Brown S, Eubanks JH. The expression of methyl CpG binding factor MeCP2 correlates with cellular differentiation in the developing rat brain and in cultured cells. J Neurobiol 2003; 55: 86-96.
108. Mill J, Petronis A. Molecular studies of major depressive disorder: the epigenetic perspective. Mol Psychiatry 2007; 12: 799-814.
109. Mill J, Petronis A. Pre- and peri-natal environmental risks for attention-deficit hyperactivity disorder (ADHD): the potential role of epigenetic processes in mediating susceptibility. J Child Psychol Psychiatry 2008; 49: 1020-1030.
110. Oh G, Petronis A. Environmental studies of schizophrenia through the prism of epigenetics. Schizophr Bull 2008; 34: 1122-1129.
111. Petronis A. Human morbid genetics revisited: relevance of epigenetics. Trends Genet 2001; 17: 142-146.
112. Petronis A. Epigenetics and bipolar disorder: new opportunities and challenges. Am J Med Genet C Semin Med Genet 2003; 123C: 65-75.
113. Mill J, Tang T, Kaminsky Z, Khare T, Yazdanpanah S, Bouchard L, Jia P, Assadzadeh A, Flanagan J, Schumacher A, Wang SC, Petronis A. Epigenomic profiling reveals DNA-methylation changes associated with major psychosis. Am J Hum Genet 2008; 82: 696-711.
114. Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, Sharma S, Pearson D, Plotsky PM, Meaney MJ. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science 1997; 277: 1659-1662.

Figure legends

Figure 1. Epigenetic reprogramming during development. Preimplantation is characterised by genome-wide demethylation; gastrulation is characterised by genome-wide *de novo* methylation [16]. 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 [35]. 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 DNA-methylation.

Figure 1