Preimplantation alcohol exposure and developmental programming of FASD: An epigenetic perspective.

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**Preimplantation Alcohol Exposure and Developmental Programming of FASD:
An Epigenetic Perspective.**

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Draft

1 **Abstract**

2 Alcohol exposure during *in utero* development can permanently change the
 3 developmental programming of physiological responses, thereby increasing the
 4 risk of childhood neurological illnesses and later adverse health outcomes associated with
 5 fetal alcohol spectrum disorders (FASD). There is an increasing body of
 6 evidence indicating that alcohol exposure during gestation triggers lasting epigenetic
 7 alterations in offspring long after the initial insult; together, these studies support the role
 8 of epigenetics in FASD etiology. However, we still have little information about how
 9 ethanol interferes with the fundamental epigenetic reprogramming wave (*e.g.*, erasure and
 10 re-establishment of DNA methylation marks) that characterizes preimplantation embryo
 11 development. This article will review key epigenetic processes occurring during
 12 preimplantation development and especially focus on the current knowledge regarding
 13 how a prenatal alcohol exposure during this period could affect the developmental
 14 programming of the early stage preimplantation embryo. We will also outline current
 15 limitations of studies examining the *in vivo* and *in vitro* effects of alcohol exposure on
 16 embryos as well as underline the next critical steps to be taken if we want to better
 17 understand the implicated mechanisms in order to strengthen the translational potential
 18 for non-invasive epigenetic diagnosis markers and the treatment of newborns that have
 19 higher risks of developing FASD.

20

21 **Keywords:** Epigenetics, DNA methylation, Preimplantation Embryos, Prenatal Alcohol
 22 Exposure, Developmental Programming.

23

1. Introduction

Adverse environmental exposures during critical periods of *in utero* development can permanently change developmental programming, which in turn can alter physiological responses and thereby increase the risk of adverse health effects later in life. These developmental programming changes can occur via the dysregulation of complex epigenetic mechanisms (*e.g.*, DNA methylation, histone modifications, non-coding RNAs), which are heritable modifications occurring on the chromatin structure that can affect gene expression and can be remodelled according to external influences. These dynamic and reversible processes, tightly interrelated with the regulation of gene expression, are crucial for the guidance of cell fate and identity. Although the epigenome is susceptible to dysregulation throughout life, it is highly vulnerable to environmental factors during *in utero* development, especially during early embryogenesis in response to environmental cues from the maternal environment (Perera and Herbstman 2011). Alcohol is among known sources of prenatal exposure altering the developmental programming and is responsible for fetal alcohol spectrum disorders (FASD) (Clarren and Smith 1978; Ramsay 2010). In recent years, research focusing on mechanisms underlying the adverse alcohol-induced neurobiological, behavioral, and health outcomes have provided evidence that alterations in epigenetic modifications correlated with the development of FASD phenotypes (Ramsay 2010; Thomas, Warren, and Hewitt 2010; Kleiber et al. 2014). Still, we have very limited knowledge regarding if and how alcohol dysregulates early embryonic epigenetic profiles that are being reprogrammed (*i.e.*, DNA methylation) and remodeled (*e.g.*, histone modifications, miRNAs) during the

preimplantation period and if these perturbations are responsible for the teratogenic effects observed later in development following a preimplantation alcohol exposure.

2. FASD

FASD is an umbrella term used to collectively describe the range of cognitive and physical disabilities observed in children born to mothers who consumed alcohol at any time during pregnancy. Although FASD is the most common preventable cause of birth defects as well as intellectual and neurodevelopmental disabilities, the prevalence of FASD is estimated to reach 2–5% among school-aged children (May et al. 2009; May et al. 2014). The range and severity of FASD-related conditions differ, with some children afflicted to a far greater degree than others. For instance, children and youth affected by FASD may present severe birth defects (*e.g.*, craniofacial malformations), while others are normal in physical appearance but suffer from a variety of persistent cognitive and behavioral impairments (*e.g.*, learning, attention and memory deficits, intellectual and developmental delays) (Clarren and Smith 1978; Astley and Clarren 1996; Chudley et al. 2005; May et al. 2014; Kobor and Weinberg 2011). There is a strong association between the neurobehavioral outcomes seen in FASD and the amount, the pattern (continuous *vs.* binge drinking), and the developmental timing of alcohol exposure. About 10% of children severely affected by prenatal ethanol exposure have a condition known as Fetal Alcohol Syndrome (FAS) (Mattson et al. 1998). These children all have similar features ranging in magnitude of expression or severity: 1) growth retardation; 2) central nervous system damage or dysfunction; and 3) characteristic facial dysmorphologies (Chudley et al. 2005; Cook et al. 2016). To date, no molecular test is available for detection of

FASD/FAS and their associated risks. The diagnosis of FASD requires a multidisciplinary team of expert practitioners and involves complex physical and neurodevelopmental assessments (e.g., 4-Digit Diagnostic Code) (Chudley et al. 2005; Astley and Clarren 2000; Astley 2013).

2.i. Modeling FASD

Modeling the effects of prenatal alcohol exposure in animals has been extremely important for the FASD research field, as it has led to breakthroughs, which would have been difficult if not nearly impossible to achieve with human subjects. The use of *in vivo* models has allowed researchers to gain valuable insights into the action of alcohol on various cell types, especially brain cells, in the developing embryo and offspring, providing a level of control for various factors (e.g., genetics, environment, pattern, timing and dosage of alcohol exposure, maternal diet and interaction with drugs) not possible in a real-world clinical setting. Alternatively, *in vitro* models have allowed detailed analyses of molecular mechanisms and pathways implicated in cell type specific responses to the teratogenic and neurotoxic effects of ethanol. Although this review focuses on the impacts of prenatal alcohol exposure on the developmental programming of preimplantation embryos, in this section, we will briefly highlight the importance and implication of choosing the proper FASD model to reflect conditions needed to answer specific research questions.

Reminiscent of the variations observed in the severity of symptoms in FASD-afflicted children, FASD models show a wide range of developmental abnormalities, which do not always lead to mutually consistent findings and conclusions. One of the principal reasons for that is the stage of brain development at which the alcohol exposure

92 takes place. Specific stages of brain development occur during all three trimesters of
 93 pregnancy in both humans and rodents, but they differ in timing. In humans, all three
 94 stages happen prenatally, whereas the developmental period corresponding to the human
 95 third trimester occurs postnatally in rodents (Clancy et al. 2007; Stiles and Jernigan
 96 2010). During the first trimester, formation of the neural tube occurs and basic brain
 97 structure begins to form. The second trimester is a critical period for neuronal
 98 proliferation, migration and differentiation. Finally, in the third trimester, the brain is
 99 developing at its fastest rate and is characterized by large amounts of growth and
 100 differentiation (Clancy et al. 2007; Stiles and Jernigan 2010). The timing of prenatal
 101 alcohol exposure certainly alters the programming of different brain cell types at different
 102 stages of their development. For instance, when the same binge-like exposure
 103 (subcutaneous injection of two doses of 2.5g/kg ethanol at 2 hour intervals) was given in
 104 a mouse model at different time points (E8/E11, E14/E16, or P4/P7) corresponding to the
 105 three different trimesters of human pregnancy, they observed gene expression alterations
 106 and micro RNAs (miRNAs) expression changes associated with specific molecular
 107 pathways and cellular functions, as well as various cognitive and behavioural
 108 impairments, depending on the stage of exposure (Kleiber et al. 2013; Mantha, Laufer,
 109 and Singh 2014; Laufer et al. 2013). Different timing of exposure within the same
 110 trimester can also lead to different outcomes. For instance, Lipinski and al. exposed mice
 111 to two doses (2.9g/kg EtOH) 4 hours apart by intraperitoneal injection at GD7 or GD8.5
 112 (first trimester equivalent). By using different imaging technologies (*e.g.*, magnetic
 113 resonance microscopy, dense surface modeling) they observed different facial
 114 dysmorphologies and brain abnormalities at GD17 associated with the moment of

exposure (Lipinski et al. 2012). They also found brain abnormalities at GD17 that were dependent upon the moment of chronic exposure within the first trimester equivalent (Parnell et al. 2014). Alcohol exposure at specific steps of brain development alters the programming of specific cell types, which in turn affects biological and cellular processes implicated in neurodevelopmental pathways. These experiments have allowed us to improve our understanding of the disparity observed in children with FASD.

Another important consideration when modeling FASD is the method of administration of the alcohol (Patten, Fontaine, and Christie 2014). Voluntary drinking is widely utilised within chronic models of exposure to obtain levels of blood alcohol concentration (BAC) ranging from 80 to 120mg/dl (Anderson et al. 2012; Crabbe, Harris, and Koob 2011). Ethanol delivery by gavage into the stomach can be used to generate BAC greater than 200mg/dl (Patten, Fontaine, and Christie 2014). However, since this method is time-consuming, invasive and extremely stressful for the animal it can bring other sources of variability, which can make results difficult to interpret. To circumvent this, ethanol injection (subcutaneous or intraperitoneal) can be used. This method is an efficient way to mimic acute and binge-like exposures at precise periods of gestation as it allows rapid increase of BAC (>200mg/dl) with minimal stress to the animal. However, the ethanol bypasses the gastrointestinal tract metabolism and may not reproduce all of the effects observed by ingestion (DiPadova et al. 1987; Brandon-Warner et al. 2012). When modeling FASD, it is crucial to carefully define which route of administration will provide the best way to answer specific questions while minimising the variability (*e.g.*, alcohol metabolism, accuracy in level of alcohol intake, animal stress) associated with the model (Patten, Fontaine, and Christie 2014).

It is important to acknowledge the limitation of FASD animal models. For example, animals metabolize ethanol at a much higher rate than humans do. Early studies reported that mice metabolize ethanol at a rate of 550 mg/kg/h; rats, at a rate of 300 mg/kg/h; and human beings, at a rate of 100 mg/kg/h (Abel 1982). As such, one should always use blood alcohol concentration instead of the ethanol administered when comparing species. Other studies have also shown that blood alcohol concentration rose higher and faster in mice when compared to rats, with intraperitoneal injections resulting in higher blood alcohol concentration compared to intragastric gavage (Livy, Parnell, and West 2003). When alcohol enters the general blood circulation, it disperses equally into all body fluids and during pregnancy into the placenta and fetus. Nevertheless, even before the presence of the placenta, alcohol can still reach the developing preimplantation embryos. Studies in rats have shown that following alcohol ingestion, the presence of ethanol can be detected in both the oviductal and uterine luminal fluids (~ 1/3 of levels detected in blood) (Sandor et al. 1981), confirming the direct action of ethanol and its metabolites upon the preimplantation embryo.

3. Epigenetics

There is an increasing body of evidence indicating that alcohol exposure during gestation triggers lasting epigenetic alterations in offspring long after the initial insult; together, these studies support the role of epigenetics in FASD etiology. Epigenetic modifications can act independently to regulate cellular mechanisms, but their interactions can also be required to refine some regulatory processes. For example, interactions between histone modifications on the same residue, on the same histone, or within the same nucleosome are frequent (Bannister and Kouzarides 2011). Cooperation

and/or antagonism between DNA methylation and histone modifications also contribute to the complexity of epigenetic regulation mechanisms. Epigenetic marks are transmitted to daughter cells through DNA replication and cell division processes. Inducing direct epigenetic perturbations or the unfaithful transmission of epigenetic modifications during cell divisions may have long-lasting effects on the developmental program of cells, adversely impacting future gene regulation, and rendering the organism more prone to adverse health outcomes. Recent developments in the field of epigenetics are reshaping the current perspectives of the etiology of many neurodevelopmental disorders (Rangasamy, D'Mello, and Narayanan 2013; Kramer and van Bokhoven 2009; Urdinguio, Sanchez-Mut, and Esteller 2009; Graff and Mansuy 2009; Riccio 2010; Gavin and Sharma 2010) , including FASD. We will overview DNA methylation, histone modifications, micro-RNAs and non-coding RNAs, well conserved epigenetic modifications (Kim, Samaranyake, and Pradhan 2009; Woo and Li 2012).

3.i. DNA methylation

DNA methylation, or 5-methylcytosine, is the addition of methyl groups to the dinucleotides cytosine-guanine (CpG) in the genome. The human genome is composed of 30 million CpGs (20 million in mouse), with 60-80% methylation in somatic cells (Trasler 2006). DNA methylation is established and maintained by families of DNA methyltransferases (DNMTs), which are divided in 3 classes. While DNMT1 is responsible for DNA methylation maintenance through cellular divisions, DNMT2 specifically methylates residues of transfer RNAs, DNMT3A and B catalyze *de novo* methylation, and DNMT3L, which lacks methyltransferase activity, stimulates *de novo* methylation, and acts as a regulator of maternal imprint establishment (Bourc'his et al.

2001). DNA methylation marks can be removed by ten eleven translocation (TET) enzymes, which oxidize 5-methylcytosines (5mCs) into 5-hydroxymethylcytosines (5hmCs) (Tahiliani et al. 2009; Ponnaluri, Maciejewski, and Mukherji 2013). The presence of DNA methylation on the genome can directly influence the structure and compaction of the chromatin, thus promote either activation or repression of transcription depending on the genomic location, background and factors occupancy (reviewed in (Zhu, Wang, and Qian 2016). As an example, DNA methylation at CpG island promoters is often associated with repression, whereas gene body DNA methylation has recently been correlated with active expression (Yang et al. 2014).

3.ii. Histone modifications

Epigenetic modifications can occur on specific residues of histone tails. Histones H2A, H2B, H3, and H4 are proteins grouped in octamers to form the nucleosome around which DNA wraps and is compacted into chromatin. The post-translational modifications (*e.g.*, methylation, acetylation, phosphorylation) on the histone tails are affixed mostly on lysines, although other residues such as arginine and serine can be modified by the histone modifying enzymes, and have been correlated with permissive or repressive chromatin structure (Jenuwein and Allis 2001; Strahl and Allis 2000). Genome-wide chromatin analyses in mice and humans have shown that commonly repressed regions of the genome overlap with specific histone modifications (*e.g.*, H3K27me3) and DNA methylation marks, whereas active regions containing H3K4me3 and H3K27ac are essentially depleted of DNA methylation. Transcriptional activity can also precede the deposition of histone modifications (H3K4me3 and H3K27me3) as recently observed in mouse embryonic stem cells (Galonska et al. 2015). Thereby, the dynamic addition and

removal of histone marks by histone modifying enzymes is more complex and context-specific than originally believed (reviewed in (Henikoff and Shilatifard 2011)).

3iii. Micro-RNAs and non-coding RNAs

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNA) that can control the posttranscriptional expression of target genes (Huntzinger and Izaurralde 2011; Pasquinelli 2012; Hara et al. 2017). Among the identified miRNAs, many exhibit cell-specific, tissue-specific and/or developmental stage-specific expression and have functional roles in promoting or maintaining tissue identity. Some miRNAs from maternal origin are essential during embryonic development as they play a crucial role in controlling gene expression and mRNA abundance (Hossain et al. 2012). Depletion of all miRNAs during preimplantation leads to lethality (Hossain et al. 2012; Alberti and Cochella 2017), whereas the loss of some well-characterised miRNAs during this period can cause severe developmental defects (Alberti and Cochella 2017). For instance, the miR-290-295 cluster was found to play a major role during early development by regulating pluripotency, self-renewal and cell reprogramming (Yuan et al. 2017). Various miRNAs (*e.g.*, miR-698, miR-296, miR-194-3, miR-290) also have stage-specific expression during early embryogenesis, revealing their potential role in embryo development (Tang et al. 2007). Furthermore, in mouse embryonic stem cells some miRNAs, such as miR-219, can promote differentiation into neural cells (Wu et al. 2017). Many miRNAs have now been shown to play a crucial role in brain development, especially in neural development (Sun, Crabtree, and Yoo 2013; Volvert et al. 2012) and their dysregulation is associated with neurodevelopmental disorders such as Autism Spectrum Disorder (ASD) (Esteller 2011; Geaghan and Cairns 2015).

3.iv. Alcohol and epigenetics

The direct effect of alcohol on epigenetic processes was first highlighted in a mouse model of prenatal alcohol exposure, where they found that fetal cells extracted from ethanol-treated mice had lower levels of global DNA methyltransferase activity, which led to ethanol-associated alterations in DNA methylation profiles (Garro et al. 1991). This study also showed that this inhibition was associated with acetaldehyde, the main metabolite of ethanol. Within the human body, ethanol is metabolized into acetaldehyde by alcohol dehydrogenase (Cederbaum 2012). This breakdown product is a highly toxic substance, even more potent than ethanol, responsible for most of the clinical effects of alcohol. Acetaldehyde has been shown to influence various epigenetic related processes by interfering with the one-carbon metabolism, involving the folate and methionine-homocysteine cycles (Varela-Rey et al. 2013; Kobor and Weinberg 2011). Acetaldehyde down-regulates expression of transporter-reduced folate carrier 1 (RFC1) and reduces folate uptake in the intestine and kidney (Hamid, Wani, and Kaur 2009; Hamid et al. 2007). Folate is a major source of methyl groups essential during nervous system and brain development. Acetaldehyde was also found to inhibit methionine synthase activity, which prevents the conversion of homocysteine to methionine, the endogenous precursor of S-adenosylmethionine (SAM) (Barak, Beckenhauer, and Tuma 1996). By interfering with the absorption of folate and availability of SAM, the universal methyl donor in all cell types, substrates needed for methyltransferase activity in DNA methylation and histone methylation reactions are therefore reduced or inhibited by alcohol metabolism.

4. Preimplantation Embryos and FASD

4.i. Preimplantation embryos and the epigenetic reprogramming wave

254 As mentioned, the effects of alcohol exposure on preimplantation stage embryos
255 have received little attention compared to other stages of gestation, even though studies
256 have shown that prenatal alcohol exposure during this period can disrupt DNA
257 methylation and lead to brain malformations and growth retardation (Haycock 2009;
258 Padmanabhan and Hameed 1988; Checiu and Sandor 1982, 1986). Since early stage
259 embryos undergo a major epigenetic reprogramming, any constraint induced by alcohol on
260 this crucial process could directly influence the transcriptional regulation of
261 developmentally important genes and lead to various degrees of adverse health outcomes.
262 In this section, we will overview the early embryogenesis process following fertilization
263 and how proper establishment of DNA methylation profiles during this period is crucial
264 for future development.

265 Preimplantation development—the time after the oocyte has been fertilized by the
266 sperm and before the embryo implants in the uterus— represents a vulnerable 7-day
267 window as the embryo progresses from the fallopian tube to the uterus. During this
268 voyage, the embryonic environment undergoes various composition changes (*e.g.*,
269 nutrients, proteins, growth factors, pH) that have a major influence on the development
270 and viability of the embryo (reviewed in (Feuer and Rinaudo 2012; Fisher 1986)).
271 Following fertilization, the zygote, or 1-cell stage embryo, undergoes rapid and
272 synchronous divisions without increasing its total size to form a 2-cell embryo, then a 4-
273 cell embryo, and so on. In these successive cleavage rounds, each individual embryonic
274 cell (blastomere) and its cellular constituents are replicated inside a protective shell called
275 the zona pellucida. At the 8-cell stage, individual blastomeres become polarized because
276 of specific cell-to-cell interactions (Ducibella et al. 1977; Handyside 1980; Reeve and

Ziomek 1981; Pratt et al. 1982). This stage also marks the beginning of the compaction process that progresses during the next division until the boundaries between the cells are barely detectable (Ducibella et al. 1977; Handyside 1980; Reeve and Ziomek 1981; Pratt et al. 1982). Asymmetric divisions of the embryonic cells during this period will lead to the first apparent sign of two distinct cell lineages: the outer cells, which give rise to the trophoblast, the precursor of the extraembryonic tissues (*e.g.* placenta); and the inner cells, which give rise to the inner cell mass (ICM), the progenitor to the embryo and yolk sac (Fleming and Johnson 1988). Ensuing cleavage divisions will begin a process called cavitation, where trophoblast cells secrete fluid at the morula stage to form an enclosed fluid-filled cavity, the blastocoel. This cavitation process will allow the blastocyst to fully expand and hatch from the protective zona pellucida for implantation onto the uterine lining (Fleming and Johnson 1988; Stephenson, Rossant, and Tam 2012). Although these developmental progressions are well conserved in mammals, there is variation between species regarding cell division timing, the number of cells at compaction and the resulting blastocyst stage embryo.

In parallel to these morphological transformations, the embryonic chromatin undergoes dynamic and profound changes in epigenetic modifications. These very first stages of mammalian development are elegantly choreographed by successive cascades of fine molecular events that remodel the parental epigenomes to generate a totipotent embryo with the potential to develop into a multicellular organism. Prior to fertilization, global levels of DNA methylation are strikingly different between male and female germ cells, with sperm CpGs heavily methylated at roughly 85%, while oocyte CpGs presenting lower levels approximating 30% (Seisenberger et al. 2013). Upon

300 fertilization, the parental genomes, which are packed independently in separate pronuclei,
301 will undergo distinct patterns of epigenetic remodeling to remove most DNA methylation
302 signatures across the genome (Figure 1). The paternal DNA methylation marks are
303 rapidly removed in an active process implicating TET-family enzymes shortly after
304 zygote formation and globally demethylated prior to DNA replication during first cell
305 division (Reik, Dean, and Walter 2001; Guo, Zhu, et al. 2014; Guo, Li, et al. 2014;
306 Wossidlo et al. 2011; Shen et al. 2014). Meanwhile in the maternal genome, TET-family
307 enzymes only partially contribute to the demethylation process, as DNA methylation is
308 mainly lost across subsequent embryonic divisions that continues until the blastocyst
309 stage. The protection of the maternal genome from this widespread active demethylation
310 process is accomplished through the presence of the maternal factor PGC7/Dppa3/Stella,
311 thought to directly interfere with the localization or activity of TET3. (Reik, Dean, and
312 Walter 2001; Guo, Zhu, et al. 2014; Guo, Li, et al. 2014; Wossidlo et al. 2011; Shen et al.
313 2014; Wu and Zhang 2017; Bian and Yu 2014; Nakamura et al. 2012). This
314 reprogramming wave removes most DNA methylation signatures inherited from the
315 gametes, except for imprinted genes and some types of repeat sequences (McGraw et al.
316 2013; McGraw et al. 2015; Messerschmidt, Knowles, and Solter 2014; Reik, Dean, and
317 Walter 2001). Although the vast exclusion of DNMT1 proteins from the nucleus is in part
318 responsible for the passive DNA demethylation, the nuclear presence of DNMT1
319 isoforms (DNMT1s: somatic, DNMT1o: oocyte specific, only maternally expressed) is
320 indispensable to maintain DNA methylation at imprinted regions (Hirasawa et al. 2008;
321 Toppings et al. 2008; Howell et al. 2001; Cirio et al. 2008; McGraw et al. 2013). Total
322 lack of DNMT1o-dependent methylation, only active at the 8-cell stage during

preimplantation, causes profound and variable phenotypic abnormalities in mid-gestation embryos including hindbrain, midbrain, and forebrain defects as well as placental integrity and imprinted X chromosome inactivation (Toppings et al. 2008; McGraw et al. 2013). These embryonic phenotypes have been associated with the disruption of inherited DNA methylation imprints and sequences outside of known imprinted gDMRs (Toppings et al. 2008; Howell et al. 2001; Cirio et al. 2008; McGraw et al. 2013; McGraw et al. 2015). In the days following implantation, DNA methylation is then re-acquired in a sex-, cell-, and tissue-specific manner by the combined action of DNMT3a and DNMT3b (Messerschmidt, Knowles, and Solter 2014; Auclair et al. 2014). The cells derived from the ICM rapidly undergo *de novo* DNA methylation; however, *de novo* methylation is less intense in the trophoctoderm and derived cells remain hypomethylated (Santos et al. 2002; Fulka et al. 2004; Smith et al. 2014; Guo et al. 2013; Robinson and Price 2015). This epigenetic inequality in DNA methylation levels arising from the ICM and TE will be upheld for the entire pregnancy between the placenta and the embryo. During this embryonic reprogramming wave, modifications on histone residues are also being remodeled. For instance, the paternal chromatin undergoes rapid H3K4 and H3K27 mono- and tri-methylation, while the maternal genome exhibits stable levels of those histone marks (Figure 2). This asymmetry disappears after several divisions but is thought to be important for the chromatin remodeling process. In the blastocyst, the appearance of the two subpopulations of cells, the trophoctoderm (extraembryonic) and the inner cell mass (embryonic), coincides with the re-emergence of divergent levels of histone marks, which is associated with gene activation in lineage specification (Marcho, Cui, and Mager 2015). During this early period, miRNAs also appear to play a key role in

the reprogramming wave (Figure 3), as they are implicated in clearing the transcriptional landscape during this developmental transition in order to facilitate the establishment of novel cellular states (reviewed in (Giraldez 2010)).

4.ii. Preimplantation embryo epigenetic susceptibility to adverse exposure

It was once assumed that the maternal environment had little effects on the embryo prior to embryonic implantation (when the embryo adheres to the uterine wall to receive oxygen and nutrients from the mother); however, many studies have now shown that this maternal environment can influence proper development as the preimplantation embryo is in direct contact with the mother's reproductive tract cells. For instance, in animal models, exposition of preimplantation embryos to anticancer chemotherapy drugs such as cyclophosphamide can induce various congenital malformations (Rengasamy 2016). However, most studies on the impacts of preimplantation exposure have been done in *in vitro* models.

In humans, many studies suggest that manipulation of preimplantation embryos during artificial reproductive technology (ART) treatments and procedures (*e.g.*, *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI)) increases the rate of imprinting disorders, such as Angelman syndrome and Beckwith-Wiedemann (reviewed in (McGraw, Shojaei Saadi, and Robert 2013)). The genomic imprinting disorders seen in children conceived using ART were accompanied in many cases by a loss of maternal DNA methylation at imprinted loci. A recent study also reported small but significant genome-wide DNA methylation decrease in 4730 CpGs in umbilical cord blood from

children conceived from ICSI (El Hajj et al. 2017). Promoter regions of genes like *SNORD114-9*, located in the imprinted *DLK1-DIO3* cluster, were affected and validated in another cohort of samples (El Hajj et al. 2017). Another recent study by Ghosh et al. revealed that global genome methylation levels, assessed by the DNA methylation of LINE1 repeat elements, differed between placentas from natural conceptions compared to placentas conceived by IVF (Ghosh et al. 2017).

Such studies revealed that early preimplantation embryos are responsive to their environment, and that adverse exposures can directly impact the epigenome, which in turn could alter the normal developmental programming and be detrimental for the future outcome of the offspring.

4.iii. In vitro models of alcohol exposure during preimplantation

Surprisingly, the first studies on the effect of ethanol on preimplantation embryos were accomplished to determine whether it could improve *in vitro* embryo culture and development. Publications from the early '90s showed that the addition of ethanol to the culture media either prevented or increased normal development in a dose-dependant and embryonic stage-specific manner. Addition of 1.6% (w/v) ethanol to the media of 1-cell and 2-cell embryo decreased overall blastocyst formation and hatching, whereas 0.4% ethanol exposure at the 1-cell stage inhibited further development (Leach, Stachecki, and Armant 1993). When 1-cell and 2-cell embryos were exposed to 0.1% ethanol, the rate of blastocyst formation significantly increased, stimulated the successful hatching of the embryos from the zona pellucida, and increased the implantation rate after embryo transfer. Another study also showed that exposure to 0.05% and 0.1% ethanol at the morula stage improved the process of cavitation (i.e., secretion of fluid by trophoblast

cells to create the internal cavity found in the blastocyst) (Stachecki et al. 1994). Nevertheless, these studies only evaluated the deleterious or beneficial effects of ethanol on the rate of development, but alterations in gene transcription and epigenetic modifications as on future brain functions were not investigated. Recently, using a similar approach, the Sirard lab showed that a 0.2% ethanol exposure during the entire preimplantation period is detrimental for blastocyst formation rate in porcine embryos. This exposure also led to a significant reduction in mitochondrial membrane potential, an early event of the apoptosis process, and aberrantly activated pathways associated with oxidative stress and nervous system damage (Page-Lariviere, Campagna, and Sirard 2017). Overall, such studies (Table 1) suggest that the undifferentiated cells of preimplantation embryos are highly sensitive to ethanol. Although these culture models did not evaluate the impact of alcohol metabolites such as acetaldehyde residues, they reinforce the need to improve our understanding of how the exposure dysregulates the epigenome and perturbs the early developmental program.

4.iv. In vivo models of maternal alcohol exposure during preimplantation

Many FASD models initiate their alcohol treatment during the preimplantation period, but most of them mirror exposure periods that span until the end of the first trimester, before birth (2nd trimester equivalent), or days following birth (3rd trimester equivalent). While these studies have allowed for a better understanding of the impact of chronic exposure on epigenetic alterations and transcriptional modulations in brain cells, as well as brain structure and functions (summarized in Table 2), these exposure paradigms still preclude the precise representation of early preimplantation exposure.

414 In the '80s, a collection of papers investigated various aspects of prenatal
 415 exposure on preimplantation embryos, such as type and duration of exposure (acute,
 416 chronic) in different models (mice, rat, *in vitro*) to better understand the impact of a
 417 mother's intake of alcohol during the earliest stages of pregnancy. Among their many
 418 descriptive findings, they found that alcohol caused retardation on development and
 419 increased morphological abnormalities, implantation rate, as well as both placental and
 420 fetal weights (Checiu and Sandor 1981, 1982, 1983, 1986, 1987, 1988; Fazakas-Todea,
 421 Checiu, and Sandor 1985; Sandor et al. 1980). For example, following an acute alcohol
 422 exposure at GD4.0, they observed delays or inhibition of implantation in ~25% of GD6.0
 423 embryos (Checiu and Sandor 1982) (Figure 1). In one of their publications, they also
 424 highlighted the specific role of acetaldehyde, the main sub-product from ethanol
 425 metabolism, by showing that the direct administration of acetaldehyde via intravenous
 426 injection induces similar and/or more severe effects on embryos than ethanol. Defects
 427 were even more pronounced when they increased blood acetaldehyde levels by blockage
 428 of its further metabolization by ANTALCOL (an aldehyde-dehydrogenase blocking
 429 compound) (Checiu, Sandor, and Garban 1984). In another early study by Padmanabhan
 430 and Hameed, acute alcohol exposure (0.02mL/g or 0.03mL/g of 25% EtOH) by
 431 subcutaneous injection during one of the first six days following fertilization was
 432 associated with marked adverse outcomes at GD15.0, including increased resorption,
 433 fetal death, craniofacial abnormalities, increased placental weight, and decreased fetal
 434 weight (Padmanabhan and Hameed 1988). These defects included hydrocephaly, thinner
 435 neural wall, microtia, mandibular and maxillary hypoplasia, and palatal defects. When
 436 they injected a dose of 0.03mL/g on GD2 and GD6, they observed similar results,

437 including severe growth retardation at GD14.0. Embryos collected at GD18 that
438 underwent the same treatment were found to have an accelerated compensatory growth
439 mechanism. These late stage gestation embryos showed less growth retardation and were
440 in the same developmental stage as their controls when compared to those collected at
441 GD14 (Padmanabhan and Hameed 1988). Acute exposures (2.9g/kg equivalent of
442 0.015mL/g of 25% EtOH) given by gavage on two successive days (GD1.5 & GD2.5)
443 induced embryonic and placental growth retardation and weight reduction at GD10.5
444 (Figure 1). In this study, epigenetic profiles were for the first time investigated in a
445 preimplantation alcohol exposure model and they showed that for the imprinted gene
446 H19, loss of DNA methylation was observed on the paternal allele of ethanol exposed
447 placentae, whereas it was unaffected in ethanol-exposed embryos (Haycock 2009). They
448 also established a correlation between the placental weight and the DNA methylation
449 levels of H19 on the paternal allele (Haycock 2009). In placental trophoblast cells, H19
450 controls cell proliferation through the action of a miRNA (i.e., miR-675) embedded in its
451 first exon (Gao et al. 2012; Keniry et al. 2012). Non-mammalian FASD models (e.g.,
452 Japanese rice fish) have also provided evidence that genes implicated in epigenetic
453 mechanisms are altered by early embryonic alcohol exposure. For instance,
454 Dasmahapatra and Khan have observed that following fertilization, embryos exposed to
455 300mM of ethanol for 48h have abnormal *Dnmt1* expression levels after 48h and 96h,
456 whereas, *Dnmt3a* and *Dnmt3b* expression levels remained unchanged (Dasmahapatra and
457 Khan 2015).

458 Although limited, these studies confirmed that administration of acute and/or
459 binge-like alcohol doses during the initial stages of embryo development clearly triggers

the onset of morphological and growth defects observed later in development. Furthermore, some studies also unveiled that the placenta will be affected by such early exposures, since a portion of the undifferentiated cells of preimplantation embryos will give rise to cells that compose the placenta. We still do not fully understand the mechanism leading to this ensemble of defects. Nonetheless, since the insult occurs during the epigenetic reprogramming wave, we can hypothesize that epigenetic dysregulations are involved and are in part responsible for the long-term disrupting effects on neurobiological processes.

5. Limitations of current studies

As highlighted in this review, models of maternal alcohol exposure targeting preimplantation embryos have revealed the vulnerability of embryos during this developmental window. However, we still do not comprehend the direct impact of preimplantation alcohol exposure on the epigenetic reprogramming wave and subsequent alterations in the developmental program of early embryonic cells. At this time, the aftermath of such exposure has only been described in later stages of development. Most, if not all, of the work related precisely to the preimplantation period has been accomplished *in vitro* (Table 1), and although these studies have uncovered highly valuable information, the fact that this experimental model bypasses the alcohol metabolism pathway reinforces the need for comprehensive *in vivo* studies. Most of the *in vivo* pathological effects arising from early preimplantation alcohol exposures have been researched at later stages of pregnancy (*e.g.*, GD10.5 to GD19) and these results are mostly based on implantation rates, growth retardation, gross morphological defects, and embryonic and placental weights (Table 3). Currently, we lack information on how these

483 alcohol-induced ill effects will have long-term consequences on brain development and
484 function and how such consequences can be associated with neurodevelopmental
485 disorders found in children with FASD. The distinct phenotypic brain abnormalities
486 observed *in utero* suggest that future brain function will be altered, but the extent of the
487 neurodevelopmental outcomes in young offspring and adults remain to be
488 comprehensively detailed. We know from *in vitro* models that alcohol exposure during
489 preimplantation will modify transcriptomic profiles at the blastocyst stage (Page-
490 Lariviere, Campagna, and Sirard 2017) however, such studies have been limited and
491 superficial due to previous technical limitations associated with low cell number in
492 preimplantation embryos. Furthermore, we do not know to what extent individual cells
493 are affected, and if cells giving rise to either the distinct cell lineages of the blastocyst,
494 the ICM, and trophectoderm are equally affected by the early exposure. Modulation in the
495 regulation of gene expression also strongly suggests possible alterations in the epigenetic
496 reprogramming wave, which could impact future developmental programs and brain cell
497 functions in offspring. We know from chronic or binge-like exposure models targeting
498 post-implantation periods that maternal alcohol exposure induces epigenetic alterations in
499 neural systems, and similar detrimental effects were also observed in neural stem cell
500 cultures (Veazey et al. 2013; Sathyan, Golden, and Miranda 2007; Tyler and Allan 2014;
501 Kleiber et al. 2014; Chater-Diehl et al. 2016; Carnahan et al. 2013; Veazey et al. 2015).
502 At this time, the impact of preimplantation alcohol exposure on the epigenetic
503 reprogramming wave and the developmental program of early embryos has not been
504 investigated. We do know that alcohol exposure during this period will alter the DNA
505 methylation status of imprinted gene H19 at GD10.5 in the placenta (Haycock 2009).

However, since only one genomic region was investigated we cannot conclude that either ethanol-exposed embryos or placentas are exempted of genome-wide epigenetic abnormalities. In such studies, the distinct phenotypic brain abnormalities observed *in utero* suggest that the developmental program and epigenome will be altered, but the extent and the neurodevelopmental outcomes in young offspring and adults remain to be comprehensively investigated.

Various human studies have shown that adverse exposures during preimplantation development can lead to persistent changes in DNA methylation in peripheral tissues of children. For instance, as mentioned in [section 4.ii](#), recent studies show that ART associated treatments and procedures caused slight but significant changes in thousands of CpGs in either cord blood or placenta. However, in such studies, definitive conclusions on which alterations are only associated with ART and could serve as a biomarker to improve protocols are difficult to substantiate due to all the variables and confounders (*e.g.*, parental age, infertility) involved. Similarly, studies aiming at discovering novel biomarkers for FASD diagnostic purposes using cells obtained from peripheral tissues of known FASD-affected children have recently been published. In one study, using buccal epithelial cells from a cohort of 110 FASD affected children, a total of 658 CpGs with subtle but significant differences in DNA methylation of which 41 CpGs had a >5% methylation change when compared to the control cohort (Portales-Casamar et al. 2016) with regions gaining methylation enriched for neurodevelopmental processes and diseases. However, the validity of these alterations as potential diagnostic markers remains to be further investigated and tested in separate FASD cohorts. For the above-mentioned ART and FASD studies, although it was not assessed, we can assume that the

529 mostly modest changes in DNA methylation levels observed, especially outside
530 regulatory regions, would have little immediate impact on gene transcription and
531 functions in peripheral cells. To determine the existing concordance between the
532 peripheral tissues and brain cells as well as to determine the implication of lasting
533 epigenetic defects on development and function, animal models will need to be used. In
534 this regard, a study using buccal swabs from a discovery cohort of 6 FASD patients
535 identified 269 CpGs with significant differences in DNA methylation levels, and a
536 portion of these results were validated in a heterogeneous group composed of six other
537 FASD children (Laufer et al., 2015). A number of alterations in DNA methylation in the
538 protocadherin cluster present in buccal DNA of FASD children were also found altered in
539 adult brain samples from a prenatal alcohol exposure mouse model (Laufer et al., 2015).
540 It remains challenging to interpret how modest or severe changes in DNA methylation
541 levels in FASD peripheral samples, especially those outside known regulatory regions,
542 correlate with transcriptional and functional changes. Even in ethanol-exposed adult brain
543 cells, derived from a chronic model of maternal ethanol consumptions, it was shown that
544 only a fraction of transcripts (16%; 21/129) and miRNAs (18%; 6/33) that showed
545 differential expression had significant DNA methylation alterations in their promoters.
546 However, with the implication of epigenetic instability marked by DNA methylation
547 alterations in diseases and aging, genome-wide ethanol-induced DNA methylation
548 alterations could potentially enhance later risk of health complications (Maegawa et al.
549 2010; Jung and Pfeifer 2015; Wilson, Power, and Molloy 2007; Robertson 2005).

550 6. The Future: What Needs to be understood?

Despite extensive evidence, it is still largely believed that alcohol exposure, like many other teratogen exposures, on preimplantation embryos will lead to an “all-or-nothing” developmental outcome, where embryo will either die or survive with no adverse side effects. To put an end to this concept and to further confirm that it is unsafe to drink alcohol during the early stage of embryo development, we need comprehensive studies using *in vivo* models to define the mechanisms, which lead to adverse neurodevelopmental outcomes in young and adult offspring.

To understand the original dysregulation events following the exposure to alcohol during preimplantation, we need to define the initial alterations in the developmental program happening during the preimplantation period through the dysregulation of intricate epigenetic interactions. This period of development is very challenging to investigate due to the limited number of cells (1-cell following fertilization up to 128/256-cells in a 4.5d mouse hatch blastocyst (Fleming, Javed, and Hay 1992). However, recent protocols for single-cell methylation landscapes have been released and can be used to quantify methylation levels of up to 48% of the CpG sites across the genome (Schwartzman and Tanay 2015; Smallwood et al. 2014; Yong, Hsu, and Chen 2016). Furthermore, new protocols demonstrate the feasibility of sequencing both the methylome and transcriptome from an individual cell (Hu et al. 2016). Such technological progress will further enhance our understanding of ethanol exposure at the cellular level. Investigation of histone modifications is more challenging since in a single preimplantation embryo, such studies are almost exclusively accomplished by fluorescent immunostaining to quantify the presence or absence of individual histone modifications. For the localization of abundant histone marks, such as H3K4me3 and H3K27me3, new

574 developments in micro chromatin immunoprecipitation (ChIP) assays now allow for a
575 much smaller number of cells (200-1000 cell range) (Dahl et al. 2016; Brind'Amour et al.
576 2015; Liu et al. 2016). These protocols offer promising alternatives; however, in most
577 cases pooling of many embryos is still required. Droplet-based microfluidic approaches
578 have recently been used to investigate H3K4me2 and H3K4me3 in single embryonic
579 stem cells. However, the initial pull-down experiments must be accomplished on
580 thousands of cells (Rotem et al. 2015). Following these altered epigenetic profiles
581 throughout *in utero* and postnatal brain development would provide crucial information
582 on the abnormal modulation of genes implicated in neuronal circuits and function. We
583 know from later prenatal alcohol exposure models, that long-lasting epigenetic
584 dysregulation leads to abnormal functioning of the hypothalamic-pituitary-adrenal (HPA)
585 axis (Hellemans et al. 2010; Rachdaoui and Sarkar 2013) or abnormal expression of non-
586 coding RNA (miRNAs and snoRNAs) in brain cells (Laufer et al. 2013). However, we
587 still do not know what specific pathways or cell types will be affected during brain
588 development or in adults following an early embryonic exposure. In this respect, detailed
589 morphological evaluations throughout brain development need to be accomplished by
590 histopathologic evaluation and immunofluorescence staining in order to identify specific
591 abnormalities in neurogenesis and neuronal differentiation processes. By identifying
592 specific brain regions or cell types affected by the early exposure will allow future
593 analyses in epigenomics, transcriptomics, and proteomics to be more targeted.

594 Although earlier *in vitro* studies might have shown that low amounts of ethanol
595 improve implantation rates (Stachecki et al. 1994; Leach, Stachecki, and Armant 1993),
596 we currently do not know if a single or prolonged exposure to much lower alcohol levels

within the range of normal social drinking during the preimplantation period can affect brain development and cause FASD-related neuro-disabilities in children. Most studies in *in vivo* models have used acute or binge-like exposures. Repeating some of these earlier experiments with low alcohol exposures using *in vivo* models, and measuring both epigenetic programming and neurodevelopmental consequences would allow us to determine if better early embryonic viability correlates with improved long-term outcomes, or if it actually leads to FASD-associated consequences.

Groundwork studies in both animal models and cohorts of children with FASD indicate that epigenetic markers in peripheral tissues could be used to determine if silent neurodevelopmental disabilities are at risk for developing FASD (Portales-Casamar et al. 2016; Laufer et al. 2015). These alcohol-induced epigenetic alterations still need to be validated in larger cohorts; however, the main challenge in these studies remains the heterogeneity in terms of ethnic background, ethanol exposure (i.e., amount, duration and developmental timing) as well as the range of neurodevelopmental impairment among cohorts of children with FASD. As suggested by others in the field, the use of epigenetic biomarkers may provide superior risk assessment of FASD if they are used in combination with other screening strategies (e.g., MRI, eye tracking, physical and mental health diagnostics, and immune markers), to portray the array of deficits associated with FASD (Lussier, Weinberg, and Kobor 2017). Since preimplantation alcohol exposure has also been associated with abnormal placental development, cells from this organ could also be used as part of the strategy for early diagnosis. With recent advances in cell-free fetal DNA—which largely originates from placental cells—recovered from maternal blood (Bischoff, Lewis, and Simpson 2005; Liao, Gronowski, and Zhao 2014), such

620 placental epigenetic biomarkers could be translated into non-invasive clinical prenatal
621 screening diagnostics offered to mothers that inadvertently subjected their embryos to
622 alcohol and believe that their unborn child is at risk for FASD. Identification of valid
623 biomarkers for the early diagnostic of FASD should be a priority in this research field,
624 since early identification of infants before age of 6 is a strong protective factor that can
625 reduce the impact of secondary disabilities (*e.g.*, mental health issues, addiction,
626 inappropriate sexual behaviors, unlawful conduct) (Streissguth 1997; Streissguth et al.
627 2004).

628 Finally, intervention studies in animal models using dietary supplementation of
629 methyl-donors, such as folate or choline, have shown that it can reduce the severity of
630 behavioural deficits and malformations associated with prenatal alcohol exposure (Young
631 et al. 2014). A recent pilot study on children with FASD showed that choline
632 supplementation had beneficial effects and could be used as an intervention to improve
633 memory (Wozniak et al. 2015). In these studies, the improvements from using dietary
634 methyl-donor supplementation on alcohol-induced epigenetic perturbations received little
635 to no attention. However, a recent study reported that choline supplementation given
636 simultaneously with the PAE (E7 to E21) normalized alcohol-induced changes in DNA
637 methylation and gene expression in adult rat brains (Bekdash, Zhang, and Sarkar 2013).
638 Nevertheless, the impact of these dietary supplementations remains to be investigated
639 using genome-wide epigenetic approaches. In some other studies, when such treatments
640 were given to children, no positive influence was observed (Nguyen et al. 2016).
641 However, the supplementation's potential could have been reduced or impeded because
642 of severe brain structure damages inflicted by the direct neurotoxic and teratogenic nature

of alcohol on the developing brain cells. Influence of maternal nutrition, especially methyl-donor enriched diets, during pregnancy has been widely investigated (Geraghty et al. 2015). Although epigenetic studies indicate a correlation between maternal methyl-donor intake during gestation and offspring DNA methylation patterns, in some cases, it had little or no impact on global or specific loci DNA methylation profiles (Boeke et al. 2012). Thus far, the potential preventative (during gestation) and/or corrective (following birth) effects of methyl-donor supplementations on the developmental programming of brain cells following an early preimplantation alcohol exposure have not been investigated. At-risk populations could benefit from these dietary treatments, as they could rectify DNA methylation imbalances related to neuronal development and function while the epigenetic profiles are being reorganized throughout *in utero* and postnatal neurogenesis processes.

7. Conclusion

Major accomplishments have been made in the FASD field, especially regarding the complex interplay between genetic and epigenetic factors. However, the detrimental effects of alcohol on first week embryos are still poorly understood and have received little attention compared to other periods of gestation. Perhaps it is because it encompasses only a short period of development or perhaps many still falsely believe that it is an “all-or-nothing” outcome. Since about 40% of worldwide births are unplanned, many women inadvertently subject their early embryos to alcohol during this time of pregnancy (Sedgh, Singh, and Hussain 2014). Furthermore, several studies suggest that FASD may be on the rise in coming years due to increasing rates of alcohol use. Binge

drinking (>4 drinks within 2 hours) rates rose 17.5% in women between 2005 and 2012 (Dwyer-Lindgren et al. 2015), and drinking during pregnancy increased among young women (18-34 years old) in a number of countries (Gruza et al. 2008; Lim et al. 2012; Popova, Lange, Probst, Parunashvili, et al. 2016; Popova, Lange, Probst, Shield, et al. 2016; Tan et al. 2015; Thomas 2012; Wilsnack, Wilsnack, and Kantor 2013). Research using early preimplantation alcohol exposure models will fill knowledge gaps that currently exist in the FASD field. Nonetheless, samples from prospective cohort studies are needed to validate our findings and further our comprehension of the mechanisms leading to FASD.

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Figure 1. Susceptibility of alcohol-induced developmental programming alterations following maternal alcohol exposure during the epigenetic reprogramming wave.

Following fertilization, both the maternal (red line) and paternal (blue line) genomes undergo a genome-wide loss of DNA methylation. After implantation, DNA methylation profiles are re-established by DNMT3A/3B in a cell specific manner, with embryonic cells derived from the inner cell mass (yellow line) gaining higher levels of methylation compared to extraembryonic cells derived from the trophectoderm (green line). Specific regions of the genome, including imprinting genes and imprinted-like regions must resist this genome-wide demethylation wave and maintain DNA methylation via a DNMT1s (somatic form) and DNMT1o (embryo-specific form) dependant process (dotted line). Defects associated specifically with maternal alcohol exposure during preimplantation are summarized on the right. Figure adapted from (Reik, Dean, and Walter 2001; McGraw, Shojaei Saadi, and Robert 2013).

Figure 2. Temporal changes in histone marks occurring during the preimplantation period.

Blue bars represent the global level of specific histone marks for the maternal and paternal genomes from fertilization to implantation. (Shi and Wu 2009; Cantone and Fisher 2013; Burton and Torres-Padilla 2014; Marcho, Cui, and Mager 2015; Zhou and Dean 2015)

705 **Figure 3. Relative amount of microRNAs during preimplantation embryo**
706 **development.** Recent studies show an increase in the global amount of miRNAs through
707 the different stages of the early embryo until the implantation. miR-698, miR-296,
708 mir_194-3 and miR-290 are examples of miRNAs highly expressed at specific phases of
709 the preimplantation and are respectively detected after fertilization, at 2-cell stage, 8-cell
710 stage and blastocyst stage. Certain non-stage specific miRNAs are also constantly
711 expressed and account for a considerable part of the total quantity of miRNAs detected
712 during preimplantation, as shown at the bottom of the graph (Tang et al. 2007).

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Figure 1. Susceptibility of alcohol-induced developmental programming alterations following maternal alcohol exposure during the epigenetic reprogramming wave. Following fertilization, both the maternal (red line) and paternal (blue line) genomes undergo a genome-wide loss of DNA methylation. After implantation, DNA methylation profiles are re-established by DNMT3A/3B in a cell specific manner, with embryonic cells derived from the inner cell mass (yellow line) gaining higher levels of methylation compared to extraembryonic cells derived from the trophoctoderm (green line). Specific regions of the genome, including imprinting genes and imprinted-like regions must resist this genome-wide demethylation wave and maintain DNA methylation via a DNMT1s (somatic form) and DNMT1o (embryo-specific form) dependant process (dotted line). Defects associated specifically with maternal alcohol exposure during preimplantation are summarized on the right. Figure adapted from (Reik, Dean, and Walter 2001; McGraw and Trasler 2013).

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Figure 2. Temporal changes in histone marks occurring during the preimplantation period. Blue bars represent the global level of specific histone marks for the maternal and paternal genomes from fertilization to implantation. (Shi and Wu 2009; Cantone and Fisher 2013; Burton and Torres-Padilla 2014; Marcho, Cui, and Mager 2015; Zhou and Dean 2015)

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Figure 3. Relative amount of microRNAs during preimplantation embryo development. Recent studies show an increase in the global amount of miRNAs through the different stages of the early embryo until the implantation. miR-698, miR-296, mir_194-3 and miR-290 are examples of miRNAs highly expressed at specific phases of the preimplantation and are respectively detected after fertilization, at 2-cell stage, 8-cell stage and blastocyst stage. Certain non-stage specific miRNAs are also constantly expressed and account for a considerable part of the total quantity of miRNAs detected during preimplantation, as shown at the bottom of the graph (Tang et al. 2007).

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Table 1. *In vitro* models of preimplantation alcohol exposure.

Exposure		Model		Ref	
Time	Dosage	Species	Duration	Summary of key findings	
Blastocyst	0.1%	Cultured mouse embryo	5 min exposition	↑ c-Myc mRNA level	(Leach et al. 1999)
Blastocyst	0.1%	Cultured mouse embryo	5 min exposition	↑ Intracellular calcium level	(Stachecki et al. 1994)
1-cell, 2- cell or 4-cell	0.1%, 0.4% or 1.6%	Cultured mouse embryo	24h exposition	↓ Blastocyst rate (1.6% 1-cell, 2-cell) Inhibition of development (0.4% 1-cell) ↑ Blastocyst rate (0.1% 1-cell, 2-cell)	(Leach, Stachecki, and Armant 1993)
2-cell	0.05% to 1% (Various alcohol type)	Cultured mouse embryo	24h exposition	Dose-dependent ↓ blastocyst rate (butanol, propanol, isopropanol, methanol)	(Kowalczyk et al. 1996)
Morula	0.1% to 1% (Various alcohol type)	Cultured mouse embryo	24h exposition	↑ Intracellular calcium level (0.1% or 1% ethanol, 1% butanol)	(Kowalczyk et al. 1996)
2-cell	0.1% or 1%	Cultured mouse embryo	8 days	↓ Blastocyst rate (1%)	(Wiebold and Becker 1987)
2-cell	0.1% or 1%	Cultured mouse embryo transfer in vivo at day 4	8 days	↑ Implantation rate (0.1%) ↓ Implantation rate (1%) ↓ Fetal survival (1%)	(Wiebold and Becker 1987)
1-cell	0.2%	Porcine cells	7 days	↓ Blastocyst rate Changes in normal gene expression Mitochondrial dysfunctions Activated pathways related to nervous system damage and oxidative stress	(Page-Lariviere, Campagna, and Sirard 2017)

Table 2. Chronic *in vivo* models of alcohol exposure starting during the preimplantation period.

Exposure			Model				Ref
Time	Dosage	Method	Species	Age	Tissue	Summary of key findings	
GD0.5 to GD8.5	10% EtOH	Voluntary drinking	Mouse	P87	Hippocampus	↑ miR 135a, 135b, 467b-5p, 487b expression ↑ Slc17a6 expression ↓ DNA methylation at Slc17a6 promoter ↑ H3K4me3 at Slc17a6 promoter ↓ Vglut2 protein	(Zhang et al. 2015)
GD0.5 to GD8.5	10% EtOH	Voluntary drinking	Mouse	P87	Hippocampus	↑ SLC17a6 expression	(Zhang and Chong 2016)
All gestation and 10 days after	10% EtOH	Voluntary drinking	Mouse	P70	Whole brain	↓ Reflex and coordination development, learning ↑ Anxiety ↓ Glra1, Grin2c	(Kleiber, Wright, and Singh 2011)
All gestation and 6 days after	10% EtOH	Voluntary drinking	Mouse	P60, P100		↓ Contextual fear ↑ Time inspecting a novel object	(Allan et al. 2003)
GD1 to GD22	4.5g/kg	Gavage	Rat	P21	Hippocampus	↑ DNMTs activity Gene expression changes in Dnmt1, Dnmt3a and MeCP2	(Perkins et al. 2013)
GD1 to GD16	4% EtOH	Liquid diet	Mouse	GD17, P7	Hippocampus	Altered DNA methylation (staining) Retarded hippocampal formation ↓ Proliferation and maturation of hippocampal cells	(Chen, Ozturk, and Zhou 2013)

GD0.5 to GD8.5	10% EtOH	Voluntary drinking	Mouse	P28, P60	Hippocampus	<p>↑ Expression of 14 genes + 2 micro-RNA (P28)</p> <p>↓ Expression of 9 genes + 1 micro-RNA (P28)</p> <p>CpGs specific DNA methylation changes in Vmn2r64, Olfr110, Olfr601 (P28)</p> <p>Asymmetry of brain structure (P60)</p>	(Marjonen et al. 2015)
GD1 to GD18	5g/kg	Liquid diet	Rat	GD21	Astrocytes, brain	<p>↓ GFAP immunoreactivity, transcription and mRNA level and stability (astrocytes)</p> <p>↑ Methylation GFAP (brain)</p>	(Valles et al. 1997)
GD1 to GD20	6.0 g/kg per day	Intubation	Rat	GD21, GD33	Olfactory bulb	<p>↓ BDNF mRNA level (GD21, GD33)</p> <p>↓ Number of olfactory bulbgranule cells (GD33)</p> <p>↑ Methylation of BDNF DNA</p>	(Maier et al. 1999)
GD0.5 to GD8.5	10% EtOH	Voluntary drinking	Agouti mouse	P21	DNA from tail	<p>↑ DNA methylation on Avy allele</p> <p>↓ Pups weight</p>	(Kaminen-Ahola et al. 2010)
GD1 to GD22		Liquid diet	Rat	P21, P55	Hippocampus and Whole brain	<p>↑ Homocysteine and methionine concentrations</p> <p>↓ Mtr, Mat2a whole brain mRNA level (P21)</p> <p>↓ Hippocampal Mtr and Cbs mRNA level (P55, males)</p> <p>↑ Mtr, Mat2a, Mthfr, and Cbs hippocampal mRNA level (P55, females)</p>	(Ngai et al. 2015)
All gestation	10% EtOH	Voluntary drinking	Mouse	P70	Brain	Changes in DNA methylation (6660	(Laufer et al. 2013)

and 10 days after						promoters)	
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Table 3. *In vivo* models of maternal alcohol exposure targeting specifically preimplantation embryos.

Exposure			Model			Ref	
Time	Dosage	Method	Species	Age	Tissue	Summary of key findings	
GD1.5 & GD2.5	2.9g/kg	Gavage	Mouse	GD10.5	Embryo & Placenta	Growth retardation (embryo and placenta) ↓ Methylation H19/Igf2 paternal allele (placenta)	(Haycock and Ramsay 2009)
One of the first 6 GD (GD1.5- GD6.5)	0.02mL/g or 0.03mL/g 25% EtOH	Injection	Mouse	GD15	Embryo & Placenta	↑ Resorption and fetal death ↓ Fetal body weight (0.03ml/g group) ↑ Placental weight ↓ Cord length Craniofacial and systematic abnormalities	(Padmanabhan and Hameed 1988)
GD2 and GD6	0.03mL/g	Injection	Mouse	GD14 ;16 ;18	Embryo & Placenta	Severe growth retardation (GD14) ↓ Fetal body weight (All time-point)	(Padmanabhan and Hameed 1988)
GD4	NA	IV injection	Mouse	GD6	Blastocyst	↓ Implantation	(Checiu and Sandor 1982)
GD3 and GD4	NA	IV injection	Mouse	GD19	Embryo & Placenta	↓ Fetal and placental weight ↓ Implantation rates Impaired embryo transfer in oviduct Retardation of development.	(Checiu and Sandor 1986)
GD0 to GD3	25% EtOH	Obligatory drinking	Mouse	P0	Embryo & Placenta	Abnormal morphological features No difference in litter size and litter weight	(Wiebold and Becker 1987)

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