Exploring the use of folic acid and GM-CSF in the prevention of epigenetic and birth defects associated with assisted reproduction

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Table of Contents

Abstract 4

Résumé 6

Acknowledgements 9

Contribution of Authors 10

Abbreviations 11

List of Figures 15

List of Tables 16

Chapter 1: Introduction 17

1.1 Assisted Reproduction 17

1.1.1 Infertility and assisted reproductive technology 17

1.1.2 Adverse outcomes associated with human ART 18

1.1.3 Dynamic DNA methylation waves and ARTs 19

1.1.3.1Epigenetic reprogramming during gametogenesis 19

1.1.3.2 Epigenetic reprogramming during preimplantation 20

1.1.3.3 Imprinted genes 21

1.2 Advantage of animal models to study assisted reproduction 22

1.2.1 ART and embryonic development 22

1.2.2 Alterations in epigenetics associated with ART in animal models 23

1.2.2.1 Superovulation 23

1.2.2.2 *In vitro* fertilization and embryo culture 25

1.2.2.3 Cumulative effect of ARTs 26

1.3 Methods to prevent epigenetic defects associated with ART 26

1.3.1 Folic acid and neural tube defects 26

1.3.1.1 Folic acid and pregnancy outcomes using animal models 27

1.3.1.2 Folic acid and DNA methylation 28

1.3.2 Granulocyte-macrophage colony stimulating factor 29

1.3.2.1 Role in embryo and placental development 29

1.3.2.2 DNA methylation and GM-CSF 30

1.3.2.3 Effects of GM-CSF examined in *in vitro* studies 30

1.4 Effects associated with ART and maternal folic acid supplementation 31

Rationale and objectives 33
Chapter 2: Materials and Methods 37
2.1 Animals and diets 37
2.2 Tissue collection and DNA isolation 38
2.3 PCR sexing of embryos 39
2.4 DNA methylation analysis 39
2.5 ART protocol 39
2.6 Blood collection 41
2.7 Statistical analysis 42
Chapter 3: Results 44
3.1 Effect of folic acid supplementation on naturally cycling mice 44
3.1.1 Effect of folic acid supplementation on maternal body weight 44
3.1.2 Effect of folic acid supplementation on pregnancy outcomes 44
3.2 Effect of folic acid supplementation on placental DNA methylation in pregnancies conceived by ART 45
3.3 Optimization of a new ART protocol 47
Chapter 4: Discussion 66
4.1 Modulation of natural mating pregnancy outcomes by moderate folic acid supplementation 67
4.2 Modulation of DNA methylation in the placentas of ART-conceived embryos by folic acid supplementation 68
4.3 Optimization of ART protocol 71
4.4 Future directions 73
4.5 Conclusions 75
References 77

Abstract

The use of assisted reproductive technology (ART) overlaps with crucial dynamic DNA methylation waves taking place during gametogenesis and embryonic development. A number of studies have shown that ART procedures are associated with alterations in embryo development and epigenetic marks. Folic acid supplements have been proposed to be beneficial in preventing embryonic and epigenetic defects in normal and ART-conceived pregnancies, but further dose and mechanistic studies are still required. The goals of the thesis were to use a mouse model to: 1) examine effects of diets supplemented with different levels of folic acid on embryonic development in naturally cycling outbred CF1 mice often used as controls in ART experiments, 2) examine effects of folic acid supplemented diets on the methylation of two imprinted and one non-imprinted gene in the context of ART, and 3) attempt to improve embryo culture conditions by the addition of the growth factor granulocyte-macrophage colony stimulating factor (GM-CSF) to prevent negative impacts of ART not corrected with folic acid. For the first goal, naturally mating mice were fed a control diet containing the daily required intake (DRI) of folic acid for mice (CD) or a moderate dose folic acid supplemented diet containing 4-fold the DRI (4FASD). Embryos and placentas were collected at mid-gestation. Both CD and 4FASD groups showed similar rates of embryonic abnormalities, 4.8% and 4.6% respectively. Pregnancy outcomes between the females on CD or 4FASD were not significantly different. For the second goal, females were exposed to three different folic acid diets (CD, 4FASD, 10FASD), ART was performed, and placentas from female embryos were selected for DNA methylation analysis of a non-imprinted gene, *Casz1*, and two imprinted genes, *Nnat* and *Gnas*. DNA methylation of *Casz1* was not affected by ART or either dose of folic acid supplementation. DNA methylation of *Nnat* was not affected by ART or folic acid supplementation, however the increase in DNA methylation variance due to ART

showed evidence of correction with both doses of folic acid supplementation. For *Gnas*, ART resulted in a decrease in mean DNA methylation and an increase in DNA methylation variance for both regions analyzed, and both changes were partially corrected by the 4FASD. For the third goal, an ART protocol was designed to combine both folic acid supplementation of mothers and GM-CSF in the embryo culture medium. Adjusting the hormone doses to the weight of the mice for the superovulation process increased the number of oocytes ovulated. Washing the mineral oil increased both the *in vitro* fertilization (IVF) and *in vitro* development (IVD) rates. The third goal, however, was not further pursued due to poor *in vivo* development following embryo transfer attributed to issues with the mineral oil. Taken together, this study shows that the CD diet is sufficient for normal development of offspring in naturally cycling CF1 mice, ART affects imprinted and non-imprinted genes differently, and mouse ART protocols can often require optimization. The study provides further evidence that moderate folic acid supplementation, similar to the doses used in human pregnancies, can only partially correct altered DNA methylation associated with ART, suggesting that further efforts are required to improve factors such as embryo culture conditions, including pursuing the use of promising growth factors such as GM-CSF.

Résumé

L'utilisation de la procréation médicalement assistée (PMA) coïncide avec des vagues dynamiques de méthylation d'ADN, cruciales pour la gamétogenèse et le développement embryonnaire. De nombreuses études ont démontré que les procédures de PMA sont associées à des altérations du développement embryonnaire et des marques épigénétiques. Il a été proposé qu'une supplémentation en acide folique prévient de façon efficace ces altérations mais de plus amples études sont nécessaires pour mieux évaluer les doses et cerner les mécanismes. Cette étude avait pour but d'examiner chez un modèle murin : 1) l'effet de la supplémentation en acide folique à différentes doses sur le développement embryonnaire lors de la reproduction naturelle chez la souche de souris CF1, souvent utilisée comme contrôle pour les expériences de procréation assistée. 2) l'effet de différents niveaux de supplémentation en acide folique sur la méthylation de trois gènes, dont deux soumis à empreinte, dans le contexte de la procréation assistée et 3) l'amélioration des conditions de culture embryonnaire par l'ajout du facteur de stimulation des colonies de granulocytes-macrophages (GM-CSF) afin de prévenir les effets négatifs associés à la procréation assistée qui ne sont pas corrigés par l'acide folique. Pour le premier objectif, les souris CF1 s'accouplant naturellement ont reçu une diète contrôle correspondant à l'apport quotidien recommandé d'acide folique chez la souris (CD) ou quadruple (4FASD). Les embryons et les placentas ont été collectés à la mi-gestation. Les deux régimes CD et 4FASD ont démontré des taux similaires d'anomalie morphologique (4,8% et 4,6% respectivement). Les résultats de la grossesse chez les souris sous CD et 4FASD n'étaient pas significativement différent. Pour le deuxième objectif, les souris CF1 femelles ont été soumises à trois niveaux de supplémentation en acide folique (CD, 4FASD, 10FASD) et suite à la procréation assistée, des placentas d'embryons femelles ont été sélectionnés pour l'analyse de méthylation de l'ADN du gène *Casz1* et celle de

deux gènes soumis à empreinte, *Gnas* et *Nnat*. La méthylation de l'ADN de *Casz1* n'a pas été affectée par la procréation assistée ou le régime alimentaire. La méthylation de l'ADN du gène *Nnat* n'a pas été altérée par la PMA ou la supplémentation en acide folique mais celle-ci, aux deux doses utilisées, a démontré une tendance à corriger la variabilité des niveaux de méthylation engendrée par la procréation assistée. Finalement, la PMA a engendré une baisse du niveau de méthylation et une augmentation de la variabilité dans deux régions étudiées du gène *Gnas*, et ces changements ont été partiellement corrigés par une dose modérée d'acide folique (4FASD). Pour le troisième objectif, un protocole de procréation assistée a été élaboré pour combiner à la fois la supplémentation maternelle en acide folique et la présence de facteur de croissance GM-CSF dans le milieu de culture embryonnaire. L'ajustement du dosage hormonal au poids des souris pour le processus de superovulation a augmenté avec succès le nombre d'ovocytes ovulés. Le lavage de l'huile minérale a amélioré à la fois le taux de fécondation *in vitro* (IVF) et de développement *in vitro* (IVD). Le troisième objectif n'a cependant pu être poursuivi à cause des faibles taux de développement *in vivo* obtenus suite au transfert d'embryon dans les souris porteuses, attribuables aux problèmes d'instabilité de l'huile minérale. L'ensemble des résultats indique que la diète contrôle en acide folique est adéquate pour l'obtention d'un développement embryonnaire normal chez les souris CF1 lors de la reproduction naturelle, que la procréation assistée affecte de façon différente les gènes soumis à empreinte et ceux non-soumis à empreinte et que les protocoles de PMA nécessitent aussi une optimisation minutieuse chez la souris. Cette étude apporte de nouvelles preuves qu'une supplémentation maternelle en acide folique à dose modérée, similaire à celle utilisée chez l'humain, ne corrige que partiellement les altérations de la méthylation d'ADN associées à la procréation médicale assistée, suggérant que de plus amples efforts sont nécessaires

pour améliorer les conditions de culture embryonnaire, incluant l'utilisation de facteurs prometteurs comme le GM-CSF.

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Contribution of Authors

The research in this thesis was performed by the candidate with assistance from lab members.

In aim 1, natural mating was performed by the candidate. DNA extraction and sexing of the yolk sacs were performed by the candidate. Embryo and placenta collections were done in collaboration with a research associate in the lab, Ms. Josée Martel.

In aim 2, tissue samples were provided by Ms. Sophia Rahimi and Ms. Josée Martel (Rahimi et al., 2019). Sample subset selection was completed by the candidate in collaboration with Ms. Josée Martel. DNA extraction and bisulfite conversions of placental tissues were performed by the candidate. The candidate carried out bisulfite pyrosequencing of embryos and placentas.

In aim 3, hormone injections were performed by the candidate. *In vitro* fertilization, embryo transfer, embryo collections, and tissue grinding were performed by the candidate in collaboration with Ms. Josée Martel. Blood collections were done by both the candidate and Ms. Josée Martel. Sexing of the yolk sacs was performed by the candidate.

Data analysis was performed by the candidate with assistance from Ms. Josée Martel. All statistical analyses were performed by the candidate.

Abbreviations

% – percent

< – less than

≤ – less than or equal to

= – equals to

ºC – degrees Celsius

4FASD – 4-fold folic acid supplemented diet

10FASD – 10-fold folic acid supplemented diet

ART – assisted reproductive technology

AS – Angelman syndrome

BHMT – betaine-homocysteine methyltransferase

BWS – Beckwith-Wiedemann syndrome

Casz1 – castor zinc finger 1

CD – folic acid control diet

CD-ART – assisted reproductive technology-folic acid control diet

CF1 - Non-Swiss Albino-Carworth Farms-1

CL – corpora lutea

CpG – cytosine-phosphate-guanine

CO_2 – carbon dioxide

COC – cumulus-oocyte complexes

Ddx3 – DEAD-box helicase 3 X-linked

DHF – dihydrofolate

DNA – deoxyribonucleic acid

DNMT – DNA methyltransferase

dpc – days post coitum

E – embryonic day

EDC – endocrine disrupting chemicals

EDTA – ethylenediaminetetraacetic acid

gDMR – gamete differentially methylated region

GM-CSF – granulocyte-macrophage colony-stimulating factor

Gnas – guanine nucleotide-binding protein

GV – germinal vesicle

h – hours

H19 – *H19* imprinted maternally expressed transcript

hCG – human chorionic gonadotropin

HTF – human tubal fluid

ICM – inner cell mass

ICR – imprinting control region

ICSI – intracytoplasmic sperm injection

IP – intraperitoneal

IU – international unit

IVF – *in vitro* fertilization

Kcnq1ot1 – *Kcnq1* overlapping transcript 1

KSOM½AA - potassium simplex optimization medium (1X) with ½ amino acids

LH – luteinizing hormone

MII – metaphase II

MAPK – mitogen-activated protein kinase

mL – milliliter

MS – methionine synthase

MTHFR - methylenetetrahydrofolate reductase

Myog – myogenin

N_2 – nitrogen gas

NAT-CD – natural mating-folic acid control diet

Nnat – neurotanin

No. – number

NSET – non-surgical embryo transfer

NTD – neural tube defects

O_2 – oxygen gas

PCR – polymerase chain reaction

Peg1 – paternally expressed gene

PGC – primordial germ cells

PGC7 – developmental pluripotency associated 3

PMSG – pregnant mare serum gonadotropin

PRAMEL7 – preferentially expressed antigen in melanoma-like protein 7

PWS – Prader-Willi syndrome

R – resorbed embryo

RBC – red blood cells

SAM – S-adenosyl methionine

Snrpn – small nuclear ribonucleoprotein polypeptide N

SRS – Silver-Russell syndrome

STAT3 – signal transducer and activator of transcription 3

STAT5 – signal transducer and activator of transcription 5

TE – trophectoderm

THF – tetrahydrofolate

TRIM28 – tripartite motif containing 28

TSH – thyroid stimulating hormone

ug – microgram

UHRF1 – Ubiquitin like plant homeodomain and RING ringer domain 1

Zfy1 – zinc finger y-chromosomal protein 1

ZPF57 – zing finger protein 57 homolog

List of Figures

Figure 1. Overlap of ARTs and DNA methylation programming during gametogenesis and embryo development …….…….…… 35

Figure 2. One carbon metabolism cycle …….……..…… 36

Figure 3. Weights of females mated ……..50

Figure 4. Effect of maternal folic acid supplementation on pregnancy outcomes …….51

Figure 5. Effect of maternal folic acid supplementation on embryonic abnormalities and delays……52-53

Figure 6. Embryonic crown rump lengths with folic acid supplementation ……54

Figure 7. Comparison of sample subset containing placentas from normal and abnormal female embryos to the original placenta sample set ……...……57

Figure 8. Effect of ART and folic acid supplementation on DNA methylation of a non-imprinted gene *Casz1* in the female placenta sample subset …….……58

Figure 9. Effect of ART and folic acid supplementation on DNA methylation of an imprinted gene *Gnas* in the female placenta sample subset ……...……59-60

Figure 10. Effect of ART and folic acid supplementation on DNA methylation of an imprinted gene *Nnat* in the female placenta sample subset ……...……61-62

Figure 11. Experimental design ……....…65

List of Tables

Table 1. Primers for bisulfite pyrosequencing used to assess DNA methylation in mice ……... 43

Table 2. Characteristics of CD litters ……55

Table 3. Characteristics of 4FASD litters ……56

Table 4. Summary of results of all ART experiments …… 63-64

Chapter 1: Introduction

1.1 Assisted Reproduction

1.1.1 Infertility and assisted reproductive technology

Infertility is defined by the failure to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochshild et al., 2009). External factors can contribute to the overall increase in both male and female fertility such as exposure to endocrine disrupting chemicals (EDCs) present in the environment (Anway et al., 2005; reviewed by Patel et al., 2015). Infertility affects as many as 186 million people worldwide. Since 1990, 1.9% of women 20 to 44 years old were unable to give birth to their first child and 10.5% were unable to give birth to their second child (Mascarenhas et al., 2012). In Canada alone, it is estimated that 11.5-15.7% of couples are infertile (Bushnik et al., 2012).

Couples who suffer from infertility resort to conceiving children using assisted reproductive technologies (ARTs). ARTs consist of many techniques such as *in vitro* fertilization manipulation of oocytes, sperm, and embryos to overcome infertility and achieve pregnancy (Zegers-Hochshild et al., 2009). Using assisted reproduction to conceive is a relatively expensive procedure, therefore, developed countries have higher rates of ART use (Connolly et al., 2010). From all children conceived in developed countries, 0.7-6.2% are born as a result of ART (Calhaz-Jorge et al., 2017). ART consist of multiple procedures that help overcome both male and female infertility. ART can be a combination of multiple techniques including ovarian stimulation (superovulation), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), and *in vitro* embryo culture.

1.1.2 Adverse outcomes associated with human ART

The majority of children conceived with ART may not display health complications at birth, but there are many negative outcomes that are associated with the use of assisted reproduction. ART has been associated with higher risk for preterm birth, birth weight, and smaller size for gestational age (Declercq et al., 2015). Children conceived using ART having an increased risk for birth defects was confirmed by a meta-analysis of 46 studies (Zhao et al., 2020).

One area of concern is the impact of ART on children later in life. Unlike birth defects, neurodevelopmental disorders are observed in the later stages in life. A number of studies have suggested an association between the use of ART and an increased risk of neurodevelopmental disorders in children. A meta-analysis consisting of 11 studies from Europe, America, and Asia concluded that children conceived with ART had a significantly greater risk of autism spectrum disorder (Liu et al., 2017). Singletons born using ART also have a small increase in risk of intellectual disability, with a double in risk if the children were born preterm (Hansen et al., 2018). The use of IVF has a small but significant increase in risk in developing attention deficit/hyperactive disorder (Källén et al., 2011). The method of fertilization also had an impact on neurodevelopment. Singletons conceived using ICSI had an increased risk of borderline delayed cognitive development when compared to singletons conceived with IVF (Goldbeck et al., 2009).

The relationship between the use of ART and its impact on metabolism or endocrine disruption in the offspring still remains unclear. However, there is evidence that there is an association between use of ART and metabolic and endocrine disruption in children (Vrooman et al., 2017). A meta-analysis concluded that children born with assisted reproduction had a small significant increase in fasting insulin levels, vessel thickness, and blood pressure (Guo et al., 2017).

ART conceived children had a significant elevation of serum thyroid stimulating hormone (TSH) and mild TSH resistance when compared to naturally conceived children (Sakka et al., 2009).

Along with impacts on neurodevelopment, metabolism, and hormone function, ART has been associated with higher incidences of imprinting disorders in the offspring. From a meta-analysis including 23 studies, Silver-Russell syndrome (SRS) had the highest odds ratio of 11.3, while other disorders such as Beckwith-Wiedemann syndrome (BWS), Angelman syndrome (AS), and Prader-Willi syndrome (PWS) had odds ratio of 5.8, 4.7, and 2.2 respectively (Cortessis et al., 2018). BWS has been strongly associated with ART, with many studies reporting that the overgrowth and stunted growth respectively are greater in children conceived with ART compared to naturally conceived children (Mussa et al., 2017; DeBaun et al., 2003; Lim et al., 2009).

1.1.3 Dynamic DNA methylation waves and ARTs

ART technologies overlap with crucial dynamic DNA methylation waves throughout development (Figure 1). DNA methylation is highly dynamic during gametogenesis, the process of germ cell development, and early embryonic development (reviewed by Marchal et al., 2004; Santos et al., 2002). Epigenetic reprogramming and the erasure and establishment of epigenetic information across the genome occurs during the dynamic waves.

1.1.3.1 Epigenetic reprogramming during gametogenesis

The first period of DNA methylation reprogramming takes place during gametogenesis, where DNA methylation is erased genome-wide and re-methylated, with the timing of acquisition between oocytes and sperm occurring at different developmental times (reviewed by Messerschmidt et al., 2014). In male germ cells, DNA methylation is acquired prenatally and is

mostly completed by birth (reviewed by Denomme and Mann et al., 2012; Saitou et al., 2012). In female germ cells, however, DNA methylation is acquired later in life during the oocyte growth phase and is completely re-established by metaphase II (reviewed by Denomme and Mann et al., 2012; Saitou et al., 2012). Oocytes enter the growth phase throughout the reproductive lifespan. In mice, the first oocytes can enter the growth phase shortly after birth whereas in human, the first oocytes do not enter the growth phase until adolescence at the time of puberty (Schultz and Wassarman, 1977; reviewed by Hunt et al., 2008). In mouse oocytes, the acquisition of methylation imprints is established asynchronously during the oocyte growth phase. Some genes gain DNA methylation in the earlier stages of oogenesis (eg. *Snrpn* and *Peg3*) while other genes are methylated closer to ovulation (eg. *Peg1*) (Lucifero et al., 2004; Hiura et al., 2006).

1.1.3.2 Epigenetic reprogramming during preimplantation

The second period of DNA methylation reprogramming takes place during the preimplantation period after fertilization in the early embryo (reviewed by Messerschmidt et al., 2014). Global DNA methylation dynamics during the preimplantation period for both humans and mouse are similar (Smith et al., 2014; Santos et al., 2002). During this second wave of reprogramming, there is a genome-wide demethylation and re-methylation with the exception of imprinted genes and certain repeat elements, which are not altered (Brandeis et al., 1993; Lane et al., 2003). In both human and mouse, the paternal genome is actively demethylated within hours of fertilization while the maternal genome is passively demethylated after the two-cell stage; remethylation occurs at the time of embryo implantation in the uterus (Haaf et al., 2006).

1.1.3.3 Imprinted genes

Imprinted genes are genes that are expressed in a parent-of-origin manner, where one parental copy of the gene is expressed while the other copy is silenced (Barlow and Bartolomei., 2014). Mice have approximately 150 identified imprinted genes and the vast majority are found in clusters (Barlow and Bartolomei., 2014). Clusters of imprinted genes are located on several chromosomes (Peters and Beechey., 2003). Imprinted gene clusters contain an imprinting control region (ICR) which inherits DNA methylation imprints from a parental gamete (Wan and Bartolomei, 2008; Barlow and Bartolomei., 2014). *H19* is an example of a paternally methylated gene while *Snrpn*, *Kcnq1ot1*, and *Peg1* are maternally methylated, which are all located on the chromosome 7 cluster (Reese et al., 2007; Shemer et al., 1997; Mitsuya et al., 1999; Lefebvre et al., 1997).

Imprinted genes acquire their methylation during gametogenesis and this methylation is maintained throughout development (Surani et al., 1984). The methylation statuses of imprinted genes are unaltered during the second wave of reprogramming due to the activity of an epigenetic modifier complex formed by ZPF57 (Quenneville et al., 2011) and TRIM28 (Alexander et al., 2015). The DNA methyltransferase, DNMT1, is responsible for maintaining DNA methylation through mitosis during embryo development (Liu et al., 2013). Maintaining DNA methylation of imprinted genes is crucial for proper embryo development. Embryos with two maternal or paternal genomes resulted in abnormal embryo development and both types of embryos were not able to survive to term (Surani et al., 1984; McGrath and Solter., 1984).

1.2 Advantage of animal models to study assisted reproduction

Animal models are a great tool to assess the impacts of ART on the offspring more accurately, examining the impacts on birth and epigenetic defects. Animal models allow researchers to control for many confounding factors that can influence human ART studies such as diet, environment, and underlying infertility.

1.2.1 ART and embryonic development

Superovulation and its effects on oocyte quality and embryonic development have been extensively studied in animal models. The way superovulation affects embryo development is two-fold: it directly impacts the oocyte/embryo quality and alters the uterine environment (Ertzeid and Storeng, 2001). In 1987, Young and colleagues demonstrated that oocytes collected from rats superovulated with two doses of pregnant mare serum gonadotropin (PMSG) had higher percentages of oocytes with abnormal morphologies in a dose dependent response (Yun et al., 1987). Van Der Auwera et al. (2001) looked at the blastocysts and embryos at midgestation and found that superovulation resulted in delayed embryonic development, an increased number of abnormal blastocysts, fetal growth retardation, and an increased number of resorption sites (Van Der Auwera et al., 2001). Superovulation also disrupted DNA methylation in both the maternal and paternal pronucleus of zygotes and resulted in delays in the onset of zygotic DNA replication (Diken et al., 2018). It has recently been shown that an altered uterine environment caused by superovulation, and not fresh or frozen embryos, resulted in pups that were small for their gestational age (Weinerman et al., 2017). Superovulation also has been shown to impact trophoblast differentiation, expansion, and invasion, resulting in impaired embryo and placental development (Mainigi et al., 2014).

In vitro culture was shown to impact the developmental capacity of the embryos in comparison to embryos grown in utero, but not to the extent of a superovulation-stimulated oviductal environment (Van der Auwera et al., 1999).

Cumulative effects of ARTs have been shown on placental development and the live pups conceived. ART has been linked with higher levels of placental inflammation, with higher levels of pro-inflammatory cytokines, and oxidative stress (Raunig et al., 2011). In addition, placentas from embryos conceived using ART resulted in overgrowth and led to abnormal placentation, negatively impacting embryo growth and weight (Chen et al., 2015).

1.2.2 Alterations in epigenetics associated with ART in animal models

ARTs are performed during many crucial stages of germ cell and preimplantation development, which also overlap with the major waves of DNA methylation programming. Therefore, ARTs may cause alterations in epigenetics (Denomme and Mann, 2012), which can contribute to the negative outcomes associated with ARTs.

1.2.2.1 Superovulation

Superovulation, an ART which induces the ovulation of multiple oocytes by administering exogenous hormones, coincides with female germ cell development (Behringer et al., 2013). In murine models, superovulation is performed through intraperitoneal (IP) injection of PMSG, which is analogous to follicle-stimulating hormone and promotes follicular growth and maturation. Exogenous human chorionic gonadotropin (hCG) is administered in the same manner 46 to 48 hours after the PMSG administration but before the release of endogenous luteinizing hormone

(LH) to maximize oocyte yield. The hCG injection mimics the effect of the LH and stimulates ovulation (Behringer et al., 2013).

Oocytes acquire imprints only during the growth phase as discussed above. Different oocytes enter the growth phase at different times throughout reproductive life, and with every growth phase, the imprinting for that oocyte is established. Multiple studies have shown that superovulation can alter epigenetic patterning. Superovulation allows for the ovulation of oocytes that would have not been normally ovulated due to incomplete epigenetic maturation (Huo et al., 2020). Superovulation resulted in a disruption of methylation of *Snrpn*, *Peg3*, *Kcnq1ot1*, and *H19* imprinted loci in blastocysts in a dose-dependent manner, higher doses resulted in a greater disruption of methylation (Market-Velker et al., 2010). The expression of DNA methyltransferases (DNMT), the enzymes involved in methylation of DNA, was also altered in oocytes and early embryos conceived after superovulation (Uysal et al., 2018). Alterations in methylation of *Peg1* and *Snrpn*, both maternally methylated, imprinted loci have been shown to persist in germ cells of male offspring until the second generation (Stouder et al., 2009). Disruptions in DNA methylation persists later in development. Our group determined that placentas from 9.5 days post coitum (dpc) embryos conceived after superovulation had a greater proportion of placentas with biallelic expression of *Snrpn* and *H19* compared to those conceived naturally (Fortier et al., 2008). A follow up study revealed that the altered imprinted gene expression was normalized later in gestation (Fortier et al., 2014). Therefore, the effect of superovulation on genomic imprinting are established as early as during gametogenesis and can persist into later stages of development.

Females undergo superovulation, therefore, alterations of imprinted loci in oocytes due to superovulation can occur during imprint acquisition and maintenance during preimplantation. From the studies presented above, paternally methylated imprinted loci are also affected by

superovulation. The impact on both maternally and paternally methylated imprinted loci are most likely due to disruptions in DNA methylation maintenance during preimplantation (Denomme et al., 2011; Market-Velker et al., 2010).

1.2.2.2 *In vitro* fertilization and embryo culture

During IVF, oocytes and sperm are transferred into culture to promote fertilization. The resulting embryos are commonly cultured *in vitro* until the blastocyst stage, before they are transferred to the uterus. The manipulations of IVF, *in vitro* fertilization and embryo culture, overlap with crucial windows of development from fertilization and until preimplantation.

IVF alone is known to cause alterations in *H19* methylation in blastocysts (Fauque et al., 2007). Some changes in *H19* methylation still persisted in IVF conceived mice at birth, 3 weeks, and 1.5 years of age (Le et al., 2013). Similar to IVF, imprinted expression and methylation of *H19* was also affected by the type of culture medium the embryos were cultured in (Khosla et al., 2001; Doherty et al., 2000). Market-Velker et al (2012) observed the effects of different types of commercial culture media in blastocyst-stage embryos; all culture media resulted in aberrant DNA methylation at imprinted regions and their relative expression (Market-Velker et al., 2012). It has also been reported that the culture medium induced loss of DNA methylation at imprinted loci and aberrant allelic expression of *H19* and *Snrpn* persisted post-implantation in midgestation placental tissues (Mann et al., 2004). Another study by de Waal et al. (2014) supports that placenta from IVF embryos presented an increased frequency of abnormal DNA methylation and imprinted gene expression compared to the embryo (de Waal et al., 2014).

1.2.2.3 Cumulative effect of ARTs

Multiple studies have demonstrated that individual ARTs negatively impact the methylation state of imprinted genes. Epigenetic alterations in the blastocysts caused by embryo culture, however, can be exacerbated by the addition of superovulation (Market-Velker et al., 2012). Imprinting defects seen in midgestation embryos caused by superovulation and embryo transfer were aggravated with the addition of embryo culture to the ART protocol (Rivera et al., 2008). More recently, in 18.5 dpc embryos conceived with superovulation alone and in combination with embryo transfer alterations in the epigenomes of the placentas were not seen, but the addition of *in vitro* fertilization and embryo culture resulted in hypomethylation at imprint control regions and abnormal imprinted gene expression from the normally repressed allele (de Waal et al., 2015).

1.3 Methods to prevent epigenetic defects associated with ART

1.3.1 Folic acid and neural tube defects

Neural tube defects (NTDs) and other major birth defects are present in up to 5% of children born in Canada (Andres et al., 2008). NTDs are congenital abnormalities that result from the improper closure of the neural tube during early embryonic development (Campbell et al., 1990). The clinical types of NTDs depends on the site at which the neural tube closed improperly (Van Allen et al., 1993; Sakai et al., 1989). Many studies have shown that environmental factors, such as chemical exposure and maternal nutrition, can influence the prevalence of NTDs (Sadler et al., 2002; Holmes et al., 2001; Kaneko et al., 1999). Links between maternal nutrition and the prevalence of NTDs are currently unknown, however, the one carbon metabolism pathway is thought to be a key factor.

One carbon metabolism is highly important for producing methyl groups that are required for proper acquisition and maintenance of methylation marks. A key component of this pathway is folate, a source of one-carbon methyl units. Folate is an essential B vitamin that is naturally found in leafy green vegetables, broccoli, spinach, lentils, and many more (Wilson et al., 2015). Humans do not have the ability to synthesize folate and must consume it through dietary means or through supplementation of its synthetic counterpart, folic acid. (Mitchell et al., 2004).

1.3.1.1 Folic acid and pregnancy outcomes using animal models

Animal models have been extensively used to study the effects of folic acid supplementation. Folic acid supplementation has been shown to have beneficial effects for multiple embryonic outcomes. The prevalence of NTDs in a murine model was significantly lower with maternal folic acid supplementation during gestation (Shin et al., 1999; Zhao et al., 2014). Embryos lacking the reduced folate carrier, which transports 5-methyltetrahydrofolate to mammalian cells, on their own died in utero midgestation; however, maternal folic acid supplementation was able to rescue the embryos and aided development until full term (Zhao et al., 2001).

Folic acid supplementation has been shown to benefit embryo development and reduce the risk of NTDs in the offspring. Studies are still being conducted to determine the optimal dose of folic acid supplementation. A folic acid-supplemented diet containing 20-fold and 10-fold higher than recommended intake, fed to mice, was associated with embryonic loss, embryonic developmental delays, placental defects, and altered heart development (Mikael et al., 2012; Pickell et al., 2010). Detrimental effects of 20-fold maternal high dose folic acid supplementation included altered behaviour in offspring and impaired reversal learning (Henzel et al., 2017).

Maternal folic acid deficiency on the other hand resulted in reduced plasma and red blood cell (RBC) folate levels, abnormal reproductive outcomes, and embryonic delays in the palate and heart (Burgoon et al., 2002). Embryos of mothers with maternal folic acid deficiency had an increased number of apoptotic cells and a decreased number of progenitor cells in the ventricular zones of the brain of (Craciunescu et al., 2004). The negative outcomes from both high dose folic acid supplementation and deficiency add importance to determining optimal folic acid supplementation.

1.3.1.2 Folic acid and DNA methylation

Folates are crucial in methylation reactions as they are involved in the production of S-adenosyl methionine (SAM), the universal methyl donor (Anderson et al., 2012). When folic acid enters the cell, it is converted to dihydrofolate (DHF) then is used to synthesize tetrahydrofolate (THF). THF serves as a single carbon donor and is enzymatically converted to 5,10-methylene-THF. The enzyme, methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylene-THF into the major circulatory folate, 5,10-methyl THF. This product transfers its methyl group to homocysteine, forming methionine, which is converted to SAM (Figure 2). The transfer of the methyl group from SAM to the 5' carbon of a cytosine residue at a CpG dinucleotide is catalyzed by DNMTs (Anderson et al., 2012).

Alterations in the availability of methyl donors, such a folate, can lead to an imbalance the production of SAM, affecting the ability to properly acquire and maintain DNA methylation, which is crucial for embryo development (Smith et al., 2013).

1.3.2 Granulocyte-macrophage colony stimulating factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a growth factor that is secreted by the uterus in both murine and human during preimplantation development (Robertson et al., 1992). The presence of GM-CSF during embryo development is crucial for proper development and warrants investigation in the context of ART.

1.3.2.1 Role in embryo and placental development

GM-CSF is a key regulator of the immune response in the uterine endometrium. The presence of antigens in semen triggers an inflammatory response, resulting in the expression of GM-CSF in the endometrial stroma and myometrium of the uterus (Robertson et al., 2000). Insufficient GM-CSF has been shown to impair the generation of T-cell mediated immune tolerance and dendritic cell profiles, which is evident in infertility, miscarriage, and preeclampsia (Moldenhauer et al., 2010; Askelund et al., 2004; Miwa et al., 2005; Scholz et al., 2008)

GM-CSF plays a crucial role in both embryo and placental development. Blastocysts from GM-CSF null mutants showed a reduction in total cell number when compared to blastocysts from GM-CSF wild-type mice (Robertson et al., 2001). When embryos from the GM-CSF null mutants were cultured *in vitro* in media containing GM-CSF, there was an increase in total cell number (Robertson et al., 2001). GM-CSF is part of a vast network of cytokines and growth factors that all interact to regulate the development of the placenta. A lack of GM-CSF altered the morphogenesis of the placenta, with visible defects in both the labyrinthine and junctional zone layers (Robertson et al., 1999). GM-CSF in combination with other factors drive an essential pathway that regulates trophoblast cell differentiation and the invasion of maternal tissues and blood vessels, which are crucial for proper nutrient and waste exchange (Morrish et al., 1998).

1.3.2.2 DNA methylation and GM-CSF

As mentioned previously, ART is known to be associated with epigenetic alterations. Our lab has shown that not only does ART alter DNA methylation, the altered DNA methylation (predominantly hypomethylation) was associated with a significant female bias (Rahimi et al., 2019). Females require more methyl groups because females require inactivation of an X chromosome through DNA methylation for proper development (McGraw et al., 2013). DNA hypomethylation, such as that seen with ART, can result in an increased expression of PRAMEL7 which ubiquitinates UHRF1, a key mediator in DNA methylation. The lower levels of active UHRF1 lead to altered DNA methylation maintenance and negative downstream effects (Bostick et al., 2007; Graf et al., 2017). GM-CSF stimulates upstream of the MAPK signaling pathway by inducing the phosphorylation of STAT3 and STAT5, resulting in the activity of UHRF1 and proper DNA methylation maintenance even in the presence of PRAMEL7 (Al-Shami et al., 1998). Thus it is possible that GM-CSF could reverse some of the DNA hypomethylation seen in ART studies.

1.3.2.3 Effects of GM-CSF examined in *in vitro* studies

A number of studies have looked at the effect of GM-CSF containing culture media on both murine and human *in vitro* embryo development. When two-cell embryos from GM-CSF null mutant females were cultured in media containing GM-CSF *in vitro*, the resulting blastocysts had an increase in total cell number (Robertson et al., 2001). GM-CSF was also seen to promote cell proliferation in the inner cell mass (ICM) and trophectoderm (TE) and suppressed stress response gene expression (Chin et al., 2009).

Like the results seen in the mouse model, human embryos grown in culture media containing GM-CSF resulted in an increased number of TE and ICM cells (Sjoblom et al., 1999). In a more recent randomized human clinical trial, embryos cultured in media containing GM-CSF and low concentrations of HSA had a significantly increased implantation and survival rate of transferred embryos (Ziebe et al., 2013). The commonality in the studies described above is that the beneficial effects of GM-CSF were seen when GM-CSF was present at 2ng/mL in the culture media (Robertson et al., 2001; Chin et al., 2009; Ziebe et al. 2013). The beneficial effects of GM-CSF in *in vitro* development, however, is still controversial within the literature. For instance, Elaimi et al. (2012) showed that low concentrations of GM-CSF (1-2ng/mL) did not affect blastulation potential or cell counts, but high concentrations of GM-CSF (5-10ng/mL) decreased blastulation rates and cell counts (Elaimi et al., 2012). No studies looking at the effects of GM-CSF in culture media have examined whether there are possible beneficial effects on DNA methylation profiles in cultured embryos.

1.4 Effects associated with ART and maternal folic acid supplementation

Many studies mentioned in sections 1.1 and 1.2 demonstrate that ART is associated with aberrant DNA methylation. Our lab has recently examined the effects of ART and maternal folic acid supplementation on embryonic development and DNA methylation (Rahimi et al., 2019). A combination of ARTs increased the rate of embryonic delay and altered DNA methylation in the four imprinting control regions (ICR) observed. CF1 females in the naturally mating group were consuming a control diet containing the recommended folic acid level for rodents. Unexpectedly, the baseline embryonic abnormality rate (31.3%) was high compared to an average embryonic

abnormality rate (6.9%) of CF1 females on a chow diet (6 mg folic acid/kg diet) (Reeves et al., 1997; Rahimi et al., 2019).

In the Rahimi study, ART did significantly increase the rate of embryonic delay but did not affect the rate of embryonic abnormalities. To determine if ART led to an increase in embryonic abnormalities, numbers of morphological abnormalities were compared between the ART and naturally cycling groups (Rahimi et al., 2019). The baseline embryonic abnormality rate of the naturally cycling group was high and the change in embryonic abnormality rate due to ART was difficult to see and would have required a much larger sample size. This result led to a crucial unanswered question: whether the control diet (containing 2 mg folic acid/kg diet) contained enough folic acid for the naturally cycling CF1 mice used in the study.

Along with changes in embryonic outcomes, ART also affected DNA methylation. Changes in DNA methylation (predominantly hypomethylation) affected the placentas more drastically than the embryos (Rahimi et al., 2019). In addition, ART was associated with a significant female bias, where more female embryos had methylation defects at the *Kcnq1ot1*, *Snrpn*, and *H19* ICRs compared to male embryos (Rahimi et al., 2019). Interestingly, a moderate periconceptional folic acid supplementation of the females undergoing ARTs reduced the proportion of developmentally delayed embryos and the DNA methylation variance for *Kcnq1ot1* and *H19* ICRs in the embryos (Rahimi et al., 2019). Although this study answered many questions on the effects of ARTs on DNA methylation and potential rescuing with folic acid supplementation, there was still another question that needed to be answered: is there something that can be done in combination with maternal folic acid supplementation that can increase the level of rescue for altered DNA methylation caused by ARTs?

Rationale and objectives

Our lab has looked at the effects of ART on embryonic development and epigenetic alterations. Naturally mated CF1 females on a regular chow diet (6 mg folic acid/kg of diet) have an average rate of 6.9% for embryonic abnormalities, while our CF1 females on the control folic acid-supplemented diet had a rate of 31.3% (Cebral et al., 2007; Rahimi et al., 2019). The higher rate of abnormalities seen by Rahimi et al. (2019) could have been the result of the control folic acid-supplemented diet (CD - 2 mg folic acid/kg of diet) not containing enough folic acid for the CF1 strain (Rahimi et al., 2019). To determine if the control diet is sufficient for naturally mating CF1 females, we compared the pregnancy outcomes of naturally mated mice on CD or 4-fold folic acid-supplemented diet (4FASD).

We hypothesize that firstly, moderate maternal folic acid supplementation in naturally cycling mice will reduce the baseline rate of morphologically abnormal embryos without altering DNA methylation. To test the first hypothesis, in aim 1, naturally cycling mice were fed a CD or a 4FASD. Pregnancy outcomes, embryo morphology and staging, were examined.

To assess the effect of ART and folic acid supplementation on epigenetic alterations, our lab recently looked at the ICRs of 4 imprinted genes (Rahimi et al., 2019). To understand the effects associated with ART for a greater number of genes, DNA methylation of both imprinted genes (differing from those already analyzed) and non-imprinted genes was analyzed.

We hypothesize that secondly, moderate maternal folic acid supplementation can prevent epigenetic alterations caused by ARTs in both imprinted and non-imprinted genes. To test the second hypothesis, in aim 2, a representative sample subset consisting of the placentas of naturally conceived morphologically normal and abnormal female embryos conceived using ARTs from Rahimi et al. (2019) were examined (Rahimi et al., 2019). DNA methylation was analyzed for a

non-imprinted gene *Casz1*, which was shown to have altered gene expression in a folic acid deficiency model, and the imprinted genes *Gnas* and *Nnat*, which were shown to have altered DNA methylation in response to ART (Carroll et al., 2012; Rahimi et al., 2019; Choufani et al., 2019).

Both doses of folic acid supplementation were not able to rescue the DNA methylation alterations completely (Rahimi et al., 2019). Due to the beneficial effects of embryo culture in media containing GM-CSF, a combination of moderate folic acid supplementation and an ART protocol containing GM-CSF has the potential to increase the level of rescue seen with folic acid supplementation alone.

We hypothesize thirdly, a combination of moderate maternal folic acid supplementation and GM-CSF in the embryo culture media can further counter the effects associated with ART. In aim 3, a new ART model was developed to look at the effects of moderate maternal folic acid supplementation and embryo culture containing GM-CSF on embryo outcomes. The ART protocol consisted of superovulation, *in vitro* fertilization, embryo culture to blastocyst, and embryo transfer. The modified ART protocol could not be pursued likely due to issues with the NSET or mineral oil.

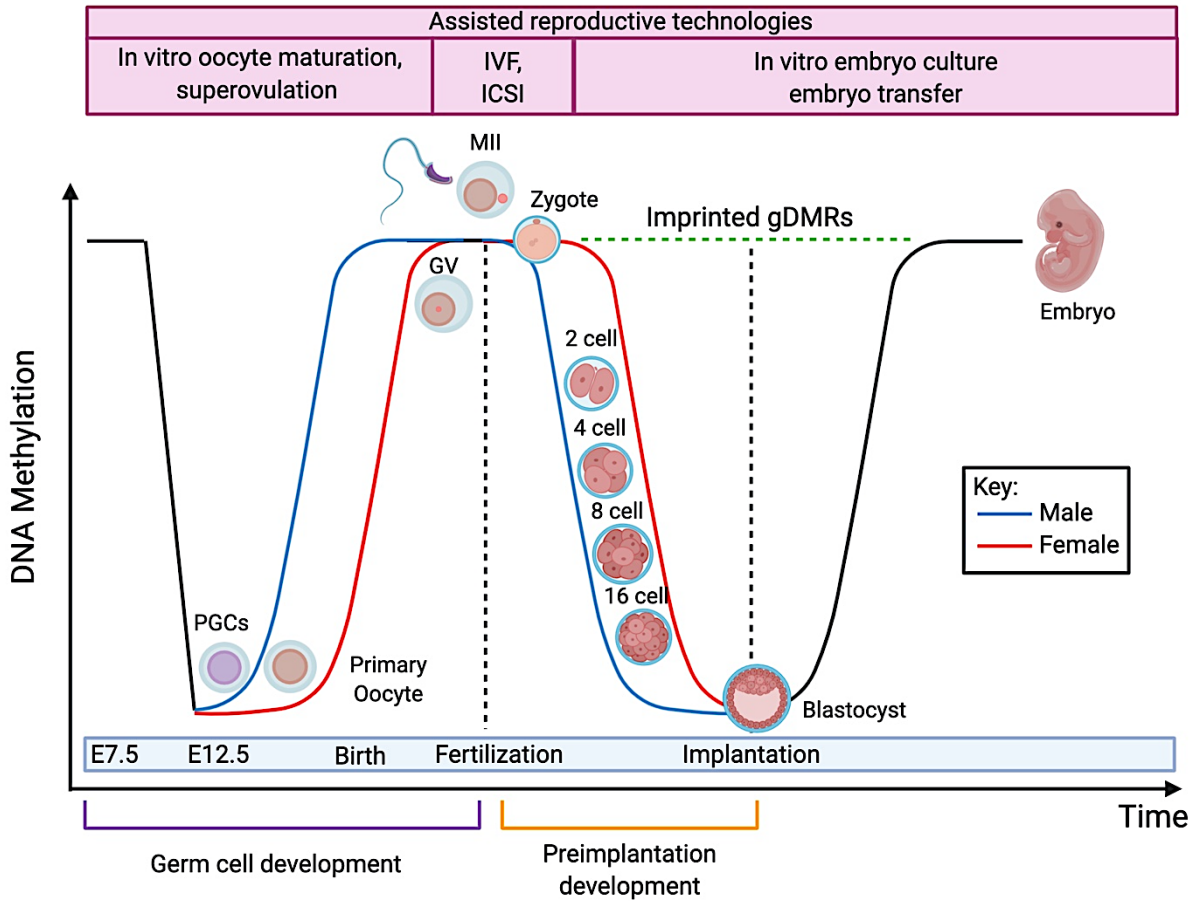


Figure 1. Overlap of ARTs and DNA methylation programming during gametogenesis and embryo development. The timing of ART procedures overlaps with the dynamic waves of DNA methylation reprogramming during germ cell and early embryo development. GV, germinal vesicle; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; MII, metaphase II, PGCs, primordial germ cells.

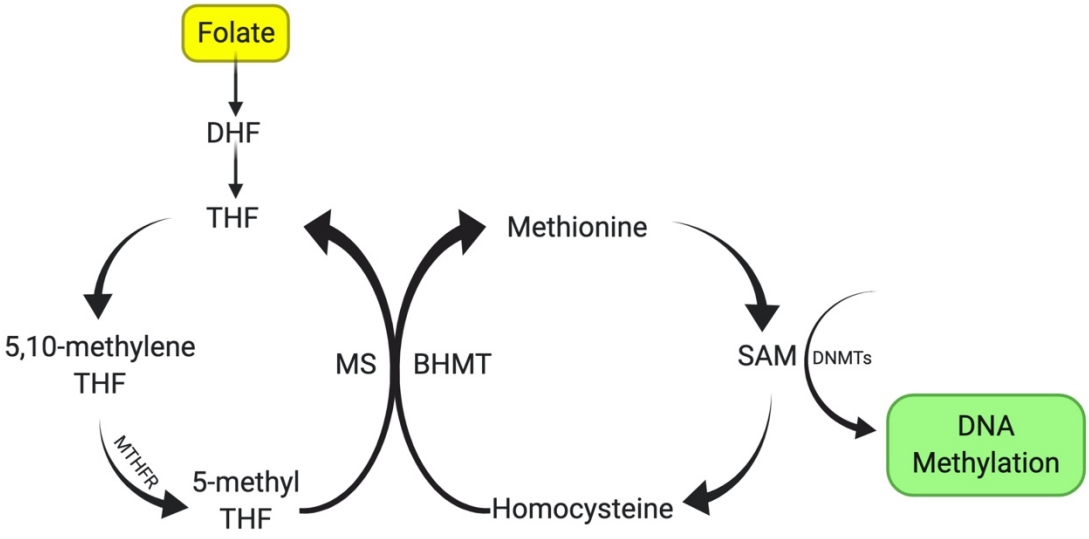


Figure 2. One carbon metabolism cycle. The one carbon metabolism pathway is required for DNA methylation. Disruptions in the pathway could lead to alterations in S-adenosyl methionine (SAM) availability and affect downstream DNA methylation. Folates are methyl donors and are essential for the proper establishment of DNA methylation patterns. BHMT, betaine-homocysteine methyltransferase; DHF, dihydrofolate; DNMTs, DNA methyltrasnferases; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

Chapter 2: Materials and Methods

2.1 Animals and diets

All animal experiments complied with the guidelines set by the Canadian Council on Animal Care and the experimental protocol was approved by the Animal Care Committee at the Research Institute of the McGill University Health Centre (RI-MUHC). All mice were housed in a sterile animal facility at the RI-MUHC in Montreal, Quebec. Mice were placed on a 12-hour light:12-hour dark cycle with free access to food and water.

Mice in aim 1 and aim 3 were Non-Swiss Albino-Carworth Farms-1 (NSA™(CF1), herein after referred to as CF1; Charles River Canada) females and were fed one of two amino-acid defined diets (Envigo Teklad): a folic acid control diet (CD, 2 mg/kg diet, TD.130565) containing the recommended level of folic acid for rodents (Reeves, 1997), or a 4-fold folic acid-supplemented diet (4FASD, 8 mg/kg diet, TD.160058). Mice in aim 2 were Non-Swiss Albino-Carworth Farms-1 (NSA™(CF1), herein after referred to as CF1; Harlan Laboratories) and were fed one of three amino-acid defined diets (Envigo Teklad): a folic acid control diet, 4-fold folic acid-supplemented diet, or a 10-fold folic acid-supplemented diet (10FASD, 20mg/kg diet, TD160059). Diets were irradiated and therefore, the initial formula contained 50% more folic acid to account for the amount that would be lost during the irradiation process. Females were fed either diet for 6 weeks prior to natural mating or assisted reproduction. Diets were continued throughout gestation until the day of embryo collections. B6SJLF1/J males (Jackson Laboratory) and CD1 vasectomized males (Charles River Canada) were fed regular mouse chow diet (6 mg folic acid/kg diet).

Natural mating females were exposed to B6SJLF1/J male bedding for two consecutive days prior to mating. All females were mated with males for only one night. After one night of mating,

vaginal plugs were not always visible. The morning after females were placed in the male cages was deemed E0.5 whether a vaginal plug was observed or not. Pregnancy was not confirmed using a vaginal smear due to the possibility of females becoming pseudo-pregnant (Cora et al., 2015). At 10.5 dpc, naturally conceived embryos and placentas were collected.

2.2 Tissue collection and DNA isolation

Midgestation embryos obtained by natural mating were staged according to previously published criteria (Theiler, 1972; Kaufman, 1992). Crown-rump lengths (length of the embryo) were measured during collection. Embryos were also assessed for morphological abnormalities including neural tube defects, craniofacial malformations, anterior posterior discordance, heart defects, and vascularity. Embryos obtained by natural mating delayed by 1.5 days or more (staged E9.0 or less) were considered developmentally delayed. Placentas were isolated and their widths, lengths, and total areas were measured. Placentas were also assessed for vascularity, colour, and relative thickness. Yolk sacs were also collected to allow for determination of the embryo sex. All samples were snap frozen in dry ice at the time of collection. Embryos and placentas were stored at -80ºC and yolk sacs at -20ºC until further use.

Placentas collected previously by Rahimi (2019) were used in aim 2 (Rahimi et al., 2019). For the natural mating group, placentas from 6 morphologically normal and not delayed samples were used. For the ART groups, placentas from 10 morphologically normal and 2 morphologically abnormal samples were used.

Frozen embryonic or placental tissue was homogenized in frozen tissue lysis buffer (Qiagen, Buffer ATL) using mortars and pestles kept on dry ice. Genomic DNA was isolated using Qiagen DNease Blood & Tissue kit (Qiagen, cat. #69506) as per manufacturer's protocol.

2.3 PCR sexing of embryos

Two different primer sets for two separate PCR reactions were used to perform a multiplex PCR on yolk sac DNA to determine the sex of each embryo. The first reaction primer set required primers for *Zfy1* (5'AAGATAAGCTTACATAATCACATGGA3' and 5'CCTATGAAA-TCCTTTGCTGCACATGT3') and primers for *Myog* (5'TTACGTCCATCGTGGACAGC3' and 5'TGGGCTGGGTGTTA-GTCTTA3') (Nagamine et al., 1989; McClive and Sinclair, 2001). The second reaction set, used to confirm the results obtained using the first PCR reaction, required primers for *Ddx3* (5'CCGATCTAGAGTCCGTCCTT3' and 5'TCCTTAGGAAAAGTAGCAC-TAAACA 3').

2.4 DNA methylation analysis

Genomic DNA (1ug) isolated from midgestation placentas was bisulfite treated using the Qiagen EpiTect Bisulfite kit (Qiagen cat. #59104). DNA methylation was measured at the DMRs of one non-imprinted gene (*Casz1*) and two imprinted genes (*Gnas* and *Nnat*) using bisulfite pyrosequencing as previously described (Whidden et al., 2016). Primer sequences for all assays are presented in Table 1. DNA methylation for *Gnas* and *Nnat* were each analyzed at two DMRs. For *Gnas*, assay 1 targeted the region more upstream (Figure 9B) and assay 2 targeted the region downstream (Figure 9C). For *Nnat*, assay 1 targeted the region within the gDMR (Figure 10B) and assay 2 targeted the region upstream of the gDMR (Figure 10C).

2.5 ART protocol

The ART protocol is adapted from the Jackson Laboratory method (Byers et al., 2006; Behringer et al., 2013; Rahimi et al., 2019). Nine-week-old CF1 females underwent superovulation

by intraperitoneal (IP) injections of 6.0-10.0 IU pregnant mare serum gonadotropin (PMSG, Cedarlane cat. CLHOR272-2) followed by 6.0-10.0 IU human chorionic gonadotropin (hCG, EMD Millipore cat. 230734-1MG). Hormone injections were given 48 hours apart.

Spermatozoa of B6SJLF1/J males (12 to 22 weeks of age) were isolated from the cauda epididymides and vasa deferentia in a drop of EmbryoMax® human tubal fluid (HTF; EMD Millipore MR-070-D) media covered in a layer of embryo tested mineral oil (Sigma) 12 hours after hCG injection. After 90 minutes of sperm capacitation in the incubator (37ºC, 5% CO2, 20% O2). HTF containing sperm were added to a new drop of HTF, achieving a final concentration of $2.5x10^6$ sperm/mL.

After superovulated CF1 females were sacrificed 13.5 hours after the hCG injection, cumulus-oocyte complexes (COCs) were released from both ampullae with a 30Gx1/2 needle under mineral oil and dragged into the drop of HTF media containing the capacitated spermatozoa. This method of dragging the COCs under the mineral oil was from the Cosmo Bio IVF protocol (Cosmo Bio, 2017). Fertilization proceeded for 6 hours at 37ºC in a reduced oxygen environment (5% CO_2, 5% O_2, 90% N_2). Remaining spermatozoa and cumulus cells were washed away from the zygotes in HTF media then transferred to a clean drop of EmbryoMax® potassium simplex optimization medium (1X) with ½ amino acids (KSOM½AA; EMD Millipore MR-106-D) with or without 2ng/mL of GM-CSF under embryo tested mineral oil. The washed zygotes were cultured at 37ºC in a reduced oxygen environment (5% CO_2, 5% O_2, 90% N_2) for 4 days.

After 4 days of embryo culture, 10 blastocyst-staged embryos (all from a single CF1 donor female) were transferred to a 2.5 dpc pseudo-pregnant CF1 recipient female using the Non-Surgical Embryo Transfer (NSET) device (ParaTechs). The candidate performed embryo transfers using the NSET alongside an experienced research associate. Roughly half of the transfers were

done by the candidate and the other half by the research associate. Pseudo-pregnant females were inferred if a vaginal plug was observed after they were mated with a vasectomized CD1 male (Charles River Canada) for one night. If a female produced more than 10 blastocysts, more than one recipient could receive blastocysts from a single female. Transferring blastocysts from a single female to more than one recipient was rare and only occurred in three out of the thirteen trials (indicated on Table 4 with an *). The day of embryo transfer was marked E3.5 and midgestation embryos and placentas were collected 8 days later (details in section 2.2). Pressure fluctuations during the embryo transfer alone can cause morphological changes, embryo development delay, and implantation delay (Grygoruk et al., 2012; Grygoruk et al., 2011; Behringer et al., 2013). Manipulated E3.5 blastocysts are transferred to the uterus of a 2.5 dpc pseudopregnant recipient because the developmental and implantation delay caused by the embryo transfer results in a loss of a day in embryo development. This asynchronous transfer (E3.5 blastocysts to a 2.5 pseudopregnant female) produces embryos with the same developmental stage as the pseudopregnant female (gestational day 10.5) (Behringer et al., 2013). Delayed implantation has been proven by multiple studies since the 1950s and is now a convention in modern mouse ART protocols (Behringer et al., 2013; Tarkowski., 1958; McLaren and Michie., 1956)

2.6 Blood collection

Right before COCs were collected through c-section, CF1 females were under isoflurane and 500μl of whole blood was collected by cardiac puncture. Whole blood was transferred into Microvette® 500µl, K3 EDTA (Sarstedt 20.1341.100) tubes and kept on ice. Within 1 hour of

collection, whole blood samples were fractionated by centrifugation, separating both the plasma and the red blood cells (RBC). Both plasma and RBC samples were stored at -80ºC

2.7 Statistical analysis

Data were analyzed using GraphPad Prism 8 (GraphPad Software). Data are presented as mean ± SEM or absolute values, as indicated. For aim 1, comparison of means between CD and 4FASD groups was performed using an unpaired student's t-test. Absolute value data were compared using Fisher's exact test. For aim 2 comparison of means between NAT-CD and CD-ART groups was performed using an unpaired student's t-test. Comparisons of means between CD-ART, 4FASD, and 10FASD were compared by one-way ANOVA with Tukey's multiple comparisons test. Methylation variance at each region of analysis was determined by averaging individual variances at all CpGs within diet groups. $P<0.05$ was considered significant for all analyses.

Table 1. Primers for bisulfite pyrosequencing used to assess DNA methylation in mice.

ICR	# CpGs analyzed	Primer sequences	Reference
Kcnq1ot1	4	Fwd: 5'-AGGTTTTGGTAGGTGGTTT Rev: 5'-biot CCTAACTAAACCAAAATACACCATCATA Seq: 5'-GTTAGGAGGAATAGTTGTTTTA Amplification location: Chr7:143,296,460-143,296,581	de Waal et al., 2014
Casz1	5	Fwd: 5'-GTTTTTTTTGGGAGGTTTGAGAA Rev: 5'-biot TCCAACTCTTAAAACATCACTTCTCTTATA Seq: 5'-GAGGAGGTGGATGGT Amplification location: Chr4:148,734,845-148,734,986	
Gnas (1)	5	Fwd: 5'-TGATGAGTAGGAAGAAGGGGTTA Rev: 5'-biot CCTCTCCTCCATAAAATCCTTAAC Seq: 5'-GTTATAGGTTGTTTTTAGTGGTT Amplification location: Chr2:174,299,873-174,300,081	
Gnas (2)	5	Fwd: 5'-GAGGGGTTTTTTGGAGAGT Rev: 5'-biot CCACCCCAACAATTCTAAACTCA Seq: 5'-ATGTTTTTTAGGGAGAAAAG Amplification location: Chr2:174,300,383-174,300,549	
Nnat gDMR	5	Fwd: 5'-biot AGGTGGTAAGAGGGTATTTAAGG Rev: 5'-ACCCAAAACCCCAATACATACTCACCT Seq: 5'-AAACCCCAATACATACTCACCTA Amplification location: Chr2:157,560,065-157,560,285	
Nnat (2)	3	Fwd: 5'-biot GAAGGGAAAAGTGTTGGGTATA Rev: 5'-CTCAACTACCCTTCCCTCCTCTCTAC Seq: 5'-CCTCTCTACCAAAAACATATTAAAT Amplification location: Chr2:157,506,992-157,57,135	

Fwd, forward primer; Rev, reverse primer; Seq, sequencing primer

Chapter 3: Results

3.1 Effect of folic acid supplementation on naturally cycling mice

3.1.1 Effect of folic acid supplementation on maternal body weight

Maternal body weight was measured the day of mating. Body weight is a good indicator of the overall health status of the animal. The body weights of all female mice mated in the 4FASD group were significantly greater than the mice in the CD group ($p<0.001$, Figure 3A). Comparing the body weights of only the females with embryos collected, the weights of the mice in the 4FASD group were also significantly greater than the mice in the CD group ($p<0.01$, Figure 3B).

3.1.2 Effect of folic acid supplementation on pregnancy outcomes

To determine the effects of different concentrations of folic acid supplementation on pregnancy outcomes, mice were fed either the CD or 4FASD diets and underwent natural mating. Detailed information on all collected litters from both CD and 4FASD groups can be found in Tables 2-3. The number of ovulated oocytes for both diet groups were not significantly different (Figure 4A). The mean number of implantation sites for both diet groups did not differ, but the distribution was statistically different ($p<0.05$, Figure 4B). All groups demonstrated similar rates of pre- and post-implantation losses, embryo viability, and litter size (Figure 4C-F).

Next, morphological assessment of midgestation embryos was performed. Embryos were analyzed for presence or absence of neural tube defects, craniofacial malformations, laterality defects, anterior posterior discordance, and heart defects. The proportions of morphologically abnormal embryos per litter for both the CD (4.6%) and the 4FASD (4.8%) groups were similar (Figure 5A). The presence of morphologically abnormal embryos was not biased to a single male

used in the mating process and was evenly distributed between all the males (Figure 5C). The distribution of the types of morphological abnormalities for both diet groups was comparable (Figure 5E). These results indicate that folic acid supplementation did not impact the abnormality rate.

Along with morphological assessments, midgestation embryos were also staged within 0.25-day intervals to assess for developmental delays. Embryos with a 1.5-day delay or more (Staged E9.0 or less) were considered developmentally delayed. Both the CD (1.1%) and the 4FASD (0.8%) diet groups resulted in similar proportions of developmentally delayed embryos (Figure 5B). The presence of developmentally delayed embryos was evenly distributed between all the males used (Figure 5D). Embryo crown rump length for all three embryonic staging increments was not affected by folic acid supplementation (Figure 6A-C). Overall, the results indicate that folic acid supplementation at the dose used here did not affect embryonic developmental delays in naturally cycling mice.

3.2 Effect of folic acid supplementation on placental DNA methylation in pregnancies conceived by ART

To determine the effects of ARTs and folic acid supplementation on non-imprinted and imprinted genes, DNA methylation was assessed in a placenta sample subset. The sample subset consisted of placentas from both normal and morphologically abnormal but not developmentally delayed female embryos in a similar proportion to the original sample set used by Rahimi et al. (2019) (Figure 7A) (Rahimi et al., 2019). DNA methylation of *Kcnq1ot1* was assessed by bisulfite pyrosequencing on the sample subset of midgestation placentas (n=6 for NAT-CD, n=12 per ART group). The mean DNA methylation, distribution of the points, and variance for both the original

sample set and the sample subset were similar, concluding that the sample subset was representative of the original sample set (Figure 7B-C).

Casz1, a non-imprinted gene, has been previously shown to have altered expression levels in an *in vitro* folic acid deficiency model (Carroll et al., 2012). Four CpG sites located upstream of exon 1 were examined (Figure 8A). Both the mean DNA methylation and variance were not affected by ART or folic acid supplementation (Figure 8B).

Gnas and *Nnat*, both imprinted genes, were shown to be affected in an ART outlier group from human female placenta samples (Choufani et al., 2019). Dramatic findings were evident for the imprinted gene *Gnas*. Regions of *Gnas* consisting of 5 CpG sites both upstream and downstream of exon 1 were examined (Figure 9A). ART resulted in a significant decrease in mean DNA methylation (relative to the NAT-CD group, $p<0.0001$ Figure 9B, $p=0.0003$ Figure 9C) and an increase in variance for both regions of *Gnas* (relative to the NAT-CD group, $p=0.0452$ Figure 9B, $p=0.001$ Figure 9C). The 4FASD group resulted in an increase in variance, trending towards correction (relative to the CD-ART group, $p=0.0089$, Figure 9B). The mean DNA methylation of all ART groups were similar (Figure 9C). The 10FASD decreased the variance in the opposite direction of correction (relative to the CD-ART group, $p=0.0475$, Figure 9C).

For *Nnat*, 5 CpG sites within the germ line differentially methylated region (gDMR) and 3 CpGs located upstream of the gDMR were analyzed (Figure 10A). ART resulted in no change to the mean DNA methylation of the gDMR of *Nnat* but resulted in a significant increase in variance (relative to the NAT-CD group, $p=0.0302$, Figure 10B). The 10FASD group showed a decrease in variance (relative to the CD-ART group, $p=0.0099$) but showed a trend towards hypermethylation (Figure 10B). ART resulted in a small significant increase in only the mean DNA methylation in the region upstream, consisting of 3 CpG sites, of the gDMR and no change in variance (relative

to the CD-ART group, p=0.0234, Figure 10C). Both *Gnas* and *Nnat* had outliers with lower DNA methylation averages compared to the rest of the samples (Figure 9B-C, Figure 10B-C). Upon further investigation, the outliers from the DNA methylation analysis of *Gnas* (Figure 9B-C) were not the same as the outliers of *Nnat* (Figure 10B-C).

Overall, these results indicate that ART affects non-imprinted and imprinted genes differently, with imprinted genes affected more dramatically. Both doses of folic acid supplementation do not restore the aberrant placental methylation associated with ART. Comparing both doses of folic acid supplementation, the 4FASD seems to have a corrective effect while the 10FASD does the opposite.

3.3 Optimization of a new ART protocol

The ART protocol consisted of many steps that required optimization to achieve the best pregnancy outcomes. Prior to starting the newly designed ART protocol, all steps within the protocol needed to be optimized (Figure 11). To control for the success of the NSET transfers, the embryo transfers using the NSET were performed by both an experienced research associate and the candidate. In the final aim, we assessed the impacts of alterations of multiple aspects of the ART process by assessing the *in vitro* fertilization rate (IVF), *in vitro* developmental rate (IVD), and the development of the embryos in the recipient female (Table 4). Table 4 is broken down below.

Following the protocol previously used by Rahimi (2019), the first trial consisted of superovulation of 9-week-old CF1 females using 6IUs of PMSG and hCG (Rahimi et al., 2019). This hormone dosage resulted in a low average of 12.5 oocytes per female. Upon further investigation, the dosage of hormones required to achieve superovulation in mice depends on the

weight of the female (Luo et al., 2011). In the second trial, increasing the hormone dosage to 9IU, accommodating the greater body weight, increased the average oocytes per female to 40.6.

Trials 2 to 4 still had low or no embryo implantation. Mineral oil was the last material in contact with the recipient female and was thought to contribute to the low implantation rates. Trial 6 and onward were used to test the effects of washing the mineral oil prior to embryo culture dish set up. The improvement of oocyte fertilization and embryo development can be seen when specifically comparing the IVF and IVD rates from trial 5, where the mineral oil was not washed, and trial 8, where the same batch of mineral oil was washed. Washing the mineral oil with water or culture media resulted in similar IVF rates (90.7% and 88% respectively) and IVD rates (86% and 82% respectively). In trial 5, the same batch of mineral oil unwashed resulted in no *in vitro* fertilization and consequently no *in vitro* development. Both the IVF and IVD rates were dramatically improved by washing the mineral oil. Washed mineral oil improved both IVF and IVD rates, but the stability of the washed oil also needed to be determined. Despite the improved IVD and IVF rates, only one female in trial 8 had a single embryo.

Trials 10-13 used the same batch of washed mineral oil to test the washed oil stability. When the oil was not in use, it was stored covered in aluminum foil in the refrigerator, to prevent degradation caused by light and heat, and brought to room temperature in the dark on the day of embryo culture dish set up. With time, the IVF rates had a slow decline while the IVD rates were inconsistent but had a larger decline compared to the IVF rates. This demonstrated that despite washing the mineral oil and storing it properly, the rate of degradation was still too high. For trials 11 to 13, different methods of removing the mineral oil coating the NSET were also examined. Oil was removed from the NSET by wiping the tool, rinsing in a rinse dish equilibrated for 1.5 hours,

or rinsing in a rinse dish equilibrated for 4 days. No recipient females for all three trials had viable embryos or resorbed embryos and thus we could not compare the different methods of oil removal.

Overall, these results show that multiple factors affect the outcomes of ARTs. Increasing hormone dosage depending on the weight of the female resulted in a higher average number of oocytes ovulated per female. From the earlier trials, the mineral oil was identified as a potential factor that impacted IVF, IVD, and implantation. Damage done to the embryos during culture by the mineral oil overlay might have been enough to prevent implantation despite different methods of oil removal. Washing the mineral oil with water or culture media increased the IVF and IVD rates but was still not optimal for *in vitro* development. Due to the poor implantation rates, the ART experiments to test the effects of GM-CSF were not pursued.

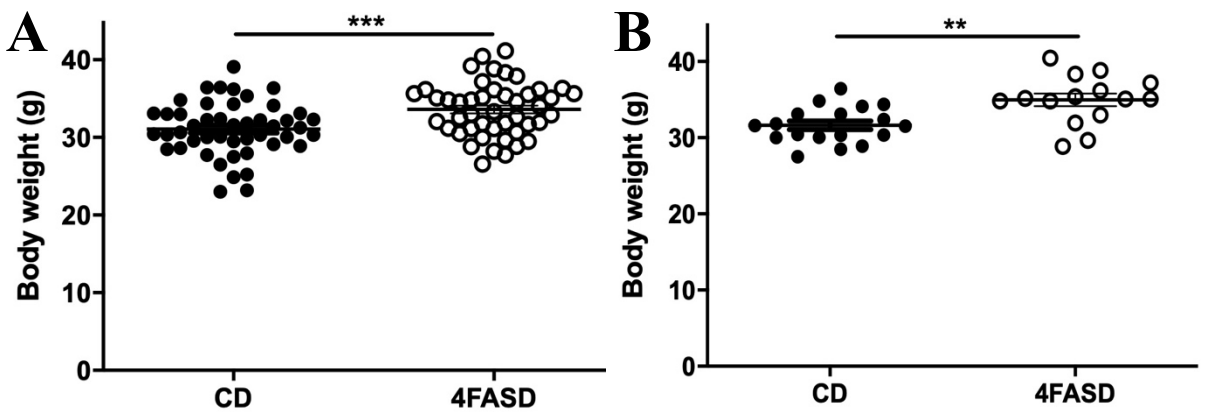


Figure 3. Weights of females mated. (A) Weights (n=47 for CD, n=46 for 4FASD) of all females mated, (B) weights (n=18 for CD, n=15 for 4FASD) of females with embryos collected. All weights were measured on the day of mating. Data are presented as mean + SEM. Comparison was performed by unpaired t-test; **p<0.01, ***p<0.001. Statistical significance is set at $p \leq 0.05$

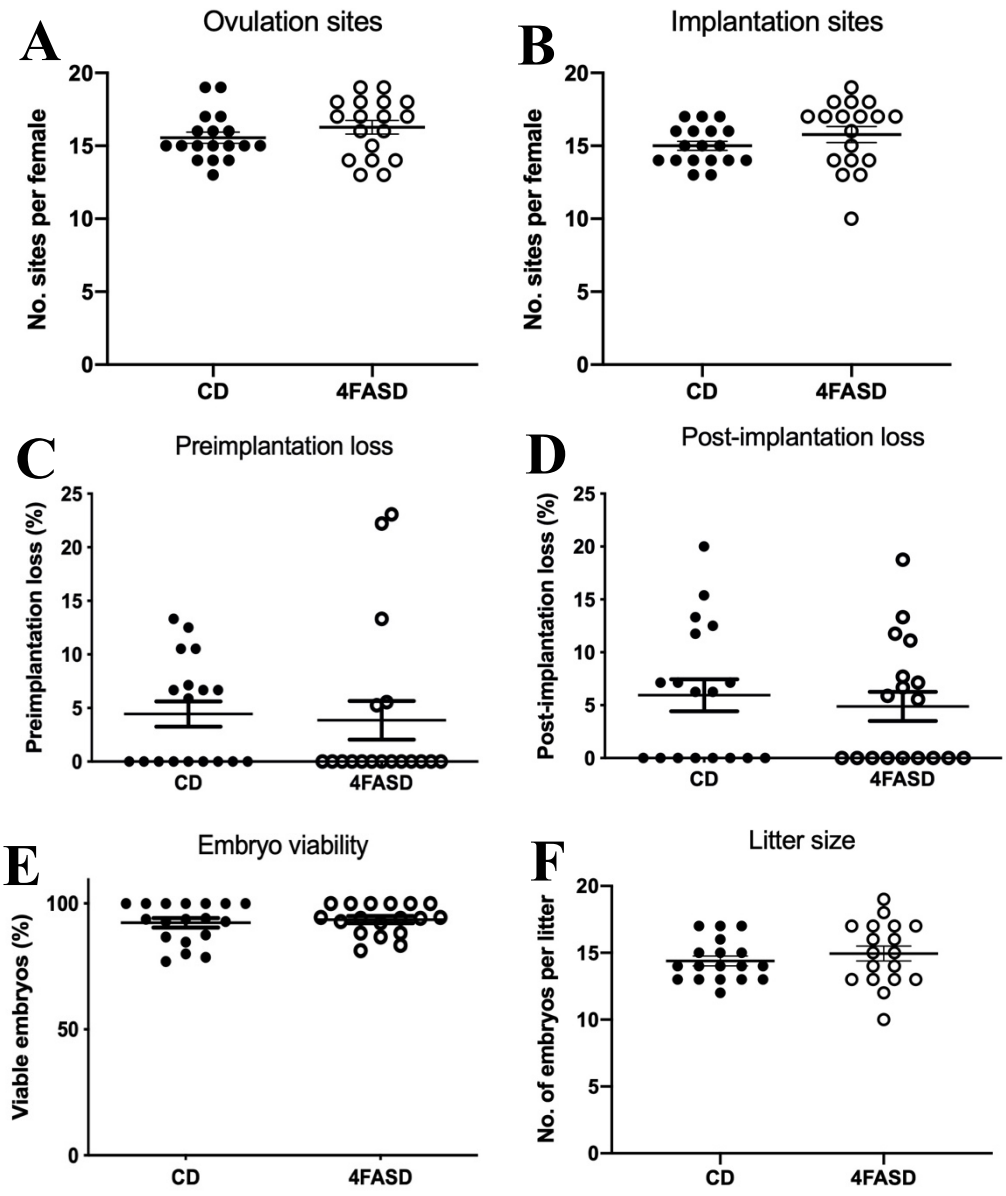
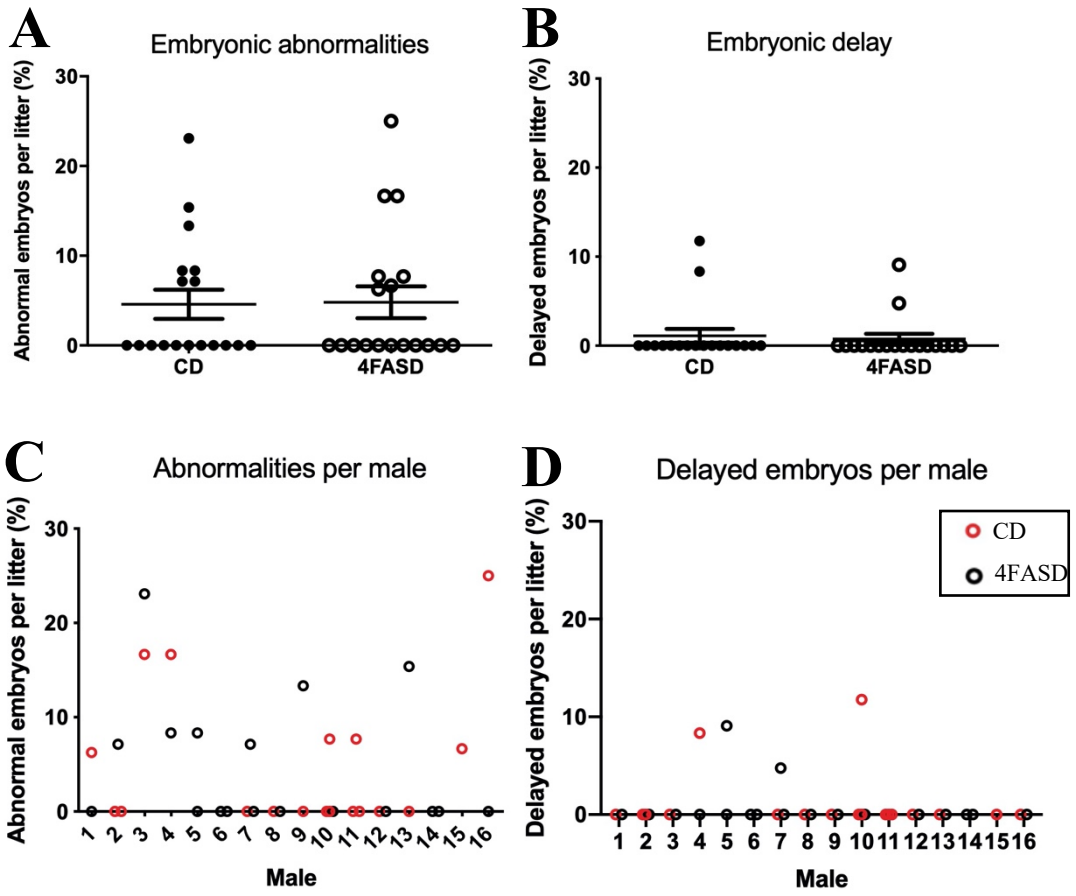


Figure 4. Effect of maternal folic acid supplementation on pregnancy outcomes. (A-B) Number of total ovulation and implantation sites, (C-D) rates of pre- and post-implantation loss, (E) embryo viability, and (F) average litter size, n=18 for both diet groups. Data are presented as mean + SEM. Comparison was performed by unpaired t-test; statistical significance is set at $p \leq 0.05$



E

Diet group	No. of Abnormal Embryos	No. of Normal Embryos	Types of abnormalities				
			Neural Tube	Craniofacial	Laterality	Anterior Posterior Discordance	Heart
CD	8	215	3	3	5	5	0
4FASD	11	230	2	4	2	4	1

Figure 5. Effect of maternal folic acid supplementation on embryonic abnormalities and delays. (A) Percentage of morphologically abnormal embryos per litter in each diet group, (B) percentage of delayed embryos per litter in each diet group, embryos staged E9.0 or less were considered developmentally delayed, (C) percentage of morphologically abnormal embryos per litter per male, (D) percentage of delayed embryos per litter per male, and (E) frequency of each abnormality within diet groups, several embryos can present more than one abnormality, n=18 for

both diet groups (A-B). Data (A-B) are presented as mean + SEM. Comparison was performed by unpaired t-test(A-B) and Fishers exact test (C); statistical significance is set at $p \leq 0.05$

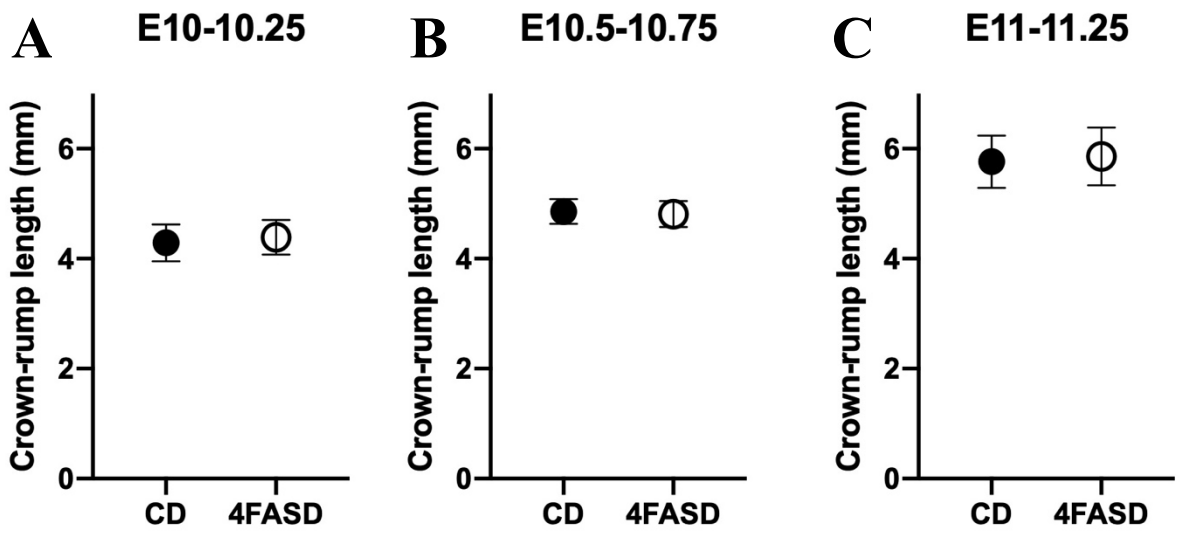


Figure 6. Embryonic crown rump lengths with folic acid supplementation. Crown rump lengths of embryos staged as embryonic day (E) (A) 10-10.25 (n=33 for CD, n=54 for 4FASD), (B) 10.5-10.75 (n=108 for CD, n=141 for 4FASD), and (C) 11-11.25 (n=64 for CD, n=50 for 4FASD). Data are presented as mean + SD. Comparison was performed by unpaired t-test; statistical significance is set at $p \leq 0.05$

Table 2. Characteristics of CD litters

Litter	CL[a]	Implantation sites	Resorbed E	Live E	Normal E	Abnormal E	Delayed E	N/A[b]	Damaged /lost[c]
1	15	13	2	11	8	3	0	0	1
2	19	17	2	17	14	1	0	1	0
3	15	15	3	13	12	0	0	1	0
4	14	14	0	14	12	0	0	2	0
5	16	16	0	16	15	0	0	1	0
6	15	15	2	12	9	2	1	1	1
7	17	16	1	15	12	1	2	2	0
8	13	15	0	15	12	0	0	3	0
9	19	17	0	17	14	0	0	3	0
10	14	13	0	10	9	1	0	0	3
11	17	17	0	16	14	1	0	1	1
12	15	14	1	13	13	0	0	0	0
13	15	14	0	11	10	0	0	1	3
14	16	14	1	13	12	0	0	1	0
15	15	16	1	15	13	0	0	2	0
16	15	14	1	14	10	2	0	0	0
17	14	14	0	14	13	0	0	1	0
18	16	16	2	14	13	0	0	1	0

E – embryo

CL[a] signifies the number of corpora lutea on both ovaries, indicating the number of ovulated oocytes.

N/A[b] signifies the embryos that could not be accurately assessed for morphological abnormalities.

Damaged or lost[c] signifies embryos that were damaged or not found and could not be accurately assessed for viability, morphological abnormalities, or staging.

Table 3. Characteristics of 4FASD litters

Litter	CL[a]	Implantation sites	Resorbed E	Live E	Normal E	Abnormal E	Delayed E	N/A[b]	Damaged /lost[c]
1	17	17	2	15	14	0	0	1	0
2	16	17	0	17	15	0	0	2	0
3	18	17	0	16	13	0	0	3	1
4	15	13	1	12	11	1	1	0	0
5	14	14	1	13	10	0	0	3	0
6	18	14	0	14	14	0	0	0	0
7	18	18	2	15	15	0	0	0	1
8	13	13	0	13	13	0	0	0	0
9	14	16	3	13	11	2	0	0	0
10	17	17	1	16	13	0	0	3	0
11	18	18	0	17	15	0	0	2	1
12	17	17	0	16	12	1	1	3	1
13	13	10	0	10	9	0	0	1	0
14	17	17	2	15	13	1	0	1	0
15	19	19	0	19	18	0	0	1	0
16	16	15	1	13	11	1	0	1	1
17	14	14	0	14	10	3	0	1	0
18	19	18	1	17	13	2	0	2	0

E – embryo

CL[a] signifies the number of corpora lutea on both ovaries, indicating the number of ovulated oocytes.

N/A[b] signifies the embryos that could not be accurately assessed for morphological abnormalities.

Damaged or lost[c] signifies embryos that were damaged or not found and could not be accurately assessed for viability, morphological abnormalities, or staging.

A

	No. of samples	Range of embryo staging	No. of normal embryos	No. of abnormal embryos
NAT-CD	6	E10.5	6	0
CD-ART	12	E9.0-11.5	10	2
4FASD	12	E10.0-10.5	10	2
10FASD	12	E10.0-10.75	10	2

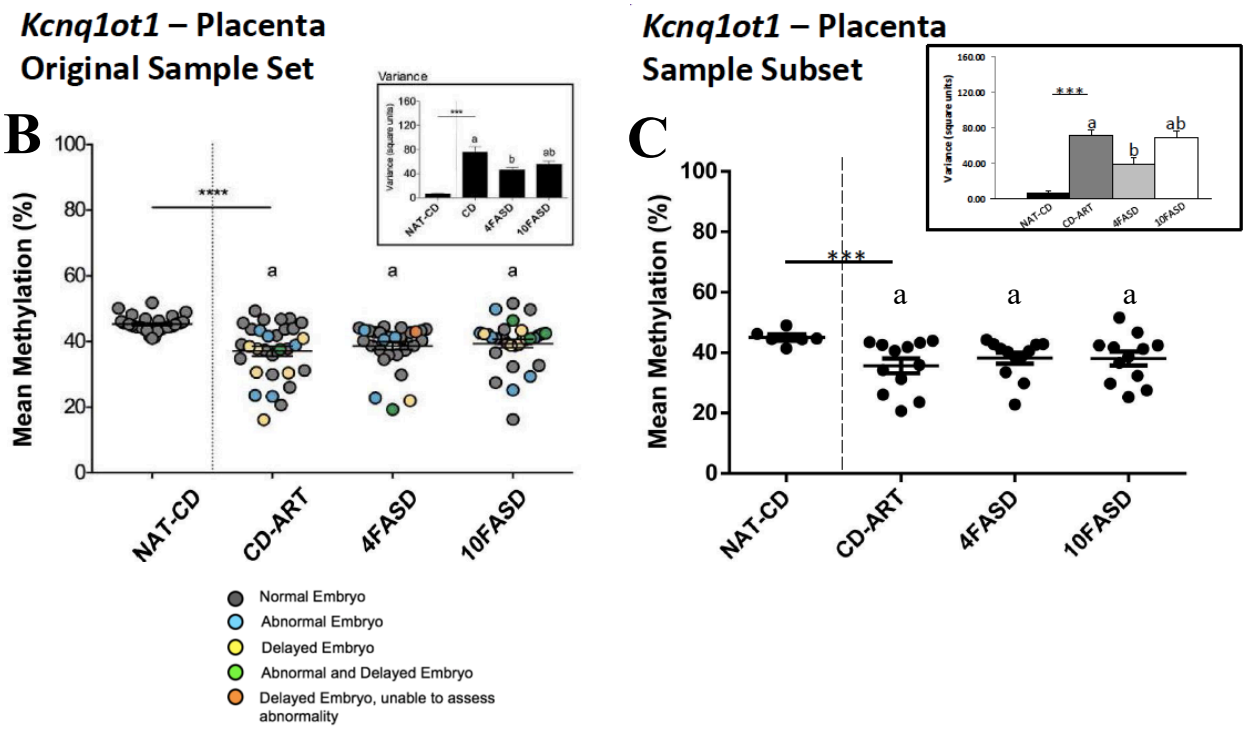


Figure 7. Comparison of sample subset containing placentas from normal and abnormal female embryos to the original placenta sample set. (A) Breakdown of characteristics of the placenta sample subset, (B) mean DNA methylation and variance for *Kcnq1ot1* of original sample (n=31-32 per group). (C) mean DNA methylation and variance of *Kcnq1ot1* (4 CpG sites) of sample subset (n=6 for NAT-CD, n=12 for CD-ART, 4FASD, and 10FASD). Each point on the scatterplot represents the average methylation across the imprinting control regions (ICR) for a single sample. Data are presented as mean ± SEM. Comparison between NAT-CD and CD groups was performed by unpaired t-test; ***p<0.001, ****p<0.0001. Comparison between ART groups (CD, 4FASD and 10FASD) was performed by one-way ANOVA with Tukey's multiple comparisons test; groups without a common letter significantly differ, statistical significance is set at $p \leq 0.05$.

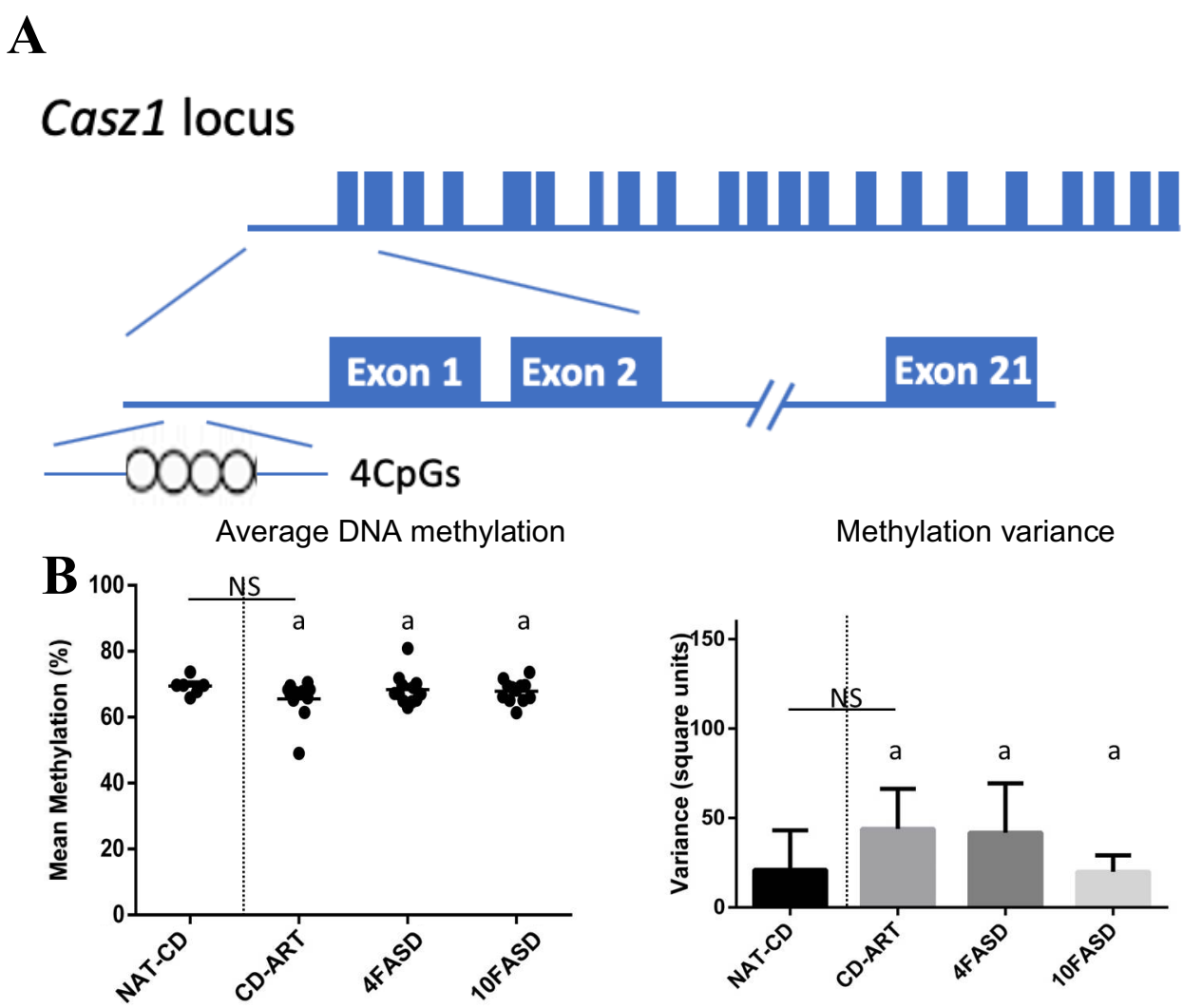


Figure 8. Effect of ART and folic acid supplementation on DNA methylation of a non-imprinted gene *Casz1* in the female placenta sample subset. (A) Region of CpGs analyzed within the locus, (B) average DNA methylation and variance of *Casz1* (n=6 for NAT-CD, n=12 for CD-ART, 4FASD, and 10FASD). DNA methylation was assessed using bisulfite pyrosequencing. Each point on the scatterplot represents the average methylation for a single sample. Comparison between NAT-CD and CD-ART groups was performed by unpaired t-test; ns represents a non-significant difference. Comparison between ART groups (CD-ART, 4FASD and 10FASD) was performed by one-way ANOVA with Tukey's multiple comparisons test; groups without a common letter significantly differ, statistical significance is set at $p \leq 0.05$.

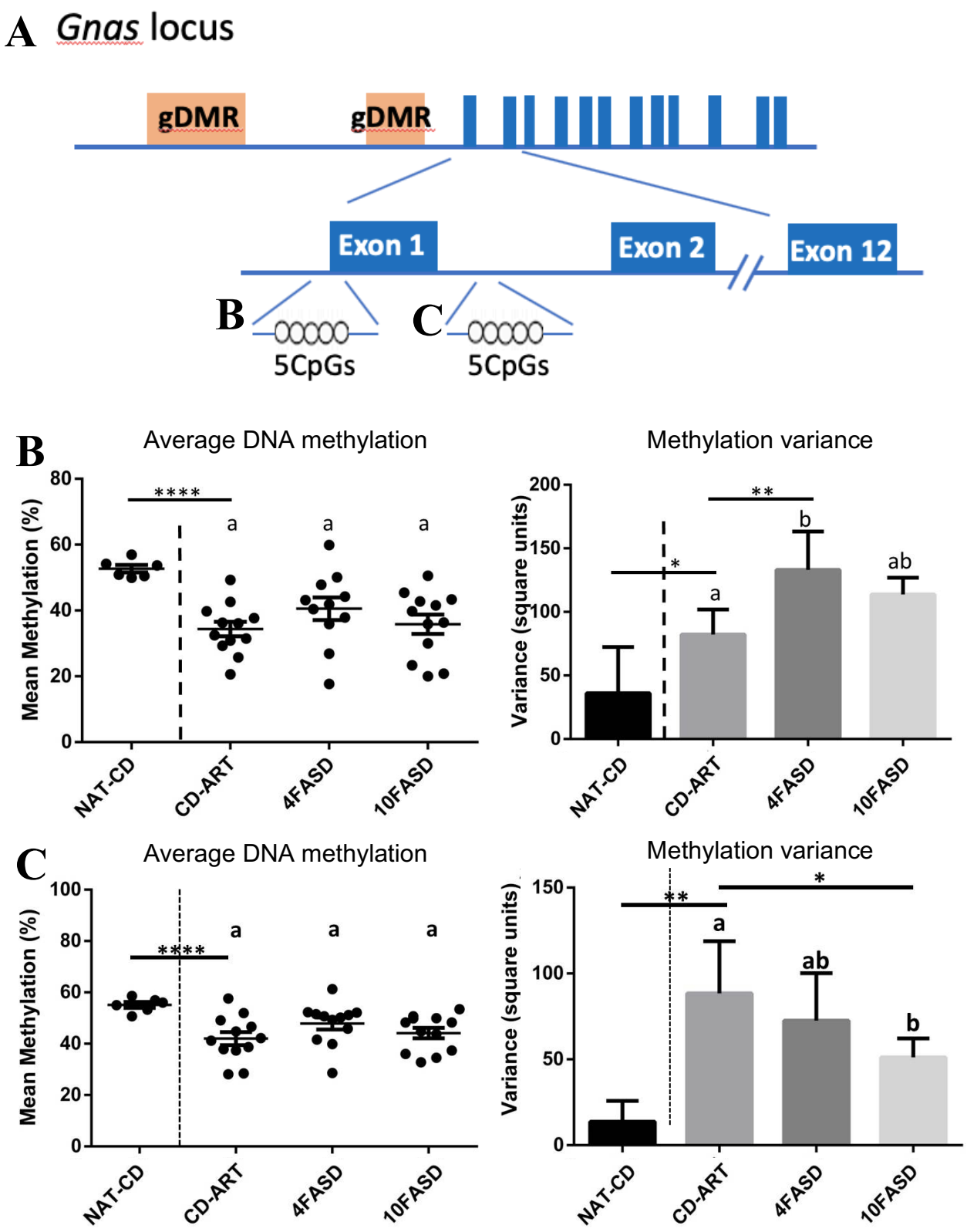

A Gnas locus
gDMR
gDMR
Exon 1
Exon 2
Exon 12
B
5CpGs
C
5CpGs
B
Average DNA methylation
Mean Methylation (%)
80
60
40
20
0
****
a
a
a
NAT-CD
CD-ART
4FASD
10FASD
Methylation variance
Variance (square units)
200
150
100
50
0
**
*
b
ab
a
NAT-CD
CD-ART
4FASD
10FASD
C
Average DNA methylation
Mean Methylation (%)
100
80
60
40
20
0
****
a
a
a
NAT-CD
CD-ART
4FASD
10FASD
Methylation variance
Variance (square units)
150
100
50
0
**
*
a
ab
b
NAT-CD
CD-ART
4FASD
10FASD

Figure 9. Effect of ART and folic acid supplementation on DNA methylation of an imprinted gene *Gnas* in the female placenta sample subset. (A) Region of CpGs analyzed within the locus, (B-C) average DNA methylation and variance of *Gnas* (5 CpG sites per region of analysis), (n=6 for NAT-CD, n=12 for CD-ART, 4FASD, and 10FASD). DNA methylation was assessed using bisulfite pyrosequencing. Each point on the scatterplot represents the average methylation for a single sample. Comparison between NAT-CD and CD groups was performed by unpaired t-test; *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001. Comparison between ART groups (CD, 4FASD and 10FASD) was performed by one-way ANOVA with Tukey's multiple comparisons test; groups without a common letter significantly differ, statistical significance is set at $p \leq 0.05$.

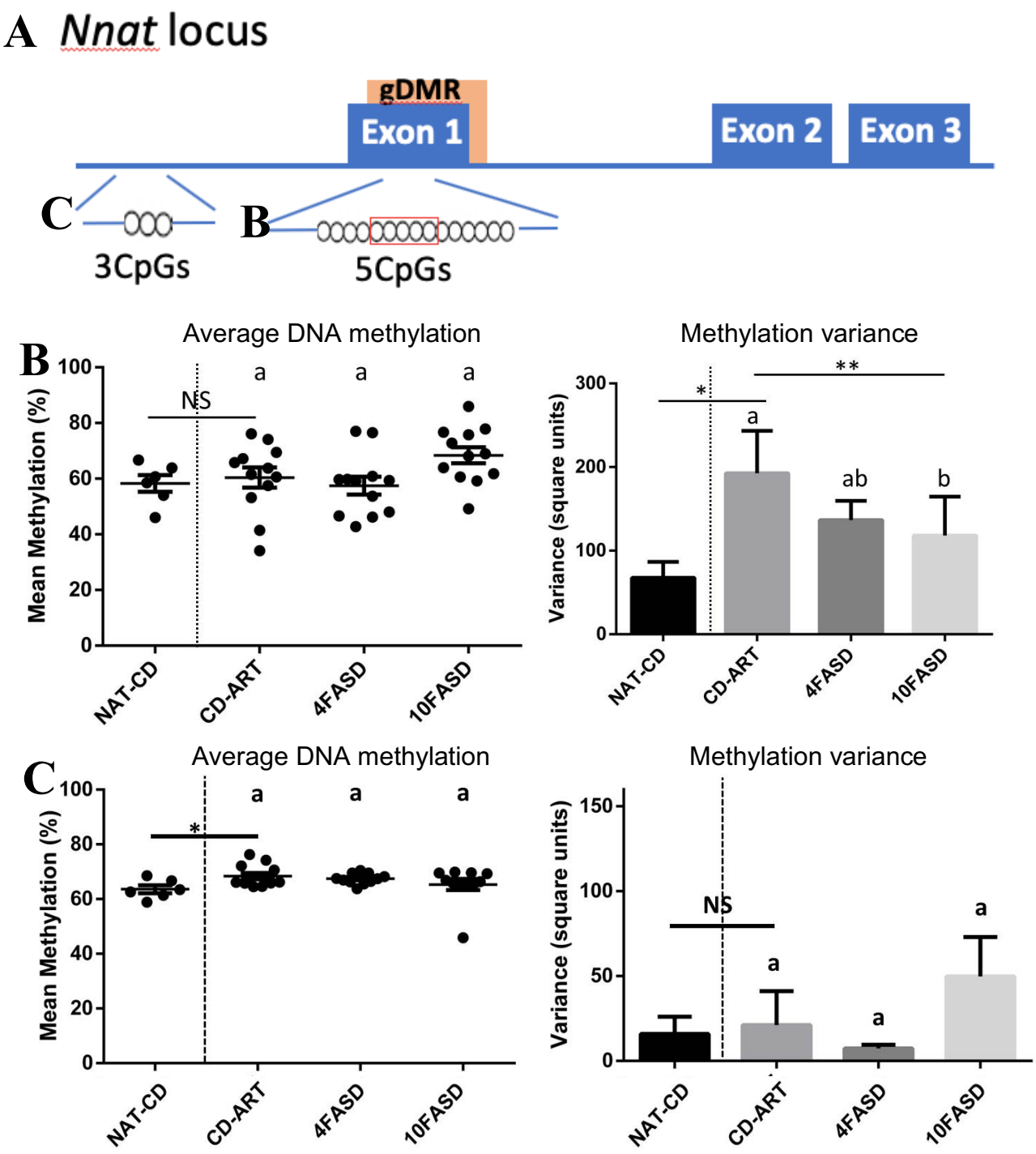


Figure 10. Effect of ART and folic acid supplementation on DNA methylation of an imprinted gene *Nnat* in the female placenta sample subset. (A) Region of CpGs analyzed, (B-C) DNA methylation and variance of *Nnat* (5 CpGs and 3CpGs respectively), (n=6 for NAT-CD, n=12 for CD-ART, 4FASD, and 10FASD). DNA methylation was assessed using bisulfite pyrosequencing. Each point on the scatterplot represents the average methylation for a single

sample. Comparison between NAT-CD and CD-ART groups was performed by unpaired t-test; *$p<0.05$, **$p<0.01$, ns represents a non-significant difference. Comparison between ART groups (CD-ART, 4FASD and 10FASD) was performed by one-way ANOVA with Tukey's multiple comparisons test; groups without a common letter significantly differ, statistical significance is set at $p \leq 0.05$.

Table 4. Summary of results of all ART experiments

Trial Code	PMSG and hCG dose (IU)	Average oocytes no. per female	IVF (%)	IVD (%)	Females with resorptions or embryos	Mineral Oil used		
1*	6	12.5	83.75	16.5	½ (0E, 1R)	MKCC6258		
Changes for next experiment -Dose of PMSG and hCG increased from 6IU to 9IU								
2	9	40.6	84.6	77	1/3 (0E, 1R)	MKCC6258		
Changes for next experiment -Oocytes washed in KSOM instead of HTF (for all experiments following)								
3	9	32	95.5	69.75	0/3	MKCC6258		
4	9	57.6	68	5.7	na	MKCC6258		
Changes for next experiment -Different mineral oils used to cover culture media drops -Human tested oil (LiteOil from Life Global Group) given by Mitra Cowan								
			Human tested oil			Oil (MKCH0156)		
Trial Code	PMSG and hCG dose (IU)	Average oocytes no. per female	IVF (%)	IVD (%)	Females with resorptions or embryos	IVF (%)	IVD (%)	Females with resorptions or embryos
5	9	32	0	na	na	0	na	na
Changes for next experiments -Implemented the use of a KSOM rinse dish right before embryo transfer -Rinse dish equilibrated in incubator for 1.5 hours -Washed oil given by Mitra Cowan								
			Oil washed (M5310)			Oil (MKCC6258)		
6	9	43.4	96	34.5	1/1 (4E, 2R)	92.7	73	2/2 (4E, 1R)
Changes for next experiments -Mineral oil washed in small batch – (35mL oil : 6mL H_20)X3 -Rinse dish equilibrated in incubator for 1 hours								
			Water washed oil (MKCH0156)			Not washed oil (MKCC6258)		
7	9	33.6	100	61.5	1/3 (0E, 2R)	99	68.5	0/2
Changes for next experiments -Mineral oil washed in medium batch – 130mL oil : 20mL H_20 vs. 130mL oil : 20mL KSOM -Rinse dish equilibrated in incubator for 30 minutes								
			Water washed oil (MKCH0156)			Media washed oil (MKCH0156)		
8	9	43.4	90.7	86	0/2	88	82	½ (1E, 0R)
Changes for next experiments -Mineral oil washed in medium batch – 130mL oil : 20mL H_20 -Rinse dish equilibrated in incubator for 4 days								
			Water washed oil (MKCC6258 + MKCH0156)			Water washed oil (MKCJ3950)		
9	9	37.7	94	61	1/1 (0E, 1R)	95.7	59	0/2

Changes for next experiments -Mineral oil washed in large batch – 450mL oil : 100mL H_20 (stored at 4ºC when not in use) -Rinse dish equilibrated in incubator for 4 days							
			Water washed oil (MKCJ3950)			Days since oil wash	
10*	9	35.5	92.8	67.6	4/4 (9E, 4R)	0	

Changes for next experiments -Remove of oil on NSET before embryo transfer done by: NSET tool wipe or rinse in media equilibrated for 1.5h or rinse in media equilibrated for 4 days -Dose of PMSG and hCG increased from 9IU to 10IU (adjusted) -Mineral oil used: large scale water wash-batch MKCJ3950								
	Water washed oil (MKCJ3950)				Days since oil wash	No. of females with resorptions or embryos		
Trial Code	PMSG and hCG dose (IU)	Average oocytes no. per female	IVF (%)	IVD (%)		NSET tool wipe	Rinse in media equilibrated for 1.5h	Rinse in media equilibrated for 4 days
11*	10	40.4	96	80	7	0/3	0/3	0/2
12	10	38.75	95.2	40.6	14	0/2	na	0/1
13	10	48	93	59.3	21	0/2	na	0/1

E – embryo

R – resorbed embryo

* – blastocysts from a single female were transferred to more than one recipient

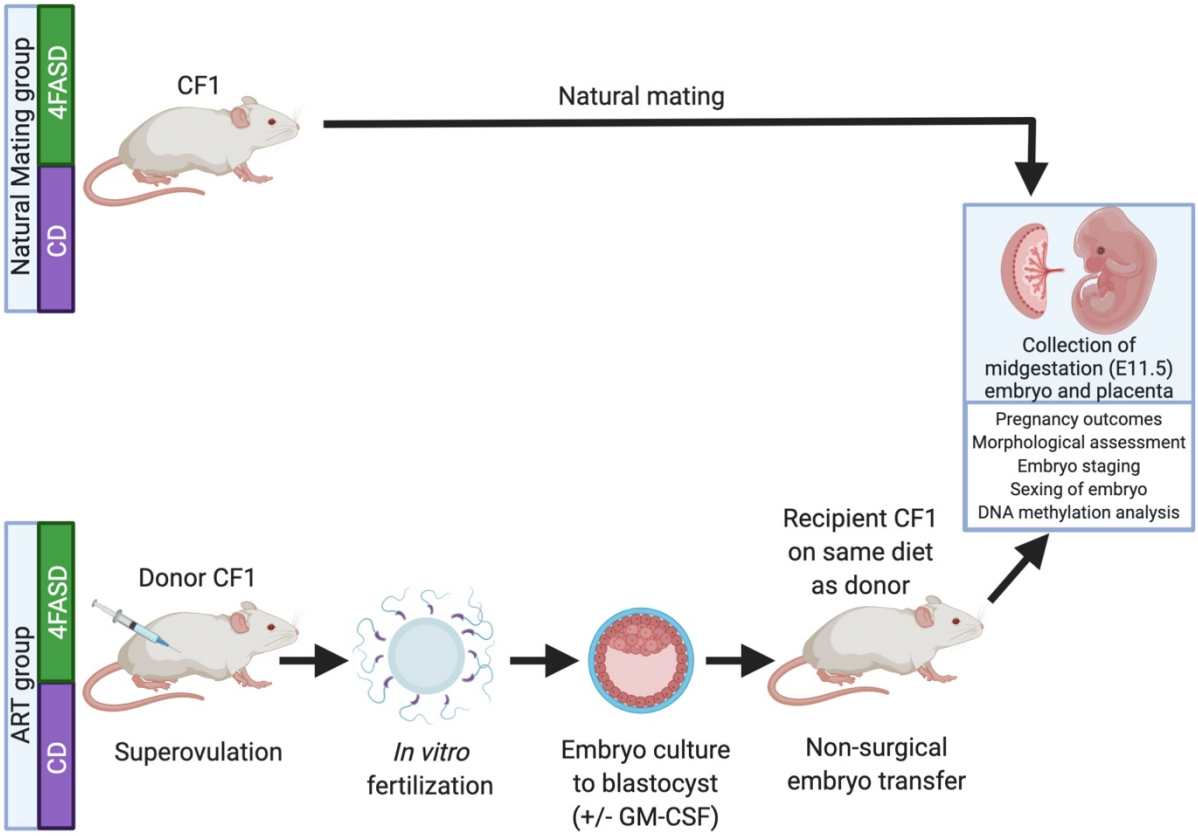


Figure 11. Experimental design. For natural mating group, CF1 female mice will be fed a folic acid control diet (CD) or 4-fold folic acid supplemented diet (4FASD) for 6 weeks than naturally mated to B6SJF1/J males. For the ART group, donor CF1 females will be fed a folic acid control diet (CD) or 4-fold folic acid supplemented diet (4FASD). Diets will be continued throughout gestation. ART will include a combination of superovulation, *in vitro* fertilization, embryo culture for 4 days, and non-surgical embryo transfer to a pseudo-pregnant recipient CF1 female (fed the same diet as the donor female for the equivalent amount of time). Embryos and placentas will be collected at midgestation and assessed for: pregnancy outcomes, morphological assessment, embryonic staging, sexing of embryos, and DNA methylation analysis of embryos and placentas. ART, assisted reproductive technologies. (ART Protocol adapted from: (Byers et al., 2006; Behringer et al., 2013))

Chapter 4: Discussion

Assisted reproduction is commonly used to aid couples who suffer from infertility and often results in the birth of a healthy child; however, there is an increased risk for negative outcomes such as neurodevelopmental disorders, metabolic or endocrine disruptions, and imprinting disorders (Liu et al., 2017; Vrooman et al., 2017; Guo et al., 2017; Mussa et al., 2017; DeBaun et al., 2003; Lim et al., 2009). Imprinting disorders in particular are associated with DNA methylation defects due to the overlap of ART procedures and key dynamic DNA methylation waves that occur during gametogenesis and early embryonic development (reviewed by Marchal et al., 2004; Santos et al., 2002). Maternal nutrition during pregnancy has been shown to greatly impact embryo and placenta development and undernutrition can lead to negative postnatal outcomes (Sun et al., 2014; Watkins et al., 2008; Luzzo et al., 2012, Coan et al., 2009). The proper acquisition and maintenance of DNA methylation depends on the availability of methyl donors consumed through the diet. Although folic acid supplementation is shown to be beneficial in moderate doses, high dose folic acid supplementation during natural pregnancies was associated with embryonic loss, embryonic developmental delays, and impairments in the surviving offspring (Mikael et al., 2012; Pickell et al., 2010; Henzel et al., 2017). Along with maternal supplementation, other avenues of improving the ART protocol itself is a topic of interest. GM-CSF, a growth factor naturally secreted by the uterus, activates the MAPK signalling pathway and aids in maintaining DNA methylation during early embryo development (Al-Shami et al., 1998). Our lab has recently demonstrated that moderate folic acid supplementation resulted in only a partial correction of the epigenetic alterations associated with ART (Rahimi et al., 2019). Therefore, our goal was to determine whether folic acid supplementation can prevent negative embryonic outcomes in naturally mating mice, prevent epigenetic alterations associated with the use of assisted

reproduction in different types of genes, and determine if folic acid supplementation along with GM-CSF in the ART protocol results in a greater degree of epigenetic rescue. All of our goals were performed using an outbred mouse strain to mimic the genetic variety observed in the human population.

4.1 Modulation of natural mating pregnancy outcomes by moderate folic acid supplementation

In our study, moderate folic acid supplementation (4FASD) led to an increase in body weight of the females. Previous studies using a rat model consuming a higher dose folic acid-supplemented diet resulted in changes in body weight in a similar manner (Cho et al., 2013; Hoile et al., 2012). Studies have shown a connection between high intake of folic acid and an increase in colorectal cancer rates (Mason et al., 2007). Increased levels of plasma folic acid among women who took folic acid supplements were associated with a decrease in circulating natural killer cells, which are important in destroying arising neoplastic cells (Janeway et al., 1999). The increase in rate of cell growth and division due to the higher dose of folic acid in the 4FASD diet could explain the increase in female body weight.

Aside from the change in weight, our rates of abnormalities for both the CD and 4FASD diet groups (4.6% and 4.8% respectively) were comparable to the rate of abnormalities of mice on a regular chow diet (6.9%) (Cebral et al., 2007). To confirm that rate of abnormalities of both diet groups is similar despite the different dose of folic acid supplementation, blood folate levels need to be analyzed. Folate levels were measured previously in our lab but was not completed for this experiment due to the COVID-19 situation (Rahimi et al., 2019). If the blood folate levels are higher in the 4FASD group, it would demonstrate that a moderate dose does not impact embryonic

outcome in naturally mated mice and the CF1 sub-strain from Charles River on the CD diet would be a useful model for follow-up experiments.

The rate of embryonic abnormalities of the mice consuming the CD diet in this experiment (4.6%) were lower than what was previously observed in our lab (31.3%) despite the mice in both experiments being the same strain, CF1, and consuming the same diet manufactured from the same company (Rahimi et al., 2019). The only difference between these two experiments was that the mice used in Rahimi et al. (2019) were CF1 mice from Envigo, while the mice used in this experiment were CF1 mice from Charles River Canada. Even though the mouse strain used in the experiments were identical, research has shown that a big liability of outbred stocks is that the extent of genetic variation in an individual strain is not well known, which could result in the development of company based sub-strains of mice, which could explain the differences seen in the pregnancy outcomes of Rahimi et al. (2019) and the this experiment (Chia et al., 2005; Rahimi et al., 2019). In addition, no changes between the CD mice and the 4FASD mice were observed in pregnancy outcomes and rates of abnormalities and developmental delays in the embryos.

4.2 Modulation of DNA methylation in the placentas of ART-conceived embryos by folic acid supplementation

Since DNA methylation is dynamic during gametogenesis and embryo development, our lab has recently looked into the effects of ART on the DNA methylation status of 4 imprinted genes (reviewed by Marchal et al., 2004; Santos et al., 2002; Rahimi et al).

To expand the analysis by looking at more genes, a sample subset was selected. All 4 imprinted genes, *H19*, *Snrpn*, *Peg1*, and *Kcnq1ot1*, have shown to be affected by ART, with the placentas more affected compared to the embryos and there was a female bias, where ART had a

greater impact on females (Rahimi et al., 2019). Taking all of this into consideration, the sample subset used here consisted of placentas from morphologically normal and abnormal embryos. Sample composition and DNA methylation analysis of *Kcnq1ot1* confirmed the female placenta sample subset was representative of the original sample set.

DNA methylation and variance of *Casz1* was not affected by ART or both doses of folic acid supplementation. DNA methylation of *Casz1* has not previously been shown to be affected by ART but expression levels have been altered in both an *in vitro* and *in vivo* model in response to folic acid deficiency (Carroll et al., 2012; MacFarlane et al., manuscript in preparation). *Casz1*, unlike imprinted genes, can correct altered DNA methylation during the wave of DNA methylation reprogramming during preimplantation. This can explain why *Casz1* was not affected by ART or folic acid supplementation.

Unlike *Casz1*, imprinted genes are required to maintain their DNA methylation during DNA reprogramming during early embryo development. Many factors are involved in imprinted gene maintenance. PGC7, expressed in gametes, primordial germ cells (PGCs), and zygotes, exerts a maintenance function for imprinted genes by binding and inhibiting the catalytic domain of the enzymes responsible for demethylation (Bian et al., 2014). Proteins, like ZFP57, bind to methylated regions and protect them from demethylation (Strogantsev et al., 2015). Methylated imprinted genes are also marked with repressive histone modifications (Lewis et al., 2004).

Both regions of the imprinted gene *Gnas* demonstrated aberrant mean DNA methylation and variance following ART. Previous studies have shown that *Gnas* is vulnerable to changes induced by ART and *Gnas* has been shown to have lower DNA methylation in an ART outlier group enriched for females (Choufani et al., 2019; Melamed et al., 2015). For both regions of analysis, the ART group with moderate folic acid supplementation resulted in a slight rescuing

effect, where the mean methylation and a higher number of samples were closer to the natural mating group. The opposite was observed in the ART group high folic acid supplemented, suggesting that assisted reproduction leads to DNA methylation perturbations and the moderate dose folic acid supplementation can slightly rescue these negative effects.

Similar to *Gnas*, the same human study has shown that *Nnat* is also vulnerable to changes induced by ART and *Nnat* has been shown to have lower DNA methylation in an ART outlier group enriched for females (Choufani et al., 2019). The gDMR ART resulted in a slight decrease in mean methylation and a slight increase in variance. However, *Nnat* was not affected as dramatically by ART or folic acid supplementation as *Gnas* was. Similar to our results, a mouse study demonstrated that embryonic stem cells with a loss of DNMT1, a protein crucial for maintaining DNA methylation, resulted in a large decrease in DNA methylation of 18 regions within the *Gnas* locus and 8 regions within the *Nnat* locus (McGraw et al., 2015). The high dose folic acid supplementation resulted in aberrant mean methylation. This suggests that assisted reproduction in combination with high dose folic supplemented diet lead to perturbations and displayed a trend deviating away from that natural mating group.

Despite folic acid supplementation, alterations in DNA methylation for both *Gnas* and *Nnat* are present in placentas at midgestation. Studies have shown that imprinted genes from preimplantation embryos, conceived naturally but cultured *in vitro*, have altered DNA methylation (Doherty et al., 2000; Mann et al., 2004). Our results support that ART can induced errors in DNA methylation maintenance despite maternal moderate folic acid supplementation.

There were two commonalities between the effects seen in *Gnas* and *Nnat*. Firstly, there was a presence of outlier samples where the errors in DNA methylation are not corrected with the 4FASD. The term ‘outliers’ has shown up in both mouse and human ART studies (de Waal et al.,

2015; Ghosh et al., 2016). The presence of outliers suggests that there are susceptible embryos, but most embryos are able to escape, compensate, or correct epigenetic alterations (de Waal et al., 2015; Ghosh et al., 2016). Secondly, the 10FASD led to a suggestion of deleterious effects (change in DNA methylation and variance in a direction away from the natural mating group). Intake of 10FASD has been associated with elevated unmetabolized folic acid in the plasma (Pickell et al., 2010; Mikael et al., 2012). Excess folic acid supplementation during natural pregnancies has been shown to exhibit pseudo-MTHFR deficiency in the mother and impaired embryonic growth and DNA methylation profiles (Bahous et al., 2017a; Bahous et al., 2017b). In addition, we suggest that the 10FASD may disrupt the one carbon metabolism pathway, impacting DNA methylation in both imprinted and non-imprinted loci.

4.3 Optimization of ART protocol

The ART protocol contains many steps, which can all impact successful development and implantation of the embryo. We were interested in optimizing the protocol before going on to examine the effects of moderate folic acid supplementation and GM-CSF in the embryo culture media.

Following the protocol previously used in our lab, ovarian stimulation using 6IU of both PMSG and hCG resulted in a relatively low number of oocytes per female. Luo et al. (2011) reported that adjusting the hormone doses depending on strain and weight increased the number of oocytes ovulated (Luo et al., 2011). We saw a similar increase in the number of oocytes when the hormone doses were increased depending on the weight of the female.

A major component of the ART protocol is the use of mineral oil. Mineral oil has been used for embryo cultures since 1963 (Brinster et al., 1963). Embryo culture dishes are covered

with an oil layer to prevent evaporation of the culture medium and appropriate osmotic pressure. Depending of the manufacturer, lot number, and storage conditions, mineral oil can deteriorate and contain toxins harmful to embryo development (Otsuki et al., 2007). Oils from the same company but different lots resulted in a dramatic decrease in the number of two-cell embryos that survived to the blastocyst stage (Morbeck et al., 2010). Like the results seen by Morbeck et al. (2010), washing our mineral oil with water or culture media resulted in a dramatic increase in IVF and IVD rates but still had batch to batch differences (Morbeck et al., 2010).

We considered two main explanations for the lack of implantation after multiple trials of embryo transfer, failure of the NSET embryo transfer or quality of the mineral oil. To control for the success of the embryo transfer using the NSET, the procedure itself was performed by an experienced research associate, who has had a number of years of experience successfully carrying out embryos transfers with NSET (e.g. Rahimi et al., 2019) and by the candidate (Rahimi et al., 2019). Another control that could have been implemented, to confirm the success of the embryo transfer in the experiments carried out in the thesis, would have been to transfer non-manipulated freshly collected blastocyst stage embryos from a naturally mated female to a pseudopregnant female.

The other possible factor that could have impacted implantation was the quality of the mineral oil. During embryo culture, volatile organic compounds from the mineral oil can transfer into the culture medium (Martinez et al., 2017). Although washing the mineral oil improved IVF and IVD rates, the transfer of the volatile organic compounds to the culture media and the exposure of the developing blastocysts to those compounds might have been enough to prevent implantation despite the removal of all oil traces during the embryo transfer. Other labs that also perform mouse ART, Mitra Cowen at the McGill Integrated Core for Animal Modeling and Dr. Marisa Bartolomei

at the University of Pennsylvania, were contacted. At the same time we were having problems, both labs also had difficulty with the success of their mouse ART which they could attribute to the mineral oil as they had simultaneous success of implantation with old batches of oil. When contacting Sigma-Aldrich, they stated that majority of their embryo-grade mineral oil had been on back order for a couple months because their batches were not passing quality control.

Washed mineral oil improved the IVF and IVD values but requires proper storage to prevent degradation caused by heat and light, which is detrimental to embryo development (Otsuki et al., 2007). In between the last three trials, the washed mineral oil was stored at 4ºC covered in aluminum foil to prevent oil degradation and only brought to room temperature in the dark on the day of embryo culture dish preparation. Despite the extra measures, the IVF and IVD rates continued to decline, suggesting that, even with washing, oil degradation continues to occur.

Overall, our results suggest that many factors can influence the outcomes of ART. Hormone doses needs to be adjusted to the individual and issues like success of NSET and quality of mineral oil may influence the success of mouse ART.

4.4 Future directions

The first part of the study demonstrates that a control diet containing folic acid (2 mg/kg of diet) is sufficient for naturally mating mice and a moderate folic acid-supplemented diet does not negatively impact the pregnancy outcomes. These results do not inform as to whether moderate folic acid supplementation impacts DNA methylation profiles of the offspring that are naturally conceived.

As a follow-up study, both plasma and red blood cell folate levels would need to be examined to confirm that the moderate folic acid-supplemented diet is reflective as to what our lab

has seen previously (Rahimi et al., 2019). Along with folate levels, the impact of different doses of folic acid supplementation needs to be assessed at the DNA methylation and global methylation levels. DNA methylation statuses of *H19*, *Snrpn*, *Peg1*, and *Kcnq1ot1* will allow us to determine whether the moderate folic acid supplementation impacts DNA methylation of well-known ICRs and compare our results with a previous study from our lab (Rahimi et al., 2019). There is a possibility that none of the ICRs have altered DNA methylation. To confirm that overall DNA methylation profiles are unaffected, a global DNA methylation analysis should also be completed.

The second part of the study demonstrated that *Casz1*, a non-imprinted gene, was not affected by ART or folic acid supplementation while *Gnas* and *Nnat*, both imprinted genes, were. Like previously shown by our lab, a moderate folic acid-supplemented diet showed a trend towards rescue. These results do not inform as to whether significance was seen due to the smaller sample size or due to the sample subset containing only females, which were shown to be more affected by ART (Rahimi et al., 2019).

To properly assess the impact of ART and all three doses of folic acid supplementation, a larger and more representative sample of placentas and embryos of both males and females similar to Rahimi et al. (2019) needs to be selected (Rahimi et al., 2019). Along with a larger sample size, each group of analysis must contain both male and female samples along with representation of normal, delayed, and abnormal samples. *Casz1*, *Gnas*, and *Nnat* along with imprinted like genes would be reanalyzed with the larger sample size. Imprinted like genes determined by McGraw et al. (2015) are genes that are known not to be imprinted but have similar characteristics as imprinted genes, where after a loss and regain of DNMT1 resulted in DNA methylation statuses are not fully rescued (McGraw et al., 2015). Analyzing the effects of ART and folic acid supplementation on a

broader context would give a better understanding to the extent ART and folic acid supplementation affects DNA methylation and which genes are more susceptible.

Maternal folic acid supplementation has been shown to have beneficial effects on the embryos conceived with ART. Aside from maternal nutrition, the final study demonstrated that the ART protocol itself requires many modifications to optimize positive embryonic outcome.

As a follow-up study, it would be interesting to examine the effects of GM-CSF containing culture media with the optimized ART protocol. Females being fed the control folic acid-supplemented diet and the moderate dose folic acid-supplemented diet will undergo ART. The females consuming the moderate dose of folic acid will need to be separated into two groups, embryos cultured with and without GM-CSF. Along with pregnancy outcomes, DNA methylation will also need to be analyzed to assess the impact of ART, folic acid supplementation, and GM-CSF. DNA methylation will need to be examined by assessing individual imprinted, imprinted like, and non-imprinted genes along with a genome-wide approach.

4.5 Conclusions

Overall, the 4FASD did not change pregnancy outcomes for embryos conceived naturally. For embryos conceived with ART, the corresponding placentas showed no alterations in DNA methylation for non-imprinted genes but were altered in the imprinted genes at loci within and away from the gDMRs. The 4FASD was beneficial and show a trend towards correction in loci for both mean DNA methylation and the corresponding variance. However, the 10FASD was associated with changes in mean DNA methylation away from the naturally mating group. Looking at the ART protocol in depth, the mineral oil used to overlay the culture dishes was determined to be the key factor in impacting embryonic development. Washing the oil improved

the fertilization and development rate of embryos *in vitro* but did not enough to result in implantation.

Therefore, this study reports no effect of moderate folic acid supplementation on pregnancy and embryonic outcomes for mice naturally mated. There is a dose-dependent effect of folic acid supplementation on epigenetic outcomes associated with ART, with evidence supporting that the moderate dose of folic acid is protective while the high dose of folic acid is detrimental. Along with folic acid supplementation, the ART protocol itself affects embryo development. Mineral oil used in the culture dishes is a key factor and can impact embryonic development.

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