Abnormal Placental DNA Methylation and Gene Expression Associated with Assisted Reproduction: Early Detection and Effect of Folic Acid Supplementation

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Table of Contents

Abstract

In Canada, as many as one in six couples experience infertility, defined as the inability to conceive after 12 months or more of regular unprotected sexual intercourse. As a result, an increasing proportion of couples resort to the use of assisted reproductive technologies (ARTs) to achieve pregnancy. Today, more than 8 million children worldwide have been conceived by ARTs. Among them, a higher incidence of adverse perinatal outcomes, birth defects and imprinting disorders, often associated with DNA methylation perturbations, has been reported. Indeed, periods of germ cell and early embryo development are particularly sensitive to ARTs because DNA methylation patterns are highly dynamic. To establish and maintain proper DNA methylation profiles, methyl donors such as folate are essential. Using a mouse model of ARTs, our group recently found that at midgestation, ARTs were associated with increased developmental delay, decreased DNA methylation at certain imprinted loci as well as genome-wide alterations. In addition, these DNA methylation defects were more pronounced in placentas versus embryos and partially corrected by folic acid supplementation. Using the same ART model, our collaborators at the University of Pennsylvania reported placental morphological defects at the end of gestation. Thus, we hypothesized that ARTs perturb DNA methylation and gene expression during early placenta development leading to defects at the end of gestation. In addition, we postulated that folic acid supplementation could rescue these defects. To test this hypothesis, outbred female mice were placed on one of three folic acid-supplemented diets for six weeks: either a control diet (CD; 2 mg folic acid/kg), a 4-fold folic acid-supplemented diet (FAS4; 8 mg folic acid/kg) or a 10-fold folic acid-supplemented diet (FAS10; 20 mg folic acid/kg). Mice were then subjected to ARTs similar to those used in humans, which were a combination of superovulation, in vitro fertilization, embryo culture and transfer. Diets were continued throughout gestation until the collection of midgestation embryos and placentas. We performed RNA sequencing on placentas from E10.5 embryos and found 41 and 28 differentially expressed genes (DEGs) in female and male placentas, respectively. Many DEGs were involved in placenta development, angiogenesis, and spermatogenesis; some DEGs clustered at known imprinting regions. In both sexes, FAS4 partially corrected DEGs while FAS10

exacerbated differential expression in male placentas. Using droplet digital PCR in E10.5 placentas, gene expression was validated for a subset of 5 DEGs involved in early placenta development: *Phlda2, EphB2, Igf2*, *Peg3 and L3mbtl1*. Moreover, gene expression was assessed in placentas from developmentally delayed and abnormal embryos. Notably, *Phlda2* and *Igf2* gene expression were lower in female delayed placentas compared with female E10.5 and abnormal placentas. Since imprinted genes rely on DNA methylation for imprinted expression, we assessed DNA methylation at the *Kcnq1ot1* ICR which regulates *Phlda2* expression using bisulfite pyrosequencing. In both sexes, we observed a positive correlation between *Phlda2* expression and *Kcnq1ot1* DNA methylation. ARTs were associated with sex-specific imprinting defects in placental tissues and FAS4 but not FAS10 exhibited partial correction of mean methylation levels in E10.5 placentas. Folic acid supplementation decreased methylation variance in female placentas only. Taken together, ARTs perturbed the expression of key genes involved in early placenta development and function. These alterations may contribute to adverse placental defects observed at the end of gestation in the mouse. Moderate but not high dose folic acid supplementation may be more beneficial in ART pregnancies using a mouse ART model.

Résumé

Au Canada, un couple sur six souffre d'infertilité, définie comme l'incapacité de concevoir après 12 mois ou plus de rapports sexuels réguliers non protégés. Par conséquent, une proportion croissante de couples recourt aux techniques de reproduction assistée (TRA) pour concevoir. Aujourd'hui, plus de 8 millions d'enfants dans le monde ont été conçus grâce à ces techniques. Parmi eux, on signale une incidence plus élevée de conséquences périnatales incluant des malformations congénitales et des troubles de croissance et de l'empreinte génomique, souvent associés à des perturbations de la méthylation de l'ADN. En effet, les périodes de développement des cellules germinales et de l'embryon coïncident avec des vagues dynamiques de méthylation de l'ADN et sont particulièrement sensibles aux TRA. Pour établir et maintenir des profils de méthylation de l'ADN appropriés, les donneurs de groupes méthyle tels que l'acide folique sont essentiels. En utilisant un modèle murin de TRA, notre groupe a récemment découvert qu'à la mi-gestation (jour embryonnaire 10.5 ; E10.5), les TRA étaient associés à un retard embryonnaire accru, à une diminution de la méthylation de l'ADN au niveau de certaines régions contrôles d'empreinte (ICR) ainsi qu'à des altérations dans la méthylation d'ADN à l'échelle du génome. De plus, ces défauts de méthylation d'ADN étaient plus prononcés dans les tissus placentaires que dans les embryons et partiellement corrigés par une supplémentation en acide folique. En utilisant le même modèle murin de TRA, nos collaborateurs de l'Université de Pennsylvanie ont rapporté des défauts morphologiques placentaires à la fin de la gestation. Ainsi, nous avons émis l'hypothèse que les TRA perturbent la méthylation de l'ADN et l'expression des gènes au cours du développement précoce du placenta, entraînant les défauts observés à la fin de la gestation. De plus, nous avons postulé qu'une supplémentation en acide folique pourrait corriger ces défauts. Pour tester cette hypothèse, des souris femelles non-consanguines ont été placées sous l'un des trois régimes supplémentés en acide folique pendant six semaines : soit un régime de contrôle (CD ; 2 mg d'acide folique/kg), un régime supplémenté 4 fois en acide folique (FAS4 ; 8 mg d'acide folique/kg) ou un régime supplémenté 10 fois en acide folique (FAS10 ; 20 mg d'acide folique/kg). Les souris ont ensuite été soumises à des TRA similaires à celles utilisées chez l'homme, soit une

combinaison de superovulation, de fécondation in vitro, de culture embryonnaire et de transfert de blastocystes. Les régimes alimentaires ont été poursuivis tout au long de la gestation jusqu'à la collecte des embryons et des placentas à mi-gestation. Après avoir effectué un séquençage de l'ARN sur des placentas d'embryons E10.5, nous avons trouvé 41 et 28 gènes exprimés de manière différentielle (DEG) dans les placentas d'embryons femelles et mâles, respectivement. De nombreux DEGs étaient impliqués dans le développement du placenta, l'angiogenèse et la spermatogenèse alors que certains DEGs étaient regroupés dans des régions d'empreinte génomique connues. Chez les deux sexes, FAS4 a partiellement corrigé la majorité des DEGs tandis que FAS10 a exacerbé l'expression différentielle, particulièrement dans les placentas d'embryons mâles. En utilisant la technique PCR digitale en gouttelettes sur les placentas collectés à mi-gestation, nous avons validé l'expression génétique de 5 DEGs impliqués dans le développement précoce du placenta, soit *Phlda2, EphB2, Igf2, Peg3* et *L3mbtl1*. En outre, l'expression de ces gènes a été évaluée dans deux autres groupes : les placentas d'embryons anormaux et ceux présentant un retard embryonnaire. Notamment, l'expression des gènes *Phlda2* et *Igf2* était plus faible dans les placentas de femelles retardées par rapport aux placentas de femelles E10.5 et anormales. Puisque les gènes soumis à l'empreinte parentale dépendent de la méthylation de l'ADN, nous avons utilisé une technique de pyroséquençage pour évaluer la méthylation de l'ADN au niveau de l'ICR *Kcnq1ot1* qui contrôle l'expression du gène à empreinte *Phlda2*. Chez les deux sexes, nous avons observé une corrélation positive entre l'expression de *Phlda2* et la méthylation de l'ADN du *Kcnq1ot1* ICR. Les TRA ont été associés à des défauts d'empreinte spécifiques au sexe dans les tissus placentaires et FAS4, mais pas FAS10, a apporté une correction partielle des niveaux de méthylation dans les placentas E10.5. De plus, la supplémentation en acide folique a diminué la variance de la méthylation dans les placentas femelles uniquement. Dans l'ensemble, les TRA ont perturbé l'expression de gènes clés impliqués dans le développement et la fonction précoces du placenta. Ces altérations peuvent contribuer aux défauts placentaires indésirables observés à la fin de la gestation chez la souris. D'après nos résultats obtenus en utilisant un modèle de TRA chez la souris, une supplémentation en acide folique à dose modérée, mais non à forte dose, pourrait être plus bénéfique pour les grossesses conçues par TRA.

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Contribution of authors

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

Placenta tissue samples were provided by Ms. Sophia Rahimi and Ms. Josée Martel[1]. Sample subset selection of placentas from E10.5, delayed and abnormal embryos was performed by the candidate in collaboration with Ms. Josée Martel. Tissue grinding of placenta samples as well as DNA extraction and sexing were performed by the candidate.

Total RNA extraction from placenta samples was performed by Ms. Josée Martel and RNA sequencing was performed by Novogene (Beijing, China). Among differentially expressed genes (DEGs) induced by ARTs, selection of a subset of four DEGs for validation was performed by the candidate with assistance from Ms. Josée Martel. For gene expression analysis using droplet digital PCR (ddPCR), cDNA synthesis and aliquoting to minimize freeze-thaw cycles were performed by the candidate with assistance from Ms. Josée Martel due to high volume of samples. Optimal reference genes identification was performed by the candidate. Forward and reverse primer designs, validation, and optimization for four DEGs selected as a subset as well as two reference genes were performed by the candidate. Preparation of ddPCR reaction log-sheets and mixes for droplet generation were completed by the candidate. Data retrieval from the QX200 reader and analysis using the QX Manager v1.2 Standard software were also performed by the candidate.

Pyrosequencing primer design, bisulfite conversions of all placenta samples and bisulfite pyrosequencing were carried out by the candidate. All data and statistical analysis were completed by the candidate.

All chapters of this thesis, including the manuscript, were written by the candidate. Given that ART procedures and RNA extraction/sequencing of female E10.5 placentas were performed prior to the candidate's arrival in the lab, Ms. Josée Martel revised the material and methods section with minor corrections for accuracy.

List of figures

SUPPLEMENTAL FIGURE 2. Expression of ART-induced DEGs in midgestation male and female placentas.

SUPPLEMENTAL FIGURE 3. Chromosomal distribution of differentially methylated tiles (DMTs) induced by ARTs in placenta.

SUPPLEMENTAL FIGURE 4. Sex-specific response to folic acid supplementation of female ART DEGs in midgestation placentas following ART procedures.

SUPPLEMENTAL FIGURE 5. Sex-specific response to folic acid supplementation of male ART DEGs in midgestation placentas following ART procedures.

List of tables

Abbreviations

% - Percent

> - greater than

< - less than

ºC - degrees Celsius

Adm - adrenomedullin

ARTs - assisted reproductive technologies

AS - Angelman syndrome

Asb4 - Ankyrin Repeat and SOCS Box-Containing 4

BHMT - betaine-homocysteine methyltransferase

BS – bisulfite

BWS - Beckwith-Wiedemann syndrome

CD - folic acid control diet

cDNA – complementary deoxyribonucleic acid

Ceacam1 - carcinoembryonic antigen-related cell adhesion molecule 1

CF1 - Non-Swiss Albino-Carworth Farms-1

CH3 - methyl group

CO_2 - carbon dioxide

COC - cumulus-oocyte complex

CpG - cytosine-phosphate-guanine

CTCF - CCCTC-binding factor

Csf2rb - colony Stimulating Factor 2 Receptor Subunit Beta

Cysltr2 - cysteinyl leukotriene receptor 2

DEG – differentially expressed gene

ddPCR – droplet digital polymerase chain reaction

DHF - dihydrofolate

DMG - dimethylglycine

DMR - differentially methylated region

DMT - differentially methylated tile

DNA - deoxyribonucleic acid

DNAme – DNA methylation

DNMT - DNA methyltransferase

Dpc - days post coitum

E - embryonic day

EPC – ectoplacental cone

EphB2 - ephrin B receptor 2

EXE - extraembryonic ectoderm

FA – folic acid

FAS4 - 4-fold folic acid-supplemented diet

FAS10 - 10-fold folic acid-supplemented diet

FGR – fetal growth restriction

Fos - FBJ osteosarcoma oncogene

FSH - follicle stimulating hormone

GC – glycogen cell

h - hour

hCG - human chorionic gonadotropin

Htatip2 - HIV-1 Tat Interactive Protein 2

HTF - human tubal fluid

ICM - inner cell mass 11

ICR - imprinting control region

ICSI - intracytoplasmic sperm injection

IUGR – intrauterine growth restriction

Igf2 - insulin-like growth factor 2

Igf2r - insulin-like growth factor 2 receptor

IP - intraperitoneal

Iqcg - IQ Motif Containing G

IU - international unit

IUI – intrauterine insemination

IVF – *in-vitro* fertilization

JZ – junctional zone

Kcnq1ot1 - *Kcnq1* overlapping transcript 1

Klf14 - Krüppel Like Factor 14

KRAB - Krüppel associated box

KSOM+AA - potassium simplex optimization medium (1x) with ½ amino acids

L3mbtl1 – Lethal (3) malignant brain tumor-like protein 1

LH - luteinizing hormone

lncRNA - long non-coding ribonucleic acid

mg - milligram

MII - metaphase II

mL - milliliter

mRNA – messenger ribonucleic acid

MTR - methionine synthase

MTHFR - methylenetetrahydrofolate reductase

N2 - nitrogen gas

NAT_CD - natural mating folic acid control diet

Nodal - Nodal Growth Differentiation Factor

NSET - non-surgical embryo transfer

NTD - neural tube defect

O2 - oxygen gas

PCR - polymerase chain reaction

PE – preeclampsia

Peg3 - paternally expressed gene 3

PGCs - primordial germ cells

Phlda2 - Pleckstrin Homology Like Domain Family A Member 2

PMSG - pregnant mare serum gonadotropin

PWS - Prader-Willi syndrome

qRT-PCR - Real-Time Quantitative Reverse Transcription PCR

RBC - red blood cell

Rhox13 - reproductive homeobox 13

RNA - ribonucleic acid

Rpl13a - Ribosomal Protein L13a

Rpl39l - Ribosomal Protein L39 Like

Rps18 - Ribosomal Protein S18

RRBS - Reduced-representation bisulfite sequencing

SAH - S-adenosylhomocysteine

SAM - S-adenosylmethionine

SEM - standard error of the mean

sFLT1 - Soluble fms-like tyrosine kinase-1

Slc38a4 - Solute Carrier Family 38 Member 4

Smoc1 - SPARC related modular calcium binding 1

Snrpn - small nuclear ribonucleoprotein polypeptide N

SPT - spongiotrophoblast

SRS - Silver-Russell syndrome

Synt - syncytiotrophoblast

TB – trophoblast

TGC – trophoblast giant cell

THF - tetrahydrofolate

TE - trophectoderm

TET - ten-eleven translocation

TRIM28 - tripartite motif-containing 28

ug - microgram

VEGF - vascular endothelial growth factor

XCI - X chromosome inactivation

ZFP57 - zinc finger protein 57

Zrsr1 - zinc finger (CCCH type), RNA binding motif and serine/arginine rich 1

CHAPTER 1: Literature review

Since the birth of the first IVF-conceived baby; Louise brown in 1978; millions of children have been born worldwide using assisted reproductive technologies (ARTs). Indeed, although most children are healthy, there has been concern over the short-term and long-lasting effects of ARTs. Over the years, reports of adverse perinatal outcomes and birth defects have emerged concerning ARTs. Thus, studies must be conducted to assess the health of ART offspring.

1.1 Introduction

The Developmental Origins of Health and Disease (DOHaD) hypothesis states that parental and environmental factors can have an impact on embryonic development and offspring health persisting until adulthood, where diseases may originate from the time in-utero[2]. This is especially relevant to the field of assisted reproduction given that ART procedures, which are performed during early embryonic development, have been associated with several pregnancy complications and adverse conditions in children born using ARTs. Since ARTs overlap with key periods of DNA methylation reprogramming, epigenetic instability has been proposed as a potential mechanism explaining adverse effects[3].

Given that methyl donors such as folic acid are essential to establish and maintain DNA methylation patterns during embryonic development, our study looked to investigate whether FA supplementation could rescue the epigenetic instability induced by ARTs. Particularly, we were interested in examining placentas since fetal development is completely dependent on the placenta for nutrient and gas exchange, immune protection, and endocrine function amongst other roles. Studies have linked placenta maldevelopment to adverse fetal health and have provided evidence that ARTs can disrupt embryonic health by dysregulating placenta development and function, through genomic imprinting and other perturbations[4-6].

In Canada, it has been reported that women of reproductive age often exceed the recommended level of FA intake, as prenatal supplements prescribed and/or marketed typically contain ≥ 1mg/day, in addition to dietary folate [7,8]. This is of particular concern because evidence from human[9-11] and animal[12,13] studies suggests that high folate leads

to potential adverse consequences. For this reason, our study further investigated whether the effects of FA supplementation on ART placentas was dose dependent, using clinically relevant moderate and high FA doses in an outbred mouse strain, representative of the genetic variability found in the humans. In addition, the mouse model was used because of the notable structural and functional similarities between rodent and human placentas[14].

Altogether, our study focused on investigating the effects of folic acid supplementation on the placenta during early gestation, following assisted reproduction. Over the next few pages, I will introduce and cover current knowledge on assisted reproductive technologies, DNA methylation reactions and folic acid supplementation, before diving into the rationale and hypothesis of this study.

1.2 Assisted reproductive technologies (ARTs)

1.2.1 Infertility and rise of ARTs

Over the past decades, there has been a significant increase in the use of ARTs to achieve pregnancy. Recent data shows that more than 8 million children have been conceived by ARTs[15]. Infertility, or the inability to conceive, remains a problem of global concern, affecting as many as 186 million people worldwide[16], and approximately 1 in 6 couples In Canada[17].

There are several uniparental or mixed factors contributing to the current prevalence of infertility[18]. In addition, sexually transmitted diseases, or environmental exposures adversely affect fecundity[19]. For these reasons, couples experiencing infertility frequently turn to ARTs as medical treatment. ART procedures comprise a group of techniques including intrauterine insemination (IUI), superovulation, in-vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), frozen embryo transfer and embryo culture.

1.2.2 Health outcomes in human studies

Though most children conceived with ARTs are born healthy, increasing evidence from epidemiological studies suggests that ART pregnancies may be at higher risk of adverse perinatal outcomes compared to those naturally conceived, including preterm birth, low birth weight, small for gestational age, stillbirth, and perinatal mortality[20,21]. In

addition, ART pregnancies have been associated with obstetric complications such as hypertensive disorder in pregnancy, gestational diabetes, and placental complications amongst others[22,23]. The first and second-risk trimester (FASTER) trial found that women who had with ART pregnancies were at higher risk (2.7-fold) of preeclampsia (PE) than those who conceived naturally [24,25].

Bonduelle and coworkers defined birth defects associated with ARTs as major malformations causing functional impairment and requiring surgical correction[26]. Along with other studies [27,28], a meta-analysis by Hansen and coworkers found that infants conceived with ARTs had a 32% increased risk of birth defects compared with naturally conceived infants[29]. In addition, a meta-analysis by Qin and colleagues found that the risk of congenital malformations was higher in ART-conceived children [30].

Multiple studies have investigated the effects of ARTs on neurodevelopmental health. Attention deficit hyperactivity disorder (ADHD) was associated with IVF in a Swedish study[31]. Furthermore, Fountain and colleagues reported an increased incidence of autism spectrum disorder (ASD) in multiples and singletons conceived by ARTs[32]. Moreover, an elevated risk of cerebral palsy was detected among 5680 children born after IVF[33].

In addition, the impact of ART on cardiovascular and metabolic health has been the subject of multiple studies, recently summarized in a meta-analysis by Guo and colleagues[34]. Findings indicate that IVF/ICSI children display a slight yet significant increase in blood pressure and vessel thickness compared to those naturally conceived, without alterations in their lipid metabolism by early adulthood[34]. Limited findings suggest that ARTs may be associated with suboptimal cardiovascular function[35,36].

Moreover, ARTs have been associated with an increased risk of imprinting disorders[37-39]. The Silver-Russel (SRS), Beckwith-Wiedemann (BWS), Angelman (AS) and Prader-Willi (PWS) syndromes have been associated with ARTs, with a combined odds ratio of 3.67 [95% CI 1.39–9.74][40]. Similarly, a recent meta-analysis by Cortessis and colleagues demonstrated a positive correlation between ARTs and all four imprinting disorders (SRS, BWS, PWS, AS), where the BWS had an odds ratio of 5.8 [95% CI 3.1–11.1][41].

1.3 DNA methylation

Infertility and parental factors combined with ARTs could be among the many factors contributing to ART-associated abnormal outcomes. Though the reason and exact mechanism(s) for increased adverse outcomes associated with ARTs remain unknown, studies have shown that ART procedures are performed during germ cell and preimplantation development when DNA methylation patterns are highly dynamic (**INTRODUCTION FIGURE 1**)[3]. Thus, ARTs may cause epigenetic instability during early embryo development leading to adverse outcomes[3].

As such, DNA methylation patterns undergo two major epigenetic reprogramming waves, during gametogenesis and early embryonic development, involving de novo DNA methylation and demethylation[42]. In mammalian development, DNA methylation is essential for meiotic progression[43] and plays important roles in transposons silencing, X-chromosome inactivation, genomic imprinting and transcriptional regulation by recruiting proteins or by inhibiting binding of transcription factors to DNA[42,44].

1.3.1 DNA methylation reactions

DNA methyltransferase enzymes (DNMTs) catalyze the DNA methylation reaction by transferring a methyl group from S-adenyl methionine (SAM); the universal methyl donor; to the fifth carbon of a cytosine residue to form 5-methylcytosine (5mC)[45]. DNMT3a and DNMT3b are referred to as de novo DNMTs because they establish new DNA methylation patterns onto unmodified naked DNA. Additionally, enzymatically inactive DNMT3L interacts with de novo DNMTs and stimulates their activity. Unlike de novo DNMTs, DNMT1, recruited by the ubiquitin-like protein (UHRF1), preferentially methylates hemimethylated DNA and is therefore responsible for DNA methylation maintenance[42,46,47]. Both de novo and maintenance DNMTs are essential for proper development as DNMT1 or DNMT3B knockout mice die in utero during gestation, while DNMT3a knockout mice die approximately within 4 weeks after birth[48,49]. Active DNA demethylation is carried out by Ten-eleven translocation (TET) enzymes[45].

1.3.2 DNA methylation reprogramming during development

Similar to humans, the first major event of DNA methylation reprogramming in the mouse occurs during gametogenesis, where genome-wide DNA demethylation occurs at imprinted differentially methylated regions (DMRs) and other genomic sequences in primordial germs cells (PGCs)[50]. DNA demethylation is complete by embryonic day (E) 13.5 [50] and remethylation appears to be sex-specific, occurring at different times in oocytes and sperm[3] (**INTRODUCTION FIGURE 1**). In male germ cells, DNA methylation marks are re-established around E15-E16 and completed by birth[50-53] whereas in female germ cells, remethylation takes place after the onset of puberty; during oocyte growth; and is completed by metaphase II [50,53-55]. It is important to note that methylation imprints are acquired asynchronously during oocyte growth[55]. As such, certain imprinted genes – expressed from only one parental allele – acquire their methylation marks earlier (e.g., *Kcnq1*) or later (e.g., *Peg1*) during oocyte growth, and therefore may be differently susceptible to disruption by ARTs (e.g., superovulation) as discussed by Fortier and colleagues[56].

The purpose of the second wave is for the embryo to acquire totipotency: after fertilization and during early embryogenesis, gamete-inherited methylation marks are erased in preimplantation embryos and embryonic methylation marks are established upon implantation[57]. Recent data suggests that DNA demethylation of the paternal genome is actively catalyzed by TET3 enzyme, while loss of 5mC in the maternal genome is passive, mainly due to the lack of DNMT1 in the oocyte nuclei [45]. Despite the extensive demethylation, imprinted genes maintain their methylation. This is possible due to the action of DNMT1 required for the maintenance of methylation imprints [45,57]. At the eight-cell stage of preimplantation embryos, DNMT1o inherited from oocytes is the predominant DNMT1 isoform and is responsible for maintaining imprinted methylation[58]. Additionally, the ZFP57-TRIM28 complex plays a critical role maintaining imprinted methylation by binding to specific genetic motifs (TGCCGC) present at imprinting control regions (ICRs)[57]. Li and colleagues demonstrated that knockout of the maternal effect gene ZFP57 (encodes a KRAB zinc finger protein) in mouse zygotes results in embryonic lethality because of failure to maintain maternal and paternal methylation imprints after fertilization at multiple imprinted regions[59].

1.3.3 Genomic imprinting

Genomic imprinting refers to the monoallelic expression of an imprinted gene due to the differential DNA methylation patterns established in the two parental germlines; these imprinted genes withstand genome-wide demethylation that occurs during preimplantation development[45]. In 1984, experiments by McGrath and Solter and by Surani et al. determined that maternal and paternal genomes were not identical in their contributions to embryonic development[60,61]. This was concluded after mammalian parthenogenetic and androgenetic embryos containing two sets of maternal or paternal chromosomes respectively, showed different phenotypes and died shortly before implantation[60,61]. The earliest imprinted genes to be discovered were *Igf2*, *Igf2r* and *H19* [62-65].

1.3.3.1 Imprinted genes

Studies on imprinted genes have revealed that they mostly regulate embryonic, placental, and neonatal growth[66]. As such, paternally expressed genes such as *Igf2*, *Peg1* and *Peg3* are growth promoters whereas maternally expressed genes including *Cdkn1c* and *H19* often function as growth repressors[67]. For instance, inheritance of an *Igf2* variant in humans resulted in growth restriction[68] and silencing of maternally expressed *Cdkn1c* was associated with the BWS[69].

There are hypotheses surrounding the function and origin of genomic imprinting. The first hypothesis is the “parental conflict” theory which states that paternally expressed genes promote embryonic growth by maximizing maternal resources whereas maternally expressed genes suppress fetal growth to allow for maternal resource preservations for future pregnancies[70]. The second hypothesis is referred to as “trophoblast defense”. The theory proposes that if oocyte or sperm activation occur spontaneously, the maternal genome is more at risk of ovarian trophoblast disease than the paternal genome given the oocyte anatomical equipment in comparison to the sperm[71]. Therefore, genomic imprinting activates the expression of genes repressing placental development and inhibits the expression of genes promoting this process[71]. Though none of these hypotheses have been confirmed, they indicate a role of imprinted genes in fetal and placental development [66].

1.3.3.2 Regulation mechanisms of imprinted genes

To date, approximately 150 imprinted genes have been identified in the mouse and the majority are clustered into genomic regions mapped across 17 chromosomes including the X chromosome[72]. Many of the imprinted gene clusters are coregulated by a cis-acting ICR, able to control the expression of imprinted genes[73]. There are two main mechanisms involved in genomic imprinting control, previously discussed by Wan and Bartolomei[74]. The first is the enhancer-insulator model, where the insulator can block enhancer-promoter interactions when placed between them[74]. This model applies to the *H19*/*Igf2* locus located at the human 11p15.5 imprinted region. In this case, the *H19* ICR - located between *H19* and *Igf2* - is paternally methylated and maternally unmethylated. The CTCF insulator protein binds to the unmethylated *H19* ICR on the maternal allele resulting in silencing of *Igf2* (blocks *Igf2* promoter and enhancer interactions) and preferential expression of *H19* from the maternal allele. The CTCF is unable to bind the paternal chromosome due to methylation which allows for expression of *Igf2* and silencing of *H19* from the paternal allele[74-76].

The second mechanism of regulation of imprinted genes is mediated by non-coding RNAs (ncRNA)[74]. The *Kcnq1* locus is a cluster of well-studied imprinted genes (i.e., *Cdkn1c*, *Phlda2*, and *Kncq1*) regulated by the *Kcnq1ot1* long (>60kb) ncRNA (lncRNA)[77]. *Kcnq1*ot1 expression relies on the DNA methylation status of its promoter contained within the KvDMR1 ICR[78,79]. KvDMR1 ICR (also referred to as *Kcnq1ot1* ICR in Chapter II) is methylated on the maternal allele and unmethylated on the paternal allele; thus, paternally expressed[78]. Loss of maternal-specific methylation at the KvDMR1 ICR results in biallelic expression of *Kcnq1*ot1 and silencing of maternally expressed genes such as *Phlda2* across the domain[77].

1.4 Genomic imprinting disorders in humans

ART-induced epigenetic defects at imprinting control regions have been associated with imprinting disorders such as the BWS and SRS[80,81]. The BWS is an overgrowth syndrome characterized by features such as macroglossia (enlarged tongue), and anterior abdominal wall defects [82]. In the general population, the incidence of BWS is estimated to be 1/13700 [83]. Tenorio and colleagues studied 156 patients with BWS

born after ARTs or conceived naturally (ART: 139; conceived naturally: 17)[84]. They demonstrated that the odds ratio of having BWS after ARTs was 7 times higher than when conceived naturally[84]. Moreover, they found that 88% of BWS patients born after ART had hypomethylation at the *Kcnq1*ot1 ICR compared to 50% in BWS patients conceived naturally[84]. Overall, 50% of BWS patients exhibit loss of methylation at the maternal *Kcnq1*ot1 ICR while 5-10% show gain in methylation at the maternal *H19*/*Igf2* ICR[83,85].

The Silver-Russel syndrome (SRS) has also been studied for its association with ARTs and imprinted genes at 11p15.5[81]. SRS is characterized by pre- and post-natal growth restriction[86]. The incidence of SRS varies between 1/30000 to 1/100000 in the general population[87]. Among SRS patients, hypomethylation at the *H19*/*Igf2* ICR resulting in the silencing of *Igf2* from both parental alleles was observed in 30% of cases[88]. However, some reports have studied the association between SRS with ART, namely IVF and ICSI, with no conclusive results[89].

Taken together, imprinting disorders have been associated with ARTs and characterized by epigenetic disruption, as evidenced by DNA methylation abnormalities following assisted reproduction.

1.5 ARTs in animal models

Animal models have been used to separate the effects of ARTs from the underlying impact of confounding factors such as infertility[22]. Factors such as age, genetics, environment, and reproductive disease can affect perinatal outcomes. In fact, a large study in the Netherlands found that birth weight was independently associated with both parental and ART characteristics[20]. Given that infertility/subfertility and ARTs influence pregnancy outcomes, animal models are essential to determine the effects of ARTs.

1.5.1 Adverse developmental outcomes associated with ARTs in animal models

The effects of ARTs have been studied in various animals including mice to eliminate confounding effects such as infertility and advanced maternal age [90]. These models offer an alternative way to investigate ART effects and improve ART procedures.

Ertzeid and Storeng found that superovulation impaired oocyte/embryo quality and development while also altering the uterine environment and receptivity[91]. As such,

superovulation of female mice was associated with increased post-implantation mortality, increased proportion of abnormal preimplantation embryos [92,93], fetal growth retardation [92-94]and lower implantation rates[94]. In addition, similar results were reported by Fortier et al., as superovulation was associated with increased developmental delay[56].

Schwarzer and colleagues identified embryo culture components influencing development, including composition of culture media, oxygen levels, pH, temperature, and culture dish elasticity[95]. In their study, 13 human ART culture protocols were compared for their effects on embryo development, gene expression and cell lineage composition of mouse zygotes[95]. They found culture-medium specific differences in blastocyst and fetal development, litter sizes and transcriptome profiles, indicating that embryo culture environment affects mouse embryonic development[95].

Moreover, IVF and ICSI fertilization methods were compared using a mouse ART model[96]. In this study, female mice were super-ovulated by injection of the pregnant mare serum gonadotropin (PMSG), which promotes follicular growth and maturation, and the human chorionic gonadotropin (hCG) 48 hours later to stimulate ovulation of multiple oocytes[96]. To achieve IVF, oocytes and spermatozoa were collected and cultured to promote fertilization whereas oocytes were microinjected with spermatozoa during the ICSI procedure[96]. Resulting embryos were cultured until the blastocyst stage (E3.5) using two different culture mediums, before transfer into the uterus[96]. Results showed that method of fertilization more than culture medium determined the transcriptome of blastocysts[96]. ICSI was also associated with reduced blastocyst cell number. Chen and colleagues also demonstrated that embryo culture increased the incidence of large offspring syndrome - an overgrowth syndrome like the BWS - in the bovine model[97].

1.5.2 Epigenetic and long-term effects associated with ARTs in animal models

Epigenetic defects related to DNA methylation have been detected in various animal models following ARTs[98].

As described previously, superovulation involves the administration of exogenous gonadotrophins to induce growth, maturation, and ovulation of multiple oocytes[98]. Studies have reported possible effects of superovulation on imprinting maintenance [56,99]. Superovulation has been shown to alter the methylation of certain imprinted genes[56]. Yu

et al showed that superovulation perturbs the genome-wide methylation erasure process in mouse preimplantation embryos[100]. Moreover, superovulation led to a dose-dependent loss of *Snrpn*, *Peg3*, and *Kcnq1ot1* and gain of *H19* imprinted methylation in mouse blastocysts[101]. Our group demonstrated that superovulation resulted in aberrant biallelic expression of Snrpn and *H19* in midgestation placentas[56]. Although a follow-up study determined that gene expression levels of these genes were normalized later in gestation[102], epimutations in somatic cells of pups born from superovulated naturally mated female mice provided evidence of persisting effects into later stages of development.

In mice, evidence shows that embryo culture also compromises imprinting maintenance as preimplantation embryo culture led to aberrant expression and methylation of *H19* in fetal tissues and activation of normally silent genes in placental tissues[103]. To determine whether preimplantation embryos are more susceptible to epigenetic defects at specific stages, Vrooman and colleagues recently compared the effects of embryo culture performed during different windows of preimplantation development[104]. For this, 4 groups of embryo culture at different stages were defined in their experiments: 1-cell-morulae (66h culture), 4-cell-morulae (24h culture), 1-cell-blastocyst (96h culture) and morulae-blastocyst (24h culture)[104]. At 18.5, the 1-cell-morulae culture group was associated with decreased placental DNA methylation and increased variance at *H19/Igf2* and *Peg3* ICRs while 4-cell-morulae group exhibited hypomethylation at *H19/Igf2* ICR[104]. Extended culture in the 1-cell-blastocyst group induced additional placental DNA methylation changes at *H19/Igf2*, *Kcnq1ot1* and *Peg3* ICRs; however, the morulae-blastocyst culture group was not associated with significant changes[104]. These results suggested that ICRs are affected by embryo culture asynchronously during preimplantation development. Likewise, IVF-derived embryos displayed aberrant *H19* imprinting[105]. Overall, suboptimal culture conditions interfere with imprinting maintenance; thus, highlighting the importance of optimization to ensure adequate development [99].

Finally, we and others have shown that cumulative ART procedures are associated with epigenetic deregulation in mouse embryos and placentas[1,6]. In addition to phenotypic similarities between large offspring syndrome and BWS described by Chen and

colleagues[97], epigenetic defects at the KvDMR1 ICR were observed: *Kcnq1*ot1 lncRNA was biallelically expressed in some overgrown conceptuses and associated with loss of methylation at the KvDMR1 ICR on the maternal allele[97].

Animal models of ARTs can also be useful to determine the long-term effects of ARTs that are often overlooked. In mice, embryo culture increased systolic blood pressure in 21-week-old mice independent of litter size, maternal origin, or body weight and altered gene expression of enzymatic regulators of cardiovascular and metabolic physiology[106]. Moreover, IVF-conceived mice displayed unique metabolic profiles compared to those naturally conceived, resulting in pathway changes affecting glucose metabolism[107].

1.6 Placenta development

Epigenetic deregulation during placentation has been associated with ARTs and demonstrated in cases of intrauterine growth restriction (IUGR), preeclampsia (PE) and other pregnancy complications[108,109]. Given that disturbed placentation can be detrimental to both mother and child [108], it is essential to elucidate the mechanism through which ARTs lead to adverse placental outcomes.

1.6.1 Mouse placenta development

During pregnancy, the placenta is essential for optimal fetal growth and survival. It mediates implantation by anchoring the newly formed blastocyst into the uterine wall and establishes the interface for nutrient and gas exchange between maternal and fetal circulations[110]. In addition, the placenta protects the growing fetus from the maternal immune system by producing several hormones to minimize immunological rejection[111] .

In mice, the blastocyst forms at E3.5 and consists of the outer trophectoderm cells, which give rise to the placenta and the inner cell mass which later form the embryo[112]. At the time of implantation (E4.5), the outer invasive mural trophectoderm cells form trophoblast giant cells (TGCs) while those that overlie the inner cell mass differentiate into the extra-embryonic ectoderm (EXE) and the ectoplacental cone (EPC) [111,112]. With gastrulation at E6.5, cells of the EXE form the trophoblast (TB) cells of the chorion[111,112]. Around the same time, the allantois develops from the extra-embryonic mesoderm at the

posterior end of the embryo and later fuses with the chorion in a process known as the chorioallantoic attachment, essential for further pregnancy progression[111]. Chorionic TB cells eventually differentiate into syncytiotrophoblast (SynT) cells (two layers in mice and one in humans) to establish the exchange interface referred to as the labyrinth[112]. The junctional zone (JZ) is positioned between the labyrinth and the decidua; and it contains three main cell types: spongiotrophoblasts (SpT) glycogen cells (GCs) and trophoblast giant cells (TGCs) in contact with the maternal decidua[112].

At midgestation, the mouse placenta constitutes three main structures: the maternal decidua; the JZ and the labyrinth[111,113]. Mice and humans share a hemochorial type of placentation, which means that fetal trophoblasts are in direct contact with maternal blood, allowing for efficient nutrient transfer[112]. For this reason, mice have been increasingly used to study placental development.

1.6.2 Placenta-specific imprinted genes

Placenta development is regulated by several highly expressed imprinted genes[114]. Targeted gene mutation studies in mice have revealed their importance in placenta development and function[111]. A common feature is that they regulate nutrient exchange at the maternal-fetal interface; mutants exhibit fetal growth restriction caused by a failure of the placenta[111]. For instance, the function of the *H19*/*Igf2* ICR in placental development has been well studied: *Igf2* expression is driven by promoters P1, P2, P3 in TGCs, SpT, GCs and fetal endothelium, with an additional P0 transcript in labyrinthine cell layers[114]. In mice, loss of *Igf2* leads to IUGR [62,115] while overexpression leads to fetoplacental overgrowth [116].

Genes from the *Kcnq1* cluster on mouse chromosome 7 such as *Ascl2* and *Cdkn1c* have been some of the most studied for their effect on placental development. *Ascl2* was the first imprinted gene shown to be required for embryonic viability[117]. *Cdkn1c* encodes a kinase inhibitor which negatively regulated cell growth and proliferation: mouse mutants exhibit placental overgrowth with large thrombotic lesions in the labyrinth zone[118].

Taken together, current evidence highlights the important role of imprinted genes in placental development as aberrant imprinting in the placenta during gestation could have adverse effects on fetoplacental development.

1.6.3 ART-induced epigenetic and morphological alterations in placenta

Given the association between ARTs and an increased risk of PE and IUGR, often caused by placental failure, it is paramount to investigate the effects of ARTs on placental development[25,119,120]. Evidence from human and mouse studies have demonstrated that ART procedures induce epigenetic and morphological abnormalities in the placenta [121]. A recent human study by Choufani and colleagues used the 450K analysis to find genome-wide epigenetic abnormalities in matched placentas from ART and control singleton pregnancies[122]. Once ART placentas were separated by ART procedure; they found IVF or ICSI groups had distinct epigenetic profiles as compared to less invasive procedures (IUI, ovulation induction)[122]. Similarly, a human study by Song and al. found altered CpG DNA methylation levels in placentas from ART conceived children compared to those conceived naturally[123].

Evidence from two important mouse studies revealed that ARTs induce adverse outcomes on placentation. Using a mouse model of ARTs, a study by De Waal et al. showed that ART procedures induce epigenetic perturbations in extraembryonic tissues resulting in adverse placental phenotypes[124]. In this study, mice were subjected to superovulation, IVF, embryo culture and embryo transfer procedures, following which placental morphology and epigenetic profiles were assessed at E18.5[124]. De Waal et al. observed increased placental weight particularly due to JZ overgrowth and a reduced fetal-to-placental weight ratio[124]. They also found global and imprinted gene hypomethylation at *H19*/Igf2, *Peg3* and *Kcnq1*ot1 ICRs in IVF placentas[124]. Using the same model, the group also found early abnormalities in the placenta labyrinth vasculature due to embryo culture, corrected later in gestation[6]. The placenta composition of different cell lineages (TGCs and SynT cells) was also altered by E18.5[6]. Moreover, ARTs were correlated with increased levels of sFLT1, an anti-angiogenic marker implicated in PE[6]. Adding to previously mentioned epigenetic defects induced by embryo culture during preimplantation development, placenta JZ overgrowth and increased levels of parietal TGCs were observed in the 1-cell-morulae, 4-cell-morulae and 1-cell-blastocyst groups compared to controls[104]. Thus, embryo culture at different stages of preimplantation development, overlapping with genome-wide DNA demethylation, leads to JZ alterations detectable at the end of gestation in the mouse[104].

Taken together, these key studies suggest that both human and mouse placentas are highly susceptible to epigenetic modifications following ARTs. While ART procedures have also been associated with adverse placental phenotypes, the role of these epigenetic defects in these outcomes remain to be explored as they could have consequences on long-term health in children.

1.7 Folic acid supplementation

1.7.1 Folic acid and neural tube defects

Neural tube defects (NTDs), such as spina bifida or anencephaly, are birth defects of the central nervous system that arise due to incomplete closure of the neural tube during embryogenesis[125]. In 1998, folic acid (FA) fortification was made mandatory to reduce the prevalence of NTDs in Canada the incidence of NTDs dropped from 1.58 per 1000 births pre-fortification to 0.86 in the year 2000[126]. One important study that showed the benefits of FA was a double-blind randomized trial performed by the UK Medical Research Council comparing the effect of 4 mg/day FA with a multivitamin supplement (with or without FA) or a placebo in a cohort of women with a known history of NTDs-affected offspring[127,128]. The study showed a 72% decrease in the number of NTDs in the FA-supplemented group compared to the placebo group[127,128]. This study, in addition to other large studies that followed, was instrumental in proving that periconceptional FA supplementation prevents NTDs[127,129].

Despite FA fortification of certain foods, folate intake solely from food sources may not be adequate, especially for women of reproductive age[130]. Today, the pre- and periconceptional use of FA supplements is recommended by Health Canada for women of childbearing age[131]. Current guidelines recommend daily use of a multivitamin containing 0.4-1 mg FA for low to moderate risk pregnancies taken starting 2-3 months before conception[132]. Up to 4 mg/day FA is recommended for women with high-risk of NTDs taken from 3 months before gestation until 12 weeks of pregnancy; then, a lower dose of 0.4-1 mg/day FA is recommended until the end of lactation[132]. A pregnancy is considered high-risk if either partner has personal history of NTDs, or NTDs-affected children[132].

1.7.2 One-carbon metabolism and DNA methylation

Folate is the natural form of vitamin B9, an essential water-soluble B-vitamin naturally found in fruits and leafy vegetables[132]. Given that humans do not synthesize folate, adequate intake is achieved through consumption of folate-containing foods or through supplementation using folic acid[133]. Folate takes part in one-carbon metabolism by providing one-carbon units **(INTRODUCTION FIGURE 2)**[134]. This process is essential to several physiological processes including DNA methylation[135].

One-carbon metabolism is driven by methyl donors; folate and methionine; linked by methionine synthase (MS), the rate limiting enzyme that converts homocysteine (Hcy) to methionine. In the cell, folate enters the one carbon metabolism pathway through its conversion into dihydrofolate (DHF), which is enzymatically reduced to tetrahydrofolate (THF). Subsequently, THF is converted to 5,10-methyleneTHF which is reduced to 5-methytetrahydrofolate (5-mTHF) – the major circulatory form of folate - by 5, 10-methylenetetrahydrofolate reductase (MTHFR). 5-mTHF acts as a methyl donor to form methionine from Hcy. In the liver, betaine which is derived from dietary choline, can act as a methyl donor for betaine-homocysteine S-methyltransferase (BHMT) to generate methionine and dimethylglycine (DMG) as a by-product. Methionine is then converted into S-adenosyl methionine (SAM), the universal methyl donor used by multiple methyltransferases including DNMTs[136]. As a result, the availability of methyl donors such as folate is critical to ensure adequate SAM levels necessary to establish and maintain proper DNA methylation profiles during development.

1.7.3 Effects of high FA intake

Since women of reproductive age often exceed higher-than-recommended doses of FA supplementation[7,8], recent studies have investigated the effects of exposure to high levels of FA suggesting possible deleterious effects[137]. Human studies have shown that maternal folate deficiency and supplementation can alter DNA methylation status in offspring[138]. In fact, a meta-analysis from two population-based birth cohort studies by Joubert and colleagues found significant associations between maternal plasma folate levels and cord blood DNA methylation at 443 CpGs (48 after Bonferroni correction), representative of genes implicated in developmental pathways[139]. Notably, children of

mothers who were supplemented with FA periconceptionally (400mg/day) showed a 4.5% (49.5% vs. 47.4%, P = 0.014) increase in DNA methylation at the *IGF2* DMR compared with control children[140]. In addition, folate deficiency has been linked to DNA hypomethylation[141]. A study by Chang and al. found hypomethylation in the brain of fetuses with NTDs, who had lower maternal serum folate levels compared to controls[142]. In humans, impaired psychomotor development during infancy was also associated with high maternal FA intake (>5mg/day)[10].

Recent reports suggest that excessive FA supplementation mimics folate deficiency by impairing the one-carbon metabolism cycle[12,13,143]. Key mouse studies from the Rozen lab have demonstrated that maternal 5x FA supplementation equivalent to 10mg/kg diet (half of FAS10 in our study) decreased MTHFR mRNA protein levels in mothers and male offspring[143]. This effect led to behavioral perturbations and altered choline-derived metabolites in the offspring liver and cerebral cortex[143]. Consistent with these findings, additional mouse studies from the same group showed that maternal 10x FA supplementation equivalent to FAS10 dose used in our study (20mg/kg diet) led to embryonic loss and delay[13], memory impairment in offspring, reduced MTHFR protein/mRNA levels in offspring liver, perturbed choline metabolism and smaller embryos/placentas by E17.5[144]. Notably, expression alterations of enzymes involved in one-carbon metabolism were also detected in embryos[144]. Thus, excessive maternal FA supplementation seems to be impairing the one-carbon metabolism pathway by altering the expression of key enzymes involved in the pathway and therefore ultimately eliciting similar effects as folate deficiency. As such, there is a need to characterize the effects of high dose maternal FA supplementation and determine an optimal dose, especially in the context of assisted reproduction.

1.8 Study rationale, hypothesis, and objectives

Previously, we examined the impact of moderate and high dose FA supplementation on reproductive and epigenetic outcomes in a mouse model of ARTs where mouse ARTs consisted of superovulation, IVF, embryo culture and embryo transfer[1]. First, ARTs were associated with increased embryonic mortality and developmental delay[1]. While FA supplementation did not affect the rate of embryonic

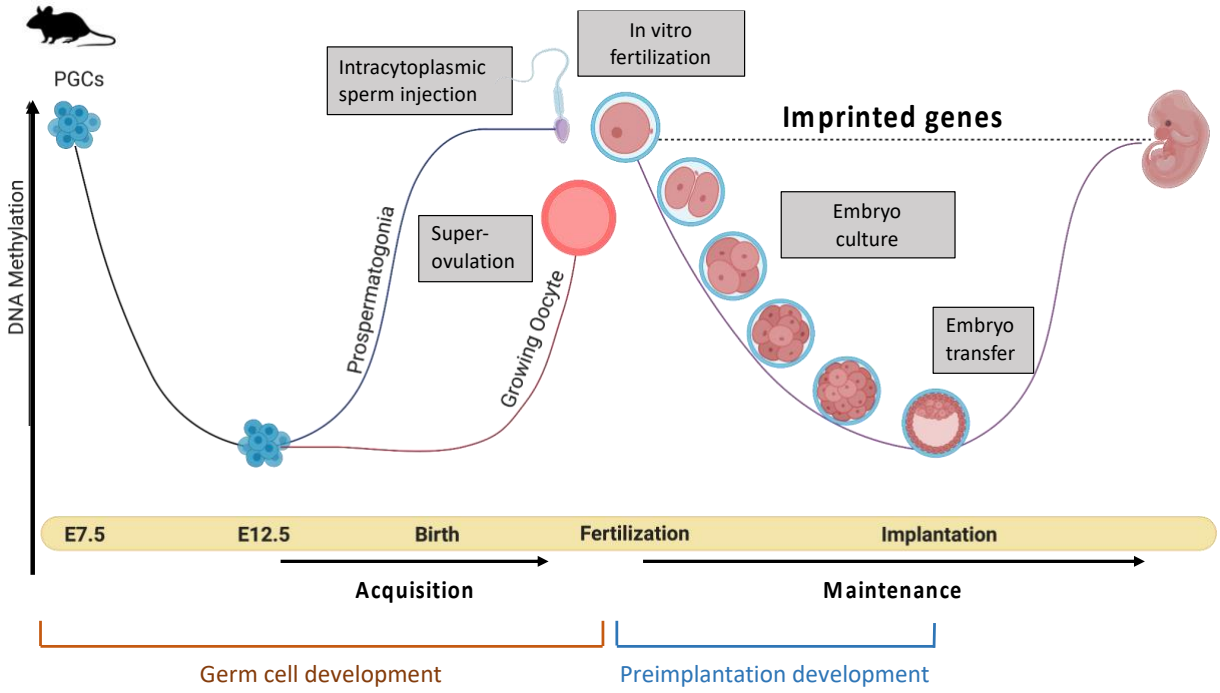
mortality, the moderate but not the high dose of FA resolved embryonic developmental delay[1]. Secondly, ARTs induced hypomethylation at the *Kcnq1*ot1, *H19*, *Snrpn* imprinted ICRs in placentas more than embryos, and in female embryos more than males[1]. Moderate FA corrected this sex-specific bias at the *Kcnq1*ot1 and *Snrpn* ICRs and only high dose FA resolved this effect at the *H19* ICR[1]. Thirdly, genome-wide DNA methylation analysis on placenta samples revealed thousands of differentially methylated tiles (DMTs) most of which were hypomethylated[1]. While FA supplementation corrected some of these defects, it also induced additional diet specific DMTs more pronounced in male placentas compared to female placentas[1].

Taken together, our previous study demonstrated that ARTs were behind some adverse developmental outcomes associated with epigenetic abnormalities[1]. In addition, the findings suggested that 1) females may be more sensitive to ART-induced epigenetic abnormalities, later improved by FA and that 2) higher doses of FA may be more deleterious in male ART-conceived embryos compared to females[1]. Therefore, a follow-up analysis using a larger sample size was suggested to confirm these findings.

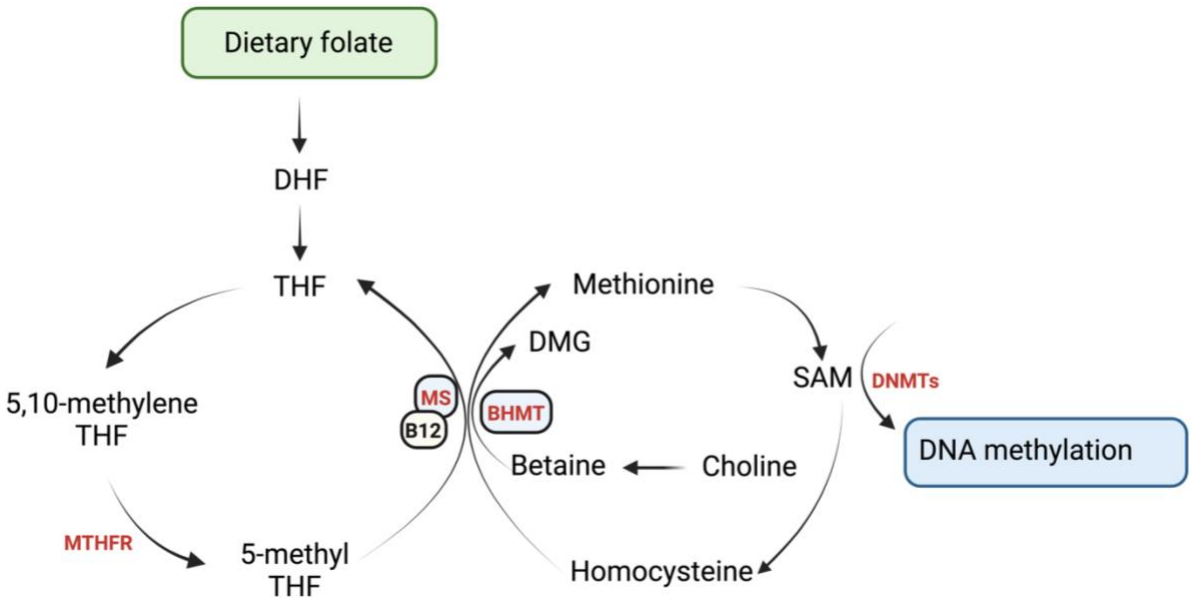
The placenta functions as the exchange interface for nutrients and oxygen between maternal and fetal circulations to ensure optimal embryonic development. While results from the Rahimi study demonstrated that ARTs affect the placental epigenome more drastically than embryos at midgestation[1], Vrooman et al. showed that cumulative ART procedures result in placental vasculature defects, overgrowth, and increased levels of PE sFLT1 marker at the end of gestation[6]. Whether placental epigenetic alterations at midgestation contribute to adverse placental phenotypes observed at E18.5 is still unclear.

In this follow-up study to Rahimi and al., we hypothesize that ARTs alter DNA methylation and gene expression during early placenta development, leading to defects at the end of gestation and we postulate that FA supplementation can rescue some of these effects. To test this hypothesis, we first aimed to assess the effect of ARTs and the previously described moderate and high doses of FA supplementation on the placental transcriptome of E10.5 male and female embryos. Next, we used these results to identify a subset of ART-perturbed genes involved in early placental development for validation in normal E10.5 placentas: we examined the targeted expression of imprinted *Phlda2*,

Igf2, *Peg3* and imprinted-like *EphB2* and *L3mbtl1*[145]. In addition, we assessed DNA methylation and gene expression of these genes in placentas from abnormal and developmentally delayed embryos. To elucidate the association between epigenetic defects and transcriptional changes, we investigated the correlation between DNA methylation at the *Kcnq1*ot1 ICR and *Phlda2* gene expression. Given the early role of *L3mbtl1* transcription factor in placentation, we also analyzed DNA methylation at *L3mbtl1* promoter.



INTRODUCTION FIGURE 1. The timing of ARTs coincides with key windows of epigenetic reprogramming during germ cell and preimplantation development. ART procedures are performed during waves of DNA demethylation and remethylation occurring during germ cell and early embryonic development. PGCs: primordial germ cells. Created with BioRender.com



INTRODUCTION FIGURE 2. The one carbon metabolism cycle. Folate plays an essential role in the establishment and maintenance of proper DNA methylation profiles by providing methyl groups. Disruption of this cycle leads to alterations in the levels of S-adenosylmethionine, the universal methyl donor, resulting in downstream effects on DNA methylation patterns. DHF, dihydrofolate; THF, tetrahydrofolate; MTHFR, 5,10-methylenetetrahydrofolate reductase; MS, methionine synthase; B12, vitamin B12; BHMT, betaine-homocysteine S-methyltransferase; DMG, dimethylglycine; DNMTs, DNA methyltransferases; SAM, S-adenosylmethionine. Created with BioRender.com

CHAPTER I References

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CHAPTER II: Protective and sex-specific effects of moderate dose folic acid supplementation on the placenta following assisted reproduction

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Abbreviations: ARTs, assisted reproductive technologies; DEG, differentially expressed genes; DNAme, DNA methylation; FA, folic acid; NAT, naturally conceived; CD, control diet; FAS4, 4-time folic acid supplemented diet or moderate dose; FAS10, 10-time folic acid supplemented diet or high dose; ddPCR, droplet digital PCR.

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ABSTRACT

Epigenetic defects induced by assisted reproductive technologies (ARTs) have been suggested as a potential mechanism contributing to suboptimal placentation. Here, we hypothesize that ARTs perturb DNA methylation (DNAme) and gene expression during early placenta development, leading to abnormal placental phenotypes observed at term. Since folic acid (FA) plays a crucial role in epigenetic regulation, we propose that FA supplementation can rescue ART-induced placental defects. To test this hypothesis, female mice were placed on a control FA diet (CD) or either a moderate 4-fold (FAS4) or high dose 10-fold (FAS10) FA-supplemented diet for six weeks prior to natural mating or ARTs (superovulation, in vitro fertilization, embryo culture and transfer), continuing throughout gestation. ARTs induced 41 and 28 differentially expressed genes (DEGs) in E10.5 female and male placentas, respectively. Many DEGs were implicated in early placenta development and associated with DNAme changes; a number clustered at known imprinting control regions (ICR). In both sexes, FAS4 partially corrected alterations in gene expression while FAS10 demonstrated male-biased deleterious effects. DNAme and gene expression for five genes involved in early placental development (*Phlda2, EphB2, Igf2, Peg3, L3mbtl1*) were followed up in placentas from normal as well as delayed and abnormal embryos. *Phlda2* and *I*gf2 expression levels were lowest after ART in placentas of female delayed embryos. Moreover, ARTs concomitantly reduced DNAme at the *Kcnq1ot1* ICR which regulates *Phlda2* expression: FAS4 partially improved DNAme in a sex-specific manner. Despite DNAme defects at the *L3mbtl1* promoter, we did not observe significant expression dysregulation at midgestation. In conclusion, placental DNAme and transcriptome alterations observed at mid-gestation are sex-specific; they may help explain adverse placental phenotypes detected at term and are partially corrected by maternal moderate dose folic acid supplementation.

Keywords (5-10): Assisted Reproductive technologies/ Folic acid supplementation / early placenta development / Sex-specific / DNA methylation / Gene expression

2.1 introduction

With infertility affecting ~15% of couples, there has been a continuous increase in the use of assisted reproductive technologies (ARTs) in developed countries[1]. Although most children born from ARTs are healthy [2], evidence suggests that ART pregnancies are at an increased risk of birth defects [3,4] and imprinting disorders [5-7]. In addition, ART pregnancies are associated with adverse pregnancy outcomes such as preeclampsia and intrauterine growth restriction [8-10]. Data from human and animal model studies support the hypothesis that suboptimal placenta function may be responsible for several adverse pregnancy outcomes associated with ARTs [11].

Animal models, particularly the mouse, that lack the confounder of parental infertility present in human studies, have been utilized to investigate the effects of ARTs on placental development [12]. Similar to humans, the mouse placenta can be separated into three structures: 1) the outer maternal layer composed of decidual cells from the uterus and the maternal vasculature that brings blood to/from the implantation site; 2) a middle "junctional zone" (JZ) consisting of spongiotrophoblasts (SpTBs), glycogen cells (GCs), and trophoblast giant cells (TGCs) in contact with the maternal decidua; and 3) an inner layer known as the labyrinth, composed of syncytiotrophoblast I and II cells responsible for nutrient exchange [12-14]. During pregnancy, the placenta ensures optimal fetal growth by mediating implantation, serving as the interface for nutrient and gas exchange [15] and protecting the growing fetus from the maternal immune system [14].

In mice, the use of multiple ART procedures (e.g. superovulation, in vitro fertilization and embryo culture), similar to protocols used routinely for human ART, induce adverse placental phenotypes observed at the end of gestation, including placental vasculature defects, overgrowth, and increased levels of the pre-eclampsia marker sFLT1 marker [16,17]. Epigenetic alterations in the placenta have been suggested as a potential mechanism to explain abnormal placental phenotypes associated with ART. Indeed, ART procedures, particularly embryo culture, are performed during preimplantation development when DNAme erasure occurs across the genome except at imprinted loci and certain repeat elements [18,19]. Imprinted genes in particular, which tightly regulate placenta development, must maintain their DNAme patterns during preimplantation development to ensure parent-of-origin specific expression[20].

Dysregulation of DNAme at imprinted loci due to ARTs may influence placentation, potentially modify nutrient transfer, and compromise offspring outcomes [11,17].

The availability of methyl donors such as folate is critical to establish and maintain proper DNAme profiles during development through the one-carbon metabolism pathway[21]. Adequate intake is achieved through consumption of folate-containing foods or through supplementation using folic acid (FA)[22]. During pregnancy, FA supplementation is recommended to reduce the risk of neural tube defects (NTDs) [23,24]. Current guidelines recommend daily use of a multivitamin containing 0.4-1 mg FA for low to moderate risk pregnancies and up to 4 mg/day FA for women with high-risk of NTDs [25]. Mouse studies indicate that both folate deficiency and high dose folic acid supplementation can have deleterious effects on development. In mice, folate deficiency has been linked to craniofacial alterations[26] whereas high dose maternal FA supplementation (10- and/or 20-fold higher than recommended) was associated with disrupted embryonic development [27-29].

To determine optimal FA doses for ART pregnancies, a previous study from our group investigated the effects of clinically relevant moderate 4-fold (~1.6 mg/day in human) and high 10-fold (~4.0 mg/day in human) FA-supplemented diets on reproductive and epigenetic outcomes at mid-gestation using a mouse model of ARTs. In this study, ART resulted in an increase in embryos with developmental delay along with genome-wide DNAme abnormalities that were particularly marked in the placenta; both outcomes were partially corrected with moderate but not high dose FA supplementation[30]. Whether the prominent placental epigenetic defects we found at mid-gestation resulted in transcriptional changes that could impact placental function remained to be explored. In the current study, we hypothesized that ARTs alter DNAme programming in preimplantation embryos leading to altered gene expression during early placenta development in the post implantation period, subsequently resulting in the placental defects that have been reported at the end of gestation[17]. In addition, we postulated that FA supplementation can rescue these effects. We show that ARTs in mice have a notable impact on the placental transcriptome at midgestation and that FA supplementation acts to partially correct these changes in a dose-dependent and sex-specific manner. We reveal that many of the genes affected by ARTs are involved in early placenta

development and differentially expressed in placentas from E10.5, delayed and abnormal embryos.

2.2 Materials and methods

2.2.1 Ethics

All animal experiments were performed in compliance with the guidelines established by the Canadian Council of Animal Care and approved by the Animal Care Committee at the Research Institute of the McGill University Health Centre (RI-MUHC).

2.2.2 Mice and clinically relevant diets

This study is a follow-up to the experiments described in Rahimi et al.,2019 [30]. Briefly, mice were placed on a 12-hour light:12-hour dark cycle with access to food and water *ad libitum*. Hsd:NSA (CF1) outbred female mice (Envigo, Indianapolis, IN, USA) were fed one of three amino-acid defined diets (Envigo) for 6 weeks prior to natural mating or ART and throughout gestation: either a folic acid control diet (CD, 2 mg/kg diet, TD.130565) containing the recommended level of folic acid for rodents[31]; a 4-fold folic acid-supplemented diet (FAS4, 8 mg/kg diet, TD.160058); or a 10-fold folic acid-supplemented diet (FAS10, 20 mg/kg diet, TD.160059). A six-week diet period was chosen for two reasons: (1) It allows stabilization of long-term intracellular folate storage, as reflected by red blood cell folate levels[32]; (2) It corresponds to three oocyte maturation cycles in mouse, equivalent to 12 months in humans, which is the usual time spent by couples trying to conceive before undergoing ARTs. B6SJLF1/J males (Jackson Laboratory, Bar Harbour, ME, USA) and CD1 vasectomized males (Charles River Laboratories, Senneville, QC, Canada) were fed a mouse chow diet (Teklad Global 18% Protein Rodent Diet, 4 mg folic acid/kg diet, Envigo).

2.2.3 ART procedures

Mouse ART procedures were adapted from the Jackson Laboratory method [33] and published articles [16,34]. Briefly, 9-week-old CF1 female mice were superovulated by IP injection of 5 IU pregnant mares serum gonadotropin (PMSG, 367222, EMD Millipore, Etobicoke, ON, Canada), followed by 5 IU human chorionic gonadotropin (hCG, 230734,

EMD Milipore)[35]. Spermatozoa were isolated from the cauda epididymis and vas deferens of B6SJLF1/J males (12 to 14-weeks-old) and capacitated in a drop of EmbryoMax Human Tubal Fluid media (1X) (HTF, MR-070-D, EMD Millipore) covered by embryo tested mineral oil (M8410, EMD Millipore). IVF was initiated by dragging cumulus-oocyte complexes using a 30G½ needle from both ampullae of superovulated CF1 females to the drop of HTF media containing capacitated spermatozoa. After 6 hours, resulting zygotes were washed in HTF media then cultured in KSOM+ ½ AA (MR-106-D, EMD Millipore) under mineral oil at 37°C in a humidified, reduced oxygen environment (5% CO_2, 5% O_2, 90% N_2) for 4 days. Following embryo culture, ten blastocyst-stage embryos (from a single CF1 superovulated donor) were transferred to a 2.5-day post-coitum (dpc) pseudopregnant CF1 recipient fed the same diet as the donor, using the Non-Surgical Embryo Transfer (NSET) device (ParaTechs, Lexington, KY, USA). Day of blastocyst transfer was defined as E3.5 and embryos and placentas were collected 8 days later.

2.2.4 Natural mating protocol

Naturally cycling CF1 females were fed the CD for 6 weeks prior to conception and mated with B6SJLF1/J males. The morning of observance of a copulation plug was considered 0.5 dpc. Diets were continued though gestation until embryos and placentas were collected 10.5 dpc. These placentas served as controls forming the NAT_CD group.

2.2.5 Tissue collection, examination, and DNA extraction

Midgestation embryos, placentas and yolk sacs were collected as previously described [30]. Embryos were staged according to published criteria [36,37] and examined for developmental delay and malformations; they were considered developmentally delayed if they were staged at E9.5 or less [30]. Samples were snap frozen in dry ice and stored at -80°C until further use. Placental tissues were homogenized in a frozen tissue lysis buffer (Qiagen, Mississauga, ON, Canada) using mortars and pestles on dry ice. Genomic DNA was extracted from E10.5 and abnormal placentas using the DNeasy Blood & Tissue kit (Qiagen) and from smaller delayed placentas using the QIAamp DNA Micro kit (Qiagen)

as per the manufacturer's protocol. Embryo sex was determined by PCR using yolk sac DNA as described previously [30], and re-confirmed in this study using the placental DNA.

2.2.6 RNA extraction and sequencing

Placentas chosen for transcriptome analysis were morphologically normal and associated with normal embryos staged at E10.5 (n=4-6 per group/sex). Total RNA was extracted from placentas using the miRNeasy micro kit (Qiagen) according to the manufacturer's instructions. cDNA library construction, sequencing on Illumina NovaSeq PE150 (6G raw data per sample) and bio-informatic analysis were performed by Novogene (Sacramento, CA, USA). Briefly, read mapping was performed with STAR_v2.6.1d, DESeq2_v1.26.0 was used for the differential analysis and genes with false rate discovery (FDR) adjusted p-value < 0.05 and |log2(FoldChange)| > 0.3 were considered as differentially expressed. GO enrichment analysis was performed using ClusterProfiler v3.8.1. (See Supplemental Tables 1 to 5).

2.2.7 Gene expression analysis using droplet digital PCR (ddPCR)

Droplet digital PCR analysis was performed according to the manufacturer's protocol and published articles [38,39]. Briefly, 500 ng total RNA was converted to complementary DNA (cDNA) using the iScript Advanced cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, Canada) as per the manufacturer's instructions. Primers were designed in regions of the mRNA sequence transcribed for all protein coding transcripts using the NCBI primer Blast tool (mouse genome GRCm39): Forward and Reverse primers for- *Phlda2:* GCTCTGGGTCCGTGAAACG/ GGGTTGGAAGCAGGTAACCA; *EphB2:* ACCATGACAGAAGCCGAGTA/ CTGTTACATACGATGGCAATGAC; *Igf2:* ACACGCTTCAGTTTGTCTGTTC/ AGTACGGCCTGAGAGGTAGAC; *Peg3*: GCACCAGCCGAGGTCTCAAA/ GGTTGCGAGCCACATCCTTG; *Rps18*: GGGAAGTACAGCCAGGTTCTG/ CAAAGGCCCAGAGACTCATTTC, *Rpl13a*: CTGCTGCTCTCAAGGTTGTTC/ TGCCTGTTTCCGTAACCTCAAG.

A pool of 12 samples from all groups/ sex was run on real-time PCR (Bio-Rad CFX96 Touch) to optimize annealing temperature, assess cDNA dilution, verify amplicon size and specificity for each primer set. Three representative samples per group/sex were

used to determine optimal reference genes using predesigned M96-well plate (Bio-Rad): *Rpl13a* and *Rps18* were identified as ideal reference genes by GeNorm software (CFX Maestro Bio-Rad) with M values < 0.5 [40] (Supplemental Dataset 1 and 2).

Serial dilutions of cDNA and primer sets were added to the QX200 ddPCR EvaGreen Supermix (Bio-Rad) according to the associated protocol. The reaction mix was converted to droplets with the QX200 droplet generator (Bio-Rad). Droplet-partitioned samples were transferred to a 96-well plate, sealed, and cycled in a C1000 Thermocycler (Bio-Rad) according to the manufacturer's protocol. The cycled plate was then transferred and read using the QX200 reader (Bio-Rad). Gene expression level measured as copies/μL was normalised to transcripts encoding *Rps18* and *Rpl13a* using QX Manager v1.2 Standard.

2.2.8 DNA methylation analysis

For each midgestation placenta sample, 1μg of DNA was treated with bisulfite using the EpiTect Bisulfite kit (Qiagen) and DNAme was quantified by bisulfite pyrosequencing at the *Kcnq1ot1* ICR (For: AGGTTTTGGTAGGTGGTTT, Rev: Biot-CCTAACTAAACCAAAATACACCATCATA, Seq: GTTAGGAGGAATAGTTGTTTTA, Ta:55°C) and the *L3mbtl1* promoter region (For: GTTGTTTATGGGTGGGAAGATTGAG, Rev: Biot-ACAAAAAAAACTACAAACCCTCATAC, Seq1: GTTAAGATATAATTTTTTTGGAA, Seq2: GTGGGTTTTAATAAAGTAGTG, Ta: 60°C) as previously described [41]. (See Supplemental Table 6).

2.2.9 Statistical analysis

Graphs and presented data were analyzed using GraphPad Prism 9 software: means ± standard error of the mean (SEM) are shown. Statistical significance was set at $P < 0.05$ for all analyses. Two tailed unpaired student's t-test was used to compare NAT_CD and CD groups and determine the effect of ART. To assess the effect of FA supplementation following ARTs, means of ART groups (CD, FAS4 and FAS10) were compared using one-way ANOVA with Tukey's correction for multiple comparisons. Percentage (%) correction of FA supplementation for individual DEGs shown in figures 2C, 2D and supplemental figures 4,5 was calculated using group means in Abbott's

formula: $[1-\left(\frac{NAT\_CD-ART\_FA}{NAT\_CD-ART\_CD}\right)]*100$, where ART_FA refers to ART_FAS4 or ART_FAS10 groups. DNAme variance was obtained by averaging variances at all CpGs within each group.

2.3 Results

2.3.1 Dose-dependent effects of folic acid supplementation on the placental transcriptome at midgestation, following ARTs

To determine the effects of folic acid (FA) supplementation on the placental transcriptome following ART, we performed a genome-wide transcriptome analysis using RNA sequencing on whole placentas from E10.5 male and female embryos (n=4-6/group/sex). Differential gene expression was determined when the FDR adjusted p-value < 0.05. We identified 41 and 28 differentially expressed genes (DEGs) in E10.5 female and male placentas, respectively (Fig. 1A, B and Supplemental Tables 1 and 4). In E10.5 female placentas, 17 genes were upregulated and 24 were downregulated (Fig 1A and Supplemental Table 1) while in E10.5 male placentas, 21 genes were upregulated and 7 were downregulated (Fig. 1B and Supplemental Table 4). In addition, diet-induced DEGs were identified in both sexes. In midgestation female placentas, moderate dose FA supplementation (FAS4) induced two downregulated and one upregulated gene (Supplemental Table 2) and a further increase in FA supplementation induced downregulation of two genes and upregulation of nine (ART_FAS10 vs ART_FAS4, Supplemental Table 3). In male placentas, only the high dose of FA (FAS10) resulted in diet-induced DEGs with two downregulated genes and two upregulated genes (Supplemental Table 5). For either sex, no DEGs were common between the three comparisons (CD vs NAT_CD; ART_FAS4 vs CD; ART_FAS10 vs CD). However, two ART-induced upregulated DEGs (*Susd2; 2410003L11Rik*) and one downregulated DEG (*Igf2os*) were common between male and female placentas (Supplemental tables 1 and 4).

Some DEGs were imprinted genes regulated by diverse epigenetic mechanisms including DNAme, long-noncoding RNAs (lncRNA) and histone modifications (H3K9me/H3K27me)[42,43]. Among DEGs identified in midgestation female and male placentas; *Sgce, Asb4 and Klf14, H19, Peg3*, *Pmaip1*, and *Igf2* rely on DNAme profiles

to ensure adequate parent-specific gene expression/regulation while *Slc22a18, Phlda2* and *Galnt6* gene expression is controlled by imprinting control region (ICR) -directed lncRNAs (Fig. 1A and B). Additionally, *Slc38a4* and *Smoc1* are known non-canonical imprinting genes which rely on H3K9me and oocyte-derived H3K27me3 marks[42,44].

Previously, genome-wide analysis using reduced representation bisulfite sequencing (RRBS)[30] revealed that ARTs induced thousands of differentially methylated tiles (DMTs) in male and female E10.5 placentas, the majority of which were hypomethylated (Fig. S3). To assess whether ART-induced DEGs were sensitive to changes in DNAme, we examined previous DNAme data obtained using RRBS or bisulfite pyrosequencing [30]. We found that some DEGs were associated with DNAme changes by identifying (1) hypomethylated or hypermethylated CpGs within corresponding genomic regions in RRBS data or (2) ICR hypomethylation or hypermethylation observed by BS pyrosequencing (Fig. 1A and B).

Next, we assessed whether FA could rescue these ART-induced gene expression alterations. FAS4 resulted in partial correction of 39 and 23 DEGs in E10.5 female and male placentas, respectively (Fig. 1A, B and 2C, D). FAS10 was more beneficial in female placentas relative to males by partially rescuing gene expression of 37 genes, though to a lesser extent than the FAS4, while 16 genes were partially corrected in midgestation male placentas (Fig. 1A, B and 2C, D). Since ARTs and FA supplementation resulted in very few DEGs, no significant gene ontology enrichment could be detected. However, as evidenced by gene knockout and overexpression studies (described in section 2.3.3), ART-induced DEGs are involved in several important functions including early placental development, angiogenesis, and spermatogenesis (Fig. 1C).

Taken altogether, these results support the hypothesis that ART results in placental transcriptomic changes during early stages of development when the placenta is being established. We show that moderate folic acid supplementation can partially rescue these effects more efficiently than the high dose.

2.3.2 DEGs respond to folic acid supplementation in a sex-specific manner and some DEGs cluster at known imprinting regions

Many DEGs identified in E10.5 female placentas were also affected in E10.5 male placentas, often exhibiting changes in the same direction. Out of 17 up- and 24 downregulated female ART genes, 16 and 22 were also up- and downregulated in male placentas, respectively (Fig. 2A). Similarly, out of 21 up- and 7 downregulated male ART genes, 21 and 4 were undergoing the same change in expression in female placentas (Fig. 2B). As shown in more detail at an individual-placenta level, most DEGs identified in female placentas showed similar dysregulation in male placentas and vice-versa: in both sexes, mean relative expression > 1 for upregulated genes and < 1 for downregulated genes (Fig. S2A).

Next, we compared the response to FA supplementation of DEGs identified in female and male placentas and found sex-specific effects. Except for 2 DEGs which were not corrected by FAS4 (i.e., *24…Rik* and *Nudt10)* and 4 DEGs for FAS10 (i.e., *Adm, Susd4, Ikzf4, Fam109b, Csf2rb*); the two doses of FA supplementation exhibited partial correction of most female ART DEGs (Fig. 2C and S4). FAS4 partially corrected 95% of the female ART DEGs, ranging from 2-103% (mean correction = 45%) while FAS10 corrected 90% of female ART DEGs to a lesser extent, from 1-74% (mean correction = 23 %). For the male placentas, FAS4 corrected 23 of the 28 ART DEGs (exception: *Slc38a4, Galnt6, 24…Rik, Pmaip1, Trpm2*), exhibiting 0-80% correction (mean correction = 18%) while FAS10 showed evidence of deleterious effects on the expression of 12/28 male ART DEGs, with a mean correction of -2% (Fig. 2D and S5). To explore this observation further, we looked in more detail at the effects of FA supplementation on female ART DEGs in male placentas and vice-versa (Fig. S4 and S5). Unexpectedly, we observed that FAS4 and FAS10 still exhibited better correction of most male ART DEGs in female placentas compared to male placentas (i.e., *Cysltr2*, *Fgl2*…) (Fig. S5A).

Chromosomal location of male and female ART DEGs revealed that while DEGs were distributed across the genome, some DEGs clustered on distal chromosome 7 and 15 (Fig. 2E). Interestingly, the mouse imprinted cluster on chromosome 7 was affected in both sexes and included syntenic genes located in human chromosome region 11p15.5, also known as ICR1 and ICR2 and associated with ART-related syndromes such as the Beckwith Wiedemann syndrome[45]. DEGs located in this cluster are well known to be primarily reliant on DNAme for parent-of-origin specific gene expression[42] suggesting that

corresponding DNAme profiles may also be affected. Altogether, both doses of FA supplementation seem beneficial to female placentas during early development whereas high dose FA may lead to deleterious effects in male placentas at midgestation.

2.3.3 Many DEGs are implicated in early placenta development

Given the broad transcriptional changes observed above, we set out to perform an extensive literature search and summarize gene expression data in relation to placental lineages (Fig. 3). The mouse placenta consists of three main tissue types: the maternal decidua composed of the maternal vasculature; the JZ, first site of trophoblast (TB) invasion which serves an endocrine function; and the labyrinth responsible for nutrient and gas exchange[14]. Ephrin receptors including *EphB2* have been suggested to play key roles in the remodeling and maturation of VEGF-induced immature vessels in the placenta[46]. Both *Adm* [47,48]; a growth promoting angiogenic factor, and *Ceacam1*[49,50]; a cell-adhesion molecule, promote trophoblast invasion and remodeling of uterine spiral arteries of the maternal decidua during placentation. Contrary to *Adm* and *Ceacam1*, *Fos* inhibits TB migration and invasion of the maternal decidua [51,52]. In addition, *L3mtbtl1* functions as a transcriptional repressor and chromatin compactor [53]. It has been suggested that *L3mbtl1* gene expression may regulate cell fate by preventing trophectoderm (TE) formation [53]. After implantation, *Nodal* signaling from the inner cell mass promotes differentiation of polar TE cells into TB stem cells essential for JZ establishment [54]. Interestingly, *Phlda2* is a placenta-specific imprinted gene responsible for regulating JZ growth: loss of *Phlda2* in mice is associated with placenta overgrowth characterized by an expansion of the JZ [20] whereas over-expression decreases JZ size and placental glycogen stores [55,56]. Similarly, *Csf2rb* is a subunit of *Csf2* receptor which regulates placental JZ structure: loss of *Csf2rb* expands the JZ with increased levels of SpTBs and GCs[57,58]. In addition, imprinted genes *Peg3* and *Igf2* play an important role in optimal JZ establishment [56]: Loss of *Peg3* results in reduced placental glycogen stores and decreased SpTBs and GCs [59] while loss of *Igf2* is associated with decreased SpTBs and GCs volume in female but not male placentas[60]. Additionally, *Igf2* is implicated in fetoplacental endothelial cell proliferation within the labyrinth [61]. Given that circulating levels of *Igf2* reflect fetal nutrient demands, the *Igf2-Igf2r* axis is responsible for regulating

placental microvasculature expansion within the labyrinth accordingly[61-63]. Amino acid transporter *Slc38a4* is also highly expressed by syncitiotrophoblasts in the labyrinth to support transfer of amino acids toward the fetal circulation[64]. Additionally, *Asb4* ubiquitin ligase promotes trophoblast differentiation in the labyrinth at midgestation[65]. Finally, *Klf14* is an imprinted transcription factor which acts to limit placenta growth in late gestation [66]. Altogether, many ART-induced DEGs play key roles in placenta cell lineages proliferation, migration, maturation, and differentiation. These early transcription changes may result in adverse placental phenotypes observed at the end of ART gestation in the mouse [16,17].

2.3.4 Gene expression analysis of key genes affected by ARTs and involved in early placenta development

Mouse studies previously showed that ARTs are associated with late gestational placental overgrowth, mainly in the JZ[16,17]. To investigate the early mechanisms at the transcriptome level, we selected two female DEGs (*Phlda2* and *EphB2*) and two male DEGs (*Igf2* and *Peg3*) that could lead to such placental phenotypes and performed gene expression analysis on an extended cohort. Notably, using previous RRBS data assessing genome-wide DNA methylation in E10.5 placentas[30], we identified ART-induced DMTs in *EphB2, Igf2 and Peg3* DEGs. For analysis of gene expression, total RNA was isolated from individual E10.5 male and female placentas (n=9-13 placentas/group/sex) and transcript levels were measured via droplet digital PCR (ddPCR) (Fig. 4). Consistent with RNA-sequencing data, *Phlda2* and *Igf2* were significantly downregulated in female and male E10.5 placentas following use of ART ($P < 0.05$; Fig. 4A, B, E, F). *EphB2* relative expression was significantly decreased in female, but not in male placentas while *Peg3* displayed significantly greater abundance in ART_CD groups of both sexes compared to controls ($P < 0.05$; Fig. 4C, D, G, H).

Next, we assessed whether FA supplementation could modify these ART-induced expression alterations. While not significant, there was a trend for FAS4 ($P=0.089$) and FAS10 ($P=0.1$) to ameliorate *Phlda2* relative expression levels in female, but not male placentas (Fig. 4A, B). However, neither level of FA supplementation significantly affected mean relative expression levels of *EphB2*, *Igf2*, or *Peg3* in either sex following ART.

Together, these results from an extended cohort are consistent with the RNA-sequencing data obtained from a subset.

2.3.5 Gene expression analysis of key genes affected by ART and involved in early placentation, in placentas from developmentally delayed / abnormal embryos

Previously, ARTs were associated with a significant increase in embryonic developmental delay; an adverse effect mitigated by moderate but not high dose FA supplementation (Fig. S1)[30]. Although not significant, ARTs were associated with female-biased developmental delay, as the proportion of delayed female embryos (staged at E9.5 or earlier) was higher than males in the ART group (Fig. S1A)[30]. In addition, embryos with abnormalities were observed in different groups, even though overall rates remained unaffected by ARTs or FA[30].

For this reason, we first set out to examine the effects of ART on gene expression in placentas from ART-conceived delayed/abnormal embryos (mutually exclusive), using the same male and female ART DEGs previously described (Fig.5). Given that ARTs significantly increased developmental delay but did not affect rates of embryonic abnormalities, we postulated that gene expression in delayed placentas will be more affected than gene expression in abnormal placentas. ART-downregulated *Phlda2* and *Igf2* also exhibited a significant decrease in female delayed and female abnormal placentas, with lower levels of expression in delayed placentas (Fig. 5A, B, I, J). In contrast, *EphB2* and *Peg3* relative expression levels were significantly decreased and increased, respectively, as they were in normal placentas, in female abnormal placentas only (Fig. 4C, G and 5F, N). Due to the small sample size of male delayed (n=1-3) and male abnormal (n=2) placentas in the CD group we did not observe significant gene expression changes (Fig. 5C, D, G, H, K, L, O) except for increased *Peg3* gene expression in male abnormal placentas, similarly to what was observed in normal placentas (Fig. 4H and 5P).

Next, we assessed whether FA supplementation could rescue these ART-induced changes. Despite lack of female delayed placenta samples in the ART_FAS4 group, we could observe that FAS10 and FAS4 corrected *Phlda2* expression in female delayed and

female abnormal placentas, respectively (Fig. 5A and B). Similarly, FAS10 significantly corrected *EphB2* expression in female abnormal placentas (Fig. 5F).

Thus, key ART DEGs involved in placenta development were similarly affected in normal, delayed, and abnormal placentas, except for *Igf2* gene expression which was more affected in female delayed placentas.

2.3.6 Sex-specific effects of FA supplementation on *Kcnq1ot1* ICR DNAme and positive correlation between *Kcnq1ot1* DNAme and *Phlda2* gene expression

ARTs have been suggested to affect gene expression through epigenetic dysregulation, particularly because ART procedures are performed during key periods of DNAme reprogramming[18]. To determine whether ART affects DNAme of DEGs, we examined DNAme at the *Kcnq1ot1* imprinting control region (ICR) which regulates *Phlda2* expression using bisulfite pyrosequencing (Fig. S6). ARTs were associated with female-biased DNAme defects as female E10.5 and abnormal placentas exhibited more decreased mean methylation relative to males as evidenced by more significant P values between the NAT_CD and CD groups in females relative to males (for females, $P<0.001$ in Fig. 6A, C; for males $P<0.05$ in Fig 6G, and P is not significant in Fig 6I). Interestingly, decreased DNAme levels were partially rescued by FAS4 but not FAS10 in female E10.5 placentas (Fig. 6A). Observed sex differences were further evidenced by significantly increased methylation variance at the *Kcnq1ot1* ICR in female placentas relative to males (Fig. 6.D-F and J-L). While FAS4 partially corrected methylation variance in female E10.5/abnormal placentas, FAS10 achieved a similar result in female delayed placentas. However, we did not observe significant changes of DNAme in delayed placentas due to the small sample size of certain groups (further discussed in study limitations).

DNAme at the *Kcnq1ot1* ICR results in expression of *Phlda2* from the maternal allele via lncRNA[67]. To examine this relationship in our samples, we performed a correlation analysis between *Kcnq1ot1* ICR DNAme and *Phlda2* gene expression, where delayed and abnormal placentas were combined. We confirmed a positive correlation for female E10.5 ($r=0.7$; $P < 0.0001$), female delayed and abnormal ($r=0.61$; $P < 0.0001$), male E10.5 ($r=0.64$; $P < 0.0001$), and male delayed and abnormal placentas ($r=0.43$; $P = 0.02$) (Fig. 6M-P). Together, the results suggest that ARTs are associated with sex-

specific placental DNA hypomethylation at the *Kcnq1ot1* ICR, which results in *Phlda2* downregulation. Moderate but not high dose FA partially rescues some of these changes in a sex-specific manner.

2.3.7 Dysregulation of *L3mbtl1* promoter DNAme may not result in transcription changes at midgestation

Although imprinted in humans, the ART-upregulated transcription repressor *L3mbtl1* is imprinted-like in mice, i.e., it requires uninterrupted DNA methyltransferase activity at each early embryonic division to be accurately inherited, as previously described[68]. Given that ARTs induced *L3mbtl1* promoter hypomethylation previously detected by RRBS[30] and considering the important role of this gene for extra-embryonic lineage differentiation (E4.5), we examined the impact of ARTs on *L3mbtl1* promoter 2 DNAme using bisulfite pyrosequencing (Supplemental Table 6) and gene expression using ddPCR, in female placentas. ARTs resulted in a marked decrease of promoter 2 DNAme in female E10.5/abnormal placentas, as demonstrated by significant differences between the NAT_CD and ART_CD groups (Fig. 7D and F). In addition, delayed female placentas displayed a trend toward a significant decrease of *L3mbtl1* promoter DNAme ($P = 0.06$). In contrast, only female abnormal placentas showed a significant increase of *L3mbtl1* relative expression. Neither level of FA supplementation affected mean DNAme or gene expression levels. In summary, ARTs altered *L3mbtl1* promoter DNAme at midgestation but whether these DNAme alterations are still resulting in transcription changes at E10.5 for this early-acting transcription factor, will require more exploration.

2.4. Discussion

Evidence suggests that ARTs induce epigenetic defects which result in severe placental phenotypes; yet the mechanism is not well understood [11].We show that ARTs induce epigenetic alterations and transcriptional dysregulation of many genes involved in early placenta development, possibly leading to adverse placental phenotypes observed at the end of gestation[17]. In both sexes, we demonstrate that moderate FA supplementation partially rescues some of these effects, while high dose FA exhibits male-biased deleterious effects.

Previously, we and others have investigated the impact of ARTs on fetal and placental epigenetic profiles [11,16,17,69]. In our previous study, we found thousands (>10 000) of DMTs associated with ARTs in midgestation placentas [30], while here, only a few genes were significantly differentially expressed overall. In fact, DNA methylation patterns are susceptible to changes in the environment, particularly during early in-utero development stimuli such as diet[70]. However, though DNA methylation changes may perturb the expression of imprinted and certain non-imprinted genes[68], DNA methylation defects do not always translate into gene expression alterations, which is tightly regulated especially for genes involved in embryonic development. In this study, given that DEGs exhibit same-direction changes in both sexes, we propose that they are critical for fetal and/or placental development as summarized in figure 3. Some DEGs were also identified as placental enriched genes affected by embryo culture including imprinted genes *Peg3* and *Slc38a4*[71]. Since fetal growth is dependent on a functioning placenta, improper expression of DEGs which tightly regulate placenta development and/or function may result in adverse fetoplacental outcomes.

In mouse and human placentas, trophoblast invasion and remodeling of maternal spiral arteries are critical for placentation and embryo viability [12,72,73]. Impaired or shallow invasion/remodeling has been implicated as a contributing factor to preeclampsia [74]. Among DEGs identified, upregulated *Fos* inhibits the invasion/spiral artery remodeling process whereas upregulated *Adm/Ceacam1* promotes it. In agreement with these findings, preeclampsia marker sFLT1 expression was increased in midgestation placentas [17], as would be predicted by *Fos* upregulation. Thus, concomitant upregulation of *Adm/Ceacam1* may indicate an adaptive mechanism of the placenta in response to ARTs.

The mouse placental labyrinth, like the chorionic villi in humans, is the exchange interface for nutrient and gas between maternal and fetal circulations [14]. Imprinted genes, particularly *Igf2*, have a central role in the regulation of maternal-fetal resource allocation[75]. *Igf2* expression in fetoplacental endothelial cells of the labyrinth promotes angiogenesis and endothelial cell proliferation [61]. Within the labyrinth, *Igf2* expression is driven by placenta-specific promoter P0 and deletion of the P0 transcript results in proportionate reduction of both the labyrinth and junctional zones [76]. Therefore, it can be

inferred that downregulation of *Igf2* following ARTs contributes to labyrinth abnormalities detected by Vrooman et al. at midgestation, presumably interfering with gas and nutrient exchange[17]. In accordance with this hypothesis, *Igf2* P0$^{+/-}$ mutants display increased expression of System A amino acid transporter genes, including imprinted *Slc38a4* [75]. Likewise, ARTs produce a similar effect by inducing upregulation of *Slc38a4* amino acid transporter in midgestation placentas. Notably, loss of imprinting of *Slc38a4* also contributes to the placental enlargement phenotype[44,77]. Thus, canonical and non-canonical imprinting dysregulation following ARTs may both contribute to placenta defects at E18.5.

The cluster of DEGs on distal mouse chromosome 7 is one of the most extensively characterized domains, controlled by the lncRNA *Kcnq1ot1* transcription which recruits repressive machinery to silence the paternally unmethylated allele and express neighboring genes only from the maternal allele [42,78]. Within this domain, ARTs significantly downregulated placenta specific-gene *Phlda2* which acts to limit the placental JZ size. In previous studies, JZ overgrowth was observed at the end of gestation and associated with embryo culture and IVF [16,17]. Tunster and colleagues found that *Phlda2* deficiency drives the expansion of the JZ, increases placental glycogen and induces fetal growth restriction [56]. In contrast, *Phlda2* overexpression drives a significant reduction of the JZ with limited glycogen stores and fetal growth restriction (FGR) at the end of gestation[55]. Though our study did not examine placental glycogen content and JZ composition in relation to transcriptional changes, we postulate that ART-induced downregulation of *Phlda2* during early placenta development contributes to JZ expansion observed at the end of gestation and causes alterations in JZ cell composition which may affect energy storage and endocrine function. This hypothesis is further supported by *Igf2* downregulation in the placenta because a key study found that loss of *Igf2* in the JZ alters cell composition and endocrine capacity[60]. Gene expression changes detected at midgestation may originate from the time of embryo culture as it was reported that even a 24h culture from the 4-cell to morulae stage leads to placenta overgrowth with the expansion of JZ and increased parietal TGCs in mouse placenta[71].

In agreement with previous studies, our results support the observation that ARTs induce DNAme defects responsible for imprinted gene dysregulation[11]. Beyond imprinted

genes, many non-imprinted and imprinted-like DEGs[68] - which require continuous DNMT1 activity to maintain adequate methylation profiles - were associated with DNAme abnormalities in their respective sequences. While developmental delay - equivalent to FGR or IUGR in human - has been observed in the context of ARTs [30,79-81]; to our knowledge, this is the first study to evaluate epigenetic and expression changes associated with ARTs in placentas from delayed and abnormal mouse embryos. Generally, placentas from E10.5, delayed and abnormal embryos were similarly affected by ARTs for the subset of DEGs selected, except for *Igf2* expression which was considerably decreased in female but not male delayed placentas due to small sample size limitation. Supporting these findings, *Igf2* expression was lower in trophectoderm cells after IVF compared with controls [82] and deletion of placenta specific *Igf2* (*Igf2* P0$^{+/-}$) led to reduced placenta growth and FGR, with the former preceding the latter[76]. In addition, *IGF2* mRNA was also significantly decreased in human IUGR placenta[83]. Therefore, adding to possible labyrinth abnormalities, severe disruption of *Igf2* expression following ART may be a potential indicator of developmental delay. As such, early detection, and intervention, perhaps using moderate FA supplementation, may optimize fetal growth. As observed in our previous study, moderate but not high FA corrected developmental delay [30], which may explain the lack of female and male delayed placentas in the ART_FAS4 groups.

Despite low sample sizes in certain groups, altered *Phlda2* expression at midgestation was accompanied by sex-specific DNAme changes at the *Kcnq1ot1* ICR with greater hypomethylation observed in female relative to male placentas. In humans[84,85], *PHLDA2* mRNA and protein expression were also significantly downregulated in term ART placentas compared with placentas from natural pregnancies and associated with upregulated *KCNQ1OT1* mRNA; DNA methylation levels at the *KCNQ1OT1* ICR showed a decreasing trend in ART placentas[84]. Furthermore, placentas from mouse embryos cultured from 1-cell to blastocyst stage exhibited hypomethylation at *Kcnq1ot1*, *Peg3* and *H19/Igf2* ICRs at E18.5[71]. These results suggest that in humans and mice, ARTs interfere with DNA methylation maintenance machinery likely during the time of global DNA methylation erasure in preimplantation embryos. Alterations persist until the end of gestation and may contribute to adverse placental outcomes and

phenotypes observed at term. As discussed by Rahimi and colleagues, similar sex-specific methylation differences were also observed in human placentas following ARTs [86]. To explain this bias, we hypothesize that since loss of DNAme is already detectable at the blastocyst stage [87-90] and DNAme is required for X chromosome inactivation (XCI) [91,92], supply of methyl donors during embryo culture when XCI takes places in female embryos is essential. During embryo culture, methyl donor availability for imprint maintenance may decrease because of its use for XCI in female embryos. As a result, females may be more predisposed to DNAme alterations detectable during gestation [41].

During placentation, transcription factors act to control key cell fate decisions in the trophoblast (TB) cell lineages [93]. The *L3mbtl1* transcription factor inhibits differentiation of cells toward the trophectoderm of the blastocyst [53] which is the precursor to the TB cell lineage of the placenta [93]. Indeed, it is reported that overexpression of *L3mbtl1* results in improper segregation and cytokinesis; thus, it may be important for adequate progression of cell division[85,94]. While DNAme changes associated with ARTs were observed at the *L3mtbl1* promoter in midgestation placentas, we did not observe significant *L3mbtl1* upregulation except for female abnormal placentas. However, we noticed an increased variance in the DNAme profiles of the NAT_CD control groups. Though *L3mbtl1* was detected in the mouse TE at E3.5[95], little is known about expression of this transcription factor in the different placental cell lineages at midgestation. Since we evaluated expression in whole placentas, it is possible that each individual placenta had a different cell composition thus accounting for differences in *L3mtbl1* DNAme levels within the control group. Given that *L3mtbl1* acts before midgestation, single-cell RNA sequencing experiments in TB cell lineages before mature placenta formation may be more informative of ART effects.

Overall, FA supplementation displayed partially corrective effects of DNAme and gene expression alterations in a dose dependent and sex-specific manner. While neither dose significantly corrected differential expression at the individual gene level, except for certain genes in delayed and/or abnormal placentas; a corrective effect of moderate FA supplementation was more apparent on a larger scale. However, female placentas appeared to benefit more from FA than males, as males were more susceptible to deleterious effects of FAS10. Notably in both sexes, there were more diet-induced DEGs

due to FAS10 compared with FAS4. Given that female embryos have an increased need for methyl donors during XCI, FAS4 and FAS10 may have more beneficial effects in females relative to males. Thus, while FAS4 improves alterations induced by ARTs in males, FAS10 may be higher than the required threshold for males and exacerbate rather than correct ART-induced changes. We propose that supplementation of moderate FA prior to conception with ARTs and during early gestation benefits placenta development by enabling early adaptive placental responses and optimizing fetal growth.

In summary, we demonstrate that ARTs lead to epigenetic and transcriptional alterations in mouse E10.5, delayed and abnormal placentas. If left unaddressed, we propose that these alterations are behind adverse placental phenotypes often associated with ARTs at the end of gestation in the mouse. Many changes are mitigated by moderate FA supplementation in both sexes, suggesting a potential adaptive response of the placenta at midgestation. Notably, high dose FA supplementation is no longer beneficial in male placentas as it exhibits deleterious effects. Further proteomics studies at various time points during gestation will be required to (1) firmly establish the association between epigenetic/transcriptional changes and adverse fetoplacental phenotypes and (2) gain more insight into the sex-specific effects of FA supplementation.

DATA AVAILABILITY

RNA-seq data are available as supplementary data at FASEB journal online. RRBS data were first used by Rahimi et al., 2019 and are available at NCBI Gene Expression Omnibus accession number: GSE123143.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

R.G.I, J.M. and J.M.T. designed the study. R.G.I., J.M. and S.R. performed the research experiments. R.G.I. and J.M. analyzed the data. R.G.I interpreted the data and wrote the manuscript. … provided advice regarding study design. J.M.T. and J.M. revised the manuscript.

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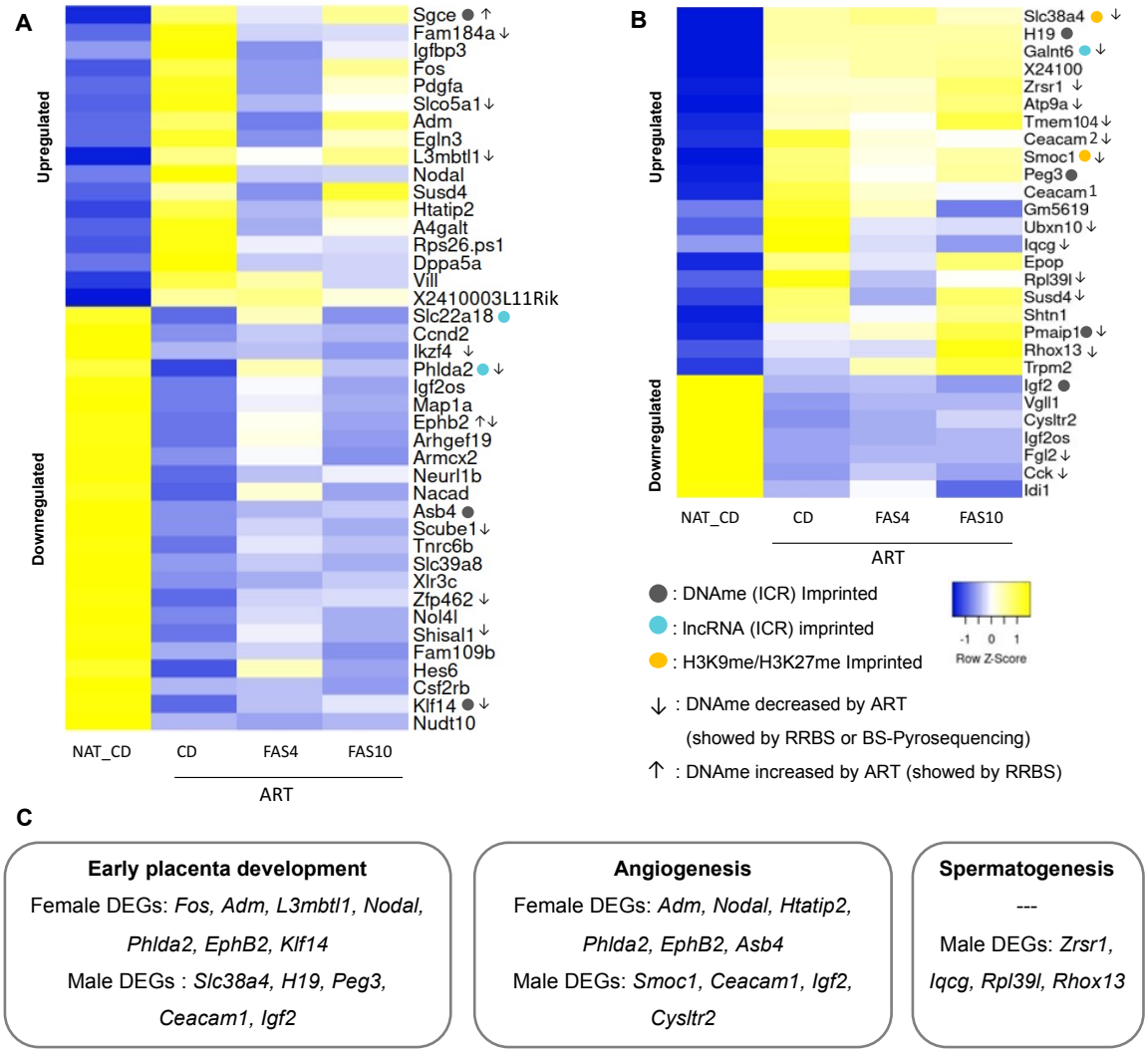


FIGURE 1. ART induces differential expression of genes in midgestation placentas of both sexes and folic acid partially rescues these effects. Heatmaps showing the relative expression of genes affected by ART, as revealed by RNA-sequencing analysis (false discovery rate P<0.05) in E10.5 normal (A) female and (B) male placentas and the effect of folic acid (FA). Each row represents an individual differently expressed gene (DEG), and each column represents one group (n=4-6/group/sex). Yellow color indicates lower levels of expression and blue color indicates higher levels of expression. Circles indicate imprinted regulation mechanism. (C) Function distribution of placental male and female DEGs involved in early placenta development, angiogenesis, and spermatogenesis.

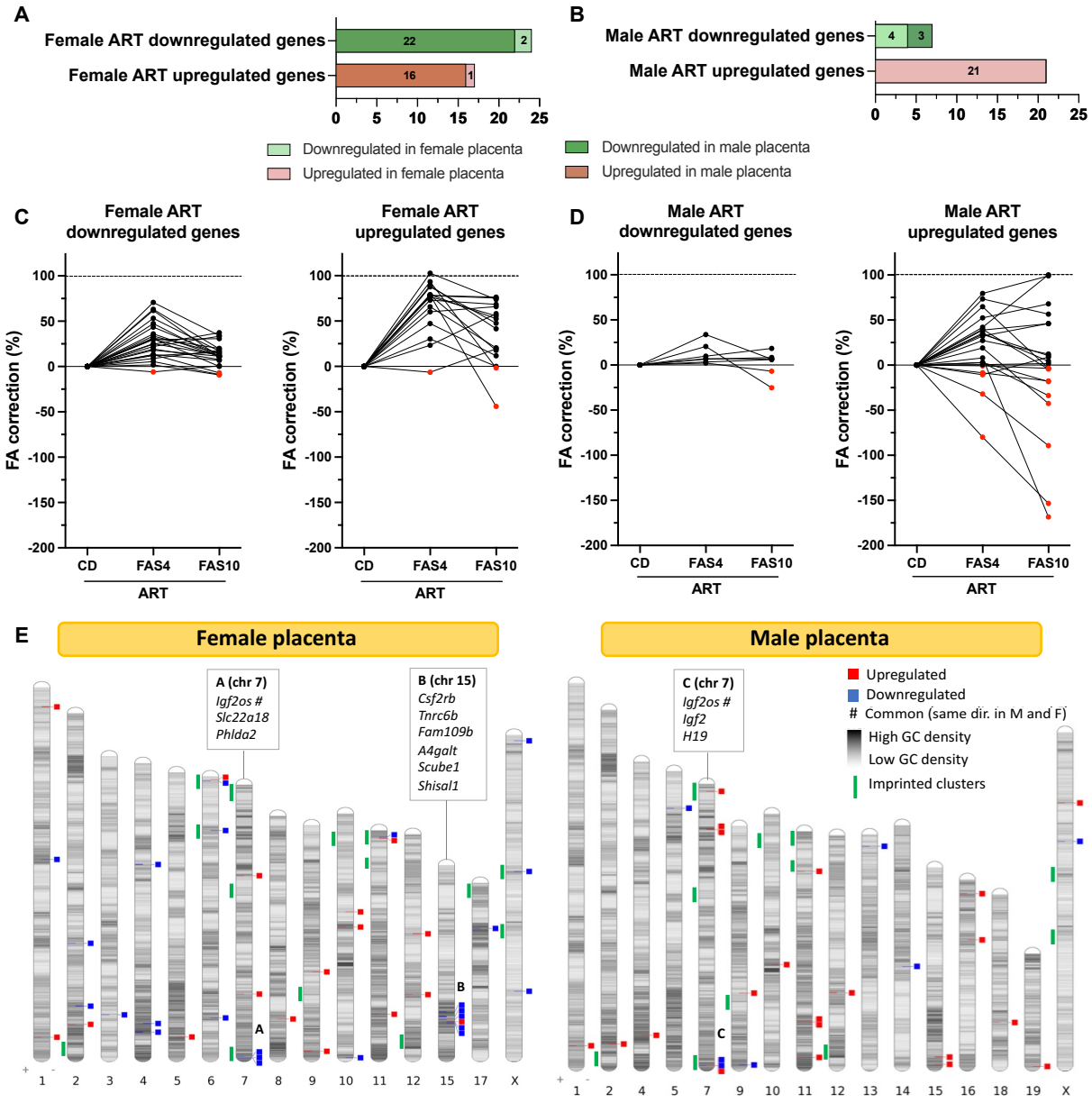


FIGURE 2. Sex-specific response of male and female DEGs to folic acid supplementation at midgestation and respective chromosomal distribution. (A) Proportion of female ART DEGs exhibiting similar expression changes in male placentas. (B) Proportion of male ART DEGs exhibiting similar expression changes in female placentas. (C) Percent correction of female ART DEGs by moderate (FAS4) and high dose (FAS10) of folic acid supplementation in E10.5 female placentas (n=4-6/group/sex). (D) Percent correction of male ART DEGs by FAS4 and FAS10 in E10.5 male placentas (n=4-6/group/sex). Black dots indicate percent correction of DEGs from 0-100% by FAS4 or FAS10 whereas red dots indicate deleterious effects of FAS4 or FAS10. For each DEG, percent correction by

folic acid was calculated using mean expression values of the DEG in the NAT_CD and ART groups (n=4-6placentas/group/sex) as follows: $[1 - \left(\frac{NAT\_CD - ART\_FA}{NAT\_CD - ART\_CD}\right)] * 100$, where ART_FA refers to either ART_FAS4 or ART_FAS10 groups. (E) Chromosomal distribution of DEGs resulting from ARTs in female and male placentas. A, B and C refer to different clusters of DEGs on chromosomes 7 and 15. # refers to common DEGs in male and female placentas following the use of ARTs. M, male; F, female; dir., direction.

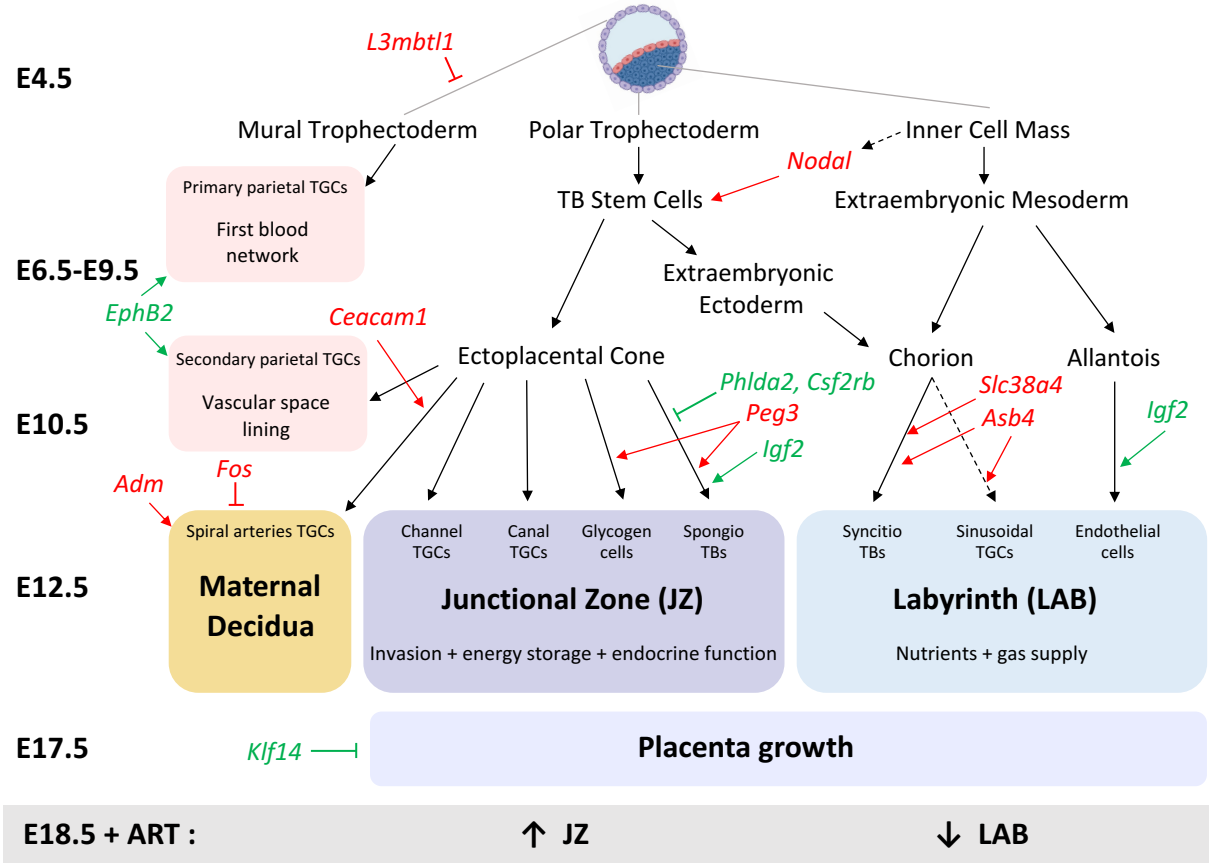


FIGURE 3. ART-induced DEGs are implicated in early placenta development. Upregulated genes are colored in red and downregulated ones in green. E4.5, Embryonic day 4.5; TGCs, Trophoblast giant cells; TBs, Trophoblasts. Adapted from Rai and Cross [54] and Tunster et al.[59].

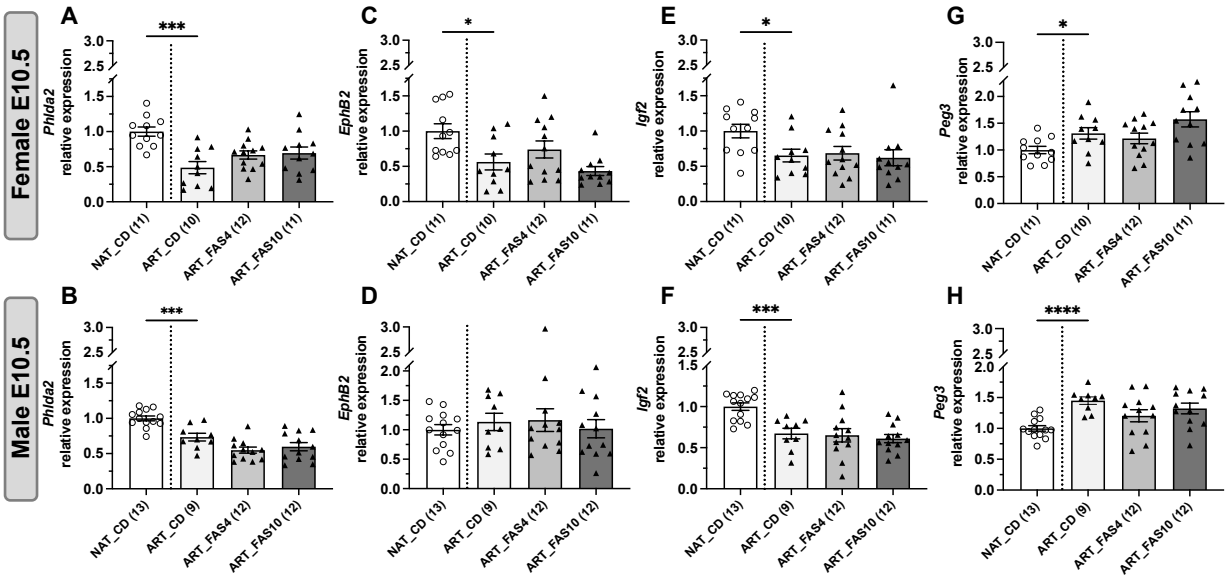


FIGURE 4. Relative expression of key genes affected by ART and involved in early placenta development, in females and male midgestation placentas. (A) *Phlda2* gene expression in placentas from E10.5 female and (B) male placentas. (C) *EphB2* gene expression in E10.5 female and (D) male placentas. (E) *Igf2* gene expression in E10.5 female and (F) male placentas. (G) *Peg3* gene expression in E10.5 female and (H) male placentas. Sample sizes for each group are indicated in parenthesis on the x-axis. Each white circle and black triangle correspond to an individual placenta in the NAT_CD and ART groups, respectively. Gene expression was normalised to transcripts encoding *Rps18* and *Rpl13a*. Data are presented as relative expression compared to controls with mean of NAT_CD E10.5 control group adjusted to 1. Means ± SEM are shown. Student's t-test compared NAT_CD and ART_CD groups; *P < 0.05, ***P < 0.001, ****P < 0.0001. One-way ANOVA with Tukey's correction for multiple comparisons was used to compare ART groups.

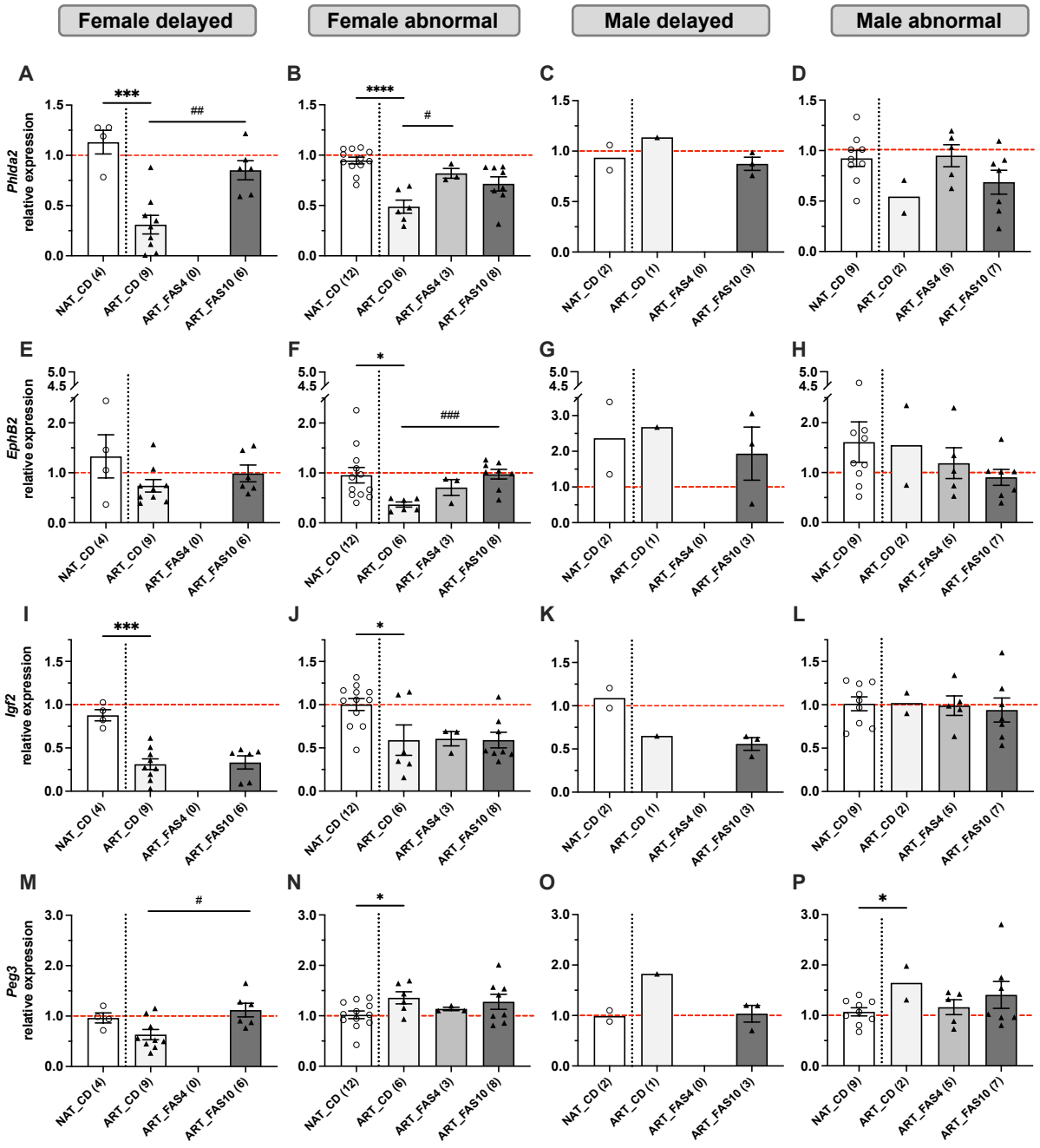


FIGURE 5. Relative expression of key genes affected by ART and involved in early placenta development, in placentas from delayed and abnormal embryos. (A) *Phlda2* gene expression in female delayed, (B) female abnormal, (C) male delayed, (D) male abnormal placentas. (E) *EphB2* gene expression in female delayed, (F) female abnormal, (G) male delayed, (H) male abnormal placentas. (I) *Igf2* gene expression in female delayed, (J) female abnormal, (K) male delayed, (L) male abnormal placentas. (M) *Peg3* gene expression in female delayed, (N) female abnormal, (O) male delayed, (P) male

abnormal placentas. Sample sizes for each group are indicated in parenthesis on the x-axis. Each white circle and black triangle correspond to an individual placenta in the NAT_CD and ART groups, respectively. Gene expression was normalised to transcripts encoding *Rps18* and *Rpl13a*. Data are presented as relative expression compared to controls. The red line indicates mean of NAT_CD E10.5 control group which is adjusted to 1 as presented in Fig. 4. Means ± SEM are shown. Student's t-test compared NAT_CD and ART_CD groups; *P < 0.05, ***P < 0.001. One-way ANOVA with Tukey's correction for multiple comparisons was used to compare ART groups; # P < 0.05, ## P<0.01, ### P<0.0001.

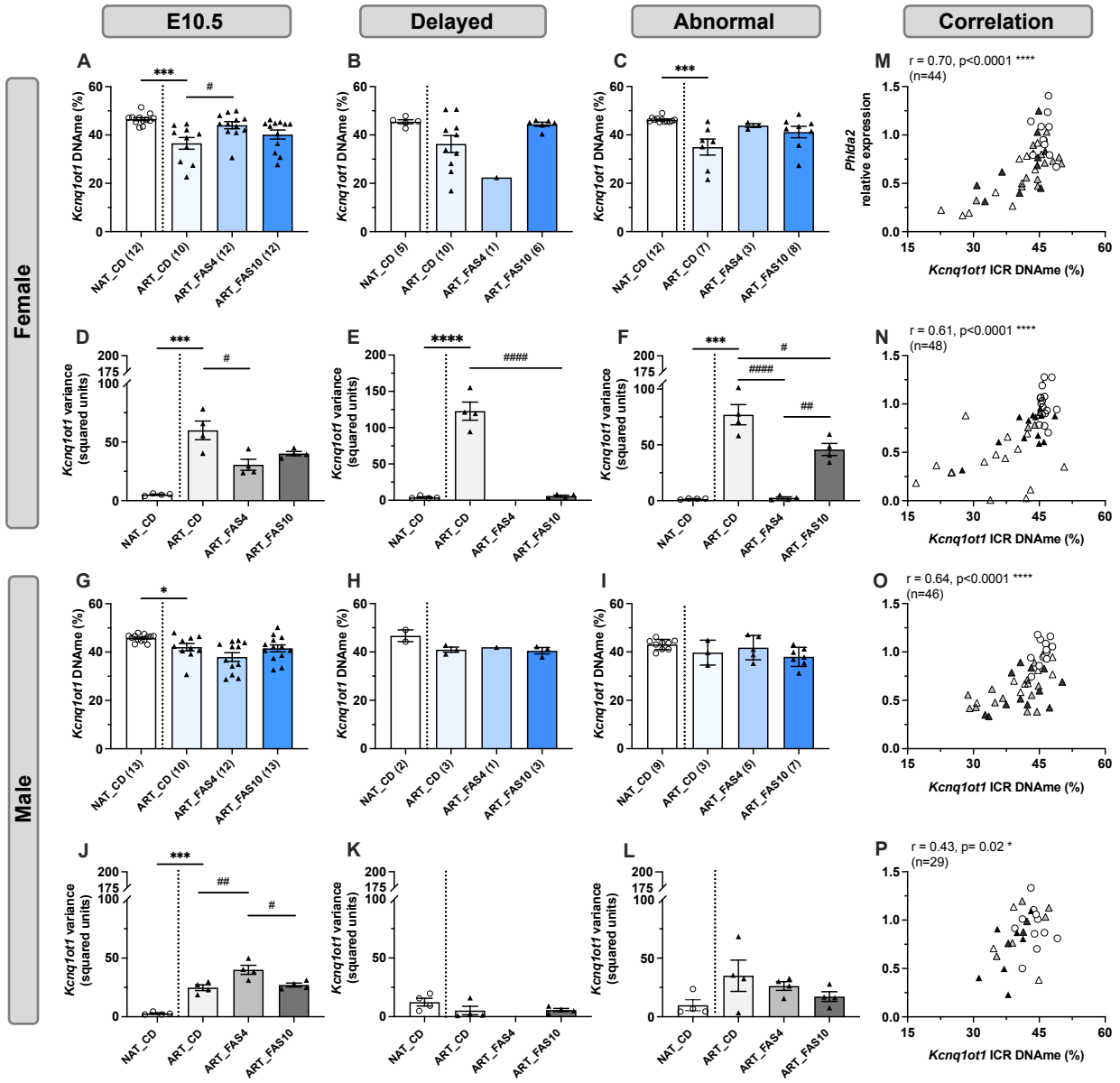


FIGURE 6. Sex-specific effects of folic acid supplementation on *Kcnq1ot1* ICR DNA methylation and correlation between *Kncq1ot1* ICR DNA methylation and *Phlda2* gene expression. (A) DNA methylation at the *Kncq1ot1* ICR in female E10.5, (B) female delayed, (C) female abnormal, (G) male E10.5, (H) male delayed and (I) male abnormal placentas. DNA methylation variance at the *Kncq1ot1* ICR in (D) female E10.5, (E) female delayed, (F) female abnormal, (J) male E10.5, (K) male delayed and (L) male abnormal placentas. Correlation analysis between *Kncq1ot1* ICR DNA methylation and *Phlda2* gene expression in (M) female E10.5, (N) female delayed and abnormal, (O) male E10.5 and (P) male delayed and abnormal placentas. For DNA methylation and variance

analysis, sample sizes for each group are indicated in parenthesis on the x-axis. Each white circle and black triangle correspond to an individual placenta in the NAT_CD and ART groups, respectively. Means ± SEM are shown. Student's t-test compared NAT_CD and ART_CD groups; *P < 0.05, ***P < 0.001, ****P < 0.0001. One-way ANOVA with Tukey's correction for multiple comparisons was used to compare ART groups; # P < 0.05, ## P<0.01, #### P < 0.0001. For correlation analysis, each white circle represents an individual placenta in the NAT_CD group. White, grey, and black triangles indicate individual placentas in the ART_CD, ART_FAS4 and ART_FAS10 groups, respectively. Pearson correlation coefficient r is shown for each analysis; *P < 0.05, ****P < 0.0001. Strong correlation: r > 0.7; Moderate correlation: 0.5 < r < 0.7; Weak correlation: r < 0.3.

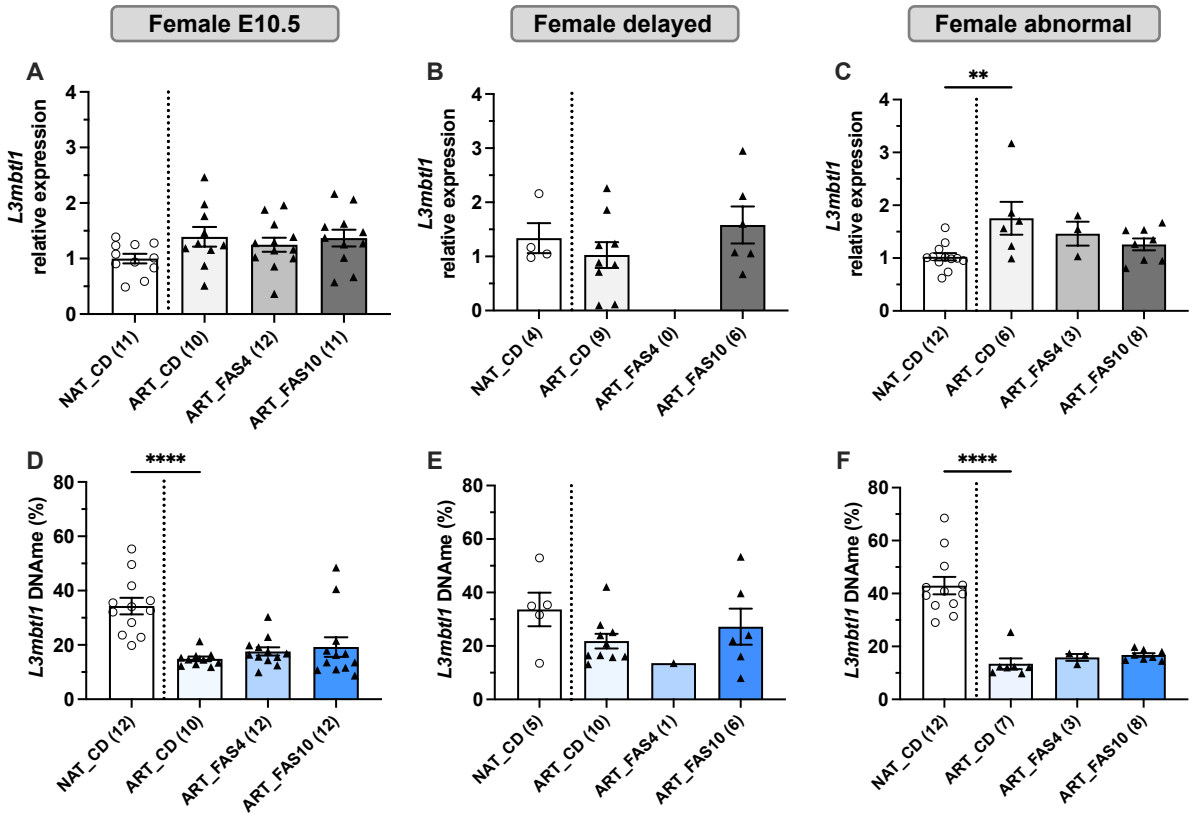
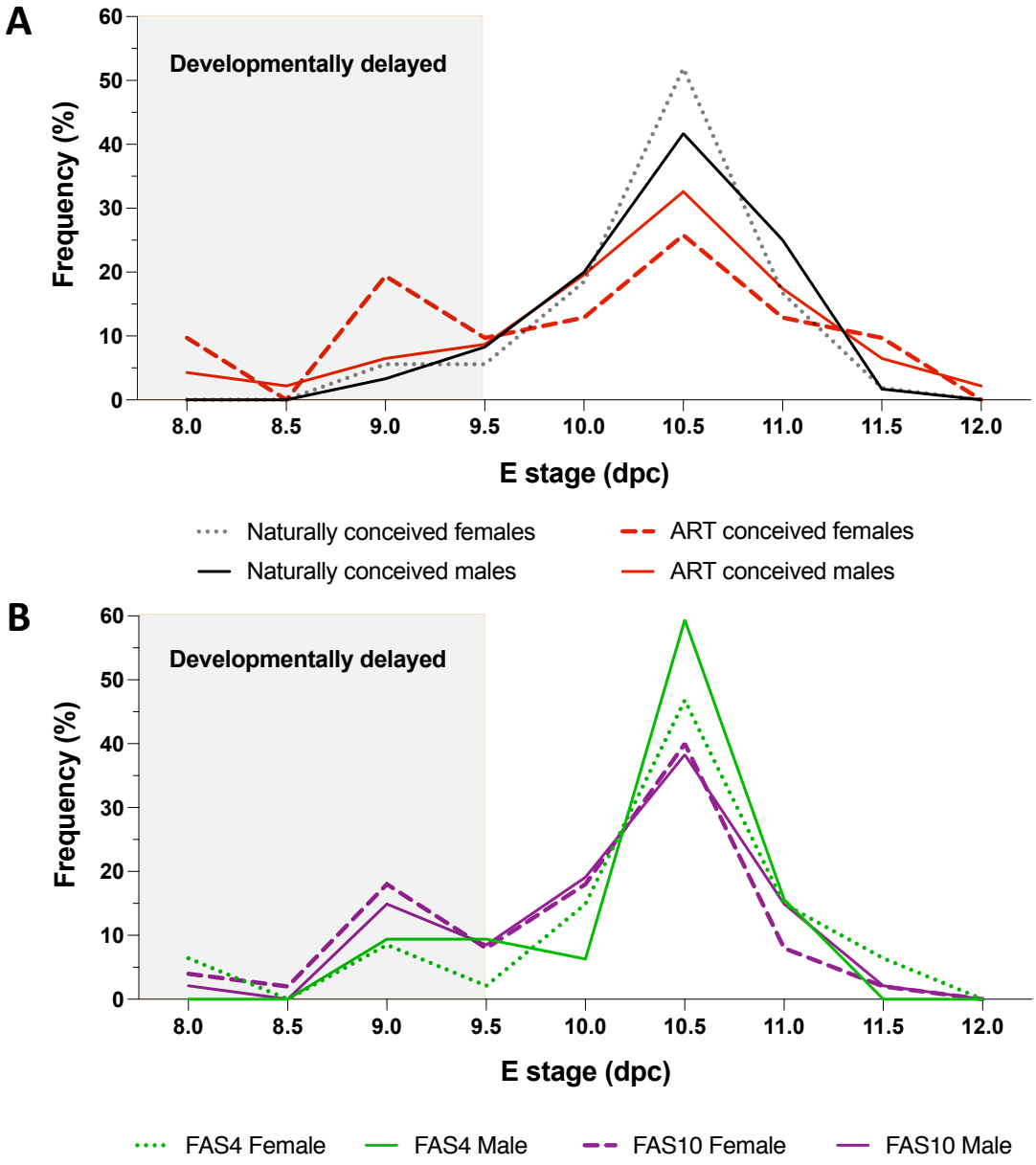
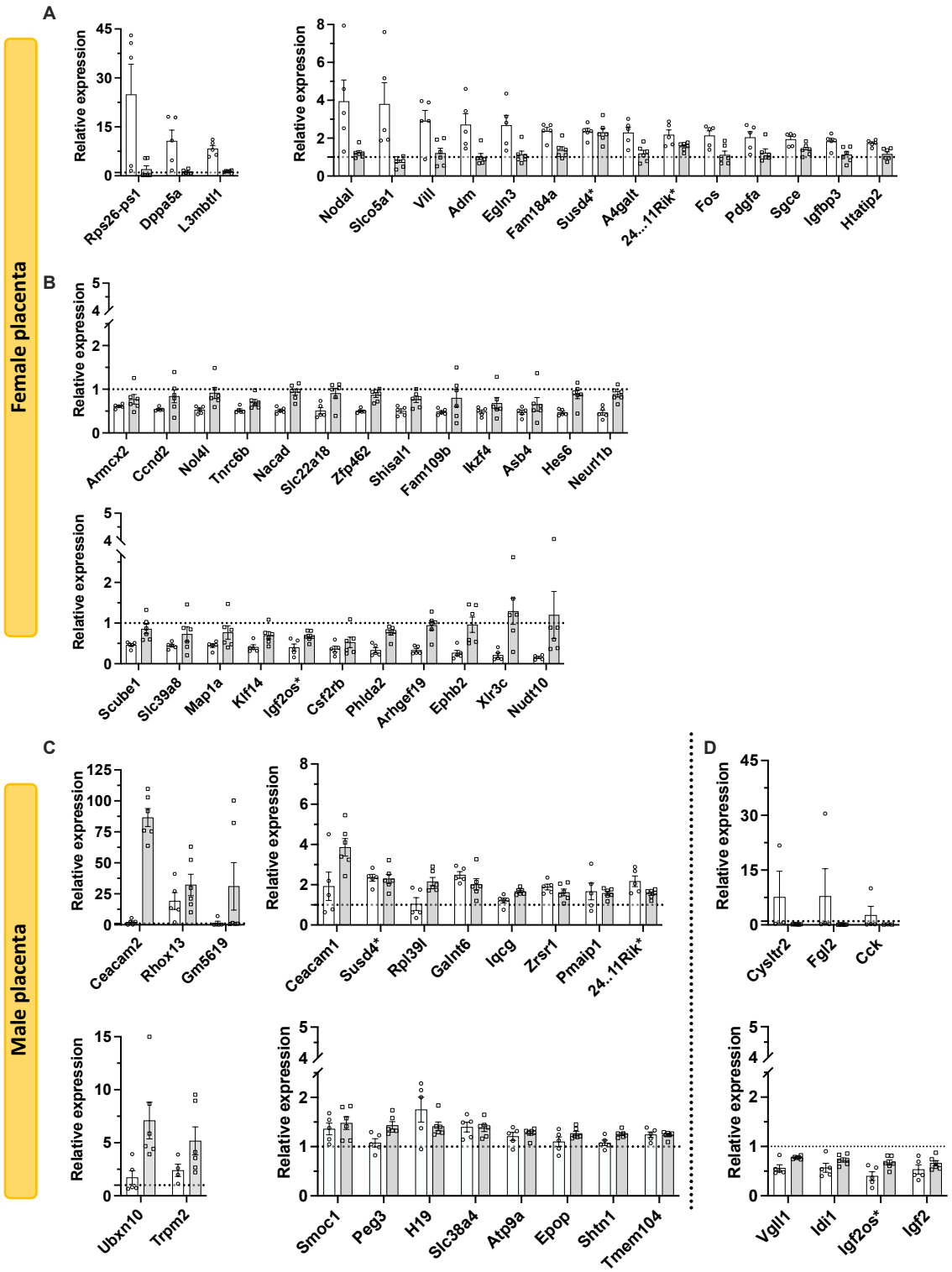


FIGURE 7. Gene expression and DNA methylation profiles of *L3mbtl1* transcription factor following ART. (A) *L3mbtl1* gene expression in female E10.5, (B) delayed and (C) abnormal placentas. (D) *L3mbtl1* promoter DNA methylation in female E10.5, (E) delayed and (F) abnormal placentas. Sample sizes for each group are indicated in parenthesis on the x-axis. Each white circle and black triangle correspond to an individual placenta in the NAT_CD and ART groups, respectively. Gene expression was normalised to transcripts encoding *Rps18* and *Rpl13a*. Gene expression data are presented as relative expression compared to controls with mean of NAT_CD E10.5 control group equal to 1. Means ± SEM are shown. Student's t-test compared NAT_CD and ART_CD groups; **P < 0.01, ****P < 0.0001. One-way ANOVA with Tukey's correction for multiple comparisons was used to compare ART groups.

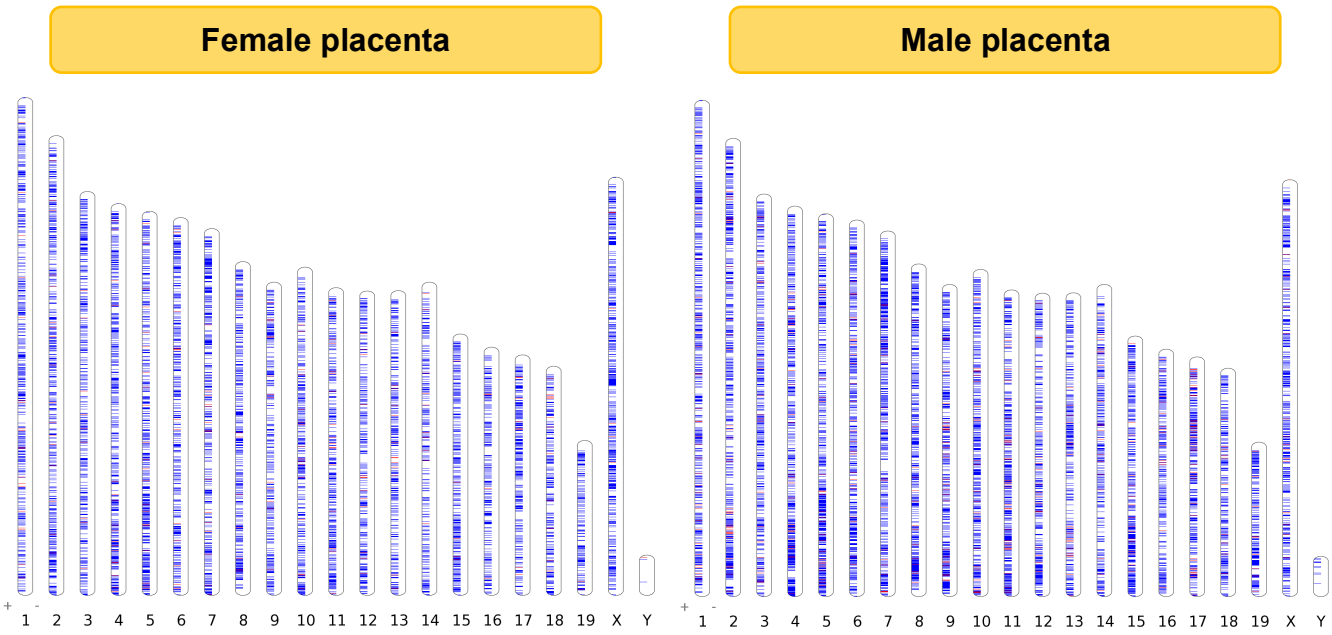


SUPPLEMENTAL FIGURE 1 Frequency distribution curves of embryonic stages per sex, for embryos collected at mid-gestation. Embryos collected from females (A) fed the control diet and (B) fed the 4x folic acid supplemented diet (ART_FAS4) or 10x folic acid supplemented diet (ART_FAS10).

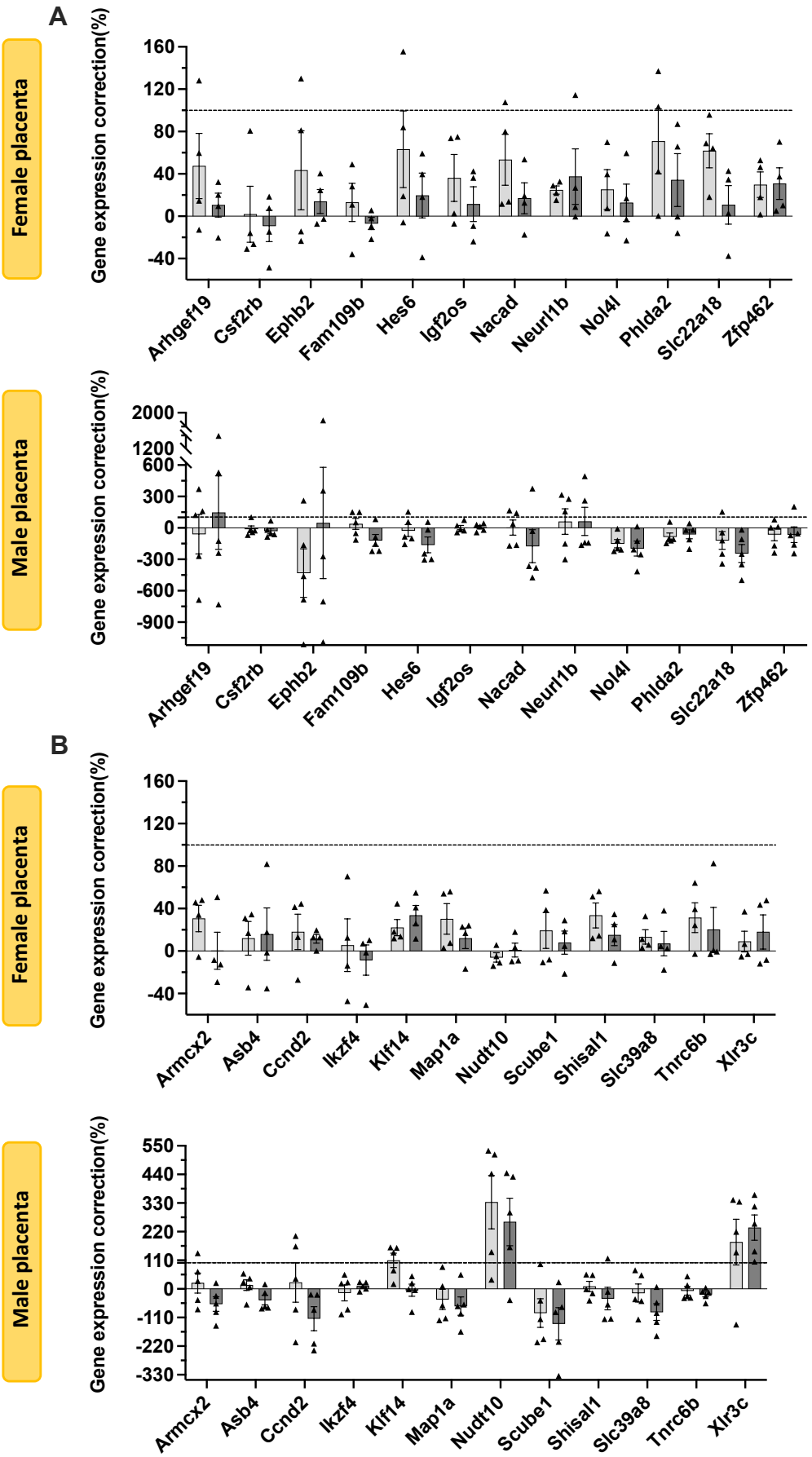


SUPPLEMENTAL FIGURE 2 Expression of ART-induced DEGs in midgestation male and female placentas. The expression of female ART (A) upregulated and (B) downregulated genes in female and male E10.5 placentas. The expression of male ART

(C) upregulated and (D) downregulated genes in female and male E10.5 placentas. Each white circle (hollow bars) and white square (grey bars) corresponds to an individual placenta sample (n= 4-6/group) in female and male placentas, respectively. An asterisk indicates common DEGs between male and female placentas. 24…11Rik: 2410003L11Rik



SUPPLEMENTAL FIGURE 3 Chromosomal distribution of differentially methylated tiles (DMTs) induced by ART in placenta. Genome-wide DNA methylation in placentas corresponding to normal E10.5 embryos were compared between the NAT_CD group and the ART_CD group (n= 4-6 per sex/group). Chromosomal distribution of DMTs identified by Reduced Representation Bisulfite Sequencing (RRBS) caused by ART in male and female placentas are shown.


A
Female placenta
Gene expression correction(%)
160
120
80
40
0
-40
Arhgef19
Csf2rb
Ephb2
Fam109b
Hes6
Igf2os
Nacad
Neurl1b
Nol4l
Phlda2
Slc22a18
Zfp462
Male placenta
Gene expression correction(%)
2000
1200
600
300
0
-300
-600
-900
Arhgef19
Csf2rb
Ephb2
Fam109b
Hes6
Igf2os
Nacad
Neurl1b
Nol4l
Phlda2
Slc22a18
Zfp462
B
Female placenta
Gene expression correction(%)
160
120
80
40
0
-40
Armcx2
Asb4
Ccnd2
Ikzf4
Klf14
Map1a
Nudt10
Scube1
Shisal1
Slc39a8
Tnrc6b
Xlr3c
Male placenta
Gene expression correction(%)
550
440
330
220
110
0
-110
-220
-330
Armcx2
Asb4
Ccnd2
Ikzf4
Klf14
Map1a
Nudt10
Scube1
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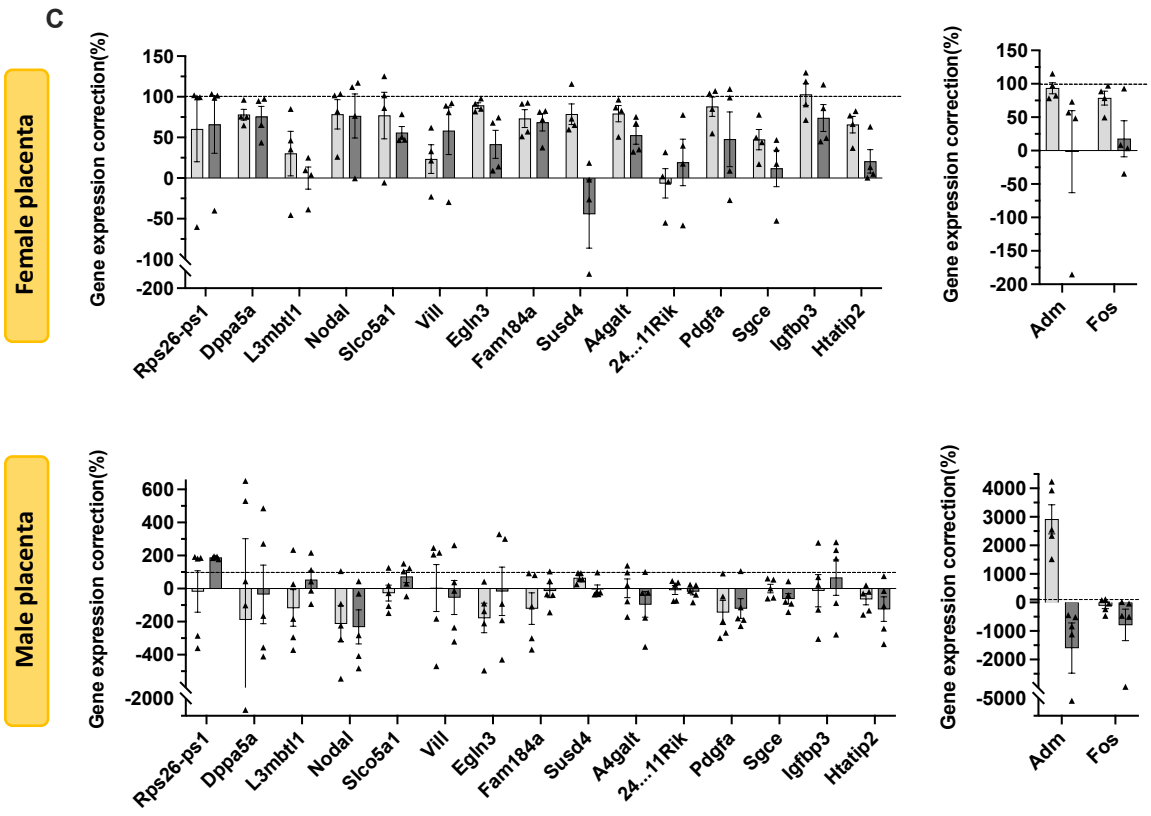
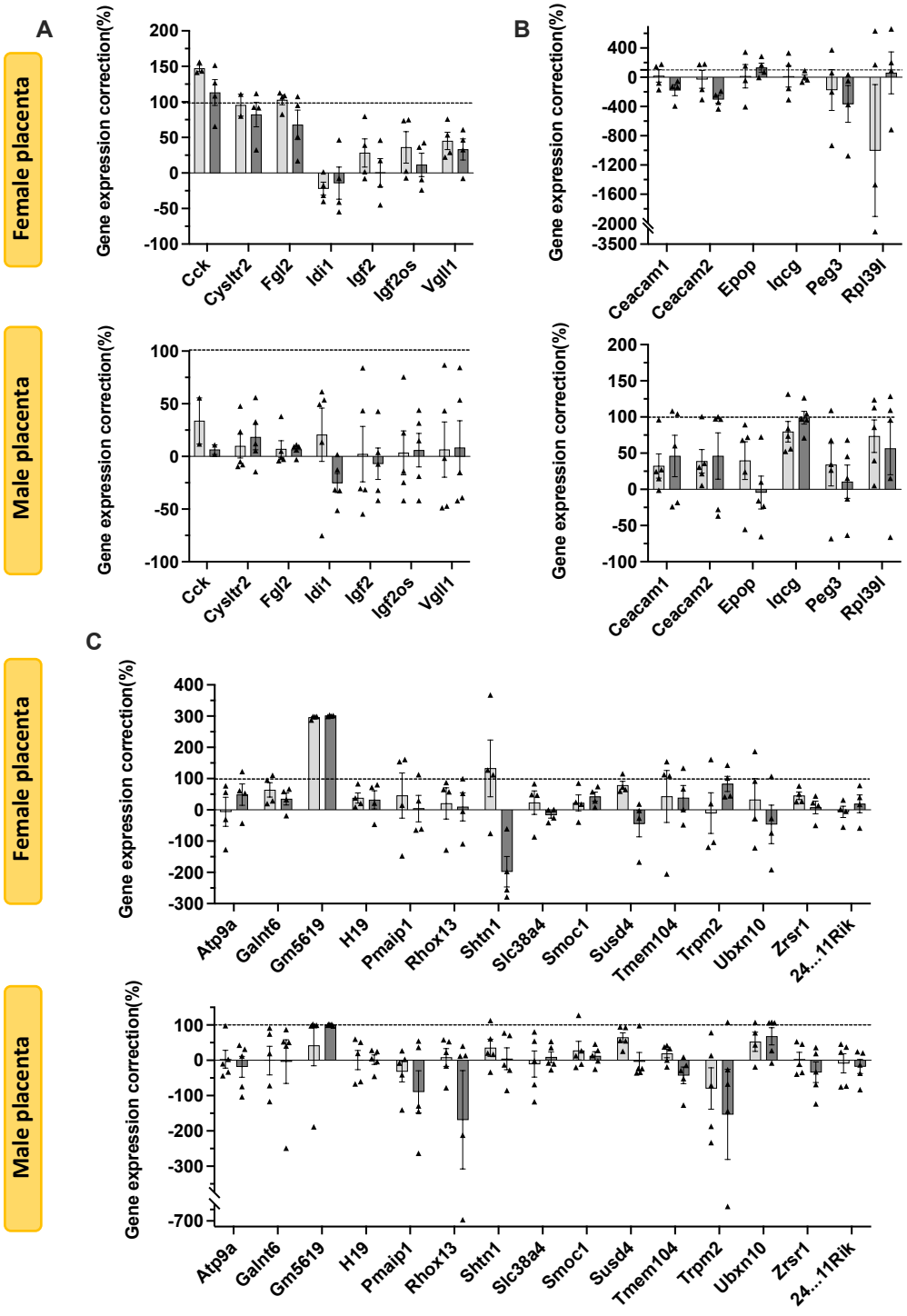


SUPPLEMENTAL FIGURE 4 Sex-specific response to folic acid supplementation of female ART DEGs in midgestation placentas following ART procedures. Correction of gene expression by moderate and high folic acid supplementation for (A/B) female ART downregulated genes and (C) female ART upregulated genes in E10.5 male and female placentas (n=4-6 per sex/group). Each filled triangle indicates an individual placenta. Light-grey filled bars indicate % correction by moderate FA supplementation (ART_FAS4) and dark-grey filled bars indicate % correction by high FA supplementation (ART_FAS10). % Correction was calculated using group means in Abbott's formula: $[1 - \left(\frac{NAT\_CD - ART\_FA}{NAT\_CD - ART\_CD}\right)] * 100$. Means $\pm$ SEM are shown. 24…11Rik: 2410003L11Rik



SUPPLEMENTAL FIGURE 5. Sex-specific response to folic acid supplementation of male ART DEGs in midgestation placentas following ART procedures. The effect of moderate and high folic acid supplementation on the gene expression correction of (A) male ART downregulated genes and (B/C) male ART upregulated genes in E10.5 male

and female placentas (n=4-6 per sex/group). Each filled triangle indicates an individual placenta. Light-grey filled bars indicate % correction by moderate FA supplementation (ART_FAS4) and dark-grey filled bars indicate % correction by high FA supplementation (ART_FAS10). % Correction was calculated using group means in Abbott's formula: $[1 - \left(\frac{NAT\_CD - ART\_FA}{NAT\_CD - ART\_CD}\right)] * 100$. Means $\pm$ SEM are shown. 24…11Rik: 2410003L11Rik

Supplemental Table 1. ART-induced DEGs in female placentas (ART_CD vs NAT_CD).

Down-regulated genes			Up-regulated genes		
Gene	**Fold change**	**FDR adjusted p-value**	**Gene**	**Fold change**	**FDR adjusted p-value**
Arhgef19	2.50	0.00019	*Susd4*	2.14	0.00044
Slc39a8	2.06	0.00019	*A4galt*	2.08	0.00139
Phlda2	2.47	0.00030	*Slco5a1*	2.62	0.00192
Tnrc6b	1.81	0.00107	*Dppa5a*	2.85	0.00310
Zfp462	1.84	0.00369	*Sgce*	1.83	0.00449
Ccnd2	1.73	0.00565	*Fam184a*	2.07	0.00523
Xlr3c	2.60	0.00737	*Egln3*	2.23	0.00523
Map1a	1.97	0.01161	*2410003L11Rik*	1.97	0.00606
Armcx2	1.58	0.01567	*Igfbp3*	1.74	0.01051
Nol4l	1.76	0.01567	*Adm*	2.20	0.01051
Scube1	1.92	0.01806	*Rps26-ps1*	2.47	0.01262
Nudt10	2.49	0.01806	*Vill*	2.24	0.01567
Fam109b	1.87	0.01815	*L3mbtl1*	2.47	0.01883
Igf2os	2.07	0.01929	*Fos*	1.90	0.02442
Nacad	1.78	0.02014	*Pdgfa*	1.85	0.03377
Hes6	1.88	0.03377	*Nodal*	2.32	0.03426
Shisal1	1.80	0.03542	*Htatip2*	1.64	0.04660
Csf2rb	2.12	0.03542			
Klf14	2.02	0.03560			
Ephb2	2.28	0.03793			
Slc22a18	1.79	0.04069			
Asb4	1.86	0.04161			
Ikzf4	1.83	0.04660			
Neurl1b	1.87	0.04701			

DEGs: Differentially expressed genes; FDR: False discovery rate

Supplemental Table 2. DEGs induced by the moderate dose of folic acid supplementation in female placentas (ART_FAS4 vs ART_CD).

Down-regulated genes			Up-regulated genes		
Gene	**Fold change**	**FDR adjusted p-value**	**Gene**	**Fold change**	**FDR adjusted p-value**
Synj2	2.05	0.00944	*Adamts4*	1.91	9.03E-05
Pvrl1	1.83	0.02546			

DEGs: Differentially expressed genes; FDR: False discovery rate

Supplemental Table 3. DEGs induced by a further folic acid supplementation in female placentas (ART_FAS10 vs ART_FAS4).

Down-regulated genes			Up-regulated genes		
Gene	**Fold change**	**FDR adjusted p-value**	**Gene**	**Fold change**	**FDR adjusted p-value**
Gjb4	2.08	0.00105	*Serping1*	2.79	0.00000
Nptx1	2.06	0.00184	*Chrd*	2.26	0.00043
			Des	2.15	0.00154
			Cd68	1.82	0.00912
			Hand2	1.96	0.01354
			Degs2	1.92	0.03037
			Gatm	1.83	0.03562
			Cd14	1.88	0.03562
			Ly6a	1.81	0.04967

DEGs: Differentially expressed genes; FDR: False discovery rate

Supplemental Table 4. ART-induced DEGs in male placentas (ART_CD vs NAT_CD).

Down-regulated genes			Up-regulated genes		
Gene	**Fold change**	**FDR adjusted p-value**	**Gene**	**Fold change**	**FDR adjusted p-value**
Igf2	1.51	0.00071	*Ceacam2*	88.30	5.20E-70
Vgll1	1.29	0.00080	*Rhox13*	31.19	3.91E-09
Cysltr2	6.06	0.00134	*Ceacam1*	3.86	3.91E-09
Igf2os	1.49	0.00293	*Susd4*	2.29	0.00014
Fgl2	11.39	0.00562	*Shtn1*	1.26	0.00024
Cck	17.27	0.02413	*Slc38a4*	1.38	0.00052
Idi1	1.38	0.02664	*H19*	1.41	0.00101
			Peg3	1.43	0.00364
			Trpm2	5.21	0.00612
			Zrsr1	1.62	0.00805
			Galnt6	2.02	0.00805
			Smoc1	1.48	0.01080
			Ubxn10	7.25	0.01238
			2410003L11Rik	1.55	0.01546
			Tmem104	1.23	0.01819
			Epop	1.27	0.01819
			Pmaip1	1.57	0.01819
			Iqcg	1.65	0.01819
			Gm5619	31.02	0.02086
			Atp9a	1.27	0.04530
			Rpl39l	2.16	0.04899

DEGs: Differentially expressed genes; FDR: False discovery rate

Supplemental Table 5. DEGs induced by the high dose of folic acid supplementation in male placentas (ART_FAS10 vs ART_CD).

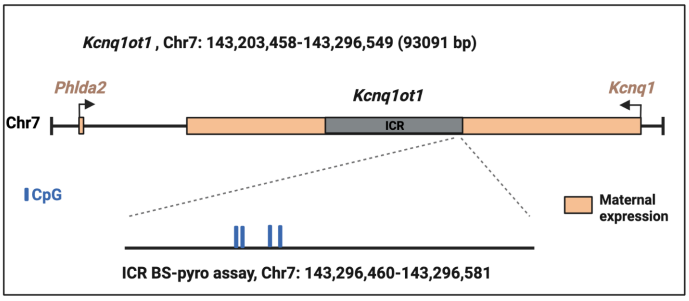
Down-regulated genes			Up-regulated genes		
Gene	**Fold change**	**FDR adjusted p-value**	**Gene**	**Fold change**	**FDR adjusted p-value**
Slc15a2	55.58	0.00150	*Ptpru*	1.51	0.02004
Hmga2	1.37	0.00572	*Cpsf7*	1.20	0.02004

DEGs: Differentially expressed genes; FDR: False discovery rate

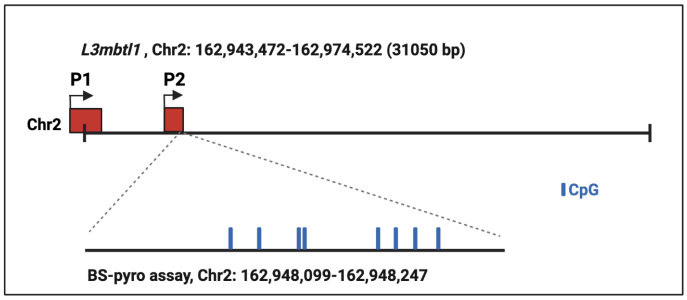
Supplemental Table 6. Primers used for BS-pyrosequencing

BS-Pyrosequencing primers

Gene analyzed	Genic region	Region coordinates (GRCm38)	No. of CGs	Ta (°C)	Primers	5'-3' sequence
Phlda2	ICR (*Kcnq1ot1*)	Chr7: 143296460-143296581	4	55	For	AGGTTTTGGTAGGTGGTTT
					Rev	Biot-CCTAACTAAACCAAAATACACCATCATA
					Seq1	GTTAGGAGGAATAGTTGTTTTA



L3mbtl1	Promoter 2	Chr2: 162948099-162948247	7	60	For	GTTGTTTATGGGTGGGAAGATTGAG
					Rev	Biot-ACAAAAAAAACTACAAACCCTCATAC
					Seq1	GTTAAGATATAATTTTTTTGGAAT
					Seq2	GTGGGTTTTAATAAAGTAGTG



BS: Bisulfite; ICR: Imprinting control region; GRCm38: Genome Reference Consortium mouse build 38 (used as reference genome); P: promoter; Ta: annealing temperature; For: forward primer; Rev: reverse primer; Seq1: sequencing primer no.1, Seq2: sequencing primer no.2

CHAPTER III: Discussion, future directions, and conclusion

3.1 Discussion

In this section, we will address additional points of discussion that were not included in the manuscript. First, we will take a closer look at the timing of folic acid supplementation in our mouse model of ARTs and suggest future experiments to tackle questions arising from our experimental design. Secondly, most studies examining epigenetic marks affected by ARTs have concentrated on DNA methylation; now evidence shows that other epigenetic marks relying on methyl donors should also be considered. Histone marks - notably H3K27me3 - are retained in preimplantation embryos and can regulate gene expression.

3.1.1 The timing of folic acid supplementation

In Canada, maternal folic acid supplementation is recommended at least three months – equal to three oocyte maturation cycles - before pregnancy and continuing during the first months of pregnancy to prevent neural tube defects and other complications. In our mouse model of ARTs, female mice were supplemented with FA for 6 weeks - equivalent to three oocyte maturation cycles in mice - prior to ARTs to reflect optimal folate status. FA supplementation continued post conceptionally (after embryo transfer) until midgestation; however no methyl donors were added during the embryo culture period which coincides with times when DNA methylation patterns are highly dynamic. When cultured blastocysts were transferred back into the uterus for implantation, this coincided with the period of re-acquisition of DNA methylation profiles in the embryonic and extraembryonic tissues which are important for gene expression regulation. Therefore, maternal FA supplementation post-implantation and during gestation is essential to provide methyl donors for optimal embryonic development.

However, the question arising from the current guidelines on maternal periconceptional use of FA is that they do not address the case of assisted reproduction and particularly supplementation during embryo culture when gamete-derived DNA methylation at imprinted gene ICRs and other sequences must be maintained post-fertilization. Thus, it is reasonable to believe that methyl donors are also needed during

embryo culture for the maintenance of DNA methylation patterns at key sequences. In 2010, Menezo and colleagues suggested that DNA methylation changes can be attributable to lack of essential methyl donors and amino acids in culture medium[1]. In fact, several authors have pointed out the need for B vitamins, including vitamin B9, in culture media as epigenetic regulators that can affect DNA methylation and genomic imprinting[2-4]. Though we did not supplement embryo culture with FA or any other methyl donor, the KSOM media used in our experiments contains approximately 50 uM/L of methionine. Commercially available culture media used in human ART display different methyl donor concentrations, with methionine concentrations usually varying from 0 - 100 uM[5]. Even though the methionine concentration in the KSOM media is within this range, studies on appropriate levels of methyl donors in culture and the impact on DNA methylation of regulatory sequences in relation to assisted reproduction are needed. We would expect that maternal use of FA starting preconceptionally and continuing during gestation combined with supplementation in culture may be optimal as it would improve oocyte quality/maturation and embryo development[6,7].

Remarkably, studies have suggested that ART-induced defects observed in placentas and offspring were primarily driven by embryo culture[8,9]. When Vrooman and colleagues compared the effects of different ART techniques in the mouse, embryo culture alone reduced fetoplacental weight ratios, increased placental JZ size, and decreased placental DNAme of imprinted loci at E18.5[9]. Previous studies have shown that loss of DNA methylation at imprinted loci is already detectable at the blastocyst stage, although the mechanism is poorly understood[10-13]. Recent evidence also shows that embryo culture decreases methylation levels of imprinted gene ICRs in an asynchronous manner[8]. Therefore, it is essential to understand methyl donor action particularly prior to the morulae stage, as culture during this sensitive time leads to placental morphological abnormalities and imprinting defects[8].

Another question arising from our experiments is whether ART-induced transcriptomic changes persist past midgestation and whether FAS4 can rescue such defects until the end of gestation. Recently, Vrooman and colleagues noted that placental DNA methylation defects at imprinted loci persisted until E18.5[9]; however, our results do not inform us whether these imprinting defects are associated with expression and

proteomic alterations at different times leading to the end of gestation. Furthermore, we do not know whether FAS4-induced ameliorations are still present at the end of gestation, especially in placentas from developmentally delayed and/or abnormal embryos. A future experiment could inform us whether FA supplementation helps these developmentally delayed and/or abnormal embryos to catch up and survive until the end gestation. Since FAS4 corrected development delay in our previous study, we expect that improvements detected at midgestation will persist until the end of gestation in the mouse and improve adverse placenta defects associated with ARTs. Therefore, an important follow-up study investigating the effects of FAS4 on ART-associated placental defects after midgestation would also be required.

As mentioned previously, adverse effects of FA over-supplementation - seen in the case of male placentas in our study - can exert the same effects as folate deficiency by downregulating enzymes involved in one-carbon metabolism. Since folic acid is a synthetic supplement, it lacks coenzyme activity and must therefore be converted into the metabolically active THF form. 5-mTHF is the most abundant form of folate and it is normally found in circulation. Studies have compared folic acid and 5-mTHF supplementation and found that 5-THF displays better performance in terms of plasma concentrations of folate [14,15]. Moreover, using 5-mTHF bypasses the catalytic reaction performed by DHFR which is slow and easily oversaturated, reduces the risk of high unmetabolized FA which can mask vitamin B12 deficiency and overcomes metabolic defects caused by a common *MTHFR* polymorphism which has been associated with increased risk of NTD and increased cardiovascular risk[14]. Thus, if the results of a potential follow-up study indicated that FAS4 does not rescue placental defects at term as hypothesized, we propose experiments on alternative maternal methyl-group donors such 5-mTHF.

3.1.2 ARTs and histone modifications; beyond DNA methylation

Recently, new studies have shed light on the reprogramming of histone modifications during early peri-implantation development[16]. After fertilization, sperm H3K4me3 and H3K27me3 histone modifications are replaced by broad domains of *de novo* H3K4me3 and H3K27me3 in zygotes[16,17]. However, mouse oocyte H3K4me3 is

inherited in embryos, persisting until the 2-cell-stage while oocyte-derived H3K27me3 reprogramming is more complex[16]. H3K27me3 is removed at promoters of developmental genes and is only restored at the blastocyst stage whereas oocyte-derived distal H3K27me3 domains are inherited in embryos and persist to the blastocyst stage[18]. During this brief time when oocyte derived H3K27me3 domains are inherited in embryos, they can function to regulate imprinted gene expression[19]. In mouse, imprinted genes marked by H3K27me3 rather than DNA methylation are known as non-canonical imprinted genes. Canonical imprinted genes are normally marked by DNA methylation but can also carry histone methylation marks as well. Notably, H3K27me3 is completely depleted before zygotic genome activation in humans and restored as early as the morulae stage, suggesting absence of H3K27me3-mediated imprinting in humans[16].

Polycomb Repressive complex (PRC2) which consists of the Embryonic Ectoderm Development (EED) protein among other interacting factors, methylates histone H3K27 to form H3K27me3[20]. Mouse experiments examining imprinting in $Eed^{-/-}$ mutant embryos have revealed that certain paternally expressed imprinted genes (PEGs) are regulated by oocyte-derived H3K27me3, which inhibits maternal gene expression of PEGs in early embryos [19,21,22]. Moreover, XCI in female preimplantation embryos is regulated by maternal H3K27me3 and dysregulation leads to developmental retardation in Eed maternal KO embryos[23]. So far, at least seven PEGs have been identified, including *Slc38a4* which was upregulated in our study following ARTs. Though PEGs rely on H3K27me3 for genomic imprinting during preimplantation development, maternal H3K27me3 marks are largely lost after implantation. Post-implantation, PEGs acquire secondary DNA methylation marks on the maternal allele, to ensure and maintain paternal expression in extraembryonic tissue[24-27]. These PEGs are also known as non-canonical imprinted genes.

Recently, new evidence has shown that non-canonical imprinted genes are essential for embryonic development and placenta growth regulation[23]. It was demonstrated that KO of *Slc38a4* severely impairs placental development, and that loss of imprinting contributes to placental enlargement[23,28,29]. Since our study identified that *Slc38a4* was upregulated in placentas at midgestation following ARTs, it is possible that oocyte-derived H3K27me3 patterns at this locus were perturbed by IVF and/or that

H3K27me3 in preimplantation embryos was altered during embryo culture. Though the exact mechanism remains unknown, *Slc38a4* upregulation in midgestation placentas observed in our study following the use of ARTs may very well contribute to the placenta overgrowth phenotype detected at the end of gestation by Vrooman and colleagues[9] and stem from altered histone methylation in the pre-implantation period. This hypothesis is further confirmed by a recent report demonstrating that *Slc38a4* upregulation/overexpression (due to loss of imprinting) contributes to placenta overgrowth by increasing amino acid transport via mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway overactivation in SCNT placentas[29]; whereas maternal loss of *Slc38a4* restores imprinting and rescues placental enlargement[29].

Given that histone methylation marks play a pivotal role in XCI and non-canonical imprinting which regulate embryonic and placental development, we must also consider such aspects in future studies. Thus, adverse placental phenotypes observed at the end of gestation in the mouse model of ARTs may be a result of alterations in canonical and non-canonical imprinted genes and perhaps other loci. Importantly, further investigation into the effects of ARTs on histone modifications is warranted, given their essential role in genomic imprinting. Since FA supplementation provides SAM which is also a methyl-donating substrate to histone methyltransferases[30,31], studies on the effects of FA supplementation on H3K27me3-mediated events taking place in early embryos following ARTs are warranted. As such, methyl donor supplementation may be able to rescue canonical and non-canonical imprinting defects following ARTs.

3.2 Conclusion

Overall, FAS4 was beneficial to both male and female placentas given that maternal supplementation improved expression levels of many ART-perturbed DEGs, variance at *Kcnq1ot1* ICR and DNA methylation levels in midgestation placentas. However, whereas FAS10 exhibited partial corrective effects of most female ART DEGs, it exacerbated the expression of close to half of the male ART DEGs.

Altogether, ARTs perturbed gene expression and DNAme in midgestation placentas. These changes may persist and contribute to adverse placental defects observed at the end of ART pregnancies in the mouse. While clinically relevant moderate

but not high dose FA supplementation was beneficial, further investigation into optimization of ART procedures and FA supplementation during ART pregnancies is warranted.

CHAPTER III references

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