INVESTIGATION INTO DNA METHYLATION AND GENE EXPRESSION PROFILES
OF IMPRINTED REGIONS IN INFANTS CONCEIVED VIA ASSISTED
REPRODUCTIVE TECHNOLOGIES

by

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Abstract

A correlation has been found between babies born through assisted reproductive technologies (ARTs) and an increased risk of genomic imprinting disorders such as Beckwith-Wiedemann syndrome (BWS), Angelman syndrome (AS), and Silver-Russell syndrome (SRS), as well as other diseases in later life such as diabetes and obesity. In order to assess whether in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) impact the establishment of DNA methylation and/or gene expression patterns of imprinted regions, cord blood and placental tissue were collected from babies born after IVF and ICSI infertility treatments, as well as from naturally conceived controls. DNA methylation levels at the PLAGL1, KvDMR1 and PEG10 differentially methylated regions (DMRs) and their corresponding gene expression levels were analyzed due to their association with known imprinting disorders and influence on fetal and placental development. Methylation at the LINE-1 repetitive element was also analyzed as an indicator of global DNA methylation.

We discovered a small, but significant increase in DNA methylation at the DMR of the tumor suppressor gene, PLAGL1, in cord blood from IVF pregnancies, as well as a lack of methylation at PEG10 in cord blood from all groups. We also found decreased levels of PLAGL1 gene expression in the IVF and ICSI groups, and decreased expression of KCNQ1OT1 and increased expression of IGF2 in IVF cord blood samples, which may suggest the disruption of an imprinted gene network involving chromosome 11p15.5. These findings indicate that the PLAGL1 DMR may be susceptible to epimutations in the ART population and could potentially lead to increased risk of imprinting disorders, intrauterine growth restriction and cancer susceptibility in ART conceived infants.
Preface

This thesis was written with input and guidance from Dr. Sai Ma as well as my supervisory committee members: Dr. Timothy Rowe, Dr. Patrice Eydoux, and Dr. Wan Lam. These projects were designed by Dr. Sai Ma, Edgar Chan Wong, and Rebecca Vincent. The experiments from Chapter 2 and 3 were performed by Rebecca Vincent and a Ma lab research assistant: All gene expression assays were conducted by Rebecca Vincent, as well as a large portion of the DNA methylation procedures. Data analysis, figures and tables were prepared by Rebecca Vincent, with assistance from Kenny Louie. Preliminary results from Chapters 2 and 3 were published in the abstract book and presented at the 71st annual American Society of Reproductive Medicine (ASRM) meeting in October 2014.

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Table of Contents

Abstract ........................................................................................................................................... ii
Preface ............................................................................................................................................... iii
Table of Contents .............................................................................................................................. iv
List of Tables ..................................................................................................................................... ix
List of Figures ................................................................................................................................... x
List of Abbreviations ......................................................................................................................... xi
Acknowledgements ........................................................................................................................... xiv

Chapter 1: Introduction ....................................................................................................................... 1

1.1 Project overview ......................................................................................................................... 1

1.2 Infertility ....................................................................................................................................... 2

1.2.1 Male factor infertility ............................................................................................................. 3

1.2.1.1 CF mutations .................................................................................................................... 4

1.2.1.2 Y chromosome microdeletions ......................................................................................... 5

1.2.1.3 Chromosomal abnormalities ............................................................................................ 6

1.2.2 Female factor infertility .......................................................................................................... 7

1.2.2.1 Advanced maternal age .................................................................................................... 8

1.3 Assisted reproductive technologies ........................................................................................... 9

1.3.1 In vitro fertilization ................................................................................................................. 10

1.3.2 Intracytoplasmic sperm injection .......................................................................................... 11

1.3.3 Pregnancy outcomes of ARTs .............................................................................................. 11

1.3.3.1 Multiple gestations ........................................................................................................... 12

1.3.3.2 Low birth weight and malformations .............................................................................. 13
1.3.3.3 Long-term outcomes ......................................................................................................................... 14
1.4 Embryogenesis ........................................................................................................................................... 15
  1.4.1 Fertilization and early embryogenesis ..................................................................................................... 16
    1.4.1.1 Origins of placental and embryonic tissues ......................................................................................... 17
    1.4.1.2 Structure and function of the placenta ................................................................................................. 19
1.5 Epigenetics, genomic imprinting, and imprinted gene network .............................................................. 21
  1.5.1 Epigenetics ............................................................................................................................................. 21
    1.5.1.1 DNA methylation ................................................................................................................................. 22
    1.5.1.2 Histone modifications ............................................................................................................................ 24
    1.5.1.3 Non-coding RNA .................................................................................................................................. 26
  1.5.2 Genomic imprinting ............................................................................................................................... 27
    1.5.2.1 Parental-Conflict Theory ....................................................................................................................... 29
    1.5.2.2 Genomic reprogramming ...................................................................................................................... 30
    1.5.2.3 Imprinting disorders ............................................................................................................................. 33
    1.5.2.4 Imprinting in the placenta ..................................................................................................................... 37
    1.5.2.5 Imprinted gene network ....................................................................................................................... 39
1.6 ART and genomic imprinting ..................................................................................................................... 43
  1.6.1 Case studies of ART and imprinting disorders ......................................................................................... 43
  1.6.2 Imprinting in phenotypically healthy children conceived by ART ......................................................... 46
1.7 Origins of adverse outcomes related to ART ............................................................................................... 49
  1.7.1 Ovarian stimulation ................................................................................................................................. 49
  1.7.2 Sperm from infertile males ....................................................................................................................... 51
  1.7.3 Fertilization, manipulation, and in vitro culturing ..................................................................................... 53
Chapter 2: DNA methylation and gene expression analysis of imprinted genes in placental chorionic villi from IVF/ICSI conceived infants versus NC controls

2.1 Introduction

2.2 Methods

2.2.1 Study participants

2.2.2 Sample preparation

2.2.3 DNA extraction from placental tissue

2.2.4 Preparation of cDNA library

2.2.5 DNA methylation analysis by pyrosequencing

2.2.6 Gene expression analysis by quantitative polymerase chain reaction (qPCR)

2.2.7 Statistical analysis

2.3 Results

2.3.1 Clinical information

2.3.2 DNA methylation of imprinted genes from IVF and ICSI placental tissue

2.3.3 Gene expression analysis of ART groups compared to NC controls

2.4 Discussion of results
Chapter 3: DNA methylation of imprinted genes in umbilical cord blood from IVF/ICSI conceived infants versus NC controls

3.1 Introduction ........................................................................................................ 83

3.2 Methods .................................................................................................................. 84
   3.2.1 Study participants ......................................................................................... 84
   3.2.2 Sample preparation ...................................................................................... 84
   3.2.3 DNA extraction from umbilical cord blood ................................................. 85
   3.2.4 Preparation of cDNA library ................................................................. 85
   3.2.5 DNA methylation analysis by bisulphite pyrosequencing ......................... 85
   3.2.6 Gene expression analysis by qPCR .............................................................. 85
   3.2.7 Statistical analysis ....................................................................................... 86

3.3 Results .................................................................................................................. 86
   3.3.1 Clinical data .................................................................................................. 86
   3.3.2 DNA methylation analysis of cord blood from ART and NC infants .......... 87
   3.3.3 Analysis of PLAG1 gene expression in ART and NC cord blood ..................... 92
   3.3.4 Analysis of imprinted gene network in IVF cord blood .......................... 93
   3.3.5 Co-regulation of PLAG1 and KCNQ1OT1 in cord blood ...................... 95

3.4 Discussion of results ............................................................................................ 96
   3.4.1 DNA methylation of imprinted regions in umbilical cord blood .............. 96
   3.4.2 Gene expression in umbilical cord blood and imprinted gene network .... 99

Chapter 4: Conclusion ................................................................................................ 103

4.1 Major findings and implications ........................................................................... 103

4.2 Limitations .......................................................................................................... 109
4.3 Future studies .................................................................................................................. 110

References .......................................................................................................................... 113
List of Tables

Table 1.1 Types of male factor infertility ................................................................. 4
Table 1.2 Summary of known imprinting Disorders and their associated genomic regions. ...... 36
Table 1.3 Imprinted genes in the placenta involved in fetal growth phenotypes ................. 39
Table 1.4 Summary of case studies examining imprinting disorders and ART .................. 45
Table 1.5 Summary of human studies examining DNA methylation in children born via ART . 48
Table 2.1 PCR and pyrosequencing primer sequences .............................................. 68
Table 2.2 Clinical information for DNA methylation analysis in placenta ...................... 71
Table 2.3 Clinical information for gene expression analysis in placenta ....................... 71
Table 2.4 Mean and Median (IQR) Methylation values of PLAGLI, KvDMR1, PEG10 and LINE-1 in placental villi from NC, IVF and ICSI pregnancies ........................................... 74
Table 2.5 Median (IQR) Methylation values for each CpG site analyzed from NC, IVF and ICSI placental chorionic villi samples .................................................................................... 75
Table 3.1 Clinical characteristics of study population in gene expression analysis ............ 87
Table 3.2 Mean and median (IQR) methylation values of PLAGLI, KvDMR1, PEG10 and LINE-1 in cord blood from NC, IVF and ICSI pregnancies ......................................................... 90
Table 3.3 Median (IQR) Methylation Values for each CpG site analyzed from NC, IVF and ICSI umbilical cord blood samples. .................................................................................. 91
List of Figures

Figure 1.1 Origins of embryonic and placental tissue .......................................................... 19
Figure 1.2 Diagram of paternally imprinted gene ................................................................ 29
Figure 1.3 Genomic reprogramming in male and female germline ........................................ 32
Figure 1.4 Imprinted gene network ....................................................................................... 41
Figure 1.5 PLAGL1 as regulator of chromosome 11p15.5 imprinted genes ......................... 42
Figure 2.1 Placental biopsy extraction sites ............................................................................ 65
Figure 2.2 DNA methylation in placental tissue from IVF, ICSI and NC infants ................... 73
Figure 2.3 Gene expression of imprinted genes and LINE-1 element in placental tissue ....... 77
Figure 3.1 DNA methylation in cord blood from IVF, ICSI and NC infants ......................... 89
Figure 3.2 DNA methylation at 4 CpG sites at PLAGL1 ..................................................... 92
Figure 3.3 Gene expression levels of imprinted genes in cord blood from IVF, ICSI and NC infants .................................................................................................................. 94
Figure 3.4 Correlation between PLAGL1 and KCNQ1OT1 in cord blood and placenta ...... 95
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ART</td>
<td>assisted reproductive technologies</td>
</tr>
<tr>
<td>AS</td>
<td>Angelman syndrome</td>
</tr>
<tr>
<td>AZF</td>
<td>azoospermia factor</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BWS</td>
<td>Beckwith-Wiedemann syndrome</td>
</tr>
<tr>
<td>CARTR</td>
<td>Canadian Assisted Reproductive Technology Registry</td>
</tr>
<tr>
<td>CBAVD</td>
<td>congenital bilateral absence of vas deferens</td>
</tr>
<tr>
<td>CDKN1C</td>
<td>cyclin-dependent kinase inhibitor 1C</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis transmembrane regulator</td>
</tr>
<tr>
<td>CG</td>
<td>cytosine-guanine</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CpG</td>
<td>cytosine-phosphate-guanine</td>
</tr>
<tr>
<td>CTCF</td>
<td>CCCTC control factor</td>
</tr>
<tr>
<td>DCN</td>
<td>decorin</td>
</tr>
<tr>
<td>ddCt</td>
<td>Delta-delta Ct</td>
</tr>
<tr>
<td>DIRAS3</td>
<td>DIRAS family, GTP-binding RAS-like protein 3</td>
</tr>
<tr>
<td>DLK1</td>
<td>delta-like homolog 1</td>
</tr>
<tr>
<td>DMR</td>
<td>differentially methylated region</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>E2</td>
<td>enhancer 2</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>GNAS</td>
<td>guanine nucleotide-binding protein alpha stimulating</td>
</tr>
<tr>
<td>GOM</td>
<td>gain of methylation</td>
</tr>
<tr>
<td>GRB10</td>
<td>growth factor receptor-bound protein 10</td>
</tr>
<tr>
<td>HATS</td>
<td>histone acetyltransferases</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HOM</td>
<td>higher order multiples</td>
</tr>
<tr>
<td>ICM</td>
<td>inner cell mass</td>
</tr>
<tr>
<td>ICR</td>
<td>imprinting control region</td>
</tr>
<tr>
<td>ICSI</td>
<td>intracytoplasmic sperm injection</td>
</tr>
<tr>
<td>IGF2</td>
<td>insulin-like growth factor 2</td>
</tr>
<tr>
<td>IGF2R</td>
<td>IGF2 receptor</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
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</table>
IUGR  intrauterine growth restriction
IUI  intrauterine insemination
IVF  *in vitro* fertilization
*KCNQ1*  KQT-like subfamily, member 1
*KCNQ1OT1*  KCNQ1-overlapping transcript 1
LBW  low birth weight
LH  luteinizing hormone
LINE-1  long-interspersed element 1
LOI  loss of imprinting
LOM  loss of methylation
LOS  large offspring syndrome
Me-CP2  methyl CpG-binding protein 2
*MEST*  mesoderm specific transcript
miRNA  microRNA
mRNA  messenger RNA
mUPD  maternal uniparental disomy
NC  natural conception
ncRNA  non-coding RNA
NOA  non-obstructive azoospermia
OA  obstructive azoospermia
PCR  polymerase chain reaction
*PEG10*  paternally expressed gene 10
*PHLDA2*  pleckstrin homology-like domain, family A, member 2
*PLAGL1*  pleiomorphic adenoma gene-like 1
pUPD  paternal uniparental disomy
PWS  Prader-Willi syndrome
RNA  ribonucleic acid
rRNA  ribosomal RNA
RT  reverse transcriptase
RQ  Relative quantitation
S1  site 1
S2  site 2
SD  Standard deviation
SGA  small for gestational age
siRNA  small inhibitory RNA
*SLC22A18*  solute carrier family 22, member 18
*SNRPN*  small nuclear ribonucleoprotein-associated protein N
*SNURF*  SNRPN upstream reading frame
SRS  Silver-Russell syndrome
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>TNDM</td>
<td>transient neonatal diabetes mellitus</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>UBE3A</td>
<td>ubiquitin protein ligase E3A</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta</td>
</tr>
<tr>
<td>ZNF331</td>
<td>zinc-finger protein 331</td>
</tr>
</tbody>
</table>
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I offer my enduring gratitude to the faculty, staff and my fellow students at UBC, who have inspired me to continue my work in this field. I owe particular thanks to Dr. Sai Ma for taking me on as a graduate student and providing endless support and guidance. I am truly grateful for the opportunities she has granted me throughout my time under her supervision. I would also like to extend my gratitude to my supervisory committee members; Dr. Timothy Rowe, Dr. Wan Lamb, and Dr. Patrice Eydoux. Thank you for taking time out of your hectic schedules and for providing constructive feedback and suggestions. Your guidance and input has been truly appreciated.

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I would like to thank my friends and family for all of their love and support throughout the years, I could not have done this without them. A very special thank you to my parents for supporting me, both financially and emotionally, throughout my education, without your constant care and patience I would not be where I am today. Finally, I would like to thank my fiancé, Gordon, for always being there for me and being a source of continuing support and encouragement.
Chapter 1: INTRODUCTION

1.1 Project overview

Infertility is estimated to afflict 11.5-15.7% of couples in Canada, and 10-15% of couples in industrialized countries worldwide (Bushnik et al. 2012). As the rates of infertility are rising, an increasing number of couples are trying to conceive using assisted reproductive technologies (ART) such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). In Canada, it is estimated that up to 4% of pregnancies are conceived using either IVF or ICSI (Bohlmann et al. 2009); and in 2012, roughly 5 million babies had been born via ART since the first assisted conception in 1978 (Cedars 2013). In recent years, the safety of these technologies has been in question and a correlation has been found between babies born by ART and adverse perinatal outcomes such as perinatal mortality, preterm birth, and intrauterine growth restriction (IUGR) (McDonald et al. 2009, Helmerhorst et al. 2004, Jackson et al. 2004, McGovern et al. 2004, Kozinszky et al. 2003). Many studies have indicated that there may be an increased risk of rare genomic imprinting disorders such as Beckwith-Wiedmann syndrome (BWS), Angelman syndrome (AS), and Silver-Russell syndrome (SRS) (Maher et al. 2003, Kobayashi et al. 2009) linked to the ART population and it is speculated that individuals conceived via ART may be at an increased risk of other diseases later in life such as diabetes and obesity (van Montfoort et al. 2012). It is postulated that hormone treatments, in vitro culturing conditions, and manipulation of the gametes during ART may produce aberrant methylation of imprinted genes leading to improper regulation of gene expression during development (Sato et al. 2007, Owen and Segars, 2009, Manipalviratn et al. 2009), which may cause disorders related to abnormal fetal and placental growth. This thesis focuses on further understanding the link between disrupted DNA methylation and gene expression of imprinted regions and ART. The study examines DNA
methylation at three imprinted regions, specifically *KvDMR1*, *PLAGL1*, and *PEG10*, and the repetitive element LINE-1 in the placentae and cord blood from phenotypically normal babies conceived via IVF, ICSI, and naturally conceived controls. Imprinted gene expression levels in both tissues will also be examined in order to determine whether there are changes in transcription of imprinted genes correlating with aberrant methylation, or in the absence of DNA methylation changes. An investigation into a proposed co-regulated genomic imprinted gene network thought to be involved in fetal and placental development will also be examined. This chapter outlines the basics of infertility, ARTs, fertilization and preimplantation development, the placenta, epigenetics and genomic imprinting, and how ART may contribute to alterations in imprinted DNA methylation and gene expression.

1.2 Infertility

The WHO defines infertility as “a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse” – (WHO-ICMART glossary). A recent study has found that the prevalence of current infertility in Canada from the years 2009-2010 was between 11.5 and 15.7% (Bushnick et al. 2012). When these values are compared to previous values of 5.4% in 1984 (Balakrishnan and Fernando 1993) and 8.5% in 1992 (Dulberg and Stephens 1993) using similar definitions, it is clear that there has been a significant increase in infertility rates in the last two decades and that this is a major concern for the current and upcoming generations. Statistics Canada determined that infertility is due to male factors in 30% of cases, female factors in 40% of cases, both partners in 20% of cases and unknown causes in 10% of cases. The recent increase in infertility
rates may be associated with increased maternal age and environmental factors such as pollution, diet, and an increasing prevalence of obesity.

1.2.1 Male factor infertility

Infertility in males is multifactorial in nature and encompasses a wide variety of disorders. Infertility is diagnosed based on the analysis of sperm from semen samples which is based on 3 main parameters: concentration, motility, and morphology (Cooper et al. 2010). Diagnoses associated with alterations in sperm parameters are listed in table 1.1. Infertility in males can be due to genetic or non-genetic causes. Non-genetic causes include hormonal imbalances, epididymal obstruction after an infection, such as chlamydia or gonorrhea (Hirsh 2003), or varicocele, which is an abnormal enlargement of the varicose veins within the scrotum. Defective sperm production is the most common cause of infertility in males, the origin of which is unknown in approximately 50% of cases (Namiki et al. 2000). Although some genetic causes leading to impaired spermatogenesis have been identified, for a majority of male infertility the reason remains unknown. This section will focus on the genetic causes of male factor infertility including cystic fibrosis (CF) gene mutations, Y chromosome deletions, and chromosomal abnormalities.
Table 1.1 Types of male factor infertility (Modified from Hirsh 2003)

<table>
<thead>
<tr>
<th>Type of Infertility</th>
<th>Semen parameters</th>
</tr>
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</table>
| Normozoospermia                             | ≥ 20 million sperm/ml
|                                             | ≥50% motile sperm
|                                             | ≥30% normal morphology                                                           |
| Oligozoospermia                             | Reduced numbers of sperm in ejaculate:
|                                             | **Mild**: 5-20 million sperm/ml
|                                             | **Severe**: <5 million sperm/ml                                                   |
| Asthenozoospermia                           | Reduced sperm motility                                                            |
| Teratozoospermia                            | Increased abnormal forms of sperm                                                 |
| Oligoasthenoteratozoospermia                | All sperm variables subnormal                                                     |
| Azoospermia                                 | No sperm in semen:
|                                             | **Obstructive Azoospermia** (OA): Normal spermatogenesis but physical blockage prevents release of sperm
|                                             | **Non-obstructive Azoospermia** (NOA): Impaired spermatogenesis                   |
| Aspermia                                    | No ejaculate                                                                      |

1.2.1.1 CF mutations

Cystic Fibrosis (CF) is an autosomal recessive disorder that affects the lungs, pancreas, liver, and intestines. CF is caused by one or more mutations of the cystic fibrosis transmembrane regulator (CFTR) gene which is involved in chloride ion transport across the respiratory epithelium (Quinton 1983). 97-99% of all men with CF also present with congenital bilateral absence of vas deferens (CBAVD) (Blau et al. 2002), resulting in obstructive azoospermia as the transport of spermatozoa is blocked. Men who carry only one mutated CFTR allele may result in infertility without displaying the CF phenotype; approximately 60% of men with CBAVD carry one mutated CFTR gene (de Braekeller and Ferec 1996). Men with obstructive azoospermia are
able to conceive via ICSI using sperm retrieved from epididymis. However, it is crucial to screen for CFTR mutations as these can be passed on to the offspring (Gazvani and Lewis-Jones 2006).

1.2.1.2 Y chromosome microdeletions

Y chromosome abnormalities are the second most common genetic anomaly observed in infertile males (Slezak and Sasiadek 2002). Large deletions of the Yq11 region were first demonstrated in men suffering from infertility, providing evidence that this region is essential for spermatogenesis and thus became known as the azoospermia factor (AZF) region (Tiepolo and Zuffardi 1976). It has since been shown that 8.2% of infertile men carry submicroscopic deletions within this AZF region (Foresta et al. 2001). The AZF locus can be subdivided into non-overlapping, distinct segments: AZFa, AZFb, and AZFc; and the occurrence of Y chromosome partial and microdeletions are caused by homologous recombination between these highly repetitive sequences (Blanco et al. 2000, Kuroda-Kawaguchi et al. 2001, Repping et al. 2002). Deletions of all three of these regions can be associated with non-obstructive azoospermia, with deletions of AZFb presenting the most severe phenotypes (Hirsh 2003). AZFc deletions cause the least severe phenotypes and sperm can be found in the ejaculate of roughly 70% of men with microdeletions in this region (Patrat et al. 2010). Deletions of the Y chromosome are observed in approximately 10% of non-obstructive azoospermia and 5% of oligozoospermia cases (Hotaling 2014). ICSI provides a means by which men suffering from infertility due to Y chromosome deletions may conceive, however there are some concerns as this process can also facilitate the transmission of the Y deletion from the father to the male offspring (Page et al. 1999, Oates et al. 2002, Repping et al. 2003, Lynch et al. 2005, Minor et al. 2007).
1.2.1.3 Chromosomal abnormalities

Chromosomal abnormalities leading to male factor infertility can be structural or numerical in nature. Robertsonian translocations, the fusion between two acrocentric chromosomes, are the most common structural chromosome rearrangements in humans. The most frequent Robertsonian translocations occur between chromosomes 14 and 21, and 14 and 13, which is the translocation most frequently associated with male infertility (Jacobs 1981). Men carrying Robertsonian translocations usually display some degree of infertility due to synaptic abnormalities during meiosis which leads to meiotic arrest. The incidence of Robertsonian translocations in men with infertility is approximately 10.3 times greater than in the general population (Morel et al. 2004). Reciprocal translocations involve the exchange of chromosome parts between nonhomologous chromosomes; the frequency of these translocations is 11.5 times greater among infertile males than in the general population (Morel et al. 2004). Depending on the nature of the translocation and chromosomes involved, carriers of translocations are at a higher risk of producing chromosomally unbalanced sperm, leading to a higher risk of miscarriage or malformations in the offspring. Translocations may also impair spermatogenesis by disrupting the fidelity of chromosome pairing during meiosis (Oliver-Bonet et al. 2005, Ferguson et al. 2008).

Numerical abnormalities occur when there is a gain or loss of a complete chromosome (aneuploidy). Aneuploidy of the sex chromosomes is associated with male factor infertility such as in Klinefelter’s syndrome or 47, XYY syndrome. Klinefelter’s has a prevalence of approximately 1 in 600 males and is the most common cause of azoospermia (Bojesen and Gravholt 2007), which occurs as genes on the extra X chromosome interfere with normal
spermatogenesis. Men with a 47, XYY genotype are at an increased risk of experiencing infertility (Chan Wong et al. 2008); however the majority of men are fertile. Interestingly, studies examining infertile males with a normal karyotype have shown that they may produce elevated levels of chromosomally abnormal sperm. Studies have found increased rates of numerical chromosomal abnormalities in sperm from men with severe oligozoospermia (Moosani 1995, Pang et al. 1999, Nishikawa et al. 2000, Tang et al. 2004, Kirkpatrick et al. 2008), as well as from sperm retrieved from the testes of men with azoospermia (Bernardini et al. 2000, Burrello et al. 2002, Rodrigo et al. 2004), which may contribute to an increased risk of chromosomal abnormalities after ICSI.

1.2.2 Female factor infertility

Female factor infertility can be due to a number of different aetiologies including disorders of ovulation, tubal damage, endometriosis, or peritoneal and uterine abnormalities. Ovulatory disorders account for roughly 20% of infertility cases, and polycystic ovarian syndrome accounts for 70% of these disorders (Adamson and Baker 2003). Disorders affecting the fallopian tubes account for 35% of infertility and can occur due to scarring from previous surgeries, sexually transmitted infections, such as chlamydia or gonorrhea, inflammatory conditions, or a congenital anomaly (Adamson and Baker 2003). Endometriosis, the presence of endometrial-like tissue present outside of the uterus, can lead to infertility by inducing chronic inflammation, scar tissue and adhesions that alter the pelvic morphology (Kennedy et al. 2005) and may even lead to an earlier onset of diminished ovarian reserve (Adamson and Baker 2003). The most commonly reported relationship between genetics and reduced fertility in women is the maternal age effect (Shah et al. 2003). In Canada, as well as in other developed nations, the
amount of time women are waiting to have their first child is increasing due to time spent establishing careers and advancements in birth control. In 2010, for the first time, the age-specific fertility rate in Canada was higher for women aged 35-39 than for women aged 20-24 and in 2011, 19.2% of births were to women aged 35 and older, compared to 4.9% in 1981 (Statistics Canada). This trend of advancing maternal age does not appear to be slowing down and the implications associated with this will be discussed below.

1.2.2.1 Advanced maternal age

A woman’s fertility begins to decline in her late 20’s and becomes more severe after the age of 35. Women have a pool of resting oocytes in the form of the primordial follicles which are established prior to birth. In the fetus at around 20 weeks gestation there are approximately 3.5x10^6 oocytes in each ovary, most of which are destroyed by apoptosis (Hsueh et al. 1996). At the time of birth there are approximately 1x10^6 oocytes left in each ovary and this number steadily declines throughout a woman’s lifetime due to atresia and recruitment towards ovulation (Gosden and Faddy 1998). Therefore, there is a limit to the overall ovarian reserve which is characterized in terms of ovarian follicle number and oocyte quality (Adamson and Baker 2003).

A study examining the number of couples who remained childless after trying to conceive for at least a year was conducted and in couples where the woman was aged 40-44 the infertility rate was 64% compared to only 6% in the women aged 20-24 and 30% in women aged 35-39 (Menken et al. 1986). Pregnant women of advanced age also have a higher risk of miscarriage and it is believed that this is due to a higher frequency of chromosomal abnormalities. Advanced maternal age has been linked with higher rates of aneuploidy and chromosomal abnormalities in human oocytes (Dailey et al. 1996, Pellestor et al. 2003) and the presence of aneuploidies of
chromosomes X, Y, 13, 18, and 21 have been shown to increase from 4% in 8-cell stage embryos from women 25-34 years of age to 37% in women over 40 years of age (Munne et al. 1995). Women of advanced age who are unable to achieve a pregnancy naturally may undergo IVF treatment. The chance of achieving a live birth via IVF with one’s own oocytes decreases with age. Women age 34 and under display live birth rates of 40-49%, whereas this rate drops between 2-6% for every year afterwards, with the live birth rate among 43 year old women being only 5% (Van Voorhis 2007). Although a large portion of women with advanced maternal age may achieve a pregnancy, the miscarriage rate among this age group is roughly 50%. When IVF is performed using donor eggs from younger women, the success rates increase drastically with approximately 50% of attempts leading to a live birth, suggesting that oocyte quality, not endometrial receptivity, is affected with age (Van Voorhis 2007).

1.3 Assisted reproductive technologies

As infertility rates are steadily increasing in western society, more couples are relying on assisted reproductive technologies in order to conceive a child. ARTs encompass any treatment modality that is used to improve fertility and establish a pregnancy, including ovarian stimulation, intrauterine insemination (IUI), IVF, and ICSI. In Canada, it is estimated that up to 4% of pregnancies are conceived using either IVF or ICSI (Bohlmann et al. 2009) and over 5 million babies have been born via ART since the first assisted conception in 1978 (Cedars 2013). This section will focus on ovarian stimulation, IVF, and ICSI, as these are the most commonly used procedures and the focus of our study, as well as the pregnancy outcomes after ART.
1.3.1 *In vitro* fertilization

IVF is a procedure used to overcome female factor or mild male factor infertility. In order to obtain multiple oocytes for fertilization, the women must undergo superovulation, which is the administration of hormones in order to stimulate the development and ovulation by multiple ovarian follicles. These hormones work by stimulating the release of FSH and LH, which results in the development of multiple mature follicles, in comparison to only one mature follicle in the natural menstrual cycle. The most commonly used hormones include clomiphene citrate, human chorionic gonadotropin, human menopausal gonadotropins, and gonadotropin releasing hormone (Carlson 2004, WHO 2002). Upon maturation of the follicles, just before ovulation takes place, the follicular fluid is aspirated from each follicle from the ovary through the vaginal wall guided by transvaginal ultrasound and the mature eggs are collected from the fluid. In IVF, the mature oocytes, which have been retrieved by aspiration, are cultured *in vitro* with motile sperm from the male partner in order to achieve fertilization. In order for IVF to be successful, 10,000 to 100,000 motile sperm are required per oocyte in culture (Gardner et al. 2001). Multiple embryos are cultured to either day 3 (8-cell stage) or day 5 (blastocyst) and, if fertilization occurs, the resulting good quality embryos are selected to be transferred into the uterine cavity via catheter. If there are excess embryos of good quality left after culturing than these embryos can be cryopreserved and used in future IVF cycles (Van Voorhis 2007). In 2010, 11,806 IVF treatments were performed throughout Canada, resulting in 3,188 live births (Canadian Assisted Reproductive Technologies Registry – CARTR) for a live birth rate of approximately 28%.
1.3.2 **Intracytoplasmic sperm injection**

As a high concentration of motile sperm is necessary for IVF, men suffering from disorders of spermatogenesis, such as oligozoospermia, non-obstructive azoospermia (NOA), or obstructive azoospermia (OA), are unable to use this form of treatment. ICSI, a more recently developed technique, has been successful in the treatment of male factor infertility; representing more than 60% of IVF cycles in Canada (Gunby et al. 2011). ICSI is similar to the IVF procedure, however instead of combining eggs and sperm *in vitro* and allowing the sperm to fertilize the egg, a single spermatozoon is injected through the zona pellucida directly into the oocyte (Palermo et al. 1992, Ma and Yuen 2000). ICSI is advantageous because it requires only one spermatozoon for every oocyte (Devroy and Steirteghem 2004). For men with OA, spermatozoa can be extracted from the epididymis or the testes and, in men with NOA, ICSI can be used if spermatozoa can be retrieved from the testicular tissue (Merchant et al. 2011). The live birth rate for ICSI is similar to that of IVF and is roughly 25% per ICSI cycle (Gunby et al. 2011).

1.3.3 **Pregnancy outcomes of ARTs**

The development and improvements of ARTs over the years has been revolutionary and has helped millions of infertile couples conceive a child of their own. For the most part, the children born through ARTs are healthy (Davies et al. 2012) and do not differ from their naturally conceived peers. However, some concerns have been raised regarding the safety of these technologies as there does seem to be an increase in some obstetrical and perinatal outcomes such as preterm birth, low birth rate, pre-eclampsia, and an increased risk of rare imprinting disorders.
1.3.3.1 Multiple gestations

There are much higher rates of multiple gestation pregnancies among the ART population. The cause of this is mainly due to the common practice of transferring multiple embryos into the uterus in order to increase the possibility of implantation. However, it is interesting to note that there is also an increased rate of monozygotic twins in IVF pregnancies compared to natural conceptions (Allen et al. 2006). Multiple birth rates following ART are between 25% and 50% (Sunderam et al. 2009), compared to 1% in the general population (Reynolds et al. 2003). A retrospective population cohort study examining the rates of stillbirths, neonatal mortality, preterm births, and small for gestational age (SGA) infants out of 226,624 singletons, 6,941 twins, and 285 higher order multiples (HOM) in Australia observed that 1% of singletons, 15.4% of twins and 34.7% of HOM were conceived via ART (Chambers et al. 2014). They found that twins were 3.4 times more likely to be stillborn, 6.4 times more likely to die during the neonatal period, 18.7 times more likely to be born preterm, and 3.6 times more likely to be SGA than singletons (Chambers et al. 2014). In terms of HOM infants, they were 9.6 times more likely to be stillborn, 36.7 times more likely to die during the neonatal period, 525.1 times more likely to be preterm, and 2.8 times more likely to be SGA (Chambers et al. 2014). Multiple pregnancies increase the risk to the mother and the fetus, and also place increased strain on the healthcare system, as hospital costs for each baby in a multiple pregnancy is 4.8 times more costly than a singleton (Canadian Institute for Health Information 2009). However, in recent years efforts have been made to reduce the number of embryos transferred in one IVF cycle in order to reduce the rates of multiple births. In Canada in 2008, 68% of all IVF/ICSI cycles
transferred one or two embryos, whereas in 2012 this figure increased to 88%, 44% of which were single embryo transfers (CARTR 2008/2012).

### 1.3.3.2 Low birth weight and malformations

It has been shown that babies born via ART are more likely to have adverse perinatal outcomes; although multiple gestation pregnancies do contribute to this effect, singleton pregnancies conceived via ART are also at an increased risk (Schieve et al. 2004). Many studies have observed higher risks of low birth weight (LBW), younger gestational age, premature delivery, prenatal mortality, and hospital admissions in ART-conceived infants when compared to NC controls (Bower and Hansen 2005, Henningsen et al. 2011, Sazonova et al. 2011). A meta-analysis examining infants conceived via ART compared to natural conceptions found an approximately 2 fold increase in the risk of LBW, preterm birth, and perinatal mortality, as well as a 50% increased risk of being SGA and 30%-35% increase in birth defects in ART infants (Bower and Hansen 2005). A more recent meta-analysis focusing on IVF/ICSI singleton pregnancies also found an increased rate of congenital anomalies, low birth weight, perinatal mortality, preterm delivery, and SGA infants among the IVF/ICSI group, using data from 20 matched and 10 unmatched cohort studies (Pandey et al. 2012).

Several studies have suggested a potential role for subfertility in relation to adverse outcomes for both the mother and child (Doornbos et al. 2007). Studies have shown that women who take longer than 1 year to conceive have a 1.4-1.8 fold increase in risk for preterm delivery and a 3 fold increased risk of perinatal mortality, when compared to women who experienced no delay in conception (Allen et al. 2006). Davies et al. (2012) examined the outcomes of 308,974 births, 6,163 of which were ART, in South Australia and concluded that a history of infertility,
with or without ART, was significantly associated with birth defects in this cohort (Davies et al. 2012).

1.3.3.3 Long-term outcomes

As the development and popularity of ARTs is still relatively new, there are not many long-term follow-up studies looking at the outcomes of these children. However, as the first children conceived via IVF are now reaching adulthood, more attention has been directed towards the long-term consequences of ARTs. Several studies have been conducted and are generally reassuring. However, there are some concerns as LBW and preterm birth are more prevalent among this population and it has been suggested that this may predispose these individuals to increased disease risk in adulthood (Barker 2007).

Current literature suggests that there are potential differences in metabolic manifestations and potentially increased chance of malignancies in children born via ART (Ludwig et al. 2006, Halliday et al. 2007, Steel and Sutcliffe 2009, Wilson et al. 2011), however, the data regarding these risks after ART has been somewhat conflicting. There does not appear to be a higher prevalence of overweight or obese children compared to natural conceptions (Beydoun et al. 2010), however significantly more peripheral fat deposits have been noted in studies examining ART children when controlling for antenatal, maternal, and paternal factors (Ceelen et al. 2007). In a Belgium cohort of ICSI children, boys had a tendency toward higher percent body fat while girls had a significantly higher BMI, percent body fat mass, skin folds, and waist circumference compared to NC controls (Belva et al. 2012). Furthermore, one study observed differences in blood pressure between ART and NC children (average age of 8.8 years) in a small case-control study (Sakka et al. 2009).
Studies examining rates of childhood cancers among the ART population also produce inconsistent findings. Three cohort studies, one from Holland, one from Australia, and one from Israel, found no increased risk of cancers among ART children (Bruinsma et al. 2000, Lerner-Geve et al. 2000, Klip et al. 2001). However, a meta-analysis of 11 cohort studies calculated an adjusted standardized risk ratio for cancer of 1.33 (CI: 0.62-2.85) among IVF children (Raimondi et al. 2005) and a Swedish cohort study controlling for all potential factors derived an odds ratio of 1.42 (CI: 1.09-1.87), noting an excess of haematological malignancies, particularly histiocytosis (Källén et al. 2010). Further, several studies have concluded that there does appear to be an increased risk of retinoblastoma among children born through ART (Moll et al. 2003).

Overall, children conceived by IVF and ICSI appear to be healthy, however, studies following cohorts of ART-conceived infants well into adulthood are needed in order to truly determine whether the ART population is any more susceptible to adult diseases such as cancer, cardiovascular disease, diabetes, or obesity.

1.4 Embryogenesis

In IVF and ICSI, the process of fertilization and the first 3-5 days of embryonic development occur outside of the natural environment of the female reproductive tract. As the embryo travels down the fallopian tube and into the uterus it experiences natural changes in pH and oxygen tension, as well as moving from a high pyruvate/low glucose environment into a high glucose/low pyruvate environment. It is still unclear how the difference in preimplantation environment may affect the programming of the resulting fetus. Although advances have been made to better mimic the natural environment of fertilization, it is impossible to capture the full complexities and natural milieu of the female reproductive tract which may have lasting effects.
on the developing fetus. In order to understand how the ART process may affect the
development of the resulting offspring and the placenta, the process of fertilization and the early
preimplantation stages and placentation will be discussed.

1.4.1 Fertilization and early embryogenesis

Embryogenesis is the process whereby cells divide and differentiate to form the embryo. This process begins with the fertilization of an oocyte by a spermatozoon and encompasses the
first eight weeks of development. Embryogenesis is an intricate and sensitive process, the full
complexities of which are still not fully understood. During this early phase of pregnancy, it is
crucial that all events occur in a coordinated manner and establish a favourable environment;
otherwise it could result in developmental abnormalities, pregnancy complications, or even
miscarriage. Fertilization is the process whereby 2 gametes, the sperm and the oocyte, unite and
produce a zygote. Fertilization occurs in the ampulla of one of the fallopian tubes, one or two
days after ovulation (Nowritz et al. 2001). In mammals, fertilization of the oocyte causes
dramatic changes in the organization of both paternal and maternal genomes. The oocyte, which
is arrested in metaphase II, completes meiosis by forming the haploid maternal pronucleus and
releasing the second polar body. The sperm DNA, which is tightly packed with protamines,
decondenses after penetration and protamines are exchanged for histones (Schagdarsurengin et
al. 2012); at this point, a one-cell embryo is formed containing paternal and maternal DNA.

Following fertilization, the zygote must undergo cleavage, which is a series of mitotic
divisions, in order to rapidly increase the number of embryonic cells. Cleavage divisions take
place as the zygote passes along the fallopian tube towards the uterus, with one cleavage division
occurring per day for the first two days (Carlson 2004). Three days after fertilization, the morula
is formed, which is a 16-cell embryo with an inner cell mass surrounded by a layer of cells that form the trophectoderm. During the cleavage phase there are major genomic reprogramming events taking place to regain pluripotency of the male and female germline; during this time both the male and female germlines are demethylated and this continues up until the morula stage (van Montfoort et al. 2012). As the morula enters the uterus, fluid penetrates the zona pellucida to form a fluid-filled space and at this stage the embryo is called the blastocyst. Once the blastocyst enters the uterus, the trophoblast cells begin to secrete enzymes which allow the blastocyst to “hatch” from the zona pellucida. After shedding the zona pellucida, approximately 6 days after fertilization, the blastocyst is able to attach to the endometrial endothelium and initiate implantation. During implantation, de novo methylation occurs to repress the germline expression and mediate transition to terminal differentiation (Borgel et al. 2010). During this phase the inner cell mass, which will make up the embryo, will become differentially methylated from the extraembryonic lineages. These reprogramming events will be discussed in further detail in following sections.

1.4.1.1 Origins of placental and embryonic tissues

The inner cell mass (ICM) and the trophectoderm represent two cell lines with different, independent developmental programs. Only a few cells within the ICM will differentiate to form the embryo proper, the remaining cells along with cells of the trophectoderm will form the extraembryonic tissues (Norwitz et al. 2001). The trophoblast, which makes up the major portion of the placenta, is derived from the trophectoderm and differentiates into two layers: the cytotrophoblast and the syncytiotrophoblast. The inner cytotrophoblast layer consists of mononucleated cells, whereas the outer syncytiotrophoblast layer consists of a multinucleated
protoplasmic mass in which no cell boundaries are observed (Moore and Pasaud 2008). The syncytiotrophoblast cells displace endometrial cells, which undergo apoptosis, to facilitate invasion at the implantation site and will further extend throughout the endometrium and invade the connective tissues in order to derive maternal nutrients. Cells in the primary cytotrophoblast serve as a stem cell population, actively dividing and migrating into the syncytiotrophoblast, where they lose their cell membrane and fuse together (Salder 2004). The cytotrophoblast cells will differentiate and eventually give rise to the chorionic villi of the placenta and the amnion, whereas the syncytiotrophoblast will form the lacunae which will develop into the intervillous spaces and become filled with maternal blood (Gude et al. 2004). The fibroblast-like stromal cells of the site of implantation in the maternal endometrium will become the decidua, which forms the maternal portion of the placenta.

Simultaneously, cells of the ICM differentiate into two separate layers: the hypoblast layer and the epiblast layer (Salder 2004). The hypoblast and the epiblast together form the bilaminar disc. A small cavity forms within the epiblast which enlarges to form the amniotic cavity. The epiblast will differentiate to form the embryonic ectoderm, embryonic mesoderm, and embryonic endoderm, which will provide all the cells to form the embryo proper (Carlson 2004). The dorsal cells of the epiblast (extraembryonic ectoderm) will spread across the mesoderm and give rise to the mesenchymal layer of the amniotic membrane and the amniotic epithelium will form from the embryonic ectoderm. The amnion is a membrane that surrounds the embryo and will later become filled with fluid to form the amniotic sac. The hypoblast will differentiate into the extraembryonic endoderm, which will form the yolk sac, and the extraembryonic mesoderm. The yolk sac is the first site of fetal blood formation, which later takes place in the fetal liver and then the bone marrow. The extraembryonic mesoderm will give
rise to the mesodermal core of the villi and the allantois, and will fuse with the trophoblast endoderm to form the chorion of the placenta (Bianchi et al. 1993, Carlson 2004, Robinson et al. 2002).

Figure 1.1 Origins of embryonic and placental tissue. Origins of embryonic and placental tissues from the blastocyst (adapted from Bianchi et al. 1993, Carlson 1999, Carlson 2004, Gude et al. 2004, Robinson et al. 2002).

1.4.1.2 Structure and function of the placenta

The placenta is an organ that develops alongside the embryo and is essential for proper fetal growth and development. Alterations in the development of the placenta can greatly influence fetal growth and the ability of the fetus to survive in utero. The placenta is a multifunctional organ that is critical for the development of the fetus and failure of proper
formation and maturation of this organ is associated with a wide range of adverse pregnancy outcomes, such as pregnancy loss (Kujovich 2004), pre-eclampsia, fetal growth restriction (Chaddha et al. 2004), and preterm birth (Salafia et al. 1991). The placenta has many functions crucial for the developing fetus including the transport of nutrients and oxygen, immunologic, synthetic, and waste disposal functions.

The human placenta is discoid and haemochorial, meaning that the maternal uterine epithelium and capillary endothelial layers are lost and the maternal blood flow is in direct contact with the terminal villi of the placenta (Wildman et al. 2006). This allows for transport of nutrients, respiratory gases, and waste products across the placental membrane. The placenta is unique as it is made up of tissues from two genetically distinct organisms: the mother and the fetus. The placenta is comprised of a fetal section that develops from the chorionic sac and is termed the chorionic plate in the mature placenta; the umbilical cord directly connects the fetus to the highly vascularized chorionic plate. The maternal section develops from the endometrium and is referred to as the basal plate (Gude et al. 2004). The intervillous space lies between these two plates and contains the extensively branched and closely packed villous structures, which contain the fetal blood, and are the main functional units of the placenta. The maternal blood enters the intervillous space through spiral arteries and bathes the villi, where maternal-fetal exchange occurs (Gude et al. 2004).

During pregnancy, in addition to transporting nutrients and eliminating waste from the fetus, the placenta also acts as an endocrine organ to produce hormones important for maintaining a healthy pregnancy and to prepare the mother for labour and breastfeeding. The placenta produces oestrogens, progesterone, chorionic gonadotropins, placental lactogen, and growth hormones, as well as many cytokines, chemokines, and other pregnancy-associated
proteins (Gude et al. 2004). The placenta also serves a protective function as it acts as a barrier between the mother and the fetus, not only protecting against an attack on the fetus by the maternal immune system but also serves to defend the fetus against the transmission of some bacteria, viruses, and other dangerous substances.

1.5 Epigenetics, genomic imprinting, and imprinted gene network

During ART procedures, the normal *in vivo* processes of oocyte maturation, fertilization, and the first days of embryonic development are affected by various synthetic factors from hormone stimulation to *in vitro* manipulation of gametes. It is believed that the ART process overlaps with essential epigenetic reprogramming events and may disrupt the establishment and maintenance of important epigenetic marks that regulate fetal and placental growth and development. Epigenetic alterations may provide a mechanism to explain why ART infants tend to have higher rates of LBW, IUGR, imprinting disorders, and an increase in susceptibility to diseases in adulthood. In this section, I will outline the basics of epigenetics, genomic imprinting and reprogramming, as well as the role of imprinted genes in placental development and imprinting disorders. I will also discuss the recently proposed idea of a co-regulated network of imprinted genes regarded as an “imprinting gene network”.

1.5.1 Epigenetics

The increasing knowledge in the field of epigenetics is starting to unveil the dynamic interplay between genes and the environment. Epigenetics refers to the study of heritable alterations in gene expression that are not caused by changes in the actual nucleotide sequence of the DNA, but rather by modifications that act to silence or activate transcription. In order to
complete their specialized roles within the body, each cell has a particular epigenome, a unique set of epigenetic modifications used to establish and maintain lineage-specific expression profiles (Jenuwein 2006). Epigenetic modifications are important for adaptation to the environment because genetic information is very stable and requires multiple generations to acquire mutations and evolve, whereas epigenetic modifications are reversible and able to rapidly respond to endogenous and exogenous signals. Furthermore, epigenetics are thought to orchestrate the spatial and temporal regulation of cell differentiation throughout development (Goldberg et al. 2007, Margueron and Reinberg 2010, Zaidi et al. 2010).

DNA is packaged inside the cell nucleus in a structure called chromatin. The fundamental unit of chromatin is called a nucleosome, which consists of 147 base pairs of DNA wrapped around an octamer of histone proteins comprised of duplicate copies of histone H3, H4, H2A and H2B (Kouzarides 2007). A linker histone protein (H1) helps to organize the nucleosomes into a higher-order structure which can be organized in such a way that they can be compacted to form tightly packed, transcriptionally inactive chromatin (heterochromatin) or loosely packed, transcriptionally active chromatin (euchromatin). The four main methods of epigenetic modification include DNA methylation, histone modification, non-coding RNAs (ncRNAs) and the organization of nuclear structure including chromosome replication behaviour (Qureshi and Mehler 2011).

1.5.1.1 DNA methylation

DNA methylation involves the addition of a methyl group onto the cytosine of a CG dinucleotide to produce 5-methylcytosine. DNA methylation in mammals occurs almost exclusively at CpG dinucleotides (Cytosine-phosphate-Guanine), however non-CpG methylation
does have a functional role in plants (Chan et al. 2005). CpG dinucleotides are most commonly found on the 5’ end of genes in regions called CpG islands (Bird 2002) and CpG islands are found within roughly 70% of the genome (Saxonov et al. 2006). DNA methylation is a repressive modification; the addition of methyl groups to a CpG island within the promoter of a gene acts as a barrier so that the transcriptional machinery is unable to bind and the gene is therefore unable to be transcribed. Another method in which DNA methylation inhibits transcription is by recruiting methyl CpG-binding protein 2 (Me-CP2), which then recruits histone-modifying complexes (Fuks et al. 2003). DNA methylation also plays an important role in the immobility of transposable elements (Slotkin and Martienssen 2007). When a CpG island is transcriptionally repressed due to high levels of DNA methylation this is referred to as hypermethylation, whereas if a CpG island is hypomethylated then it is thought to be free of methyl groups and therefore transcriptionally active (Bird 1995).

DNA methylation is an extremely important process for development and contributes an essential role in X-chromosome inactivation (Walsh et al. 1998, Waterland and Jirtle 2003), genomic imprinting (Reik and Walter 2001), and tissue-specific gene expression (Bird 2002). DNA methylation patterns are established during fetal development and early post-natal life and cell specification by methylation of tissue-specific genes occurs during embryogenesis. DNA is methylated by three DNA methyltransferase enzymes: DNMT1, DNMT3A, and DNMT3B. DNMT1 is a sequence-independent methyltransferase which is involved in maintenance of methylation status during DNA replication and has a preference for hemi-methylated DNA (Bestor 2000). DNMT3A and DNMT3B establish DNA methylation patterns during specific stages of gametogenesis and early development which involves sequence-specific de novo methylation (Okano et al. 1999; Bestor 2000). Knocking out any of these methyltransferase
enzymes is lethal in mice (Li et al. 1992), and therefore underlines the importance of methylation establishment and maintenance to proper mammalian development. DNA methylation was thought to be a very stable modification that was established during development and remained in place throughout the lifetime; however, recently, numerous studies have shown evidence of active demethylation and a number of potential DNA demethylases have been discovered (Lillycrop et al. 2014). Changes in methylation are associated with various pathologies such as different types of cancers, which can be caused by hypermethylation of tumor-suppressor promoter regions (Jones and Baylin 2002) or a genome-wide loss of methylation.

1.5.1.2 Histone modifications

As mentioned at the beginning of this section, histones are the proteins that bind as an octamer in order to form the nucleosomes. These core histones are subject to over 100 possible post-translational modifications including acetylation, methylation, phosphorylation, and ubiquitination at specific positions within the amino-terminal histone tails (Bernstein et al. 2007). Nucleosomes must be tightly packed in order to achieve the 10,000 – 20,000 fold compaction of DNA within the nucleus (Woodcock and Ghosh 2010); to attain this, histone tails are positively charged to interact with the negatively charged DNA. Histone acetylation was the first modification which was identified in 1961 (Philips 1963). Acetylation of the lysine residues on the tails of the histone proteins neutralizes the positive charge, weakening the interaction between the histone and the DNA or adjacent histones which then increases accessibility to transcription machinery (Zentner and Henikoff 2013). Histone lysine acetylation functions in many cellular processes that require DNA access such as DNA replication. During DNA replication, histone-DNA interactions are relaxed to allow room for replication machinery and
histone acetylation occurs at double-stranded breaks as a way to increase DNA access by repair factors (Zentner and Henikoff 2013). Histones are acetylated by a set of enzymes called histone acetyltransferases (HATS) and deacetylated by histone deacetylases (HDACs) and these enzymes are associated with sites of active transcription (Wang et al. 2009). Histone acetylation is a very dynamic process and many acetylation events have half-lives of only a few minutes (Barth and Imhof 2010).

Histone tails can also be methylated and on the lysine residues it is possible to add 1, 2 or 3 methyl groups leading to mono-, di-, or tri-methylation; these modifications do not affect the positive charge of the lysine and are, therefore, thought to be less direct (Zentner and Henikoff 2013). Arginine residues on the histone tails can also be mono- or di-methylated. It is thought that methylation of the histones functions to increase the affinity of certain protein modules for histone residues (Zentner and Henikoff 2013). Methylation is also thought to enhance nucleosome stability by enhancing the affinity of certain proteins for the histone tails.

Phosphorylation of the histone tails conveys a negative charge which suggests a similar role as acetylation in terms of modulating the nucleosome dynamics (Banerjee and Chakravarti 2001). The phosphate backbone of DNA would be repulsed by a phosphorylated histone residue which could then loosen the association of histones with DNA. Studies have demonstrated that phosphorylated histones are less effective at inhibiting DNase I digestion of chromatin than unphosphorylated histones, in theory, due to the more open structure of the chromatin (Mirsky et al. 1972). Phosphorylation of histones seems to play a role in development; in the sea urchin it has been observed that after fertilization, the sperm histones are heavily phosphorylated and then are detached from the chromatin (Green and Pocca 1985). Other forms of histone modification includes ADP-ribosylation, which also imparts a negative charge and is thought to function in a
similar manner as phosphorylation, and glycosylation which is more recently discovered and believed to be involved in repression of transcription (Zentner and Henikoff 2013). As opposed to DNA methylation, which is believed to be a long-term silencing mechanism, histone modifications are believed to be more short-term and reversible and are able to repress and activate transcription (Riek 2007). There is also believed to be interactions, or cross-talk, between DNA methylation marks and histone variations.

1.5.1.3 Non-coding RNA

ncRNAs are transcribed from DNA but are not translated into protein and are involved in the processing and regulation of other RNAs such as mRNA, tRNA, and rRNA (Collins et al. 2010). Protein-coding RNA constitutes only about 2% of the total transcriptional output, while the remainder is made up of ncRNA (Peschanskey and Wahlstedt 2014). The fact that such a high percentage of the transcriptome is non-coding indicates that these molecules could play an extensive role in regulation of the genome. There is still a lot of unknowns when it comes to ncRNA, however, recent studies have implicated that they are involved in a majority of processes controlling development and differentiation (Mohammad et al. 2009). The epigenetic-related classes of ncRNA can be divided into two main groups: long ncRNAs and short ncRNAs, which include small inhibitory RNA (siRNAs) and microRNA (miRNAs) (Collins et al. 2011). Long ncRNAs are over 200 nucleotides in size, while anything under that is classified as short ncRNA. These smaller ncRNA molecules are involved in controlling gene expression through regulating target mRNA and chromatin, and are able to target the actual transcription of a gene as well as downstream processes (Peschanskey and Wahlstedt 2014). Genomic loci producing ncRNAs are subject to epigenetic regulation in the same way as protein-coding regions and are
therefore subject to changes based on their environment. There are many ways in which ncRNAs can up-regulate or down-regulate transcription, such as by interacting with genes, silencing transcription, or guiding methylation (Collins and Chen 2009, Amaral et al. 2008). ncRNAs are important for development and have critical roles in regulating X-chromosome inactivation (Chow and Herd 2009) and genomic imprinting (Royo and Cavaille 2008).

1.5.2 Genomic imprinting

Genomic imprinting refers to mono-allelic gene expression based on parent-of-origin specific silencing of one allele by repressive epigenetic modifications. Genomic imprinting is thought to have evolved along with the process of placentation as genomic imprinting is only present in eutherian mammals (mammals with long-lived placenta) (Killian et al. 2000), but not in prototherians (egg-laying mammals), reptiles, or birds (Hore et al. 2007). Genomic imprinting has also evolved independently in flowering plants (Köhler and Weinhofer-Molisch 2010), where the endosperm mirrors the function of the placenta. There are 96 known imprinted genes identified in the human genome, but there are many more predicted imprinted genes and it is believed that this number is probably much higher (geneimprint.com). In mammals, imprinted genes are crucial for placental development and fetal growth (Ferguson-Smith and Surani, 2001, Reik and Walter 2001, Tilghman 1999, Tycko and Morris 2002) and alterations in the dosage of imprinted genes can have drastic implications for the developing fetus. Imprinted genes are often organized into clusters and are controlled in cis through an imprinting control region, which is usually regulated via DNA methylation. Roughly 80% of imprinted genes are physically linked in clusters with other imprinted genes (Reik and Walter 2001). Imprinting control regions (ICRs) are segments of DNA that control the expression of imprinted genes in cis over distances that can
reach up to one megabase and they must display the following characteristics: i) must have a differentially methylated region (DMR) and chromatin confirmation status between the paternal and maternal alleles and ii) the establishment of imprinting marks must occur during gametogenesis because this is the only period in which the genomes are separated (Pateras et al. 2009). The importance of genomic imprinting was first discovered by James McGrath and David Salter when they first recognized that both maternal and paternal genomes were required for completion of embryogenesis (McGrath and Salter 1984). Their experiments included the transplantation of pronuclei between one-cell stage embryos in order to construct diploid mouse embryos with two female pronuclei (biparental gynogenones) or two male pronuclei (biparental androgenones). The ability of these embryos to develop to term was compared with control nuclear-transplant embryos in which the male or the female pronucleus was replaced with an isoparental pronucleus from another embryo (McGrath and Salter 1984). Neither the biparental androgenones nor the biparental gynogenones survived past mid-gestation and, while the androgenotes displayed retarded embryonic tissue development and well-formed extraembryonic tissue, the gynogenotes had poorly-developed extraembryonic tissue with relatively normal embryos (Barton et al. 1985, Surani and Barton 1983, Surani et al. 1984). These studies demonstrate that the maternal and paternal genomes both contribute unique characteristics in terms of development and they are both required for normal embryogenesis to occur.
1.5.2.1 Parental-Conflict Theory

It is still unclear why genomic imprinting evolved since the diploid state provides increased protection against the effects of deleterious mutations or epimutations on one allele (Ishida and Moore 2012). In order for this process to have evolved, it must confer a greater biological advantage that outweighs the risks associated with functional haploidy (Ishida and Moore 2012). There are many theories behind why genomic imprinting exists, however only one of these theories will be described as it is most commonly discussed in literature.

The most prevalent theory is called the Parental-Conflict or Kinship Theory and was proposed by Moore and Haig (1991). The Kinship Theory was originally formulated in the context of genes expressed in the fetus that involve allocating resources from its mother. It explains how allocating more of the maternal resources to the fetus may directly benefit the offspring, but this will confer a dangerous cost to the mother (Wilkins and Haig 2003). Maternal and fetal interactions across the placenta can vary the amount of nutrients available, a process...
which does not occur during the development of egg-laying animals (Skaar et al. 2012). It is suggested that genes which are paternally expressed, and maternally imprinted, function as growth-enhancing genes, whereas genes that are maternally expressed, and paternally imprinted, are growth suppressing. The idea is that it is competitive for the paternal alleles to try and obtain as much nutrients from the mother and other offspring, which could potentially be of different paternal origin, whereas for the maternal genome it is competitive to limit resources to one fetus in order to retain resources for herself, as well as her other offspring (Wilkins and Haig 2003). When the maternal and paternal imprints are correctly established and maintained this creates a harmonious environment, reaching a balance between growth-enhancing and growth-suppressing genes.

1.5.2.2 Genomic reprogramming

Genomic reprogramming is a process in which the epigenetic marks within the nucleus of a cell are erased and new epigenetic marks are re-established. Between generations there are two major reprogramming events that take place: 1) when the primordial germ cells enter the gonadal ridge until just after their entry into the incipient gonad and 2) after fusion of sperm and oocyte in the preimplantation embryo during the first cleavage divisions (van Montfoort et al. 2012). These reprogramming events occur in order to restore pluri- and toti-potency and to minimize the amount of epigenetic inheritance between the generations (Feng et al. 2010). The first phase of genomic imprinting occurs to reset the sex-specific imprints of the germ cells during gametogenesis. During this phase, genome-wide demethylation occurs as the primordial germ cells enter the gonadal ridge and the new sex-specific imprints are established; all parental imprints are erased and totipotency is re-established (Manipalviratn et al. 2009). The germline
ICRs are also demethylated during this first reprogramming event and the timing of the event is controlled individually for each imprinted gene cluster (Lee et al. 2002; Hajkova et al. 2008). In both the male and female germlines re-establishment begins in the late fetal stages (Manipaiviratn et al. 2009). In the male germline complete re-establishment of imprinting marks occurs earlier in gametogenesis and are established by birth (Manipaiviratn et al. 2009), whereas in the female germline the re-establishment of methylation marks occurs during follicle development and is not complete until right before ovulation in each oocyte over the reproductive life (Manipaiviratn et al. 2009). The re-establishment of new methylation marks has been associated with the enzymes DNMT3A in collaboration with DNMT3L (Kaneda et al. 2010).

The second phase of reprogramming occurs after fertilization and in this phase the male genome is actively demethylated through conversion of the 5’ cytosine methyl groups into hydroxymethyl groups (Wossidlo et al. 2011), while the female genome is passively demethylated due to a decrease in DNMT1 activity in the pronuclei (Cirio et al. 2008). An important aspect of this reprogramming event is that the imprinted regions maintain their methylation status while the rest of the genome experiences demethylation (Howell et al. 2001, Hirasawa et al. 2008). The protection of the DMRs of imprinted clusters from global demethylation results in transgenerational inheritance of the epigenetic states of these imprinted genes (Lane et al. 2003). At the morula stage, the passive demethylation of the maternal genome ceases, and DNMT3b catalyses de novo methylation in order to mediate transition to a phase of terminal differentiation (Borgel et al. 2010). At this time, the inner cell mass becomes differentially methylated from the extraembryonic lineages (Santos et al. 2002). Overall, there are lower levels of methylation in the extraembryonic tissues compared to the embryonic tissues.
(Santos et al. 2002). Both of the major reprogramming events discussed act as a proof reading mechanism in order to ensure that the next generation is not subject to a potentially harmful epigenetic burden from their parents (Lange and Schneider 2010).

Figure 1.2 Genomic reprogramming in male and female germlines. Summary of methylation changes during genomic reprogramming events within the female (red) and male (blue) germlines; dashed lines represent the imprinting control regions.
1.5.2.3 Imprinting disorders

In humans, the importance of genomic imprinting can be highlighted by the disorders that result from genetic or epigenetic mutations of imprinted regions. Imprinted genes play an important role in embryogenesis, particularly in visceral structure formation and development of the nervous system (Platonov and Isaev 2006), therefore alterations in imprinted gene expression, either by genetic or epigenetic mechanisms, can lead to malformations and disease (Butler 2009). Types of disruptions associated with imprinting disorders vary, but are generally linked to altered dosage of imprinted genes (Ishida and Moore 2012). Genetic mutations involve deletions of the imprinted region, uniparental disomy, or chromosomal duplications. Epigenetic mutations leading to imprinting disorders cause aberrant expression of imprinted genes without affecting the nucleotide sequence and are generally caused by disruption of methylation at the imprinting control regions (Butler 2009). These epimutations could be introduced during gametogenesis due to a failure to erase or re-establish the imprint or due to ineffective imprint maintenance post-fertilization (Ishida and Moore 2012).

Studies have suggested that there is an increased risk of rare imprinting disorders among the ART population such as Beckwith-Wiedemann syndrome (BWS), Angelman syndrome (AS), and Silver-Russell syndrome (SRS) (Maher et al. 2003, DeBaun et al. 2003, Halliday et al. 2004). The most frequently discussed in this context is Beckwith-Wiedemann syndrome (BWS), an overgrowth disorder caused by imprinting errors on chromosome 11p15 (Shuman et al. 2006). Other characteristics of BWS include macroglossia, umbilical hernia, embryonal tumours (e.g. Wilms tumour, hepatoblastoma, neuroblastoma, and rhabdomyosarcoma), neonatal hypoglycemia, and renal abnormalities. Early death may occur due to complications of prematurity, hypoglycemia, cardiomyopathy, macroglossia, or tumours. BWS is quite rare;
The incidence of the disease is roughly one in 13,700. Methylation defects account for a large proportion of BWS cases, with approximately 50% caused by a loss of methylation (LOM) at the KvDMR1 region on chromosome 11p15.5 and 5% caused by gain of methylation (GOM) on the maternal chromosome of the H19/IGF2 ICR (ICR1) (Soejima and Higashimoto 2013). Other causes of BWS are paternal uniparental disomy of chromosome 11p15 and mutations of CDKN1C (Diaz-Meyer et al. 2003).

Angelman syndrome (AS) is characterized by mental retardation, an inappropriate happy demeanour, and dysmorphic facial features. The incidence of AS is roughly 1 in 15,000 and is caused by abnormalities in chromosome 15q11-13, with less than 5% of cases being caused by imprinting defects (Williams and Driscoll 2007). Most cases of AS are due to a loss of function of the imprinted UBE3A (ubiquitin protein ligase E3A) gene; this can be due to deletions or mutations on the maternal allele or paternal UPD. In the cases where AS is caused by an imprinting defect, this is due to hypomethylation of an ICR located in the promoter of the SNURF/SNRPN gene on the maternal allele. It is important to note that most cases of AS that have been found in the ART population have been due to epigenetic imprinting errors, whereas in the general population this only occurs approximately 3% of the time (Amor and Halliday 2008).

SRS is a disease characterized by IUGR and postnatal growth deficiency with normal head circumference, short stature, fifth-finger clinodactyly, triangular faces, and limb length asymmetry. Children with SRS are also at a significant risk of developmental delay and learning disabilities (Saal 2011). SRS is a genetically heterogeneous condition with diagnosis being based on consistent clinical features. Roughly 35-50% of individuals with SRS have hypomethylation of ICR1 on the paternal chromosome 11p15.5 (Giquel et al. 2005, Netchine et al. 2007).
Maternally inherited chromosome duplications involving chromosome 11p15 ICR1 can cause SRS-like phenotype (Fisher et al. 2002, Eggerman et al. 2005), and maternally inherited duplication of chromosome 11p15.5 KvDMR1 has been reported (Schönherr et al. 2007). 5% of cases have maternal uniparental disomy of chromosome 7 (mUPD7) (Netchine et al. 2007) and approximately 30% of cases are of unknown aetiology. The specific gene, or set of genes, responsible for mUPD7 imprinting have not yet been determined. A summary of known imprinting disorders and their associated genomic locations is listed in table 1.2.
Table 1.2 Summary of known imprinting disorders and their associated genomic regions.

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Associated Genomic Locations</th>
<th>Characteristics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beckwith-Wiedemann Syndrome (BWS)</td>
<td>11p15.5</td>
<td>Overgrowth (height and weight &gt;97th percentile), macroglossia, visceromegaly, embryonal tumours, neonatal hypoglycemia</td>
<td>Shuman et al. (2005)</td>
</tr>
<tr>
<td>Silver-Russell Syndrome (SRS)</td>
<td>11p15.5</td>
<td>IUGR, postnatal growth deficiency, short stature, fifth-finger clinodactyly, distinct facial features, developmental delay, learning disabilities</td>
<td>Kagami et al. (2007), Netchine et al. (2007), Saal (2011)</td>
</tr>
<tr>
<td>Angelman Syndrome (AS)</td>
<td>15 7</td>
<td>Severe developmental and intellectual disability, seizures, jerky movements, frequent laughter, happy demeanour</td>
<td>Williams and Droscoll (2007)</td>
</tr>
<tr>
<td>Prader-Willi Syndrome (PWS)</td>
<td>15</td>
<td>Mild intellectual disability, low birth weight, poor suckling, hypotonia, obesity after weaning</td>
<td>Cassidy and Schwartz (2006), Cassidy and Driscoll, 2009</td>
</tr>
<tr>
<td>Transient Neonatal Diabetes Mellitus (TNDM)</td>
<td>6q24</td>
<td>Neonatal hyperglycemia, IUGR, macroglossia, umbilical hernia</td>
<td>Temple and Shield, (2010), Temple et al. (2007)</td>
</tr>
<tr>
<td>pUPD14</td>
<td>14q32</td>
<td>Facial anomaly, abdominal wall defects, placentomegaly, polyhydramnios</td>
<td>Kagami (2008), Kotzot and Utermann (2005)</td>
</tr>
<tr>
<td>Pseudohypoparathyroidism type 1b</td>
<td>20q13</td>
<td>Resistance to parathyroid hormone leading to hypocalcemia and hyperphosphatemia</td>
<td>Bastepe et al. (2001), Bastepe et al. (2005), Liu et al. (2005)</td>
</tr>
</tbody>
</table>
1.5.2.4 Imprinting in the placenta

The placenta is a vital organ that acts as the interface between mother and fetus and provides nutrient transport, gas and waste exchange through the maternal blood flow. Proper development of the placenta is of extreme importance to ensure appropriate prenatal growth and development; abnormal placentation is associated with pregnancy complications such as miscarriage, pre-eclampsia, and IUGR (Bourqe et al. 2010). The placenta stands out amongst other mammalian organs due to its abundant expression of imprinted genes, which are absolutely essential for proper morphology and function (Kawahara et al. 2009, Nelissen et al. 2011). It has been well established that imprinted genes are highly expressed in extraembryonic tissues and that some genes may even have placenta-specific promoters, such as *IGF2* (Constância et al. 2002). The imprinted genes *IGF2*, *PEG10*, and *CDKN1C* are highly expressed in the placenta, as well as *PHLDA2* and *PLAGL1*, which are down-regulated in most tissues (Steinhoff et al. 2009).

The *de novo* DNA methyltransferase *DNMT3L* is expressed in high levels in the chorion of the placenta (Bourc’his et al. 2001), suggesting the importance of DNA methylation in placental regulation. When *DNMT3L* is knocked out in mice, there is an absence of invasion of fetal blood vessels into the chorionic plate and a reduction in spongiotrophoblast (Arima et al. 2006). The *DNMT3L* -/- mice demonstrated arrest of proliferation of extraembryonic tissue and it is suggestive that these mutant placentae were non-functional, accounting for the manifestation of fetal death in the mutant mice (Arima et al. 2006). Other experiments have demonstrated the importance of epigenetic regulation in the placenta; when methylation patterns in rat placentae were altered by treatment with 5-azacytidine, it resulted in disturbed glycoprotein expression, as well as significantly lower placental weight (Serman et al. 2007). Furthermore, the placental
morphology was abnormal and trophoblast proliferation was poor, indicating the importance of proper imprint establishment for placental development (Serman et al. 2007).

Data from knockout or transgenic mice suggests that, generally, the paternally expressed genes tend to enhance placental growth and maternally expressed genes tend to suppress placental growth and trophoblast invasion, in agreement with the Kinship theory. When paternally expressed genes Ifg2, Peg1 and Peg3 are knocked out in mice there is a restricted growth of the labyrinthine trophoblast, blood vessels, and the spongiotrophoblast (Lefebvre et al. 1998, Li et al. 1999, Constanticia et al. 2002). Conversely, when maternally expressed Igf2r and Cdkn1c are knocked out, it results in placental hyperplasia (Ludwig et al. 1996, Takahashi et al. 2000) and it has been established that proper methylation of maternally imprinted genes is absolutely crucial for vertebrate placentation (Arima et al. 2006). Guo et al. (2008) investigated methylation and gene expression patterns of imprinted genes in SGA placentae and observed a loss of imprinting at the H19 locus, which may be a cause of poor growth of the fetus (Guo et al. 2008). From these studies, it can be concluded that proper establishment of imprinted genes is crucial to proper placental development and that aberrations in this process can lead to altered growth and invasion. Imprinted genes may play a role in providing nutrients to the fetus from the mother and disease pathologies linked to aberrant imprinting may be caused by placental inefficiency (Frost and Moore 2010).

Table 1.3 below outlines imprinted genes in the placenta that have been associated with fetal growth disorders in humans.
## Table 1.3 Imprinted genes in the placenta involved in fetal growth phenotypes (modified from Frost and Moore (2010))

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene</th>
<th>Imprinted Allele</th>
<th>Associated Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>6q24</td>
<td>PLAGL1</td>
<td>Maternal</td>
<td>Overexpression linked to TNDM and both under and over expression linked to IUGR</td>
</tr>
<tr>
<td>7p12</td>
<td>GRB10</td>
<td>Maternal</td>
<td>Overexpression linked to SRS; when knocked out in mice leads to fetal and placental overgrowth</td>
</tr>
<tr>
<td>7q21</td>
<td>PEG10</td>
<td>Maternal</td>
<td>Overexpression linked to IUGR; murine knockouts lack spongiotrophoblast</td>
</tr>
<tr>
<td>7q32</td>
<td>MEST</td>
<td>Maternal</td>
<td>Overexpression linked to SRS; murine knockouts display postnatal growth restriction</td>
</tr>
<tr>
<td>11p15</td>
<td>H19</td>
<td>Paternal</td>
<td>Overexpression linked to SRS; underexpression linked to BWS</td>
</tr>
<tr>
<td></td>
<td>IGF2</td>
<td>Maternal</td>
<td>Overexpression linked to BWS; underexpression linked to SRS</td>
</tr>
<tr>
<td></td>
<td>KCNQ1</td>
<td>Paternal</td>
<td>Underexpression linked to BWS and long QT syndrome 1</td>
</tr>
<tr>
<td></td>
<td>KCNQ1OT1</td>
<td>Maternal</td>
<td>Overexpression linked to BWS</td>
</tr>
<tr>
<td></td>
<td>CDKN1C</td>
<td>Paternal</td>
<td>Underexpression linked to BWS and umbilical hernia</td>
</tr>
<tr>
<td></td>
<td>SLC22A18</td>
<td>Paternal</td>
<td>Unknown; within BWS region</td>
</tr>
<tr>
<td></td>
<td>PHLDA2</td>
<td>Paternal</td>
<td>Overexpression linked to IUGR; underexpression associated with increased birth weight; murine knockout shows placental hyperplasia</td>
</tr>
<tr>
<td>14q32</td>
<td>DLK1</td>
<td>Maternal</td>
<td>Overexpression in subset of BWS patients; murine knockouts show fetal growth restriction</td>
</tr>
<tr>
<td>19q13</td>
<td>ZNF331</td>
<td>Paternal</td>
<td>Reduced expression linked to IUGR</td>
</tr>
<tr>
<td>20q13</td>
<td>GNAS</td>
<td>Maternal</td>
<td>Underexpression linked to growth restriction</td>
</tr>
</tbody>
</table>

### 1.5.2.5 Imprinted gene network

In recent years it has been suggested that there is a network of co-regulated imprinted genes that play an essential role in fetal and placental development. This idea was first suggested when one group observed that there were similarities in the phenotypes of infants affected by TNDM and BWS, and that the two genes CDKN1C and PLAGL1, which are associated with BWS and TNDM, respectively, shared similar expression patterns (Arima et al. 2005). Although
unable to show a direct interaction between these two genes, this group demonstrated that the transcription factor PLAGL1 on chromosome 6q24 bound to the unmethylated CpG sites within the KCNQ1OT1 promoter region on chromosome 11p15.5 in vitro (Arima et al. 2005). KCNQ1OT1 is an anti-sense RNA that negatively regulates imprinted genes on chromosome 11p15.5, including the CDKN1C gene (Arima et al. 2005). Because KCNQ1OT1 and CDKN1C are both implicated in BWS, and PLAGL1 has been shown to directly induce KCNQ1OT1 expression, this suggests the presence of an imprinted network that is associated with BWS (Arima et al. 2005). Another study knocked out the Plagl1 gene on the paternal allele of mice and found that the resulting pups displayed symptoms of IUGR, altered gross morphology and bone formation and high rates of neonatal lethality (Varrault et al. 2006). They then used a meta-analysis of 116 microarray data sets in order to examine the interactions between Plagl1 and other imprinted genes and found strong co-regulation between several imprinted genes that are involved in embryonic development (Varrault et al. 2006). They confirmed that overexpression of PLAGL1 led to induction of IGF2, CDKN1C, and H19, and that in PLAGL1-deficient liver there was down-regulation of CDKN1C, IGF2, H19, and DLK1 (Varrault et al. 2006). They also showed that PLAGL1 binds to a shared enhancer (E2) on chromosome 11p15.5 that induces expression of IGF2 and H19 (Varrault et al. 2006). Recently, a study examining PLAGL1 in IUGR placentae also found down-regulation of this gene in female infants and found a strong correlation between expression of PLAGL1 and CDKN1C, H19, and IGF2. They also observed binding of PLAGL1 to the E2 enhancer (Iglesias-Platas et al. 2014). These studies suggest that there is co-regulation between imprinted genes and that the PLAGL1 gene on chromosome 6q24 is a regulator of imprinted gene expression on chromosome 11p15.5 in both mice and humans.
A study examining the functional role of H19 in mice found that targeted deletion of the H19 gene lead to an up-regulation of at least six imprinted genes, with a recovery of normal expression in 5 of these genes upon re-expression of H19 (Gabory et al. 2009). This study suggests that H19 plays a role in the regulation of the imprinted gene network, and that PLAGL1 acts upstream to control its regulation. These mouse models support the existence of the imprinted gene network and suggest that it functions to create equilibrium by inducing up or down-regulation of oppositely imprinted genes in order to create balance between growth-activating and growth-repressing genes (Gabory et al. 2009).

Figure 1.3 Imprinted gene network. Maternally imprinted genes are in red and paternally imprinted genes are in blue. Dashed lines represent links between H19 and imprinted genes observed by Gabory et al. (2009) and solid black lines represent connections between genes found in a study by Varrault et al. (2006). (Figure was modified from Gabory et al. (2009)).
Figure 1.4 PLAGL1 as regulator of chromosome 11p15.5 imprinted genes. Proposed interactions between PLAGL1 on chromosome 6q24 and the KvDMR1 (ICR2) cluster and shared H19/IGF2 enhancer region (represented by the blue circle) on chromosome 11p15. Red lines represent genes transcribed from the maternal allele and green lines represent genes silenced on the maternal allele. Blue lines represent genes expressed from the paternal allele and orange lines represent genes silenced on the paternal allele. Grey boxes represent methylated ICRs and open boxes represent unmethylated ICRs. (Figure was modified from Smith et al. (2007))
1.6 ART and genomic imprinting

As described in previous sections, the process of ART overlaps with preimplantation embryonic development, which coincides with important genomic reprogramming events. For this reason, it is believed that the ART procedure may contribute to aberrant DNA methylation at ICRs leading to altered gene expression of the associated imprinted genes. This section will outline the current literature related to genomic imprinting disorders and ART. I will first outline the case studies that lead to the hypothesis that ART may alter the modification and expression of imprinted regions, and then review studies which have looked at imprinted regions within phenotypically healthy ART children.

1.6.1 Case studies of ART and imprinting disorders

The first set of studies which implicated IVF in imprinting related disorders was the observation that bovine, who were conceived through ART, seemed to demonstrate higher rates of an overgrowth disorder, referred to as large offspring syndrome (LOS). LOS is characterized by increased birth weight and perinatal morbidity, and was reported in animals after nuclear transfer and in vitro culturing (Sinclair et al. 2000). LOS has been associated with reduced methylation and expression of the IGF2 receptor (IGF2R) (Young et al. 2001). LOS displays similar characteristics to BWS in humans.

The first association in humans linking ART with aberrant imprinting was the observation that three children conceived by ICSI were diagnosed with AS due to aberrant methylation of chromosome 15, within the SNRPN region (Cox et al. 2002, Ørstavik et al. 2003). These results are significant because in the general population only 3% of AS cases are caused by imprinting defects (Amor and Halliday 2008), yet all 3 of these children conceived by ICSI
were due to epimutations at the SNRPN DMR. A British survey used questionnaires to determine the conception history of children diagnosed with AS and determined that 3 out of 75 (4%) were conceived via ART, one of which had an imprinting error in the SNRPN region (Sutcliffe et al. 2006). Overall, 75% of children diagnosed with AS after ART have been due to epigenetic imprinting errors, which varies significantly from the general population.

DeBaun et al. (2003) observed that seven children who were diagnosed with BWS were all conceived via ARTs; of the six children who had molecular analysis performed, 5 had hypomethylation of the KvDMR1 region and one displayed hypermethylation of the H19 DMR. This is unusual, as generally only 50% of cases of BWS are due to epimutations and the other 50% are caused by uniparental disomy or deletions of the imprinted regions. This study also observed that prevalence of ART in the Washington University BWS registry was 4.6%, compared to only 0.76% in the general US population at the time, resulting in a 6 fold increase of ART among the cohort of BWS patients (DeBaun et al. 2003). Conversely, a large cohort study undertaken to investigate the incidence of imprinting disorders after ART in the Danish population analyzed a Denmark National Registry of 25,000 children born after IVF and did not identify any cases of diagnosed imprinting disorders (Lidegaard et al. 2005). However, it is significant that in over 90% of children diagnosed with BWS after ART, it is due to an imprinting defect, compared to 40-50% in the general population, and that the majority of AS cases after ART are due to epigenetic defects, when this is quite rare otherwise.

Maternal age and underlying infertility has been thought to contribute to this effect, as a large Dutch cohort study examining children diagnosed with BWS born in the Netherlands between 1983 and 2003 observed that the maternal age of the BWS population was significantly higher than the average maternal age of the Dutch population, and that there were significantly
increased rates of underlying fertility problems among this group (Doornbos et al. 2007). The rate of ART among the BWS cohort was 5.6%, compared to 0.92% of the Dutch population at that time, and all children displayed hypomethylation of the KvDMR1 region, however once they controlled for the fertility issues among the parents, there was no significantly increased prevalence of ART (Doornbos et al. 2007). As imprinting disorders occur very rarely, it is difficult to confidently confirm any association with ART. A summary of the mentioned, and additional, case studies examining ART and imprinting disorders is presented in Table 1.4 below.

**Table 1.4 Summary of case studies examining imprinting disorders and ART**

<table>
<thead>
<tr>
<th>Study Cohort</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 AS patients born after ICSI</td>
<td>LOM at \textit{SNRPN} in 2/2 cases</td>
<td>Cox et al. 2002</td>
</tr>
<tr>
<td>7 BWS patients born after ART</td>
<td>\textit{KCNQ1OT1} LOM 5/6 cases \textit{H19 GOM} 1/6</td>
<td>DeBaun et al. 2003</td>
</tr>
<tr>
<td>149 BWS cohort</td>
<td>4% ART (3 IVF, 3 ICSI) compared to 1.0% in general population; 2/6 LOM \textit{KCNQ1OT1}, 4/6 not analyzed</td>
<td>Maher et al. 2003</td>
</tr>
<tr>
<td>149 BWS cohort</td>
<td>4% ART (4 IVF, 2 ICSI) compared to 1.3% in reference population; 6/6 LOM \textit{KCNQ1OT1}</td>
<td>Gicquel et al. 2003</td>
</tr>
<tr>
<td>1 AS ICSI child</td>
<td>LOM \textit{SNRPN}</td>
<td>Ørstavik et al. 2003</td>
</tr>
<tr>
<td>1,316,500 live births – 37 BWS (4 ART)</td>
<td>Risk of BWS in ART population is 9x higher</td>
<td>Halliday et al. 2004</td>
</tr>
<tr>
<td>12 BWS children</td>
<td>5/12 IVF, 5/12 ICSI</td>
<td>Chang et al. 2005</td>
</tr>
<tr>
<td>1680 IVF, 4372 ICSI</td>
<td>No imprinting disorders</td>
<td>Lidegaard et al. 2005</td>
</tr>
<tr>
<td>75 AS children</td>
<td>0 born after ART</td>
<td>Sutcliffe et al. 2006</td>
</tr>
<tr>
<td>SRS IVF child</td>
<td>Hypermethylation of \textit{PEG1/MEST}</td>
<td>Kagami et al. 2007</td>
</tr>
<tr>
<td>19 ART (SGA) 29 NC</td>
<td>GOM at \textit{KCNQ1OT1} in 1 ICSI child</td>
<td>Kanber et al. 2009</td>
</tr>
<tr>
<td>6 ART (1 BWS, 5 SRS)</td>
<td>10 fold increased frequency of BWS and SRS after ART; aberrant DNA methylation at multiple loci</td>
<td>Hiura et al. 2012</td>
</tr>
</tbody>
</table>
1.6.2 Imprinting in phenotypically healthy children conceived by ART

Results from the case studies summarized above have been varied, and oftentimes inconclusive, owing to the rarity of measurable disease phenotypes, as well as the variability in the types of fertility treatments and study methods used. Consequently, many researchers have turned to investigating imprinted regions in ART pregnancies that do not have any apparent complications in the hopes of finding differences in methylation which could potentially result in an increased risk of disease. As BWS and AS were found to be linked to ART, several groups focused on studying methylation among the imprinted regions associated with these disorders.

Gomes et al. (2009) examined the KCNQ1OT1 locus and observed hypomethylation in 3 out of 18 children conceived via IVF, with methylation levels reduced from 41.5% in NC controls to 14% in IVF. Another group found conflicting results, with no difference in KCNQ1OT1 methylation in the placenta, cord blood, or maternal peripheral blood (Tierling et al. 2010).

Furthermore, they looked at 8 other DMR regions (H19, SNRPN, GRB10, IG-DMR and 4 GNAS DMRs) and found no significant differences in methylation between the ART and NC infants, but found a slightly increased methylation at the MEST gene (Tierling et al. 2010).

The H19/IGF2 DMR has been one of the most studied regions in ART offspring and again, results have been conflicting. Turan et al. (2010) examined methylation of the H19/IGF2 DMR in placentae and found that aberrant methylation was more common in the in vitro group compared to the in vivo group and that expression of both H19 and IGF2 was found to be reduced in the placental tissue from ART infants (Turan et al. 2010). Two separate studies found hypermethylation of the H19/IGF2 DMR region in children conceived via ART compared to natural conceptions, along with corresponding changes in gene expression (Rancourt et al. 2012, Nelissen et al. 2013). A recent study examining DNA methylation and gene expression in the
placenta, found significantly increased expression of *H19* and *PHLDA2* in IVF/ICSI placentae compared to NC controls, with no observed difference in methylation (Nelissen *et al.* 2014). However, other studies examining placentae, cord blood, and peripheral blood have not found any significant changes at this region in ART pregnancies compared to NC controls (Chan-Wong *et al.* 2011, Oliver *et al.* 2012, Shi *et al.* 2011).

Genome-wide studies comparing DNA methylation from ART and NC have not noted a significant difference in methylation at imprinted regions (Katari *et al.* 2009). Katari *et al.* (2009) examined the methylation of more than 1500 genes, including DMRs, from IVF and NC pregnancies and found that 16% of genes displayed differences in methylation in the placenta and 23% in the umbilical cord blood; four of eleven genes tested also showed significant differences in gene expression. However, these consisted of both imprinted and non-imprinted genes. Melamad *et al.* (2013) examined genome-wide methylation from IVF and NC pregnancies and observed that there were significant differences at 2.7% of CpG sites between the two groups, however there did not appear to be significance at imprinted regions.
Table 1.5 Summary of human studies examining DNA methylation in children born via ART

<table>
<thead>
<tr>
<th>Sample size</th>
<th>Tissue Type</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 ART children 33 NC</td>
<td>Peripheral blood Cord blood Placenta</td>
<td><em>KCNQ1OT1</em> LOM 3/18 ART (2 IVF, 1 ICSI)</td>
<td>Gomes et al. 2009</td>
</tr>
<tr>
<td>18 ART children 13 NC</td>
<td>Cord blood Placenta</td>
<td>In vitro associated with small, but significant differences in methylation</td>
<td>Katari et al. 2009</td>
</tr>
<tr>
<td>112 ART 73 NC</td>
<td>Maternal blood Cord blood Placenta</td>
<td>No sig. differences at 9 DMRs (<em>KCNQ1OT1, H19, SNRPN, GRB10, MEST, 4 GNAS DMRs</em>)</td>
<td>Tierling et al. 2010</td>
</tr>
<tr>
<td>45 ART 56 NC</td>
<td>Cord blood Placenta</td>
<td><em>H19 GOM</em></td>
<td>Turan et al. 2010</td>
</tr>
<tr>
<td>29 ART twins 30 NC twins</td>
<td>Cord blood</td>
<td>No sig. changes at ICR1, KvDMR1, and <em>PEG1</em></td>
<td>Li et al. 2011</td>
</tr>
<tr>
<td>61 ICSI children</td>
<td>Cord blood</td>
<td><em>H19 LOM</em> in 3/61 children, no significant difference between controls</td>
<td>Shi et al. 2011</td>
</tr>
<tr>
<td>77 ART 12 NC</td>
<td>Placenta</td>
<td>No sig. difference between mean methylation between groups</td>
<td>Chan-Wong et al. 2011</td>
</tr>
<tr>
<td>61 IVF, 40 ICSI, 60 NC</td>
<td>Cord blood</td>
<td>No LOM at 6 imprinted DMRs</td>
<td>Zheng et al. 2011</td>
</tr>
<tr>
<td>34 IVF, 32 ICSI</td>
<td>Peripheral blood</td>
<td>No LOM at <em>H19, KCNQ1OT1</em> or <em>SNRPN</em></td>
<td>Oliver et al. 2012</td>
</tr>
<tr>
<td>59 ART 61 NC</td>
<td>Cord blood Placenta</td>
<td>Small but sig. changes in methylation at <em>KCNQ1, SNRPN</em> and <em>H19</em>. Gene expression correlation only found in <em>H19</em></td>
<td>Rancourt et al. 2012</td>
</tr>
<tr>
<td>67 ART 31 NC</td>
<td>Peripheral blood Buccal swabs</td>
<td>Possible changes at <em>IGF2</em> and <em>IGF2R</em> in buccal swabs No sig. changes in peripheral blood</td>
<td>Puumala et al. 2012</td>
</tr>
<tr>
<td>35 ART 35 NC</td>
<td>Placenta</td>
<td>Hypermethylation at <em>H19</em> and <em>MEST</em> Decreased expression of <em>H19</em></td>
<td>Nelissen et al. 2013</td>
</tr>
<tr>
<td>10 IVF 8 NC</td>
<td>Cord blood</td>
<td>Variable genome-wide changes in DNA methylation</td>
<td>Melamad et al. 2013</td>
</tr>
<tr>
<td>29 IVF 20 ICSI 86 NC</td>
<td>Buccal swabs</td>
<td>ICSI was associated with hypermethylation of <em>SNRPN</em></td>
<td>Whitelaw et al. 2014</td>
</tr>
<tr>
<td>81 IVF/ICSI 105 NC</td>
<td>Placenta</td>
<td>Increased gene expression of <em>H19</em> and <em>PHLDA2</em> in IVF/ICSI samples; no LOI</td>
<td>Nelissen et al. 2014</td>
</tr>
</tbody>
</table>
1.7 Origins of adverse outcomes related to ART

It is believed that the ART process may contribute to altered DNA methylation and imprinting defects due to the fact that the procedures overlap with two major genomic reprogramming events outlined in section 1.5.2.2. The use of hormones to stimulate the ovary for supernumerary oocyte production, the in vitro maturation of oocytes, the use of immature sperm from infertile males, the direct injection of spermatozoa into the oocyte during ICSI, and the in vitro culturing of preimplantation embryos may all have an impact on the natural establishment of genomic imprinting markers (Manipalviratn et al. 2009). ARTs by-pass many natural selection barriers and artificial environments are used to manipulate the complex and enigmatic processes of gametogenesis, fertilization, and development of the early embryo. In this section, I will outline the current literature on how ovarian stimulation, sperm from infertile males, and in vitro fertilization and culturing result in imprinting errors.

1.7.1 Ovarian stimulation

As the imprinting disorders most commonly associated with ART, AS and BWS, are due to a loss of the functional maternal allele (Maher et al. 2005), it is believed that during the ART process, the oocyte may be particularly vulnerable to imprinting defects. This may be due to the timing of hormone administration and superovulation overlapping with the establishment of DNA methylation marks at imprinting control regions (see figure 1.2). As mentioned earlier in section 1.5.2, although the imprint marks are fully established in the male germline before meiosis, in the oocyte these marks are not fully established until right before ovulation (Morgan et al. 2005). Therefore, it is possible that the ovarian stimulation procedure, which is used to superficially induce the release of multiple oocytes, may interfere with the proper establishment
of imprinting marks during oogenesis. It is believed that genes which acquire their imprinting marks later in oocyte development are more susceptible to imprinting errors (Gosden et al. 2003, Fortier et al. 2008).

In animal studies, it has been observed that superovulation with gonadotropins does negatively impact implantation and fetal development. Studies using murine models have demonstrated delayed preimplantation embryonic development, reduced implantation, reduced oocyte and embryo quality, fetal growth restriction, aberrant imprinting, and effects on the oviductal and uterine environment (Laprise 2009). In vivo fertilized two-cell embryos displayed higher rates of aberrant methylation and embryo loss during preimplantation when animals were exposed to superovulation compared to controls (Shi and Haaf 2002), providing evidence that superovulation may interfere with imprint establishment during oogenesis. Superovulation has also been shown to alter expression of imprinted genes H19, Kcnq1ot1, and Snrpn in mouse embryos and placentae (Fauque et al. 2007, Fortier et al. 2008). Furthermore, disruptions of genomic imprinting in both maternally and paternally expressed genes have been observed, suggesting that it may not only disrupt the acquisition of imprints in the growing oocytes but may also affect maternal gene products required for the maintenance of imprinting during preimplantation development (Market-Velker et al. 2010). In contrast to other studies, Denomme et al. (2011) examined imprinting status of Snrpn, Kcnq1ot1, Peg3, and H19 in oocytes from 125 female mice subjected to ovarian stimulation and found that the methylation patterns were similar to spontaneously ovulated oocytes.

A case study report of 19 BWS children conceived after ART examined the practices used by each patient, including ovarian stimulation, culture media, culturing times, and which ART procedure was used, and found the only common factor to be the use of superovulation
It has also been observed that children born after ovarian stimulation alone, without IVF, have a shorter stature compared to NC children of both fertile and infertile parents (Savage et al. 2012). However, another study examining LBW and ART concluded that the use of ovarian stimulation has no effect on birth weight (Griesinger et al. 2008). Aberrations of the imprinted genes *PEG1* and *H19* have been observed in growing oocytes from ART-treated women, and changes in methylation were also reported in superovulated mouse oocytes (Sato et al. 2007). The KvDMR1 locus in human *in vitro* cultured oocytes was found to be significantly more methylated when obtained from natural cycles compared to patients undergoing gonadotropin stimulation, indicating that hyperstimulation may recruit immature follicles which are unable to fully acquire imprinting marks during the maturation process (Khoureiry et al. 2008). In humans, due to the fact that women who undergo ovarian stimulation have lower fertility rates, an increased reproductive loss rate, and tend to be of advanced age (Shiota and Yamada 2005), it is challenging to isolate the direct effect of the ovarian stimulation from the other risk factors.

From the study of animal models, as well as observations in human oocytes, it is believed that oocytes exposed to hormonal stimulation may have a reduced ability to carry out the reprogramming events necessary for proper fetal and placental development (Shi and Haaf 2002, Haaf 2006).

### 1.7.2 Sperm from infertile males

Concerns have been raised regarding the use of sperm from infertile males to conceive a child, particularly in ICSI, as this may increase the chances of passing on genetic mutations, potential DNA damage, and genomic imprinting defects related to male factor infertility. Studies
have shown that infertile males, particularly those with oligozoospermia, have higher rates of genomic imprinting defects in their sperm and may be at an increased risk of transmitting incorrect primary imprints to their offspring (Kobayashi et al. 2007). Infertile males were found to have abnormal methylation imprints in 14% of paternal and 20% of maternal imprinted genes in their sperm (Kobayashi et al. 2007). Low sperm count has been associated with hypomethylation of the ICR1 region controlling expression of H19 and IGF2, and hypermethylation of MEST (Poplinski et al. 2010). Altered methylation profiles of H19 were also found in 0%, 17%, and 30% of men with normozoospermia, moderate oligozoospermia, and severe oligozoospermia, respectively (Marques et al. 2004). Marques et al. (2008) observed that 46.7% of patients with sperm counts below 10 x 10^6/ml displayed defective methylation of H19 and MEST imprinted genes and this effect seemed to be specific to imprinted regions as global DNA methylation was unaffected. Further, percent methylation in sperm from men with abnormal protamine ratios and oligozoospermia were shown to have significantly higher methylation at maternally imprinted genes compared to fertile controls (Hammoud et al. 2010).

Infertile men with poor semen parameters had greater levels of DNA methylation at maternally imprinted genes PLAGL1, MEST, and DIRAS3 (Houshdaran et al. 2007). Idiopathic infertile males with sperm motility below 40% and sperm morphology 5% below normal spermatozoa, also displayed hypermethylation at the MEST imprinted region (Poplinski et al. 2010). The use of immature sperm from the testes or epididymis in ICSI has also raised concerns as it is believed that some epigenetic reprogramming occurs in the epididymis (Ariel et al. 1994). Testicular spermatozoa from azoospermic men were found to carry methylation defects at the H19 locus, which has been shown to affect CTCF binding, and supports the association between disrupted spermatogenesis and imprinting errors (Marques et al. 2010). Previous studies from our
group have also shown decreased methylation at *H19* in testicular sperm from both obstructive and non-obstructive azoospermic males (Minor et al. 2011). Further research is required to understand how imprinted genes are affected in the sperm of infertile males and identify whether these epimutations are being passed on to the offspring, particularly through the use ICSI.

### 1.7.3 Fertilization, manipulation, and *in vitro* culturing

As described previously, the process of fertilization and early embryonic development is an intricate and sensitive progression which is reliant on a specific biochemical environment. During the preimplantation period, a wave of demethylation occurs as reprogramming events are initiated in order to erase gamete-specific methylation; however, imprinted regions are omitted from this process in order to maintain proper expression of imprinted genes. During the IVF process, the synchrony of the female reproductive system is absent and the embryo is exposed to artificial surroundings and manipulations that may alter the maintenance and establishment of epigenetics marks. As epigenetic modifications mediate the interaction between the environment and the genome, it is possible that alterations to the preimplantation environment could in turn alter the epigenetic status of the developing embryo.

Embryo culture may affect the establishment of DNA methylation, gene expression, and other epigenetic modifications such as histones and chromatin remodeling. Studies in mouse and rat embryos have shown that *in vitro* culturing resulted in a decreased ability to effectively demethylate the paternal genome compared to *in vivo* derived controls. Mouse studies have shown aberrant biallelic expression of *H19* and LOM at the paternal allele in cultured mouse blastocysts (Sasaki et al. 1995, Doherty et al. 2000) as well as LOM at *Snrpn* and *Peg3* (Mann et al. 2004, Sasaki et al. 1995, Doherty et al. 2000, Khosla et al. 2001, Li et al. 2010, Fauque et al.
A study examining postimplantation embryos following embryo culture noted a LOM at \textit{H19}, \textit{Snrpn}, \textit{Peg3}, and \textit{Kcnq1ot1} in extraembryonic tissues, suggesting that epigenetic alterations are maintained after implantation (Sasaki et al. 1995, Mann et al. 2004, Rivera et al. 2008). In bovine models, a LOM at \textit{Snrpn} and activation of gene expression was found in the placentae from cultured embryos (Suzuki et al. 2009). In humans, it has been suggested that \textit{in vitro} conditions could affect the maintenance of imprinting. Chen et al. (2010) found that at day 3, 19% of surplus embryos of low quality displayed hypomethylation of \textit{H19}, with no observed hypomethylation in the paternal sperm. Another study found similar results where 8 of 21 arrested embryos showed a LOM at paternal \textit{H19} DMR, without any methylation defects observed in the sperm samples (Ibala-Romdhane et al. 2011).

Studies have shown that the type of culture media used during IVF has the ability to alter DNA methylation at imprinted regions (Mann et al. 2004, Lawrence and Moley 2008). Mouse embryos cultured in different media demonstrated differential methylation and expression of \textit{H19}; a loss of methylation (LOM) was observed at this locus when cultured in Whitten’s media, whereas those embryos cultured in KSOM+AA were unaffected (Doherty et al. 2000). Another study analyzing embryos cultured \textit{in vivo}, and \textit{ex vivo} in M16 media with and without the addition of fetal calf serum, found that there were differences in expression of \textit{H19}, \textit{Igf2}, \textit{Grb10}, and \textit{Grb7} between all 3 groups (Khosla et al. 2001). A comparison of 6 different embryo culture systems (Whitten’s, KSOM+AA, HTF, Global, preimplantation/multiblast, and Glv5plus/G2v5Plus) found a loss of \textit{H19}, \textit{Snrpn}, and \textit{Peg3} methylation in all 6 systems. However, there were some differences in the severity of aberrations, where embryos cultured in KSOM+AA
were the least affected, and certain media supported higher levels of methylation at some, but not all, loci (Market-Velker et al. 2010).

*In vitro* culturing times have been shown to affect DNA methylation patterns at imprinted regions. When minimal embryo manipulations were used by transferring blastocysts that were fertilized *in vivo* and cultured *in vitro* for less than 1.5 hours, they displayed LOM on the maternal allele of the KvDMR1 locus in extraembryonic tissue (Rivera et al. 2008). However, when culturing was extended from the two-cell stage to the blastocyst stage, the number of genes with aberrations increased and both embryonic and extraembryonic tissues were affected (Rivera et al. 2008). It has also been demonstrated that unfavourable culture conditions in mice can lead to disease later in life (Fernández-Gonzalez et al. 2007), indicating that there could be long-term effects associated with the preimplantation environment.

### 1.8 Rationale

In recent generations we have begun to see a drastic increase in infertility rates due to advanced maternal age, environmental exposures, obesity, and other social and biological factors. Due to this rise in infertility, and advances in the field of reproductive biology, there is an increased demand for ARTs and the use of assisted reproduction is becoming more commonplace, with approximately 4% of babies born after IVF or ICSI (Bohlmann et al. 2009). As outlined previously in the chapter, there are growing concerns regarding the safety of ARTs, particularly due to increased rates of low birth weight and imprinting disorders observed in the ART population. Although many studies have been done to evaluate the risks of imprinting errors in pregnancies conceived via IVF and ICSI, results are often conflicting and so far inconclusive. Furthermore, the heterogeneity of the studies in terms of genes analyzed, study
participants, tissues examined, fertility treatments, and procedures used often makes it difficult to draw any major conclusions.

Because the imprinting process is still not entirely understood, this study aims to better comprehend the extent to which ART may alter DNA methylation and gene expression patterns and potentially lead to genomic imprinting disorders. The KvDMR1, PEG10, and PLAGL1 DMRs will be analyzed, along with the LINE-1 repetitive element. Following is a brief description of each region of interest and why it was chosen as a candidate region, followed by the major hypotheses and objectives.

1.8.1 KvDMR1

KvDMR1 is an imprinting control region located on chromosome 11p15.5 that regulates expression of several genes within this locus. A LOM at the KvDMR1 locus is responsible for approximately 50% of BWS and aberrant methylation of this region has also been found in the majority of BWS cases occurring after ART. There are two major ICRs on chromosome 11p15.5; the H19/IGF2 ICR (ICR1) and the KvDMR1 ICR (ICR2), which regulates expression of KCNQ1OT1, as well as other genes within the domain. The KvDMR1 region is located within the promoter of the non-coding RNA KCNQ1OT1, which acts to regulate genes involved in normal growth and development. The KCNQ1OT1 ncRNA is thought to repress expression of PHLDA2, CDKN1C, and KCNQ1 in cis on the paternal chromosome in order to maintain proper imprinting status of this region. LOM at KvDMR1 causes silencing of CDKN1C, which is over 180 kb away on the maternal chromosome and is thought to lead to BWS (Diaz-Meyer et al. 2003). In this study, KvDMR1 DNA methylation and KCNQ1OT1 gene expression levels were
analyzed due to their association with BWS and regulation of other developmentally important genes within the chromosome 11p15.5 region.

1.8.2 PEG10

PEG10 (paternally expressed gene 10) is an imprinted gene on chromosome 7 that is maternally methylated and paternally expressed. PEG10 is thought to have derived from a retroelement and has evolved into a gene required during prenatal development, particularly important for the development of the placenta. PEG10 is highly expressed in the ovary, testis, and placenta, as well as other extraembryonic tissues (Clark et al. 2007). Knocking out of Peg10 in mice is embryonic lethal by day 10.5 due to placental defects (Clark et al. 2007) and a down-regulation of PEG10 was found in third trimester fetal samples where fetal deaths had occurred (Dória et al. 2010), outlining the importance of this gene to proper embryonic and placental development. Increased expression of PEG10 has also been observed in placentae from pregnancies complicated with pre-eclampsia, suggesting an involvement in its pathophysiology (Chen et al. 2005). Recently, it was shown that both KvDMR1 and PEG10 methylation defects were observed in a subset of SRS patients (Turner et al. 2010). Further, a loss of imprinting (LOI) of the PEG10 region has been associated with human hepatocellular carcinomas (Tsou et al. 2003, Gao et al. 2010). In this study, PEG10 was included due to its involvement in placental growth and development, and its potential role in the aetiology of SRS.

1.8.3 PLAGL1

PLAG1 (pleiomorphic adenoma gene-like 1) is a paternally expressed and maternally imprinted gene found on chromosomal region 6q24 which encodes a zinc-finger protein with
transactivation and DNA binding abilities (Abdollahi et al. 1997, Kas et al. 1997, Spengler et al. 1997). *PLAGL1* exhibits tumour suppressor activities due to its capability of inducing G1 cell cycle arrest and apoptosis (Spengler et al. 1997, Varraault et al. 1998) and is a co-activator of the p53 cell cycle regulator (Huang et al. 2001). Hypomethylation of this region has been associated with transient neonatal diabetes (TNDM) (Gardner et al. 2000, Kamiya et al. 2000), a known imprinting disorder. TNDM is defined as diabetes beginning in the first 6 weeks of life with recovery by 18 months of age, most often accompanied by IUGR; a significant number of patients with TNDM will develop diabetes later in life. Changes in *PLAGL1* have also been implicated in BWS (Arima et al. 2005) and SRS (Peñaherrera et al. 2010). Both under- and over-expression of *PLAGL1* has also been linked to IUGR (McMinn et al. 2005, Mackay et al. 2010, Varraault et al. 2006, Iglesias-Platas et al. 2014). As discussed previously, *PLAGL1* has also been identified as a regulator of an important imprinted gene network and disruption of such a network may play a role in BWS and IUGR (Arima et al. 2005, Varraault et al. 2006, Iglesias-Platas et al. 2014). *PLAGL1* analysis was included in this study because it has been implicated in the imprinting disorders BWS and SRS, as well as its association with IUGR.

1.8.4 LINE-1

LINE-1 (long interspersed element 1) is a non-long terminal repeat, autonomous retroelement which is active in mammalian genomes. LINE-1 elements make up 17% of the human genome (Lander et al. 2001) presenting over 500,000 copies, and while only a small subset are active, DNA methylation plays an integral role in the silencing of their tumourigenic potential (Iskow et al. 2010). As 12% of all CpG dinucleotides fall within LINE-1 (Schmid 1996), these repetitive elements have been used as a surrogate to measure “global” changes in
DNA methylation (Yang et al. 2004, Yang et al. 2006, Weisenberger et al. 2005, Woodson et al. 2005). In this study methylation of LINE-1 repetitive elements will be analyzed in order to determine whether DNA methylation errors occur at broader levels in babies conceived via ART.

1.8.5 **Hypotheses and objectives**

Imprinting is controlled by epigenetic modifications, of which DNA methylation is the best characterized and most understood. Most imprinted genes contain differentially methylated regions (DMRs), where methylation differs between the maternal and paternal alleles. Differential DNA methylation of imprinted genes should be properly established during gametogenesis and subsequently maintained during the preimplantation stage, despite the genome-wide changes in methylation that occur in non-imprinted genes during this period. It is postulated that hormone treatments, *in vitro* culturing conditions, and manipulation of the gametes during ART, as well as the underlying subfertility of one or both parents, may result in aberrant methylation of imprinted genes leading to improper regulation of gene expression and human disease. It has been shown in mice that certain *in vitro* culturing conditions are associated with a loss of imprinting in placental tissue and reduced birth weight. It has also been shown that some men affected by severe infertility may be at risk for carrying imprinting errors in their sperm, which may be passed on to the offspring through ICSI.

There remains a lack of reliable data on the relative and overall risk of imprinting syndromes in children conceived using ART (Amor and Halliday 2008). By investigating methylation patterns in children conceived by IVF or ICSI compared to children who are naturally conceived, we are able to determine whether the process of ART alters the proper establishment of epigenetic programming within the embryo. This will allow us to determine
whether there are discrete changes in methylation occurring via the ART process which may not lead to a phenotypic imprinting disorder but may have other unknown long-term effects. This study is significant because it includes the analysis of both DNA methylation and gene expression of imprinted genes in the placenta and umbilical cord blood of ART infants and can help better understand the risks involved with ART.

Although there are a number of studies examining DNA methylation at imprinted loci in ART patients, very few studies have also examined the correlated gene expression levels in these samples. By looking at relative gene expression levels of imprinted genes controlled by important ICRs, we may get a better picture of how alterations in mean methylation levels affect the phenotype of the subjects. Furthermore, this study may allow us to gain a better understanding of the complex regulation of imprinted genes and how alterations at one locus may potentially impact other imprinted regions within the genome. It has been suggested that imprint regulation of chromosome 11p15.5 may be impacted by expression of imprinted genes on chromosome 6q24, the location of the \textit{PLAGL1} DMR. By looking at the relative gene expression levels of multiple imprinted genes located at diverse regions in different chromosomes, we aim to get a better understanding of how GOM or LOM at the DMRs actually impacts the expression of these genes. Furthermore, we aim to determine whether there is a relationship between levels of expression of the \textit{PLAGL1} gene and genes associated with the chromosome 11p15.5 region, as it is suggested that they are a part of a co-regulated imprinted gene network.

\textbf{We hypothesize that DNA methylation patterns at the DMRs of the imprinted regions} \textit{KvDMR1, PLAGL1, and PEG10}, and transcription of their associated genes, may
be altered in the placentae and cord blood samples obtained from phenotypically normal infants conceived by IVF and ICSI compared to the NC controls. As methylation acts to regulate the expression of imprinted genes, we expect that, if there are any changes in DNA methylation patterns, this should also correlate with a change in the expression of that gene, such that hypermethylation should correspond with reduced transcription and hypomethylation should result in increased transcription. As PLAGL1 has been shown to regulate imprinted genes along chromosome 11p15.5, we expect to see a positive correlation between the expression of PLAGL1 and KCNQ1OT1, CDKN1C, and IGF2.

**Objective 1a:** To compare DNA methylation at the KvDMR1, PLAGL1, and PEG10 imprinted regions and the LINE-1 retroelement in the placental tissue of children conceived via ART and NC controls.

**Objective 1b:** To compare gene expression levels of KCNQ1OT1, PLAGL1, PEG10, and L1TD1 in placental chorionic villi from ART pregnancies and NC.

**Objective 2a:** To compare DNA methylation at the KvDMR1, PLAGL1, and PEG10 imprinted regions and the LINE-1 retroelement in the cord blood from children conceived via ART and NC controls.

**Objective 2b:** To compare gene expression levels of PLAGL1 with expression of imprinted genes from chromosome 11p15.5 (IGF2, KCNQ1OT1, and CDKN1C) in umbilical cord blood from ART pregnancies to determine the potential role of an imprinted gene network.

**Objective 2c:** To examine the correlation between gene expression of PLAGL1 and several imprinted genes of chromosome 11p15.5 (KCNQ1OT1, CDKN1C, and IGF2) to confirm the presence of an imprinted gene network.
Chapter 2: DNA METHYLATION AND GENE EXPRESSION ANALYSIS OF IMPRINTED GENES IN PLACENTAL CHORIONIC VILLI FROM IVF/ICSI CONCEIVED INFANTS

2.1 Introduction

The placenta is the main means of communication and nutrient delivery to the fetus and is most likely involved in fetal homeostasis. Epigenetic regulation plays an important role in placental growth and development, and epigenetic disturbances within the placenta have been observed in cases of IUGR, SGA (Banister et al. 2011), and also in the pathogenesis of pre-eclampsia (Yuen et al. 2010). Further, the expression of imprinted genes has been shown to be altered in rat placentae with IUGR (McMinn et al., 2006), infants with LBW (Apostolidou et al. 2007, Tabano et al. 2010), and after superovulation in mid-gestation in mouse placentae (Fortier et al. 2008).

Imprinted genes are expressed in a parent-of-origin specific manner and are controlled by differential methylation between maternal and paternal alleles. Imprinted genes are essential for proper regulation of fetal and placental development, and it has been proposed that they regulate growth by controlling nutrient supply to the fetus through the placenta. Imprinted genes have been shown to be highly expressed in the placenta and disrupted methylation at imprinted regions has been shown to result in growth abnormalities in the fetus. It has been proposed that genomic imprinting evolved along with placentation, as this phenomenon has only been observed in eutherian mammals. In mice, many genes are imprinted in the extraembryonic tissue, and a number of these are in the placenta only (Wagschal and Feil 2006). Placental function follows an intricate developmental cascade during gestation and, therefore, adverse events during
the prenatal period may have a critical effect on the trophoblast and placental function, as well as on fetal programming (Gallou-Kabani et al. 2010). There appears to be a higher frequency of imprinting and growth disorders, such as LBW, in children conceived via ART, and the aetiology of these poor outcomes is still unknown. There is some evidence that ART procedures, such as superovulation and in vitro culture conditions, may alter the expression of imprinted genes, which may cause a reduction in weight. Differences in birth weight may be due to placental-mediated mechanisms, as there appears to be a higher frequency of placental-associated defects in pregnancies after ART. Previous studies examining DNA methylation at imprinted genes in the placenta from ART infants have produced conflicting results.

In order to examine whether ART procedures affect the establishment and maintenance of imprinted genes within the placenta, DNA methylation at three imprinted regions – KvDMR1, PEG10, and PLAGL1 – were analyzed in the chorionic villi in children conceived via IVF, ICSI, and naturally conceived (NC) controls. The LINE-1 repetitive element was also included in the study as an indicator of global methylation (Yang et al. 2004). Gene expression analysis of KCNQ1OT1, PEG10, PLAGL1, and LITD1 were included in this study in order to examine the effect of DNA methylation on the corresponding mRNA levels of imprinted genes. Further, as studies have shown that several imprinted genes in the placenta are not dependent on DNA methylation, but are most likely regulated by repressive histone modifications and ncRNAs (Wagschal and Feil 2006), it is important to look at transcription levels between groups in order to identify whether there may be other epigenetic alterations causing disrupted expression of imprinted genes in the ART placentae.
2.2 Methods

2.2.1 Study participants

271 samples from 249 participants were collected from pregnancies independently recruited through the Pacific Centre for Reproductive Medicine, Genesis Fertility Centre, and local hospitals throughout the Greater Vancouver area in British Columbia, Canada, including samples collected through the Regional Fertility Program in Calgary, Alberta, Canada. The 271 cases are from 249 sets of parents, with 22 sets of twins, consisting of 79 ICSI, 105 IVF, and 87 natural conception (NC) cases. Pregnant women were invited to participate and gave written informed consent to collect placenta and cord blood after detachment for research purposes.

There were no specific exclusion criteria for the ART group, and the inclusion criteria included pregnancies conceived by either IVF or ICSI. The inclusion criteria for the control group was: maternal age between 20 and 40 years with no perinatal complications or conditions such as placenta praevia, gestational diabetes, pre-eclampsia, preterm birth, use of fertility drugs, infection, fetal malformation, diagnosed depression, inherited diseases, known chromosomal abnormalities, known recreational drug use, and must not have taken over 1 year to conceive.

2.2.2 Sample preparation

The placenta was collected within 30 minutes after delivery. Two placental sites were biopsied from the fetal side by cutting and folding back the chorionic plate to excise a small (~1cm³) sample of chorionic villi which was placed into RNAlater solution (Sigma-Aldrich Corporation, St. Louis, USA) to preserve the RNA. The two placental sites are consistently taken from the same spots: site 1 being near the umbilical cord and site 2 being near the edge of the placenta (Figure 2.1). These samples are incubated at 4°C for 24 hours and then transferred to
fresh tubes for storage at -80°C. Chorionic villus tissue for DNA extraction was biopsied from fresh placentae in the same manner as mentioned above. Multiple sites were separately sampled from different areas of each placenta, including the site 1 cord insertion site and site 2 at the placental edge. Chorionic villus samples were immediately stored on dry ice during the biopsy procedure and then stored at -80°C until needed.

![Figure 2.1 Placental biopsy extraction sites. Site 1 (S1) is next to the umbilical cord insertion site (blue X) and Site 2 (S2) is near the placental edge.](image)

### 2.2.3 DNA extraction from placental tissue

DNA extraction from placental chorionic villus tissue was performed by washing the tissue several times in 1x PBS. 25mg of tissue was placed in a sterile petri dish and minced using a sterile surgical blade, tissue was then placed in lysing buffer and incubated at 56°C overnight with proteinase K in a shaking water bath. DNA extraction was then performed using the QIAamp® DNA Mini Kit (Qiagen, Mississauga, ON), according to the manufacturer’s directions with an RNase A step to remove any RNA contamination. Concentrations were then recorded using a NanoSpec spectrometer (Nanovue by General Electric Inc., CT, USA) and each sample
was diluted in a separate tube to contain 500ng of DNA. These samples were then subjected to bisulphite conversion prior to pyrosequencing analysis.

2.2.4 Preparation of cDNA library

In order to extract RNA, placental tissue was first homogenized by placing 25mg frozen chorionic villus sample into Lysing Matrix D tubes (MP biomedicals, Santa Ana, CA) in lysing solution with β-mercaptoethanol in the Bullet Blender 24 homogenizer (Next Advance, Averill Park, NY). The homogenized tissue in the lysing solution was then transferred to an RNeasy® mini spin column and RNA was extracted from these samples using the Qiagen RNeasy® Mini Kit (Qiagen, Mississauga, ON). Placental RNA samples were run on a 1.5% agarose gel (40ml TAE + 0.6g agarose + 4µl SybrSafe) using 1µl of 6x orange loading dye and 2.5µl of the RNA sample coupled with 1µl of a low range DNA ladder; these were run at 95V for 60 minutes. RNA quality was assessed by ensuring that there were two sharp bands present for the 28s and 18s rRNA, where the band for 28s rRNA should be twice as intense as the 18s rRNA band. Samples that met this requirement were converted into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA), with the following thermal profile: 10 minutes at 25°C, 2 hours at 37°C, and hold at 4°C. For placental chorionic villus samples, 1µg of RNA was used in the conversion reaction to produce the cDNA library. The cDNA products were then diluted one in fifty prior to being used for real-time PCR.

2.2.5 DNA methylation analysis by pyrosequencing

DNA methylation analyses were carried out via bisulphite modification of extracted DNA using the EZ DNA Methylation Gold kit (Zymo Research, Irvine, CA). During this
procedure, DNA is treated with sodium bisulphite; deamination of unmethylated cytosine nucleotides will convert them to uracil, whereas a methylated cytosine is protected and remains unchanged. This is followed by PCR amplification using primers specific to each region of interest (Table 2.1). During the PCR reaction, the uracil bases from bisulphite treatment will be exchanged for thymidine. Epitect methylated Human control DNA (Qiagen, Mississauga, ON) was used as a positive control and negative controls had 1µl sterilised water instead of DNA. During PCR amplification, each PCR tube contained 25µl of solution containing the following: HotStarTaq buffer (including 1.5mM MgCl2), 0.2mM dNTP, 5pmol of each of the forward and reverse primers, and 1.0U HotStarTaq DNA polymerase (Qiagen, Mississauga, ON, Canada), along with 2µl of the converted DNA. The thermal profile for the PCR amplification step was 95°C for 10 min, 44 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, followed by 72°C for 10 minutes and held at 4°C. PCR amplified samples were then run on a 1% agarose gel to check for quality along with both the positive and negative controls.

Percent methylation was analyzed via pyrosequencing using a PyroMark Q96 MD system (Biotage, Foxboro, MA) and percent methylation at each CpG site was quantified using Pyro Q-CpG software, version 1.0.9 (Biotage). Pyrosequencing is a “sequence by synthesis” method which determines percent methylation based on the C/T ratio at known CpG sites, as methylated cytosines would have been protected from deamination during the bisulphite conversion step. Pyrosequencing was performed in two replicates for each sample using 15µl of PCR product. Each run comprised of a PCR plate and pyrosequencing plate preparation. Each well on the PCR plate contained 25µl water, 38µl binding buffer, and 2µl sequencing beads (all supplied by Qiagen). The pyrosequencing wells contained 0.36µl of the appropriate primer and 11.64µl of annealing buffer (Qiagen Inc., Mississauga, ON, Canada). Sequencing primers are
listed in table 2.1. Methylation values at each CpG site were then averaged for each sample to obtain a mean methylation value, and for the placental tissue analysis, the mean methylation of the two separate sites were averaged for each case.

Table 2.1 PCR and pyrosequencing primer sequences (5’ - 3’) used for KvDMR1, PLAGL1, PEG10, and LINE-1 DNA methylation analyses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer (Biotinylated)</th>
<th>Sequencing Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>KvDMR1</td>
<td>TTAGTTTTTTTTGGTATTG</td>
<td>CCCACAAACCTCCACACC</td>
<td>TTGYGTATGTGTTTATT</td>
</tr>
<tr>
<td>PLAGL1</td>
<td>GAYGGGTTGAATGATAAA TGG</td>
<td>TCRACRCAACCATCTCTTT AACTA</td>
<td>ACRCAACCATCCTCTTA</td>
</tr>
<tr>
<td>PEG10</td>
<td>TTGTTTGGTTTTTTGAAATAG</td>
<td>TTTCCCCCTTTACTAAAT ACA</td>
<td>TTGTATTTTTAGTATTT TATGA</td>
</tr>
<tr>
<td>LINE-1</td>
<td>TTTTGGTTAGGTGTGGG ATATA</td>
<td>AAAATCAAAAAATTCCT TTC</td>
<td>AGTTAGGTGTGGGATATA GT</td>
</tr>
</tbody>
</table>

2.2.6 Gene expression analysis by quantitative polymerase chain reaction (qPCR)

The cDNA library from the placental chorionic villus samples were used to carry out real-time quantitative PCR (qPCR) using the following assays containing primers and specific FAM-labelled Taqman Probes (Applied Biosystems, Foster City, CA): PLAGL1, Hs00957794_m1, KCNJ1OT1, Hs03456562, PEG10, Hs01122877_m1, LITD1, Hs00219458_m1, CDKN1C, Hs04186044_g1, IGF2, Hs01005962_m1, and VIC-labelled YWHAZ, Hs01122451_m1 as an endogenous control (Applied Biosystems). All samples were run in duplicate on a 96-well plate with Taqman Universal PCR master mix (Applied Biosystems) on the 7500 Fast Real-Time PCR system (Applied Biosystems) with the following thermal cycling conditions: 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Each well on the PCR plate designated for the target genes contained 15µl of master mix (10µl TaqMan PCR mix, 1µl respective primer, 4µl water)
and 5µl of the specific cDNA sample. The wells for the endogenous control samples contained 17.5µl of master mix (10µl TaqMan PCR mix, 1µl \textit{YWHAZ} primer, 6.5µl water) and 2.5µl of the cDNA. Relative expression (Rq) values were determined by the ABI 7500 system software for each of the patients; these values were quantified using the incorporated control calibrators. The calibrator sample consisted of pooled naturally conceived cDNA samples. All results were analyzed using the ddCT method (Schmittgen and Livak 2008) and expressed in logarithmic scale. All plates included a control with no reverse transcriptase (–RT) to ensure there was no genomic DNA contamination as well as a water (no cDNA) control.

2.2.7 Statistical analysis

Data for statistical analysis and graphics was conducted in R statistical program (3.1.2) and RStudio (0.98.1087) with open source packages lawstat (2.4.1) and ggplot2 (2009). Significant differences in clinical information as well as DNA methylation and RNA expression ddCT values were calculated with ANOVA or Kruskal-Wallis between IVF, ICSI, and NC. When significances were found in ANOVA or Kruskal-Wallis, pairwise differences were calculated with Tukey HSD post-hoc or Mann-Whitney test, respectively. The Bonferroni correction was used to correct for multiple comparisons. A minimum significance level of 5% (95% confidence interval) was used. Twins were counted as a single data point when determining significance in tests associated with maternal age to avoid replicates.
2.3 Results

2.3.1 Clinical information

Due to difficulties in sample collection and RNA and DNA extractions, there are different subsets of groups used for the methylation and gene expression analyses and differences in numbers between the cord blood and placental tissue analyses (Tables 2.2 and 2.3). Therefore, we have divided the clinical information into two groups: 1) those used in the DNA methylation analysis and 2) those used in the gene expression analysis in placenta samples.

There was a significantly higher mean maternal age in the IVF group for both sets of data, which is to be expected (Table 2.2 and 2.3). For the methylation analysis, mean maternal age in the IVF group was 36.67 with a standard deviation (SD) of ± 3.36 years ($P = 0.000006$), 32.55 ± 5.61 years in the naturally conceived (NC), and 33.73 ± 4.50 years in the ICSI group. There was also a significant increase in maternal age for the placental gene expression patients in the IVF group (37.29 ± 3.83; $P = 0.0031$) compared to the control group (32.95 ± 5.76). Again, the maternal age in the ICSI group (35.65 ± 3.74) was not significantly different compared to controls in the placental villus analysis. In the cohort used for placental gene expression analysis, the IVF group also had significantly lower gestational age ($P < 0.05$) compared to the NC and ICSI groups (Table 2.3).
Table 2.2 Clinical information for DNA methylation analysis in placenta

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>IVF</th>
<th>ICSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>69</td>
<td>82</td>
<td>67</td>
</tr>
<tr>
<td>Mean Maternal Age</td>
<td>32.55 ± 5.61</td>
<td>36.67 ± 3.36*</td>
<td>33.73 ± 4.50</td>
</tr>
<tr>
<td>(years ± SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Gestational Age</td>
<td>276.6 ± 9.8</td>
<td>271.6 ± 15.1</td>
<td>276.8 ± 9.4</td>
</tr>
<tr>
<td>(days ± SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Birth Weight</td>
<td>3348 ± 464</td>
<td>3249 ± 707</td>
<td>3215 ± 634</td>
</tr>
<tr>
<td>(grams ± SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Girls (%)</td>
<td>48%</td>
<td>53%</td>
<td>53%</td>
</tr>
</tbody>
</table>

* significantly different between NC group P < 0.05

Table 2.3 Clinical information for gene expression analysis in placenta

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>IVF</th>
<th>ICSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>39</td>
<td>38</td>
<td>32</td>
</tr>
<tr>
<td>Mean Maternal Age</td>
<td>32.95 ± 5.76</td>
<td>37.29 ± 3.83*</td>
<td>35.65 ± 3.74</td>
</tr>
<tr>
<td>(years ± SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Gestational Age</td>
<td>274.2 ± 8.53</td>
<td>264.3 ± 16.8*</td>
<td>268.9 ± 10.5</td>
</tr>
<tr>
<td>(days ± SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Birth Weight</td>
<td>3301 ± 502</td>
<td>3116 ± 770</td>
<td>2963 ± 637</td>
</tr>
<tr>
<td>(grams ± SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Girls (%)</td>
<td>61%</td>
<td>42%</td>
<td>56%</td>
</tr>
</tbody>
</table>

* significantly different between NC group P < 0.05

2.3.2 DNA methylation of imprinted genes from IVF and ICSI placental tissue

We examined methylation levels at the differentially methylated regions of KvDMR1, PEG10, and PLAG1 as well as the repetitive LINE-1 elements in placental chorionic villus samples. The mean methylation of the regions of interest at two placental villus sites (site 1 and site 2) were used in each case.
We assessed methylation status at seven CpG sites within the KvDMR1 locus (Table 2.5, including the NotI site that is often hypomethylated in BWS patients (Smilinich et al. 1999, Weksberg et al. 2001) and used in diagnostic testing for this syndrome (Weksberg et al. 2005). There were no statistically significant changes (Kruskal-Wallis; P > 0.05) in mean methylation across conception modes with median (IQR) values of 59.85 % (56.41 - 63.16), 59.25% (56.21 - 65.31), and 59.30% (54.22 - 64.22) for NC, IVF, and ICSI, respectively (Figure 2.2, Table 2.4).

At the PEG10 DMR, six CpG sites were analyzed. There were no significant differences in median (IQR) percent methylation between the IVF [45.11% (43.05 - 54.23), P > 0.05] or ICSI [45.29 % (43.00 – 48.59), P > 0.05] placental tissues when compared to controls [47.06% (41.70 - 54.02)]. Four CpG sites were analyzed within the PLAGL1 DMR. No significant differences in median PLAGL1 methylation were found between the natural conceptions [45.63% (44.67 - 47.14)] and the IVF [46.51% (44.07 - 48.71), P > 0.05] or ICSI [45.20% (44.01 - 47.71), P > 0.05] groups (Figure 2.2, Table 2.4).

As an indicator of global DNA methylation, four CpG sites at LINE-1 were analyzed. No significant differences in methylation in mean LINE-1 methylation were observed between the ART (P > 0.05) placental tissues compared to controls with values of 49.00% (46.05 - 51.73), 46.57% (44.98 - 50.68), and 48.00% (45.93 - 51.61) for NC, IVF, and ICSI samples, respectively.
Figure 2.2 DNA methylation in placental tissue from IVF, ICSI, and NC infants. Percent methylation at KvDMR1, PEG10, PLAGL1, and one repetitive element (LINE-1). The black line represents the median value and the boxes indicate the upper and lower quartiles. IVF and ICSI groups were compared with the natural conception (NC) controls. The pink, green, and blue bars represent the ICSI, IVF, and NC groups, respectively.
Table 2.4 Mean and median (IQR) methylation values (percentages) of *PLAGL1*, KvDMR1, *PEG10*, and LINE-1 in placental villi from NC, IVF, and ICSI pregnancies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Pregnancy Type</th>
<th>DNA methylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLAGL1</strong></td>
<td>NC</td>
<td>46.32; 45.63 (44.67, 47.14) (n = 66)</td>
</tr>
<tr>
<td></td>
<td>IVF</td>
<td>46.80; 46.51 (44.07, 48.71) (n = 66)</td>
</tr>
<tr>
<td></td>
<td>ICSI</td>
<td>46.49; 45.20 (44.01, 47.71) (n = 63)</td>
</tr>
<tr>
<td><strong>KvDMR1</strong></td>
<td>NC</td>
<td>59.74; 59.85 (56.41, 63.16) (n = 67)</td>
</tr>
<tr>
<td></td>
<td>IVF</td>
<td>60.77; 59.25 (56.21, 65.31) (n = 58)</td>
</tr>
<tr>
<td></td>
<td>ICSI</td>
<td>59.05; 59.30 (54.22, 64.22) (n = 63)</td>
</tr>
<tr>
<td><strong>PEG10</strong></td>
<td>NC</td>
<td>48.83; 47.06 (41.70, 54.02) (n = 57)</td>
</tr>
<tr>
<td></td>
<td>IVF</td>
<td>47.68; 45.11 (43.05, 54.23) (n = 54)</td>
</tr>
<tr>
<td></td>
<td>ICSI</td>
<td>47.36; 45.29 (43.00, 48.59) (n = 53)</td>
</tr>
<tr>
<td><strong>LINE-1</strong></td>
<td>NC</td>
<td>49.06; 49.00 (46.05, 51.73) (n = 69)</td>
</tr>
<tr>
<td></td>
<td>IVF</td>
<td>47.92; 46.57 (44.98, 50.68) (n = 64)</td>
</tr>
<tr>
<td></td>
<td>ICSI</td>
<td>48.67; 48.00 (45.93, 51.61) (n = 63)</td>
</tr>
</tbody>
</table>
Table 2.5 Median (IQR) methylation values for each CpG site analyzed from NC, IVF, and ICSI placental chorionic villus samples.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Pregnancy Type</th>
<th>CpG 1</th>
<th>CpG 2</th>
<th>CpG 3</th>
<th>CpG 4</th>
<th>CpG 5</th>
<th>CpG 6</th>
<th>CpG 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLAGL1</td>
<td>NC</td>
<td>45.89 (44.27 – 47.16) (n = 68)</td>
<td>47.45 (45.67 – 48.96) (n = 69)</td>
<td>46.23 (45.20 – 47.51) (n = 67)</td>
<td>44.44 (42.50 – 46.92) (n = 68)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IVF</td>
<td>45.98 (43.85 – 48.35) (n = 67)</td>
<td>47.91 (45.81 – 50.86) (n = 67)</td>
<td>46.66 (44.38 – 49.51) (n = 66)</td>
<td>45.23 (42.06 – 47.67) (n = 67)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ICSI</td>
<td>45.56 (43.43 – 47.42) (n = 65)</td>
<td>47.87 (44.72 – 49.42) (n = 65)</td>
<td>45.97 (44.48 – 48.09) (n = 64)</td>
<td>44.55 (42.30 – 47.99) (n = 64)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KvDMR1</td>
<td>NC</td>
<td>60.07 (56.98 – 64.55) (n = 69)</td>
<td>62.70 (58.09 – 66.47) (n = 69)</td>
<td>58.31 (53.94 – 61.67) (n = 69)</td>
<td>59.79 (56.10 – 63.23) (n = 69)</td>
<td>60.54 (57.17 – 64.28) (n = 69)</td>
<td>59.15 (54.68 – 61.78) (n = 68)</td>
<td>57.58 (53.38 – 60.99) (n = 68)</td>
</tr>
<tr>
<td></td>
<td>IVF</td>
<td>60.14 (58.08 – 67.00) (n = 66)</td>
<td>62.34 (58.69 – 68.68) (n = 65)</td>
<td>58.18 (55.12 – 62.23) (n = 65)</td>
<td>59.36 (56.59 – 64.47) (n = 65)</td>
<td>60.72 (57.01 – 66.78) (n = 64)</td>
<td>57.87 (53.03 – 63.76) (n = 63)</td>
<td>56.41 (53.21 – 60.77) (n = 62)</td>
</tr>
<tr>
<td></td>
<td>ICSI</td>
<td>60.73 (56.52 – 66.92) (n = 65)</td>
<td>62.02 (56.80 – 67.42) (n = 65)</td>
<td>57.56 (53.80 – 61.57) (n = 64)</td>
<td>59.52 (55.22 – 64.02) (n = 65)</td>
<td>59.39 (54.86 – 65.83) (n = 64)</td>
<td>57.45 (53.14 – 62.37) (n = 65)</td>
<td>54.48 (51.58 – 61.31) (n = 65)</td>
</tr>
<tr>
<td>PEG10</td>
<td>NC</td>
<td>49.56 (42.36 – 56.99) (n = 69)</td>
<td>51.05 (44.91 – 59.47) (n = 68)</td>
<td>45.12 (40.46 – 53.38) (n = 69)</td>
<td>44.99 (38.95 – 51.47) (n = 69)</td>
<td>50.77 (46.18 – 59.96) (n = 65)</td>
<td>48.00 (42.77 – 56.59) (n = 69)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IVF</td>
<td>47.22 (42.80 – 56.43) (n = 67)</td>
<td>48.86 (43.82 – 60.89) (n = 64)</td>
<td>43.77 (40.59 – 51.42) (n = 67)</td>
<td>43.08 (39.91 – 49.56) (n = 66)</td>
<td>50.55 (46.39 – 58.50) (n = 57)</td>
<td>46.89 (42.81 – 55.62) (n = 65)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ICSI</td>
<td>46.02 (42.80 – 51.84) (n = 64)</td>
<td>48.01 (44.29 – 56.74) (n = 65)</td>
<td>43.26 (39.38 – 48.12) (n = 64)</td>
<td>42.70 (40.25 – 46.99) (n = 63)</td>
<td>50.08 (47.42 – 59.82) (n = 57)</td>
<td>46.23 (43.66 – 50.79) (n = 65)</td>
<td>-</td>
</tr>
<tr>
<td>LINE-1</td>
<td>NC</td>
<td>45.62 (42.70 – 48.88) (n = 69)</td>
<td>55.43 (52.85 – 58.35) (n = 69)</td>
<td>45.00 (41.51 – 48.03) (n = 69)</td>
<td>49.99 (47.05 – 53.04) (n = 69)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IVF</td>
<td>43.46 (40.92 – 47.39) (n = 67)</td>
<td>54.03 (52.41 – 58.27) (n = 65)</td>
<td>42.59 (39.62 – 45.80) (n = 66)</td>
<td>48.17 (45.58 – 52.05) (n = 65)</td>
<td>-</td>
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<tr>
<td></td>
<td>ICSI</td>
<td>44.84 (42.18 – 48.52) (n = 65)</td>
<td>55.70 (53.04 – 59.03) (n = 65)</td>
<td>43.67 (41.48 – 46.77) (n = 63)</td>
<td>49.37 (46.36 – 51.77) (n = 65)</td>
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</table>
2.3.3 Gene expression analysis of ART groups compared to NC controls

In order to ensure that there were no changes in transcription of the three imprinted genes and the LINE-1 repetitive element, gene expression analysis by RT-PCR was performed on placental chorionic villus samples. Due to strict guidelines for RNA samples used in the analysis, we were unable to gather data for both placental sites for each case, therefore we have analyzed the two sites separately. No significant differences were seen between the gene expression levels of PLAGL1, KCNQ1OT1, LITD1, and PEG10 in either site for ART placentae compared to controls. KCNQ1OT1 expression levels were not significantly different between the natural conceptions, IVF, and ICSI groups for S1 (log10 expression -0.111, 0.093, and -0.020 for NC, IVF, and ICSI, respectively; P > 0.05) or for S2 (log10 expression -0.053, 0.087, and -0.062 for NC, IVF, and ICSI, respectively; P > 0.05). LITD1 is the transcript regulated by the LINE-1 element, no significant differences in expression levels were observed between the 3 groups for S1 (log10 expression -0.030, 0.009, and 0.111 for NC, IVF, and ICSI, respectively; P > 0.05) or for S2 (log10 expression 0.034, -0.024, and 0.085, respectively; P > 0.05). The PLAGL1 transcript demonstrated no significant difference in expression between the groups for S1 (log10 expression -0.018, 0.146, and 0.062 for NC, IVF, and ICSI, respectively; P > 0.05) or for S2 (log10 expression 0.016, 0.145, and 0.086 for NC, IVF, and ICSI, respectively; P > 0.05). PEG10 gene expression levels also did not display any significant differences between the NC and ART groups for S1 (log10 expression -0.001, 0.014, and 0.129 for NC, IVF, and ICSI, respectively; P > 0.05) or S2 (log10 expression 0.015, -0.001, and 0.055 for NC, IVF, and ICSI, respectively; P > 0.05) (Figure 2.3). Furthermore, previous studies have shown that there is a difference in expression between placental sites, suggesting multiple sites should be used when looking at gene expression in the placenta (Avila et al. 2010). However, we did not observe any
significant differences when correlation analyses were performed between the separate sites for any of the genes analyzed (t-test; P > 0.05).

**Figure 2.3** Gene expression of imprinted genes and LINE-1 element in placental tissue. Log10 expression values of *KCNQ1OT1, L1TD1, PEG10*, and *PLAGL1* from two chorionic villus sites in the placenta (Site 1 and Site 2) from IVF, ICSI, and NC infants. The black line represents the median value and the boxes indicate the upper and lower quartiles. The pink, green, and blue boxes represent the ICSI, IVF, and NC groups, respectively.
2.4 Discussion of results

It is postulated that ART may disrupt important genomic reprogramming events and produce aberrant methylation of imprinted genes leading to improper regulation of imprinted gene transcription during development (Sato et al. 2007, de Waal 2014, Owen and Segars 2009). The complexity of imprinted domain regulation may render these regions particularly susceptible to environmental changes in the preimplantation embryo. It has been shown in mice that certain in vitro culturing conditions are associated with a loss of imprinting in placental tissue and reduced birth weight (Mann et al. 2004). As proper growth and development of the placenta is so crucial for the fetus, and imprinted genes play an important role in placental development, we examined the DNA methylation patterns of three imprinted regions, and one repetitive element, within the placental chorionic villi, as well as the corresponding gene expression levels.

We observed no significant changes in DNA methylation between the IVF and ICSI samples compared to the NC controls for KvDMR1, PEG10, PLAGL1, and LINE-1. There were no significant differences in the overall mean methylation or at any individual CpG sites for any of the analyzed regions. As there did not appear to be a difference in methylation, this suggests that the ART procedure does not affect the imprinting status of these regions within the placenta. Further, the LINE-1 repetitive element which is used to represent global methylation did not show any alterations between the ART and NC groups, suggesting that global DNA methylation in the placenta is unaffected by ART. Accordingly, when gene expression assays were run on the placental samples, no differences in mRNA levels were observed between the IVF and ICSI groups compared to the controls.

It is difficult to draw any major conclusions regarding imprinting in the placenta from ART pregnancies as there does not appear to be consistency among different studies. However,
our results regarding DNA methylation of KvDMR1 do appear to agree with the majority of previous findings. Gomes et al. (2009) examined placental tissue from 6 in vitro and 8 in vivo conceived infants and did not find any changes in KvDMR1 methylation between the groups. However, they did find hypomethylation of this region in the peripheral blood from 3/18 in vitro conceived samples. Tierling et al. (2010) also looked at DNA methylation at KvDMR1 in the placentae from IVF, ICSI, and NC pregnancies and did not observe any significant differences between the groups. These experiments also included the examination of 9 other DMRs and only a slight difference in MEST methylation in the IVF and ICSI groups was observed (Tierling et al. 2010). Another study investigating imprinted genes in ART placentae found no difference in KvDMR1 methylation, but did observe hypermethylation at MEST and H19; gene expression of H19, but not MEST, was also found to be affected (Nelissen et al. 2013). In contrast, Rancourt et al. (2012) observed small, but significant changes in methylation at the KvDMR1, SNRPN, and H19 regions in placental tissue from IVF pregnancies, however, a change in gene expression was only observed for H19. From these studies it can be presumed that KvDMR1 imprinting in the placenta does not seem to be significantly affected in ART pregnancies.

A genome-wide analysis found that overall the placentae from ART pregnancies displays lower levels of methylation compared to natural conceptions (Katari et al. 2009). Katari et al. (2009) looked at over 700 genes within the placenta and found DNA methylation differences associated with changes in gene expression of both imprinted and non-imprinted genes. Several of the disrupted genes were from pathways involved in chronic metabolic disorders such as obesity and type II diabetes. Nelissen et al. (2014) found a 1.3 fold and 1.5 fold increase in H19 and PHLDA2 gene expression, respectively, with no indication of LOI. They also did a genome-wide analysis revealing 13 significantly overrepresented pathways involved in metabolism,
immune response, transmembrane signaling, and cell cycle control, which were mostly up-regulated (Nelissen et al. 2014). However, other studies have found no significant differences in the DNA methylation at imprinted loci in ART placentae (Tierling et al. 2010, Chan Wong et al. 2011). A previous study from our group examined placental DNA methylation at the *H19/IGF2* ICR1 region in children conceived via ART and found no significant differences in methylation (Chan Wong et al. 2011). These results directly conflict with a study that found aberrant methylation of this region within the *in vitro* group, as well as lower levels of *H19* and *IGF2* mRNA (Turan et al. 2010). From the studies conducted so far, it appears that changes at the *H19/IGF2* ICR1 region and *MEST* DMR are the most commonly noted in ART placentae, and gene expression of *H19* tends to be altered, however not all studies are in agreement.

*PEG10* and *PLAGL1* have not been studied extensively in ART placentae. However, a recent study examining *PLAGL1* DNA methylation and gene expression in placental tissue from IUGR infants did note a significant decrease in gene expression in a subgroup of 12 ART participants with no changes in DNA methylation (Iglesias-Platas et al. 2014). This study suggests that the regulation of *PLAGL1* in the placenta is not solely regulated by DNA methylation and that there must be other factors contributing to the changes in gene expression between the ART and NC groups. Unfortunately, the sample size is extremely small, so it is hard to make any definitive conclusions. This finding is interesting because the *PLAGL1* gene has been implicated in the pathology of TNDM, which puts patients at a higher risk of developing diabetes later in life, and the pathways that were found to be affected in genome-wide studies are those involved in chronic metabolic diseases. Therefore, it appears that there may be a common trend between adverse environment *in utero* and metabolic disorders, which is in agreement with the “fetal programming” or “Barker” hypothesis. The Barker hypothesis suggests that insults at a
critical, sensitive period of early life has permanent effects on structure, physiology, and metabolism (Barker 2004). This theory suggests that fetal programming may result from adaptations which developed when the materno-placental nutrient supply fails to match nutrient demand.

There are some limitations when it comes to studying and comparing molecular features in the placenta. There tends to be high levels of both inter- and intra-placental variation in terms of gene expression as placentae are variable in size, structure, and cell composition. The placenta is comprised of a highly heterogeneous cell population and distribution is not equal in all sites. There are many stochastic factors that may contribute to variability between placental sites such as the relative proportion of undifferentiated and differentiated trophoblast and mesenchymal cells, and clustering of random changes due to clonal placental development (Avila et al. 2010). Studies have also suggested that there may be differences in cell composition or gene expression based on the physical location of the cells, particularly in terms of oxygen exposure (Avila et al. 2010). This is evident in our gene expression analyses, as a large amount of variability was observed in all groups. It is a possibility that any potential differences in gene expression between groups may not have been observed due to fluctuations in cell composition or systemic differences in gene expression between samples. We did try to eliminate this effect by sampling multiple sites within the placenta; however, gene expression results, even averages of multiple sites, did seem to be more variable among all groups in placental tissue compared to the cord blood samples. Interestingly, even though previous studies have demonstrated significant site-to-site variation of gene expression within the placenta (Avila et al. 2010), we did not find a significant difference between the two different sites. However, it has been noted that sampling time does tend to have a larger effect on gene expression variability (Avila et al. 2010) and all of
our samples were processed within 30 minutes of delivery, otherwise they were not used in the analysis.

From our current study it seems that DNA methylation is not affected in placentae from ART pregnancies in the analyzed regions. Correspondingly, the expression of these genes appears to be consistent among the ART and NC groups with no significant differences observed. However, it is a possibility that due to sampling techniques and the nature of placental tissues, there may have been minor differences that were not discovered in our analysis. Further, the lack of consistency among studies may be due to lab-to-lab variability in sampling procedures, such as differences in location and number of sites used, tissue cleaning and separation methods, as well as time of sampling post-delivery (Avila et al. 2010).
Chapter 3: DNA METHYLATION OF IMPRINTED GENES IN UMBILICAL CORD BLOOD FROM IVF/ICSI CONCEIVED INFANTS VERSUS NC CONTROLS

3.1 Introduction

Although studying placental DNA methylation and gene expression is valuable and provides insight into how the early preimplantation environment may affect imprinting establishment, the placenta is derived from a separate cell line than the fetus during embryogenesis. Methylation patterns in extraembryonic tissues have been shown to differ significantly from that of embryonic tissue (Katari et al. 2009). For these reasons, umbilical cord blood analyses have also been conducted, as this tissue originates from the inner cell mass during embryogenesis, and may provide better insight into the effect of ART on genomic imprinting within the fetus. Genome-wide analysis comparing cord blood and placental tissue from ART and NC pregnancies noted that generally there was higher mean methylation in the cord blood and lower mean methylation in the placental tissue (Katari et al. 2009). Rancourt et al. (2012) examined DNA methylation and gene expression in the placenta and cord blood and found that in the placenta there were changes in methylation at the H19 and MEST genes, whereas in the cord blood there were changes in KCNQ1, demonstrating that different genes may be more susceptible to epimutations in different tissues. Further, while the interpretation of placental methylation is complicated and tends to be much more variable, cord blood is a more reliable tissue when trying to observe potential changes in the offspring.

In this chapter, the DNA methylation levels at KvDMR1, PEG10, PLAGLI, and LINE-1 regions will be evaluated in cord blood samples from NC, IVF, and ICSI conceived pregnancies.
Additionally, gene expression analyses of the PLAGL1 gene and three imprinted genes of chromosome 11p15.5 – KCNQ1OT1, IGF2, and CDKN1C – will be conducted in order to examine the effect of ART on a proposed imprinting gene network. PLAGL1 has been shown to be involved in regulating the KvDMR1 cluster, the largest known imprinted gene cluster, by acting directly on KCNQ1OT1. PLAGL1, a transcription factor, binds to the unmethylated KCNQ1OT1 promoter and activates its expression, which then acts as a ncRNA to down-regulate CDKN1C (Arima et al. 2005). Further, PLAGL1 has been shown to bind to an enhancer region (E2) for the H19 and IGF2 genes, also located on chromosome 11p15.5 but under the regulation of a different ICR (ICR1) than the KCNQ1OT1 imprinted gene cluster (Varrault et al. 2006, Iglesias-Platas et al. 2014). Mouse studies have shown interactions and co-regulation between imprinted genes Plagl1, Igf2-H19, Cdkn1c, Rtl1, and Dlk1 (Gabory et al. 2009, Varrault et al. 2006) and it has been suggested that PLAGL1 is the “master regulator” gene of this imprinted network.

3.2 Methods

3.2.1 Study participants

See Section 2.2.1.

3.2.2 Sample preparation

After delivery, the cord blood was immediately extracted into EDTA vacuum tubes (BD Vacutainer®, NJ) and 2.5ml of cord blood was transferred into PAXgene™ blood RNA tubes (PreAnalytix, Switzerland) to preserve RNA. The remaining blood was stored at 4°C for DNA
extraction. The PAXgene™ blood RNA tubes were incubated overnight at room temperature and then stored at -20°C.

3.2.3 DNA extraction from umbilical cord blood

Cord blood DNA extraction was carried out using the Qiagen Puregene Blood Core Kit C (Qiagen, Mississauga, ON), according to the manufacturer’s protocol and stored at -20°C. Concentrations were then recorded using a NanoSpec spectrometer (Nanovue by General Electric Inc., CT, USA) and each sample was diluted in a separate tube to contain 500ng of DNA. These samples were then subjected to bisulphite conversion prior to pyrosequencing analysis.

3.2.4 Preparation of cDNA library

PAXgene™ tubes were incubated at room temperature overnight and 1ml of whole blood was used for RNA extraction. Cord blood RNA was extracted using the Paxgene™ Blood RNA kit (PreAnalytix, Switzerland) according to the manufacturer’s protocol and stored at -20°C. RNA integrity and cDNA conversion of cord blood samples were carried out as described in section 2.2.4, however only 0.25µg of RNA was used in the conversion reaction for each sample.

3.2.5 DNA methylation analysis by bisulphite pyrosequencing

See section 2.2.5.

3.2.6 Gene expression analysis by qPCR

See section 2.2.6.
3.2.7 Statistical analysis

The correlation between two variables was determined with the Pearson product-moment correlation coefficient, where a P-value less than 0.05 was deemed significant. The remaining statistical analyses were conducted as described in section 2.2.7.

3.3 Results

3.3.1 Clinical data

Clinical characteristics are the same for the cord blood DNA methylation analysis as for the placental DNA methylation analysis in Table 2.2. There was a significantly higher mean maternal age in the IVF group for both the DNA methylation and gene expression subcategories (Tables 2.2 and 3.1). For the methylation analysis, mean maternal age in the IVF group was 36.67 with a standard deviation (SD) of ± 3.36 years (P = 0.000006), 32.55 ± 5.61 years in the naturally conceived (NC), and 33.73 ± 4.50 years in the ICSI group. For the gene expression analysis, the maternal age of the IVF group was also significantly higher (35.46 ± 2.93, P = 0.025) compared to NC (32.76 ± 4.22), but the ICSI group was not significantly different from NC (34.72 ± 4.30). In the cohort used for gene expression analysis, the IVF group also had significantly lower gestational age (P = 0.019) compared to the NC and ICSI groups (Table 3.1).
Table 3.1 Clinical characteristics of study population in gene expression analysis

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>IVF</th>
<th>ICSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>35</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Mean Maternal Age</td>
<td>32.76 ± 4.22</td>
<td>35.46 ± 2.93*</td>
<td>34.72 ± 4.30</td>
</tr>
<tr>
<td>(years ± SD)</td>
<td></td>
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<tr>
<td>Mean Gestational Age</td>
<td>274.9 ± 10.3</td>
<td>262.0 ± 18.6*</td>
<td>273.7 ± 9.47</td>
</tr>
<tr>
<td>(days ± SD)</td>
<td></td>
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<tr>
<td>Mean Birth Weight</td>
<td>3259 ± 473</td>
<td>2896 ± 686</td>
<td>3192 ± 447</td>
</tr>
<tr>
<td>(grams ± SD)</td>
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<tr>
<td>Girls (%)</td>
<td>66%</td>
<td>50%</td>
<td>30%</td>
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</table>

* significant between NC group P < 0.05

3.3.2 DNA methylation analysis of cord blood from ART and NC infants

There was a significantly increased methylation of the PLAG1 DMR in the cord blood samples from IVF conceptions compared to naturally conceived controls (P = 0.0007). The mean methylation in the IVF group was 48.19% with a median (IQR) 48.92% (47.42 – 50.62), compared to 46.22% [45.86% (43.14 - 48.64)] in the naturally conceived group (Figure 3.1, Table 3.2). All four CpG sites analyzed within the IVF cord blood samples had increased methylation compared to the NC and ICSI groups (Figure 3.2, Table 3.3). There was no significant difference in methylation between the ICSI group 45.59% [44.92% (43.77 - 47.57)] and the naturally conceived controls (P = 0.91).

There was a lack of methylation at the PEG10 region in cord blood samples from the control, IVF, and ICSI infants with median methylation values of 2.97% (2.58 - 3.84), 2.94% (2.62 - 3.72) and 3.02% (2.73 - 3.36), respectively (Figure 3.1, Table 3.2). Despite the
unexpectedly low mean methylation values observed, there were no significant differences in
*PEG10* methylation between IVF or ICSI and the NC control groups (Kruskal-Wallis; P > 0.05).

At the KvDMR1 locus mean methylation levels for the IVF and ICSI groups were not
significantly different from controls in the cord blood analysis (P > 0.05). Methylation values
were 59.57% (56.12 - 66.93), 59.37% (55.93 - 63.47), and 60.94% (54.96 - 64.16) for the
control, IVF, and ICSI groups, respectively (Figure 3.1, Table 3.2).

For the repetitive LINE-1 element there was no difference detected between methylation
levels in cord blood of IVF or ICSI (P > 0.05) groups compared to controls and median (IQR)
methylation values were 78.32% (76.87 - 79.71), 77.22% (75.36 - 78.90), and 77.47% (76.65 –
79.01) in the control, IVF, and ICSI groups, respectively (Figure 3.1, Table 3.2).

Detailed summaries of average methylation at each individual CpG site for every gene
can be found in table 3.3.
Figure 3.1 DNA methylation in cord blood from IVF, ICSI, and NC infants. Percent methylation at KvDMR1, PEG10, PLAGL1, and one repetitive element (LINE-1) in cord blood from IVF, ICSI, and NC infants. The black line represents the median value and the boxes indicate the upper and lower quartiles. IVF and ICSI groups were compared with the natural conception (NC) controls. The asterisks represent significant differences compared to NC (P < 0.05). The pink, green, and blue boxes represent the ICSI, IVF, and NC groups, respectively.
Table 3.2 Mean and median (IQR) methylation values (percentages) of *PLAGL1*, KvDMR1, *PEG10*, and LINE-1 in cord blood from NC, IVF, and ICSI pregnancies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Pregnancy Type</th>
<th>DNA methylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLAGL1</strong></td>
<td>NC</td>
<td>46.22; 45.86 (43.14, 48.64)</td>
</tr>
<tr>
<td></td>
<td>IVF</td>
<td>48.19; 48.92 (47.42, 50.62)*</td>
</tr>
<tr>
<td></td>
<td>ICSI</td>
<td>45.59; 44.92 (43.77, 47.57)</td>
</tr>
<tr>
<td><strong>KvDMR1</strong></td>
<td>NC</td>
<td>61.69; 59.57 (56.12, 66.93)</td>
</tr>
<tr>
<td></td>
<td>IVF</td>
<td>60.13; 59.37 (55.93, 63.74)</td>
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<tr>
<td></td>
<td>ICSI</td>
<td>60.29; 60.94 (54.96, 64.16)</td>
</tr>
<tr>
<td><strong>PEG10</strong></td>
<td>NC</td>
<td>4.11; 2.97 (2.58, 3.84)</td>
</tr>
<tr>
<td></td>
<td>IVF</td>
<td>3.97; 2.94 (2.62, 3.72)</td>
</tr>
<tr>
<td></td>
<td>ICSI</td>
<td>4.26; 3.02 (2.73, 3.36)</td>
</tr>
<tr>
<td><strong>LINE-1</strong></td>
<td>NC</td>
<td>77.29; 78.32 (76.87, 79.71)</td>
</tr>
<tr>
<td></td>
<td>IVF</td>
<td>76.59; 77.22 (75.36, 78.90)</td>
</tr>
<tr>
<td></td>
<td>ICSI</td>
<td>76.88; 77.47 (76.65, 79.01)</td>
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</table>

*significant between NC group P < 0.05
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<tr>
<th>Gene</th>
<th>Pregnancy Type</th>
<th>CpG 1</th>
<th>CpG 2</th>
<th>CpG 3</th>
<th>CpG 4</th>
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<td>(n = 53)</td>
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<td>(n = 52)</td>
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<tr>
<td>PLAGL1</td>
<td>NC</td>
<td>44.60 (40.91 – 47.18)</td>
<td>47.36 (43.90 – 49.87)</td>
<td>45.84 (42.68 – 48.40)</td>
<td>46.20 (43.66 – 49.83)</td>
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<td></td>
<td>IVF</td>
<td>47.18 (45.11 – 48.68)</td>
<td>51.33 (48.52 – 53.11)</td>
<td>47.94 (45.86 – 49.89)</td>
<td>49.50 (48.10 – 51.38)</td>
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<td>ICSI</td>
<td>43.26 (41.54 – 46.82)</td>
<td>46.93 (44.60 – 47.18)</td>
<td>44.62 (43.55 – 47.48)</td>
<td>45.27 (44.27 – 48.76)</td>
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<tr>
<td>KvDMR1</td>
<td>NC</td>
<td>59.23 (55.85 – 67.96)</td>
<td>60.60 (56.39 – 64.90)</td>
<td>57.32 (53.54 – 64.18)</td>
<td>57.51 (53.50 – 64.32)</td>
<td>59.66 (55.95 – 67.88)</td>
<td>57.83 (54.35 – 64.52)</td>
<td>61.08 (56.91 – 70.14)</td>
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<td>IVF</td>
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<td>59.98 (56.85 – 64.47)</td>
<td>56.40 (54.00 – 60.27)</td>
<td>56.72 (54.14 – 61.96)</td>
<td>57.31 (54.84 – 64.28)</td>
<td>57.76 (54.22 – 62.85)</td>
<td>59.26 (56.56 – 63.60)</td>
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<td></td>
<td>ICSI</td>
<td>59.95 (54.27 – 64.48)</td>
<td>61.44 (55.67 – 66.36)</td>
<td>55.30 (51.16 – 60.71)</td>
<td>56.43 (51.92 – 62.74)</td>
<td>58.16 (53.87 – 65.03)</td>
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<tr>
<td>PEG10</td>
<td>NC</td>
<td>4.61 (4.10 – 6.65)</td>
<td>5.92 (4.84 – 7.30)</td>
<td>2.12 (1.72 – 3.15)</td>
<td>1.04 (0.82 – 2.14)</td>
<td>2.87 (2.29 – 4.12)</td>
<td>1.74 (1.36 – 2.45)</td>
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<td></td>
<td>IVF</td>
<td>4.38 (3.19 – 5.11)</td>
<td>5.54 (4.76 – 7.79)</td>
<td>2.32 (1.64 – 5.40)</td>
<td>1.25 (0.96 – 3.29)</td>
<td>2.67 (2.22 – 4.08)</td>
<td>1.46 (1.04 – 2.22)</td>
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<td></td>
<td>ICSI</td>
<td>4.55 (3.83 – 5.54)</td>
<td>6.08 (5.20 – 6.95)</td>
<td>2.06 (1.59 – 2.59)</td>
<td>1.18 (0.90 – 1.80)</td>
<td>2.84 (2.31 – 3.87)</td>
<td>1.44 (1.11 – 2.01)</td>
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<tr>
<td>LINE-1</td>
<td>NC</td>
<td>81.16 (78.25 – 82.21)</td>
<td>79.03 (76.85 – 80.94)</td>
<td>74.18 (71.61 – 76.83)</td>
<td>77.60 (75.71 – 79.03)</td>
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<td></td>
<td>IVF</td>
<td>79.86 (77.97 – 81.86)</td>
<td>78.69 (77.55 – 79.66)</td>
<td>74.42 (71.69 – 76.22)</td>
<td>76.50 (74.30 – 78.58)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ICSI</td>
<td>81.13 (78.29 – 82.83)</td>
<td>78.66 (77.76 – 79.97)</td>
<td>73.94 (71.89 – 76.10)</td>
<td>77.03 (74.85 – 78.56)</td>
<td>-</td>
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*significant between NC group P < 0.05
Figure 3.2 DNA methylation at 4 CpG sites at *PLAGL1*. Percent DNA methylation at four CpG sites within the *PLAGL1* DMR in cord blood samples from IVF, ICSI, and NC infants. The black line represents the median value and the boxes indicate the upper and lower quartiles. The asterisks represent significant differences compared to NC (P < 0.05). Pink, green, and blue boxes represent ICSI, IVF, and NC, respectively.

### 3.3.3 Analysis of *PLAGL1* gene expression in ART and NC cord blood

As the *PLAGL1* DMR had significantly higher levels of methylation in the IVF cord blood samples compared to both the naturally conceived and ICSI groups, transcription of *PLAGL1* was similarly compared. We observed a significantly lower level of *PLAGL1* mRNA in both IVF (P=0.0013) and ICSI (P=0.0155) cord blood samples when compared to naturally conceived controls (log10 expression -0.065, 0.187, and 0.160 for NC, IVF, and ICSI, respectively) (Figure 3.3). Interestingly, we did expect to see decreased expression of *PLAGL1* in
our IVF samples due to the observed increase in methylation of the ICR; however a decreased expression of *PLAGL1* in the ICSI group was unexpected.

### 3.3.4 Analysis of imprinted gene network in IVF cord blood

*PLAGL1* is a suggested master regulator of an imprinted gene network and is involved in regulating the imprinted genes on chromosome 11p15.5 (Arima et al. 2005, Varrault et al. 2006). The regulation of imprinted genes on chromosome 11p15.5 is divided into two domains. Domain 1 is regulated by ICR1 and controls expression of two genes: *H19* and *IGF2*. Domain 2 is controlled by KvDMR1 (ICR2) and regulates the genes *KCNQ1, KCNQ1OT1, CDKN1C, SLC22A18*, and *PHLDA2*. *PLAGL1* has been shown to directly bind to the promoter region of *KCNQ1OT1* (Arima et al. 2005). As KvDMR1 is located within the 10th intron of *KCNQ1OT1* (Arima et al. 2005), *PLAGL1* is important in imprinted regulation of the genes in Domain 2.

We examined *CDKN1C* expression in these groups as it is negatively regulated by the *KCNQ1OT1* anti-sense RNA transcript, and it has been shown to share similar expression patterns as *PLAGL1* (Arima et al. 2005). *IGF2* expression levels were also evaluated as it has been demonstrated that *PLAGL1* binds to an enhancer region (E2) shared by these two genes (Varrault et al. 2006).

The *KCNQ1OT1* non-coding RNA (ncRNA) was significantly decreased in the IVF (log10 expression 0.114, P = 0.00018) group but not in the ICSI group (log10 expression -0.009, P > 0.05) compared to NC controls (log10 expression 0.048). *CDKN1C* appeared to be increased in the IVF group compared to the control group, however there was no statistical significance (log10 expression -0.169, -0.075, and -0.212 for NC, IVF, and ICSI, respectively; P > 0.05). The relative expression of *IGF2* was increased in the IVF (log10 expression 0.124, P =
0.022) cord blood compared to NC controls (log10 expression -0.212), but not in the ICSI group (log10 expression 0.029, P > 0.05). Therefore, of the three imprinted genes analyzed on chromosome 11p15.5, two displayed significantly altered expression in the cord blood from the IVF participants (Figure 3.3).

Figure 3.3 Gene expression levels of imprinted genes in cord blood from IVF, ICSI, and NC infants. Log10 gene expression values of PLAGL1 and chromosome 11p15.5 imprinted genes KCNQ1OT1, CDKNIC, and IGF2 in cord blood samples from IVF, ICSI, and NC infants. The black line represents the median value and the boxes indicate the upper and lower quartiles. The asterisks represent significant differences compared to NC (P < 0.05). Pink, green, and blue boxes represent ICSI, IVF, and NC, respectively.
3.3.5 Co-regulation of *PLAGL1* and *KCNQ1OT1* in cord blood

In order to determine whether there was correlation between expression of the *PLAGL1* gene and the genes of chromosome 11p15.5, Pearson’s correlation tests were performed on the log10 RQ values from *KCNQ1OT1*, *CDKN1C*, and *IGF2* against *PLAGL1*. There was a significant correlation between *PLAGL1* and *KCNQ1OT1* expression patterns in the cord blood samples (P = 4.38x10^-5, r = 0.430) (Figure 3.4). As this correlation was noted in the cord blood samples, we evaluated the correlation within the placental tissues and found a strong significant correlation (P = 2.2x10^-16, r = 0.824) (Figure 3.4). Surprisingly, there was no significant correlation between *PLAGL1* and *CDKN1C* or *IGF2* gene expression (P > 0.05) (Figures not shown).

![Figure 3.4 Correlation between *PLAGL1* and *KCNQ1OT1* in cord blood and placenta. Pearson’s correlation between log10 expression values of *PLAGL1* and *KCNQ1OT1* in matched samples from cord blood (n=84) and from placental chorionic villi (n=173).](image-url)
3.4 Discussion of results

3.4.1 DNA methylation of imprinted regions in umbilical cord blood

Although no changes in methylation were observed in the placental tissue from ART pregnancies compared to the NC controls, studies have shown that methylation may be tissue-specific and differences in cord blood and placenta DNA methylation has been noted. Further, as extraembryonic and embryonic tissues are derived from separate origins, it is possible that imprinting may be affected in one tissue but not in the other. There were no significant differences in methylation observed at the PEG10, KvDMR1, or LINE-1 regions in the cord blood from ART infants compared to NC. The lack of changes at KvDMR1 does agree with previous studies, as Li et al. (2011) examined DNA methylation at the imprinted ICR1, KvDMR1, and PEG1 loci and did not observe any significant changes. However, Rancourt et al. (2012) did note differences at the KCNQ1 region within the cord blood samples of ART children, but this did not correspond with a change in gene expression, suggesting there was no LOI. It is interesting to note that there were no changes at LINE-1, as several studies have detected variable genome-wide changes in DNA methylation in cord blood samples from IVF and ICSI pregnancies (Katari et al. 2009, Melamad et al. 2013). This may indicate that the LINE-1 repetitive element is not an ideal representative of overall genome-wide methylation. Although many studies have shown that LINE-1 is a good indicator of global methylation (Yang et al. 2004, Yang et al. 2006, Weisenberger et al. 2005, Woodson et al. 2005), a recent study examining the effect of superovulation on methylation in mouse blastocysts found no changes in global DNA methylation during whole-genome analysis but did observe decreased methylation at LINE-1. These findings indicate that LINE-1 methylation may not always be an ideal representative of global methylation (Liang et al. 2013).
This is the first study to examine the methylation and gene expression patterns of \textit{PLAGL1} within the cord blood of ART infants versus natural conception controls. A statistically significant increase in DNA methylation at the \textit{PLAGL1} DMR in the IVF group was observed when compared to controls. However, the calculated mean value of 48.19\% is still within the expected 35-65\% methylation range for an imprinted gene (Woodfine et al. 2011). Further, there were no significant differences in cord blood DNA methylation at \textit{PLAGL1} between the ICSI group and natural conceptions. The fact that this change was only seen in the IVF group, and not the ICSI group, is somewhat perplexing as the procedures are generally the same with the added step of directly injecting a single spermatozoa into the oocyte during ICSI, which one would think would make these embryos even more vulnerable to disruptions. Therefore, the observed changes may be linked to the composition of the experimental group in terms of clinical background and/or the underlying causes of subfertility rather than the ART procedure, \textit{per se}. There are several studies suggesting that infertility itself is a risk factor for genomic imprinting disorders (Ludwig et al. 2005, Doornbos et al. 2007, Romundstad et al. 2008). It is also worth noting that the IVF group does have a statistically significantly higher mean maternal age (36.67 ± 3.36 years) compared to both the natural conception and ICSI groups (32.55 ± 5.61 years and 33.73 ± 4.50 years, respectively). Advanced maternal age has been linked with higher rates of aneuploidy and chromosomal abnormalities in human oocytes (Dailey et al. 1996, Pellestor et al. 2003); however the effect on imprinting has yet to be established. In future studies it will be critical to subdivide groups not only based on conception mode but also on the underlying cause of infertility.

It is important to point out that \textit{PLAGL1} is a maternally methylated and paternally expressed gene (Gardner et al. 2000, Kamiya et al. 2000) and therefore, if there were extra
methyl groups present, theoretically this should be of paternal origin. It has been suggested that men who suffer from severe infertility are at a higher risk of carrying imprinting errors in their sperm (Marques et al. 2004, Sato et al. 2007, Ferguson et al. 2007, Minor et al. 2007, Kobayashi et al. 2009), and it is likely that these imprinting errors are directly passed on to the offspring (Kobayashi et al. 2009). A study by Hammoud et al. (2010) examined percent methylation in sperm from men with abnormal protamine ratios, oligozoospermia, and fertile controls and found that the PLAGLI locus did display hypermethylation in the sperm from both types of infertile males compared to fertile controls. Interestingly, this group also looked at CpGs within the KCNQ1OT1 promoter region and found this locus was also significantly hypermethylated in the two infertile groups (Hamoud et al. 2010), but we saw no changes in methylation at this domain in our analysis. It would also be important to determine whether specific procedural differences in ART, such as types of embryos transferred (ie. fresh vs. frozen), would account for the differences in methylation observed in these different studies. It has been shown that frozen embryo transfer, as opposed to fresh embryo transfer, may reduce the risk of low birth weight and adverse perinatal outcomes in the offspring (Maheshwari et al. 2012), suggesting that the uterine environment may contribute to adverse outcomes, potentially through disrupted imprinting. Imprinting errors have also been associated with the use of poor quality embryos (Shi et al. 2014).

An unexpectedly low level of mean methylation was observed in the PEG10 region in the cord blood consistently among all conception modes. PEG10 has been reported as an imprinted gene (Ono et al. 2001), however our data reveals a lack of methylation among the ART and naturally conceived groups, which is unexpected for an imprinted region. It is widely accepted that except in the primordial germline, imprinting is static throughout the lifespan of the
individual. While it has been shown that loss of imprinting can occur at *PEG10* in cancer or otherwise abnormal cell populations or cell lines (Gao et al. 2010, Kim et al. 2007), and that biallelic expression of normally methylated imprinted genes is present under certain circumstances (Kamiya et al. 2000), our study has shown evidence supporting the idea that *PEG10* imprinting may occur in a tissue-specific manner as a part of normal development. Another previously published study looking at *PEG10* methylation in cord blood had similar findings (Peñaherrera et al. 2010), providing further support for this theory.

*PEG10* is derived from a retroelement that has been integrated by acquiring a cellular function and is now essential for prenatal development (Clark et al. 2007). In many cases where retroelements have been co-opted into cellular function, the region remains unmethylated and actively transcribing (Cohen et al. 2011, Matousková et al. 2006). Thus, one might hypothesize that *PEG10* cord blood demethylation represents further evolutionary relaxation of retroelement silencing as it becomes incorporated into host function. In support of this, it has been found that hypomethylation of the LINE-1 retroelement may accompany normal physiological processes in the colonic mucosa (Figueiredo et al. 2009); this may be an indication that LINE-1 elements are becoming co-opted for normal cellular processes and thus their methylation profile is beginning to change.

### 3.4.2 Gene expression in umbilical cord blood and imprinted gene network

As DNA methylation of the imprinted *PLAGL1* gene appeared to be higher in the IVF cord blood samples compared to NC, the corresponding gene expression levels were examined. Transcription levels of *PLAGL1* in cord blood samples from the IVF, ICSI, and NC groups were analyzed by RT-PCR. As methylation is generally a repressive modification, it would be
expected that hypermethylation of an ICR would result in lower levels of gene transcription. Accordingly, we did observe a significant decrease in expression of the PLAGL1 gene within the cord blood samples of the IVF group compared to the natural conceptions. Unexpectedly, we also found that the ICSI group displayed a significant down-regulation of PLAGL1 in the cord blood, although not as extensively as in the IVF group. The fact that there are decreased levels of gene expression in both of the ART groups, with the ICSI samples displaying no difference in methylation, suggests that there may be another mechanism regulating PLAGL1 expression. In fact, a recent study examining PLAGL1 in the IUGR placenta discovered significantly decreased expression from children conceived by ART compared to spontaneous conceptions with no observed change in methylation status (Iglesias-Platas et al. 2014). Unfortunately, as ART was not the focus of their study, the sample size was very small (12 children from 7 couples) and they did not differentiate between IVF or ICSI conceptions. It is plausible that the increase in mean methylation of PLAGL1 within our IVF cohort, though statistically significant, is not the leading biological factor in the down-regulation of the transcript. Gene expression levels of PLAGL1 in ART conceptions have not been extensively studied and therefore it is quite significant that our group, as well as the aforementioned group, have both detected similar patterns.

Previous studies have shown that PLAGL1 may be a “master regulator” of an important imprinted gene network (Arima et al. 2005, Varrault et al. 2006). As these regulators are believed to affect a number of different genes and processes, changes in their gene expression could have drastic and far-reaching effects (Skaar et al. 2012). We therefore decided to look at the gene expression levels of KCNQ1OT1, CDKN1C, and IGF2 in our cord blood samples as it has been shown that PLAGL1 interacts and co-regulates these genes on chromosome 11p15.5 (Varrault et al. 2006, Gabory et al. 2009), and that PLAGL1 binds to an enhancer region shared
by IGF2 and H19 (Varrault et al. 2006, Iglesias-Platas et al. 2014). We found that expression of KCNQ1OT1 was significantly lower in our ART groups compared to controls. This was expected as PLAGL1 is thought to induce expression of the KCNQ1OT1 non-coding RNA (ncRNA) by binding to the unmethylated CpG sites within the promoter region (Arima et al. 2005). When we looked at correlation between the two genes we found that there was a significant positive correlation between the expression of PLAGL1 and KCNQ1OT1 in the cord blood samples, as well as in the placental samples. The correlation between these two genes in both sets of tissues provides further evidence to support the idea of the induction of KCNQ1OT1 by PLAGL1 in vivo.

The expression of CDKN1C in our ART samples was not statistically different than our controls, as KCNQ1OT1 is thought to negatively regulate the expression of CDKN1C (Arima et al. 2005), then one would expect to find increased transcription of CDKN1C with decreased levels of KCNQ1OT1. Surprisingly, no significant correlation between these two genes was observed in the cord blood. We also ran a Pearson’s correlation test between PLAGL1 and CDKN1C transcription levels and no significant correlation was evident. Levels of IGF2 were significantly increased in our IVF cord blood samples compared to the controls, but no differences were found between ICSI and control samples. There was no significant correlation between the gene expression levels of PLAGL1 and IGF2 in these cord blood samples. Again, these results are surprising as previous studies have demonstrated a positive correlation between PLAGL1 and IGF2 gene expression in the human placenta (Iglesias-Platas et al. 2014). As our observations in the cord blood differ from previous findings in the human placenta with regards to the correlation of PLAGL1 with CDKN1C and IGF2, this may be indicative of differential regulation between tissues. However, the extremely strong correlation between PLAGL1 and
expression in both the cord blood and placenta does strongly suggest interaction between these two imprinted regions in both tissues.

The main findings of this chapter are that there are alterations of \textit{PLAGL1} gene expression in the cord blood from both IVF and ICSI infants. As there is a small, but significant, increase in methylation at the \textit{PLAGL1} region in the IVF cord blood samples, but not the ICSI samples, it is probable that, although this difference was statistically significant, it is not the biological cause of the changes in gene expression. This theory is supported by the observations in humans where the expression of \textit{PLAGL1} was shown to be altered in ART placentae despite no change in DNA methylation. It is most likely that the ART procedure and/or underlying subfertility of the parents is causing alterations in other epigenetic regulatory mechanisms, such as repressive histone modifications or ncRNAs, that is affecting the transcription of genes within this co-regulated gene network. In fact, another important component in imprinting is histone modifications, which have been shown to be key regulators in gene expression (Skaar et al. 2012).

These findings are significant because \textit{PLAGL1} has been shown to function as a central regulator of other imprinted genes, which could enable a wide influence over development and health. In the IVF samples, there appeared to also be dysregulation of \textit{KCNQ1OT1} and \textit{IGF2}, which suggests that the altered expression of \textit{PLAGL1} in this tissue has downstream effects on the transcription of other imprinted genes important for fetal development. Further studies are required to determine whether discrete changes in the expression of genes within the imprinted gene network have any lasting effects on the health of the offspring.
Chapter 4: CONCLUSION

4.1 Major findings and implications

As infertility rates continue to rise, more and more couples are beginning to rely on ARTs in order to conceive. Since the birth of Louise Brown, the first IVF-conceived infant, in 1978, there have been over 5 million babies worldwide conceived by ARTs (Cedars et al. 2013) and major advances have been made in the field of reproductive biology. ARTs are becoming more accessible and are able to overcome various fertility issues. However, there are some concerns regarding the safety of these procedures as there appears to be increased rates of rare imprinting disorders, such as BWS and AS, in the ART population (Cox et al. 2002, Maher et al. 2003, Halliday et al. 2004, Giquel et al. 2005, Hiura et al. 2012), as well as increased rates of adverse perinatal outcomes such as low birth weight and preterm delivery (Pandey et al. 2012). It has also been suggested that these children may be at an increased risk of metabolic disorders, such as obesity and diabetes, as well as certain malignancies later in life (Katari et al. 2009).

ART procedures overlap with important genomic reprogramming events that are essential for establishing epigenetic modifications at imprinting control regions within the germline. Imprinted genes are expressed in a parent-of-origin specific manner and are controlled by differential methylation at imprinting control regions (ICRs). There are 96 known imprinted genes in humans (geneimprint.com) and, although this only makes up a small portion of the genome, imprinted genes have been shown to be essential to fetal and placental development (Ferguson-Smith and Surani 2001, Reik and Walter 2001, Tycko and Morris 2002). There are two major reprogramming events that occur to erase and re-establish methylation throughout the genome: the first wave of reprogramming occurs as the primordial germ cells enter the gonadal ridge where genome-wide demethylation occurs, including at ICRs, and are reset in a sex-
specific manner, and the second event happens after fertilization to regain totipotency of the germline. During the second genomic reprogramming event, the ICRs retain their epigenetic marks, as dosage-dependent expression of imprinted genes is crucial during this phase to regulate proper development of the embryo. Genomic reprogramming events create a critical window during which the environment can have a profound effect on the epigenetic pattern of the offspring. During the ART procedure, the gametes and resulting embryos are exposed to unnatural environments and manipulations that may disrupt the establishment and maintenance of DNA methylation at imprinted regions, altering their transcription and potentially leading to developmental disorders. However, the link between imprinting disorders, genomic imprinting, and ART is still unclear (Lazaraviciute et al. 2014), and it is unknown whether the underlying subfertility of one or both parents plays a causative role (Ludwig et al. 2005, Doornbos et al. 2007).

Previous studies have investigated cohorts of ART offspring with imprinting disorders (Maher et al. 2003, DeBaun et al. 2003, Halliday et al. 2004, Cox et al. 2002, Ørstavik et al. 2003, Gicquel et al. 2003). Results have been varied, and oftentimes inconclusive, owing to the rare occurrence of imprinting disorders, as well as the inconsistency in the types of fertility treatments and study methods used. Consequently, many researchers have turned to investigating imprinted regions in ART pregnancies that do not have any apparent complications in order to determine whether ART alters the epigenome and increases the risk of disease among ART children (Katari et al. 2009, Gomes et al. 2009, Shi et al. 2011, Turan et al. 2010, Wong et al. 2011, Oliver et al. 2012). Katari et al. (2009) examined more than 700 genes from the cord blood and placenta of ART-conceived infants and found subtle changes in DNA methylation of genes associated with metabolic disorders; some of these changes were also accompanied by altered
gene expression (Katari et al. 2009). Turan et al. (2010) examined children conceived both in vitro and in vivo and discovered aberrant methylation of the H19/IGF2 ICR1 region from in vitro conceptions. However, other studies have not observed any epigenetic differences between ART infants and controls (Tierling et al. 2010, Li et al. 2011, Puumala et al. 2012, Rancourt et al. 2012). Further, many studies have examined DNA methylation of imprinted regions in ART infants but have not included gene expression assays, which is crucial as there are many layers of regulation of imprinted gene expression, including histone modifications and ncRNA expression.

In chapter 2, we examined the DNA methylation and gene expression levels of three imprinted genes – KvDMR1, PEG10, and PLAGL1 – and one repetitive element – LINE-1 – in placental tissue from IVF, ICSI, and NC pregnancies. Previous studies have examined genomic imprinting in the placentae from ART children, however results have been varied and it is difficult to draw any major conclusions. Furthermore, there has not been consistency in these studies in terms of the genes examined and the protocols used. Alteration of imprinted gene expression in the placenta can affect growth and development, as well as the ability to transfer nutrients to the fetus. We hypothesized that the ART procedure may affect the establishment and maintenance of DNA methylation at DMRs within the placenta and, therefore, alter the expression of the corresponding imprinted genes. We observed no significant difference in DNA methylation at the KvDMR1, PEG10, or PLAGL1 imprinted regions, or at the LINE-1 repetitive element within the placental tissue from IVF or ICSI derived pregnancies compared to controls. Gene expression analyses were also conducted for the four genes KCNQ1OT1, PEG10, PLAGL1, and LITD1 within the placental chorionic villi and there were no significant changes between the ART and NC groups. These results suggest that imprinting of these genes in the placenta is unaffected by the ART procedures. Further, as there were no significant differences
between DNA methylation at the LINE-1 elements, which is used as a surrogate for global DNA methylation, then this would suggest that non-imprinted genes are also unaffected in placental tissues. However, it is worth noting that, as there is extreme inter- and intra-variability in gene expression in the placenta (Avila et al. 2010), it can be difficult to make any major conclusions with confidence. Further, recent studies have observed that LINE-1 repetitive element methylation can differ from global levels of DNA methylation.

In chapter 3, we analyzed the DNA methylation and gene expression of the same imprinted genes within the umbilical cord blood from IVF and ICSI infants compared to NC controls. We found significantly increased mean methylation of PLAGL1 in the IVF cord blood samples, however the mean methylation value was still within the expected range for imprinted genes. There was a significant increase in methylation at all four analyzed CpG sites in the IVF group compared to the controls, suggesting that this alteration was consistent within the PLAGL1 DMR, and not just at one particular CpG site. Increased methylation of PLAGL1 has been correlated with pheochromocytoma (Jarmalaite et al. 2011) and capillary hemangioblastomas (Lemeta et al. 2007), and changes in PLAGL1 have also been implicated in BWS (Arima et al. 2005) and SRS (Peñaherrera et al. 2010). As increased methylation was not observed in the ICSI group, it is likely that changes in methylation are associated with the underlying clinical aspects related to causes of subfertility in the IVF group, rather than due to the ART procedures. As the maternal age was significantly higher in the IVF group, it is possible that this contributes to the observed changes. There were no significant differences between groups at the KvDMR1, PEG10, or LINE-1 regions, however the PEG10 region did display much lower than expected levels of methylation in all three groups, indicating tissue-specific imprinting of this gene.
As PLAGL1 displayed increased methylation in the IVF cord blood samples, gene expression analysis was carried out via RT-qPCR. Gene expression analysis of PLAGL1 within the umbilical cord blood samples revealed significantly decreased gene expression in both the IVF and ICSI groups compared to the naturally conceived controls. PLAGL1 is a tumour suppressor gene known to be down-regulated in many types of cancer (Bilanges et al. 1999, Cvetkovic et al. 2004, Pagotto et al. 2000, Koy et al. 2004, Basyuk et al. 2005) and both under- and over-expression of PLAGL1 has been linked to IUGR (McMinn et al. 2005, Mackay et al. 2010, Varrault et al. 2006, Iglesias-Platas et al. 2014). The observation that PLAGL1 mRNA levels were lower in both the IVF and ICSI samples, despite there only being increased methylation in the IVF group, suggests that the mechanism causing the decreased expression is not solely dependent on DNA methylation. It is likely that other epigenetic modifications are disrupted in the ART procedures causing alterations in PLAGL1 expression within the umbilical cord blood of these neonates. Imprinted genes are associated with multiple layers of epigenetic regulation, including histone tail modifications and ncRNAs (Kacem and Feil 2009). As changes in gene expression of PLAGL1 have been linked to IUGR (Varrault et al. 2006, Iglesias-Platas et al. 2014), it is possible that disruptions at this region may contribute to the observed increased rates of low birth weight in the ART population.

Microarray analyses have revealed that PLAGL1 is a member of a co-regulated group of imprinted genes known as the imprinted gene network (Varrault et al. 2006, Gabory et al. 2009). As PLAGL1 is a zinc-finger transcription factor that has been shown to bind to the KvDMR1 region and a shared H19/IGF2 enhancer region (Arima et al. 2005, Iglesias-Platas et al. 2014), it is speculated that PLAGL1 may be a “master regulator” of this imprinted gene network, particularly involved in the regulation of imprinted genes at chromosome 11p15.5. As decreased
expression of \textit{PLAGL1} was observed within the ART cord blood samples, the analysis of three imprinted genes from chromosome 11p15.5 – \textit{KCNQ1OT1}, \textit{CDKNIC}, and \textit{IGF2} – thought to be co-regulated with \textit{PLAGL1} were analyzed. We found that \textit{KCNQ1OT1} displayed significantly lower mRNA levels in the IVF samples compared to natural conception controls and, although not statistically significant, there did appear to be a trend of lower mRNA levels in the ICSI group. \textit{IGF2} expression was significantly increased in IVF cord blood samples compared to controls. There were no significant changes in gene expression levels in the ICSI group, and no significant difference in \textit{CDKNIC} expression. The correlation between \textit{PLAGL1} gene expression values with those of the three genes was measured and there was a significant positive correlation between \textit{PLAGL1} and \textit{KCNQ1OT1} in both cord blood and placental tissues, providing further evidence for their interaction \textit{in vivo}. These results suggest that there is dysregulation of an important imprinted gene network in cord blood from IVF pregnancies. As there was down-regulation of \textit{PLAGL1} in both IVF and ICSI cord blood samples, but no changes in expression of \textit{KCNQ1OT1} or \textit{IGF2} in the ICSI group, this may suggest changes were more modest in the ICSI group and therefore were not significant, or it is possible that there are other epigenetic mechanisms involved that were unaffected in the ICSI group. As the sample number was limited for the gene expression analyses, it is possible that with increased sample size there may be significance in the ICSI group, as well as in the IVF group.

Our findings in chapter 3 provide a link between ART and alterations of imprinted genes in phenotypically normal neonates. To our knowledge, this is the first study to examine DNA methylation and gene expression of the \textit{PLAGL1} imprinted gene in cord blood from humans conceived via IVF and ICSI. Our results show increased methylation and decreased expression of \textit{PLAGL1} in the IVF cord blood samples, as well as changes in \textit{KCNQ1OT1} and \textit{IGF2} mRNA
levels. We also observed decreased \textit{PLAGL1} gene expression in the ICSI group, even though no changes in methylation were detected. These findings indicate that the imprinted \textit{PLAGL1} tumour suppressor gene may be susceptible to epimutations in the ART population and could potentially lead to increased risk of imprinting disorders, IUGR, and cancer susceptibility in ART conceived infants, as well as dysregulation of an imprinted gene network involved in growth and development. These changes were not observed in the placenta, which agrees with a study in mice that noted a lack of regulation of the imprinted gene network in the placental tissue, suggesting that the regulatory mechanisms differ between extraembryonic and embryonic tissues (Gabory et al. 2009). Further, we noted extremely low levels of methylation at \textit{PEG10} in all experimental groups, which may indicate tissue-specificity of imprinting during development.

4.2 Limitations

There are some limitations to this study. Although our current study has demonstrated significant alterations of \textit{PLAGL1} within the ART groups, we have no way of knowing whether these changes are occurring throughout the actual procedures, such as during superovulation or \textit{in vitro} culturing, or whether these changes are due to the underlying subfertility of the parents. Previous studies have shown that superovulation may disrupt the acquisition of imprints in the growing oocytes and may also affect maternal gene products required for the maintenance of imprinting during preimplantation development (Market-Velker et al. 2010). Other studies examining the \textit{in vitro} culturing of oocytes have demonstrated that the type of culture media and length of \textit{in vitro} culturing may alter DNA methylation and gene expression at imprinted regions in the embryo (Mann et al. 2004, Doherty et al. 2000, Khosla et al. 2001, Li et al. 2010, Fauque et al. 2007, Market-Velker et al. 2010, Rivera et al. 2008). Further, studies have shown that
males suffering from infertility may have an increased frequency of imprinting errors in their sperm, which may be directly passed onto the offspring through ART (Kobayashi et al. 2007, Marques et al. 2008, Hammoud et al. 2010, Minor et al. 2011). Cohort studies examining children with imprinting disorders after ART have found that subfertility and maternal age contribute to the increased risk of imprinting errors after ART (Ludwig et al. 2005, Davies et al. 2012). As these studies demonstrate, there are multiple mechanisms and factors which may contribute to errors in imprinting and therefore it would be beneficial to control for these factors to narrow in on what is causing these aberrations in our ART cohorts.

As there were some difficulties in sample collection and preparation, both DNA methylation and gene expression data could not be collected for all samples. For placental tissue RNA collection, samples were only used if tissue could be properly stored within RNA-preserving media within 30 minutes of delivery, which was not always attainable. Quality of RNA samples from both cord blood and placental tissue was monitored and those samples which appeared to not be fully intact were not used in analysis, further limiting sample numbers. Additionally, for some participants it was only possible to collect the placenta or the cord blood due to complications with collection or the banking of cord blood by parents, therefore both tissues were not always available for all participants.

4.3 Future studies

In future studies, it will be critical to gather a greater number of samples and subdivide groups not only based on conception mode, such as IVF or ICSI, but also based on the underlying causes of infertility. In terms of the study group, it will be essential to match NC and ART pregnancies as closely as possible in terms of birth weight, gestational age, and maternal
and paternal ages. Further, as superovulation has been shown to induce errors in imprinting in mouse models, it might be revealing to subdivide groups based on length of stimulation. As it has been suggested that imprinting errors may originate from the sperm of infertile males, it would be informative to collect semen samples from the fathers in order to compare imprinting in paternal sperm with imprinting in the offspring.

The inclusion of intrauterine insemination samples (IUI) as an experimental group would be beneficial as it would control for the effect of in vitro culturing. IUI cases may act as a way of observing the effects of subfertility and ovulation induction on imprinting establishment. Unlike IVF and ICSI, the IUI procedure does not include any in vitro manipulation of oocytes and could therefore lead to a better understanding of how this stage affects the proper establishment of DNA methylation at ICRs within the resulting embryo. Further, it may be possible to obtain samples from women undergoing IUI who are not suffering from infertility, such as those using donor semen samples, and compare this to couples using IUI as a means of overcoming subfertility. These studies would be valuable as many studies have investigated methylation status of imprinted genes in IVF and ICSI cases, but very little data has been collected regarding IUI and no studies have been able to rule out the effect of infertility of the parents.

As PLAGL1 functions as a central regulator of imprinted genes and has been shown to be affected in the ART samples in this study, further analysis of the genes of the imprinted gene network should be conducted. In future studies, gene expression analysis of more genes known to be involved in this network should be analyzed, including H19, DLK1, RTL1, and GNAS. Further, as sample size for the cord blood gene expression analysis was limited, including more samples for the evaluation of KCNQ1OT1, CDKN1C, and IGF2 mRNA levels may produce more significant results, particularly in terms of the ICSI cohort. Additionally, similar studies
could be carried out on buccal swabs or peripheral blood samples from ART children, as these tissues may provide more revealing results on the effect of ART on long-term health of the offspring. If studies on ART children are conducted, it would be crucial to control for socioeconomic status, weight, and age, as these factors may be linked to epigenetic status.
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