Maternal Control of Genomic Imprinting: Effects of Infertility and Ovarian Stimulation in a Mouse Model

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Graduate Program in Biochemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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MATERNAL CONTROL OF GENOMIC IMPRINTING:
EFFECTS OF INFERTILITY AND OVARIAN STIMULATION
IN A MOUSE MODEL

(Thesis format: Integrated-Article)

by

Michelle M. Denomme

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
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London, Ontario, Canada

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Abstract

Gametogenesis and early embryogenesis are important stages in which genome-wide epigenetic transitions required for early mammalian development are orchestrated. This is exemplified by the occurrence of genomic imprinting, where epigenetic mechanisms lead to the monoallelic expression of a subset of genes. Parental-specific DNA methylation in the gametes results in the distinct nonequivalence of the parental genomes in the early embryo. Changes from normal gamete and embryo development by impaired fertility or assisted reproductive technologies (ARTs) may disrupt the processes of imprint acquisition and imprint maintenance. My hypothesis is that aberrant imprinted methylation arises from impaired maternal fertility or ovarian stimulation (superovulation), and that maternal effect factors involved in imprint regulation are disrupted by ARTs. To evaluate this, I developed a single cell methylation assay to determine DNA methylation patterns in individual oocytes and preimplantation embryos. I used this technique to examine the effects of compromised maternal fertility on imprint acquisition at three imprinted genes in growing oocytes, revealing that Peg1 DNA methylation acquisition was arrested in CX37-null oocytes, but not Snrpn or Peg3. I also used this technique to assess the effects of superovulation on imprint acquisition at four imprinted genes in MII oocytes, showing that imprint acquisition was unaffected at Snrpn, Kcnq1ot1, Peg3 and H19. Finally, I determined the effects of superovulation on the maternal effect factor, ZFP57, during preimplantation development. Mislocalization away from the nucleus and increased protein levels preceded a decrease in protein enrichment at five imprinted domains, Snrpn, Kcnq1ot1, Peg3, Peg1 and H19, proposing a possible mechanism for imprint methylation maintenance loss following ARTs. Data presented in this thesis suggest that infertility can predispose the oocyte to imprinting errors, but imprint acquisition is a relatively robust process and is unaffected by ARTs. Instead, superovulation disrupts one or more key maternal effects factors, including ZFP57, necessary for imprint maintenance during early embryogenesis. Future studies defining additional factors involved in the regulation of genomic imprinting, and improving current ARTs techniques to minimize effects on this pathway, will lead to a reduced incidence of disease in children born under impaired fertility and through assisted reproduction.
Keywords

Genomic Imprinting, Infertility, Assisted Reproductive Technologies, Ovarian Stimulation, Superovulation, Maternal Effect Factors, ZFP57, TRIM28, Oocyte, Preimplantation Embryo
Co-Authorship

Chapter 2: Liyue Zhang was instrumental in the development of the single oocyte bisulfite mutagenesis assay. Dr. Mellissa Mann recognized the demand for the experimental assay and co-wrote the manuscript.

Chapter 3: Carolina Gillio-Meina performed the oocyte isolations from Gja4-null females. Carlee White and Dr. William MacDonald performed diameter measurements of individual oocytes. Carlee White also provided assistance with the single oocyte bisulfite mutagenesis assays. Dr. William MacDonald was instrumental in the statistical analyses of the data. Dr. Bonnie Deroo provided the Esr2-null females and contributed to writing the manuscript. Dr. Gerald Kidder provided the Gja4-null females, conceived the project and contributed to the writing the manuscript. Dr. Mellissa Mann conceived the project and experiments, aided in analyzing the data and co-wrote the manuscript.

Chapter 4: Liyue Zhang facilitated the design of the experimental assay. Dr. Mellissa Mann conceived the project and experiments, aided in analyzing the data and co-wrote the manuscript.

Chapter 5: Liyue Zhang developed the single blastocyst chromatin immunoprecipitation assay and aided in performing the ChIP assays, developed the Western blot assay for ten oocytes or embryos at each stage, and aided in performing the Western blot experiments. Dr. William MacDonald assisted in statistical analyses of the data. Carlee White provided assistance collecting embryos for experiments. Dr. Mellissa Mann conceived the project and experiments, aided in analyzing the data and co-wrote the manuscript.
Dedications

To my parents, Mike & Jen Denomme.
For your continual love, support, and encouraging words.
For helping me succeed. I love you.

And to my fiancé Mike Tignanelli.
For being right beside me through all the highs and lows,
the late nights, the early mornings, the lost weekends.
For listening, advising, comforting. For making it all worth it.
And mostly, for believing in me. I love you.

Thank you. Truly.
Acknowledgements

I would like to start by thanking my supervisor, Dr. Mellissa Mann, for her mentorship and support throughout my candidacy, and her willingness to take a chance on me. Her passion and dedication for research will continue to be an inspiration to so many within the field.

To my graduate committee, Dr. Andrew Watson, Dr. Bonnie Deroo and Dr. Nathalie Bérubé, thank you for your continual support, advice and guidance. In particular, thank you for the many kind words and references on my behalf. I would like to make a special acknowledgement to Dr. Bonnie Deroo for accepting to read and edit my thesis.

I would also like to acknowledge Barb Green from the Department of Biochemistry, Paula Nopper from the Children’s Health Research Institute (CHRI) and the Collaborative Graduate Program in Developmental Biology, and Terri van Gulik from the CIHR Training Program in Reproduction, Early Development, and the Impact on Health (REDIH), for their administrative support through the years.

Finally, many thanks to my exceptional peers in the Mann lab; past members Dr. Brenna Market Velker, Dr. Michael Golding, Sarah Lalone, and Anne Pin and current members Liyue Zhang, Lauren Landschoot, Dr. William MacDonald, Carlee White, and Saqib Sachani. Thank you for your insight and advice, your comments and your feedback, and your invaluable help over the years. We make a good team.
Ethics Approval

All studies were performed in compliance with the guidelines set by the Canadian Council for Animal Care, and the policies and procedures approved by the University of Western Ontario Council on Animal Care.
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List Of Abbreviations

% Percent
@ At
°C Degrees Celcius
+ Plus
x Times
> Greater than
< Less than
~ Approximately
-/- null or knockout
π pi
ΔCt Delta cycle threshold
5caC 5-formylcytosine
5FC 5-carboxylcytosine
5hmC 5-hydroxymethylcytosine
5mC 5-methylcytosine
A Adenine
α-tubulin Alpha-tubulin
Ac Acetyl group
ADB Antibody dilution/wash buffer
ANOVA Analysis of variance
Apeg3 Peg3 opposite strand
ART / ARTs Assisted reproductive technology / technologies
AS Angelman Syndrome
AS-IC Angelmann Syndrome imprinting center
Ascl2 Achaete-scute complex homolog 2 (Drosophila)
ATP Adenosine triphosphate
Atp10a ATPase, class V, type 10A
B6 C57BL/6
B6(CAST7p6) C57BL/6 (CAST chromosome 7 partial 6)
bp Base pair
BSA Bovine serum albumin
BWS Beckwith-Wiedemann Syndrome
C Cytosine
cAMP Cyclic adenosine monophosphate
CAST Mus musculus castaneus
Cd81 CD81 antigen
Cdkn1c Cyclin-dependent kinase inhibitor 1C
cDNA Complementary deoxyribonucleic acid
Cep41 C entrosomal protein 41
ChIP Chromatin immunoprecipitation
Copg2 Coatamer protein complex, subunit gamma 2
COBRA Combined bisulfite restriction analysis
CpG Phosphorylated cytosine followed by guanine
CTCF CCCTC-binding factor
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<td>Connexin 37</td>
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<td>CX43</td>
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<td>D3</td>
<td>Day 3</td>
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<td>D5</td>
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<tr>
<td>ddH2O</td>
<td>Double-distilled water</td>
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<td>DMR</td>
<td>Differentially methylated region</td>
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<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Dnmt3L</td>
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<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
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<td>dpc</td>
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<td>Days post partum</td>
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<td>Enhancer</td>
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<td>eCG</td>
<td>Equine chorionic gonadotropin</td>
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<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>ERβ</td>
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<td>FSH</td>
<td>Follicle stimulating hormone</td>
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<td>G</td>
<td>Guanine</td>
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<td>gDMR</td>
<td>Gametic differentially methylated region</td>
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<td>Gja4</td>
<td>Gap junction protein, alpha 4</td>
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<td>GOM</td>
<td>Gain of methylation</td>
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<td>Grb10</td>
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<tr>
<td>H1foo</td>
<td>H1 histone family, member O, oocyte-specific</td>
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<td>hCG</td>
<td>Human chorionic gonadotropin</td>
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<td>HCl</td>
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<td>HP1</td>
<td>Heterochromatin protein 1</td>
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<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<td>HSB</td>
<td>High salt buffer</td>
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<tr>
<td>IC</td>
<td>Imprinting center</td>
</tr>
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<td>ICR</td>
<td>Imprinting control region</td>
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<td>ICSI</td>
<td>Intracytoplasmic sperm injection</td>
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<td>IF</td>
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<td>Igf2</td>
<td>Insulin-like growth factor 2</td>
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<td>Igf2r</td>
<td>Insulin-like growth factor 2 receptor</td>
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<tr>
<td>IP</td>
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<td>IPTG</td>
<td>Isopropyl beta-D-1-thiogalactopyranoside</td>
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<tr>
<td>Ipw</td>
<td>Imprinted gene in Prader-Willi syndrome region</td>
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<td>Lithium dodecyl-sulfate</td>
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<td>LMP</td>
<td>Low melting point</td>
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<td>IncRNA</td>
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<td>Loss of imprinting</td>
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<td>me</td>
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<td>MeCP2</td>
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<td>Methylated DNA immunoprecipitation</td>
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<td>min</td>
<td>Minute</td>
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<td>Mit1 / Copg2os2</td>
<td>Coatomer protein complex, subunit gamma 2,</td>
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<tr>
<td>Mkrn3</td>
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CHAPTER 1: Introduction

1.1 Epigenetics

1.1.1 Introduction to Epigenetics

The science of epigenetics has transformed over the last three quarters of a century from an intriguing phenomenon to a modern field of research. First termed by C.H. Waddington in 1942 for the study of causal mechanisms of development (Waddington 2012), epigenetics is now defined as the study of heritable and reversible chromatin modifications that alter the accessibility of genes and regulate gene expression (Jaenisch & Bird 2003, Rodenhiser & Mann 2006). From the combination of the word “genetics” and the prefix “epi”, meaning “above”, epigenetics encompasses alterations in gene function that are not brought about by conventional changes to the DNA sequence. Rather, these modifications reflect the interactions between genetic processes and their external environment, altering the “readability” of the genetic information. The mechanisms that regulate gene activation or repression are essential for development and differentiation, known as epigenetic programming; a similar concept to the “epigenetic landscape” originally described by Waddington (Waddington 2006).

1.1.2 Mechanisms of Epigenetic Regulation

Knowledge of epigenetic regulation continues to evolve as our understanding of its mechanisms expands. DNA methylation, histone modifications, and long non-coding RNAs all contribute to changes in chromatin structure and accessibility of the genetic material (Figure 1-1). These mechanisms play important roles in the establishment of epigenetic programming during gamete and embryo development.

1.1.2.1 DNA Methylation

DNA methylation is the covalent addition of a methyl group onto the C5 position of cytosine residues in CpG dinucleotides. It is typically associated with gene silencing. DNA methyltransferase enzymes DNMT3A, DNMT3B, along with DNMT3L, act as de novo methyltransferases establishing DNA methylation at unmethylated CpGs.
**Figure 1- 1 Mechanisms of Epigenetic Regulation**

The nucleosome consists of DNA (blue line) wrapped twice around an octamer of core histone proteins; H2A, H2B, H3 and H4, with H1 or H5 as linkers. In active domains, the most common histone modifications applied to histone tails (black wavy lines) include acetylation (eg. H3K9Ac, green circle), methylation (eg. H3K4Me, yellow hexagon) and phosphorylation (eg. H3S10P, red triangle). There is typically no DNA methylation at CpG dinucleotides in an active domain (white circles on DNA strands). Long non-coding RNAs (light orange wavy lines) act in *cis* or *trans* to promote gene activation, act as scaffolds, or promote chromatin changes. In repressed domains, nucleosomes are tightly compacted. The most common histone modification is methylation (eg. H3K9Me and H3K27Me, yellow hexagons), along with the presence of DNA methylation (black circles on DNA strands). Long non-coding RNAs (dark orange wavy line) act in *cis* or *trans* to suppress gene expression, act as scaffolds, or promote chromatin changes.
By comparison, DNMT1 is responsible for maintaining DNA methylation through its recognition of hemi-methylated DNA, resulting in heritable DNA methylation through successive rounds of DNA replication by adding a methyl group to the daughter strand. Upon the absence of maintenance DNMT1 activity, the act of passive DNA demethylation results in lower levels of DNA methylation following each round of replication. By comparison, the act of active DNA demethylation remained unclear until the discovery of the TET (ten-eleven translocation) family of DNA dioxygenases. The enzymatic oxidation by TET enzymes convert 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), and further into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), completing the reversion from 5caC to unmethylated cytosine (C) through passive loss (Gu et al. 2011, Inoue et al. 2011, Inoue & Zhang 2011).

1.1.2.2 Histone Modifications

The basic chromatin unit is the nucleosome, containing double-stranded DNA wrapped around an octamer of core histone proteins, two each of H2A, H2B, H3 and H4. Histones H1 or H5 act as linkers (Campos & Reinberg 2009). Chromatin is a dynamic structure that undergoes alterations in its packaging, affecting the accessibility of underlying genes (Bannister & Kouzarides 2011). Heterochromatin is a highly condensed structure that results in gene silencing, while euchromatin is a less compacted configuration supporting gene expression. Switching between active and repressive chromatin states is facilitated by covalent post-translational modifications of histone tails (Bannister & Kouzarides 2011). Histone tail modifications confer either activating or silencing functions, depending on the type of modification and the amino acid residue that is modified. A more active and open chromatin state occurs by acetylation of lysine residues and phosphorylation of serine and threonine residues, examples being H3K9Ac and H3S10P (Mahadevan et al. 1991, Schiltz et al. 1999). In comparison, modifications such as methylation, ubiquitination, and sumoylation can promote either an open or closed chromatin state depending on their location along the histone tail. For example, methylation at H3K4Me is characteristic of an active state, while H3K9Me and H3K27Me promotes a repressive conformation. Histone methylation can be present in mono-, di-, or tri- forms on both lysine and arginine residues. A potential for chromatin
bivalency in the early embryo, where both activating and repressive marks occupy the same stretch of chromatin, is likely a major factor in establishing the correct gene expression profile for embryonic development (Schultz 2002).

1.1.2.3 Long Non-coding RNAs

Owing to novel technologies including tiling arrays and genome-wide cDNA sequencing, it has been revealed that only 1% of the mammalian genome carries protein-coding genes, and in fact the majority of the mammalian transcriptome is comprised of non-coding RNAs (Lee 2012). These non-coding RNAs have been recognized as key players in epigenetic regulation, chromatin remodeling, and alternative mRNA processing (Kuramochi-Miyagawa et al. 2008, Guttman et al. 2009, Khalil et al. 2009).

Long non-coding RNAs (lncRNAs) are defined as RNAs greater than 100 nucleotides in length. They can be found in intronic, intragenic, or intergenic regions along the genome. These regulatory transcripts can be cis-acting or trans-acting, can be in a sense or antisense orientation, and can positively or negatively modify expression of their protein coding or non-coding target genes (Dinger et al. 2008), including suppression of transcriptional processes by transcriptional interference. Finally, they are reported to act as scaffolds, recruiting chromatin modifying complexes to specific loci to enable repressive or activating histone modifications (Mancini-Dinardo et al. 2006, Rinn et al. 2007).

The best-known functional lncRNAs are involved in X chromosome inactivation, an epigenetic dosage-compensation mechanism in female mammals. The inactive X-specific transcript (Xist) lncRNA is expressed from the inactive X chromosome only, and is essential for silencing the entire chromosome (Wutz 2011). The X (inactive)-specific transcript, opposite strand (Tsix), determines allelic choice by repressing Xist transcription on the active X chromosome (Lee et al. 1999, Lee & Lu 1999). Interestingly, the number of lncRNAs within the genome of an organism increases with its morphological complexity (Amaral & Mattick 2008). Many show significant evolutionary conservation (Guttman et al. 2009), as well as distinct cell type specific and developmental stage specific expression profiles (Dinger et al. 2008), supporting their functional role in the regulation of key physiological processes (Koerner et al. 2009).
1.2 Genomic Imprinting

1.2.1 Introduction to Genomic Imprinting

First termed by H. V. Crouse in 1960, an “imprint” was described as a mark that a chromosome harboured independent of its genetic information, but rather on the gametic-origin through which it had been inherited (Crouse 1960). This functional non-equivalence of the parental genomes was later demonstrated through elegant nuclear transplantation studies in the mouse. Specifically, zygotes containing two sets of maternal chromosomes (gynogenotes) result in mid-gestation conceptuses with apparent embryonic tissues but underdeveloped extraembryonic (placental) tissues. Zygotes containing two sets of paternal chromosomes (androgenotes) show opposite results, having placental tissues present but lacking embryonic tissue development. In both uniparental conditions, embryos fail to grow beyond mid-gestation (Barton et al. 1984, McGrath & Solter 1984). The authors concluded that both maternal and paternal contributions are necessary for normal mammalian development, and suggested the term “genomic imprinting” for the process that makes the two pronuclei functionally different (Surani et al. 1984). The use of mouse models carrying two copies of a chromosome pair or part of a chromosome from a single parent, termed uniparental disomy (UPD), show that these parental-specific effects map to specific genomic regions (Cattanach & Kirk 1985). These studies confirmed that imprinted genes expressed only from one of the two inherited chromosomes are important and necessary for early mammalian development.

Genomic imprinting is now defined as a specialized epigenetic mechanism that employs repressive modifications to silence one parental allele and activating modifications on the other parental allele to enable expression (Hirasawa & Feil 2010) (Figure 1-2). These imprinted genes often reside within domains, coordinately regulated in cis by a DNA element within the differentially methylated region (DMR) known as the imprinting center, or imprinting control region (ICR). To be classified as an ICR, the differentially methylated region must be deleted, either spontaneously or experimentally, resulting in loss of imprinting (Spahn & Barlow 2003). Germ line differentially methylated regions and ICRs acquire DNA methylation in the gametes and are faithfully maintained through development.
Figure 1- 2 Genomic Imprinting

Biallelic expression (green bars) from both the maternal and paternal alleles is characteristic of most genes in the genome. A small subset of genes are expressed in a monoallelic, parental-specific manner. Some are expressed from the maternally inherited active allele (red bar), and are repressed on the paternally inherited silent allele (grey bar with black circles representing DNA methylation), while oppositely, others are expressed from the paternally inherited active allele (blue bar) and repressed on the maternally inherited silent allele (grey bar with black circles representing DNA methylation).
1.2.2 Imprinted Domains of Interest

There are approximately 150 imprinted genes identified to date (Morison et al. 2005) ([http://www.mousebook.org/catalog.php?catalog=imprinting](http://www.mousebook.org/catalog.php?catalog=imprinting)). Of these, the five domains below are commonly used for genomic imprinting studies.

1.2.2.1 The H19 Imprinted Domain

The H19 domain, one of the first imprinted domains to be discovered (Bartolomei et al. 1991), is located on human chromosome 11p15.5 and mouse chromosome 7. H19 is a long non-coding RNA expressed from the maternal allele and silent on the paternal allele. Insulin-like growth factor 2 (Igf2), a second gene within the domain, is a protein-coding gene oppositely expressed from the paternal allele and silent on the maternal allele. Located downstream of H19 are enhancer elements, and upstream of H19 is an imprinting control region (ICR) (Srivastava et al. 2000). The H19 ICR acquires DNA methylation during spermatogenesis and is unmethylated in oocytes, resulting in a methylated paternal allele and an unmethylated maternal allele in embryos/offspring.

Regulation of the H19 imprinted domain results from an enhancer-insulator model (Figure 1-3). On the maternal unmethylated allele, the insulator protein CTCF binds to the CpG island forming an intrachromosomal loop. This brings the H19 promoter in close proximity to the enhancer elements, enabling transcription. At the same time, this insulator action prevents interaction of Igf2 and the enhancer elements, resulting in silencing (Hark et al. 2000, Li et al. 2008a). On the paternal allele, DNA methylation at the ICR prevents CTCF binding, enabling a chromatin confirmation that supports interaction between Igf2 and the enhancers, promoting transcription. DNA methylation at the ICR expands into the H19 promoter DMR and effectively represses H19 expression (Srivastava et al. 2000, Kaffer et al. 2001).

The H19 domain has been implicated in the overgrowth disorder Beckwith-Wiedemann Syndrome (BWS) (Shuman et al. 1993). This disorder is characterized by macroglossia, neonatal hypoglycemia, abdominal wall defects, and postnatal growth above the ninetieth percentile (Elliott et al. 1994). There is also an increased incidence of Wilm’s tumor in these patients (Shuman et al. 1993).
The H19 imprinted domain undergoes genomic imprint regulation through an insulator/enhancer model. Top and bottom strands correspond to maternal and paternal alleles, respectively. The maternal allele is unmethylated at the imprinting control center (ICR), allowing the insulator protein CTCF (orange pentagon) to bind. CTCF interacts with the ICR and the unmethylated DMR1 generating a looping structure. This prevents the interaction of the enhancer elements (E; green boxes) with the upstream Igf2 gene promoter resulting in repression, but allows for interaction with the H19 promoter resulting in expression (red box). On the paternal allele, DNA methylation (black circles) of the ICR represses H19 expression and prevents binding of CTCF, generating an alternative looping structure with the methylated DMR2 that allows the enhancer elements to interact with the Igf2 promoter, resulting in expression (blue box). Errors in the H19 domain have been reported to associate with both Beckwith-Wiedemann Syndrome and Silver-Russell Syndrome.

Figure 1-3 The H19 Imprinted Domain
An estimated 5% of BWS patients result from a gain of DNA methylation (hypermethylation) imprinting defect at the maternal H19 ICR, causing biallelic silencing of H19 and overexpression of IGF2 (Sparago et al. 2004, Prawitt et al. 2005). Interestingly, H19 is also implicated in a second imprinting disorder, Silver-Russell Syndrome (SRS). DNA hypomethylation of the H19 domain occurs in up to 44% of these cases (Eggermann et al. 2010). SRS is further discussed in the Peg1 section below.

1.2.2.2 The Kcnq1ot1 Imprinted Domain

The KCNQ1 overlapping transcript 1 (Kcnq1ot1) imprinted domain is situated in close proximity to the H19 domain, and is located on human chromosome 11p15 and on mouse chromosome 7. The Kcnq1ot1 ICR acquires gametic DNA methylation in the oocyte and is unmethylated in sperm. Thus, embryos/offspring possess a methylated maternally-inherited allele and unmethylated paternally-inherited allele. Kcnq1ot1 is a long non-coding RNA involved in imprinted regulation of the entire domain. Its promoter is embedded in the ICR and consequently its imprinted pattern corresponds accordingly; maternally repressed and paternally expressed (Mancini-DiNardo et al. 2003, Pandey et al. 2004) (Figure 1-4). On the paternal allele, the Kcnq1ot1 ICR is unmethylated, allowing expression of the Kcnq1ot1 lncRNA, which in turn represses all imprinted genes within the domain. A study published from my research group indicates that transcription of Kcnq1ot1 extends 471 kb in length on the paternal allele (Golding et al. 2011). This results in repression of all other imprinted genes within the domain. Controversy exists within the field as to whether the act of Kcnq1ot1 transcription through the domain creates transcriptional interference of downstream genes or whether the lncRNA itself plays a post-transcriptional role in coating the domain, similar to that of Xist on the X chromosome. However, the study from my colleagues supports the former. RNA-interference technology via short hairpin RNA (shRNA)-mediated Kcnq1ot1 RNA depletion in embryonic stem (ES), trophoblast stem (TS) and extra-embryonic endoderm stem (XEN) cells shows no observable effect on the imprint status of the surrounding genes in the domain (Golding et al. 2011). By comparison, DNA methylation on the maternal ICR results in repression of Kcnq1ot1 and allows tissue-specific expression of all other imprinted genes within the domain.
The *Kcnq1ot1* imprinted domain is regulated through long non-coding RNA mediated silencing. Top and bottom strands correspond to maternal and paternal alleles, respectively. On the paternal allele, the ICR is unmethylated, allowing expression of the lncRNA *Kcnq1ot1* (blue wavy line). This in turn represses all imprinted genes within the domain (grey boxes). On the maternal allele, the *Kcnq1ot1* ICR is methylated, preventing expression of *Kcnq1ot1* and facilitating an active domain where all other imprinted genes are expressed (red boxes; maternal expression in embryo and placenta, pink boxes; maternal expression in placenta). Errors in the *Kcnq1ot1* domain give rise to Beckwith-Wiedemann Syndrome.

**Figure 1-4 The *Kcnq1ot1* Imprinted Domain**

The *Kcnq1ot1* imprinted domain is regulated through long non-coding RNA mediated silencing. Top and bottom strands correspond to maternal and paternal alleles, respectively. On the paternal allele, the ICR is unmethylated, allowing expression of the lncRNA *Kcnq1ot1* (blue wavy line). This in turn represses all imprinted genes within the domain (grey boxes). On the maternal allele, the *Kcnq1ot1* ICR is methylated, preventing expression of *Kcnq1ot1* and facilitating an active domain where all other imprinted genes are expressed (red boxes; maternal expression in embryo and placenta, pink boxes; maternal expression in placenta). Errors in the *Kcnq1ot1* domain give rise to Beckwith-Wiedemann Syndrome.
Like the *H19* imprinted domain, misregulation of the *Kcnq1ot1* imprinted domain occurs in patients with Beckwith-Wiedemann Syndrome (Weksberg et al. 2001, Weksberg et al. 2005). Loss of DNA methylation on the maternal *KCNQ1OT1* ICR occurs in 50% of BWS patients, causing biallelic expression of the *KCNQ1OT1* lncRNA and biallelic silencing of the maternally expressed genes, including potassium voltage-gated channel, KQT-like subfamily, member 1 (*KCNQ1*) and cyclin-dependent kinase inhibitor 1C (*CDKN1C*) (Horike et al. 2000, Weksberg et al. 2001, Niemitz et al. 2004).

### 1.2.2.3 The *Snrpn* Imprinted Domain

The small nuclear ribonucleoprotein N (*Snrpn*) imprinted domain is located on human chromosome 15q11-13 and mouse chromosome 7. The ICR is embedded within the promoter and first exon of *Snrpn*. It has a maternal specific DNA methylation mark acquired in the oocytes, and is unmethylated in sperm (Shemer et al. 1997). On the paternal allele, the unmethylated *Snrpn* ICR facilitates transcription of a long non-coding RNA, *Snrpnlt* (*Ube3a-as*), which directs expression of the other paternally expressed genes and silences the maternally expressed ubiquitin protein ligase E3A (*Ube3a*) and ATPase, class V, type 10A (*Atp10a*) genes (Figure 1-5). Conversely, DNA methylation at the ICR on the maternal allele prevents transcription of *Snrpnlt* and enables *Ube3a* and *Atp10a* expression (Horsthemke & Wagstaff 2008).

The *SNRPN* imprinted domain is implicated in two neurological imprinting disorders, Angelman Syndrome (AS) and Prader-Willi Syndrome (PWS). Angelman Syndrome is represented by ataxia, severe mental and motor retardation with absence of speech, aggressive behaviour, excessive inappropriate laughter and seizures (Nicholls et al. 1998, Jiang et al. 1999, Nicholls & Knepper 2001). Prader-Willi Syndrome is characterized by hypotonia and failure to thrive in the neonatal period, respiratory distress, hyperphagia leading to obesity, hypogonadism, short stature, and behavioural problems associated with mental retardation (Butler 1990, Nicholls et al. 1998, Nicholls & Knepper 2001). Up to 70% of patients harbour large deletions in the *SNRPN* imprinted region (Horsthemke 1997). Uniparental disomy (Nicholls et al. 1989) and imprinting defects (Buiting et al. 1990) account for the other portion of these cases.
Figure 1-5 The *Snrpn* Imprinted Domain

The *Snrpn* imprinted domain is regulated by a bipartite ICR. Top and bottom strands correspond to maternal and paternal alleles, respectively. The AS-IC promotes expression of the maternally expressed genes (red boxes). Loss of maternal DNA methylation and loss of expression of the maternally expressed genes result in Angelman Syndrome. The PWS-IC is involved in expression of the paternally expressed genes (blue boxes), like *Snrpn*. Gain of paternal DNA methylation and loss of expression on the paternally expressed genes result in Prader-Willi Syndrome.
Two distinct regions in the \textit{SNRPN} ICR have been identified through microdeletions, characterizing it as a bipartite ICR (Horsthemke 1997). The centromeric component is involved in promoting expression of \textit{UBE3A} and \textit{ATP10A} (AS-IC) on the maternal allele. AS is caused by loss of function of these normally maternally expressed genes via paternal UPD, deletion of the AS-IC, or loss of DNA methylation at the \textit{SNRPN} ICR. One causative factor identified in the development of Angelman Syndrome is silencing of \textit{UBE3A}, an enzyme involved in the ubiquitin protein degradation system in the brain (Horsthemke & Wagstaff 2008). The telomeric component of the bipartite ICR (PWS-IC) is involved in expression of paternally expressed 12 (\textit{PEG12/FRAT3}) makorin ring finger protein 3 (\textit{MKRN3}), MAGE-like 2 (\textit{MAGEL2}), necdin, melanoma antigen family member (\textit{NDN}), and \textit{SNRPN} on the paternal allele alone (El-Maarri \textit{et al.} 2001). PWS is caused by loss of function of the paternally expressed genes and can occur through maternal UPD, deletion of the PWS-IC, or gain of DNA methylation at the \textit{SNRPN} ICR.

\subsection*{1.2.2.4 The Peg1 Imprinted Domain}

The paternally expressed gene 1 (\textit{Peg1}, also known as \textit{Mest}) imprinted domain is located on human chromosome 7q32 and mouse chromosome 6. A \textit{Peg1} ICR has not yet been molecularly delineated, but the known DMR spanning the \textit{Peg1} promoter acquires its gametic DNA methylation mark in the oocyte. This results in a methylated maternal allele and an unmethylated paternal allele in embryos/offspring (Riesewijk \textit{et al.} 1997, Nishita \textit{et al.} 1999). Also located in the domain are two maternally expressed genes, Kruppel-like factor 14 (\textit{Klf14}) and coatmer protein complex, subunit gamma 2 (\textit{Copg2}), and a paternally expressed gene, \textit{Copg2} opposite strand 2 (\textit{Mit1}) (\textbf{Figure 1-6}). Regulation of this domain remains largely unknown, although a study has shown that TRIM28 and its interaction with HP1 are essential for maintaining repression on the methylated maternal allele (Riclet \textit{et al.} 2009). Maternal UPD harbouring the \textit{PEG1} imprinted domain arises in 5\% of Silver-Russell Syndrome patients (Kotzot \textit{et al.} 1995, Eggermann \textit{et al.} 2010). SRS is an imprinting disorder characterized by intrauterine and post-natal growth retardation (Wollmann \textit{et al.} 1995), low birth weight, triangular shaped face and body asymmetry (Silver \textit{et al.} 1953, Russell 1954).
Figure 1-6 The Peg1 Imprinted Domain

The Peg1 imprinted domain. Top and bottom strands correspond to maternal and paternal alleles, respectively. The Peg1 DMR is methylated on the maternal allele, silencing Peg1 (also known as Mest) and enabling expression of Copg2 and Klf14 (red boxes). On the paternal allele, the Peg1 DMR is unmethylated, enabling expression of Peg1 (blue box) while Copg2 and Klf14 are silent. Imprinted genes are interspersed among non-imprinted genes (green boxes) that are expressed from both parentally inherited alleles. Silver-Russell Syndrome has been linked to imprinting errors at this locus.
1.2.2.5 The Peg3 Imprinted Domain

The paternally expressed gene 3 (Peg3) imprinted domain is situated on human chromosome 19q13.4 and mouse chromosome 7. An identified ICR has not yet been delineated, but the Peg3 DMR is differentially methylated in the gametes, acquiring DNA methylation only in the oocytes while in sperm the Peg3 DMR is unmethylated. This differential methylation is carried into adulthood (Li et al. 2000, Huang & Kim 2009). As the ICR is embedded in the Peg3 promoter and first exon, Peg3 is silenced accordingly on the maternal allele and expressed paternally (Figure 1-7). The transcriptional start site of ubiquitin specific peptidase 29 (Usp29) lies close to that of Peg3 and is also paternally expressed, along with zinc finger protein 264 (Zfp264) located further upstream. Zinc finger imprinted 1, 2, and 3 (Zim1, Zim2, Zim3, respectively) are conversely maternally expressed. To date, no human imprinting disorders has been linked to errors at this locus. However, loss of paternal Peg3 expression in mice results in growth retardation, lower metabolic rate, delayed development (Curley et al. 2005), increased apoptosis in the brain (Broad et al. 2009), as well as aberrant maternal behaviour (Champagne et al. 2009).

1.2.3 Epigenetic Programming

Gamete and early embryo development are important stages when genome-wide epigenetic transitions are orchestrated. During oogenesis and spermatogenesis, previous somatic epigenetic modifications are erased, and new sex-specific epigenetic marks are acquired (Hajkova et al. 2002, Kageyama et al. 2007). Paternal DNA methylation acquisition occurs by DNMT3B/3L during prenatal stages of spermatogenesis and is completed by birth (Saitou et al. 2012). During spermiogenesis, protamines replace the majority of histones (Carrell 2012), and the resulting effect is tight compaction of chromatin. Histone-containing chromatin, which is situated at spermatogenic, developmental, microRNA and paternally expressed imprinted gene promoters, harbors active histone modifications (H3Ac, H4Ac, and H3K4me2/3) or bivalency marks (H3K4me3 and H3K27me3) and is hypomethylated, while that at maternally expressed imprinted genes possess repressive modifications (H3K9me2/3 and DNA methylation) (Hammoud et al. 2009, Brykczynska et al. 2010, Carrell 2012, Nakamura et al. 2012).
Figure 1- 7 The Peg3 Imprinted Domain

The Peg3 imprinted domain. Top and bottom strands correspond to maternal and paternal alleles, respectively. The Peg3 imprinted domain is methylated at the DMR on the maternal allele, silencing genes nearby, like Peg3 and the A Peg3 lncRNA, but enabling expression of periphery genes (red boxes). On the paternal allele, the DMR is unmethylated, enabling expression of nearby genes (blue boxes) and the A Peg3 lncRNA (blue wavy line). No human imprinting disorder has been linked to errors at this locus, but some characteristics coincide with known syndromes.
During oogenesis, acquisition of maternal DNA methylation begins comparatively later (puberty) in primary to antral stage follicles and is mostly complete in MII-ovulated oocytes (Saitou et al. 2012). The MII oocyte genome also possesses repressive histone modifications (H3K9me2/3 and H4K20me3) (Lepikhov et al. 2010, Hales et al. 2011) (Figure 1-8).

Following fertilization, global epigenetic reprogramming occurs again with a switch from a gamete-specific to embryonic state. The pronuclear paternal genome is rapidly and actively demethylated by the enzymatic oxidation of TET3, undergoing protamine to histone replacement, and further acquiring active histone modifications (H4Ac, H3Ac, and H3K4me2/3) (Lepikhov et al. 2010, Hales et al. 2011). By comparison, the maternal pronuclear genome contains active (H4Ac, H3Ac, and H3K4me2/3) and repressive modifications (H3K9me2/3, H3K27me2/3, and H4K20me3) and becomes passively demethylated during preimplantation development at each replicative cycle in the absence of DNA methylation maintenance activity. By the four-cell stage, the paternal genome acquires repressive histone modifications (H3K9me2, H3K27me2, and H3K27me3) and is globally no longer distinguishable from the maternal genome (Hales et al. 2011). Thus, before and after fertilization, the paternal and maternal genomes have acquired asymmetric epigenetic modifications.

The two parental genomes undergo extensive changes in global DNA methylation during preimplantation development, and post-implantation, de novo DNA methylation gradually increases in accordance with cellular differentiation (Monk et al. 1987). DNA methylation at imprinting domains is excluded from the global DNA methylation events during preimplantation development; the ICR of the methylated allele remains methylated and protected from demethylation, while the unmethylated allele remains unmethylated (Morgan et al. 2005). Maintenance of these methylation marks through preimplantation development requires expression of DNMT1 (Howell et al. 2001, Cirio et al. 2008, Hirasawa et al. 2008, Kurihara et al. 2008), as well as changes in histone modifications, including H3K9me2 and H3K27me3 enrichment on the repressed allele (Kim & Ogura 2009) and repressive proteins including DPPA3, ZFP57, and TRIM28 (discussed below) (Figure 1-9).
In mature sperm, protamines tightly compact chromatin into toroids (90–99% chromatin) that are punctuated by histone solenoids (1–10% chromatin). Sperm DNA is hypermethylated (red; DNA me) except at regions bearing active and bivalent histone modifications. By comparison, histones compact chromatin in the mature oocyte. Chromatin is further condensed into loops that are bound to spindle fibers. Oocyte chromatin is hypermethylated and carries repressive histone modifications (Denomme & Mann 2012).
Figure 1- 9 DNA Methylation Asymmetry

Paternal (blue line) and maternal (red line) DNA methylation is erased in primordial germ cells. Acquisition of DNA methylation by de novo DNMTs (red and orange circles) occurs earlier in male compared to female germ cell development. Following fertilization, the paternal genome is rapidly and actively demethylated (light blue line) while the maternal genome (light red line) is passively demethylated at each replication cycle. Differential DNA methylation at imprinted genes is protected from demethylation. DPPA3 (yellow circle) protects against active demethylation in the zygote. ZFP57 (pink circle), TRIM28 (blue circle), and DNMT1 (purple circle) protect against passive demethylation during preimplantation development. Infertility/subfertility and various assisted reproductive technologies (ARTs) may cause epigenetic instability at the erasure, acquisition, and maintenance stages (Denomme & Mann 2012)
1.3 Programming of Genomic Imprinting

1.3.1 Maternal Effect Factors

During oocyte growth, transcripts from the maternal genome accumulate. While most are translated into proteins by oocyte machinery, some are sequestered and remain dormant until after fertilization (Racki & Richter 2006). The majority of maternal transcripts (90%) are degraded within the zygote after completing their oogenic function. However, a subset of maternal transcripts are required for early developmental programming prior to embryonic gene activation (Sirard 2012). This pivotal event occurs at the 2-cell stage in mice, 4-cell stage in humans and 8–16-cell stage in cattle (Bruce 2013). Thus, the health of the early preimplantation embryo is largely dependent on the oocyte for many crucial aspects of embryonic development, requiring transcription factors, pluripotency-enabling factors and chromatin remodelling factors, among many others (Li et al. 2010). Since imprinted methylation marks must be maintained during the oocyte-to-embryo transition, maternal-effect genes may also be expected to play a role in genomic imprint regulation (Figure 1-9).

One of the first hypotheses proposing that maternal-effect factors play a role in embryonic epigenetic regulation was by Marilyn Monk (Monk 1990). She postulated that reduced levels of global DNA methylation in oocytes compared with that in spermatozoa would permit increased expression of a repertoire of stored molecules needed to support development after fertilization. In accordance with this hypothesis, high concentrations of DNA methyltransferases present in the oocyte would ensure propagation of imprinted methylation patterns during preimplantation development. In a second hypothesis of ‘mother knows best’ (Miri & Varmuza 2009), it was posited that the engulfing of the maternal pronuclear chromatin by the nuclear membrane would generate a protective shield around it, allowing selective transport of critical proteins. By comparison, the paternal genome, which is bathed in ooplasm, would be exposed to oocyte remodeling systems, permitting epigenetic transformation of the paternal genome.

Since these hypotheses were proposed, four maternal-effect proteins have been identified that protect imprinted methylation sites during preimplantation development:
(i) developmental pluripotency-associated 3 (DPPA3; also known as STELLA/PGC7) (Nakamura et al. 2007); (ii) DNA methyltransferase 1 oocyte-specific isoform (DNMT1o) (Hirasawa et al. 2008); (iii) zinc finger protein 57 (ZFP57) (Li et al. 2008b); and (iv) tripartite motif-containing 28 protein (TRIM28, also known as KAP1/TIF1b) (Messerschmidt et al. 2012).

1.3.1.1 The Maternal Effect Factor DPPA3

DPPA3 was first described as a primordial germ cell marker in the ovary and testis (Goto et al. 2002). Although it is not required for germ cell specification in mice, homozygous null females have reduced fertility because early embryos undergo precocious compaction and fail to reach the blastocyst stage (Payer et al. 2003, Bortvin et al. 2004). Thus, maternally supplied DPPA3 is important during cleavage stages of preimplantation development. Embryonic gene activation from the paternal DPPA3 allele at the 2-cell stage does not rescue the abnormalities resulting from maternal deletion. This outcome indicates that a crucial function of DPPA3 must occur after fertilization but before the 2-cell stage (Nakamura et al. 2007). During this time, the zygote undergoes active demethylation of the paternal genome, whereby TET3 methylcytosine dioxygenase oxidizes 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (Nakamura et al. 2007, Nakamura et al. 2012). Zygotes lacking maternal DPPA3 actively demethylate both the paternal and maternal genomes (Nakamura et al., 2007), demonstrating that DPPA3 plays a role in protecting the maternal genome from active demethylation following fertilization. This protection is mediated by DPPA3 binding to H3K9me2-enriched nucleosomes to produce a change in chromatin structure, reducing TET3 chromatin affinity and thereby inhibiting 5mC conversion to 5hmC (Nakamura et al., 2012). Thus, specificity is conferred by H3K9me2–DPPA3 interactions on the maternal but not the paternal genome.

The effects on the whole genome brings into question how the paternal DNA methylation at imprinted genes survive. The protective function of DPPA3 also extends to imprinted genes harbouring H3K9me2 (Nakamura et al. 2012). These imprinted genes include not only those with maternally inherited methylation (Peg1, Peg3, Peg10) but also those with paternally inherited methylation (H19, Rasgrf1). In consequence, zygotes
generated from DPPA3-null oocytes have significantly reduced DNA methylation levels at imprinted genes (Nakamura et al. 2007). Thus, DPPA3 is a maternal-effect protein that acts to protect the maternal genome, as well as imprinted genes inherited from both parents, from active demethylation in zygotes.

1.3.1.2 The Maternal Effect Factor DNMT1o

The DNA methyltransferase family catalyses both de novo and maintenance methylation. DNA methyltransferase 3A (DNMT3A) and its accessory protein DNMT3L are required for acquisition of DNA methylation at unmethylated cytosines in both male and female germ lines (Bourc'his et al. 2001, Kaneda et al. 2004). By comparison, DNMT1 preferentially recognizes and methylates hemimethylated DNA, maintaining methylation at each replicative cycle, including at imprinted regions (Yoder et al. 1997). Two DNMT1 isoforms are present in mature oocytes and preimplantation embryos: oocyte-specific (DNMT1o) and somatic (DNMT1s) isoforms. DNMT1o accumulates to high concentrations during oocyte growth (Bao et al. 2000) and is the abundant form expressed in oocytes and preimplantation embryos (Mertineit et al. 1998). DNMT1o displays an intriguing localization pattern during preimplantation development. While predominantly cytoplasmic, it transiently localizes to the nucleus at the 8-cell stage (Howell et al. 2001), although this localization pattern is controversial (Hirasawa et al. 2008, Kurihara et al. 2008). Maternal- and embryonic-derived DNMT1s is also present in preimplantation embryos, albeit at much lower concentrations than DNMT1o (Cirio et al. 2008, Kurihara et al. 2008). Both forms of DNMT1 play a role in protecting imprinted genes from passive demethylation. DNMT1o functions as a maternal-effect protein, since DNMT1o deficiency does not affect methylation acquisition in the oocyte, but instead produces loss of imprinted methylation in mutant embryos derived from Dnmt1o-null oocytes (Howell et al. 2001, Cirio et al. 2008). For the insulin-like growth factor 2 receptor (Igf2r) imprinted gene, methylation loss occurred in 8-cell, morula, and blastocyst but not 4-cell mutant embryos. Analysis of morula, day-7.5 and day-9.5 mutant embryos also showed Snrpn and H19 DNA methylation loss with variable levels of methylation loss among embryos (Howell et al. 2001, Cirio et al. 2008). Depletion of maternal DNMT1s via microinjection of oocytes with anti-DNMT1s antibody similarly
produced a partial loss of *H19* imprinted methylation in morula (Kurihara *et al.* 2008). Deficiency of maternal DNMT1o and 1s also caused a partial loss of *H19, Rasgrf1, Peg3* and *Snrpn* DNA methylation at the blastocyst stage (Hirasawa *et al.* 2008). These results indicate that loss of maternal DNMT1o and/or DNMT1s produces a partial loss of imprinted methylation. By comparison, lack of maternal + embryonic DNMT1 generated complete demethylation of the *H19, Rasgrf1, Peg3* and *Snrpn* ICR in blastocysts (Hirasawa *et al.* 2008). Thus, maternal and embryonic DNMT1 provide protection from preimplantation passive demethylation, which occurs in part by selective targeting of DNMT1 to imprinted regions through ZFP57 hexanucleotide binding and TRIM28 repressive complex recruitment (Quenneville *et al.* 2011).

### 1.3.1.3 The Maternal Effect Factor ZFP57

ZFP57, a Krüppel-associated box (KRAB) domain-containing zinc finger protein originally identified as a marker of undifferentiated mouse embryonic stem cells (Okazaki *et al.* 1994), is an essential maternal–embryonic-effect gene required for effective imprint maintenance during preimplantation development. Functionally, ZFP57 plays a role in gene repression by binding to methylated DNA at the hexanucleotide motif, TGCCGC (Quenneville *et al.* 2011). This hexanucleotide is present in mouse and human DMRs. During preimplantation development, maternally derived ZFP57 is likely the sole source of this protein. According to expression arrays, embryonic *Zfp57* transcription does not begin until at least the blastocyst stage (Zeng *et al.* 2004), a time at which embryonic ZFP57 protein has been detected (Li *et al.* 2008b). Examination at earlier stages is still required to determine when embryonic ZFP57 protein is first produced. To untangle the roles of maternally derived and embryonically produced ZFP57 protein, targeted deletion and mating strategies were engineered to obtain maternal, embryonic or maternal + embryonic deficiencies. Deletion of maternal + embryonic ZFP57 resulted in embryonic lethality (Li *et al.* 2008b). While ablating embryonic ZFP57 produced partial neonatal lethality, maternal ZFP57 deletion was not lethal due to rescue by embryonic ZFP57. With respect to imprinted gene regulation, mid-gestation embryos with loss of maternal + embryonic ZFP57 failed to maintain DNA methylation at multiple maternally methylated DMRs (*Snrpn, Peg1, Peg3, Peg5*) as well
as at the paternally methylated Gtl2 ICR (Li et al. 2008b). Embryos with maternal ZFP57 deficiency exhibited hypomethylation of Snrpn at day 3.5, but methylation levels were rescued at day 13.5 by embryonic ZFP57. By comparison, embryos with an embryonic ZFP57 mutation experienced a partial loss of DNA methylation that varied among embryos (Li et al. 2008b). In humans, embryonic ZFP57 mutations also affected DNA methylation at several imprinted regions (PLAGL1, GRB10, PEG3) (Mackay et al. 2008). As stated, ZFP57 binds to its methylated hexanucleotide recognition site in imprinted genes (Quenneville et al. 2011), and therefore may contribute to the imprinting errors observed in infertility and ARTs patients.

1.3.1.4 The Maternal Effect Factor TRIM28

TRIM28 is a central element in a heterochromatin-inducing macromolecular complex that was recently shown to protect imprinted regions from demethylation. TRIM28 recruits heterochromatin protein 1 (HP1), DNMT1 and the H3K9me3-catalyzing histone methyltransferase SETDB1, among others (Schultz et al. 2001, Schultz et al. 2002, Iyengar & Farnham 2011). The specificity of this complex is achieved through TRIM28 interactions with KRAB zinc finger proteins (Schultz et al. 2001, Schultz et al. 2002), a role taken by ZFP57 in imprinted genes, in which repression occurs via ZFP57–TRIM28 interactions (Zuo et al. 2012). TRIM28 is a maternal-effect protein (Messerschmidt et al. 2012), as indicated by the partial embryonic lethality resulting from maternal TRIM28 deficiency. In mid-gestation Trim28 maternal mutant embryos, loss of imprinted methylation is observed at the H19, Snrpn and Gtl2 ICRs, which vary between embryos (Messerschmidt et al. 2012). Although embryonic TRIM28 activation occurs at the 2-cell stage with the protein appearing at the 4-cell stage, H19 imprinted methylation is still lost in 4- and 8-cell Trim28 maternal mutant embryos, demonstrating a role for maternal TRIM28 in protection from passive demethylation.

Overall, these studies indicate that epigenetic reprogramming within the preimplantation embryo necessitates the recruitment of specialized maintenance complexes to imprinted regions to ensure epigenetic integrity, both directly after fertilization and throughout cleavage-stage development. Misregulation of this pathway
may explain loss of imprinting regulation among those with infertility and following assisted reproduction.

1.4 Infertility and Assisted Reproduction

1.4.1 Introduction to Infertility

Worldwide, approximately 1 in 6 couples experience some form of impaired fertility (Wright et al. 2005, Sunderam et al. 2009), defined as an inability to conceive after one full year of regular unprotected sexual intercourse, and reduced to half a year for women above 35 years of age. Infertility/subfertility can arise from an array of genetic, epigenetic, endocrine, environmental, and physical factors. Treatment for impaired fertility encompasses the use of assisted reproductive technologies (ARTs).

1.4.2 Female Infertility and Genomic Imprinting

The most common variable influencing natural conception in modern society is reproductive age. Advanced maternal age (>35 years) is directly related to a decline in female fecundity with a reduced oocyte reserve and poor oocyte quality (Liu & Case 2011). This leads to a plethora of embryonic issues including compromised embryo quality, reduced blastocyst formation and expansion rate (Janny & Menezo 1996), poor implantation efficiency and an increase in spontaneous abortion rates (Menken et al. 1986). The decline arises from chromosomal abnormalities, deviation in levels of maternal-effect factors from the oocyte, and altered patterns of gene expression, among others (Janny & Menezo 1996).

An increased incidence of imprinting disorders, like Beckwith-Wiedemann Syndrome (BWS) and Angelman Syndrome (AS) in the ARTs population leads to the question of whether infertility predisposes embryos to imprinting errors. An examination of 16 AS children born to subfertile couples found 4 to arise by sporadic imprinting defects, including 2 from couples without assisted reproduction (Ludwig et al. 2005). In addition, a case of two BWS children in the same family, one born via ARTs and the other born naturally, suggests that impaired fertility may be associated with mechanisms leading to imprinting disorders (Strawn et al. 2010).
A Dutch study evaluating ARTs and parental infertility reported the same relative risk of AS and BWS in subfertile couples with and without ARTs (Doornbos et al. 2007), indicating that the increased prevalence of imprinting disorders can be explained by compromised fertility. Significantly, advanced maternal age is more frequent in these AS and BWS mothers compared to the general population, suggesting that advanced maternal age may decrease fertility and increase the risk of imprinting disorders.

Barriers to a thorough evaluation of human infertility exist, including the ethical and social issues involved in using human oocytes and embryos. Thus, validating a link between female infertility and genomic imprinting is proven difficult. Similar to studies on ARTs techniques, animal models can assist in addressing this issue. The mouse is advocated as the model system of choice for studies of early embryo development and its molecular regulation (Quinn & Horstman 1998, Summers & Biggers 2003). In mouse, advanced maternal age compromises post-implantation development, although age-related change in imprinted DNA methylation is not detected at Snrpn, Kcnq1ot1, Peg1, H19, Igf2r, and zinc finger (CCCH type), RNA binding motif and serine/arginine rich 1 (Zrsr1) in blastocysts and mid-gestation conceptuses (Lopes et al. 2009). As imprints are acquired during oocyte growth, studies are required to determine how genomic imprinting acquisition in the oocyte changes under female infertility conditions. A complex endocrine signaling pathway is active in the ovary regulating follicle and oocyte development (Drummond & Fuller 2012). In addition, it is known that development of healthy oocytes is dependent on interactions between the growing oocyte and surrounding follicular cells (Kidder & Vanderhyden 2010). Compromised female fertility through an impaired endocrine pathway and/or disrupted intercellular communication may lead to a molecular mechanism causing imprinting acquisition errors in the infertile population.

1.4.3 Introduction to Assisted Reproductive Technologies

Owing to the pioneering work of R. G. Edwards and the first assisted conception in 1978, it is estimated that assisted reproductive technologies (ARTs) has enabled the birth of ~5 million children worldwide from couples with infertility/subfertility (International Committee for Monitoring Assisted Reproductive Technologies). ARTs
encompasses any treatment modality that is used to improve fertility and establish a pregnancy, including ovarian stimulation (superovulation), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and in vitro embryo culture (IVC) as well as the experimental procedures such as in vitro oocyte maturation (IVM), and oocyte and ovarian tissue cryopreservation. ARTs can lead to adverse prenatal and postnatal outcomes, including increased risk of intrauterine growth restriction, premature birth, low birth weight, congenital anomalies, and genomic imprinting syndromes (Savage et al. 2011). To reduce these risks, it is paramount to determine which aspects of treatment lead to adverse effects so they may be modified for improved safety. Given that both impaired fertility and ARTs alter the gamete and embryo environment and coincide with crucial epigenetic events during those periods, epigenetic instability may be the primary determinant of these suboptimal outcomes (Figure 1-9).

1.4.4 Imprinting Syndromes and Assisted Reproductive Technologies

Disruptions in the asymmetric parental states during gametogenesis and/or embryogenesis can have severe consequences for growth and development. Of significance is that many imprinted genes play critical roles in the development of the embryo, and their misregulation is linked to the development of human imprinting disorders, including Angelman Syndrome and Beckwith-Wiedemann Syndrome (Hirasawa & Feil 2010). While generally healthy, evidence indicates that children conceived by assisted reproduction may be at increased risk for imprinting errors and the development of these rare genomic imprinting disorders (Denomme & Mann 2012). The concern with ARTs is that the timing of these procedures coincides with crucial epigenetic reprogramming events in gametes and early embryos, leading to their possible disruption. This concern prompts the investigation of many procedures to determine their effects on imprinted gene regulation (Denomme & Mann 2012).

An increased incidence of the human imprinting disorders AS and BWS with the use of ARTs is observed (Cox et al. 2002, DeBaun et al. 2003, Gicquel et al. 2003, Maher et al. 2003, Orstavik et al. 2003, Halliday et al. 2004, Ludwig et al. 2005, Rossignol et al. 2006, Sutcliffe et al. 2006). More specifically, the incidence of AS in the general population is approximately 1 in 16,000 births, with 5% of these cases related to
imprinting abnormalities (Cox et al. 2002, Van Buggenhout & Fryns 2009). As the prevalence of AS is low, large-scale studies containing sufficient numbers of patients is difficult to achieve. However, seven cases of AS following the use of ARTs are reported to date, five of which display imprinting abnormalities (Cox et al. 2002, Chang et al. 2005, Ludwig et al. 2005, Sutcliffe et al. 2006). This is a significantly higher proportion in the ARTs-population compared with the non-ARTs population.

BWS is also reported in the ARTs population. This syndrome affects about 1 in 13,700 children (Weksberg et al. 2010). Parents of children with BWS are 4 to 9 times more likely to have undergone fertility treatments than the general population, and a higher incidence of BWS is seen in ART children compared with naturally-conceived children (DeBaun et al. 2003, Gicquel et al. 2003, Maher et al. 2003, Halliday et al. 2004, Chang et al. 2005, Ludwig et al. 2005). Taken together, ARTs may impose inherent risk for normal imprinted gene regulation resulting in imprinting disorders.

1.4.5 Ovarian Stimulation and Genomic Imprinting

To produce increased oocyte numbers for assisted reproduction, protocols incorporate large gonadotropin doses. Ovarian stimulation, or superovulation, alone as a fertility treatment can result in BWS and AS (Chang et al. 2005, Ludwig et al. 2005). This leads to investigations of superovulation as a potential imprinting disruptor. Individual mouse 16-cell embryos recovered from superovulated (7.5 IU eCG/hCG) females have paternal H19 loss of DNA methylation in 2/10 embryos, maternal Snrpn loss of DNA methylation in 2/10 embryos, and maternal H19 gain of DNA methylation in 1/10 embryos. This frequency of imprinting errors is not statistically different from controls. However, since only 12-24% of DNA strands per gene per embryo are recovered, additional perturbations may be missed (El Hajj et al. 2011). Alternatively, methylation perturbations may initiate at or after the 16-cell stage, since imprinted methylation errors are present in blastocysts after superovulation. Following low (6.25 IU eCG/hCG) and high (10 IU eCG/hCG) hormone regimes, a study from my research group reports imprinted DNA methylation perturbations in individual mouse blastocysts at maternal alleles of Snrpn (loss of DNA methylation in 4/10 low, 9/10 high blastocysts), Peg3 (loss of DNA methylation in 4/9 low, 5/9 high blastocysts), Kcnq1ot1 (loss of DNA methylation in 4/9 low, 5/9 high blastocysts).
methylation in 2/6 low, 5/9 high blastocysts), and H19 (gain of DNA methylation in 1/10 low, 4/10 high blastocysts) (Market-Velker et al. 2010b). As paternal H19 loss of DNA methylation is also seen (3/10 low, 7/10 high blastocysts), my colleagues concluded that superovulation impairs both imprint acquisition in oocytes and imprint maintenance in early embryos, in a dose-dependent manner. Moreover, multi-locus imprinted methylation perturbations are greater in the high hormone group (10/10 blastocysts) compared to the low hormone (4/10 blastocysts) and control groups (1/10 blastocysts). Others have reported that at midgestation, mouse conceptuses produced via low hormone (5 IU eCG/hCG) treatment show altered allelic expression of Snrpn, H19 and Igf2, but not Kcnq1ot1 in placentas but not embryos (Fortier et al. 2008). Additionally, 3/8 superovulation-derived mice (5 IU eCG/hCG) show loss of DNA methylation at H19 and Peg3, but not Snrpn, in brain and liver tissues (de Waal et al. 2012). These studies indicate that superovulation can lead to imprinting maintenance errors.

As ovarian stimulation is administered during oogenesis, it may also disrupt imprint acquisition. However, few studies have examined the effects of ovarian stimulation on imprint acquisition in oocytes. Following low hormone treatment (5 IU eCG/hCG), methylation of Snrpn, Peg3, Igf2r, and H19 is unaffected in mouse MII oocyte pools (14-18 DNA strands analyzed per gene) (Anckaert et al. 2009a). These results contrast with mouse oocytes collected after sequential hormone treatment (3 days 7.5 IU eCG/1 day 5 IU hCG) where H19 exhibits gain of DNA methylation (26-37%) in pooled MII oocytes, although normal methylation acquisition is present at Peg1, Kcnq1ot1 and pleiomorphic adenoma gene-like 1 (Plagl1/Zac1) (Sato et al. 2007). As zona pellucidae are not removed from pooled oocytes, this H19 gain of DNA methylation may be the result of cumulus cell contamination.

Following ovarian stimulation in humans, individual MI oocytes show PEG1 loss of DNA methylation (2/7 oocytes) and H19 gain of DNA methylation (2/3 oocytes) (Sato et al. 2007), and in the last study, pooled MI oocytes exhibit maternal KCNQ1OT1 loss of DNA methylation (2/19 strands) (Khoeiry et al. 2008). With respect to the human MI oocyte study, PEG1 may still be in its acquisition phase. Alternatively, human oocytes may be more prone to epigenetic errors and/or encounter more stressors, such as multiple hormone administration, advanced maternal age, and inherent infertility.
Overall, the effect of superovulation on the establishment of maternal genomic imprints in oocytes is still unresolved. Considering the frequency of epigenetic perturbations in blastocysts compared to oocytes, ovarian stimulation may also have an adverse impact on maternal-effect factors involved in imprint regulation.

1.5 Rationale

Gametogenesis and early embryogenesis are important stages when genome-wide epigenetic transitions are orchestrated, including erasure, establishment and maintenance of genomic imprinting. Changes from the normal maternal environment by inherent infertility or the incorporation of assisted reproductive technologies may disrupt the processes of imprint acquisition, imprint maintenance, and/or the factors required for imprinting regulation. Furthermore, the discovery of maternal-effect genes has brought new insights into the regulation of genomic imprinting. Understanding how an assisted reproductive technology may disrupt those maternal-effect products that regulate imprinting will be essential for maximizing the safety of assisted reproductive technologies to ensure healthy embryonic development.

1.6 Hypothesis

My overall hypothesis is that maternal control of genomic imprinting is compromised independently by impaired fertility and by ovarian stimulation. Specifically, the process of imprint acquisition is disrupted in a maternal infertility/subfertility model, and imprint acquisition is also disrupted upon superovulation. Furthermore, I hypothesize that superovulation disrupts the function of essential maternal-effect factors required for the regulation of genomic imprinting within the critical periods of oocyte and preimplantation embryo development.

Importantly, the field of genomic imprinting has begun to adopt analyses at the individual embryo level based on the stochastic nature of imprint loss between embryos and between imprinted loci within each embryo. To undergo my objectives, it is necessary to first modify the current bisulfite mutagenesis assay that facilitates analysis at the individual blastocyst stage to enable analysis at the individual oocyte stage.
1.7 Objectives

My objectives are as follows:

(1) Design a bisulfite mutagenesis assay to enable analysis of imprinted DNA methylation at the individual oocyte level.

(2) Determine the effects of maternal subfertility and infertility on imprinted DNA methylation acquisition in oocytes.

(3) Determine the effects of ovarian stimulation on imprinted DNA methylation acquisition in oocytes.

(4) Determine the effects of ovarian stimulation on imprint regulation by the maternal-effect factor ZFP57.
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2 CHAPTER 2: Single Oocyte Bisulfite Mutagenesis Assay

The work in this chapter originates from the following peer-reviewed article:


2.1 Introduction

Epigenetics encompasses all heritable and reversible modifications to chromatin that alter gene accessibility, and thus are the primary mechanisms for regulating gene transcription (Jaenisch & Bird 2003). DNA methylation is an epigenetic modification that acts predominantly as a repressive mark. Through the covalent addition of a methyl group onto cytosines in CpG dinucleotides, it can recruit additional repressive proteins and histone modifications to initiate processes involved in condensing chromatin and silencing genes (Rodenhiser & Mann 2006). DNA methylation is essential for normal development as it plays a critical role in developmental programming, cell differentiation, repression of retroviral elements, X-chromosome inactivation and genomic imprinting.

One of the most powerful methods for DNA methylation analysis is bisulfite mutagenesis. Sodium bisulfite is a DNA mutagen that deaminates cytosines into uracils. Following PCR amplification and sequencing, these conversion events are detected as thymines. Methylated cytosines are protected from deamination and thus remain as cytosines, enabling identification of DNA methylation at the individual nucleotide level (Frommer et al. 1992). Development of the bisulfite mutagenesis assay has advanced from those originally reported (Clark et al. 1994, Feil et al. 1994, Raizis et al. 1995) towards ones that are more sensitive and reproducible (Patterson et al. 2011). One key advancement was embedding smaller amounts of DNA in an agarose bead, thereby protecting DNA from the harsh bisulfite treatment (Olek et al. 1996). This enabled methylation analysis to be performed on pools of oocytes and blastocyst-stage embryos (Mann et al. 2004). The most sophisticated bisulfite mutagenesis protocol to date is for individual blastocyst-stage embryos (Market-Velker et al. 2010b). However, since
blastocysts have on average 64 cells (containing 120-720 pg of genomic DNA), this method is not efficacious for methylation studies on individual oocytes or cleavage-stage embryos.

Taking clues from agarose embedding of minute DNA amounts including oocytes (Meng et al. 2008), here we present a method whereby oocytes are directly embedded in an agarose and lysis solution bead immediately following retrieval and removal of the zona pellucida from the oocyte. This enables us to bypass the two main challenges of single oocyte bisulfite mutagenesis: protecting a minute amount of DNA from degradation, and subsequent loss during the numerous protocol steps. Importantly, as data are obtained from single oocytes, the issue of PCR bias within pools is eliminated. Furthermore, inadvertent cumulus cell contamination is detectable by this method since any sample with more than one methylation pattern may be excluded from analysis (Denomme et al. 2011). This protocol provides an improved method for successful and reproducible analyses of DNA methylation at the single-cell level and is ideally suited for individual oocytes as well as cleavage-stage embryos. The video component of this article can be found at [www.jove.com/video/4046/](http://www.jove.com/video/4046/).

### 2.2 Protocol

**DAY 1**

Prepare the following solutions fresh on the day of oocyte collection with sterile, distilled water such as GIBCO water. To reduce the chance of DNA contamination, change gloves often and use filter tips. Keep tubes angled away when open, and recap all tubes when not in use. We recommend that solutions are made as n+1.

#### 3% LMP Agarose

30 mg Low Melting Point (LMP) Agarose

up to 1 mL GIBCO H$_2$O

dissolve @ 70°C
**Lysis Solution**
8 μl lysis buffer
1 μl proteinase K
1 μl 10% IGEPAL
place on ice until ready for use

**2:1 Agarose:Lysis Solution** (10 μl per individual oocyte, amount is for 3 oocytes)
20 μl 3% LMP agarose
10 μl Lysis Solution
mix @ 70°C

**SDS Lysis Buffer** (501 μl per individual oocyte)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x TE pH 7.5</td>
<td>450 μl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50 μl</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>1 μl</td>
</tr>
</tbody>
</table>

**2.2.1 Oocyte Collection**

- Place the dissected mouse oviducts in M2 media, and tear the ampulae to extract the cumulus cell complex
- Separate the oocytes from the cumulus cell complex using 0.3 mg/mL hyaluronidase solution in a 30 μl drop of M2 media. Keep the oocytes in solution only as long as it takes to remove the cumulus cells, as lengthy exposure may damage them. Wash the oocytes 3x in 30 μl drop of M2 media, removing cumulus cells periodically.
- Remove the zona pellucida using acidic tyrode’s solution. Place the oocytes in one 30 μl drop of solution first, and then transfer to another 30 μl drop, as any media carried along will dilute the acid and reduce its efficiency. Keep the oocytes in solution only as long as it takes to remove the zona, as lengthy exposure may damage them. Note: an increased concentration of acidic tyrode’s
solution or pronase may be used for human samples, as the human zona pellucida is more resistant to treatment with acidic tyrode’s solution than the mouse.

- Wash the oocytes once more in a 30 μl drop of M2 media.

### 2.2.2 Agarose Embedding and Lysis

- To perform agarose embedding, place the lysis solution on a 70°C heatblock. Add the preheated LMP agarose to the lysis solution, producing a 2:1 agarose:lysis solution.

- Place a single oocyte onto a clean glass slide in minimal M2 media. Take up 10 μl of the agarose:lysis solution into a pipette tip, and (under a microscope) gently expel a small amount (~1 μl or less) onto the glass slide, allowing it to mix with the minimal media. Gently pick up the oocyte into the pipette tip and put all 10 μl into an eppendorf tube with 300 μl mineral oil so the bead forms a sphere. Note: this process must be done fairly quickly as the agarose will harden if the temperature drops as little as 5°C below 70°C.

- Incubate the tube on ice for 10 minutes. To perform lysis, remove the 300 μl mineral oil and add 500 μl of the SDS lysis buffer. Incubate overnight in a 50°C waterbath. Note: Lysis solution may also be used for this purpose.

### DAY 2

Prepare the following solutions fresh on the day of bisulfite mutagenesis. To reduce chance of DNA contamination, change gloves often and use filter tips. Keep tubes angled away when open, and recap all tubes when not in use. We recommend that solutions are made as n+1.

- **3 M NaOH**: 2.4 g NaOH in 20 mL autoclaved ddH₂O
- **0.1 M NaOH**: 0.5 mL of 3M in 14.5 mL autoclaved ddH₂O
- **0.3 M NaOH**: 1.5 mL of 3M in 13.5 mL autoclaved ddH₂O
2.5 M Bisulfite Solution
(a) 3.8 g sodium bisulfite
   5.5 mL GIBCO distilled H₂O
   1 mL 3 M NaOH
dissolve @ room temperature
(b) 110 mg Hydroquinone
   1 mL GIBCO distilled H₂O
dissolve @ 90°C (for only as long as it takes to dissolve, mix regularly)

When fully dissolved, mix solution (a) and (b)

*Keep away from light*

2.2.3 Bisulfite Mutagenesis

- Fully remove the 500 μl SDS lysis buffer and add 300 μl mineral oil (~20 hours). Any lysis buffer remaining will dilute the agarose when it is heated and the bead will be more susceptible to dissolving in the subsequent steps. Proceed with bisulfite mutagenesis immediately, or store at -20°C for up to 5 days.
- If applicable, remove oocytes from the freezer and let thaw (only until agarose bead is relatively translucent). Incubate for 2.5 minutes on a 90°C heat block, following which Incubate on ice for 10 minutes. Note: Do not mix or stir, extend longer than 2.5 minutes, or fluctuate temperature.
- To perform denaturation, remove the mineral oil and add 1 mL 0.1M NaOH to each tube, flick and invert 5-6 times.
- Incubate for 15 minutes in a 37°C waterbath, inverting every 3-4 minutes. The bead should float in the NaOH.
- To perform bisulfite treatment, spin the tube gently, then remove the NaOH and add 300 μl mineral oil and 500 μl bisulfite solution. Incubate the tube for 3.5 hours in a 50°C waterbath. *Keep away from light* Note: Length of incubation may need to be empirically determined for gene of interest.
To perform desulfonation, incubate on ice for 3 minutes, then remove the mineral oil and the bisulfite solution, spin gently, and add 1 mL 0.3 M NaOH. Flick and invert 5-6 times.

Incubate for 15 minutes in a 37°C waterbath, inverting every 3-4 minutes. The bead should float in the NaOH.

Wash the samples, by first spinning gently, then remove the NaOH and add 1 mL 1x TE pH 7.5. Shake for 5-10 minutes at room temperature (on a shaker). Spin gently again, then remove the 1x TE. Repeat this washing process twice.

Add 1 mL autoclaved ddH2O. Shake for 5-10 minutes at room temperature (on a shaker). Spin gently, then remove the H2O. Repeat ddH2O wash twice.

Check the pH of the supernatant; it should be pH 5.0. If still too basic, wash again with H2O. Remove all supernatant, leaving only the agarose bead.

### 2.2.4 PCR amplification

**Prepare 1st round PCR mix **while washing**

- 10 μM Primer Forward Outer 0.5 μl
- 10 μM Primer Reverse Outer 0.5 μl
- 240 ng/mL tRNA 1 μl
- H2O 13 μl

Add to Illustra Ready-to-Go Hot Start PCR beads

Carefully slide the solid agarose bead into the PCR tube (~10 μl)

Heat to 70°C and mix

Add 25 μl mineral oil

Total: 50 μl

Amplify. Note: An example of cycling conditions for mouse Snrpn is denaturation for 2 minutes at 94°C, followed by 40 cycles of 30 seconds at 94°C, 1 minute at 50°C, and 1 minute at 68°C; and a final 10 minute elongation step at 68°C. Annealing temperature for 1st round PCR for mouse H19 and Peg3 is 50°C.
Prepare 2nd round PCR mix

- 10 μM Primer Forward Inner 0.5 μl
- 10 μM Primer Reverse Inner 0.5 μl
- H₂O 19 μl

Add to Illustra Ready-to-Go Hot Start PCR beads

Add 5 μl 1st Round product as a template. Be sure to pipette below the layer of mineral oil.

Add 25 μl mineral oil

Total: 50 μl

Note: Nested primer sequences for Snrpn, H19, and Peg3 can be found in (Market-Velker et al. 2010b, Denomme et al. 2011).

- Amplify. Note: Cycling conditions for mouse Snrpn is denaturation for 2 minutes at 94°C, followed by 40 cycles of 30 seconds at 94°C, 1 minute at 50°C, and 1 minute at 68°C; and a final 10 minute elongation step at 68°C. Mouse H19 and Peg3 require a 50°C annealing temperature for 2nd round PCR.
- As a diagnostic test, second round samples can be cut with a restriction enzyme that is methylation- or strain-specific.

2nd Round product 4 μl
Restriction Enzyme 1 μl
Buffer 1 μl
H₂O 4 μl

- Electrophorese the digestion products on an 8% acrylamide gel. Any heterogeneous bands represent more than one sequence.

2.2.5 TA Cloning and Colony PCR

- To clone 2nd round product, ligate into vector using the Promega pGEM-T Vector System (Fisher Scientific Cat#A1360).
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2nd Round PCR 1 μl
pGEMT-EASY vector 1 μl
Ligase 1 μl
H2O 2 μl
2x Ligation Buffer 5 μl

Incubate overnight @ 4°C in PCR machine.

- Thaw competent E.coli cells on ice for 15 minutes (Zymo Research Corp Cat#T3009). Add 3 μl ligation reaction to 8 μl E.coli and incubate ligation on ice for 15 minutes.
- Heat shock for 40 seconds in a 42°C waterbath, and incubate on ice for 2 minutes. Add 60 μl S.O.C. medium and incubate at 37°C for 1 hour (in shaker).
- Place all of the reaction mix on an LB/Agar/IPTG/Xgal/Amp plate and incubate plate at 37°C overnight.

Prepare colony PCR mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 μM M13 Forward Primer</td>
<td>0.7 μl</td>
</tr>
<tr>
<td>20 μM M13 Reverse Primer</td>
<td>0.7 μl</td>
</tr>
<tr>
<td>Taq 10x PCR Buffer</td>
<td>3.5 μl</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>0.7 μl</td>
</tr>
<tr>
<td>50 mM MgCl2</td>
<td>1.05 μl</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>0.28 μl</td>
</tr>
<tr>
<td>H2O</td>
<td>28.07 μl</td>
</tr>
<tr>
<td></td>
<td>35 μl Total</td>
</tr>
</tbody>
</table>

Add 35 μl Colony PCR master mix into a PCR tube.

- Pick a white bacterial colony from the plate with a pipette tip, and swirl it into the PCR reaction.
- Amplify with denaturation for 10 minutes at 94°C, followed by 30 cycles of 45 seconds at 94°C, 30 seconds at 57°C, and 1 minute at 72°C; and a final 10 minute elongation step at 72°C. Electrophorese 4 μl on a 1.5% agarose gel. Send ~30 μl
of the PCR product for sequencing. Note: For oocytes, 5 colony PCR products are sequenced.

- Once sequencing results are obtained, methylation patterns can be read. Any original CG that remained as a CG was methylated, and any original CG that is now a TG was unmethylated.

2.3 Representative Results

In our work, we assay imprinted methylation in individual oocytes and embryos (Figure 2-1). Following nested PCR amplification using bisulfite converted primers, it is possible to confirm a successful conversion by visualizing a correct fragment size on an agarose gel (Figure 2-2). An individual oocyte represents one parental allele, and in theory, has one imprinted methylation pattern. As such, second round PCR products can be tested for unintentional contamination. A restriction enzyme sensitive to DNA methylation (such as HinfI or DpnII) can be used to digest the second round PCR product to assess whether it contains a methylated or unmethylated allele (Figure 2-3). A methylated C within the enzyme recognition sequence is cleaved while an unmethylated C that is converted to T is no longer recognized by the enzyme and is uncut. Any MII oocyte sample containing both methylated and unmethylated alleles should be discarded, as it is indicative of cumulus cell contamination. While it may instead be inclusion of a polar body having a dissimilar methylation pattern at the cut site, cumulus cell contamination cannot be ruled out at this point. (Figure 2-3). Following ligation and transformation, successful colony PCR amplification can be visualized on an agarose gel to ensure samples with the correct product size are sent for sequencing (Figure 2-4). Finally, the sequence of five individual clones from an MII oocyte should produce five identical methylation patterns (Figure 2-5a). Any samples that contain more than one pattern should be discarded (Figure 2-5b). Since ovulated MII oocytes have two chromosome copies or an attached polar body, there is a possibility for obtaining two similar sequence patterns (Figure 2-5c). We recommend discarding data from oocytes that have highly dissimilar methylation patterns since cumulus cell contamination cannot be ruled out.
Figure 2-1 Schematic of the Single Oocyte Bisulfite Mutagenesis Assay

1) Retrieve oocytes, remove cumulus cells, remove zona pellucida. Place individual oocyte on a glass slide.

2-3) Embed in 2:1 agarose/lysis solution. Place in tube under mineral oil then on ice to harden. Remove mineral oil. Lyse overnight at 50°C.


\[ C > U > T \]
\[ mC > C > C \]

12-14) Ligate, clone and sequence.

Clone 1: ⚫⚫⚫⚫⚫⚫⚫ 100%
Figure 2- 2 Representative results from the 2nd round amplification of Snrpn from a single MII oocyte on a 1.5% agarose gel

Lanes 1-4 are four single MII oocytes and lane 5 is a negative control (no oocyte). Expected amplicon size for Snrpn is 420 bp. L, ladder.
Figure 2-3 Representative results from the 2nd round methylation-specific restriction digestion for Snrpn from a single MII oocyte on an 8% acrylamide gel

HinfI diagnostic restriction digestion shows unmethylated DNA which harbors a T that abolishes the restriction site (420bp, lane 1) or methylated DNA which contains a C within recognition site (cut, 262, 103, and 54 bp, lane 2). Digestion showing both methylated and unmethylated restriction enzyme sites (cut & uncut bands, lane 3) are indicative of cumulus cell contamination. L, ladder.
Figure 2- 4 Representative results for colony PCR amplification for *Snrpn* from a single MII oocyte on a 1.5% agarose gel

Expected amplicon size following ligation of *Snrpn* into the pGEM-T Easy vector and using M13 forward and reverse primers is 656 bp. Lane 1-8, amplicons from clones 1-8. Clone 5 has an incorrect amplicon size and should not be sent for sequencing.
Figure 2- 5 Representative sequencing results for Snrpn from a single MII oocyte

Snrpn is methylated in oocytes. Black circles indicate methylated CpGs. White circles indicate unmethylated CpGs. CpG number and placement is representative for a B6 strain female mouse. a) Expected sequencing results for Snrpn from a single MII oocyte. Only a single strand of DNA should amplify in all five clones. Oocytes with a single methylation pattern and identical non-CpG conversion pattern (percentage indicated to the right, based on number of non-CpG cytosines converted to thymine as a percentage of total non-CpG cytosines) should be included in analyses. b) Sequencing results for Snrpn from a single MII oocyte with cumulus cell contamination. Note the dissimilarity between methylation states and conversion patterns indicating multiple strand amplification. c) Sequencing results for Snrpn from a single MII oocyte with both chromosome copies or polar body inclusion.
2.4 Discussion

This single oocyte assay contains many steps with a number that are critical and require special care. The first is oocyte washing. It is particularly important to wash each oocyte multiple times in fresh medium drops following hyaluronidase treatment to remove as many cumulus cells as possible. Moreover, when transferring oocytes to acidic tyrode’s solution for zona pellucida removal make sure surrounding medium is clear of cumulus cells. The oocyte is very sticky following zona removal, and any surrounding cumulus cells can easily become stuck to the oocyte. It is very difficult to remove a cumulus cell that is stuck to a zona-free oocyte. While this protocol allows for detection of cumulus cell contamination at later time points, it is not constructive, nor economical, to undergo the full protocol on oocytes that will likely be discarded.

The second critical step is oocyte embedding. When embedding the oocyte in the agarose and lysis solution, it is important to note that low melting point (LMP) agarose will harden at temperatures as little as 5°C below 70°C. As such, we recommend mixing the agarose and lysis solution at 70°C, and then placing the individual oocyte on the glass slide in minimal medium. Once ready, pipette the agarose/lysis solution and embed the oocyte into a prepared eppendorf tube under mineral oil, in a careful yet timely manner.

It is critical that heat inactivation of the proteinase K be performed at 90°C for 2.5 minutes. Deviation from this is not recommended. Higher temperatures or longer times may damage the DNA, while lower temperatures or shorter times may not inactivate the proteinase K.

As a reminder, sodium bisulfite and hydroquinone are light sensitive. Solutions should be prepared and then wrapped in foil, amber tubes and/or placed in a dark drawer until use. Once the bisulfite solution has been added to the tube, the tube should be covered with foil or a dark covered bag when incubating at 50°C. We use empty foil bags from GE Healthcare that originally contained the hot start PCR beads.

Finally, as with most PCRs, we recommend preparing each round in a timely manner. Excessive delays, particularly after mixing at 70°C, will reduce success rate of amplification.
At the current time, one limitation of the protocol is that the success rate of bisulfite converted DNA amplification from individual oocytes ranges from 40% to 65% depending on the gene fragment amplified. While additional trouble-shooting may increase this percentage, there is a trade-off between the bisulfite conversion treatment being too long or harsh and not having sufficient conversion rates (>85-90%). A second limitation is that, currently bisulfite mutagenesis cannot distinguish between 5-methylocytosine (5mC) and 5-hydroxymethylcytosine (5hmC). While 5mC is predominantly a repressive mark and is used to signify silenced alleles, 5hmC is a possible intermediate in the demethylation pathway (Tahiliani et al. 2009) and thus, results at this time cannot differentiate between these two potentially opposing functions.

Several modifications may be required based on gene or region of interest. The bisulfite conversion time may require optimization for the highest conversion percentage with the lowest DNA damage. We suggest a range of 2.5 to 4 hours (half hour increments) for bisulfite treatment. Further optimization can involve PCR primer design for converted sequences of interest (for example http://www.urogene.org/methprimer/), as well as optimization of PCR programs based on fragment length and CG content (see Patterson et al for additional bisulfite mutagenesis optimization options (Patterson et al. 2011)). A final modification is that gel extraction of the 2nd round PCR product may be required if there is abundant primer-dimers or non-specific amplicons that will integrate into the vector when cloned.

We have previously shown that this protocol is effective for individual MII oocytes (Denomme et al. 2011). We have also tested its efficiency on growing oocytes at a range of different oocyte diameters, with the same rates of successful amplification of converted DNA. Future applications may include analysis of DNA methylation patterns in single cells of any origin, including in vivo-derived and cultured cells. Additionally, we have developed an individual blastocyst assay that recovers DNA and RNA from the same embryo, allowing for both expression and methylation data to be obtained. However, the blastocyst is the latest stage in preimplantation development, and this assay was not feasible for methylation studies on smaller cell numbers. Analysis of individual oocytes and early embryos is essential for reproductive and developmental biology studies, specifically in relation to assisted reproduction and other techniques that involve
manipulations of germ cells and early embryos. A future application will involve modifying the single oocyte assay to recover RNA for expression analyses as well as DNA for methylation assays. To note, this assay has a possibility of undetected cumulus cell contamination when the cumulus cell has the same expected methylation pattern as the oocyte. A future modification would include optimization of duplex assays within the same oocyte, similar to the method described by El Hajj et al (El Hajj et al. 2011), which would allow for methylation detection at a second gene with opposing expected methylation pattern as the gene of interest.

DNA methylation analyses can range from genome-wide to locus-specific. Genome-wide methods such as methylated DNA immunoprecipitation (MeDIP) in conjunction with microarrays or sequencing typically require abundant amounts of material. Locus-specific methods that include combined bisulfite restriction analysis (COBRA) using methylation-specific restriction enzymes, or the MethylDetector kit (Active Motif), are less than optimal for single blastocyst analyses, resulting in insufficient recovery of DNA, PCR bias and lack of reproducibility. Recently, Hajj et al utilized the EZ-DNA Methylation kit (Zymo Research) along with a limited dilution bisulfite pyrosequencing technique (El Hajj et al. 2011). The authors used this method to analyze methylation at four loci within individual 2-cell mouse embryos, however their allele recovery percentage was low (13-25%). When analyzing bovine oocytes, again the authors recovered only 34% of oocyte DNA (and 8% polar body DNA). These low percentages may be due in part to the use of columns and elution buffers, as kits are known to reduce DNA recovery. Diluting and then transferring samples following bisulfite mutagenesis would also likely result in some loss of the minute amount of DNA.

Bisulfite mutagenesis is the gold standard for analyzing DNA methylation, and it is clear that the analysis of single cells is of great advantage. Agarose embedding permits smaller sample sizes to be analyzed with bisulfite treatment without the use of kits, as agarose protects against DNA degradation and prevents DNA loss during numerous protocol steps. However, when compared to the blastocyst, a single oocyte has approximately 3-6 pg of genomic DNA. Meng et al utilized LMP agarose to embed the oocyte prior to bisulfite mutagenesis (Meng et al. 2008). Our modified protocol, which embeds single oocytes in agarose containing lysis buffer, prevents DNA loss beginning at
the initial lysis step and moderates the harsh bisulfite treatment. In summary, the advantage of single oocyte analysis is that it allows detection of inadvertent cumulus cell contamination as well unmasking rare events and eliminating any biases undetected in pooled samples. Altogether, this modified protocol provides quality data on the methylation state of individual oocytes and early embryos.
2.5 References


Patterson K, Molloy L, Qu W & Clark S 2011 DNA methylation: bisulphite modification and analysis. *J Vis Exp*.


3 CHAPTER 3: The Effects of Maternal Infertility and Subfertility on Imprint Acquisition

The work in this chapter originates from the following peer-reviewed article:


3.1 Introduction

The tight regulation of monoallelic gene expression based on gametic origin is termed genomic imprinting (Bartolomei & Ferguson-Smith 2011). This dynamic process relies on epigenetic modifications such as DNA methylation to mark, or “imprint”, one of the two parental alleles, resulting in differential gene expression in progeny (Verona *et al.* 2003). Gametogenesis encompasses the critical period of heritable epigenetic reprogramming for imprinted genes. Imprinted DNA methylation is first erased in primordial germ cells, subsequently allowing for *de novo* differential methylation at imprinted loci in oocytes and sperm (Li & Sasaki 2011). In males, *de novo* DNA methylation acquisition occurs during the prenatal stages of spermatogenesis, beginning in prospermatogonia and is completed by birth (Kafri *et al.* 1992, Davis *et al.* 1999, Davis *et al.* 2000, Ueda *et al.* 2000). In females, *de novo* DNA methylation is acquired after oocytes enter the growth phase of follicular development, from the primary to antral follicle stage (Luciferro *et al.* 2004, Hiura *et al.* 2006, Sato *et al.* 2007, Song *et al.* 2009). Importantly for oocytes, imprinted methylation acquisition is dependent on oocyte size and not oocyte age, with methylation levels increasing as oocyte diameter increases.

The correct establishment of germline imprints is significant as disruptions to this process can result in the development of imprinting disorders such as Beckwith-Wiedemann Syndrome (BWS), Silver-Russell Syndrome (SRS), and Angelman Syndrome (AS). BWS is an overgrowth disorder that is caused by imprinting defects that
result in a gain of maternal methylation at the H19 imprinting control region (ICR) or a loss of maternal-specific methylation at the KCNQ1OT1 (KCNQ1 overlapping transcript 1) ICR (Weksberg et al. 2010). SRS is an intrauterine growth restricted imprinting disorder with imprinting defects at the H19 and possibly at the paternally-expressed gene 1 (PEG1) imprinted domains (Eggermann 2010). AS is a neurological disorder that is caused by loss of maternal-specific methylation at the small nuclear ribonucleoprotein N (SNRPN) ICR (Mabb et al. 2011). Sporadic epigenetic errors resulting in these disorders are reported to occur more frequently in the assisted reproductive technologies (ARTs) population (Cox et al. 2002, DeBaun et al. 2003, Gicquel et al. 2003, Maher et al. 2003, Orstavik et al. 2003, Halliday et al. 2004, Chang et al. 2005, Ludwig et al. 2005, Rossignol et al. 2006, Azzi et al. 2009, Bliek et al. 2009, Lim et al. 2009, Lennerz et al. 2010, Turner et al. 2010). For AS, patients at the highest risk for an imprinting defect have parents with prolonged infertility undergoing infertility treatment (Ludwig et al. 2005, Doornbos et al. 2007). This raises the question as to whether imprinting errors in ART patients are associated with parental infertility/subfertility. While studies have been conducted to determine the effects of ARTs on genomic imprinting, investigations of how impaired infertility may contribute to imprinting errors are lacking. In this study, we queried whether impaired fertility arising during oogenesis could lead to imprinting defects.

Development of healthy oocytes is dependent on interactions between the growing oocyte and surrounding follicular cells (Kidder & Vanderhyden 2010). Oocytes play an important role in regulating granulosa cell development, proliferation, and differentiation, as well as steroid hormone production. In turn, follicular cells play a critical role in oocyte growth, meiotic progression, and transcriptional activity and chromatin remodeling of the oocyte genome. This synergistic partnership is facilitated by endocrine and paracrine signaling, and intercellular gap junctional communication, ensuring meiotic and developmental competence of the oocyte. In this study, we specifically examined the effects of aberrant signaling and communication on imprint acquisition.

A complex endocrine signaling pathway is active in the ovary that regulates follicle and oocyte development. 17β-estradiol acting through nuclear estrogen receptor
beta (ERβ) augments the actions of follicle stimulating hormone (FSH). In the ovary, ERβ is expressed primarily in granulosa cells and at low levels in the oocyte (Drummond & Fuller 2011). Female mice bearing a targeted deletion of the ERβ (Esr2) gene are subfertile, producing fewer oocytes following superovulation, as well as litters with fewer pups (Krege et al. 1998, Couse et al. 2000, Dupont et al. 2000, Couse et al. 2003, Couse et al. 2005, Emmen et al. 2005). Attenuated differentiation of granulosa cells following gonadotropin stimulation in Esr2-null mice leads to decreased antrum formation, delayed follicle maturation, and reduced follicular rupture, producing greater numbers of atretic follicles and fewer preovulatory oocytes. In addition, vascularization of the thecal layer, which is required for follicular growth, is impaired (Inzunza et al. 2007). Mechanistically, ERβ is required for optimal cAMP production in mouse granulosa cells following gonadotropin stimulation (Deroo et al. 2009). ERβ deficiency causes disruption of cAMP second messenger signaling in granulosa cells in response to FSH, producing aberrant FSH-regulated gene expression, decreased response to luteinizing hormone, and impaired ovulation and fertility.

Gap junctions are specialized channels composed of six membrane proteins termed connexins (CX). These channels are essential for communication between neighbouring cells (Harris 2001). In the mouse, CX37 and CX43 are the only connexins known to be required in developing follicles (Kidder & Vanderhyden 2010). CX43 localizes to gap junctions in the granulosa cell membranes, enabling granulosa cell to granulosa cell communication. By comparison, CX37 constitutes the gap junctions coupling the oocyte with surrounding granulosa cells and is specifically located at the interface between the oocyte and the first layer of granulosa cells (Simon et al. 1997). Gap junctions allow the transport of nutrients, metabolites and second messengers, such as cAMP, between the granulosa cells and the oocyte (Kidder & Vanderhyden 2010). Targeted deletion of the CX37 (Gja4) gene causes arrested folliculogenesis at the early antral stage, impaired oocyte development and meiotic competency, and premature luteinization of the follicles (Simon et al. 1997, Carabatsos et al. 2000).

In this study, we employed the Esr2−/− and Gja4−/− genetic models to interfere specifically with endocrine signaling and gap junctional communication, compromising fertility. We hypothesized that inhibition of the ERβ pathway and/or oocyte-granulosa
cell gap junctional communication would lead to perturbations in imprinted methylation acquisition. To compare mutant oocytes to control oocytes, DNA methylation acquisition was first examined in individual, 20-80 μm control oocytes at three imprinted genes, Snrpn, Peg3 and Peg1 (also known as Mest). Similar to previous studies (Lucifero et al. 2004, Hiura et al. 2006, Sato et al. 2007, Song et al. 2009), we observed that each gene had its own size-dependent acquisition kinetics. To determine whether compromised endocrine signaling and gap junctional communication disrupted de novo methylation acquisition, preovulatory oocytes from Esr2−/− females, and early antral stage oocytes from Gja4−/− mice were assessed for DNA methylation establishment at Snrpn, Peg3 and Peg1. We observed no aberrant or delayed acquisition of DNA methylation at Snrpn, Peg3 and Peg1 in preovulatory oocytes from ERβ deficient females. Similarly, we found no perturbation of Snrpn and Peg3 de novo methylation in oocytes from CX37-null follicles. However, Peg1 methylation acquisition was lost or delayed in Gja4-deficient oocytes compared to controls. We attribute this to the late establishment of DNA methylation at the Peg1 gene. These results indicate that compromised fertility though impaired intercellular communication can lead to imprinting acquisition errors. Further studies are required to determine the postfertilization effects of subfertility/infertility originating from impaired signaling and intercellular communication during oogenesis.

3.2 Material and Methods

3.2.1 Oocyte Isolation

3.2.1.1 Control Oocyte Collections

Ovaries were obtained from C57BL/6 female mice (Charles River) at 10, 14, 21 and 28 days post partum (dpp), and placed in Waymouth MB 752/1 medium (Invitrogen) supplemented with 10% fetal bovine serum (Li et al. 2007). For further follicle separation, ovaries were digested in the same medium containing 2 mg/mL type I collagenase (Sigma-Aldrich) at 37°C. Primary, secondary and early tertiary (antral) follicles were liberated by repeated aspiration and expulsion with a 1 mL pipette. Follicles were washed several times in culture medium without collagenase. For oocyte isolation, follicles were centrifuged for 5 minutes at 4,000 rpm, re-suspended and
digested in 0.05% Trypsin/EDTA in a culture dish for 15 minutes at 37°C. Oocytes were
dissociated from the granulosa cells by repeated aspiration and expulsion with a 1 mL
pipette. Oocytes were retrieved through mouth pipetting and placed in 30 μL drops of M2
medium (Sigma) for further analysis.

3.2.1.2 Gja4-null Oocyte Collections

Ovaries were removed from Gja4<sup>−/−</sup> female mice (C57BL/6 background) at 21 and
28 dpp, and placed in Waymouth MB 752/1 medium (Invitrogen) supplemented with
10% fetal bovine serum. Gja4-null oocytes were retrieved through the same collection
method as control oocytes and placed in 30 μL M2 medium (Sigma) for further analysis.

3.2.1.3 Esr2-null Oocyte Collections

Ovaries were removed from Esr2<sup>−/−</sup> females (C57BL/6 background) at 28 dpp and
placed in a 100-mm cell culture dish containing 15 mL ice-cold M199 medium (Sigma)
supplemented with 1 mg/mL BSA (Invitrogen) and 2.5 g/mL gentamicin (Invitrogen)
(Deroo et al. 2009). Follicles were released by manual puncture with 25-gauge needles
and subsequent pressure applied with a sterile spatula. Oocytes were retrieved through
mouth pipetting and transferred to 30 μL drops of M2 medium (Sigma) for further
analysis.

3.2.2 Single Oocyte Bisulfite Mutagenesis and Sequencing

Processing, embedding and bisulfite mutagenesis of individual oocytes was
performed as previously described (Denomme et al. 2011). Briefly, oocytes were treated
with 0.3 mg/mL hyaluronidase (Sigma) to remove any surrounding cumulus cells (if
present), washed three times in 30 μL drops of M2 medium (Sigma), and then imaged
using the Olympus IX81 microscope. Oocyte diameter was measured using
Macnification v.1.8 (Orbicule). Following treatment with acidic Tyrode’s solution
(Sigma) to remove the zona pellucida (if present), oocytes were washed twice in M2
medium, then individual oocytes were embedded in 10 μL of 2:1 LMP agarose and lysis
solution [100 mM Tris–HCl, pH 7.5 (Bioshop), 500 mM LiCl (Sigma), 10 mM EDTA,
ph 8.0 (Sigma), 1% LiDS (Bioshop), and 5mM DTT (Sigma)], 1 μL 2 mg/mL proteinase
K [Sigma], and 1 μL 10% Igepal [Sigma] under 300 μL of mineral oil (Sigma), and placed on ice for 10 min for the agarose to harden. Mineral oil was replaced with 500 μL SDS lysis buffer [450 μL 1X Tris EDTA (TE), pH 7.5 (10mM Tris (Bioshop), 1mM EDTA), 50 μL 10% SDS (Bioshop), 1 μL 2 mg/mL proteinase K] and incubated at 50°C overnight. Following overnight incubation, lysis buffer was replaced with 300 μL mineral oil and oocytes were either immediately treated for bisulfite conversion or frozen at -20°C for up to five days. Firstly, samples were placed at 90°C for 2.5 min to heat inactivate the proteinase K, and then DNA was denatured using 0.1 M NaOH (Sigma) at 37°C for 15 min. Treatment with 2.5 M bisulfite solution (0.125 M hydroquinone [Sigma], 3.8 g sodium hydrogen sulfite [Sigma], 5.5 mL water, and 1 mL 3 M NaOH) at 50°C for 3.5 hours was followed by desulfonation using 0.3 M NaOH at 37°C for 15 min. Samples were washed twice in 1X TE pH 7.5 and twice in water, and then added directly to a Ready-To-Go PCR bead (GE) consisting of 15 μL water, gene-specific primers and 1 μL of 240 ng/mL transfer RNA as a carrier, with 25 μL mineral oil overlay. Negative controls (no oocyte) were processed alongside each bisulfite reaction. PCR amplification of the Snrpn ICR, Peg3 DMR and Peg1 DMR was performed as previously described (Market-Velker et al. 2010b). Following ligation into the PGEM-T easy vector (Promega) and cloning, 30 μL of colony PCR product was sent to Bio-Basic Inc. (Markham, Ontario, Canada) for sequencing. For each sample, five clones were sequenced. As MI oocytes have not extruded the first polar body, both alleles were successfully amplified in some oocytes, and only one allele was detectable in other oocytes. Oocytes with more than two clones having very different methylation patterns and different non-CpG conversion rates were excluded from analysis, as cumulus cell contamination could not be ruled out. Table 3-1 gives the number of oocytes included and excluded from analysis per gene.

3.2.3 Statistical Analysis

For each imprinted gene, significant difference of CpG methylation percentage was determined by a two-tailed Mann-Whitney test between mutant oocytes and control oocytes matched for size.
Table 3-1 Number of Oocytes Included and Excluded from Analysis

<table>
<thead>
<tr>
<th></th>
<th>Snrpn</th>
<th></th>
<th>Peg3</th>
<th></th>
<th>Peg1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IN</td>
<td>EX</td>
<td>%EX</td>
<td>IN</td>
<td>EX</td>
<td>%EX</td>
</tr>
<tr>
<td>WT</td>
<td>55</td>
<td>1</td>
<td>1.8</td>
<td>56</td>
<td>6</td>
<td>9.7</td>
</tr>
<tr>
<td>Esr2−/−</td>
<td>12</td>
<td>1</td>
<td>7.7</td>
<td>11</td>
<td>1</td>
<td>8.3</td>
</tr>
<tr>
<td>Gja4−/−</td>
<td>31</td>
<td>5</td>
<td>13.9</td>
<td>20</td>
<td>1</td>
<td>4.8</td>
</tr>
<tr>
<td>Total</td>
<td>98</td>
<td>7</td>
<td>6.7</td>
<td>87</td>
<td>8</td>
<td>8.4</td>
</tr>
</tbody>
</table>

IN, included oocytes with one to two clone patterns; EX, excluded oocytes with 3 or more clone patterns.
A diameter range of 6 to 80 μm was used to compare Esr2-deficient oocytes to control oocytes, while the 35 to 60 μm diameter range (including KO468 for Snrpn with a diameter of 60.5 μm) was used to compare the Gja4-deficient to control oocytes. A P-value less than 0.05 was taken to be statistically significant.

3.3 Results

3.3.1 Methylation Acquisition in Control Oocytes correlates with Oocyte Diameter

In female mammals, imprinted DNA methylation has been shown to arise during follicle growth from the primary to the antral stage in correlation with oocyte diameter (Lucifero et al. 2004, Hiura et al. 2006), with gene-specific kinetics for imprint acquisition. However, these analyses were performed with pooled oocytes of different sizes. To compare individual mutant oocytes to control oocytes, we first needed to examine imprinted DNA methylation acquisition in individual control oocytes. C57BL/6 oocytes were collected at 10, 14, 21 and 28 dpp to obtain oocytes with a diameter range of 20 to 80 μm. Oocytes that were collected at 10 dpp displayed a diameter range of 20 to 80 μm, those at 14 dpp were 40 to 80 μm in diameter, at 21 dpp ranged from 0 to 80 μm, and at 28 dpp ranged from 60 to 80 μm in diameter.

Analysis of _de novo_ methylation acquisition at the Snrpn ICR showed mean methylation levels of 8.7% in 20-40 μm, 12.6% in 40-45 μm, 3.7% in 45-50 μm, 37% in 50-55 μm, 82.7% in 55-60 μm, 97.0% in 60-65 μm, 86.2% in 65-70 μm, 93.8% in 70-75 μm, and 100.0% in 75-80 μm oocytes (Figure 3-1 and 3-2). Likewise, mean methylation levels at the Peg3 DMR were 1.6% in 20-40 μm, 11.2% in 40-45 μm, 16.1% in 45-50 μm, 22.9% in 50-55 μm, 51.5% in 55-60 μm, 58.1% in 60-65 μm, 81.0% in 65-70 μm, 90% in 70-75 μm, and 95.8% in 75-80 μm oocytes (Figure 3-3 and 3-4). For the Peg1 DMR, mean methylation levels were 4.9% in 20-40 μm, 6.8% in 40-45 μm, 12.7% in 45-50 μm, 15.9% in 50-55 μm, 45.5% in 55-60 μm, 51.6% in 60-65 μm, 93.3% in 65-70 μm, 100.0% in 70-75 μm, and 95.0% in 75-80 μm oocytes (Figure 3-5 and 3-6). Thus, we observed that each gene had its own acquisition kinetics. DNA methylation acquisition began first for Snrpn at ~50 μm and was near completion at >55 μm.
The *Snrpn* ICR region analyzed contains 16 CpGs. Black circles indicate methylated CpGs while white circles indicate unmethylated CpGs. Each row represents an individual oocyte (designation indicated to the left). Methylation percentage and diameter for each oocyte is shown at the right. Oocytes are grouped into cohorts ranging from 20 μm to 80 μm diameters in 5 μm increments. Oocytes with one methylation pattern represent one of the two parental alleles detected. Oocytes with two methylation patterns represent detection of both parental alleles.

![Figure 3-1 Methylation Analysis of the *Snrpn* ICR in Control C57BL/6 Oocytes](image)

The *Snrpn* ICR region analyzed contains 16 CpGs. Black circles indicate methylated CpGs while white circles indicate unmethylated CpGs. Each row represents an individual oocyte (designation indicated to the left). Methylation percentage and diameter for each oocyte is shown at the right. Oocytes are grouped into cohorts ranging from 20 μm to 80 μm diameters in 5 μm increments. Oocytes with one methylation pattern represent one of the two parental alleles detected. Oocytes with two methylation patterns represent detection of both parental alleles.
For oocytes with two parental alleles, each allele was graphed separately. Blue diamonds represent oocytes from control females, red circles represent oocytes from $Esr2^{-/-}$ females, and green triangles represent oocytes from $Gja4^{-/-}$ females.

Figure 3- 2 Methylation Percentage at the $Snrpn$ ICR in Relation to Oocyte Diameter
The Peg3 DMR region analyzed contains 23 CpGs. Details are described in Figure 3-1.
Figure 3-4 Methylation Percentage at the Peg3 ICR in Relation to Oocyte Diameter

Oocytes from control females, $Esr2^{-/-}$ females and $Gja4^{-/-}$ females are represented by blue diamonds, red circles and green triangles, respectively.
The Peg1 DMR region analyzed contains 15 CpGs. Details are described in Figure 3-1.
Figure 3-6 Methylation Percentage at the Peg1 DMR in Relation to Oocyte Diameter

Oocytes from control females, Esr2<sup>−/−</sup> females and Gja4<sup>−/−</sup> females are represented by blue diamonds, red circles and green triangles, respectively.
Next was Peg3, where DNA methylation acquisition was initiated at ~45 μm and nearly complete at >65 μm, which was followed by Peg1, where DNA methylation acquisition began at ~55 μm and was near completion by >70 μm. Snrpn had the shortest acquisition interval while Peg3 had the longest.

3.3.2 Methylation Acquisition in ERβ-deficient Oocytes

Ovaries deficient in Esr2 produce a reduced number of maturing oocytes, but those that do mature appear to not be developmentally compromised (Krege et al. 1998). Consistent with this, we recovered a small number of oocytes from 28 dpp females, ranging in diameter size from 61 μm to 82 μm, corresponding to the preovulatory stage in oocyte growth. To investigate the role of reduced hormone signaling on imprint acquisition, we analyzed the progression of DNA methylation acquisition in developing oocytes from mice deficient in Esr2. For the Snrpn ICR, mean methylation levels were 72.8% for 65-70 μm, 98.8% for 70-75 μm, and 100.0% for 75-80 μm oocytes (Figure 3-2 and 3-7). For the Peg3 DMR, mean methylation was 100.0% in 65-70 μm, 99.4% in 70-75 μm, and 98.7% in 75-80 μm oocytes (Figure 3-4 and 3-8). For the Peg1 DMR, mean methylation levels were 96.5% for 65-70 μm, 95.1% for 70-75 μm, and 96.5% for 75-80 μm oocytes (Figure 3-6 and 3-9). Thus, oocytes from Esr2-null females had comparable DNA methylation levels to control oocytes, indicating that imprint DNA methylation acquisition was unaffected by Esr2 deficiency.

3.3.3 Methylation Acquisition in CX37-deficient Oocytes

Previous analyses have shown that oocytes in CX37 null ovaries arrest development before reaching meiotic competence, around the time the antrum begins to form (~21 dpp) (Simon et al. 1997, Carabatsos et al. 2000, Li et al. 2007). We collected and analyzed oocytes from Gja4–null 21 dpp females, which ranged in diameter sizes from 35 to 55 μm and from 28 dpp females, which ranged in size from 50 to 60.5 μm. The maximum diameter obtained was 60.5 μm, consistent with previous studies (Simon et al. 1997, Carabatsos et al. 2000). To explore the relationship between gap junction loss and imprint acquisition, we analyzed the progression of DNA methylation establishment in developing oocytes from Gja4-deficient mice.
### Figure 3-7 Methylation Analysis of the *Snrpn* ICR in *Esr2*-null Oocytes

Details are described in **Figure 3-1**.
**Figure 3- 8 Methylation Analysis of the Peg3 DMR in Esr2-null Oocytes**

Details are described in Figure 3-1.
Figure 3-9 Methylation Analysis of the Peg1 DMR in Esr2-null Oocytes

Details are described in Figure 3-1.
Figure 3-10 Methylation Analysis of the Snrpn ICR in Gja4-null Oocytes

Details are described in Figure 3-1.
Details are described in Figure 3-1.

Figure 3-11 Methylation Analysis of the Peg3 DMR in Gja4-null Oocytes
Figure 3- 12 Methylation Analysis of the Peg1 DMR in Gja4-null Oocytes

Details are described in Figure 3-1.
At the Snrpn ICR, mean methylation levels were 6.3% in 35-40 μm, 14.0% in 40-45 μm, 17.4% in 45-50 μm, 45.8% in 50-55 μm, 80.8% in 55-60 μm, and 88.0% in 60-65 μm oocytes (Figure 3-2 and 3-10). No significant difference was observed in methylation levels between Gja4-null and control oocytes.

Analysis at the Peg3 DMR showed mean methylation levels of 2.7% for 35-40 μm, 74.0% in 40-45 μm, 28.0% for 45-50 μm, 50.7% for 50-55 μm, and 55.3% for 55-60 μm oocytes (Figure 3-4 and 3-11). No significant difference was observed in methylation levels between Gja4-null and control oocytes. For the Peg1 DMR, mean methylation levels were 1.8% in 35-40 μm, 1.4% in 40-45 μm, 9.7% in 45-50 μm, 14.3% in 50-55 μm, and 19.1% in 55-60 μm oocytes (Figure 3-6 and 3-12). Statistical analysis of Peg1 showed a significant difference in methylation acquisition between control and Gja4-deficient oocytes (P = 0.0006). Because Gja4-null oocytes stop growing and are eventually lost from the follicles, it could not be determined whether this is a delay or a disruption in Peg1 DNA methylation acquisition.

3.4 Discussion

Growth and maturation of oocytes within follicles requires bidirectional signaling and exchange of nutrients, metabolites and second messengers through gap junctions between the oocyte and granulosa cells (Matzuk et al. 2002, Gilchrist et al. 2008, Su et al. 2009). Aberrant endocrine signaling and loss of gap junctional communication between the oocyte and granulosa cells leads to compromised folliculogenesis, oocyte maturation and oocyte competency, consequently impairing fertility. Given that oocyte-specific DNA methylation establishment at imprinted genes occurs during this growth phase, we determined whether compromised endocrine signaling and gap junctional communication would disrupt de novo methylation acquisition. Individual oocytes from Esr2- and Gja4-deficient mice were assessed for DNA methylation establishment at Snrpn, Peg3 and Peg1. We observed no aberrant or delayed acquisition of DNA methylation at Snrpn, Peg3 or Peg1 in oocytes from Esr2-deficient females, and no perturbation in Snrpn or Peg3 de novo methylation in oocytes from Gja4-null females. However, Gja4-deficiency resulted in a loss or delay in methylation acquisition at Peg1. One possible explanation for this difference between the three loci analyzed is the late
establishment of DNA methylation at the Peg1 gene. These results indicate that compromised fertility though impaired intercellular communication can lead to imprinting acquisition errors. Further studies are required to determine whether subfertility/infertility originating from impaired signaling and intercellular communication during oogenesis has an effect postfertilization on imprint maintenance in the preimplantation embryo.

3.4.1 Gene-specific Methylation Acquisition according to Oocyte Size

This study is the first to investigate imprint methylation acquisition of Snrpn, Peg3, and Peg1 in individual oocytes. We observed that each gene has its own size-dependent acquisition kinetics. Snrpn had the shortest acquisition interval with de novo methylation beginning at ~50 μm and near completion at >55 μm. Peg3 had the earliest and longest acquisition interval. DNA methylation acquisition was initiated at ~45 μm and was nearly complete at >65 μm. Peg1 had the latest acquisition of de novo methylation, beginning at ~55 μm and near completion by >70 μm. Previous studies reported similar findings using pooled oocytes where methylation level increased with days postpartum, follicular stage or with oocyte diameter/size, and initiation of acquisition was gene-specific (Lucifero et al. 2004, Hiura et al. 2006, Sato et al. 2007, Song et al. 2009). Oocyte-specific de novo methylation was also found to occur differentially with the maternal allele acquiring methylation prior to the paternal allele for Snrpn, Zac1 and Peg1 (Lucifero et al. 2004, Hiura et al. 2006). Our data are consistent with this observation. Firstly, in oocytes for which two alleles were successfully amplified, one allele possessed higher and the other allele lower methylation levels, indicative of maternal and paternal contribution, respectively. For example, Snrpn WT563 oocyte had 81% and 50% methylation (Figure 3-1). Secondly, for oocytes within each diameter range (see Peg3 control oocytes between 60 and 65 μm; Figure 3-3), a subset of oocytes had high methylation percentages (68, 71, 87 and 96%, indicative of the maternal allele) while others had low methylation percentages (18, 28, 48, 52%, indicative of the paternal allele). Finally, scatter plots show two distinct cohorts within the same range of diameter measurements. For example, Peg1 control oocytes between 55 and 65 μm grouped into 0-40% methylation and 75-100% methylation (Figure 3-6).
3.4.2 Compromised Fertility leads to Loss or Delayed Peg1 Methylation Acquisition

While Gja4-deficient oocytes ceased development and did not achieve mature size, analyses indicated that they were not compromised in their ability to catalyze DNA methylation as de novo DNA methylation was initiated for the Snrpn and Peg3 imprinted genes. The failure to initiate Peg1 methylation acquisition may simply be due to the fact that oocytes lacking CX37 never reach the size necessary for de novo methylation to commence at late-acquiring loci. However, control oocytes of comparable size (55–60 μm) displayed initiation of de novo Peg1 methylation. This suggests that Peg1 methylation acquisition was lost or delayed in mutant oocytes. Alternatively, CX37 null oocytes may have reduced stores of methyl donors or other metabolites required for DNA methylation that would normally be transported from granulosa cells to the oocyte via gap junctions. If this is the case, then there must have been sufficient availability of methyl donors in mutant oocytes for Snrpn and Peg3 de novo methylation, but oocytes lacking junctional coupling with the granulosa cells may have exhausted their methyl donors during oocyte growth, preventing de novo methylation at late-acquiring genes like Peg1. To investigate the requirement for methyl donors during follicle development, Anckaert and colleagues (2010) cultured preantral follicles in medium with low methyl donors. While this led to impaired antrum development and polar body formation, it did not impede the acquisition of DNA methylation at the Snrpn ICR and the Peg3 DMR (Anckaert et al. 2010). However, a reduced level of DNA methylation was found at the Peg1 DMR. This provides support for the argument that gap junctional communication provides important metabolites for DNA methylation acquisition. To better understand the mechanism leading to loss or delayed methylation acquisition, further studies are required to assess the level of methyl donors, amount of S-adenosylmethionine, and ability to carry out global and gene-specific methylation in 55–60 μm CX37-null or CX37-depleted oocytes. Furthermore, methylation studies should be carried out using F1 females. For Peg1 CX37 oocytes between 45 and 60 μm, oocytes possessed 0–53% methylation. DNA methylation acquisition was likely initiated on the maternal Peg1 allele in some oocytes, while other oocytes lacked methylation on both parental alleles.
Thus, loss or delayed Peg1 methylation acquisition may preferentially lead to a failure of the paternal allele to become methylated. Further studies are required to investigate this potential grandpaternal effect.

Peg1 may also be more susceptible to perturbation by assisted reproductive technologies. Loss of Peg1/PEG1 methylation was observed in mouse oocytes following in vitro maturation (Kerjean et al. 2003), and human oocytes following ovarian stimulation (Sato et al. 2007). Further studies are required to determine whether the susceptibility of Peg1 to perturbation relates to its late acquisition of methylation or whether a different epigenetic regulatory mechanism(s) operates at this gene. Superovulation also caused imprinting errors in the mouse preimplantation embryo (Market-Velker et al. 2010b). Although imprinted methylation acquisition was not perturbed in mouse oocytes by exogenous hormone treatment (Anckaert et al. 2009a). We hypothesized that superovulation disrupts maternal-effect gene products required for imprint maintenance during embryo development. Thus, impaired fertility may not only disrupt Peg1 methylation acquisition but may also lead to inadequate stores of maternal products, including those from granulosa cells, that may disrupt imprint maintenance at Peg1 as well as at Snrpn and Peg3 during preimplantation development. Extending studies to preimplantation embryos generated from fertilized ERβ-deficient and CX37-depleted oocytes will be required to determine their effects on imprint maintenance. In addition, further studies are required to determine whether assisted reproductive technologies, such as in vitro oocyte maturation and superovulation, lead to aberrant endocrine and paracrine signaling as well as granulosa cell–oocyte gap junctional communication.

It is important to understand granulosa cell-oocyte communication as technological advances move forward. Procedures such as slow-freezing cryopreservation and ultra-fast vitrification of oocyte-enclosed follicles, which employ cryoprotectants and very low temperatures, may permanently or temporally disrupt actin- or tubulin-rich projections that extend from granulosa cells through the zona pellucida to the oocyte (Kidder & Mhawi 2002). Slow-freezing of mouse, rhesus monkey and human preantral follicles disrupted projections and uncoupled the oocyte and granulosa cells (Barrett & Albertini 2010). Temporal disruption of oocyte-granulosa cell contacts was
also observed following vitrification (Trapphoff et al. 2010). Thus, transfer of molecules between the two compartments may be temporarily disturbed. While low levels of imprinting errors were detected in a subset of oocyte pools following vitrification (Trapphoff et al. 2010), further studies are required to determine whether disruption of oocyte-granulosa coupling leads to errors in imprint acquisition and/or maintenance.

Continued studies in animal models and in humans are required to understand the molecular mechanisms regulating genomic imprinting acquisition and maintenance as well as how impaired fertility and assisted reproductive technologies induce epigenetic changes and disease.
3.5 References


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4 CHAPTER 4: The Effects of Maternal Ovarian Stimulation on Imprint Acquisition

The work in this chapter originates from the following peer-reviewed article:


4.1 Introduction

The prevalence of Assisted Reproductive Technologies (ARTs) is rapidly increasing in developed countries as effective treatments for infertile couples. However, the consequences of manipulating germ cells and early embryos are not fully known. Accumulating evidence indicates that ART children are at increased risk for intrauterine growth retardation, premature birth, low birth weight, and genomic imprinting disorders (Doyle *et al.* 1992, Shiota & Yamada 2005).

Genomic imprinting is a transcriptional regulatory mechanism controlling unequal gene expression based on parental-origin (Reik & Walter 2001, Jaenisch & Bird 2003). While the majority of genes within the genome are biallelically expressed, imprinted genes have restricted expression to either the maternally or paternally inherited allele. The opposite parental copy is silenced by epigenetic mechanisms including DNA methylation, where a methyl group is covalently linked to cytosines within CpG dinucleotides (Verona *et al.* 2003). Germ cell and preimplantation embryo development are critical periods in genomic imprinting regulation. During spermatogenesis, paternal methylation imprints are acquired by the post-natal stage (Shamanski *et al.* 1999). During oogenesis, maternal methylation imprints are acquired during maturation from primordial to antral follicle in correlation with oocyte diameter (Lucifero *et al.* 2002, Lucifero *et al.* 2004, Hiura *et al.* 2006). Importantly, the timing of ARTs coincides with these critical stages and presents an opportunity for the disruption of imprinting acquisition and maintenance. Aberrant imprinting can lead to developmental disorders such as Beckwith-
Wiedemann Syndrome (BWS) and Angelman Syndrome (AS) (Weksberg et al. 2005), which are characterized by loss of maternal methylation at the *Kcnq1ot1* and *Snrpn* imprinting control regions (ICR), respectively (Cox et al. 2002, DeBaun et al. 2003, Gicquel et al. 2003, Maher et al. 2003, Orstavik et al. 2003).

Superovulation, or ovarian stimulation, is an ART procedure that enables increased oocyte production. It has become common practice to incorporate some form of ovarian stimulation to treat infertility in humans. The use of high doses of exogenous hormones has recently caused much debate surrounding their effects on oocyte maturation (Van der Auwera & D’Hooghe 2001, Krisher 2004). Typically, multiple ARTs procedures are performed together, and may have accumulating effects on the mechanisms of imprinting regulation. However it has been shown that superovulation alone as a fertility treatment can cause aberrant imprinting in offspring. As imprint acquisition has been shown to occur relatively late in oogenesis (Obata et al. 1998, Lucifero et al. 2004, Hiura et al. 2006), the establishment of these imprints may be susceptible to exogenous hormone treatments. Evidence from mice includes delayed in vitro preimplantation development, fetal growth retardation, and increased number of abnormal blastocysts and number of resorptions (Ertzeid & Storeng 2001, Van der Auwera & D’Hooghe 2001). At the molecular level, embryos derived from superovulated females exhibit a higher occurrence of abnormal global methylation patterns, presenting either complete loss of methylation in one or both nuclei, or complete methylation in one or both nuclei (Shi & Haaf 2002). Likewise, for both BWS and AS human imprinting disorders, patients have been identified having ovarian stimulation as the only form of ARTs used (Young et al. 1998, Chang et al. 2005, Ludwig et al. 2005). This questions the impact of superovulation on the ability of the oocyte to establish maternal imprints.

We have previously shown that superovulation alone affects genomic imprinting in blastocyst stage embryos at four imprinted genes (*Snrpn, Kcnq1ot1, Peg3, H19*) in a hormone dosage-dependent manner (Market-Velker et al. 2010b). A greater frequency of aberrant methylation occurs at the high hormone dosage. As disruption of maternal-inherited as well as paternal-inherited methylation was observed, we proposed that superovulation had dual effects during oogenesis, firstly acting on imprint acquisition within the oocyte, and secondly on maternal-effect gene products later required for
imprint maintenance within the embryo (Market-Velker et al. 2010b). To distinguish between these two effects, we compared the methylation levels in spontaneously ovulated and superovulated oocytes. We hypothesized that superovulation increases the risk of developing embryonic imprinting perturbations by affecting maternal imprint acquisition. To address this, we analyzed the methylation patterns of Snrpn, Kcnq1ot1, Peg3, and H19 following the same high and low exogenous hormone treatments, as our previous study (Market-Velker et al. 2010b). Contrary to our hypothesis, we observed that superovulation does not affect methylation acquisition at any examined imprinted loci regardless of hormone treatment dosage. As such, our results indicate that superovulation disrupts maternal-effect gene products required for imprint maintenance during embryo development.

4.2 Materials and Methods

4.2.1 Ovarian Stimulation and Oocyte Collection

C57BL/6(CAST7p6) [B6(CAST7p6)] females were crossed with C57BL/6 (B6) males to obtain B6(CAST7p6)XB6 F1 females, which inherited one Mus musculus castaneus chromosome 7 and partial chromosome 6 (modified from Mann et al. 2003) from the mother, and one B6 chromosome set from the father. Strain-specific polymorphisms between these mice allow identification of grandmaternal and grandpaternal alleles within oocytes. To recover superovulated oocytes, F1 females were injected with a single dose of equine Chorionic Gonadotropin (eCG, Intervet Canada) followed by the same dose of human serum Chorionic Gonadotropin (hCG, Intervet Canada) 46-48 hours later. Two hormone doses, 6.25 IU (low) and 10 IU (high) were used to stimulate ovulation (Market-Velker et al. 2010b). Oocyte-cumulus cell complexes were recovered from ampullae into M2 media (Sigma) the following day at noon (22 hours post-hCG). Control oocyte-cumulus cell complexes were collected at noon from untreated females in the estrous stage of spontaneous ovulation cycles. Experiments were performed in compliance with the guidelines set by the Canadian Council for Animal Care, and the policies and procedures approved by the University of Western Ontario Council on Animal Care.
Oocytes were dissociated from surrounding cumulus cells using 0.3 mg/mL hyaluronidase (Sigma) and were washed three times in 30 μL of M2 media. Two diameter measurements were taken using the Olympus IX81 microscope, averaged then halved for the radius. Oocyte volume was calculated $V=\frac{4}{3}\pi r^3$. To compare oocyte diameter and volume, ANOVA and t-test were performed. Following removal of the zona pellucida using Acidic Tyrode’s solution (Sigma), oocytes were washed twice in 30 μL M2 media and then placed individually in minimal media onto a glass slide. At 70°C, the oocyte was gently mixed with a small amount of 2:1 agarose:lysis solution [20 μL 3% low melting point agarose (Sigma), 8 μL lysis buffer (100 mM Tris–HCl pH 7.5 (Bioshop)), 500 mM LiCl (Sigma), 10 mM EDTA pH 8.0 (Sigma), 1% LiDS (Bioshop), 5 mM DTT (Sigma)], 1 μL 2mg/mL proteinase K (Sigma), 1 μL 10% Igepal (Sigma)] and then embedded in 10 μL of the 2:1 agarose:lysis solution under 300 μL mineral oil (Sigma), producing an agarose-embedded oocyte. Following a 10-minute incubation on ice, the mineral oil was removed and 500 μL lysis buffer was added. Embedded oocytes were incubated overnight in a 50°C waterbath. The following day, embedded oocytes were either processed immediately, or stored in 300 μL mineral oil at -20°C for up to five days. Superovulation and oocyte collection were performed on eight 6.25 IU treated females and seven 10 IU treated females. For the untreated group, oocytes were collected from fifteen spontaneously ovulating females.

4.2.2 Bisulfite Mutagenesis of Individual Oocytes

Bisulfite mutagenesis involving individual embedded oocytes was performed as previously described with modification (Market-Velker et al. 2010b). In short, following overnight incubation, lysis buffer was removed and the agarose beads were covered with 300 μL mineral oil, incubated for 2.5 minutes at 90°C to heat inactivate the proteinase K, then placed on ice for 10 minutes. DNA was denatured in 0.1 M NaOH (Sigma) at 37°C for 15 minutes with periodic shaking. Agarose beads were placed in a 2.5 M bisulfite solution [0.125 M hydroquinone (Sigma), 3.8 g sodium hydrogensulfite (Sigma), 5.5 mL water, 1 mL 3 M NaOH] at 50°C for 3 (Kcnq1ot1) or 3.5 hours (remaining genes). Following bisulfite mutagenesis, samples were desulfonated with 0.3 M NaOH at 37°C for 15 minutes with shaking. Agarose beads were washed twice each with TE pH 7.5 and
water, then directly added, as a solid bead, to a Ready-to-Go PCR bead (GE) containing gene-specific primers and 1 μL of 240 ng/mL tRNA as a carrier in a 15 μL solution with 25 μL mineral oil overlay. Negative controls (no oocyte) were processed alongside each bisulfite reaction. From the first round product, 5 μL was added into the second round PCR reaction with 25 μL mineral oil overlay. PCR amplification of Snrpn, Kcnq1ot1, Peg3 and H19, and cloning was performed as described (Market-Velker et al. 2010b). Approximately 30 μL of colony PCR reaction was sent to Bio-Basic Inc. (Markham, ON, Canada) for sequencing. For each sample, 5 clones were sequenced. Since only one strand of oocyte DNA is expected to amplify, any samples with more than one methylation pattern were excluded from analysis, as this might be indicative of cumulus cell contamination and/or polar body inclusion (Supplementary Figures 4-1s and 4-2s in Appendix 1). Polymorphisms between the B6 and B6(CAST7p6) alleles allowed distinction of grandparental identity for each sequence.

4.3 Results

To determine the effects of superovulation on imprint acquisition, we examined the methylation status of the Snrpn ICR, the Kcnq1ot1 ICR, and the Peg3 differentially methylated region (DMR), which acquire methylation during oogenesis as well as the H19 ICR, which remains unmethylated in oocytes. Oocytes were obtained from females undergoing spontaneous and induced ovulation. For the spontaneously ovulating group, 106 oocytes were subjected to bisulfite mutagenesis. Successful amplification was observed for 45% of individual oocytes (48/106) (Table 4-1). Of these, 83% showed one methylation pattern (40/48) for the imprinted gene of interest. The remaining eight oocytes had more than one methylation pattern and were excluded from further analysis under the assumption of cumulus cell contamination. Ten oocytes were examined for each of the four genes. We found that the Snrpn ICR, the Kcnq1ot1 ICR and the Peg3 DMR were fully methylated in individual oocytes (Figure 4-1), similar to a previous study (Lucifero et al. 2004). Likewise, the H19 ICR was unmethylated in individual oocytes.

For the induced ovulation group, we examined 301 individual oocytes. Successful amplification was observed in 48% (76/157) of the 6.25 IU treated oocytes and 56%
(81/144) of 10 IU treated oocytes (Table 4-1). Of these, 79% (60/76) and 80% (65/81), respectively, displayed one methylation pattern for the imprinted gene of interest. At least fifteen oocytes for each imprinted gene were examined at each dosage.

Contrary to our hypothesis, we observed that the Snrpn ICR, the Kcnq1ot1 ICR and the Peg3 DMR were fully methylated, while H19 was unmethylated, whether the hormone treatment was low (Figure 4-2) or high (Figure 4-3). A single oocyte from the 10 IU hormone group showed an unmethylated Kcnq1ot1 ICR. We attribute this to a spontaneous loss of maternal methylation, cumulus cell contamination, or a rare imprinting error caused by ovarian stimulation. Furthermore, as both grandmaternal (B6(CAST7p6)) and grandpaternal (B6) alleles displayed similar hypermethylation, our results do not demonstrate differential allelic susceptibility to perturbations by superovulation. Finally, analysis of oocyte diameter, volume, or morphology revealed no significant difference between oocytes derived from spontaneously and induced ovulating females (data not shown).

4.4 Discussion

In a previous study, we examined the effects of superovulation on imprinted methylation of Snrpn, Kcnq1ot1, Peg3 and H19 in blastocyst-stage embryos recovered from spontaneously and induced ovulating females (Market-Velker et al. 2010b). We observed a loss of Snrpn, Kcnq1ot1 and Peg3 maternal methylation and a gain of maternal H19 methylation. This aberrant imprinting occurred in a dose-dependent manner, with perturbations more frequent following a high than a low hormone dosage. As superovulation was anticipated to affect oocytes, imprinting perturbations could be expected on the maternal, oocyte-derived allele. Surprisingly, we also observed loss of H19 methylation on the paternal, sperm-contributed allele. This led us to propose that superovulation had dual effects during oogenesis, disrupting imprint acquisition during oogenesis, and producing changes in maternal-effect gene products that are later required for imprint maintenance in resulting embryos (Market-Velker et al. 2010b).
Table 4-1 Single Oocyte Analysis from Spontaneous and Induced Ovulation Groups

<table>
<thead>
<tr>
<th>Ovulation group</th>
<th>Oocytes amplified</th>
<th>Five clones one pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous ovulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snrpn</td>
<td>58 (11/19)</td>
<td>91 (10/11)</td>
</tr>
<tr>
<td>Kcnq1tot1</td>
<td>36 (10/28)</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td>Peg3</td>
<td>31 (10/32)</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td>H19</td>
<td>63 (17/27)</td>
<td>59 (10/17)</td>
</tr>
<tr>
<td>Total</td>
<td>45 (48/106)</td>
<td>83 (40/48)</td>
</tr>
<tr>
<td>6.25 IU induced ovulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snrpn</td>
<td>54 (19/35)</td>
<td>79 (15/19)</td>
</tr>
<tr>
<td>Kcnq1tot1</td>
<td>44 (17/39)</td>
<td>88 (15/17)</td>
</tr>
<tr>
<td>Peg3</td>
<td>45 (18/40)</td>
<td>83 (15/18)</td>
</tr>
<tr>
<td>H19</td>
<td>51 (22/43)</td>
<td>68 (15/22)</td>
</tr>
<tr>
<td>Total</td>
<td>48 (76/157)</td>
<td>79 (60/76)</td>
</tr>
<tr>
<td>10 IU induced ovulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snrpn</td>
<td>63 (19/30)</td>
<td>79 (15/19)</td>
</tr>
<tr>
<td>Kcnq1tot1</td>
<td>40 (16/40)</td>
<td>100 (16/16)</td>
</tr>
<tr>
<td>Peg3</td>
<td>51 (20/39)</td>
<td>85 (17/20)</td>
</tr>
<tr>
<td>H19</td>
<td>74 (26/35)</td>
<td>65 (17/26)</td>
</tr>
<tr>
<td>Total</td>
<td>56 (81/144)</td>
<td>80 (65/81)</td>
</tr>
</tbody>
</table>

Note: Values are percentage (number).

Figure 4-1 Methylation Analysis in Individual Spontaneously Ovulating Oocytes

Methylation analysis at the *Snrpn* ICR (A), *Kcnq1ot1* ICR (B), *Peg3* DMR (C), and *H19* ICR (D). For each gene, n=10. Black circles indicate methylated CpGs. White circles indicate unmethylated CpGs. U, untreated; C, CAST allele; B, B6 allele. Each row represents an individual oocyte; designation indicated to the left. The *Snrpn* ICR region analyzed contains 16 CpGs; a polymorphism eliminates CpG dinucleotide 1 in the CAST allele. The *Kcnq1ot1* ICR region analyzed contains 20 CpGs. The *Peg3* DMR region analyzed contains 24 CpGs; a polymorphism eliminates CpG dinucleotide 22 in the B6 allele. The *H19* ICR region analyzed contains 17 CpGs; a polymorphism eliminates CpG dinucleotide 8 in the B6 allele.
Methylation analysis at the *Snrpn* ICR (A), *Kcnq1ot1* ICR (B), *Peg3* DMR (C), and *H19* ICR (D). For each gene, n=15. Details are as described in Figure 4-1.
Methylation analysis at the *Snrpn* ICR (A), *Kcnq1ot1* ICR (B), *Peg3* DMR (C), and *H19* ICR (D). For each gene, n=15-17. Details are as described in Figure 4-1.

**Figure 4-3 Methylation Analysis in Individual High Dose (10 IU) Superovulated Oocytes**
To distinguish between these effects, in our current study, we analyzed methylation acquisition in individual MII ovulated oocytes at the same imprinted genes following the same superovulation dosage regimens. To our surprise, there were no superovulation-induced imprinting errors. We conclude that ovarian stimulation leads to disruption of maternal-effect gene products that are later required for imprint maintenance during embryo development. Support for this comes from a study examining global methylation following superovulation. They found that as early as the 2-cell stage, embryos from superovulated females had aberrant global methylation patterns twice as often as embryos from naturally ovulated females (Shi & Haaf 2002).

To date, investigations of the effects of superovulation on imprinting in oocytes have been limited and have produced conflicting results. Consistent with our findings, Anckaert et al. observed normal methylation patterns at Snrpn, Peg3 and H19 loci, along with the Igf2r locus in pooled oocytes from superovulated female mice (Anckaert et al. 2009a). In contrast to our results, Sato et al. found an aberrant gain of DNA methylation at the normally unmethylated H19 locus in pooled mouse oocytes, although no change in methylation was observed for Peg1, Kcnqlot1 and Zac. Additionally, they found that individual human oocytes from women undergoing multiple hormone stimulations possessed aberrant imprinting at both the PEG1 and H19 loci (Sato et al. 2007). By analyzing individual mouse oocytes, our study obtained results from a greater number of oocytes, and eliminated any biases based on oocyte pools and undetected cumulus cell contamination. It is important to acknowledge that human oocytes may be more susceptible to ovarian stimulation, although multiple ART procedures and advanced maternal age may be confounding factors (El-Maarri et al. 2001, Borghol et al. 2006, Geuns et al. 2007).

During oogenesis, methylation acquisition occurs in a parental-specific manner. For Snrpn, the maternal allele is re-methylated prior to the paternal allele acquiring a maternal methylation identity (Lucifero et al. 2004). Thus, the original grandparental identity of the oocyte chromosomes was an important consideration in the design of this study as the paternal allele may be more susceptible to imprint disruption, given its later acquisition. Our results showed no difference in susceptibility to maternal imprint acquisition between the grandparental alleles at either hormone dosage in MII oocytes.
Analysis of early embryos will be required to determine whether there is differential susceptibility to loss of imprint maintenance.

Our study argues against methylation acquisition as the origin for imprinting errors following superovulation, instead supporting the notion that ovulation induction via exogenous hormones causes aberrant methylation maintenance during preimplantation development. Acceleration of oocyte maturation by superovulation may result in inefficacy to synthesize and store sufficient amounts of these maternal factors (Li et al. 2010). Our findings have significant bearing on procedures involving manipulation of oocytes, such as in vitro follicle culture and oocyte maturation, as well as oocyte cryopreservation and vitrification. A number of studies have argued that these methods do not increase the risks of abnormal imprinting acquisition in oocytes (Anckaert et al. 2009a, Anckaert et al. 2009b, Trapphoff et al. 2010). However, this study together with our previous results on the effects of superovulation on imprint maintenance supports the importance of more in-depth investigations into the effects of ARTs by extending studies of oocyte manipulations to the embryo.
4.5 References


CHAPTER 5: The Effects of Maternal Ovarian Stimulation on the Maternal-Effect Factor ZFP57

The work in this chapter originates from the following manuscript:


5.1 Introduction

Genomic imprinting is a phenomenon that leads to monoallelic expression of a subset of genes based on parental-origin. These genes are subject to epigenetic modifications, such as DNA methylation, that inhibit transcription of one parental allele and others, such as active histone modifications, that permit transcription of the other parental allele (Bartolomei & Ferguson-Smith 2011). Genomic imprinting is a two-generation phenomenon whereby sex-specific epigenetic modifications are inherited in the gametes of parents and these modifications are maintained in offspring to direct monoallelic gene expression. As such, gametogenesis and preimplantation embryo development are critical periods for imprinted DNA methylation acquisition and maintenance, respectively. During spermatogenesis, paternal methylation imprints are established by the post-natal stage (Saitou et al. 2012). During oogenesis, maternal methylation imprints are acquired during maturation from primordial to antral follicle in correlation with oocyte diameter (Lucifero et al. 2004, Hiura et al. 2006, Denomme et al. 2012). Following fertilization, differential methylation between alleles is reliably maintained despite a global wave of genomic DNA demethylation during the preimplantation period.

Disruption to genomic imprinting can lead to developmental disorders such as Beckwith-Wiedemann Syndrome (BWS) and Angelman Syndrome (AS) (Weksberg et al. 2005, Horsthemke & Wagstaff 2008), which are characterized by genetic and epigenetic
errors at the \textit{KCNQ1OT1}, \textit{H19} and \textit{SNRPN} imprinting control regions (ICRs), respectively. Importantly, these syndromes have an increased prevalence in the Assisted Reproductive Technologies (ARTs) community, suggesting a link between ARTs and imprint dysregulation (Cox \textit{et al.} 2002, DeBaun \textit{et al.} 2003, Gicquel \textit{et al.} 2003, Maher \textit{et al.} 2003, Orstavik \textit{et al.} 2003). The timing of ARTs coincides with the critical stages of imprint acquisition and imprint maintenance, and thus may be susceptible to disruption. Within the last few decades, ARTs have become standard treatment of care for infertility in developed countries. These techniques encompass manipulation of gametes and embryos, such as ovarian stimulation (superovulation), and \textit{in vitro} embryo culture.

The impact of ARTs on imprinting regulation has become the focus of intense investigation by a number of groups. We and others have shown that DNA methylation acquisition during oogenesis is a robust process, and interventions such as superovulation or \textit{in vitro} oocyte maturation do not perturb imprint acquisition (Anckaert \textit{et al.} 2009a, Denomme \textit{et al.} 2011, Denomme \& Mann 2012). However, superovulation and/or embryo culture result in loss of imprinted DNA methylation as early as the blastocyst stage of embryo development (Sasaki \textit{et al.} 1995, Doherty \textit{et al.} 2000, Mann \textit{et al.} 2004, Market-Velker \textit{et al.} 2010a, Market-Velker \textit{et al.} 2010b, Market Velker \textit{et al.} 2012), as well as at postimplantation time points (Mann \textit{et al.} 2004, Fortier \textit{et al.} 2008). Thus, imprint maintenance is more susceptible than imprint acquisition to the adverse effects of these techniques. This led to our hypothesis that ARTs affects a maternal-effect factor(s) that is produced in the oocyte but is required for imprint maintenance in the embryo.

Many transcripts from the maternal genome accumulate during oocyte growth. While the majority are used by the oocyte, some are required post-fertilization for critical aspects of development. Since imprinting marks must be maintained through this oocyte-to-embryo transition, maternal factors likely contribute to its regulation. To date, a handful of maternal factors have been shown to play essential roles. Protection from active zygotic demethylation occurs by binding of the maternal effect protein developmental pluripotency-associated 3 (DPPA3/STELLA/PGC7) to H3K9me2. This inhibits 5mC conversion to 5hmC by TET3 (Nakamura \textit{et al.} 2007, Nakamura \textit{et al.} 2012). Protection from passive demethylation occurs via the maternal effect proteins, zinc finger protein 57 (ZFP57), tripartite motif 28 protein (TRIM28/KAP1), and DNA
methyltransferase 1 (DNMT1) (Iyengar & Farnham 2011, Iyengar et al. 2011, Messerschmidt et al. 2012). ZFP57 and TRIM28 bind to CpG methylation at imprinted genes, attracting repressive macromolecular complexes that include DNMT1 and the H3K9me3-catalyzing histone methyltransferase SETDB1 (Li et al. 2008, Quenneville et al. 2011, Zuo et al. 2012). In this current study, we examined ZFP57 transcript and protein abundance as well as ZFP57 localization in spontaneously ovulated and superovulated oocytes and resulting embryos. We observed that superovulation leads to increased protein levels and mislocalization away from the nucleoplasm in the early stages of preimplantation development, and increased cytoplasmic levels remained in subsequent preimplantation stages. This misregulation of ZFP57 preceded a decrease in protein enrichment at imprinted domains, proposing a possible mechanism for imprint methylation maintenance loss following ARTs.

5.2 Materials and Methods

5.2.1 Ovarian Stimulation and Oocyte Collection

Oocytes were obtained from B6(CAST7p6) (CAST chromosome 7 partial chromosome 6 on a C57BL/6 background) female mice and embryos were obtained from B6(CAST7p6) females and C57BL6 (B6) males to enable allelic identity. B6(CAST7p6)xB6 F1 and B6 females were also used for immunocytochemistry and Western blot analyses where allelic identity was not required. Female mice were injected with a single dose of 10 IU eCG (Intervet Canada) followed by a single dose of 10 IU hCG (Intervet Canada) 44-48 hours later. To produce embryos, females were then mated to B6 males the same night following hCG. To obtain spontaneously ovulated embryos, control females were checked for estrous during their natural cycle and then mated with B6 males. Females were checked the following day for the presence of a vaginal plug. Oocytes and 1-cell embryos were harvested from the ampulae at 0.5 days post coidum (dpc) and dissociated from surrounding cumulus cells using 0.3 mg/mL hyaluronidase (Sigma). Oviducts were flushed for 2-cell embryos at 1.5 dpc, 4-cell embryos at 2 dpc, and 8-cell embryos at 2.5 dpc. Morula-stage embryos were flushed from uteri at 3 dpc.
and blastocysts at 3.5 dpc. Embryos were washed several times in 30 μL M2 media (Sigma).

5.2.2 Gene Expression Analysis

Embryos were immediately stored at -80°C in individual 0.2 mL tubes. To generate a cDNA library, 10 μL of Dynabead Lysis Buffer (Invitrogen) was added to pre-equilibrated oligo-dT Dynabeads (Invitrogen), which were then added to each 0.2 μL tube containing an individual oocyte or embryo. RNA-oligo-dT hybridization was conducted for 10 minutes at room temperature with shaking. On the magnetic rack, the supernatant was removed and the samples were washed twice with Dynabead Wash Buffer A (Invitrogen) and three times with Dynabead Wash Buffer B (Invitrogen). Samples underwent reverse transcription, generating a cDNA library as previously described (Market-Velker et al. 2010a, Market Velker et al. 2012). Each embryo cDNA library sample was stored in ITT (50 μL 10% IGEPAL, 50 μL 10% Tween 20, 50 mL TE) buffer at 4°C.

Zfp57 transcript abundance was performed using the cDNA library generated for each oocyte and embryo, with Proteasome 26S subunit, non-ATPase 4 (Psmd4) serving as the internal control. Primers and PCR parameters can be found in Table 5-1. Second-strand synthesis was performed using the forward primers of both Zfp57 and Psmd4, and amplification products were then split into separate reactions for RT-PCR. Psmd4 was chosen as an internal control transcript, since this gene has been shown to exhibit relatively equal transcript abundance throughout all seven stages analyzed (Kouadjo et al. 2007). Amplification was performed on at least ten biological replicates for each stage and treatment group with SYBR Green PCR supermix (BioRad) using the BioRad Opticon Monitor Real-Time PCR machine and software. On account of primer-dimers produced with such low amounts of cDNA, semi-quantitative analysis of gel images was performed using Image J densitometry and ZFP57 values were normalized to those of the internal control gene, Psmd4. Significant differences were calculated using the Student’s t-Test statistical test between spontaneously ovulated and superovulated samples. Error bars represent the standard error of the mean.
Table 5-1 List of Primers and PCR Parameters

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>PCR Program</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zfp57</td>
<td>Forward: CTGGAATAGAAGTCAACGCC</td>
<td>Second strand synthesis: 94°C 2 min 94°C 30 sec 56°C 30 sec 72°C 30 sec 94°C 10 min</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGCTTTGTGGATTTGTGAAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Psmd4</td>
<td>Forward: CATCTTCAGTAGGGCTTCATC</td>
<td>Semi-Quantitative RT-PCR: 95°C 4 min 94°C 30 sec 56°C 30 sec 72°C 30 sec 45 cycles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: GACAGTGCTCTCTGGACCTA</td>
<td>Melting curve: 94°C 2 min 30°C 2 min 55°C-95°C, read every 1°C</td>
<td></td>
</tr>
<tr>
<td>Snrpn</td>
<td>Forward: CACAGCTCTGACTTCCAGGAG</td>
<td>Quantitative RT-PCR: 94°C 2 min 94°C 15 sec 56°C 15 sec 72°C 15 sec 45 cycles</td>
<td>ChIP</td>
</tr>
<tr>
<td></td>
<td>Reverse: ATTAGTGAGCAATCTTTGGG</td>
<td>Melting curve: 94°C 2 min 30°C 2 min 65°C-94°C, read every 1°C</td>
<td></td>
</tr>
<tr>
<td>Kcnq1ot1</td>
<td>Forward: GACCACTGGACTTCCAGGAG</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: ATTCATGAGCCGCCTCTTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peg3</td>
<td>Forward: CGATTTCTCTCTCGTACCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: CGTCTGCAGAGTGTCAGGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peg1</td>
<td>Forward: TATCATGGGCTAAGGGCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: AGAGATCCCTCCCAACATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H19</td>
<td>Forward: GGTTCACAAATGCACCTAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: GGGTCACAAATGCCACTAGG</td>
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</tbody>
</table>
5.2.3 Western Blot Analysis

Embryos were washed in 200 μL filtered 1X PBS and were stored in pools of 10 embryos in a 0.2 mL tube at -80°C. Each embryo pool was mixed with 10 μL cell lysis buffer (1X TE pH 7.5, 10% SDS (Bioshop)) and 1 μL proteinase inhibitor cocktail (Sigma) and placed on ice for 10 minutes. WB dye (3 μL; 100 mM Tris-HCl, 200 mM DTT, 4% SDS, 0.2% Bromophenol blue, 20% glycerol) was added, and the samples were mixed and placed at 70°C for 10 minutes. Following an additional 3 minutes on ice, the samples were loaded into a 15-well mini 8% SDS-PAGE gel and run for 1 hour. The gel was electrotransfered to an immuno-blot PVDF membrane (BioRad) for 2 hours covered in ice. The membrane was then blocked in 4% milk-TBST (tris-buffered saline, Tween 20) buffer at room temperature for 1 hour, and primary antibody was added in 4% milk-TBST, incubated at 4°C overnight for ZFP57 and TRIM28, and at room temperature for 1 hour for histone 3 (H3) and α-tubulin. The following morning, the membrane was washed in TBST three times, and a secondary antibody was added in 4% milk-TBST at room temperature for 1 hour. The membrane was again washed three times in TBST, and then incubated in Western Lightning Plus ECL solution (PerkinElmer) at room temperature for 3-5 minutes. The membrane was then allowed to expose to an x-ray film. Intensity measurements were made using Image J densitometry. Protein levels for ZFP57, TRIM28 and H3 were normalized to internal α-tubulin, which exhibits comparable protein levels throughout preimplantation with a slight decrease at the blastocyst stage. Significant differences were calculated using the Two Factor ANOVA with replication statistical test. Error bars represent the standard error of the mean. Antibody dilutions can be found in Table 5-2.

5.2.4 Immunocytochemistry

Immunocytochemistry was performed as described with modification (Bell et al. 2009). Following washes in M2 media (Sigma), embryos were washed in 200 μL 1X PBS in a glass dish, then fixed in 200 μL 4% PFA for 30 minutes. Following an additional wash in 1X PBS, fixed embryos were either processed immediately or stored in 1X PBS at 4°C for no more than 5 days.
### Table 5-2 List of Antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Dilution</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ZFP57 (Abcam; ab45341)</td>
<td>1:200</td>
<td>IF</td>
</tr>
<tr>
<td>Anti-ZFP57 (Abcam; ab45341)</td>
<td>1.5 μg</td>
<td>WB</td>
</tr>
<tr>
<td>Anti-ZFP57 (Abcam; ab45341)</td>
<td>4.5 μL</td>
<td>ChIP</td>
</tr>
<tr>
<td>Anti-KAP1 (Abcam ab22553)</td>
<td>1:200</td>
<td>IF</td>
</tr>
<tr>
<td>Anti-KAP1 (Abcam; ab22553)</td>
<td>1 μg</td>
<td>WB</td>
</tr>
<tr>
<td>Anti-H3 (Abcam; ab1791)</td>
<td>1:5000</td>
<td>WB</td>
</tr>
<tr>
<td>Anti-H3 (Abcam; ab1791)</td>
<td>2.5 μL</td>
<td>ChIP</td>
</tr>
<tr>
<td>Anti-a-tubulin (Sigma; T5168)</td>
<td>1:600</td>
<td>WB</td>
</tr>
<tr>
<td>Normal IgG</td>
<td>2.5 μL</td>
<td>ChIP</td>
</tr>
<tr>
<td>Anti-rabbit IgG-Alexa488 (Invitrogen; A11008)</td>
<td>1:200</td>
<td>IF</td>
</tr>
<tr>
<td>Anti-mouse IgG-Alexa594 (Invitrogen; A11032)</td>
<td>1:200</td>
<td>IF</td>
</tr>
</tbody>
</table>
Fixed embryos were permeabilized for 40 minutes in 200 μL permeabilizing buffer (0.5% Triton X-100 in 1X PBS) and then blocked overnight at 4°C in 200 μL blocking buffer (5% normal goat serum in 1X PBS). Embryos were incubated with primary antibody overnight in an Antibody Dilution/wash Buffer (ADB; 0.005% Triton X and 1% normal goat serum in 1X PBS). Negative controls were incubated in ADB alone without primary antibody. Table 5-2 lists antibody dilutions and catalogue numbers. The following morning, embryos were washed in fresh ADB for 6-8 minutes at 37°C four times, and then incubated in secondary antibody in ADB for 1 hour at 37°C. Following one wash for 20 minutes in ADB with Hoechst 33342 dye (1:200 dilution) at 37°C, embryos were washed three more times for 6-8 minutes in fresh ADB at 37°C.

Embryos were placed in 4 μl M2 media in a glass-bottom culture dish under mineral oil for imaging. Embryos were imaged at 20X magnification using the Fluoview 1000 laser scanning confocal microscope (Olympus Corp.), using the 20X objective (Olympus super apochromat 0.75) and Fluoview V10-ASW 2.1 software. At least 20 samples were analyzed for each embryonic stage and treatment group. Fluorescence intensity was calculated using Volocity Demo 6.3 by drawing a perimeter enclosing each nucleus as well as each embryo and determining the sum intensity within the enclosed areas, expressed in arbitrary units. Intensity value for nucleoplasm was calculated as sum nuclear staining divided by volume of nuclear staining. Intensity value for cytoplasm was calculated as sum embryo staining minus sum nuclear staining divided by volume of embryo staining minus volume of nuclear staining. Statistical significance was determined by the Student’s t-Test between spontaneously ovulated and superovulated samples.

5.2.5 Micro-scale Chromatin Immunoprecipitation

Micro-scale ChIP was performed as described with modification for single blastocyst stage embryos (O’Neill et al. 2006, Dahl & Collas 2009, Kernohan et al. 2010). Blastocysts were fixed in 200 μL 1% PFA at room temperature for 8 minutes in a glass dish. Crosslinking was stopped by placing the embryos in 200 μL 1X glycine (125mM) at room temperature for 5 minutes. Embryos were then washed twice in 200 μL
1X PBS, and transferred individually to the bottom of a sonication tube in less than 5 μL
1X PBS. Individual blastocysts were either processed immediately or stored at -80°C.

For solution pre-treatment, 8 μL Dynabeads-Protein G (Invitrogen) was washed
on ice with 100 μL cool Dilution Buffer in a 0.2 mL tube, and then 4.5-5 μL ZFP57
antibody, 2.4 μL Histone H3 antibody or 2.5 μL normal IgG antibody in 100-150 μL
Dilution Buffer (0.01% SDS, 1% Triton-100, 2 mM EDTA, 20 mM Tris-HCl (pH8.1),
160 mM NaCl) was added. Dynabeads-Protein G-antibody was rotated at 4°C for 2 hours,
following which the supernatant was removed and Dynabeads-Protein G-antibody was
washed once with Dilution buffer for 5 minutes on rotator.

During this time, 5 μL acid Tyrode’s solution (Sigma) was added to each fixed
blastocyst sample on ice to remove the zona pellucida. Three μL 1% SDS lysis buffer
(1% SDS, 10mM EDTA, 50mM Tris-HCl (pH8.1)), 16 μL 1X PBS (on ice), and 1 μL
proteinase inhibitor cocktail (Sigma) was added. Samples were incubated on ice for 3-5
minutes, and then were sonicated for 13 cycles (30 sec/cycle) in cool water. Samples
were spun down after 7 cycles and after 13 cycles. A total of 10 μL of the sample
(chromatin lysate) was added to a 0.2 mL PCR tube, and stored at -80°C overnight as
“Input DNA”. To the remaining chromatin lysate, 180 μL Dilution buffer and 2 μL
cocktail was added, mixed and then transferred to the 0.2 mL tube containing the
Dynabeads-Protein G-antibody complex. Samples were then rotated overnight at 4°C.
The following morning, the supernatant was removed and samples were washed 2X for
ZFP57 and 1X for H3 in 200 μL low salt buffer (LSB; 0.1% SDS, 1% Triton X-100, 2
mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl) for 4 minutes, 1X for ZFP57 and
3X for H3 in 200 μL high salt buffer (HSB; 0.1% SDS, 1% Triton X-100, 2 mM EDTA,
20mM Tris-HCl (pH 8.1), 500 mM NaCl) for 4 minutes, 1X in 200 μL TE for 5 minutes,
and 1X in 200 μL water for 1 minute, resulting in “ChIP DNA”. InstaGene Matrix
(BioRad; Chelex-100 beads) (40 μL) was then added to the ChIP DNA on ice. After
removing the Input DNA from the freezer, 40 μL Chelex-100 beads were added in the
same fashion. Samples were heated to 100°C for 10 minutes, and cooled to room
temperature. After the addition of 1 μL proteinase K (Sigma), the samples were incubated
at 55°C for 30 minutes, rotating in a hybridization chamber, then heated again to 100°C
for 10 minutes. Supernatant was drawn into a non-stick tube, generating the total ChIP DNA and Input DNA (~40 μL each).

Primers were designed to span two or three ZFP57 hexanucleotide motifs (TGCCGC) within each DMR. Primer details and PCR parameters can be found in Table 5-1. Input DNA or ChIP DNA was added to SYBR Green PCR supermix (BioRad) containing gene-specific primers. Amplification was performed on biological replicates using the BioRad Opticon Monitor Real Time PCR machine and software. Percent enrichment over input was calculated as follows: \( \Delta Ct = (\text{ChIP Ct}) - (\text{Input Ct}) \), Power = \( 2^{-\Delta Ct} \), Ratio (%) = (Power / [ChIP DNA (μL) / Input DNA (μL)]) \times 100\%. Error bars represent the standard error of the mean. The statistical significance of enrichment was determined by Student’s t-test between spontaneously ovulated and superovulated samples.

5.3 Results

5.3.1 Superovulation did not affect Zfp57 Transcript Abundance

Since we hypothesized that superovulation adversely affects a maternal effect imprint protector protein, we first assessed Zfp57 mRNA expression in at least 10 individual spontaneously ovulated and 10 IU induced oocytes and resulting preimplantation embryos. In spontaneously ovulated samples, Zfp57 transcript abundance was high in oocytes and 1-cells, which is characteristic of a sequestered maternal mRNA (Figure 5-1). At the 2-cell stage, there was a dramatic decrease in Zfp57 transcript abundance, with transcript levels remaining low through the 4-cell, 8-cell and morula stages, consistent with maternal ZFP57 function. Zfp57 transcript abundance increased in blastocyst stage embryos, indicating that embryonic gene activation occurs late in preimplantation development. Comparison of spontaneously ovulated and 10 IU induced groups revealed no difference in transcript abundance at any stage, indicating that superovulation does not affect transcript abundance in oocytes or embryos.
Zfp57 transcript abundance was assessed by RT-PCR in individual oocytes and preimplantation embryos. Ten biological replicates were performed for each stage of spontaneously ovulated (white bars) and 10 IU induced (black bars) samples. Semi-quantitative intensity measurements of each gel band were calculated using ImageJ software, and Zfp57 transcript abundance was normalized to that of Psmd4. No significant differences were observed between control and 10 IU induced samples. Oo, oocytes; 1c, 1-cell; 2c, 2-cell; 4c, 4-cell; 8c, 8-cell Mo, morula; Bl, blastocyst stage embryos.
5.3.2 Superovulation Increased ZFP57 Protein Levels

Next, we quantified ZFP57 protein levels in oocytes and preimplantation embryos to assess the effects of superovulation. Western blot analysis was performed on triplicate pools of ten oocytes, 1-cells, 2-cells, 4-cells, 8-cells, morula and blastocyst stage embryos following spontaneously and 10 IU induced ovulation using antibodies for ZFP57, as well as Histone H3 as a control. We observed that relative ZFP57 protein levels were equal in both groups at the oocyte and zygote stages.

However, while ZFP57 levels decrease in the spontaneous group at the 2-cell stage, increasing at the blastocyst stage consistent with embryonic gene activation, ZFP57 protein levels remained at levels similar to 1-cell embryos in the 10 IU induced group at the 2-cell through to morula-stage. Higher protein levels were also present at the blastocyst stage in the 10 IU induced group compared to controls (Figure 5-2). These data indicate that there was greater stability of the ZFP57 protein in embryos in the superovulated group. In comparison, no difference was observed in oocytes or embryos for the control, Histone H3, in spontaneous ovulated and 10 IU induced groups (Figure 5-3). Since ZFP57 recruits its co-regulator TRIM28, we also analyzed TRIM28 protein levels in spontaneous ovulated and 10 IU induced groups. We observed similar TRIM28 levels in the control and 10 IU induced samples (Figure 5-4), which is perhaps not surprising since TRIM28 is recruited by and interacts with many zinc finger proteins. Using a Two Factor ANOVA With Replication statistical test, a significant difference was observed between preimplantation stages \( P < 0.05 \) for ZFP57, TRIM28 and H3, but no statistical difference was observed between treatment groups at any stage for any of the proteins analyzed.

5.3.3 Superovulation Perturbs ZFP57 Protein Localization

Given the difference in protein levels, we used immunocytochemistry to determine ZFP57 protein localization and intensity within oocytes, 1-cells (zygotes), 2-cells, 4-cells, 8-cells, early morulae, late morulae and blastocyst stage in spontaneously ovulated and 10 IU hormone treatment groups. In spontaneously ovulated oocytes, ZFP57 is cytoplasmic in localization (Figure 5-5, 5-6).
ZFP57 protein levels were assessed in oocytes and preimplantation embryos by Western blot analysis. Three biological replicates were performed for each stage of spontaneously ovulated (white bars, 0 IU dosage) and 10 IU induced (black bars) samples. Intensity measurements of each band were calculated using ImageJ software, and were normalized to α-tubulin. Relative ZFP57 protein levels were equal in both groups at the oocyte and 1-cell stages. While ZFP57 protein levels decrease in the spontaneous group at the 2-cell stage, they remain at levels similar to 1-cell embryos in the 10 IU induced group, having higher protein levels at all subsequent preimplantation stages in the 10 IU induced group compared to controls. Oo, oocytes; 1c, 1-cell; 2c, 2-cell; 4c, 4-cell; 8c, 8-cell Mo, morula; Bl, blastocyst stage embryos.

Figure 5-2 ZFP57 Protein Levels in Spontaneously Ovulated and 10 IU Induced Oocytes and Embryos
Histone 3 (H3) protein levels were assessed in oocytes and preimplantation embryos by Western blot analysis. Three biological replicates were performed for each stage of spontaneously ovulated (white bars, 0 IU dosage) and 10 IU induced (black bars) samples. Intensity measurements of each band were calculated using ImageJ software, and were normalized to α-tubulin. No significant differences were observed between control and 10 IU induced samples. Oo, oocytes; 1c, 1-cell; 2c, 2-cell; 4c, 4-cell; 8c, 8-cell Mo, morula; Bl, blastocyst stage embryos.

Figure 5- 3 H3 Protein Levels in Spontaneously Ovulated and 10 IU Induced Oocytes and Embryos
TRIM28 protein levels were assessed in oocytes and preimplantation embryos by Western blot analysis. Three biological replicates were performed for each stage of spontaneously ovulated (white bars, 0 IU dosage) and 10 IU induced (black bars) samples. Intensity measurements of each band were calculated using ImageJ software, and were normalized to α-tubulin. No significant differences were observed between control and 10 IU induced samples. Oo, oocytes; 1c, 1-cell; 2c, 2-cell; 4c, 4-cell; 8c, 8-cell Mo, morula; Bl, blastocyst stage embryos.

Figure 5-4 TRIM28 Protein Levels in Spontaneously Ovulated and 10 IU Induced Oocytes and Embryos
Figure 5-5 ZFP57 and TRIM28 Immunofluorescence Staining in Spontaneously Ovulated and 10 IU Induced Oocytes and Embryos

ZFP57 (Green) and TRIM28 (Red) were co-stained by immunocytochemistry in oocytes (OO) (n=23-25), 1-cell (1C) (n=28-31), 2-cell (2C) (n=20-29), 4-cell (4C) (n=23-24), 8-cell (8C) (n=20-25), early morula (EM) (n=44-50), late morula (LM) (n=20-43), early blastocyst (EB) and late blastocyst (LB) (n=33-33) stage embryos. Hoechst dye (Blue) was used to stain genomic DNA. ZFP57 staining is reduced in the pronuclei of the 1-cells and the nuclei of the 4-cell stage 10 IU induced embryos, and ZFP57 cytoplasmic staining is increased in 8-cells, early morulae, late morulae and blastocysts in comparison to spontaneously ovulated embryos. TRIM28 staining is increased in the nuclei of 10 IU induced 1-cells, but decreased in 10 IU induced 4-cells, late morulae and blastocysts.
Figure 5-6 ZFP57 Protein Localization in Spontaneously Ovulated and 10 IU Induced Oocytes and Embryos

A) ZFP57 intensity staining in the nucleoplasm was categorized as low (light green bars), equal (medium green bars), or high (dark green bars) in nucleoplasm compared to cytoplasm of each sample. Each category was calculated as a percentage of total samples in spontaneous (0) and 10 IU induced (10 IU) oocytes (Oo), and 1-cell (1c), 2-cell (2c), and 4-cell (4c) stage embryos. B) ZFP57 intensity staining in the cytoplasm was categorized as low (light purple bars), medium (medium purple bars), or high (dark purple bars) cytoplasmic staining. Each category was calculated as a percentage of total samples in spontaneous (0) and 10 IU induced (10 IU) 8-cell (8c), early morula (EM), late morula (LM), and blastocyst (Bl) stage embryos.
In 16/28 (57%) zygotes, ZFP57 was found in the pronuclei in addition to the cytoplasmic staining. In control 2-cell stage embryos, there was low nuclear staining in 15/20 (75%) compared to cytoplasmic levels. However, at the 4-cell stage, ZFP57 nuclear intensity levels were higher than cytoplasmic levels in 23/24 (96%) control embryos. ZFP57 continued this dual cytoplasmic and nucleoplasmic staining in the 8-cell stage, with 24/25 (96%) having medium or low cytoplasmic staining. In the early morula stage of development, there was a reduction in cytoplasmic staining of ZFP57 in all 44 (100%) embryos, with primarily nuclear only staining in all 20 (100%) late morulae and 34 (100%) blastocysts.

In comparison to controls, 10 IU induced oocytes also exhibited ZFP57 staining in the cytoplasm. While overall staining intensity between the two zygotic groups did not differ, examination of individual embryos showed that while 16/28 (57%) of spontaneous zygotes possessed pronuclear staining, 0/31 (0%) 10 IU induced zygotes showed staining in the pronuclei. At the 2-cell stage, staining levels between the two groups remained unchanged, having 15/20 (75%) spontaneously ovulated and 20/29 (70%) induced ovulated 2-cell stage embryos showing lower staining levels in nucleoplasm compared to cytoplasm. Intriguingly, a statistically significant decrease in nuclear staining was observed in the 10 IU induced 4-cell stage embryos ($P < 0.01$ in nucleoplasm). Examination of individual embryos showed that while 23/24 (96%) of spontaneously ovulated 4-cell embryos showed higher nuclear intensity levels than cytoplasmic levels, this was only observed in 3/23 (13%) of 4-cell stage embryos in the 10 IU induced group.

The level of staining in both the cytoplasm and the nucleoplasm of the 8-cell stage embryos was significantly higher in the 10 IU induced group ($P < 0.001$ in the nucleoplasm, $P < 0.001$ in the cytoplasm), having 14/20 (70%) superovulated embryos with high cytoplasmic staining compared to 1/25 (4%) in spontaneous embryos. This observation carried through to the early morula stage ($P < 0.001$ in the nucleoplasm, $P < 0.001$ in the cytoplasm), where the number of embryos with strong cytoplasmic staining was higher in 33/50 (66%) of the 10 IU induced embryos compared to 0/44 (0%) of the unstimulated controls. By the late morula stage, 10 IU induced embryos exhibited significantly higher cytoplasmic staining in comparison to control embryos ($P < 0.05$ in the cytoplasm). Embryos in the spontaneously ovulated late morulae group exhibited high...
nucleoplasmic ZFP57 staining and low cytoplasmic staining, having 0/20 (0%) remaining high in the cytoplasm, while superovulated late morulae showed 11/43 (26%) remaining high in the cytoplasm. By the blastocyst stage however, no significant difference was observed between cytoplasmic and nuclear staining of ZFP57 with respect to localization or intensity (Figure 5-5, 5-6). Thus, In comparison to controls, 1- and 4-cell embryos in the high hormone-treated group possessed primarily cytoplasmic localization of ZFP57. Furthermore, 8-cell, early morula and late morula stage embryos displayed aberrant cytoplasmic staining. This indicates that superovulation leads to aberrant ZFP57 localization with delayed or defective nuclear import until the 8-cell stage combined with increased ZFP57 protein stability.

5.3.4 Superovulation Alters TRIM28 Protein Intensity in the Nucleus

ZFP57 was co-stained with TRIM28 in each oocyte and embryo and examined by immunofluorescence. TRIM28 staining was undetectable in both spontaneous and 10 IU induced oocytes (Figure 5-5). In 1-cell zygotes, TRIM28 was localized to both parental pronuclei, and remained in the nuclei at all subsequent preimplantation stages. Nuclear staining in the 10 IU induced group was statistically higher than controls at the 1-cell stage ($P < 0.01$). By the 4-cell stage, TRIM28 nuclear staining was lower in the 10 IU induced group compared to the spontaneous ovulated 4-cell stage group. Similarly, lower TRIM28 nuclear staining was present in late morula and blastocysts in the 10 IU induced group compared to control embryos ($P < 0.01$). In individual blastomeres undergoing mitosis, TRIM28 staining is observed throughout the cytoplasm.

5.3.5 ZFP57 Binding is Reduced at Imprinted DMRs

The above analyses address the global effects of superovulation on ZFP57. To directly assess the effects of superovulation on ZFP57 activity we investigated ZFP57 binding at the ZFP57 hexanucleotide motif(s) within the Snrpn, Kcnq1ot1 and H19 ICRs, and at the Peg3 and Peg1 DMRs. Micro-scale chromatin immunoprecipitation was performed on 20-24 individual blastocysts in the spontaneously ovulated and 10 IU induced groups. Histone H3 was also examined at each locus as a control. Compared to the spontaneously ovulated group, a significant reduction in ZFP57 enrichment was
observed at all five imprinted domains in the 10 IU induced group (Figure 5-7). In contrast, no significant difference was observed for H3 binding at ICRs/DMRs between blastocysts in spontaneously ovulated and 10 IU groups (Figure 5-8).

5.4 Discussion

Imprint maintenance in the early embryo is susceptible to ART manipulations, including superovulation (Market-Velker et al. 2010b, El Hajj et al. 2011). Yet, imprint acquisition in the oocyte is unaffected (Anckaert et al. 2009a, Denomme et al. 2011). Thus, we hypothesized that a maternal factor, accumulating in the oocyte as exogenous hormones are applied, is instead affected and can no longer properly maintain genomic imprinting post-fertilization.

In this study, we set out to determine whether superovulation affected the maternal factor ZFP57, known to play an essential role in genomic imprinting regulation. In the 10 IU induced group of embryos, we observed an increase in ZFP57 protein levels initiating at the 2-cell stage. At the same time, ZFP57 was mislocalized away from the nucleus, particularly at the superovulated 4-cell stage. The majority of increased cytoplasmic staining was opaque and clouded, however some embryos from both treatment groups displayed a punctate staining, which occurred more frequently in the 10 IU induced embryonic group (Supplementary Figure 5-1s, Appendix 2, (Denomme & Mann 2013), reminiscent of autophagy activity.

However, while protein levels were increased, we observed a significant decrease in ZFP57 protein enrichment at all five imprinted domains examined (Kcnqlot1, Snrpn, Peg3, Peg1, H19). As protein levels were higher in the 10 IU induced group at multiple stages of development, while transcript abundance was unaffected, it is possible that hormone induction alters ZFP57 protein folding. This could generate increased protein stability, but at the same time affect access to the critical zinc fingers or KRAB box domain involved in DNA methylation maintenance.
ZFP57 protein enrichment at imprinted DMRs was assessed by micro-scale chromatin immunoprecipitation in individual blastocysts. At least twenty biological replicates (n=20-24) were performed for each imprinted loci in spontaneously ovulated (white bars) and 10 IU induced (black bars) blastocysts. Significant differences were calculated using the Student’s t-Test statistical test, (a, $P < 0.001$; b, $P < 0.05$).

Figure 5- 7 ZFP57 Protein Enrichment at Imprinted Loci in Spontaneously Ovulated and 10 IU Induced Blastocysts
Figure 5- 8 H3 Protein Enrichment at Imprinted Loci in Spontaneously Ovulated and 10 IU Induced Blastocysts

Histone 3 (H3) protein enrichment at imprinted DMRs was assessed by micro-scale chromatin immunoprecipitation in individual blastocysts. At least twenty biological replicates (n=20-24) were performed for each imprinted loci in spontaneously ovulated (white bars) and 10 IU induced (black bars) blastocysts. Significant differences were calculated using the Student’s t-Test statistical test.
Alternatively, it may compromise the nuclear localization signal (NLS), including the many phosphorylation sites and other necessary post-translational modifications (Alonso et al. 2004), affecting the ability for ZFP57 to be properly and timely transported into the nucleus. Going forward, we hypothesize that delayed or defective ZFP57 nuclear localization at the 4-cell stage leads to passive loss of imprinted DNA methylation. This may result in failure to recruit TRIM28 and in turn DNMT1. We observed lower levels of TRIM28 in 10 IU induced 4-cell embryos. This compromised ability to protect imprinted genes from global demethylation processes would be expected to lead to a passive loss of imprinted methylation initiating at the 8-cell stage. In alignment with this premise, El Hajj et al. examined 7.5 IU induced 16-cell stage embryos and reported loss of Snrpn maternal methylation in 2/10 mouse embryos, loss of H19 paternal methylation in 2/10 embryos, and gain of H19 maternal methylation in 1/10 embryos (El Hajj et al. 2011). Aberrant loss and gain of methylation was observed in a greater number of embryos and imprinted loci by the blastocyst stage (Market-Velker et al. 2010b). However, since ZFP57 binds to a methylated recognition sequence, it is possible that DNA methylation maintenance is compromised prior to the 4-cell stage. Reduced imprinted methylation would lead to reduced enrichment of ZFP57 at ICRs. Resolving these possibilities will require investigations into when imprinted methylation is lost during preimplantation development following superovulation.

Targeted deletion and mating strategies were previously used to resolve the roles of maternally derived and embryonically produced ZFP57 in the early embryo (Li et al. 2008). The authors reported embryonic lethality by midgestation upon deletion of both forms. Ablating embryonic ZFP57 alone produced partial neonatal lethality, but deletion of maternal ZFP57 alone was not lethal due to embryonic ZFP57 rescue (Li et al. 2008). During preimplantation development, maternally derived Zfp57 is the sole source of this protein, demonstrated in our results by RT-PCR analysis, as well as through expression arrays (Zeng et al. 2004). Maternal ZFP57 deficiency resulted in hypomethylation of Snrpn at embryonic day 3.5, confirming the status of ZFP57 as an essential maternal effect factor. However, embryonic ZFP57 was able to rescue the reduced methylation levels by day 13.5 (Li et al. 2008).
The same study demonstrated that maternal ZFP57 is required for the establishment of methylation imprints at Snrpn in the oocyte (Li et al. 2008). However, germline methylation acquisition occurred normally at other DMR regions examined, Peg1, Peg3, and Peg5. Effects from assisted reproductive technologies on ZFP57 function likely do not present themselves in the oocyte, as we and others have described no effect on proper imprint acquisition at maternally imprinted domains from superovulation (Anckaert et al. 2009a, Denomme et al. 2011), in vitro oocyte maturation in culture (Anckaert et al. 2009a, Anckaert et al. 2009b, Anckaert et al. 2010), or oocyte vitrification (Trapphoff et al. 2010). Similarly, total Zfp57 mRNA levels in mature oocytes were comparable following in vitro follicle culture and in vivo stimulated development (Anckaert et al. 2013). Although, a second study reports a significant decrease in Zfp57 transcripts upon administration of hCG (Agca et al. 2013) compared to pre-ovulatory levels in control oocytes. While more studies are required to delineate Zfp57 transcript levels throughout oogenesis and upon meiotic resumption, there does not appear to be a difference between induced ovulation and spontaneous ovulation.

The partial loss of DNA methylation observed in blastocyst stage embryos at individual genes and the stochastic nature of this loss among blastocysts from stimulated mothers (Market-Velker et al. 2010b) and cultured embryos (Market-Velker et al. 2010a) is reminiscent of previously reported stochastic loss between embryos with maternal Zfp57, Trim28 and Dnmt1 deletion (Hirasawa et al. 2008, Li et al. 2008, Messerschmidt et al. 2012). While ZFP57 and TRIM28 have demonstrated roles as protectors, they did not function at all imprinted genes. Maternal and embryonic ZFP57 deletion did not lead to H19 DNA methylation loss (Li et al. 2008); and TRIM28 deletion did not lead to Peg3 DNA methylation loss (Messerschmidt et al. 2012). One possible explanation for these differences is that sufficient numbers of embryos may not have been examined.

A recent study in mouse ES cells reported the functional interchangeability between human and mouse ZFP57 in maintaining DNA methylation at the Snrpn, Zac1, and Dlk1-Dio3 imprinted loci (Takikawa et al. 2013). This functional equivalence suggests that the human ZFP57 protein may also be susceptible to ovarian stimulation or other ARTs techniques and may affect genomic imprinting maintenance during human embryonic preimplantation development.
Interestingly, ZFP57 mutations have recently been shown to be the causative factor in 5% of Transient Neonatal Diabetes Mellitus 1 (TNDM1) patients. This disease arises from hypomethylation at the TNDM1 locus, which lies within the imprinted promoter of the major candidate gene \textit{PLAGL1/ZAC1} (Mackay et al. 2008). Additional hypomethylation is reported at maternally imprinted loci \textit{PEG3} and \textit{GRB10}, with variable and partial hypomethylation at \textit{PEG1}, \textit{KCNQ1OT1} (Mackay et al. 2008), as well as \textit{NESPAS/GNAS} (Boonen et al. 2013) in some patients. In addition to transient neonatal diabetes, the key clinical features in patients with TNDM1 can include intrauterine growth retardation, macroglossia, heart defects, and developmental delay. These variable phenotypes are reminiscent of the known imprinting disorders BWS and SRS (Elliott et al. 1994, Kotzot et al. 1995, Weksberg et al. 2001, Eggermann et al. 2010). However, no correlation has yet been described between ZFP57 mutations and these known imprinting disorders (Spengler et al. 2009, Boonen et al. 2012).

For decades, ART techniques have been correlated with imprinting errors. However, the pathway to these errors is unclear, and the regulatory proteins directly disrupted by these manipulations are still unknown. The recent discovery of numerous maternal effect genes has brought new insight into genomic imprinting regulation. In this study, we have begun to shed light on the pathway affected by assisted reproductive technologies. As the significance of ZFP57 in imprinting regulation has only recently been reported, and its susceptibility to ovarian stimulation is highlighted in this study, further studies should be performed in detail with human IVF clinics to ensure safe procedures.
5.5 References


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6 CHAPTER 6: Discussion

6.1 Opening Discussion

Genomic imprinting is a transcriptional regulatory process controlling monoallelic gene expression (Bartolomei & Ferguson-Smith 2011). This dynamic process relies on epigenetic modifications such as DNA methylation to mark, or “imprint”, and silence one of the two parental alleles. Mechanisms must operate within both developing parental germ cells and in the preimplantation embryo to first establish and then maintain these allele-specific differential marks. The question of gamete and embryo predisposition to infertility- and ART-induced epigenetic defects is of critical importance to maximize the safety of fertility treatments. Therefore, identifying the factors involved in the imprinting regulatory pathway, and understanding how they are affected by assisted reproduction, will help reduce the occurrence of imprinting disorders in the ART population.

The field of genomic imprinting has been limited by the technical difficulties involved in investigating such small amounts of genetic material, and in the past, studies were carried out using pools (often hundreds) of oocytes or preimplantation embryos. Work from my research group has substantially furthered the field by facilitating DNA methylation analyses in individual blastocysts (Velker et al. 2012) and in individual oocytes (Denomme et al. 2012b). We now have the ability to evaluate the effects of infertility and ARTs at the single cell level, identifying the frequency of perturbations among samples as well as among imprinted loci within individual samples. In addition, we have revolutionized conventional Western blot and chromatin immunoprecipitation (ChIP) protocols to examine small cell numbers, specifically ChIP analysis using single blastocysts and Western blot analysis using pools of ten oocytes. Importantly, this new level of investigation is approaching relevancy for application in the human clinic.

Using these modified assays, the experiments within this thesis provide a comprehensive analysis of the maternal control of genomic imprinting at the single oocyte and preimplantation embryo level and uncover a part of the molecular mechanism that is disrupted by ARTs. I examined the effects of maternal infertility and subfertility on imprint acquisition in growing oocytes. I also examined the effects of maternal
ovarian stimulation as an ART treatment on imprint acquisition in MII oocytes. Finally, I investigated the effects of maternal ovarian stimulation on the mechanisms of imprint regulation via the maternal factor ZFP57.

Work presented in this thesis has brought new insights into the maternal control of genomic imprinting regulation: (a) maternal infertility can predispose imprinted loci, namely the late-acquiring Peg1 locus, to imprinting errors in the female gamete, (b) in comparison, ovarian stimulation does not disrupt the process of imprint acquisition in the female gamete; (c) instead, ovarian stimulation disrupts the protein localization, protein abundance, and protein binding (at imprinted loci) of the maternal effect factor ZFP57, a specific and central component of the imprinting regulatory pathway.

6.2 Pathways Leading to Imprinting Instability

Multiple avenues of investigation are required to delineate the effects of infertility and assisted reproduction on epigenetic gene regulation. Foremost, studies are required to determine the molecular and cellular mechanisms giving rise to epigenetic errors following maternal environmental perturbations. This includes the identification of maternal factors involved in epigenetic regulation during embryo development. Adaptation or stress induced by a suboptimal fertile environment, or acceleration of oocyte maturation or recovery of atretic oocytes by superovulation, can all affect the ability of the oocyte to properly synthesize and store sufficient amounts of maternal factors (Li et al. 2010).

6.2.1 Maternal Factors Regulating Genomic Imprinting are disrupted by ARTs

Recent reports have begun to shed light on the roles played by maternal factors during embryo development, namely during the oocyte-to-embryo transition period as whole genome reprogramming occurs. This has brought new insight into the mechanisms involved in genomic imprinting regulation, where the recruitment of specialized maintenance complexes to imprinted regions is necessary to ensure epigenetic integrity, both directly after fertilization and throughout cleavage-stage development. DPPA3 is one protein identified to protect imprinted genes from active DNA demethylation, while
the triumvirate of proteins, ZFP57, TRIM28, and DNMT1, appears to provide protection from passive DNA demethylation during preimplantation development.

Understanding how ARTs may disrupt maternal effect products that regulate imprint maintenance will be essential for maximizing the safety of their procedures and ensuring healthy embryonic development. In this thesis, I characterized the maternal effect product ZFP57 throughout preimplantation development under *in vivo* conditions, and identified how it is susceptible to ARTs. I demonstrated that ZFP57 protein levels were markedly higher in superovulated embryos in comparison to spontaneous controls at the 2-cell stage and subsequent preimplantation stages. As no change was noted in *Zfp57* transcript abundance, it is possible that protein stability is affected by superovulation. ZFP57 protein localization undergoes a cytoplasmic to nucleoplasmic transition during preimplantation development, and this was disrupted and/or delayed in stimulated embryos, particularly evident at the 4-cell stage. This misregulation of ZFP57 preceded a significant decrease in protein enrichment at imprinted ICRs in blastocysts, proposing a mechanism for imprint methylation maintenance loss following ARTs. My current model utilizing this data postulates that if ZFP57 cannot enter the nucleus at the appropriate stage, it cannot recruit its co-factor TRIM28 and the repressive complexes to imprinted regions. The ability to protect imprinted genes from global demethylation processes is compromised, and imprinted DNA methylation maintenance cannot be sustained, leading to a passive loss of methylation initiating at the 8-cell stage. To support this, aberrant loss of *Snrpn* and *H19* methylation was reported in some alleles of superovulated 16-cell embryos (El Hajj et al. 2011) and a greater number of embryos and imprinted loci were affected by the blastocyst stage (Market-Velker et al. 2010b). By elucidating the effects of superovulation on the imprint regulator ZFP57, I have uncovered an essential component of the molecular mechanism that is disrupted by ARTs.

### 6.2.2 Infertility Predisposes Imprinting Loci to Errors

The increased incidence of imprinting disorders in the ART population has led to the question of whether infertility alone predisposes embryos to imprinting errors. To imitate female subfertility, I used ERβ deletion mice with a compromised endocrine signaling pathway. However, normal DNA methylation acquisition was observed at
Snrpn, Peg3, and Peg1 in oocytes from these Esr2-deficient females. In comparison, as a means of simulating female infertility, I used connexin37 deletion mice to determine whether compromised gap junctional communication between the oocyte and the cumulus cells would disrupt *de novo* DNA methylation acquisition (Denomme *et al.* 2012a). Connexin37 deficiency resulted in loss or delayed methylation acquisition at the late-acquiring gene Peg1, suggesting that stored methyl donors or other metabolites normally transported from granulosa cells to the oocyte may have been exhausted during oocyte growth in these Gja4-deficient females.

Barriers to a thorough evaluation of human infertility exist, and so validating a link between female infertility and genomic imprinting in human oocytes is proven difficult. However, numerous studies have reported a predisposition to imprinting errors upon male infertility in human sperm (Manning *et al.* 2001, Kobayashi *et al.* 2007, Marques *et al.* 2008, Boissonnas *et al.* 2010, Minor *et al.* 2011, Sato *et al.* 2011). The principal question, however, is whether oocyte- or sperm-imprinting errors are transmitted to offspring. An analysis of aborted conceptuses from males with moderately-to-severely low sperm concentration reported 7/17 (41%) cases with abnormal DNA methylation in the aborted conceptus and the identical alterations present in the paternal sperm. (Kobayashi *et al.* 2009), indicating that imprinting errors from the gametes can be transmitted to the developing embryo.

Taken together, impaired fertility in the gametes can predispose imprinting loci to imprinting errors. Further studies are required to delineate the process in which this occurs, and whether maternal factors are involved.

### 6.2.3 ARTs Predisposes Imprinting Loci to Errors

Paramount to assisted reproduction is that the timing of ARTs coincides with critical epigenetic events during gametogenesis and early embryogenesis. Understanding how ARTs cause epigenetic disruption is crucial for maximizing their efficacy and safety. In both BWS and AS human imprinting disorders, patients have been conceived by ovarian stimulation alone, having no other form of ARTs used (Chang *et al.* 2005, Ludwig *et al.* 2005). This led to the investigation of ovarian stimulation as a potential imprinting disruptor. These disorders typically exhibit aberrant loss or gain of maternal
methylation at their imprinting control regions (DeBaun et al. 2003, Gicquel et al. 2003, Maher et al. 2003, Orstavik et al. 2003). Because exogenous hormones are administered during oocyte development, I hypothesized that the acquisition of imprints would be disrupted in oocytes. Surprisingly, Snrpn, Kcnq1ot1 and Peg3 were hypermethylated, while H19 was hypomethylated, regardless of low or high hormone treatment (Denomme et al. 2011), indicating no effect. A single oocyte (1/125) from the 10 IU hormone group contained a hypomethylated Kcnq1ot1 ICR. I attributed this to a spontaneous loss of maternal methylation, cumulus cell contamination, or possibly a rare imprinting error predisposed by ovarian stimulation. With this caveat in mind, the study suggests that ovarian stimulation does not greatly impact DNA methylation acquisition.

Molecular evidence from an earlier mouse study showed a higher occurrence of abnormal global methylation patterns as early as the 2-cell stage in embryos derived from superovulated females (Shi & Haaf 2002). At imprinted loci, aberrant loss or gain of DNA methylation was observed in superovulated 16-cell stage embryos (El Hajj et al. 2011). Likewise, a study from my research group reported a greater number of embryos with loss or gain of DNA methylation at multiple imprinted genes by the blastocyst stage (Market-Velker et al. 2010b). Thus, taken together, these studies indicate that superovulation instead leads to imprinting maintenance errors post-fertilization.

Earlier studies from my research group indicated that a second ART technique, in vitro embryo culture, also compromises imprint maintenance when analyzed at the blastocyst stage of development (Market-Velker et al. 2010a). In a subsequent study on developmental rates of embryos in culture, imprint maintenance mechanisms were disrupted by rapid development in culture prior to the 8-cell stage, producing imprinting errors at subsequent embryonic stages. Greater numbers of embryos with fast rates of development had Snrpn and H19 loss of methylation compared with those that developed slower in culture (Market Velker et al. 2012). As a result, I propose that different ARTs techniques, including those that take place during oogenesis and those that take place during embryogenesis, converge on a common imprinting regulatory pathway involving maternal-effect genes.
6.2.4 Complexity of Multiple ARTs and Infertility: Relation to the Human Clinic

In human clinics, multiple ARTs are employed, and so it is difficult to discern the origin of imprinting anomalies. Moreover, ethical and social limitations of using human embryos prevent a thorough evaluation of ARTs techniques, especially the inability to acquire and analyze control oocytes and embryos. With this in mind, several groups used animal models to conduct research distinguishing the effects of various ARTs (Fauque et al. 2007, Rivera et al. 2008, Market-Velker et al. 2010a). Taken together, these studies demonstrate an additive effect with combined ART procedures.

Human embryos produced via ARTs are also the product of underlying infertility/subfertility. This has led to questions regarding the origin of epigenetic instability; does it come from underlying infertility in gametes/embryos, does it come from gamete/embryo manipulations, or does it come from a combination of infertility and ARTs. The relationship between impaired fertility, ARTs and epigenetic stability is unquestionably complex. However, the possibility exists that ARTs and infertility may disrupt the same mechanistic pathways involving maternal-effect genes that lead to epigenetic errors. If this is the case, perturbations induced by infertility may be exacerbated by gamete or embryo manipulation, similar to combined ART treatments. This would be consistent with ART-associated BWS children where variable ART procedures were reported to be used, including different embryo culture media, day of transfer, and specific ART method used (IVF, ICSI), in addition to the cause of infertility (Gicquel et al. 2003, Chang et al. 2005).

Overall, if embryo response to infertility and ARTs is stochastic, then the leading question remains: which embryo will be least compromised by ovarian stimulation, or other manipulations. As fast rates of development in culture correlate with loss of imprinting, it was proposed that embryos that adapt or respond the least to their suboptimal environment will likely develop in the most normal fashion (Baumann et al. 2007, Leese et al. 2007, Market Velker et al. 2012). Modification of ARTs techniques that minimize the need for increased adaptation will likely have a corresponding decrease in epigenetic perturbations and thus, increased overall health of the embryo. Thus,
limiting ovarian stimulation protocols through single oocyte retrieval and single embryo transfer is one way to minimize effects of stress by ARTs.

6.3 Experimental Limitations

Research on individual oocytes and embryos provides us with information at the single cell level, emphasizing the stochastic nature of genomic imprinting loss and minimizing any biases based on pools. Nevertheless, working with this minute amount of material can be limiting in the types of experiments performed, and statistical analyses can be problematic. Significant results by quantitative analyses can be particularly difficult to obtain at the later stages of preimplantation development, where the number of blastomeres varies between embryos as they develop at different rates. The variability in ZFP57 protein levels between replicates in the Western Blot experiment may have concealed statistical significances that would otherwise be observed in larger pools of samples. In my examination of Zfp57 transcript abundance, unavoidable primer-dimers were produced with such low amounts of cDNA, preventing quantitative analysis between treatment groups through conventional ΔCt methods. While my research group has modified the bisulfite mutagenesis, Western blot and micro-scale ChIP assays to accommodate small cell numbers, other techniques like co-immunoprecipitation, mass spectrometry, and high-performance liquid chromatography (HPLC) are not yet available. These experiments would be useful in understanding protein-protein interactions, like those with ZFP57, in the early embryo. Cell culture techniques using ES cells, TS cells and XEN cells can overcome many of these experimental limitations driven by cell number requirements, but do not provide information for the earlier stages in embryonic development that are so dynamic and critical to understand.

6.4 Future Directions

6.4.1 Maternal Infertility

Impaired fertility can predispose imprinting loci in the gametes to imprinting errors. However, further studies are required to delineate the process in which this occurs, namely the molecular mechanisms leading to imprinting perturbations. I proposed that
CX37-null oocytes have exhausted their methyl donors during oocyte growth, preventing \textit{de novo} methylation at late-acquiring genes like \textit{Peg1}. Further studies are required, like HPLC on pools of pre-antral stage oocytes, to determine if there is a reduction in s-adenosylmethionine (SAM), the primary methyl donor in oocytes. Otherwise, as CX37-null oocytes arrest at the pre-antral stage of oogenesis, I cannot currently decipher between a disruption and a delay in methylation acquisition at \textit{Peg1}.

Studies are also required to determine whether female subfertility/infertility, like those originating from impaired endocrine signaling and intercellular communication during oogenesis have an effect post-fertilization on imprint maintenance in the embryo. As CX37-deficiency leads to infertility, the current mouse model does not facilitate these studies. However, utilizing heterozygote females or shRNA-mediated \textit{Gja4} RNA depletion would possibly promote oocyte maturity and fertilization and lead to investigations on imprint maintenance post-fertilization. As ERβ-deficient oocytes acquired \textit{de novo} DNA methylation without errors, it is important to discern if the effects manifest post-fertilization, similar to those observed with superovulation. Furthermore, to clarify if infertility and ARTs converge on a common imprinting regulatory pathway, it is necessary to elucidate the effects of infertility/subfertility on the regulation of maternal-effect factors like ZFP57 during oogenesis and preimplantation development.

Finally, to truly delineate the risk of epigenetic errors resulting from infertility vs. ARTs in the human population, investigations are required on gametes, embryos and children from couples seeking ARTs in the absence of compromised fertility such as for preimplantation genetic diagnosis and same-sex couples.

### 6.4.2 Ovarian Stimulation

While my study demonstrates that maternal DNA methylation acquisition at imprinted loci is not disrupted by superovulation, loss of methylation maintenance is observed post-fertilization. This has been reported at the 16-cell stage (El Hajj \textit{et al.} 2011) and the blastocyst stage (Market-Velker \textit{et al.} 2010b). However, it is currently unknown at what stage DNA methylation maintenance is initially lost. Resolving this will require investigations at each preimplantation stage following superovulation.
DNA methylation has been correlated with allelic expression of many imprinted genes (Verona et al. 2003). However, studies on the effects of ovarian stimulation alone on imprint expression are limited (Fortier et al. 2008), and should be investigated in more detail in the preimplantation embryo and at multiple post-implantation stages to determine if and when loss of imprinted methylation results in a corresponding loss of imprinted expression.

Human oocytes may respond differently to ovarian stimulation in comparison to animal models. As an increased proportion was reported to exhibit a loss or gain of DNA methylation (Sato et al. 2007, Khoueiry et al. 2008) they may be more prone to epigenetic errors and/or encounter more stressors. Future studies should be directed toward larger numbers of human oocytes to fully elucidate the effects. Additionally, to truly delineate the risk of imprinting errors resulting from ARTs, and specifically ovarian stimulation, investigations are required on control unstimulated human gametes and embryos.

6.4.3 Maternal Effect Factors

Perturbations to the maternal environment can predispose the oocyte to imprinting errors, likely manifesting post-fertilization. ZFP57, a key player in the regulation of genomic imprinting, is significantly misregulated by ovarian stimulation. Based on observations from my research group following superovulation and in vitro embryo culture on imprinted methylation at the blastocyst stage (Market-Velker et al. 2010a, Market-Velker et al. 2010b, Market Velker et al. 2012), I propose that different ARTs techniques converge on a common imprinting regulatory pathway involving maternal-effect factors. Thus, to support this hypothesis, it is imperative to investigate whether ZFP57 responds in a similar manner to alternative ARTs procedures including in vitro oocyte maturation and in vitro embryo culture.

My current model postulates that the delayed or defective ZFP57 nuclear localization at the 4-cell stage prevents imprint maintenance and leads to a passive loss of imprinted DNA methylation, initially occurring at the following 8-cell stage. However it is possible that DNA methylation maintenance is compromised by superovulation prior to the 4-cell stage. Since ZFP57 binds to a methylated recognition sequence, reduced
imprinted methylation would also explain the observed reduced enrichment of ZFP57 at ICRs in the blastocyst. To resolve these possibilities, investigations are again required to determine what stage imprinted DNA methylation maintenance is initially lost during preimplantation development following superovulation, as well as micro-scale ChIP analyses at each preimplantation stage to determine when ZFP57 protein enrichment is initially reduced at imprinted ICRs.

The maternal factor ZFP57 is only one of many proteins involved in imprint maintenance, and many questions remain, as the pathway from hormone induction to ZFP57 misregulation is still unknown. Superovulation accelerates the growth rate of ovarian follicles, and may erroneously rescue and promote ovulation of atretic oocytes. This accelerated growth has been thought to result in a decrease in maternal mRNA stores, which would fall below a necessary threshold for maintenance at imprinting loci prior to embryonic Zfp57 gene activation. Yet, Zfp57 transcript abundance following superovulation appears to be equal to that of spontaneously ovulated oocytes and embryos. If superovulation does not affect the synthesis and storage of Zfp57 transcripts in the oocyte, it is possible that hormone induction compromises ZFP57 protein folding, affecting the critical zinc fingers that physically bind to the methylated DNA, or the KRAB box domain involved in recruiting TRIM28 and other repressive proteins to promote maintenance of DNA methylation. Experimental techniques that enable evaluation of protein folding currently require large quantities of concentrated protein and would be difficult to evaluate in preimplantation embryos, where the discrepancy is observed.

Superovulation may alternatively compromise the nuclear localization signal (NLS) or the numerous phosphorylation and glycosylation sites affecting the ability for ZFP57 to be properly and timely transported into the nucleus. It may instead affect a maternal factor(s) upstream of ZFP57 in the imprint regulatory pathway. As ZFP57 does not enter into the nucleus at both the 1-cell and the 4-cell stage in many superovulated embryos, attractive candidates are the nuclear transport proteins, a number of which are maternally provided. The transport of proteins from the cytoplasm into the nucleus through the nuclear pore complex is an active process mediated by members of the importin/karyopherin family (Lusk et al. 2007). RanBP5 is a nuclear transport shuttle
protein that has previously been shown to bind to DPPA3 and mediate its transport into the maternal pronucleus of the zygote, protecting against active DNA demethylation (Nakamura et al. 2007). It is likely that either this protein or a related importin protein binds to the NLS locus on ZFP57 and is required for the transport of ZFP57 into the nucleus during preimplantation development. Interestingly, the embryonic transcript of RanBP5 is activated at the 8-cell stage (Zeng et al. 2004), which corresponds to the first stage of increased ZFP57 localization in the nuclei of superovulated embryos. If the maternal RanBP5 protein is affected by superovulation, it may not be able to properly transport its cargo proteins into the nucleus prior to the 8-cell stage. Future studies should begin with co-immunoprecipitation of ZFP57 and RanBP5 to determine if there is an interaction in the early embryo. Additional studies on Ranbp5 transcript abundance by RT-PCR as well as protein localization by immunofluorescence and protein abundance by Western blot will elucidate the effects of superovulation on the properties of this protein. One caveat is that RanBP5 may prove to be unaffected by superovulation or may not be the nuclear importer of ZFP57. Future experiments should also involve immunoprecipitation and mass spectrometry to identify all proteins that interact with ZFP57, although this experiment may not currently be feasible in the early embryo and may require the use of ES cells. Similar studies would then be required to determine their role in imprint maintenance, as well as how they respond to superovulation and other techniques during preimplantation development.

Work in this thesis has only begun to decipher the pathway involved in imprint regulation and how it is affected by ARTs. ZFP57 and TRIM28 have demonstrated roles as protectors, however they are not reported to function at all imprinted genes. In addition, they do not account for aberrant gain of DNA methylation. My research group previously reported a gain of maternal DNA methylation for the normally unmethylated maternal H19 allele in four out of ten 10 IU induced blastocysts (Market-Velker et al. 2010b). The repressive proteins involved in protection against demethylation currently do not explain this gain of DNA methylation observed following ARTs. Thus, further studies are required to delineate additional mechanisms that act as protectors, against both aberrant demethylation and aberrant methylation, involved in the regulation of the genomic imprints. H1foo, Zar1, Npm2, Nlrp5, and Setdb1 all have been implicated in
epigenetic regulation during the oocyte-to-embryo transition and are primary candidates (Burns et al. 2003, Maeda et al. 2008, Fernandes et al. 2012). However, no direct link between these maternal factors and genomic imprint maintenance has been described to date. As mitochondria generated in the oocyte is the sole source of acetyl groups, methyl groups and ATP (which powers chromatin-remodeling complexes) in the preimplantation embryo, it may also contribute to the unknown pathway involved in imprint regulation. Identification of these additional regulators is critical to understanding the complete mechanism of genomic imprinting in the early embryo, and elucidating the pathway from hormone administration to imprinting errors.

Finally, apparent lack of remodeling of imprinted regions during preimplantation has led to the argument that epigenetic disruption by ARTs is restricted to imprinted genes. However, other genes have now been identified with differential gametic DNA methylation that is retained through early preimplantation development (Smallwood et al. 2011, Kobayashi et al. 2012), termed “transient gDMRs”. These non-imprinted transient gDMRs later acquire methylation on the unmethylated allele, described either at mid-gestation or in adult tissues. Genome-scale studies are needed to determine the scope of epigenetic instability at non-imprinted genes in gametes and embryos as a result of infertility and ARTs to truly understand the breadth of their effects.

6.5 Conclusion

Assisted reproduction will continue to be a critical medical intervention for infertile couples. To maximize the safety of these techniques, it is imperative to understand how mechanisms involved in epigenetic regulation are affected by impaired fertility and ARTs. The work in this thesis demonstrates the maternal control of genomic imprinting regulation, and stresses the importance of extending studies of oocyte manipulations to the embryo. While imprints are acquired normally, maternal effect factors required for imprint maintenance are misregulated.

Epigenetic programming within the early embryo necessitates the recruitment of specialized maintenance complexes, namely these maternal effect factors, to imprinted regions to ensure epigenetic integrity. Continued studies in animal models and in humans are required to fully understand the molecular mechanisms of these proteins in the
regulation of imprints as well as how impaired fertility and ARTs induce epigenetic changes and disease. This will lead to preventative measures, reducing the occurrence of epigenetic perturbations and maximizing the safety of ARTs to ensure normal embryonic development and healthy children born through assisted reproduction.
6.6 References


Appendix 1: Supplementary Material - Chapter 4

The following figures were presented as supplemental data to the experiments presented in Chapter 4, published as:

Methylation analysis of individual oocytes without cumulus cell contamination.

Only a single strand of oocyte DNA was expected to amplify, either a methylated B6 or CAST *Snrpn*, *Kcnq1ot1* or *Peg3* allele, and either an unmethylated B6 or CAST *H19* allele. For each sample, 5 clones were sequenced. Oocytes (indicated to left) with a single methylation pattern, a single genotype (B6 or CAST), and identical non-CpG conversion pattern (% indicated to the right) were included in the analysis. Representative oocytes shown. Black circles indicate methylated CpGs. White circles indicate unmethylated CpGs.
Supplementary Figure 4- 2s Methylation analysis of individual oocytes with cumulus cell (CC) contamination and/or polar body (PB) inclusion.

Expected methylation patterns for females and their oocytes for *Snrpn, Kcnq1ot1* and *Peg3* (top left) and *H19* (top right) are shown. Only a single strand of oocyte DNA was expected to amplify, either a methylated CAST (CM) or B6 (BM) *Snrpn, Kcnq1ot1* or *Peg3* allele, and either an unmethylated CAST (CU) or B6 (BU) *H19* allele. For each sample, 5 clones were sequenced. Oocytes (designation indicated to left) with multiple methylation patterns, both genotypes (B6, B, and CAST, C), and/or multiple non-CpG conversion patterns (% indicated to the right) indicative of multiple strand amplification, were excluded from the analysis. Representative oocytes shown. Black circles indicate methylated CpGs. White circles indicate unmethylated CpGs.
Appendix 2: Supplementary Material - Chapter 5

The following figure is presented as supplemental data to the experiments presented in Chapter 5, in preparation as:


Supplementary Figure 5- 1s ZFP57 Localization in Mouse Morula Embryos from Spontaneously Ovulated and 10 IU Induced Females

The morula derived from a spontaneously ovulated oocyte has ZFP57 immunofluorescence staining (green) colocalized with Hoechst staining (blue) within the nucleoplasm of blastomeres. The morula derived from an oocyte induced to ovulate by 10 IU hormone treatment possesses clouded and punctate ZFP57 immunofluorescence staining (green) that is mislocalized to the cytoplasm of blastomeres, in addition to nuclear staining. Re-used with permission from (Denomme & Mann 2013).

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Gordon Research Conference: Mammalian Gametogenesis & Embryogenesis
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Publications

PUBLISHED REFEREED PAPERS

Denomme MM & Mann MR (2013) Maternal control of genomic imprint maintenance, Reproductive Biomedicine Online 27 629-636 (DOI: 10.1016/j.rbmo.2013.06.004)

Denomme MM & Mann MR (2012) Genomic imprints as a model for the analysis of epigenetic stability during ARTs, Reproduction 144 393-409 (DOI: 10.1530/REP-12-0237)


PUBLISHED BOOK CHAPTERS


SUBMITTED MANUSCRIPTS


MANUSCRIPTS IN PROGRESS


Conference Presentations

1. **Denomme MM**, Zhang L, MacDonald WA, White CR, Mann MR. Superovulation causes loss of genomic imprint maintenance by misregulation of ZFP57 in the mouse preimplantation embryo, CIHR Training Program in Reproduction, Development, and the Impact on Health (REDIH) Meeting, Ottawa ON, Dec 2013, Poster Presentation

2. **Denomme MM**, Zhang L, MacDonald WA, White CR, Mann MR. Superovulation causes loss of genomic imprint maintenance by misregulation of ZFP57 in the mouse preimplantation embryo, UWO Biochemistry Graduate Student Seminar, London ON, Oct 2013, Oral Presentation

3. **Denomme MM**, Zhang L, MacDonald WA, White CR, Mann MR. Superovulation causes loss of genomic imprint maintenance by misregulation of ZFP57 in the mouse preimplantation embryo, SSR 46th Annual Meeting, Montreal QC, Jul 2013, Poster Presentation


18. **Denomme MM**, Zhang L & Mann MR. Embryonic imprinting perturbations do not originate from superovulation-induced defects in DNA methylation acquisition, Great Lakes Mammalian Development Conference, Toronto ON, Mar 2011, Poster Presentation


20. **Denomme MM**, Zhang L & Mann MR. Embryonic imprinting perturbations do not originate from superovulation-induced defects in DNA methylation acquisition, Epigenetics Symposium, Toronto ON, Jul 2010, Poster Presentation
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