Investigation of confined placental mosaicism and aberrant *H19*/ *IGF2* imprinting in pregnancies conceived by in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI)

by

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Abstract

In-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are two of the most common forms of assisted reproductive technologies (ARTs) used to treat human infertility. Presently, over two million children have been conceived by ART. The majority of these children are born healthy. However, IVF and ICSI have been associated with increased risks of adverse perinatal health outcomes. The higher risk of low birth weight, intrauterine growth restriction, and small for gestational age are of special concern due to its association with adult-onset diseases. In this thesis project, the effect of ART on the genetic and epigenetic status of the placenta, and their associations with fetal growth restriction are examined.

In the first objective, placentas from IVF and ICSI pregnancies were investigated for the presence of confined placental mosaicism (CPM). Of all cases, CPM was not detected in IVF and ICSI placentas that were appropriate for gestational age (AGA). CPM was not detected in any of the IVF or ICSI cases that were small for gestational age (SGA). By pooling the results from this study, and previous results from this lab, it was determined that the prevalence of CPM in ART pregnancies (3.7%) was higher, but not significant, when compared to the reported literature on natural conceptions (2.22%; p=0.23).

In the second objective of this study, DNA methylation at the imprinting control region 1 (ICR1) of the genes *H19* and *IGF2*, was investigated in the placentas from IVF, ICSI and natural conceptions. SGA pregnancies were also compared to AGA pregnancies. A multiple comparison analysis did not reveal any significant differences in mean DNA methylation levels in IVF, ICSI, or natural conceptions that were AGA or SGA (p=0.49). Although hypomethylation was detected in all groups, they did not appear to bear a significant clinical effect. Hypomethylation was defined as a value of less than 2 standard deviations from the mean methylation value at each CpG site analyzed.

These results demonstrate that placental abnormalities may not be more prevalent in ART conceptions than natural conceptions. Further study of other plausible biological mechanisms in causing adverse perinatal health outcomes in ART is recommended.

Preface

Co-authorship statement

I wrote this thesis in its entirety with input and guidance from Dr. Sai Ma. This thesis was revised with input from Dr. Sai Ma, and members of my supervisory committee: Dr .Wendy Robinson, Dr. Hélène Bruyère, and Dr. Anthony Perks.

In objective 1, investigating the role of confined placental mosaicism in IVF and ICSI pregnancies, I contributed to 85% of the data. I collected the placentas from the hospitals where consenting patients delivered their babies and performed the placental biopsies and DNA extraction. I performed all the methods needed to produce G-banded metaphase slides from each umbilical cord blood sample. I performed CGH analysis on 50 of the 54 placentas. The remaining contribution (15%) consisted of karyotype analysis by different members of Dr. Sai Ma's lab, which were: Andrew Wilson, Lina Wang, Agata Minor, and Dr. Sai Ma. CGH analysis on 4 of the 54 placentas was conducted by Andrew Wilson. With permission from Dr. Sai Ma, the results included contributions from other members of the lab, namely, Agata Minor, Chiho Hatakeyama, and Andrew Wilson. In the results, data from a publication (Minor, et al. 2006) under Dr. Ma's supervision were included to increase the statistical power of the sample size.

In objective 2, I performed all the methylation assays, including bisulfite conversion, PCR, and MS-SNuPE. MS-SNuPE was performed in Dr. Wendy Robinson's lab. I prepared all the samples for Ruby Jiang, who performed the capillary electrophoresis using the ABI genetic

analyzer. I performed all the data collection and subsequent analyses under the guidance of Dr. Sai Ma, Dr. Wendy Robinson, and Dr. Maria Penanhererra.

In both objectives, separate manuscripts will be prepared for future publications. These manuscripts will include sections of this thesis that are relevant for publication, including methods, results, and conclusions for each objective found in chapters 2, 3, and 4.

Statement of research ethics approval

The entirety of the research in this thesis was conducted under ethical approval from the University of British Columbia, BC Children's and Women's Health Centre of British Columbia, Research Ethics Board (H06-70324 and H06-03668).

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List of Abbreviations

AGA	appropriate for gestational age
ART	assisted reproductive technologies
AS	Angelman syndrome
BW	birth weight
BWS	Beckwith-Wiedemann syndrome
CHD	congenital heart defect
CPM	confined placental mosaicism
CpG	cytosine-phosphate-guanine dinucleotide
CVS	chorionic villi sampling
DMR	differentially methylated region
DNA	deoxyribonucleic acid
GA	gestational age
GnRH	gonadotropin releasing hormone
hCG	human chorionic gonadotropin
FSH	follicle stimulating hormone
ICR	imprinting control region
ICSI	intracytoplasmic sperm injection
IGF2	gene: *Insulin-like Growth Factor 2*
IUGR	intrauterine growth restriction
IVF	in-vitro fertilization
LBW	low birth weight
LH	luteinizing hormone
LOS	large offspring syndrome
MI	meiosis I
MII	meiosis II
Ms-SNuPE	methylation-sensitive single nucleotide primer extension
ncRNA	non-coding RNA
OA	oligoasthenozoospermia
OAT	oligoasthenoteratozoospermia
PCR	polymerase chain reaction

PWS	Prader-Willi Syndrome
RNA	ribonucleic acid
SD	standard deviation
SGA	small for gestational age
STS	sequence tagged site
TM	trimester
UPD	uniparental disomy

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To my friends and family, thank you for your unconditional love and support. To my Mother and Father, thank you for instilling in me the importance of education and allowing me the freedom to pursue my interests. To my three siblings, thank you for much love and support. And to my late grandparents, I will never forget the life lessons that you have taught me.

1. Introduction

1.1 Overview

Infertility, over the past two decades, has become a global health issue leading to scientific advancements in diagnosis and treatment. The most significant development is that of assisted reproductive technology (ART), which has allowed infertile couples to conceive their own genetically-related children. ART includes techniques that involve the handling of gametes outside of the human body, namely in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Over 2 million children have been born worldwide. Despite the success of ART, concerns have always been present about its effect on the health of the conceived child. Retrospective analyses have shown that ART is associated with greater risks of adverse perinatal pregnancy outcomes. The causes of these risks, including low birth weight and intrauterine growth restriction, have become the focus of researchers in the field of reproductive health. In this thesis, an investigation of the placenta from IVF and ICSI pregnancies was conducted to determine the role of confined placental mosaicism and imprinting abnormalities in infants born appropriate for gestational age and small for gestational age. This study of genetic and epigenetic origins offers a two-prong approach to further the understanding of the effects of ART on pregnancy outcome. In this chapter, the fundamental biological mechanisms of human reproduction, placental development and physiology, confined placental mosaicism, genomic imprinting, and infertility are reviewed. Following, a summary of a literature review on the effects and possible causes of adverse perinatal health outcomes in ART pregnancies is provided in support of this thesis project.

1.2 Human Reproduction

Human reproduction involves gametogenesis, fertilization, and processes involved in establishing pregnancy or implantation. Gametogenesis is the process in which the gametes (sex cells) are produced through the specialized cell division called meiosis. Gametogenesis is distinctly different between the male and the female, which are respectively described as spermatogenesis and oogenesis. Fertilization involves the fusion of an egg with a sperm to generate an embryo. Establishment of pregnancy involves processes in the embryo and physiological adaptations of the maternal uterine environment. The goal of this section is to provide the reader an overview of these processes as these concepts are important to the understanding of human infertility.

1.2.1 Gametogenesis

Gametogenesis is described in four phases of: 1) primordial germ cell migration to the male or female gonad; 2) proliferation by mitosis; 3) meiosis; 4) maturation into sperm or egg (Carlson 1999). In the first phase, primordial germ cells are produced at around the third week of gestation in the endoderm of the yolk sack and migrate to the developing gonads of the embryo by the sixth week (Carlson 1999). In the gonads, they undergo several series of mitotic divisions, exponentially increasing in population to millions of cells. These mitotically active germ cells are called spermatogonia in the male, and oogonia in the female. These germ cells then proceed to either spermatogenesis or oogenesis, involving meiosis. Meiosis is a two-stage division process of reduction of the number of chromosomes from diploid to haploid. Prior to meiosis, at interphase of the cell cycle, the cell's DNA becomes compacted into chromosomes of maternal and paternal copies. Each copy gets replicated to form sister chromatids that are joined

together. At the first meiotic division, the maternal and paternal chromosomes are then independently sorted and redistributed through recombination, forming two daughter cells. Each daughter cell then undergoes a second meiotic division, whereby the sister chromatids are separated to produce four haploid cells that mature into gametes. In spermatogenesis, each spermatogonium undergoes meiosis to eventually produce four spermatozoa. However, in oogenesis, each oogonium only gives rise to one oocyte.

1.2.2 Spermatogenesis

Spermatogenesis is a complex process that involves three major stages: 1) mitosis, for the proliferation of spermatogonia; 2) meiosis; and 3) spermiogenesis, the formation of mature spermatozoa. At the developing testis of the male embryo, the migrated primordial germ cells are called spermatogonia (Carlson 1999). Spermatogonia are mitotically active early in the embryonic testes and throughout life in the basal compartment of the seminiferous tubules of the mature testes (Carlson 1999). The mitotically active spermatogonia are classified as type A spermatogonia. Type A spermatogonia either divide to produce more type A cells or to produce type B spermatogonia. Type B spermatogonia aren't produced until the onset of puberty. Type B spermatogonia undergo mitosis to produce primary spermatocytes, which then enter meiosis. Thus, meiosis does not begin until after puberty (Carlson 1999). Through each successive meiotic division, primary spermatocytes become secondary spermatocytes and then round spermatids. Spermatids undergo spermiogenesis, a cytological transformation, to form mature spermatozoa (Carlson 1999). The major processes involved are nuclear condensation and relocation to the periphery of the cell, formation of the acrosome at the surface of the nucleus, and development of microtubules and axoneme to form the flagella (Carlson 1999). In addition, the histones of the sperm DNA are replaced by protamines which result in a higher degree of

DNA compaction (Carlson 1999). The time it takes to produce mature sperm beginning at meiosis is approximately 64 days (Carlson 1999).

Spermatogenesis is regulated by endocrine and paracrine mechanisms. Endocrine control of spermatogenesis involve hypothalamic secretion of gonadotrophin releasing hormone (GnRH) in a pulsatile fashion that directly stimulates the anterior pituitary to release luteinising hormone (LH) and follicle stimulating hormone (FSH) (Carlson, 1999). LH and FSH are released into the peripheral circulation and arrive in the testicular environment (Carlson, 1999). Leydig cells, of mesenchymal origin, in the testis are stimulated by LH to generate and secrete testosterone (Carlson, 1999). Testosterone is secreted into blood circulation and affects development of sexual characteristics and behaviour (Carlson, 1999). In the testicular environment, testosterone is important in the maintenance of spermatogenesis, including spermatogonial mitosis and meiosis (Sun et al. 1990). Spermatogonia do not have receptors for FSH and testosterone (Sun et al. 1990). However, Sertoli cells express the appropriate receptors and thus take up testosterone to eventually activate functions of these cells through protein synthesis pathways (Carlson 1999). A high level of testosterone in the testicular environment is important for the maintenance of normal spermatogenesis. The Sertoli cells also have FSH receptors, and FSH binding up-regulates the production of proteins that include androgen receptors and androgen binding proteins (Carlson 1999). In addition, FSH stimulation of Sertoli cells also activates negative feedback to down-regulate FSH production in the pituitary through the inhibin-B pathway (O'Connor and De Kretser 2004). The Sertoli cells, in addition to the blood-testis barrier, play a major role in creating and maintaining the testicular environment for spermatogenesis. Sertoli cells surround and interact with the germ cells at all stages and function as paracrine support for spermatogenesis (Carlson 1999).

1.2.3 Oogenesis

In contrast to spermatogonia, oogonia are only mitotically active in the embryonic ovary from the second through the fifth month of pregnancy. In this time, about seven million oogonia are produced by mitosis (Gilbert 2000). This number declines drastically as most oogonia proceed to cell death and by the seventh month, roughly two million cells are left. Shortly after birth, oogonia enter meiosis I to become the primary oocytes. However, unlike spermatogenesis, meiosis II does not follow. Instead, the primary oocytes become arrested at the diplotene stage of meiosis I until puberty. During this period of arrest, the oocytes undergo steady changes of low level ribosomal DNA amplification, RNA accumulation, and cortical granule formation in the preparation for embryonic needs. At approximately 12 years of age the female enters puberty and the first group of primary oocytes resumes meiosis I (Gilbert 2000). It is approximated that in each menstrual cycle, 10 to 30 primary oocytes complete meiosis I (Gilbert 2000). However, only one mature oocyte is released per menstrual cycle, which is referred to as the dominant follicle. Approximately 400 oocytes are ovulated in a female's lifetime, while the remaining primary oocytes undergo atresia (Gilbert 2000). Primary oocytes can be maintained for up to 50 years during a woman's lifetime (Gilbert 2000). At the completion of meiosis I, two unequal cellular progeny are produced: a small polar body, and a larger secondary oocyte. The nature of this division is due to the placement of the metaphase plate at the periphery of the oocyte. The secondary oocyte subsequently begins meiosis II and arrests at metaphase until fertilization. When meiosis II is completed, a similar unequal division occurs, resulting in a second polar body and one haploid oocyte (Gilbert 2000).

At the onset of meiosis, the primary oocytes are surrounded by follicular cells of the ovary (Carlson 1999). The unit of primary oocyte and its surrounding follicular cells is referred

to as a follicle. Folliculogenesis is the process in which a follicle develops, which begins and ends in a span of about 375 days (Carlson 1999). It consists of five phases of development, from the primordial follicle to the pre-ovulatory follicle, which coincides with the developmental stages of the oocyte. The primordial follicle is the most undeveloped, containing only the immature oocyte and one layer of granulosa cells (Carlson 1999). With the formation of the primary follicle, a non-cellular membrane, called the zona pellucida, between the oocyte and the follicular cell develops (Carlson 1999). The pre-ovulatory follicle consists of an oocyte surrounded by the zona pellucida and an adjacent fluid-filled cavity called the antrum (Carlson 1999). Oocyte maturation is dependent on paracrine pathways with the follicular cells. Follicular cells develop microvilli and gap junctions to facilitate connections with the primary oocyte. Gap junctions are important for transporting meiosis inhibiting factors (Carlson 1999). Growth and differentiation factors are secreted by the follicular cells to stimulate oocyte growth. Folliculogenesis is mediated by the endocrine system.

GnRH secreted by the hypothalamus stimulates the secretion of FSH and LH in the anterior pituitary (Carlson 1999). The granulosa cells express FSH receptors, and the theca cells express FSH-induced LH receptors (Carlson 1999). FSH stimulates different responses in different types of granulosa cells that ultimately affect follicular growth (Carlson 1999). LH induces the production of androgens which are aromatized by granulosa cells to generate estrogens (Carlson 1999). Alongside FSH and LH secretion, estrogen secretion also increases in a progressive fashion (Carlson 1999). Inhibin, also produced by the granulosa cells, serves as a negative feedback to the rise in FSH (Carlson 1999). When estrogen increases sharply, a surge of LH and a smaller burst of FSH induce ovulation (Carlson 1999). Ovulation involves the release of the oocyte from the follicle. In post-ovulation (luteal phase), the ruptured follicle becomes the corpus luteum (Carlson 1999). The function of the corpus luteum is to

predominantly secrete progesterone (Carlson 1999). Progesterone inhibits the maturation of more follicles, and stimulates the growth of the uterine wall in preparation for implantation (Carlson 1999).

1.2.4 Fertilization

Fertilization is initiated when the sperm attaches to the zona pellucida to undergo the acrosomal reaction (Carlson 1999). The zona pellucida contains receptor proteins for sperm glycoproteins, and components involved in fertilization and embryogenesis (Carlson 1999). The enzymes of the sperm acrosome allow for penetration of the ovum. Depolarization and cortical reactions also occur as a mechanism to prevent the penetration of other sperm (Carlson 1999). Depolarization is characterized by the activation of potassium channels. Cortical reactions involve calcium influx into the ooplasm and modification of surface glycoproteins of the ovum. Fertilization triggers the completion of meiosis II in the oocyte, generating a haploid pronucleus and a second polar body (Carlson 1999). The polar bodies are extruded from the ovum, and the remaining genetic material now consists of the female and male pronuclei. Fusion of the pronuclei occurs, the zygote forms, and mitotic division subsequently begins. At three days, the zygote has proliferated into the morula, which travels down the fallopian tube to the uterine cavity (Carlson 1999). Implantation begins at the blastocyst stage, where the morula has differentiated into an inner cell mass and an outer cell layer of trophoblast cells (day 6) (Carlson 1999). Only a portion of the inner cell mass gives rise to the fetus, while the remaining cells give rise to the supporting structures of the fetus, including the placenta and extra-embryonic membranes (Carlson 1999).

1.3 The placenta

The placenta is a vital component to fetal growth and development, as it serves many physiological functions in place of the developing fetal organs. Thus, proper placental development, which first occurs at the implantation stage of pregnancy, is important for a healthy pregnancy.

1.3.1 Development, Structure and Function

The human placenta is classified as discoid and haemochorial, whereby chorionic villi are arranged in a circular plate and come into direct contact with maternal blood (Carlson 1999). The structure of the mature placenta consists of fetal tissue (chorionic plate) originating from the chorionic sac, and maternal tissue (basal plate) from the uterine endometrium (Figure 1.1). The umbilical cord extrudes from the chorionic plate and attaches to the developing fetus. The fetal membranes of the placenta consist of the fetal-facing amnion and the maternal facing chorion (Bryant-Greenwood 1998). A third structure of the placenta, which lies between the chorionic and basal plates, is the intervillous space. The intervillous space contains the main functional units of the placenta, the chorionic villi, in which fetal blood is separated from maternal blood. Chorionic villi consist of complex branching of an outer layer of trophoblast cells, and an inner layer of a mesenchymal core containing fetal blood vessels (Figure 1.1) (Carlson 1999). The development of the placenta begins as soon as the blastocyst implants into the uterus at around the 6^{th} day of pregnancy (Norwitz et al. 2001).

After the blastocyst implants into the endometrium, the trophoblast cell layer proliferates and differentiates into two major pathways: villous and extravillous. An inner layer of

cytotrophoblast and an outer layer of syncytiotrophoblast are first formed. Cytotrophoblasts are mitotically active, undifferentiated and mononucleated (Carlson 1999). In the villous pathway, some of the cytotrophoblast undergo post-mitotic fusion and differentiation into an outer layer of syncytiotrophoblast (Carlson 1999). Syncytiotrophoblast invade the connective tissues of the endometrial surface. By day 9 -12 of pregnancy, the syncytiotrophoblast has eroded the endometrium and formed lacunar cavities between the cells that will provide the first wave of maternal blood to establish utero-placental circulation (Aplin 2000). In the extravillous pathway, the cytotrophoblast proliferate and penetrate through the syncytiotrophoblast at day 13-14 (Aplin 2000). This is followed by lateral expansion to form cytotrophoblast columns and shell. These cells are also known as extravillous cytotrophoblast (EVT). EVTs differentiate into endovascular trophoblast that invade and remodel uterine arteries, and interstitial trophoblast that will form the placental bed giant cells through migration into the decidua and myometrium (Carlson 1999). Maternal blood begins to flow into the intervillous space by the 10^{th} to 12^{th} day of pregnancy, characterized by a rise in oxygen tension at the 12^{th} day (Jaffe et al. 1997, Jauniaux et al. 2000).

The chorionic villi are formed from both syncytiotrophoblast and non-migratory cytotrophoblast, beginning late in the second week of pregnancy (Carlson 1999). The syncytiotrophoblast makes up the outer epithelial layer, while the cytotrophoblast forms the core (Carlson 1999). Initially, syncytiotrophoblast are penetrated by cytotrophoblast to form the primary villi. Secondary villi are formed when mesenchyme proliferates into the cytotrophoblast, forming the mesenchyme core (Carlson 1999). Eventually, fetal capillaries develop within the mesenchyme core to form tertiary villi (Carlson 1999) (Figure 1.1). The tips of the villi are anchored to the basal plate through the invasive cytotrophoblast cell columns (Carlson 1999).

The placenta's main functions aretransport and metabolism, immune protection to the fetus, and endocrine support throughout pregnancy (Carlson 1999). The placenta provides oxygen, water, carbohydrates, amino acids, lipids, vitamins, minerals, and other nutrients to the fetus. Carbon dioxide and waste products from the fetus are also removed via the placenta. The placenta provides immunity to the fetus from maternal infection and disease. Maternal immunoglobulin G (IgG) antibodies can be transported across the placenta to provide passive immunity (Pitcher-Wilmott et al. 1980). In addition, the placenta expresses different export pumps in the syncytiotrophoblast that function to remove drug metabolites from fetal circulation (Marin et al. 2003). However the placenta is not protective to all pathogens, as some bacteria, protozoa, and viruses can cross into fetal circulation. The placenta also has endocrine, paracrine, and autocrine functions that allow it to adapt to physiological changes during pregnancy, including metabolism, fetal growth, and parturition. The placenta produces estrogen, progesterone, human chorionic gonadotrophin (hCG), growth factors, cytokines and chemokines, and various other signalling molecules (Keelan et al. 2003, Malassine and Cronier 2002, Nayak and Giudice 2003).

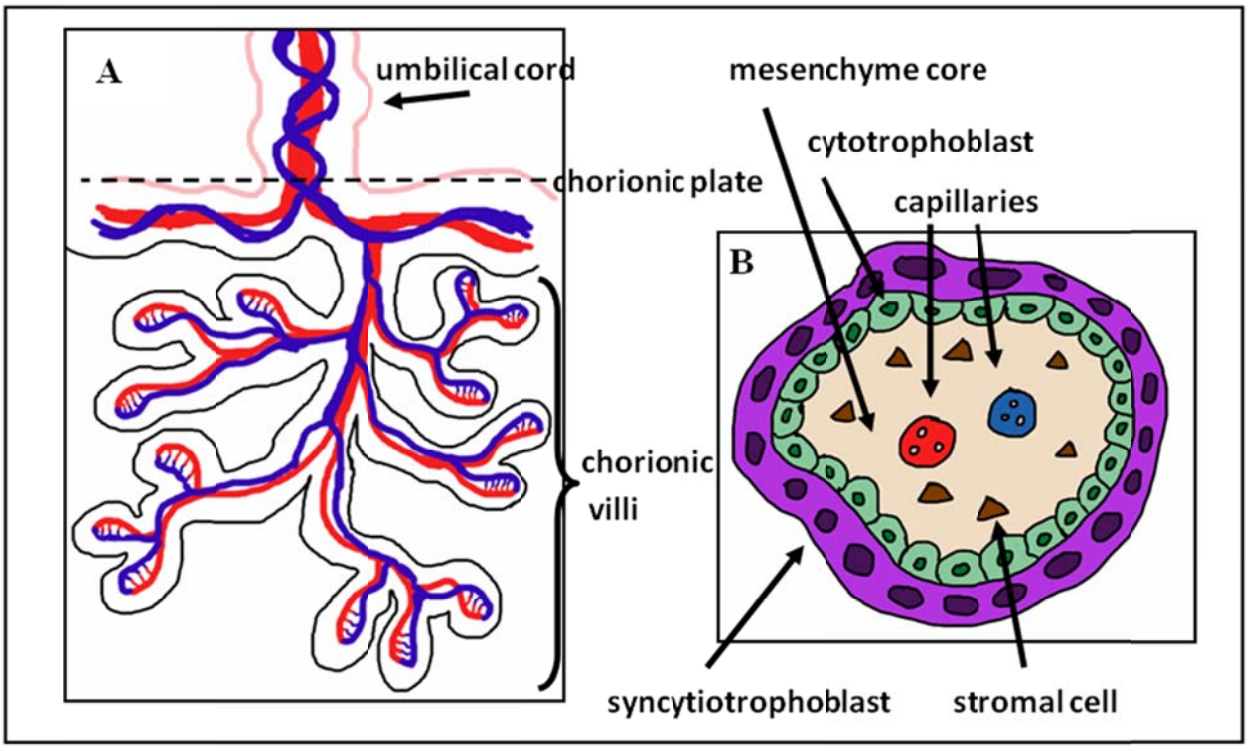

Figure 1.1 Functional unit of the placenta: chorionic villi.

A) The functional unit of the placenta consists of the chorionic villus which expands from the chorionic plate (fetus-facing). Fetal blood circulation flows through the umbilical cord and into the chorionic villi. The umbilical cord consists of one large artery (arterial blood indicated in red) and two veins (venous blood indicated in blue). In the chorionic villi, the circulation branches out into capillaries. B) A cross section of the chorionic villi is depicted. The syncytiotrophoblast (purple) forms the outer layer of cells, followed by the trophoblast (green), and the mesenchymal core (pink) and the capillaries (blue and red). This figure was adapted from Gude et al. 2004.

B)

1.4 Confined Placental Mosaicism (CPM)

The presence of cells with an abnormal genetic constitution in the placenta can be detrimental to placental function, physiology, and the health of the fetus. The condition known as confined placental mosaicism (CPM) will be discussed in this section.

1.4.1 Definition of CPM

Confined placental mosaicism (CPM) is defined as a chromosomal discrepancy that is limited only to the placenta while absent in the fetus. In prenatal diagnosis by chorionic villi sampling (CVS) in the first trimester of pregnancy, CPM is detected in 2% of the cases (Kalousek and Vekemans 1996). CPM usually involves trisomy, with chromosomes 2, 3, 7, 8, 11, 13 and 22 being the most prevalent (Wolstenholme 1996). There is no specific nomenclature for CPM, however, it is commonly accepted that the use of CPM followed by the chromosome refers to an abnormality such as trisomy for that chromosome. For instance, CPM for trisomy 2 is conventionally written as "CPM2". Partial trisomies, deletions, and polyploidy can also occur in CPM.

1.4.2 Classifications and Origins of CPM

CPM was first classified by Kalousek *et al.* into three types (I, II and II) according to the type of placental cell lineage affected (Kalousek and Vekemans 1996). CPM can affect only the cytotrophoblast (type I), chorionic stroma or mesenchyme (type II), or both lineages (type III) (Kalousek and Vekemans 1996). Type I CPM and type II CPM occur with similar frequencies (0.8%, 0.9%; respectively), and type III CPM occurs less frequently (0.2%) (Wang et al. 1993). When type III CPM is present with a fetus of normal chromosome constitution, it is likely that the zygote was abnormal to begin with (meiotic origin) (Kalousek and Vekemans 1996).

CPM can arise from either non-disjunction during meiosis (gametes) or mitosis (post-fertilization). In CPM of meiotic origin, the fertilized zygote is abnormal as a result of a chromosomally abnormal gamete (oocyte or sperm). The abnormal gamete originates from

meiosis I or meiosis II non-disjunction. During embryo development trisomic rescue may occur in one cell, where the extra chromosome is subjected to anaphase lag and jettisoned, resulting in a normal complement of chromosomes (Robinson et al. 1997). The normal cells can then be preferentially selected to proliferate and differentiate into fetal cells, while the remaining abnormal cells eventually form the makeup of the placenta and extra-embryonic tissues, thus leading to CPM. In meiotic CPM, high levels of aneuploidy are implicated and type I or type III CPM is most likely to occur (Wolstenholme 1996). Relative to mitotic origin, the level of trisomic cells from meiotic origin was reported to be significantly higher (Robinson et al. 1997).

In mitotic CPM, non-disjunction occurs in the placental lineage of early embryo. The timing of this occurrence and the cell lineage involved will determine the proportion of the placenta affected, and the type of CPM (Kalousek and Vekemans 1996). Mitotic errors give rise to all the three types of CPM, but type I and II are usually expected (Wolstenholme 1996). Type III CPM would be expected to occur when non-disjunction occurs before the differentiation of the embryo into different cell lineages, which would be before the blastocyst stage or in early blastocyst development. Nonetheless, the effect of mitotic CPM on the developing fetus and extra-embryonic tissues likely depends on the distribution of abnormal cells and whether they are selected against (Wolstenholme 1996).

The majority of trisomic CPMs have been reported to be of maternal origin, correlating with research revealing that up to 20% of oocytes in women are aneuploid (Hunt and Hassold 2008). However, paternal origin in trisomic CPM has also been reported (Shaffer et al. 1996).

1.4.3 Uniparental Disomy (UPD)

In addition to CPM, trisomic rescue can also result in uniparental disomy (UPD) in the fetus and/or the placenta. UPD occurs when both homologous chromosomes originate from the same parent. UPD is theorized to occur in 33.3% of trisomic rescue events. The rate of UPD in CPM cases is reported to be 14.2% (Kotzot 2002). UPD has been associated with multiple congenital defects and intrauterine growth restriction (Kalousek and Vekemans 1996, Hansen et al. 1997). Other studies have not shown such an association (Gibbons et al. 1997, Shaffer et al. 1996). It is likely that the clinical effect of UPD is dependent on which chromosome involved. For example, UPD in chromosomes that contain imprinted genes may be more detrimental as these genes follow a parent-of-origin specific pattern of expression

1.4.4 Presence of CPM on Pregnancy Outcome

The clinical severity of CPM most likely depends on the lineage of cells affected, the level of abnormal cells in the placentas, and the chromosome involved. Prenatally diagnosed CPM persists to term in up to 90% of the cases, and may be associated with low birth weight, intrauterine growth restriction (IUGR), pregnancy loss, maternal hypertension and gross placental pathology. The rate of prenatal or perinatal complications in pregnancies with CPM is between 16% and 21%. The rate of spontaneous abortion in CPM cases diagnosed through CVS is between 5 – 33%, compared to the range of 1.6% to 5% in non-CPM cases (Eiben et al. 1986, Griffin et al. 1997, Hogge et al. 1986, Johnson et al. 1990, Qumsiyeh 1998, Rehder et al. 1989, Warburton et al. 1978). The rate of stillbirth and neonatal death in CPM cases is reported to be 4.8% and 2.4%, respectively (Johnson et al. 1990).

Over 20% of IUGR pregnancies are associated with CPM when birth weight is ≤ 5th percentile (Lestou and Kalousek 1998). In CPM detected in term placentas, 8% to 60% of cases have been diagnosed with IUGR (Lestou and Kalousek 1998). However, the rate of CPM is lower in IUGR pregnancies of known etiology, such as maternal smoking. Type III CPM is more common in IUGR than type I or II. CPM can lead to placental dysfunction that affects fetal growth, which may explain its association with IUGR (Kalousek et al. 1991). IUGR is defined as a pathological condition during pregnancy that prevents the fetus from reaching its growth potential. In trisomic CPM cases, placental weight was also found to be significantly lower than non-CPM placentas, but the level of trisomy did not have a direct effect on placental growth (Yong et al., 2009). However, the exact mechanism by which CPM affects fetal growth is unknown. It may be complicated by the fact that various chromosomes have been linked to CPM-associated IUGR pregnancies, including CPM 2, 7, 8, 9, 13, 15, 16, 20, 21, and 22 (summarized in Table 1.1).

CPM16 is most commonly associated with IUGR (Robinson et al. 1997, Wolstenholme al. 1994). The majority of CPM16 cases are due to a maternal meiotic error (Robinson et al. 1997, Wolstenholme 1996). In the absence of UPD16, CPM16 is highly associated with IUGR, which suggests that the level of trisomic cells is associated with IUGR (Kalousek and Barrett 1994). CPM2 has been associated with intrauterine growth restriction, elevated maternal serum hCG levels, and oligohydramnios (Ariel et al. 1997, Gibbons et al. 1997, Hansen et al. 1997, Shaffer et al. 1996). The fetus or infant diagnosed with CPM15 may present hypogenitalism, cryptorchidism, dolichomegacolon, severe intrauterine growth restriction (IUGR), and two-vessel umbilical cord (Christian et al. 1996, Kalousek et al. 1991). However, CPM involving chromosomes 2, 3, 7, 8, and the sex chromosomes are usually associated with normal fetal

development (Farra et al. 2000, Wolstenholme 1996). Overall, CPM when diagnosed prenatally can be an important indicator of aberrant fetal growth and development.

Table 1.1 CPM detected by CVS in IUGR pregnancies.

Reference	Abnormality	% of cells affected	
		Direct (trophoblast)	Cultured (mesenchyme)
Kalousek et al. 1991	+2 +7 +7 +15 tetraploidy	2 53 24 10 33	7 96 73 0 0
Schuring-Blom et al. 1993	+8 45,X	100 100	- -
Kennerknecht et al. 1993	+18 Tetraploidy +6,+21,+22	100 100 -	- - 3.2
Wolstenholme et al. 1994	+2,+15 +9 +16 +16 +16 Del (13)(q13) +22	70 100 - 30 100 100 100	- 100 - - - - -
Leschot et al. 1996	+3 +8 +13 +13 +16 +22 45,X 45,X +20,+21 +5,+13 46,XX,der(5)	31 100 54 13 100 95 89 100 53 100 13	- - - - - - - - - - -
Robinson et al. 1997	+2 +7 +8 +16 +16 +16 +16 +16 +16 +16 +22 +22	100 100 0 100 100 100 100 100 100 - 100 70	43 - 100 100 100 100 - 100 100 - 66
Farra et al. 2000	+2 +2 +13,+16	40 30 48	- - -

1.4.5 Methods to Assay CPM

Chorionic Villus Sampling (CVS)

Although prenatal diagnosis is primarily used to diagnose fetal chromosome abnormalities, the results can also be used to determine CPM (Kalousek et al. 1987). The majority of studies on CPM have used results of first trimester prenatal diagnosis by chorionic villus sampling (CVS) (as shown in Chapter 1.4.4; Table 1.1). The placental tissue retrieved by CVS is then subjected to short or long-term culturing (McKinlay and Sutherland 2003). After harvesting, metaphase cells are analyzed to determine numerical and structural chromosomal abnormalities. Short term culture, or direct preparation, allows for the selection of trophoblast lineage to be analyzed (McKinlay and Sutherland 2003). Long term culture, allows for the mesenchyme lineage to be analyzed (McKinlay and Sutherland 2003). To identify the type of CPM, both short and long term culturing are applied in order to identify a mosaicism between the extraembryonic and the embryonic lineages (Kalousek et al. 1987).

Post-pregnancy analysis of the placenta

A post-pregnancy analysis of the term placenta combined with analysis of fetal cells which can be obtained from umbilical cord blood does not result in any additional health risk to the mother or the fetus experienced during labour and delivery. Culturing of placental cells and cord blood to produce karyotypes, or the use of comparative genomic hybridization (CGH) can effectively identify CPM (Barrett et al. 2001). CGH involves the fluorescent labelling of genomic DNA by nick translation (Minor et al. 2006). Using a known normal reference that is labelled with a different fluorophore, the test and reference DNA are competitively hybridized to

metaphase spreads on a microscope slide. By analyzing the fluorescent ratios between test and reference, deletions and duplications can be identified (Minor et al. 2006).

1.5 Genomic Imprinting

An imprinted gene is one that is expressed either exclusively from the paternal allele or the maternal allele. This exclusivity is heritable and reversible. Imprinted genes are established during gametogenesis in early embryonic development, and are maintained throughout life (Reik and Walter 2001). However, there is also tissue-specific imprinting, in which some genes are not imprinted in some tissue types and some genes are imprinted in only a specific tissue type. The majority of imprinted genes are implicated in growth and development, and their sex-specific expression is established through epigenetic modification in the germ line. A summary of imprinted genes that have a role in growth and development is listed in Table 1.2. Genomic imprinting has been shown to exist in flowering plants, insects, and mammals. Imprinting in mammals was first demonstrated in various nucleus transplantation experiments in mouse zygotes that resulted in grossly abnormal embryonic development (McGrath and Solter 1984). When zygotes with only two maternal genomes (parthenogenetic or gynogenetic) develop into embryos, a lack of growth and extra-embryonic development were observed (McGrath and Solter 1984). When zygotes with only two paternal genomes (androgenetic) developed into embryos, overgrowth of extra-embryonic tissues and developmental arrest were observed (McGrath and Solter 1984).

The majority of imprinted genes are clustered together in the genome, and their imprinting is regulated through common imprinting control regions (ICR) that are differentially methylated (Reik and Walter 2001). The presence of ICRs suggests that imprinting regulation

occurs at the chromosome-level, not at an individual gene-level (Buiting et al. 1995). Imprinting is also tissue-specific, species-dependent, and dependent on developmental stage (Morison and Reeve 1998). In addition, an imprinted gene's expression may not only depend on the imprinting status of each allele, but also on other genes in the same cluster (Wolf et al. 2008).

Table 1.2 Imprinted genes / domain implicated in fetal growth

Chromosome/ genes	**Function**
Chromosome 6	
ZAC/PLAGL1	Implicated in transient neonatal diabetes mellitus and IUGR Potential tumor suppressor (Varrault *et al.*,1998)
7p13-p11.2	Candidate region for Silver-Russell Syndrome
GRB10	Growth inhibitor (Hitchins et al., 2002)
7q32	Candidate region for Silver-Russell Syndrome
PEG1/MEST	Implicated in IUGR and post-natal growth restriction
MEST1T1	Untranslated transcript
COPG21T1	Untranslated transcript
CPA4	Candidate gene for prostate cancer aggressiveness
11p15.5	
CDKN1C	Negative regulator of cell proliferation
KCNQ1	Unknown role in growth or development
KCNQ1OT1/ LIT1/ KvDMR1	Untranslated transcript
PHLD2/IPL/TSSC3	Elevated expression is associated with low birth weight
SLC22AIL/IMPT1/TSSC5	Tumor suppressor candidate Growth factor, important for fetal growth
IGF2	Untranslated transcript, possible regulator of *IGF2*
H19	
14q	mUPD14 causes pre and postnatal growth restriction, early onset of puberty, skeletal deformities, small extremities and motor delay (Kotzot 2004).
14q32	
MEG3/GTL2	Implicated in cellular signalling
PEG9/DLK1	Cellular differentiation
15q11-q13	Prader-Willi Syndrome (PWS) and Angelman Syndrome region.
SNRPN	PWS may present with IUGR, hypotonia, mild retardation, learning disabilities and behavioural problems
UBE3A	
Chromosome 16	Maternal UPD16 is commonly reported with CPM16, and is associated with IUGR

1.5.1 Epigenetic Features of Imprinted Genes

Genomic imprinting is a result of epigenetic modifications that occur in germ line cells in a dynamic fashion. Thus, imprints are established in the gametes and are maintained after fertilization and throughout embryonic development (reviewed by Reik et al. 2001). Epigenetics refers to the change in the expression of a gene in the absence of changes to its DNA sequence (reviewed by Reik et al. 2001). The most studied epigenetic modifications include DNA methylation and post-translational modification of histones. DNA methylation is the most studied mechanism in imprinting, as its profile for the same imprinted gene is different in oocytes and spermatozoa. The first evidence for a role of DNA methylation in imprinting was shown in imprinted transgenes, where foreign DNA was methylated when maternally derived but non-methylated when paternally derived (Saski et al. 1993). DNA methylation involves the action of DNA methyltransferases (DNMTs) which methylated the cytosine (meC) in a sequence of cytosine-guanine (CpG; p – phosphodiester bond). When CpGs occur in high frequency in a defined genomic region, it is called a CpG island. Imprinted genes are richer in CpG islands compared to non-imprinted genes (Paulsen et al. 2000).

DNA methylation is heritable and reversible. When maternal and paternal genomes are combined, their respective DNA methylation profiles at imprinted genes are maintained. However, in the germ line, the DNA methylation profiles are erased and re-established according to the sex of the embryo (Reik et al. 2001). This dynamic property of genomic imprinting is described as a cycle of erasure, establishment and maintenance, which occur during two critical windows of development – gametogenesis and post-fertilization. In the early primordial germ cells, the DNA methylation patterns of imprinted genes are erased during genome-wide de-methylation (Figure 1.2). DNA methylation is then established at different developmental time

periods. In the male germ line, this takes place prior to the development of spermatogonia (Reik et al. 2001). In the female germ line, imprints are not established until oocyte maturation, which begins at the onset of puberty (Reik et al. 2001). These DNA methylation-mediated imprints are then maintained, and are protected from a wave of genome wide de-methylation that occurs after fertilization (Figure 1.3) (Reik et al. 2001). The imprints are maintained throughout subsequent cell divisions. Errors at any of these critical moments that affect epigenetic modifications on imprinted genes can result in their aberrant expression.

DNA methylation is mediated by DNA methyltransferases. DNMT1 is responsible for maintenance of methylation through restoration of hemi-methylated DNA strands that result during DNA replication in mitosis (Reik et al. 2001). The de novo establishment of DNA methylation is primary performed by DNMT3A and DNMT3B, which are highly expressed during specific developmental times in early development and germ cells (Reik et al. 2001). DNMT3A is more specific to imprinted genes and interspersed repetitive elements, while DNMT3B is required for methylation at pericentric satellite repeats (Kaneda et al. 2004, Okano et al. 1999). DNMT3L does not have methlytransferase activity; however it is thought to play a regulatory role in de novo DNA methylation (Hata et al. 2002).

The mechanisms in which DNA methyltransferases act are currently not fully understood, which includes how targeted sequences are recognized. It has been suggested that chromatin modifications are likely involved in setting the accessibility of the targeted loci for DNMTs. Other suggestions include the involvement of DNA binding proteins, non-coding RNAs (ncRNAs), and direct recognition of DNA sequences. In d*e novo* DNA methylation, it has been shown that DNMT3a and DNMT3b have intrinsic preference for DNA sequences that flank targeted CpGs (Wienholz et al. 2010). Furthermore, Wienholz *et al.* have also demonstrated that

DNMT3L, which has chromatin binding properties, direct *de novo* methylation at chromatinized DNA which may allow a greater range of action for DNMT3A and DNMT3B (Wienholz et al. 2010).

In addition to DNA methylation, epigenetic modifications can also be achieved through covalent modification of histones. In the nucleosomes, histone octamers (H2A, H2B, H3, and H4) are bound by 146 base pairs of DNA. Modifications of the N-terminal tails of histone subunits can alter the accessibility of DNA to transcriptional machinery. Selected amino acids are modified by enzyme-catalyzed methylation, acetylation, phosphorylation or ubiquitination and alter the accessibility of the chromatin to the transcriptional machinery (Turner 2002). Lysine acetylation is highly correlated with transcriptional activity. While lysine methylation can either promote or repress transcription, depending on which lysine residue is modified. Di- or trimethylation at lysine 4 of histone 3 (H3K4m2, H3K4me3) is associated with transcription, whereas di- or tri-methylation at H3K9 and trimethylation at H3K27 are associated with repression. Histone modification is thought to function in short-term reversible silencing to allow for developmental plasticity, whereas DNA methylation is suggested to function as a long-term silencing mechanism (Reik 2007).

Imprinting is also regulated by non-coding RNAs (ncRNAs). The majority of the transcribed mammalian genome has been found to consist of ncRNAs (Carninci 2008). ncRNAs can act in *cis* or in *trans* to regulate gene expression. *Cis* acting ncRNAs are associated with macro ncRNAs that range in length from a few hundred to a few thousand nucleotides. *Trans* acting ncRNAs are described as short ncRNAs, which includes short interfering (si), micro (mi), piwi-interacting, and short nucleolar (sno) RNAs that are 21 to 300 nucleotides in length (Koerner et al. 2009). In imprinted gene clusters that contain ICRs, one or more genes coding

for ncRNAs are usually present and are active on the unmethylated allele of the ICR (Koerner et al. 2009). In the maternally imprinted *Kcnq1* cluster, the macro ncRNA *Kcnq10t1* mediates transcriptional silencing in *cis* of the imprinted genes *Kcnq1*, *Cdkn1c*, *Cd81*, *Ascl2*, and *Osbpl5* (Pandey et al. 2008). Genes for ncRNAs have also been localized to other imprinting clusters, including *Igf2* and *Igf2r*. Although the mechanism in which ncRNAs act in imprinting regulation is not fully understood, several transcriptional-silencing models have been proposed (Pauler et al. 2007).

Overall, epigenetic modification can be a result of multiple interactions between DNA methylation, histone modification, and ncRNAs. The acquisition of these different types of modifications can be interdependent. However, the understanding of these interactions is currently derived from a few published studies.

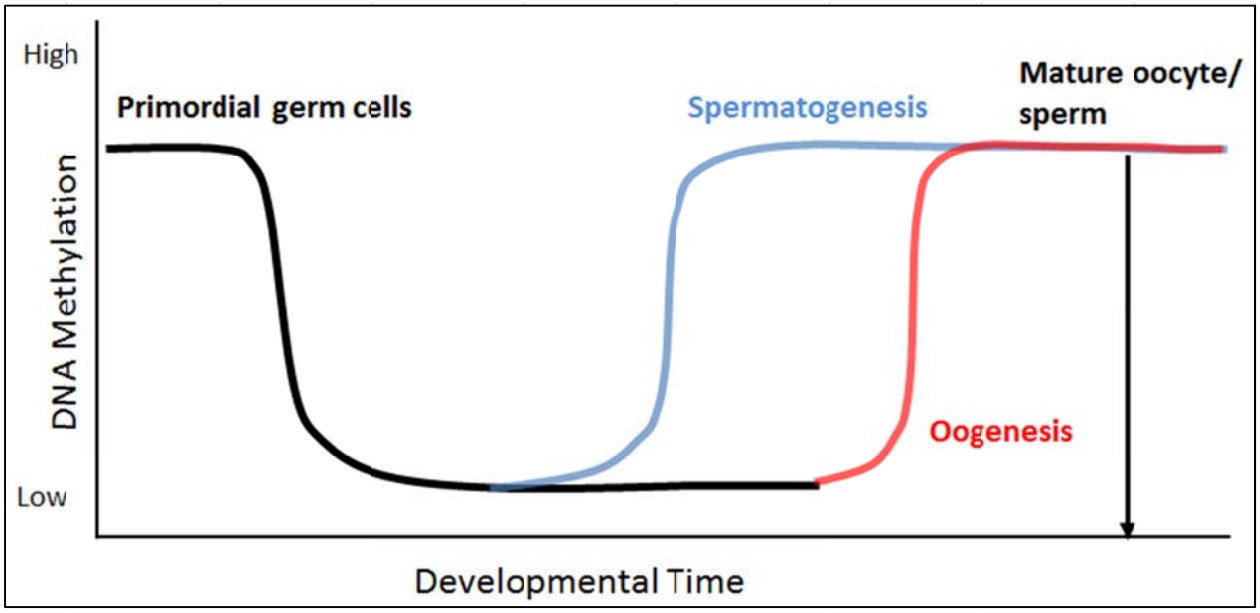

Figure 1.2 DNA methylation erasure and establishment.

As demonstrated in the mouse, primordial germ cells undergo DNA methylation erasure which is the re-established in a sex-specific manner. Remethylation begins in prior to the development of spermatogonia in male germ cells, and after birth in the developing oocytes. This figure was adapted from Reik et al. 2001.

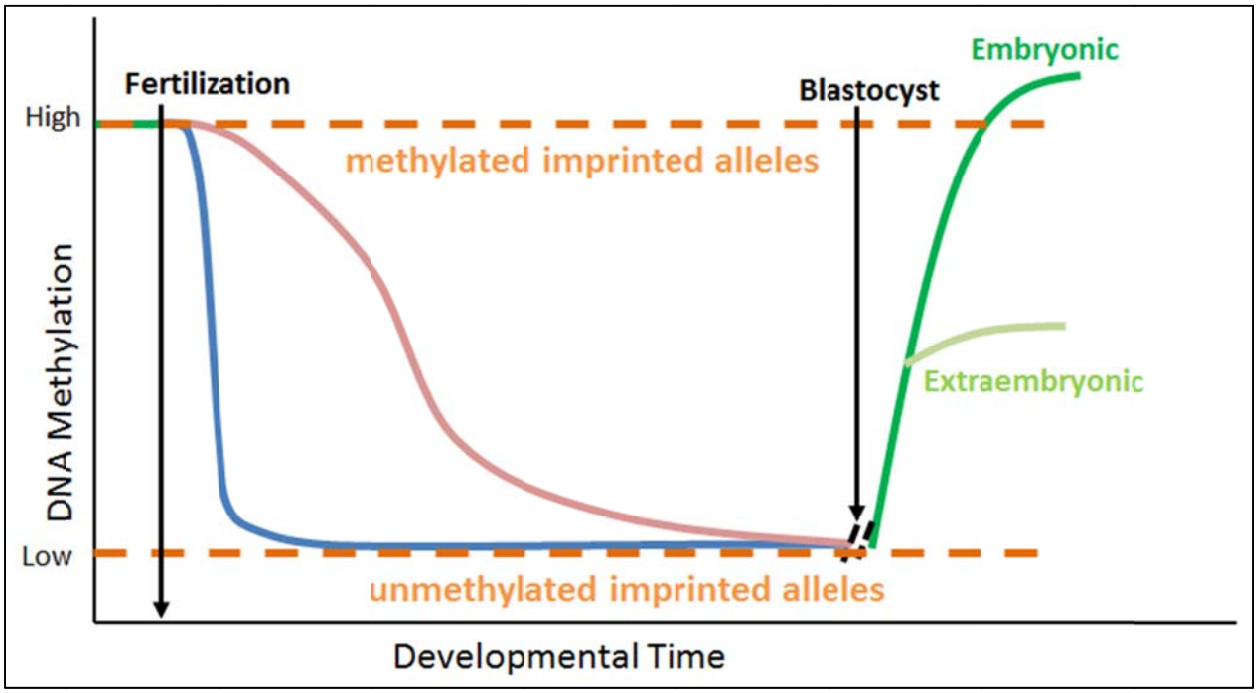

Figure 1.3 Genome-wide de-methylation and re-methylation in the pre-implantation embryo.

DNA at imprinted genes is protected from the genome-wide de-methylation and re-methylation in the pre-implantation embryos. After fertilization, the paternal genome (blue) is actively de-methylated and the maternal genome (red) is passively de-methylated. Both genomes are then re-methylated at around the time of implantation (blastocyst stage). In global DNA methylation, the extra-embryonic lineages are less methylated than the embryonic lineages. This figure was adapted from Reik et al. 2001.

1.5.2 *H19* and *IGF2*

One of the most studied pairs of imprinted genes are *H19* and *IGF2*. These genes share a common imprinting control region that is differentially methylated in a sex-specific manner.

H19 is a maternally-expressed non-coding RNA transcript that is implicated in development through regulation of *IGF2* (Weber et al. 2001). *IGF2*, insulin-like growth factor 2, is paternally-expressed fetal growth factor that is important for fetal growth and placental development (Constancia et al. 2002). *H19* and *IGF2*, separated by 70 kb, are located on chromosome 11p15.5, which also contains another imprinting cluster. The two imprinting control regions (ICRs) on 11p15.5 are defined as ICR1 and ICR2 (Figure 1.4). These regions were first found in the mouse distal chromosome 7, and the majority of studies on imprinted genes and imprinting regulation were conducted on the mouse model.

At the ICR1, which regulates imprinting of *H19* and *IGF2*, the paternal allele is methylated and the maternal allele is non-methylated. ICR1 is located -2 to -4 kb upstream of *H19* and is paternally methylated (Olek and Walter 1997, Tremblay et al. 1995, Tremblay et al. 1997). ICR1 controls imprinting through insulating and silencing activities. Contained within the ICR1 are a series of conserved sequences that signal binding sites for an insulator protein, CTCF. The binding of CTCF is methylation-sensitive and occurs when the ICR1 is unmethylated. Thus, on the maternal allele, CTCF binding occurs and insulates against *IGF2* transcription through enhancer blocking.

H19 and *IGF2* share enhancers that are located downstream of *H19*. CTCF binding allows the enhancers to activate *H19* expression. On the paternal allele, the ICR1 is methylated and CTCF binding does not occur, allowing enhancer activity on *IGF2*. Also, *H19* expression is attenuated by methylation of its promoter. This dynamic interaction of the ICR1 has mainly been shown through studies on mice (which is also referred to as the DMR, differentially methylated region). Disruption of CTCF-binding sites at the ICR1 results in ectopic expression of *Igf2* (Engel et al. 2006, Pant et al. 2003, Pant et al. 2004, Schoenherr et al. 2003, Szabo et al.

2004). Conversely, when mutations were inserted into the CTCF-binding site that promoted CTCF-binding, *Igf2* expression was down regulated. Therefore, the most critical region for imprinting control of *H19* and *IGF2* lies in the CTCF-binding sites. Using the mouse model, it has been proposed that the presence of CTCF recruits factors involved in chromatin looping which positions the enhancers away from *Igf2* (Figure 1.5) (Sasaki et al. 2000). Although differential methylation at the ICR1 is to regulate *H19* and *IGF2* imprinting, the mechanism marking each parental allele for methylation is unclear.

Aberrant methylation at the ICR1 can result in phenotypic abnormalities related to growth. Hypermethylation of ICR1 leads to biallelic expression of *IGF2*, resulting in a fetal overgrowth syndrome (Beckwith Wiedemann Syndrome, BWS) in humans and large offspring syndrome (LOS) in other mammals (Reik and Walter 2001). Conversely, hypomethylation results in loss of *H19* transcription, which is associated with Wilm's tumor and other types of cancers (Niemitz et al. 2005). Both *H19* and *IGF2* are also imprinted in the placenta, suggesting that their role in fetal growth and development are also exerted through the placenta. In the mouse placenta, *Igf2* null mutations resulted in reduced placental and fetal size (Constancia et al. 2002).

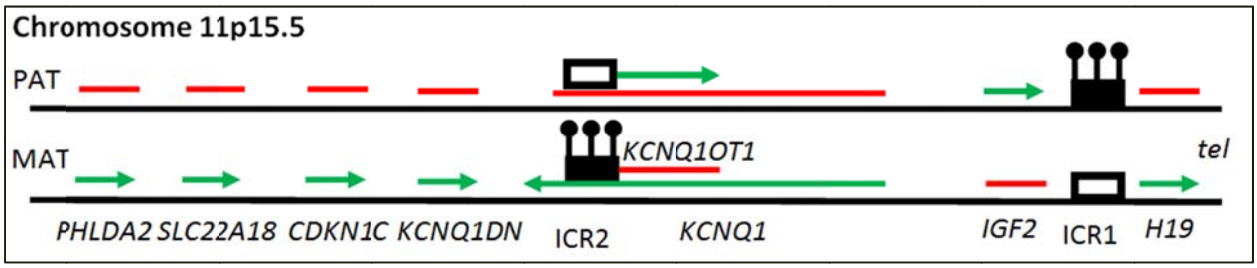

Figure 1.4 Differential methylation at ICRs of chromosome 11p15.5.

Human chromosome 11p15.5 contains two ICRs: ICR1 and ICR2. ICR1 controls imprinting at *IGF2* and *H19*. ICR2 is responsible for imprinting of 6 imprinted genes: *PHLDA2, SLC22A18,*

CDKN1C, KCNQ1DN, and *KCNQ10T1*. Green arrows represented expression. Red lines represent suppression. Black rectangles represent methylated ICRs. White rectangles represent unmethylated ICRs. Adapted from Smith et al. 2007.

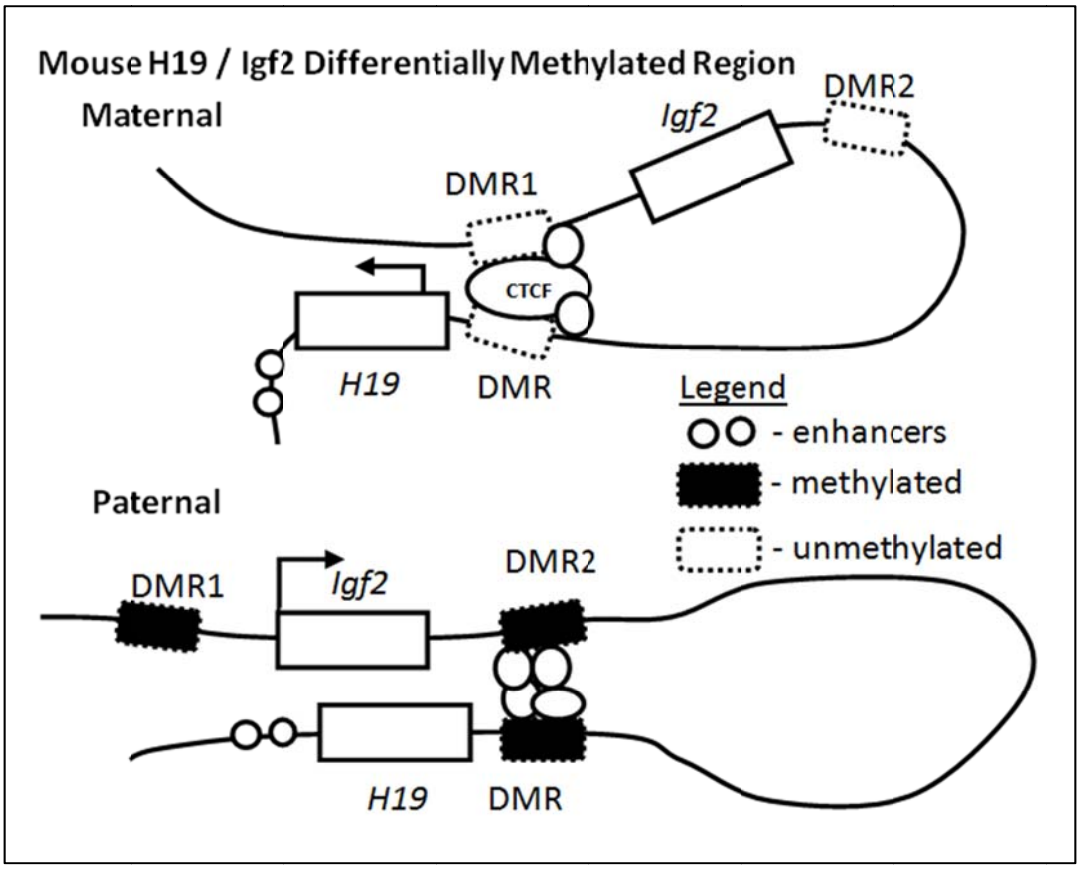

Figure 1.5 Postulated chromatin configuration at mouse *H19* DMR.

At the mouse *H19* DMR (or ICR), CTCF binding to the unmethylated DMR promotes a chromatin looping configuration that prevents the enhancers from acting on *Igf2*. Absence of CTCF changes this configuration that allows the enhancers to act on *Igf2*. Adapted from Sasaki et al. 2000.

1.5.3 Imprinting in the Placenta

As discussed earlier in chapter 1.3, the placenta is important in fetal growth and development during pregnancy. It is understood that proper regulation of the placenta is

important to a healthy fetus and mother during pregnancy. In light of research on epigenetics, it has been shown that placental development and function relies on epigenetic regulation. These include DNA methylation, histone modifications, and non-coding RNAs that affect gene expression, including those of imprinted genes. Although all d*e novo* global DNA methylation occurs in pre-implantation development, the extraembryonic lineages have lower levels of methylation than the embryonic lineages (Santos et al. 2002). Although the placenta is globally hypomethylated compared to the embryo, DNA methylation is still crucial for proper development of the extraembryonic tissues. In pregnant rats the administration of 5'-aza'2'deoxycytidine, which inhibits DNA methylation, adversely affects trophoblast differentiation (Serman et al. 2007, Vlahovic et al. 1999). The placentas of *Dnmt1*$^{-/-}$ and *Dnmt3L*$^{-/-}$ knockout mice have multiple morphological abnormalities, such as a lack of syncytiotrophoblast formation (Arima et al. 2006, Bourc'his et al. 2001, Li et al. 1992). Loss of imprinting (LOI) was observed after ablation of *Dnmt1*, which also suggests that some imprinted genes are involved in proper placental development.

Imprinted genes are important for placental function and development, as evident by the presence of imprinted genes in the placenta and placenta-specific imprinting in the mouse (Ferguson-Smith et al. 2006, Hemberger 2007, Reik et al. 2001, Steinhoff et al. 2009, Tycko 2006). Imprinted genes that are shown to play a role in fetal growth are listed in Table 1.3. Of the imprinted genes in humans, to our knowledge, none are specifically imprinted in the placenta unlike in the mouse. However, genes that are imprinted in the placenta and in the embryo are highly conserved between the mouse and humans. These genes may have synergistic functions in fetal growth and placental development. For example, in addition to affecting fetal growth, *Igf2* is also implicated in nutrient supply regulation that affects the permeability capacity of the placenta (Constancia et al, 2002; Sibley et al. 2004).

Imprinting in the placenta is dynamic throughout pregnancy as some genes experience loss of imprinting in the later stages of pregnancy and some genes don't acquire monoallelic expression until later in pregnancy. As a result, in any study investigating imprinting in the placenta, there must be considerations about the dynamic nature of placental imprinting in selecting which imprinted genes to investigate in the human placenta.

Table 1.3 Imprinted genes in the placenta implicated in fetal growth.

Gene	Name	Location	Reference	Imprinting	Placental-specific
Paternally-expressed					
DLK1	Delta-like I homolog	14q32	Pozharny *et al.* (2010)	1st TM, Term	no
IGF2	Insulin-like growth factor 2	11p15.5	Pozharny *et al.* (2010) Apostolidou *et al.* (2007), Lambertini *et al.* (2008)	1st TM Term	no
MEST/PEG1 isoform 1	Mesoderm-specific transcript homolog	7q32	Pozharny*et al.* (2010) Apostolidou *et al.* (2007), Lambertini *et al.* (2008)	1st TM Term	no
MEST/PEG1 isoform 2	Mesoderm-specific transcript homolog	7q32	McMinn et al. (2006)	3rd TM, Term	no
PEG3	Paternally expressed 3	19q13.4	Pozharny *et al.* (2010) Pozharny*et al.* (2010), Lambertini *et al.* (2008)	1st TM Term	no
PEG10	Paternally expressed 10	7q21	Pozharny *et al.* (2010) Lambertini *et al.* (2008)	1st TM Term	no
SNRPN	Small nuclear ribonucleoprotein polypeptide N	15q11.2	Pozharny *et al.* (2010) Lambertini *et al.* (2008), Pozharny *et al.* (2010)	1st TM Term	no
PLAGL1/ZAC	Pleiomorphic adenoma gene-like 1	6q24-q25	Pozharny *et al.* (2010) Lambertini *et al.* (2008), Pozharny *et al.* (2010)	1st TM Term	no
Maternally-expressed					
CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57)	11p15.5	Monk *et al.* (2006)	Term	no
H19	Imprinted maternally expressed transcript	11p15.5	Pozharny *et al.* (2010), Yu *et al*. (2009) Lambertini *et al.* (2008), Yu *et al.* (2009)	1st TM Term	no

Gene	Name	Location	Reference	Imprinting	Placental-specific
Maternally-expressed					
MEG3	Maternally expressed 3, transcript	14q32	Pozharny *et al.* (2010) Lambertini *et al.* (2008), Pozharny *et al.* (2010)	1st TM Term	no
PHLDA2	Pleckstrin homology-like domain	11p15.5	Pozharny *et al.* (2010) Apostolidou *et al.* (2007), Monk *et al.* (2006), Diplas *et al.* (2009)	1st TM Term	no
SLC22A18	Solute carrier family 22, member 18	11p15.5	Pozharny *et al.* (2010) Monk *et al.* (2006), Diplas *et al.* (2009), Pozharny *et al.* (2010)	1st TM Term	no
SLC22A18AS/SLC22AILS	Solute carrier family 22 (organic action transporter), member 18 antisense	11p15.5	Monk *et al.* (2006)	Term	no
TP73	Tumour protein p73	1p36.3	Pozharny *et al.* (2010) Lambertini *et al.* (2008) Pozharny *et al.* (2010)	1st TM Term	no

TM - trimester

1.5.4 Methods to Identify DNA Methylation

At the time of planning of this thesis project, techniques to study DNA methylation were at the early stages of development. The available techniques either allowed a single CpG or a sequence of DNA to be queried. To differentiate between methylated and unmethylated cytosines residues, bisulfite treatment of genomic DNA is commonly used. Sodium bisulfite converts unmethylated cytosines to uracils but not methyl-cytosines (Frommer et al. 1992). There are commercially-available kits that provide an efficient conversion, such as those provided by Zymo Research (Orange, CA).

Bisulfite sequencing of DNA (direct or by cloning)

In bisulfite sequencing, genomic DNA is treated by bisulfite conversion (Zhang et al. 2009). A known region is then amplified by PCR. The products are either sequenced directly or cloned and then sequenced. The dideoxynucleotide DNA method is used in sequencing (Zhang et al. 2009)(Laird 2010){{400 Laird,P.W. 2010}}. In cloning, PCR products are ligated into vectors, transformed into bacteria, and plated to produce colonies (Zhang et al. 2009). Cloning allows individual strands of DNA to be sequenced, and can also be useful when only small amounts of DNA are available (Zhang et al. 2009). However, it is a time consuming and expensive method that would not be suitable for high-throughput analyses.

Methylation-sensitive restriction enzyme digest

This method allows for a single CpG site that also serves as the cleavage site for methylation-sensitive restriction enzymes (Shames et al. 2007). These enzymes such as *HpaII*

will cleave at the CpG site only when it is methylated. Amplification of this region will then allow a quantitative assay to measure the level of methylated to unmethylated cytosines (Shames et al. 2007). This is done by comparing digested to undigested samples of DNA. A variant of this method, using bisulfite conversion based PCR with restriction digest has been used in bisulfite sequencing (COBRA; combined bisulfite restriction analysis) (Shames et al. 2007).

Methylation-sensitive single-nucleotide primer extension (MS-SNuPE)

MS-SNuPE is a variation of bisulfite sequencing that allows one CpG site to be queried at a time. This method was adapted from sequencing of single nucleotide polymorphisms (SNPs). Upon PCR amplification of bisulfite converted DNA, bisulfite specific primers designed for the CpG of interest are used in a dideoxynucleotide sequencing method whereby the polymerase only extends the primer by one nucleotide (C or T) (Sievers et al., 2007). The ratio of C to T is then measured to determine the level of DNA methylation (Sievers et al., 2007). MS-SNuPE has been shown to be a reliable and accurate technique (Sievers et al., 2007). Although multiple CpGs have to be queried in separate reactions, it is more cost-effective and less time consuming than traditional bisulfite sequencing. MS-SNuPE was used in the experiments of this thesis.

Pyrosequencing

Pyrosequencing is a recently developed method of bisulfite sequencing. It allows for high-throughput and cost-effective analyses of DNA methylation (Shames et al. 2007). Its uniqueness lies in the detection of pyrophosphate release during nucleotide incorporation, rather

than sequencing by dideoxynucleotide termination (Ahmadian et al. 2006). Thus, a region of interest is sequenced by synthesis from the starting nucleotide to the end nucleotide.

Genome-wide methylation assays

Microarray-based methods have also been recently developed to analyze bisulfite treated DNA in a genome-wide application. Bisulfite-specific oligonucleotide pairs targeting CpG sites of interest are designed from regions of interest across the genome and spotted onto microarrays. Various commercial companies have developed micro-array based assays for DNA methylation, including the Illumina Methylation Assay (Laird 2010). Microarrays can also be custom designed for specific regions of interests, such as a panel of imprinted genes. When combined with single-gene or region assays, this method can be useful for identifying candidate genes (Shames et al. 2007).

1.6 Infertility

Infertility affects approximately one in every six couples. The causes of infertility can be multi-factorial, affecting the male and /or the female partner. Both male and female infertility can be due to different factors that ultimately result in defective gamete production or function. In the female, hindrances in fertilization or implantation can result in infertility. The initial treatments for infertility involved methods such as the aspiration of sperm into the fallopian tubes, and hormonal stimulation to stimulate oocyte maturation.

1.6.1 Male Infertility, and Causes

The diagnosis in male infertility involves an assessment of medical history, a physical exam of the genital system, and genetic and hormonal tests. Additionally, a semen analysis is most often used in diagnosis. This includes the measurements of sperm concentration, motility, and morphology. Guidelines for determining optimal semen parameters have been established by the World Health Organization (WHO). Low sperm count, or oligozoospermia, is defined as a sperm concentration of less than 20 x 10^6 / mL of semen (World Health Organization 1999). Low motility, or asthenozoospermia, is defined as the presence of motile sperm in less than 50% of the semen sample (World Health Organization 1999). Azoospermia is defined as the absence of sperm in the semen, and is categorized into non-obstructive azoospermia (NOA) and obstructive azoospermia (OA). The cause of male infertility can be multi-factorial. This includes suboptimal testicular environment that affects spermatogenesis, defects in the genital system such as vas deferens obstruction, a inadequate support of the testicular environment such as endocrine deficiency, lifestyle or sexual dysfunction, exposure to environmental toxins, and idiopathic causes.

However, azoospermia and endocrine deficiencies are one of the rarest causes of male infertility. Azoospermia can be due to hypothalamic-pituitary failure, spermatogenesis failure, or obstruction. NOA is the most uncommon result of infertility, and can be due to cryptorchidism, Klinefelter syndrome (47, XXY), Y chromosome deletions, or radiation treatment (Hirsh 2003). About 15-30% of men with NOA have a sex chromosome aneuploidy or Y chromosome deletions (Hirsh 2003). However, most NOA cases are idiopathic (Hirsh 2003). Men with OA usually have normal spermatogenesis and the testicular obstruction can be due to absence of the vas deferens, epididymal obstruction after contracting chlamydia or gonorrhoea, and a

vasectomy procedure (Hirsh 2003). Treatment for OA can involve surgical reconstruction or testicular biopsy to retrieve sperm for assisted conception. Endocrine insufficiency, such as hypogonadotrophic hypogonadism can usually be treated by administration of gonadotrophin releasing hormones, in which fertility is restored by one year's time (Hirsh 2003).

Most causes of infertility are likely to result from an indirect genetic component (Shah et al. 2003). However, specific genetic abnormalities are also associated with infertility, which include mutations at the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene, *AR* (androgen receptor) gene mutations, Y chromosome deletions, and numerical and structural chromosome abnormalities. The Y chromosome contains the essential genes for spermatogenesis that are located at Yq11 and described as three non-overlapping regions called the azoospermic factors (AZFa, b, and c) (Vogt et al. 1996). The effect on infertility depends on the degree of deletion in the AZF regions, ranging from oligospermia to azoospermia. In addition to specific gene defects, the occurrence of aneuploidy in the sperm of infertile men with suboptimal semen parameters has been shown to be significantly greater than those of fertile controls (Ferguson et al. 2007, Kirkpatrick et al. 2008, Machev et al. 2005, Miharu 2005, Rives 2005, Shi and Martin 2001, Vidal et al. 2001, Wong et al. 2008). Aneuploidy is described as the loss or gain of a whole chromosome, such as trisomy or monosomy, respectively. In men with teratozoospermia, higher rates of sperm aneuploidy were observed (Machev et al. 2005, Tang et al. 2010). Sperm aneuploidy was also more prevalent in men with severe infertility, such as oligoasthenoteratozoospermia (Kirkpatrick et al. 2008). Men with abnormal karyotypes have also been reported to produce sperm with higher rates of sperm aneuploidy (Kirkpatrick et al. 2008, Shi and Martin 2001). Abnormal meiotic recombination in infertile men has been shown to be associated with sperm aneuploidy, as it has been demonstrated that absence of sex chromosome recombination is associated with higher rates of sex chromosome disomy in sperm

(Ferguson et al. 2007). Fertilization of an egg by an aneuploid sperm would most likely lead to an aneuploid embryo, which would greatly impact normal development. For example, most trisomic embryos do not survive to term, and thus end in implantation failure or spontaneous abortions (Jacobs and Hassold 1995).

1.6.2 Female Infertility, and Causes

The most common causes of female infertility are tubal and uterine conditions, and age-related decline in oocyte quality and population. The ultimate effect of these conditions is either disrupted ovulation or failure to sustain a pregnancy. Tubal conditions include blockage and adhesions in the fallopian tubes that interfere with oocyte transport or access by sperm, which account for 35% of infertility cases (Adamson and Baker 2003). Some common conditions that cause tubal factor infertility are sexually transmitted infections, surgical intervention for ectopic pregnancy, or other intra-abdominal conditions (Adamson and Baker 2003). A healthy uterine cavity is important for embryo implantation and sustenance of pregnancy, thus uterine conditions such as adhesions, polyps, fibroids, and abnormal anatomy can lead to infertility. In addition, the uterine lining undergoes proliferation and shedding during the proliferative and luteal phases of the menstrual cycle. These changes are brought on by responses to progesterone and estrogen. Thus, when the uterine lining does not receive adequate endocrine support, or it is not responsive to estrogen or progesterone, it can lead to infertility (Adamson and Baker 2003). Outside of the uterine cavity, endometriosis is a commonly associated with infertility (Adamson and Baker 2003). It is characterized by the presence of endometrial-like cells outside of the uterus that behave like the uterine endometrium and respond to hormonal changes in the menstrual cycle. As a result, endometriosis can lead to lesions and adhesions that can affect tubal and ovulatory function (Adamson and Baker 2003).

Aside from conditions associated with infertility, the effect of age on fertility decline has been studied extensively. A woman's ovarian reserve is characterized by the number of ovarian follicles and the quality of the oocytes. Depletion of the ovarian reserve leads to decline in fertility, which is especially prevalent in women who are over 35 years of age (Dunson et al. 2002). This is also characterized in women with premature ovarian failure, in which the onset of menopause occurs before the age of 40. The percentage of women between the ages of 20-24 and 25-29 who experience infertility is about 6% and 9%, respectively (Menken et al. 1986). This frequency increases to 15%, 30% and over 64% in women between the ages of 30-34, 35-39, and >40, respectively (Menken et al. 1986). In insemination programs using donor sperm, pregnancy rates have been shown to decline with increasing age (Schwartz and Mayaux 1982). However, the most significant effect of increasing maternal age is early pregnancy loss. Older women have a greater risk of having a spontaneous abortion, which occurs at a rate of 15%, 29% and 43% in women <35, at 40, and at 42 years of age, respectively (Speroff 1994). Abnormalities in the oocyte including defective meiotic spindle arrangement and composition, and aneuploidy have been associated with increasing age (Angell 1994, Benadiva et al. 1996). In oocytes obtained for in-vitro fertilization programs, the overall rate of aneuploidy is 39.5% in women between the ages of 40-44 which is significantly greater than in women <24 years old (8.5%) (Pellestor et al. 2003). Clinically, the risk of a conception with Down syndrome (trisomy 21) has also been shown to increase along with maternal age (Cuckle et al. 1987). This risk is about 1/1,300 when maternal age is 25 years, 1/365 at age 35, and increases exponentially to 1/30 at age 45 (Cuckle et al. 1987).

1.6.3 Unexplained Infertility

Approximately 25-30% of infertility cases are unexplained (Gleicher and Barad 2006). Although male and female infertility are well-defined, a specific diagnosis can be difficult and thus dependent on a physician's knowledge and experience. Endometriosis, tubal disease, premature ovarian failure, and immunological infertility are often misdiagnosed as unexplained infertility (Gleicher and Barad 2006). Furthermore, infertility may be multi-factorial and a treatment of one specific factor may not alleviate its signs. Infertility diagnosis usually involves multiple assessments of both partners, and treatments options such as assisted reproductive technology are decided based on the severity of each finding.

1.7 Assisted Reproductive Technology

The term, assisted reproductive technology (ART), refers to the use of reproductive technology to handle sperm or egg to treat infertility. ART consists of artificial insemination (or intrauterine insemination) or techniques that facilitate fertilization outside of the mother (in-vitro fertilization and intracytoplasmic sperm injection). In addition to treating infertility, ART is also used in fertile couples at high risk of transferring genetic or certain communicable diseases to their offspring. When prescribed fertility medications, which mainly involve stimulation of ovarian follicle development, do not result in pregnancy, ART become the most common treatment of infertility. Over two million babies have been conceived by ART since its initial use (International Committee for Monitoring Assisted Reproductive Technology et al. 2009). In the year 2002, over 196,000 babies were conceived worldwide, which is an increase of 12% compared to the year 2000 (International Committee for Monitoring Assisted Reproductive Technology et al. 2009).

1.7.1 In-vitro Fertilization (IVF)

In-vitro fertilization (IVF) is defined as the process in which an oocyte is fertilized by sperm outside the body. IVF was pioneered by Robert G. Edwards, the recipient of the 2010 Nobel Prize in Medicine. The first successful birth occurred in 1978, a female baby named Louise Brown. The fundamental processes of IVF treatment consist of hormone-mediated induction of oocyte maturation (ovarian stimulation), retrieval of mature oocytes prior to ovulation, and incubation of sperm and oocyte to facilitate fertilization. The fertilized oocyte is then cultured into the embryo and transferred to the uterus between three to five days after oocyte retrieval. Embryos that were cultured until the blastocyst stage have shown greater success of pregnancy than earlier stage embryos, especially when good quality embryos are available on the third day (Papanikolaou et al. 2005).

Specifically formulated media is used in each step of the in-vitro procedures. Aspects of the culture system, such as levels of oxygen and carbon dioxide and the type of incubation chambers, have been shown to affect the proper development of pre-implantation embryos (Lane and Gardner 2007). The components of culture media are dependent on the developmental stage of the embryo, and primarily include a source for energy, salt, amino acids and other supporting macromolecules (Lane and Gardner 2007). The levels of salts, glucose, amino acids, ammonium, EDTA, and macromolecules such as albumin and hyaluronan have beneficial or detrimental effects depending on the stage of embryo development and its physiological state (Lane and Gardner 2007). For example, in pre-compaction stage embryos (cleavage stage), the embryo has a lower ability to maintain cellular homeostasis and relies on pyruvate-based metabolism for energy (Lane and Gardner 2007). The addition of glucose to culture medium can lead to developmental arrest of cleavage stage embryos, especially in the absence of phosphate

and amino acids (Lane and Gardner 2007). Conversely, post-compaction embryos rely on glucose-based metabolism and have developed a transporting epithelium that allows for maintenance of cellular homeostasis (Lane and Gardner 2007). Thus, the addition of glucose in culture medium of post-compaction embryos has a beneficial effect. Due to the dynamic changes in the physiology of the embryo, commercial culture systems developed for use in IVF consist of monoculture and sequential culture media (Lane and Gardner 2007). In monoculture systems, only one specific medium formulation is used and can support development up to the blastocyst stage (Lane and Gardner 2007). However, the use of sequential culture media has resulted in significant increase in implantation rates during blastocyst transfer (Blake et al. 2004). Sequential media systems involve two different formulations, one used for support of embryo development until the cleave-stage, and the second used for extended culture to the blastocyst stage (Lane and Gardner 2007). Therefore, sequential culture media is recommended for use in extended embryo culture.

Ovarian stimulation involves a regiment of GnRH agonist for pituitary suppression and a high dose of exogenous FSH. In addition, GnRH antagonists, clomiphene citrate and aromatase inhibitors have been used. GnRH antagonists are used to prevent LH surge (Verberg et al. 2009). Clomiphene citrate inhibits the estrogen feedback loop to allow greater pituitary FSH secretion (Verberg et al. 2009). Aromatase inhibitors act to inhibit the conversion of androgens to estrogens to reduce estrogen feedback (Verberg et al. 2009). Throughout ovarian stimulation, follicular development is monitored by ultrasonography. Oocyte retrieval begins with administration of hCG, which acts as an analogue of LH. Approximately 10 to 30 follicles can be retrieved from one round of ovarian stimulation (Verberg et al. 2009).

1.7.2 Intracytoplasmic Sperm Injection (ICSI)

Although IVF became a successful method of infertility treatment, when sperm with suboptimal parameters were used fertilization rates were lower. This led to the development of ICSI, which involves the direct injection of a single sperm into the ooplasm (Palermo et al. 1992). Prior to the ICSI procedure, conventional methods used in IVF to obtain mature oocytes from the female partner. Conventional embryo culturing methods were also maintained once ICSI is performed. With the development of ICSI, only a single viable sperm is needed to fertilize one oocyte. Fertilization rates achieved by ICSI were also similar to IVF, and were greatly enhanced in cases where infertility was either due to the male, unexplained, or infertility due to repeated IVF failure (Lewis and Klonoff-Cohen 2005). In patients that have dysfunction in spermatogenesis, ICSI can be a successful treatment for infertility if sperm can be retrieved from the testicular environment.

1.7.3 Perinatal Health Outcomes of Children Conceived by ART (IVF and ICSI)

Since its use, numerous studies have been published on the health outcomes of pregnancies conceived by ART as compared to those of natural conception. In these studies, the majority reported higher incidences of twins, preterm birth, low birth weight, birth defects and malformations, and other adverse health outcomes. In contrast, there are also studies that did not observe differences in birth defects between IVF/ ICSI pregnancies and natural conceptions. Differences in these studies can be confounded by data collection methods such as differences in selection criteria and controlling for maternal age, and data analysis methods. In addition to individual studies, various meta-analyses have been published to extrapolate these studies to address overall health risks of ART.

McDonald *et al.* conducted a systematic review and meta-analyses of the literature reports on preterm birth and low birth weight among singletons from IVF or ICSI pregnancies (McDonald et al. 2009). The authors used inclusion criteria for case-control and cohort studies that had matching maternal age or adjustment for maternal age, and excluded studies that included multiple pregnancies or selective reduction pregnancies. From a review of 361 reports, 17 met their inclusion criteria, which in total compared 31,032 IVF and ICSI singletons to 81,118 naturally conceived singletons. Compared to natural conception singleton pregnancies, IVF/ ICSI singletons had increased risks in pre-term birth (<37 weeks, RR 1.84, 95% CI 1.54-2.21) and low birth weight (RR 1.60, 95% CI 1.29-1.98) (McDonald et al. 2009). The risk of IUGR was also significantly higher among IVF/ ICSI singletons (RR 1.45, 95% CI 1.04-2.00) (McDonald et al. 2009). Overall, singletons conceived by IVF/ICSI had lower birth weights (-97 g, 95% CI -161 to -24 g) and shorter gestational periods (-0.6 weeks, 95% CI -0.9 to -.04 weeks) (McDonald et al. 2009). Other meta-analyses have also shown that IVF and ICSI pregnancies have greater risks of small for gestational age and caesarean delivery (summarized in Table 1.4) (Helmerhorst et al. 2004, Jackson et al. 2004, McGovern et al. 2004).

The rate of twining in IVF and ICSI pregnancies is 26.9% and 26.2%, respectively (World collaborative Report on ART, 2000). These rates are approximately 10-fold higher than observed in the heterogeneous American general population, which were between 1.9% and 2.9% between 1980 and 1999 (Martin et al. 2010). However, recent population data have shown a rise of 2% per year in the twinning rate between 1990 and 2004, which is most likely due to the increasing use of ART (Martin et al. 2010). In ART twin pregnancies, reports of associations with preterm birth, low birth weight, malformation, and perinatal death showed mixed results (summarized in Table 1.5). Helmerhorst *et al.* demonstrated no relative risks of pre-term birth (RR 0.89, 95% CI 0.74-1.07) and low birth weight (RR 1.27, 95% CI 0.97-1.65), but

significantly greater risk of small for gestational age (RR 1.21, 95% CI 1.11-1.32) compared to twins from natural conception (Helmerhorst et al. 2004). In a separate meta-analysis by McDonald *et al.*, which included 12 reviewed studies with matched controls, IVF / ICSI twins had significantly increased risks for pre-term birth (RR 1.63, 95% CI 1.09-1.41), low birth weight (RR 1.14. 95% CI 1.06-122) (McDonald et al. 2010). However, risks of intrauterine growth restriction, and difference in duration of gestation were not significantly greater compared to twins from natural conception (McDonald et al. 2010). In general, health risks in ART twins when compared to naturally conceived twins are not as severe as in singleton pregnancies. This smaller difference is most likely due to the fact that twins in general are at greater risks of adverse health outcomes than singletons.

Despite the increased perinatal risks in ART pregnancies, the majority of infants conceived by ART are healthy. In ICSI-conceived children up to 1.4 years of age, physical health, including congenital abnormalities, and mental development were not different from matched children conceived naturally (Sutcliffe et al. 2003). In both ICSI and IVF children at one year of age, similar levels of mental development were observed when compared to naturally-conceived children (Bowen et al. 1998). In comparing IVF-born with ICSI-born children, no differences were observed in psychomotor development at 2 years of age (Bonduelle et al. 2003). In ICSI and IVF singletons that were not born preterm differences in congenital malformations, adverse health outcomes, growth, and medical consumption were not observed when compared to each other, and to natural conception children between 5 and 8 years of age (Knoester et al. 2008). However, Bonduelle *et al.* have reported that ICSI and IVF children are more likely to have had a significant childhood illness, surgical intervention, and hospital admission within the first five years after birth (Bonduelle et al. 2005). Although ART

pregnancies are at greater risks of poor perinatal health outcomes, the limited data have shown that these infants experience catch-up in physical development and normal neurodevelopment.

Table 1.4 Perinatal outcomes of IVF/ICSI singletons.

IVF/ ICSI Singletons				
Reference	Helmerhorst et al. 2004	Jackson et al. 2004	McGovern et al. 2004	McDonald et al. 2009
# of studies	14	15	27	17
Preterm birth (<37w)	RR 2.04 (1.89-2.32)	OR 1.95 (1.73-2.20)	RR 1.98 (1.77-2.22)	RR 1.84 (1.54-2.21)
Very preterm birth (<32w)	RR 3.27 (2.03-5.28)		RR 2.49 (0.86-7.21)	RR 2.27 (1.73-2.97)
Low birth weight (<2500g)	RR 1.40 (1.15-1.71)	OR 1.77 (1.40-2.22)	-	RR 1.60 (1.29-1.98)
Very low birth weight (<1500g)	RR 1.70 (1.50-1.92)	OR 2.70 (2.31-3.14)	-	RR 2.65 (1.83-3.84)
Small for gestational age	RR 1.54 (1.44-1.66)	OR 1.60 (1.25-2.04)	-	-
IUGR	-	-	-	RR 1.45 (1.04-2.00)
C Section	RR 1.27 (1.16-1.40)	-	-	-

RR = relative risk; OR = odds ratio

Table 1.5 Perinatal outcomes of IVF/ICSI twins

IVF/ ICSI Twins		
Reference	Helmerhorst et al. 2004	McDonald et al. 2010
# of studies	10	12
Preterm birth (<37w)	RR 1.07 (0.99-1.08)	RR 1.23 (1.09-1.41)
Very preterm birth (<32w)	RR 0.95 (0.78-1.15)	RR 1.63 (1.17-2.27)
Low birth weight (<2500g)	RR 1.03 (0.99-1.08)	RR 1.14 (1.06-1.22)
Very low birth weight (<1500g)	RR 0.89 (0.74-1.07)	RR 1.28 (0.73-2.24)
Small for gestational age	RR 1.27 (0.97-1.65)	-
IUGR	-	RR 1.06 (0.72-1.55)
C-section	RR 1.21 (1.11-1.32)	-

RR = relative risk

1.7.4 Low Birth Weight in ART Pregnancies

As discussed previously, ART is associated with higher incidences of low birth weight (LBW), SGA, and IUGR, especially in singleton pregnancies. As LBW is associated with various adult-onset diseases that were previously mentioned, it has become an important topic of research. The aetiology of the greater prevalence of LBW in ART pregnancies largely remains unknown.

Research has demonstrated that low birth weight babies are at a higher risk of congenital heart defects (CHD), stroke, type II diabetes mellitus, and hypertension during their adult lives (Barker 1995, Osmond et al. 1993). Death rates from CHD increase when birth weight decreases in both men and women (Barker 1995). This remains evident when gestational age is taken into account (Barker et al. 1993). Children born with low birth weight are more susceptible to obesity and diabetes due to reduced numbers of pancreatic beta cells, which are responsible for insulin production (Barker 1995). Regardless of other social behaviours that have been associated with adult diseases, such as smoking and drug use, these associations of low birth weight and adult diseases remain highly statistically significant (Barker 1995). At over nine weeks of pregnancy, the fetus undergoes rapid growth and organ function maturation that are defined by critical periods of growth (Barker 1995). For example, the kidney's critical growth period occurs in the weeks prior to birth (Barker 1995). Fetal growth largely depends on oxygen and nutrients, that when limited in supply during pregnancy cell division slows down resulting in growth deficiency (Barker 1995).

Other adverse health conditions that have been associated with LBW include cerebral palsy, vision and hearing impairment, and increased rates of hospitalization (Escobar et al. 1991, Hack et al. 1995, McCormick et al. 1993). In addition to health outcomes, LBW children experience greater difficulties in school, and have increased developmental learning disabilities and have lower academic performance (Avchen et al. 2001, Hediger et al. 2002, Pinto-Martin et al. 2004, Resnick et al. 1999). The higher prevalence of low birth weight, especially among singleton births, in pregnancies conceived by ART is a cause of concern as research has shown an association with adult onset diseases and developmental difficulties.

1.7.5 Risks of Chromosome Abnormalities in ART Pregnancies

As discussed previously in chapter 1.4, infertility can be due to specific or unknown genetic abnormalities. As ART bypasses physiological barriers in natural conception, it has been suggested that genetic or chromosomal abnormalities present in one or both parents can be passed on to the child. The frequency of prenatal diagnoses in ART pregnancies is between 7% and 47% (Gjerris et al. 2008). The indications for prenatal diagnoses in ART pregnancies include advanced maternal age, and concerns over chromosome abnormalities associated with infertility (Gjerris et al. 2008). The reported rates of abnormal karyotypes identified by prenatal diagnoses in ICSI pregnancies are between 2.7% and 12.7%, while IVF pregnancies is at 1.9% (Gjerris et al. 2008). Chromosome abnormalities are detected more frequently in ICSI pregnancies than IVF pregnancies in both prenatal screens and postnatal testing (Gjerris et al. 2008, Lathi and Milki 2004). However, studies of first-trimester abortuses have also shown similar rates of chromosome abnormalities between IVF and ICSI, and that they were not different from natural conceptions (Kushnir and Frattarelli 2009, Ma et al. 2006). The rate of chromosome aneuploidy in first trimester abortions in both IVF and ICSI pregnancies increases

with maternal age (Kushnir and Frattarelli 2009). Autosomal aneuploidy account for up to 4.2% of prenatal diagnoses, while sex chromosome aneuploidy and autosomal structural abnormalities account for up to 7.0% and 1.8% respectively (Gjerris et al. 2008). The overall rate of abnormal karyotypes in singletons is significantly higher than twins (0.8% versus 0.5%) (Gjerris et al. 2008). However, the rate of trisomy 21 (Down syndrome) is similar in singleton and twin pregnancies (0.3% and 0.2%, respectively) (Gjerris et al. 2008).

In comparison to naturally conceived pregnancies, chromosome abnormalities in ICSI pregnancies are significantly more prevalent (Allen et al. 2006). De novo and inherited autosomal abnormalities were most frequently detected in ICSI pregnancies (1.6 % and 1.4%, respectively) (Bonduelle et al. 2002). The particular association of ICSI pregnancies with chromosome abnormalities can be attributed to increased levels of sperm chromosome abnormalities in infertile men that are either chromosomally normal or abnormal (Bonduelle et al. 2002, Ferguson et al. 2007, Kirkpatrick et al. 2008, Tang et al. 2004, Tang et al. 2010, Wong et al. 2008). In abnormal cases, paternal origins of the abnormalities have been shown, which suggests that ICSI allows paternal transmission to the fetus (Bartels et al. 1998, Ma et al. 2003, Ma et al. 2003, Tang et al. 2004, Van Opstal et al. 1997). Studies of chromosome abnormalities resulting from IVF pregnancies are less common. The incidence of chromosome abnormalities in IVF pregnancies is not significantly greater than that of natural conceptions (0.7% vs. 1.0%) (Allen et al. 2006).

1.7.6 Imprinting Abnormalities in ART Pregnancies

There is a greater representation of imprinting disorders in the ICSI-conceived population compared to the general population, specifically in Beckwith Wiedemann Syndrome (BWS) and

Angelman Syndrome (AS) (Allen and Reardon 2005). BWS is a fetal overgrowth disorder characterised by a birth weight that is large for gestational age (Maher 2005). In 60% of cases, BWS is due to epigenetic changes by DNA methylation in the chromosome region 11p15.5 (Maher 2005). The rest of BWS cases are due to deletion, mutation, or duplication of the same region, or paternal uniparental disomy of chromosome 11 (Maher 2005). AS is characterized by severe developmental delay and an excitable personality in the affected child (Maher 2005). The majority of AS cases are due to deletion involving the maternal chromosome region 15q11-13 (Maher 2005). In the majority of the reported cases of BWS conceived by IVF and ICSI, DNA hypomethylation (14/19 cases) were the cause. In all of the three reported cases of AS conceived by ICSI, epigenetic defects were the cause (Allen and Reardon 2005). Although the absolute number of reported cases of imprinting abnormalities in ART pregnancies is low, it has raised concerns about the effect of ART or infertility on proper genomic imprinting.

1.7.7 Causes of Adverse Perinatal Outcomes in ART Pregnancies

The increased risk of perinatal adverse outcomes as compared to natural conceptions is concerning. The possible explanations for these risks include infertility and the couple`s medical history, procedures involved in ART, undetected vanishing twins, higher emphasis on monitoring ART pregnancies and higher tendency from physicians to conduct interventions, or a combination of these factors. The majority of the published studies have not controlled for these factors. In one study, in which infertility was controlled for, a higher risk of poor perinatal health outcomes was found (Bergh et al. 1999). In ART procedures, studies in animal models and human cells have been performed, including the effects of ovarian stimulation and embryo culturing. It has been suggested that ovarian stimulation may account for pre-term births, as it causes an increase in circulating relaxin (Johnson et al. 1991, Kristiansson et al. 1996, Weiss et

al. 1993). It is suggested that an increase in relaxin can affect maintenance of the cervical connective tissue, which can lead to a vulnerable pregnancy (Goldsmith and Weiss 2009). In comparison of 162 IVF and 263 patients that were only treated with ovarian stimulation, no differences in preterm birth rates were observed (Olivennes et al. 1992). The higher rate of preterm birth may also account for the higher risks of LBW, but not for the higher risk of SGA. Thus, it has been suggested that ovarian stimulation may be the primary cause of adverse perinatal outcomes (Ombelet et al. 2006).

1.7.7.1 Aneuploidy in ART-conceived Embryos

ART has also been shown to be associated with high frequencies of embryo aneuploidy (Baart et al. 2006, Munne and Cohen 1998, Wells and Delhanty 2000). In day 3 embryos, the level of aneuploidy appears to be associated with ovarian stimulation (Baart et al. 2006). In milder stimulation conditions, the level of aneuploidy is less severe when compared to conventional stimulation conditions (Baart et al. 2007). The clinical significance of aneuploid embryos is unknown, and it is unclear whether they would lead to a viable pregnancy if used in ART. It is likely that the level of aneuploidy, the chromosome involved, and which progenitor cells are affected would determine an embryo's viability and pregnancy outcome. One possible explanation for the higher incidence of fetal-growth related outcomes, such as low birth weight, small for gestational age, and intrauterine growth restriction, may be due to aneuploidy that persists in the extra-embryonic cells that form the majority of the placenta. This pathology is described as confined placental mosaicism (CPM), which has been associated with intrauterine growth restriction (Lestou and Kalousek 1998, Wilkins-Haug et al. 2006). In ART pregnancies, the prevalence of CPM remains unclear as only few studies have been conducted. Jacod *et al.* investigated CPM in IVF and ICSI pregnancies through retrospective analysis of karyotype

results from CVS (Jacod et al. 2008). No significance was detected in the rate of CPM between 235 IVF/ICSI pregnancies and 20,650 natural conceptions (Jacod et al. 2008). In contrast, In't Veld *et al.* have previously reported a significantly higher rate of CPM in 80 IVF pregnancies diagnosed by CVS (6.2%) (In't Veld et al. 1995). However, both of these studies consisted of retrospective analyses and term placentas were not analyzed to determine the rate of false positive CVS results. Minor *et al.* analyzed term placentas from ICSI pregnancies and also did not show a significant difference in the rate of CPM when compared to published reports of the general population (Minor et al. 2006). Nonetheless, given the present review of the literature, infertile couples who carry a higher risk of having aneuploid gametes would also have a higher risk of an aneuploid conception, which may account for the increased prevalence of adverse perinatal outcomes in pregnancies conceived by ART.

1.7.7.2 Evidence for Disruption of Normal Genomic Imprinting

As described previously, imprinting abnormalities in ART pregnancies have also raised the concern about the effects of in-vitro or infertility itself in altering genomic imprinting. The majority of imprinted genes are implicated in embryonic development and fetal growth. Thus, it is also conceivable that imprinting abnormalities as a result of ART or infertility can account for the greater adverse perinatal risks. It has been suggested that hormonal stimulation and in-vitro manipulation of oocytes can introduce imprinting abnormalities. In the mouse model, in-vitro manipulation of embryo culturing conditions and superovulation can introduce aberrant DNA methylation at imprinted genes (Doherty et al. 2000). In embryos cultured in Whitten's medium from the 2-cell stage to the blastocyst, aberrant expression of the normally silent paternal allele of *H19* was detected (Doherty et al. 2000). These embryos also showed a loss of methylation at the *H19* imprinting control region was determined to be the cause (Doherty et al. 2000). In

embryos cultured in a different media, KSOM+AA, aberrant methylation and expression were not detected (Doherty et al. 2000). It was also determined that this effect is not due to alterations in imprinting maintenance mechanisms, and may be restricted to the *H19* ICR (Doherty et al. 2000). In another study of mouse embryos, the growth related imprinted genes *H19, Igf2, Grb10 and Grb7* were expressed at significantly different levels when culture media composition was altered (Khosla et al. 2001). In embryos cultured in M16 medium supplemented with fetal calf serum (M16+FCS), *H19, Igf2, and Grb7* expression was significantly decreased and *Grb10* was significantly increased when compared to the control, which did not involve in-vitro culturing (Khosla et al. 2001). Conversely, no significant changes in expression of these genes were observed in embryos cultured in M16 medium alone (Khosla et al. 2001). In effect, embryos from M16+FCS were less viable and the pups were born with significantly reduced weight when independently compared to control and M16 pups (Khosla et al. 2001). It has also been reported that different media composition can also affect embryo cell cycle kinetics in addition to methylation at *H19* (Fauque et al. 2007). Fauque *et al.* also reported that ovarian stimulation disrupted *H19* expression in blastocysts, and the level of disruption was greater in G1.2/G2.2 medium than M16 medium (Fauque et al. 2007). In comparing *in vitro* to *in vivo* conceived mouse embryos, altered imprinting at the *H19* imprinting control region and an increase of paternal histone H3K4 dimethylation and maternal H3K9 trimethylation were observed (Li et al. 2005). Overall, the evidence from studies on mouse embryos and oocytes has demonstrated that certain imprinted genes are vulnerable to aberrant epigenetic changes when exposed hormonal stimulation and *in vitro* culturing.

In human oocytes and embryos, the effect of ovarian stimulation and *in vitro* culturing are unknown. In ovarian stimulated immature human oocytes, hypermethylation of *H19* and hypomethylation of *PEG1* have been observed (Sato et al. 2007). However, the epigenetic status

of mature oocytes was not examined. The immature oocytes may not have acquired maternal-specific methylation patterns. MI-arrested oocytes have been shown to have altered patterns of methylation; while in the majority of MII oocytes, normal patterns of methylation at the *H19* ICR was observed (Borghol et al. 2006). Nonetheless, further study is required to determine whether human oocytes and embryos experience similar vulnerabilities to *in vivo* and *in vitro* procedures involved in ART.

Studies on sperm from infertile men with suboptimal semen parameters have found an association with aberrant DNA methylation at imprinted genes (Boissonnas et al. 2010, Kobayashi et al. 2007, Kobayashi et al. 2009, Marques et al. 2008, Marques et al. 2008, Marques et al. 2010, Marques et al. 2010). Compared to men with normal semen parameters, reduced methylation at the 6th CTCF binding site of the *H19* differentially-methylated region (ICR1) and/or at the *IGF2* DMR2 was found in the sperm of infertile men with teratozoospermia (Boissonnas et al. 2010). The majority of men with OAT were also found to have hypomethylation at the *H19* DMR, which was also correlated with sperm concentration (Boissonnas et al. 2010). In infertile men with moderate or severe oligozoospermia, aberrant DNA methylation at paternally and maternally imprinted genes were more frequent than in men with normal semen parameters (Kobayashi et al. 2007). The genes affected include *H19, GTL2, PEG1, LIT1, ZAC, PEG3, and SNRPN* (Kobayashi et al. 2007). Similarly, Marques *et al.* have also demonstrated the presence of aberrant DNA methylation at *H19* and/or *MEST* in 7 of 17 infertile men with oligozoospermia (Marques et al. 2008). Subsequently, Marques *et al.* have shown disruption in *H19* methylation in testicular sperm of men with abnormal spermatogenesis (Marques et al. 2010). The majority of the studies have found aberrant DNA methylation at the ICR of *H19*. Thus, imprinting of *IGF2*, a paternally expressed fetal growth factor that shares the same ICR may also be affected. In using sperm with aberrant imprinting at *H19* and IGF2 for

ART, there is a potentially adverse effect on fetal growth and development. However, the effect on pregnancy outcome by using aberrantly imprinted sperm is presently unknown. It has been suggested that studies on imprinting in ART-conceived children are needed to further examine these effects.

1.8 Objectives and Hypotheses

The overall objective of this thesis is to determine whether ART pregnancies are associated with placental abnormalities, and whether this association is prevalent in growth restricted babies. These effects may explain the underlying causes of the increased risk of adverse perinatal health outcomes in ART-conceived infants.

1.8.1 Specific Objectives*:*

Hypotheses:

1. CPM would be observed in IVF and ICSI pregnancies more frequently than in natural conceptions. Due to its association with growth restriction, CPM would be more prevalent in SGA pregnancies conceived by IVF and ICSI.

   As reviewed in Chapter 1, there is evidence to suggest that ART conceptions are at greater risk of chromosome abnormalities. In studies of pre-implantation embryos, there is a high prevalence of aneuploidy and mosaicism (Baart et al. 2006, Munne and Cohen 1998, Silber et al. 2003). This suggests that meiotic or post-zygotic abnormalities are remarkably frequent in early human development. Infertility may contribute to aneuploidy in the embryos derived from ART, as both oocytes and sperm have been linked to chromosome

abnormalities in infertility. Due to selection against aneuploid cells, aneuploidy may manifest as confined placental mosaicism. The presence of CPM has been suggested to affect placental physiology with a downstream effect on fetal growth. Thus, CPM may account for the greater prevalence of SGA in ART pregnancies.

2. Aberrant imprinting of *H19* and *IGF2* is more prevalent in IVF and ICSI conceptions than natural conceptions. Due to its role in fetal growth and placental development, aberrant imprinting of these two genes may account for SGA in IVF and ICSI pregnancies.

   H19 and *IGF2* are two imprinted genes involved in fetal growth and placental development. In chapter 1, it has been reviewed that procedures involved in ART, including ovarian stimulation and embryo culturing, and infertility itself may affect the DNA methylation at imprinted genes. The imprinting control region of *H19* and *IGF2* is the most frequently affected in mouse embryos, human oocytes, and sperm from infertile men.

Objective 1: To investigate the role of confined placental mosaicism in pregnancies conceived by IVF and ICSI and its association with SGA.

Objective 2: To investigate the status of DNA methylation at the imprinting control region of *H19* and *IGF2* in the placenta from pregnancies conceived by IVF and ICSI.

Objective 2b: To investigate the prevalence of aberrant imprinting at *H19* and *IGF2* in SGA pregnancies conceived by IVF and ICSI.

2. Objective 1: CPM in ART Pregnancies

In this chapter, the methodology and results of Objective 1 are presented. The presence of confined placental mosaicism was investigated by comparative genomic hybridization and flow cytometry for the analysis of polyploidy.

2.1 Methodology

2.1.1 Patient Ascertainment

For objective 1, patients that have conceived by IVF or ICSI were recruited from seven IVF centres across Canada. The majority of the study participants (over 80%) were recruited from the UBC Centre for Reproductive Health, in Vancouver, Canada. Informed consent was received from each participant prior to sample collection. The placenta and a sample of umbilical cord blood (2 x 5mL) were collected at birth. The placenta was sampled for chorionic villi at three sites: one at the site of cord insertion, one at the periphery of the placenta, and the other at a site away from the first two. These sites were chosen to represent the entire placenta. The sampled tissues were then stored and frozen at -20°C until use. The sample of umbilical cord blood, collected in heparinized vacutainers, was freshly prepared for standard lymphocyte culturing to obtain cells at metaphase.

2.1.2 Definition of Small for Gestational Age (SGA)

In this study, we used SGA as an indicator of IUGR. SGA has been used to define IUGR. SGA is defined as a birth weight below the 10^{th} percentile for a given gestational age (Greer 1998). This was determined for each subject using a reference chart from the Canadian Perinatal Surveillance System (Kramer et al. 2001). Each birth weight was converted to a Z-

score: (patient value – expected mean)/ population SD. The Z-scores were used to determine SGA (Z-score < -2.0) or appropriate for gestational age (AGA; Z-score ≥ -2.0).

2.1.3 DNA Extraction

DNA was extracted from each placental site. A ~1cm^3 sample of each site was subjected to DNA extraction using standard salt-out methods (Minor et al. 2006). Each sample was washed in PBS several times to remove blood from the tissue. 50 µL of proteinase K (5mg/mL) (Invitrogen, Carlsbad, CA) was added to the sample immersed in tissue lysis buffer (100mM Tris; 40mM EDTA; 500mM Nail) and 300 µL of 10% sodium dodecyl sulphate (SDS). The tissue digestion and cell lysis were carried out by incubation overnight at 55°C. 6M NaCl was then added at 1/3 volume of the lysis mixture and mixed vigorously to precipitate proteins. Centrifugation was carried out at 4000 rpm at 4°C for 45 minutes to separate the precipitate. The supernatant was then transferred to a 15mL tube containing cold 100% ethanol to precipitate DNA. Once aggregated at the top of the tube, the DNA was transferred to a micro centrifuge tube and washed in 70% ethanol (1mL). The DNA was then pelleted by centrifugation and air-dried. TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) was used to reconstitute the DNA to appropriate concentration. UV spectrophotometer was used to determine DNA concentration and purity.

2.1.4 G-banding of Metaphase Cells from Umbilical Cord Blood

Umbilical cord blood was cultured using a standard 72-hour protocol for lymphocytes. . In each culturing flask, 8.5mL of RPMI 1640 media (Invitrogen) 1.7mL of fetal bovine serum (Invitrogen), 0.25mL of phytohemagglutinin (PHA) (Invitrogen), 0.025mL of sodium heparin (Sigma-Aldrich), 0.083mL of penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO), and

0.5mL of fresh whole blood. The cultures were then incubated at 37°C for 3 days. At 18 hours before harvest, 0.1mL of 4μM 5-fluoro-deoxy-uridine (FUdR) was added. At 4 hours before harvest, 0.2mL of thymidine ($2.4x10^{-4}$g/mL) was added. Finally, at 30 minutes before harvest, 0.05mL of colcemid (10mg/mL) was added. Harvesting involved treating the cells in a 0.06M KCl solution for up to 12 minutes at 37°C , and fixation using a 3:1 methanol:glacial acetic acid mixture. Fixated cells were spread onto microscope slides and allowed to age for up to 1 week at room temperature. The slide was then G-banded using trypsin digestion and staining solution of Giemsa and Leishman's stains. Metaphase spreads were captured using a Zeiss microscope coupled to a CCD camera and karyotyped using Cytovision (Applied Imaging International, Santa Clara, CA).

2.1.5 Comparative Genomic Hybridization (CGH) on Placental Tissue

Comparative genomic hybridization (CGH) was used to determine the presence of trisomy, partial duplication, or partial deletions in placental tissue. CGH was performed using a previously established protocol in the lab (Minor et al. 2006). 2μg of test DNA isolated from whole chorionic villi was labelled by nick translation, using dNTPs and fluorescein-12-dUTP (FITC) (Roche Diagnostics, Penzberg, Germany), 1.5μL of nick translation enzyme mix (Roche Diagnostics), and 0.8μL of DNA polymerase I (New England Biolabs, Ipswich, Germany). This was carried out at 15°C for 2h45m to obtain optimal fragment lengths of 600bp – 3kb. Reference DNA from a sample of normal genomic content (46, XX) was labelled with tetramethylrhodamine-5-dUTP (TRITC) (Roche Diagnostics). The size of the fragment lengths were checked by electrophoresis in a 2% agarose gel using a lambda-HindIII size standard. Equal amounts of test and fluorescent nick translated products, as estimated by band intensities, were co-precipitated with human 20μL of Cot-1 DNA (Invitrogen) using 200 μL of 100%

ethanol and 5µL of 4M LiCl overnight at -20°C . The precipitated DNA was then reconstituted in hybridization buffer (50% formamide/10% dextransulfate/ 2x sodium chloride sodium citrate (SSC)) and hybridized to a metaphase spread prepared with normal male 46,XY karyotype for three days in a humid chamber at 37°C . Prior to hybridization, the slides were pre-treated in RNAse (0.125mg/mL; Sigma-Aldrich, St. Louis, MO) in 2xSSC solution, fixed in 10% buffered formalin, and denatured in 70% formamide/2xSSC at 72°C for 5 minutes. Post hybridization, the slides were washed in 0.4xSSC/0.3%NP-40 at 72°C for 2 minutes, 2xSSC/0.1% NP-40 at room temperature for 1 minute, and counterstained with 4',6-Diamidine-2'-phenylindole (0.2 µg/mL; Sigma-Aldrich). Each slide was mounted with anti-fade solution (Vectashield, Vector Laboratories, Burlington, CA) and a coverslip. The slides were initially stabilized and stored at -20°C .

Standard protocols were used for capture and analysis for CGH. A Zeiss Axioplan epifluorescent microscope coupled with a CCD camera was used in conjunction with Cytovision capture and image analysis software (Applied Imaging International, Santa Clara, CA). Image capture and analysis was performed according to the software manufacturer's recommendations. Per metaphase, three fluorescent images were captured (DAPI, FITC, and TRITC). For each metaphase, hybridization quality was determined by meeting the threshold criteria for parameters such as dynamics and intensity of each fluorescent signal. Metaphases that did not pass each parameter were discarded for analysis. A CGH profile was then generated from 6-12 metaphases, and the gains or loss of DNA was determined by shifts in the ratio of FITC to TRITC fluorescence. A 1:1 ratio indicated a balance of genetic content, while a significant deviation from this ratio was determined using a 95% confidence interval using the analysis module built-into Cytovision.

2.1.6 Flow Cytometry

Flow cytometry was used to determine the ploidy level in the cells of each placental sample. This was performed using a previously established protocol with some modifications (Minor et al. 2006). Chorionic villi from identical sites used for CGH were separated into single-cell suspensions of trophoblast and stroma by enzymatic digestion. To obtain trophoblast cells, villi were digested in collagenase 1A (1mg/mL) (Sigma-Aldrich) at 37°C for 20 minutes and agitated periodically. The supernatant was collected, and the tissue was washed twice in 3mL of Hanks' Balanced Salt solution with calcium and magnesium (HBSS) (Invitrogen).

To obtain mesenchyme (stroma) cells, the remaining tissue was then acidified in 0.9% NaCl (pH 1.5) and digested with 0.5% pepsin (Sigma-Aldrich) for up to 15 minutes at 37°C while agitating periodically. Each cell suspension was passed through a 40μm cell strainer (Becton Dickson, Franklin Lakes, NJ) and fixed in pre-chilled 70% ethanol and stored overnight at 4°C. The cell suspension was then washed and re-suspended in pre-chilled PBS and the concentration was determined using a haemocytometer. Appropriate dilutions were made to obtain a concentration of 1 million cells per mL in a 500 μL preparation. The cells were stained in propidium iodide (PI) (40 μg/mL; Sigma-Aldrich) and pre-treated with RNAse A (20μg/mL; Sigma-Aldrich).

Each cell suspension was run through a FACS Calibur flow cytometer (Becton Dickson) at the immunology core facility at the Child and Family Research Institute (Vancouver, BC, Canada). Data acquisition and analysis was done using CellQuest (Becton Dickson) and FlowJo software (Tree Star, Ashland, OR) with the assistance of Dr. Lixin Xu. The instrument was calibrated using known diploid and tetraploid samples. A total of 20,000 cells were analyzed.

The level of ploidy was determined by plotting fluorescence area with cell count. Doublets and cell aggregates which are discriminated by plotting fluorescence area and width, were removed from the analysis. Ploidy was determined by using cell-cycle parameters described by Rua *et al.* The control diploid sample was used to calibrate the FACS whereby the G0/G1 peak was positioned at 200 in fluorescence area, and the G2/M peak at 400. The tetraploid control sample was then run, each time showing the G0/G1 peak at 400 and an octaploid peak representing G2/M phases of tetraploid cells at 800. Tetraploidy is considered when the G2/M peak is greater than 20% and an octaploid peak (representing G2/M) was present. Triploid was indicated by a shift in fluorescence area of the G0/G1 peak to between 130 and 160. Cases with suspected triploidy or tetraploidy were repeated..

2.1.7 Genotyping by Microsatellite Repeats Analysis

The parent-of-origin of a chromosome abnormality was determined by genotyping microsatellite repeats in the affected chromosome. Differences in repeat sizes can be detected by capillary electrophoresis after amplification with fluorescently-labelled primers. In one case of a 47,XXY infant, the origin of the X chromosomes was deduced from genotyping both parents and the child. Genomic DNA from the peripheral blood of the parents and the infant were used. Genotyping of the X chromosome was determined by using the following microsatellite regions: DXS987, DXS1226, DXS1068, DXS1106, DXS8055 and DXS1047. These repetitive and unique regions are also known as sequence tagged sites (STS). The primer sequences for each microsatellite region were obtained from the UniSTS database from the National Centre for Biotechnology Information (NCBI). In all primer sets, the forward primer was tagged with a fluorescein dye at the 5' end, either 6-FAM (6-carboxyfluorescein) or HEX (hexachlorofluorescein). Each STS was amplified by the standard protocol using HotStar Taq

polymerase (Qiagen, Venlo, Netherlands), for 35 total cycles, for a 25 μL reaction volume containing 200ng of genomic DNA. The annealing temperature used was dependent on the melting temperature of both primers. In this case, an annealing temperature of 53°C was used for all primers. Each STS was amplified in a separate PCR reaction. Amplification was verified by running 5μL of PCR product on a 1% agarose gel to determine appropriate band size using a 100bp ladder (Invitrogen).

The PCR products were run on an ABI 3130 genetic analyzer using the services of the CMMT/CFRI Bioanalyzer Core Facility. PCR products were submitted to the facility for analysis. Briefly, the products were prepared according to the manufacturer's instructions (Applied Biosystems). Similar to a standard band from electrophoresis, the data was represented in the form of fluorescence intensity and band size in base pairs. Differentially sized peaks represented heterozygosity, while the presence of a single peak represented homozygosity. By comparing peak sizes that were informative, where each parent had differently sized alleles, the origin of abnormality in the infant can be determined.

2.1.8 Statistical Analysis

Comparison of means (birth weight, gestational age, maternal age) between IVF and ICSI pregnancies were done using the Student's t-test. The chi-square test was used to compare categorical data, such as the prevalence of CPM in IVF and ICSI pregnancies. GraphPad Prism 5 was used to perform all statistical analyses (GraphPad Software, La Jolla, CA).

2.2 Results

2.2.1 Perinatal and Clinical Outcomes

In total, 54 placentas were analyzed for CPM (IVF n= 31; ICSI n=23). Twins accounted for 8 of the IVF cases, and 4 of the ICSI cases. In comparison of IVF singletons to ICSI singletons, there were no differences in maternal age, gestational age at birth, and birth weight (summarized in Table 2.1). In singletons, SGA was accounted for in 4 of 23 IVF (17.4%) and 2 of 19 ICSI (10.5%) pregnancies. In twins, SGA was detected in one set of IVF twins that also had maternal hypertension (2/8; 25.0%). Overall, the frequency of SGA for IVF and ICSI pregnancies combined is 14.8% (8/54). Between IVF and ICSI pregnancies, there were no significant differences in maternal age between twin and singleton pregnancies, respectively (t-test). When compared to IVF singletons, IVF twins had significantly lower birthweight and gestational age ($p=0.038$, $p=0.02$; respectively, t-test). ICSI twins were also significantly lower in birthweight than ICSI singletons ($p<0.001$, t-test).

Perinatal complications were reported in six cases (Table 2.2). With the exception of one twin SGA pregnancy with maternal hypertension, none of the other twin cases had any reported perinatal complications.

Table 2.1 Perinatal characteristics in IVF and ICSI conceptions.

Pregnancy	n	Mean maternal age (years ± SD)	Mean G.A at birth (weeks ± SD)	Mean birthweight (grams ± SD)
IVF singletons	23	36.4 ± 5.1	39.1 ± 1.6	3352.6 ± 581.2
IVF twins	8	35.0 ± 7.0	36.7 ± 2.3	2493.7 ± 351.1
ICSI singletons	19	34.6 ± 4.0	39.6 ± 1.5	3514.7 ± 449.1
ICSI twins	4	34.5 ± 0.71	35.5 ± 2.1	2413.8 ± 448.4

Table 2.2 Description of perinatal complications.

Complications	Conception
Heart malformation, trisomy 21	Singleton ICSI-AGA
Maternal bleeding at 6 months	Singleton ICSI-AGA
Transverse arrest	Singleton ICSI-AGA
Controlled gestational diabetes	Singleton ICSI-AGA
Spontaneous rupture of the membranes	Singleton IVF-AGA
Asymmetrical IUGR	Singleton IVF-SGA
Maternal hypertension	Twin IVF-SGA

2.2.2 Fetal chromosome abnormalities

In this data, two infants had abnormal karyoytpes: 47, XY+21 (Down syndrome) and 47,XXY (Klinefelter Syndrome). The infant with trisomy 21 was conceived by ICSI, with a birth weight of 2858g at 37 weeks of gestation, which was appropriate for gestational age. The origin of trisomy 21 was previously determined to be of maternal origin (unpublished). The age of this infant's mother was 37 years. The 47, XXY infant was conceived by IVF with a birthweight of 3782g at 40 weeks of gestation (also AGA). The age of the infant`s mother at the time of birth was 40 years. Therefore, the overall frequency of fetal chromosomal abnormalities is 3.7% (2/54).

2.2.3 Rate of CPM in IVF and ICSI pregnancies

In the results of the placentas collected in this study, one CPM case for chromosome 13 was identified (1/54), giving a rate of 1.85% (Table 2.3). To increase the statistical power, this data was combined with data that was previously produced in this lab using the same methods. This additional data consisted of 69 ICSI pregnancies, of which a subset was previously published (Minor et al. 2006) (Table 2.3). The combined incidence of CPM was increased, due to the addition of 3 ICSI pregnancies (4 out of 69 cases) for which CPM was detected, giving an

overall frequency of 3.73% (5 out of 134 cases) (Table 2.3). This frequency was not statistically different from data on natural conceptions (2.22%; 5 out of 255 cases) published in the literature (p=0.23; Chi-Square Test) (Amiel et al. 2002, Barrett et al. 2001, Robinson et al. 2010). Since IUGR is usually used interchangeably with SGA, the prevalence of CPM in SGA from IVF and ICSI pregnancies were compared to the published literature that reported on IUGR pregnancies (12/59, 20.3%) (Amiel et al. 2002, Barrett et al. 2001, Robinson et al. 2010). In the referenced data, the rate of CPM in IUGR pregnancies ranged from 15% to 50% (Amiel et al. 2002, Barrett et al. 2001, Robinson et al. 2010). The data from Robinson *et al.* accounted for 43 cases, where 4 of 43 (9.3%) were implicated as CPM (Robinson et al. 2010). Robinson *et al.* also defined IUGR as a birthweight of less than the 3rd percentile for gestational age or a birthweight of less than the 10th percentile plus the presence of pre-natal findings that were indicative of growth restriction (Robinson et al. 2010). However, Amiel *et al.* did not report their criteria used to characterize IUGR. Nonetheless, CPM was not detected in IVF-SGA or ICSI-SGA groups in the combined data; therefore, its prevalence would not be greater than in natural conceptions. It is also noteworthy that 3 of the 9 cases of SGA from Minor *et al.* were prenatally diagnosed as IUGR (2006).

Table 2.3 Rate of CPM in IVF and ICSI pregnancies.

Pregnancy outcome	Rate of CPM (%)				
	This study	Minor *et al.* 2006	Hatakeyama,2006 (unpublished)	Combined	Controls*
IVF	1/25 (4.0)	-	-	1/25 (4.0)	5/303 (1.65)
ICSI	0/21 (0.0)	3/42 (7.14)	1/28 (3.57)	4/91 (4.4)	
IVF/ ICSI	1/46 (2.17)	3/42 (7.14)	1/28 (3.57)	5/116 (4.31)	
IVF-SGA	0/6 (0.0)	-	-	0/6 (0.0)	12/59 (20.3)
ICSI-SGA	0/2 (0.0)	0/9 (0.0)	0/2 (0.0)	0/13 (0.0)	12/59 (20.3)
Total	1/54 (1.85)	3/51 (5.88)	1/30 (3.33)	5/135 (3.7)	5/225 (2.22)

*(Amiel et al. 2002, Barrett et al. 2001, Robinson et al. 2010)

2.2.4 Examination of a double trisomy in the placenta

One rare case of a double trisomy was observed in the placenta of a 47, XXY (Klinefelter syndrome) infant that was conceived by IVF. Both placental and fetal abnormalities were considered *de novo* as both parents had normal karyotypes. In addition, the father had normal semen parameters and the mother's age was 35 years at the time of pregnancy. The child was born at 40 weeks of gestation weighing in at 3782 grams, which was appropriate for gestational age. A karyotype of 47, XXY was initially detected in the umbilical cord blood, and later confirmed by a blood sample from the infant. In addition to the presence of XXY in all sampled sites of the placenta, a small area of the placenta also contained trisomy 13 (2 of 5 sites analyzed) (Figure 2.1). Thus this case was considered to have mosaic trisomy 13 confined to the placenta on an XXY background. Genotyping six microsatellite markers on the X chromosome of maternal, paternal, and infant DNA from peripheral blood showed that both X chromosomes were of maternal origin (Table 2.4). A loss of heterozygosity in the infant's X chromosomes suggests a maternal meiosis II error (Table 2.4). Furthermore, the infant was heterozygous at the distal ends of the X chromosomes, suggesting that two recombination events occurred during maternal meiosis (Figure 2.2).

2.2.5 Detection of polyploidy by Flow Cytometry

Flow cytometric analysis was performed in 41 of the 54 cases in this study. Of the 13 cases that were not analyzed for polyploidy, abnormalities were not detected by CGH. Of the 41 cases analyzed by flow cytometry, neither triploidy nor tetraploidy were detected. For each run, a known tetraploid sample was included. In data previously produced in this lab, 2 cases of

mosaic diploidy/ tetraploidy in the placentas were found in ICSI pregnancies (Minor et al. 2006). When all the datasets were combined, the overall rate of tetraploidy in ICSI pregnancies was 2/69 (2.9%).

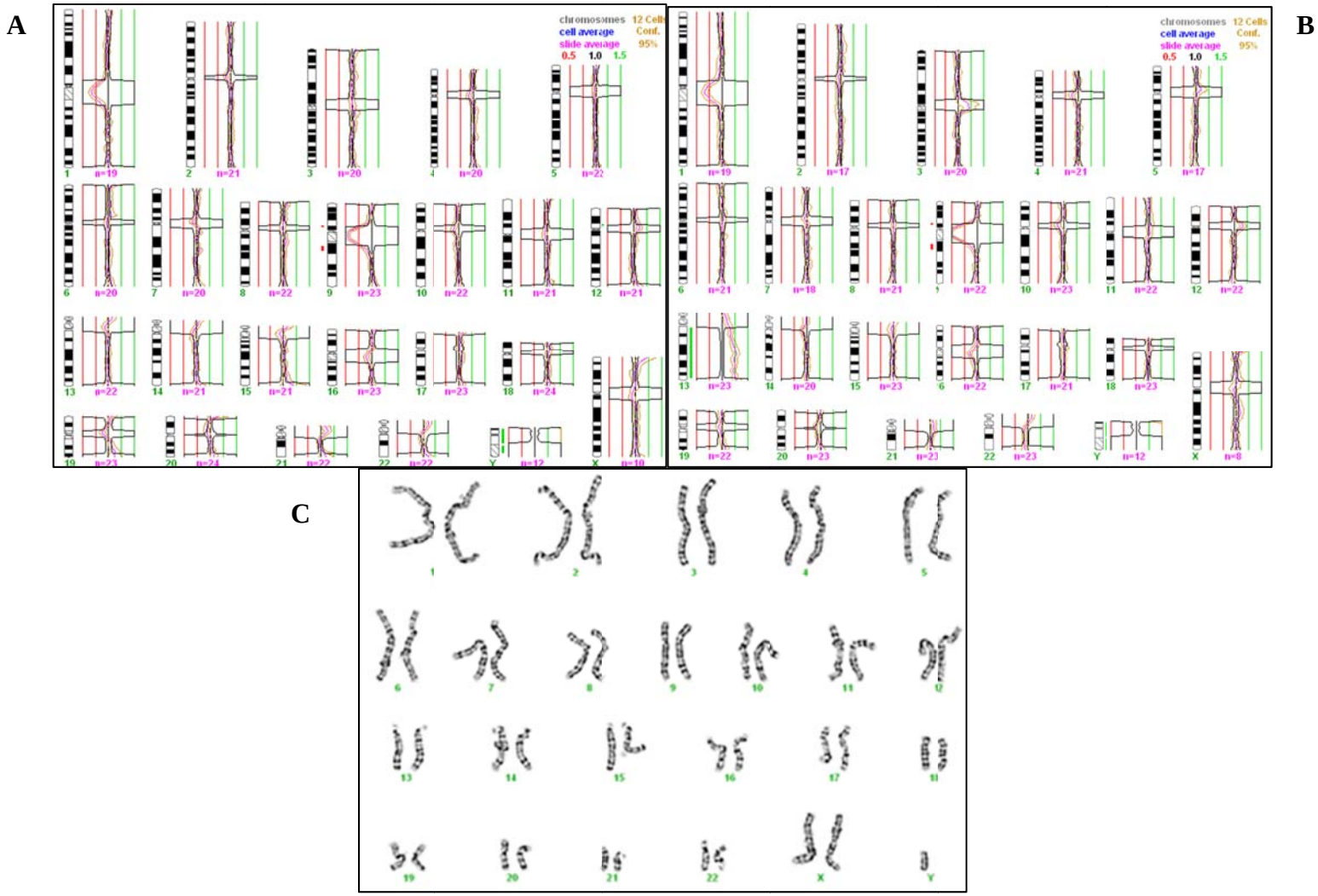

Figure 2.1 A) CGH profile of placental site showing gain of X (XXY); B) CGH profile of placental site showing gain of X and 13 (XXY+13); C) Karyotype of 47,XXY infant.

Table 2.4 Genotyping of an infant with 47, XXY

Marker	Location	Paternal allele (bp)	Infant alleles (bp)	Maternal allele (bp)	Origin
DXS987	Xp22.22	166	152, 166	152, 166	Uninformative
DXS1226	Xp22.1	199	214	214	Maternal
DXS1068	Xp11.4	255	250	250, 259	Maternal
DXS1106	Xq22.2	176	180	176, 180	Maternal
DXS8055	Xq23	158	158	158, 160	Uninformative
DXS1047	Xq26.1	199	199, 205	196, 205	Maternal

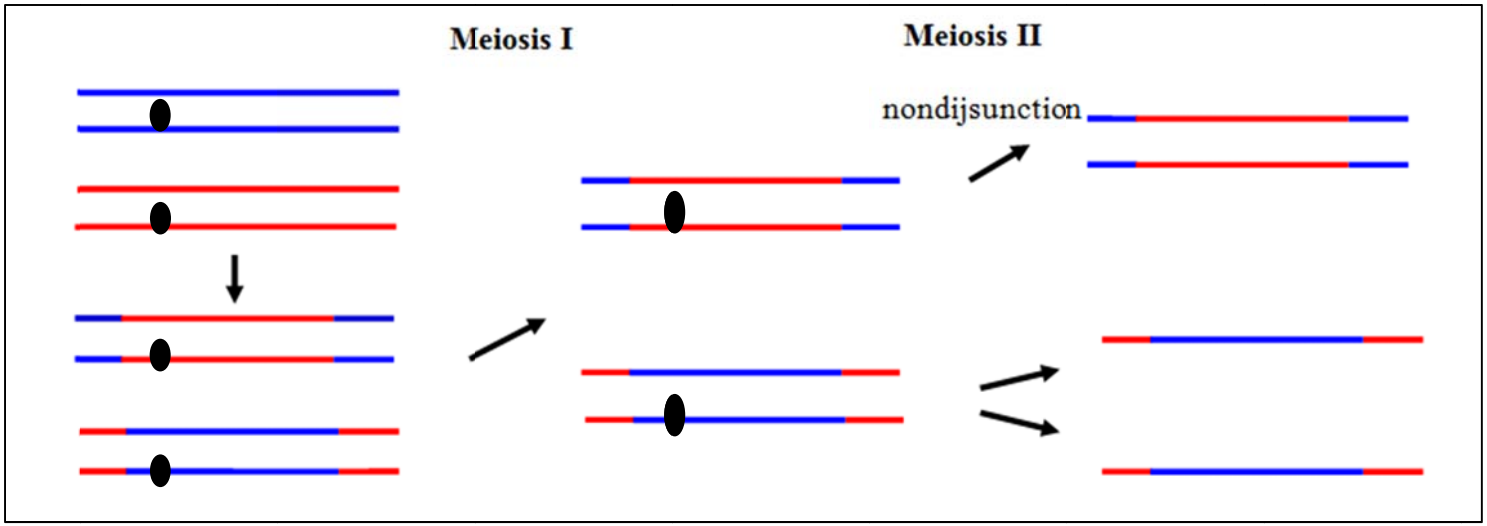

Figure 2.2. Diagram of meiosis II non-disjunction after double recombination

This figure is a hypothetical depiction of meiosis I in the maternal germ cell that led to the maternal origin of the X chromosome in the 47, XXY infant. The two replicated X chromosomes (blue and red) are aligned at metaphase of meiosis I. A double recombination event, which can involve one or both (as shown) sister chromatids, followed by nondisjunction at meiosis II is proposed to have occurred as suggested by the data on genotyping of the infant, and the infant's parents.

3. Objective 2: Investigation of *H19* and *IGF2* imprinting in the placenta

In this chapter, the methodology and results of objective 2 are discussed. DNA methylation at the imprinting control region 1 (ICR1) of *H19* and *IGF2* was investigated in placentas from IVF, ICSI, and natural conceptions. DNA methylation levels were compared in small for gestational age (SGA) and appropriate for gestational age (AGA) pregnancies. The methylation-sensitive single nucleotide primer extension assay was used (MS-SNuPE) was used to interrogate two CpG sites of the *H19* ICR1.

3.1 Methodology

3.1.1 Patient Ascertainment

Women that have conceived by IVF (n=32) or ICSI (n=45) were recruited from seven clinics across Canada. These cases include those used in objective 1 (Chapter 2). The majority of participants were recruited from the UBC Centre of Reproductive Health in Vancouver, British Columbia, Canada. Additionally, women that have conceived naturally (NC, n=12) were recruited from the BC Women's Hospital and Health Centre in Vancouver, which served as the controls for this study. The control placentas included appropriate for gestational age pregnancies that did not present any prenatal complications (grouped as IVF-AGA, ICSI-AGA, and NC-AGA), and intrauterine growth restricted pregnancies (grouped as IVF-SGA, ICSI-SGA, and NC-SGA). For each case, the whole placenta and a sample of umbilical cord blood were obtained post-delivery. The placenta was sampled for whole villi at two sites (~1-2cm^3 per site), and subjected to DNA extraction using standard salt-out methods. The umbilical cord blood was

cultured using standard cytogenetic techniques to determine the fetal karyotype by G-banding of metaphase lymphocytes.

Informed consent was obtained from all participants in this study. This study has been approved by the University of British Columbia Ethics Boards.

3.1.2 Definition of Small for Gestational Age (SGA)

Intrauterine growth restriction (IUGR) is most accurately diagnosed via ultrasound measurements during pregnancy. By definition, IUGR describes the failure of a fetus to reach its genetic growth potential. Alternatively, small for gestational age (SGA) is also used interchangeably with IUGR. In this study, we used SGA as an indicator of IUGR. SGA is defined as a birth weight below the 10^{th} percentile for a given gestational age (Greer 1998). This was determined for each subject using a reference chart from the Canadian Perinatal Surveillance System (Kramer et al. 2001). Each birth weight was converted to a Z-score: (patient value – expected mean)/ population SD. The Z-scores were used to determine SGA or appropriate for gestational age (AGA).

3.1.3 Methylation-Sensitive Single Nucleotide Primer Extension Assay (MS-SNuPE)

The MS-SNuPE assay has been shown to generate reproducible results without amplification biases for the ICR1 assay (Bourque et al. 2010, Sievers et al. 2005). Repeat analysis using distinct bisulfite conversions have also been shown to be correlated shown ($r=0.8$, $p<0.0001$) (Penaherrera et al. 2010). DNA from two placental sites for each case was analyzed for methylation at the *H19* ICR1 using the MS-SNuPE assay as previously described (Sievers et al. 2005). 300 to 500 ng of genomic DNA was bisulfite modified (DNA Methylation Gold,

Zymo Research, Orange, CA, USA). Each PCR reaction contained 1X PCR buffer (Invitrogen), 300nM of each primer, and 1U of Taq polymerase (Invitrogen Canada). The cycling conditions for both steps were 30 cycles at an annealing temperature of 61°C. The final PCR products were purified (DNA Clean & Concentrator-5, Zymo Research).

The Snapshot Multiplex Kit (Applied Biosystems, Foster City, CA, USA) was used for the single nucleotide primer extension of CpG site 10 and 12 of the ICR1 in two separate reactions. The manufacturer's instructions were followed and an annealing temperature of 52°C was used. C12 is contained in CTCF binding site 6, while the upstream C10 is outside of this boundary. Termination was performed by the addition of 1U of calf intestinal phosphatase (CIP) (Invitrogen). The products were analyzed by capillary electrophoresis on the ABI Prism 310 sequencer (Applied Biosystems). GeneScan was used to measure peak areas, representing the degree of methylation (Applied Biosystems). Percent methylation was calculated using the mathematical formula: area of methylated peak/(area of methylated + unmethylated peaks) x 100. A positive control consisting of normally methylated sperm DNA was included in each batch of samples in each step of this experiment.

3.1.4 Statistical Analysis

One-way ANOVA was used to compare mean methylation values in all experimental and control groups. The Fisher's exact test was used to compare the frequency of cases showing hypomethylation. The Student's t test was used to compare the difference in methylation values between placental DNA and umbilical cord blood DNA. Statistical analyses were done using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

3.2 Results

3.2.1 Inter-placental DNA methylation values

Two CpG sites of the *H19* ICR1 were analyzed by MS-SNuPE in whole villi from two sites of each placenta. A correlation analysis comparing DNA methylation index between C10 and C12 at each placental site showed a significant deviation from zero ($r=0.70$, $p<0.0001$) (Figure 3.1). The intra-placenta mean methylation values between the two sites sampled were also correlated ($r=0.72$, $p<0.0001$). Given these results, subsequent analyses of DNA methylation comparing each patient group and control group were done using a combined mean of C10 and C12 at both placental sites.

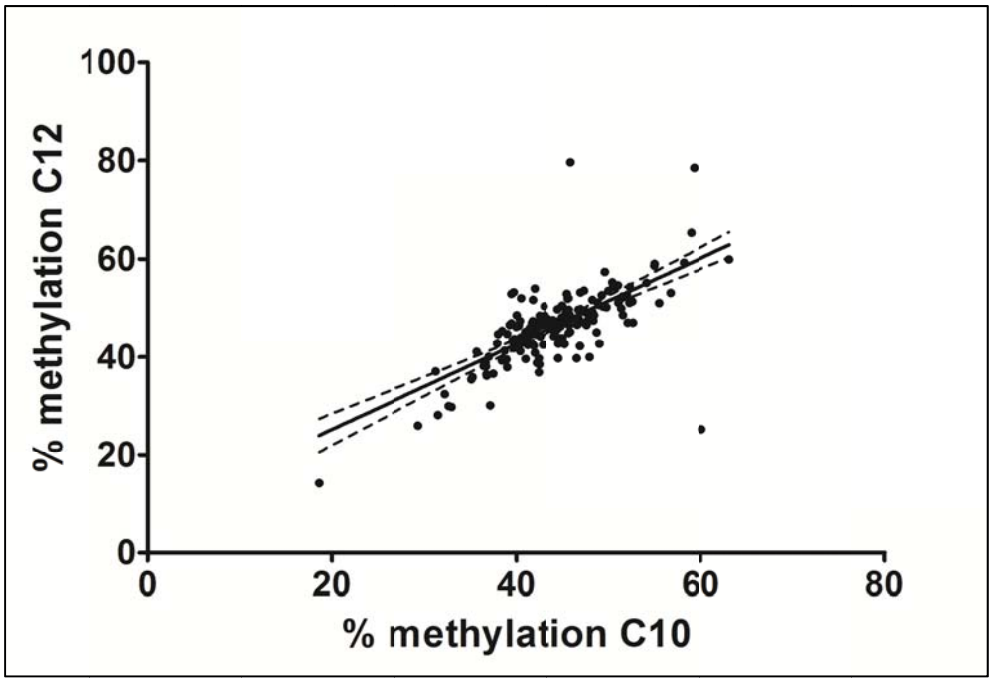

Figure 3.1 Correlation of percent methylation values measured at C10 and at C12 of the H19 ICR1.

Each point represents the value at C10 and C12 in of each sampled site of the placenta. The values at C10 and C12 showed a positive correlation ($r=0.70$, $p<0.0001$).

3.2.2 Mean methylation

The mean percent methylation in IVF-AGA (n=25), IVF-SGA (n=7), ICSI-AGA (n=32), and ICSI-SGA (n=13) groups were 45.52 ± 4.86, 47.25 ± 5.77, 45.64 ± 6.06, and 42.73 ± 4.39, respectively (mean ± SD) (Figure 3.2). The mean percent methylation in the control groups, NC-AGA (n=7) and NC-SGA (n=5), were 44.68 ± 4.18 and 44.63 ± 3.60, respectively (mean ± SD) (Figure 3.2). Using a one-way analysis of variance (ANOVA), no significant difference was detected in the multiple comparison of mean methylation values across all sample groups (p=0.49). In all samples, there were no significant correlation between mean methylation in the placenta and birth-weight corrected gestational age using the Z-score (r=-0.16, p=0.23). Thus, there were no significant differences in mean methylation at ICR1 in the placentas of pregnancies conceived by IVF, ICSI, and natural conception. In addition, placentas from SGA pregnancies in both test (IVF and ICSI) and control (NC) groups did not differ in mean methylation compared to those that were appropriate for gestational age.

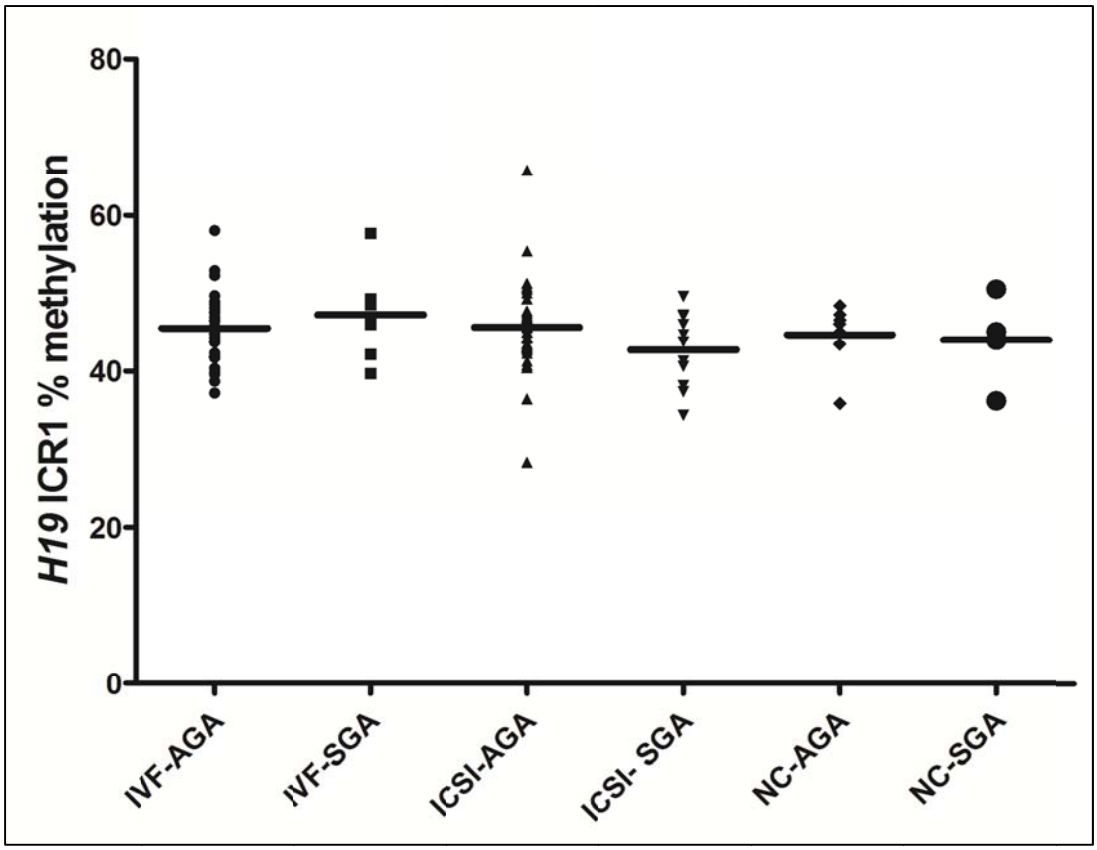

Figure 3.2 Percent methylation at H19 ICR1 in the placenta between pregnancy groups.

Each data point represents the mean methylation of C10 and C12 of the *H19* ICR1 in two sampled sties of one placenta. The mean methylation for each sample group is represented by the bar in each respective column. No significant differences in mean methylation between groups were observed (p=0.49).

3.2.3 Frequency of hypomethylation

Because no significant differences in mean methylation values were detected between assisted and natural conceptions, the methylation values of all samples were combined to generate a mean percentage value of 45.16 ± 6.83 (mean ± SD). Hypomethylation was then defined as a mean value, measured at C10 and C12, below 31.5% using a cut-off of 2 standard deviations below the mean. Using the same criteria, hypomethylation at each individual CpG was defined as below 31.7% for C10 and 33.2% for C12 when considered individually. For a normal distribution, two standard deviations from the mean would account for 95% of the

sampled population. One case with a mean methylation of less than 31.5% was observed from the ICSI-AGA group (28.41%, ICSI-AGA case 7, Table 3.1). However, hypomethylation in this case was confined to one placental site where both C10 and C12 loci were hypomethylated (18.63% and 14.32%, respectively) (Table 3.1). The other placental site assayed showed lower methylation than the mean value for the ICSI-NBW group (45.64 ± 6.06%; mean ± SD), but was not considered to be hypomethylated (37.91% and 42.77% for C10 and C12, respectively).

Although intraplacental methylation levels at both C10 and C12 were correlated, there were eight cases that had at least one CpG at one locus hypomethylated (Table 3.1). Hypomethylation at an individual CpG was not restricted to the IVF and ICSI cases (IVF-AGA, n =1; IVF-SGA, n=1; ICSI-AGA, n=1; ICSI-SGA, n=3), as it was also observed in natural conception cases (NC-AGA, n=1; NC-SGA, n=1) (Table 3.1). Of these cases, two showed hypomethylation at both C10 and C12 (ICSI-AGA case 3; ICSI-SGA case 7) (Table 3.1). However, this was only observed at one of the two placental sites that were analyzed for each case. One other case showed hypomethylation only at C12 in both placental sites analyzed (IVF-SGA case 2; 25.19% and 30.89%, respectively) (Table 3.1). The frequency of hypomethylation in ART (IVF and ICSI) placentas were not significantly greater than that of natural conception placentas ($p=0.24$, Fisher's exact test). Moreover, most could be considered as only moderately hypomethylated, with only one ICSI case exhibiting <20% methylation. However, the frequency of hypomethylation in each type of ART pregnancy was too low to conduct any meaningful statistical analysis.

Table 3.1 DNA hypomethylation at individual CpGs (C10 and C12) of the ICR1 at two sites of each placenta.

Hypomethylation at C10 and C12 is defined as a value below 31.7% and 33.2% methylation, respectively. Hypomethylation overall is defined as a value below 31.5% methylation. Hypomethylation was observed in all sample groups, IVF-AGA, IVF-SGA, ICSI-AGA, ICSI-SGA, NC-AGA, and NC-SGA. *hypomethylated.

Type of Conception	Site 1 (% methylation)		Site 2 (% methylation)		Mean (% methylation)
	C10	C12	C10	C12	
IVF-AGA (n=1)					
Case 17	31.28*	37.07	40.97	39.6	37.23
IVF-SGA (n=1)					
Case 2	37.48	25.19*	60.06	30.89*	39.38
ICSI-AGA (n=1)					
Case 7	37.91	42.77	18.63*	14.32*	28.41*
ICSI-SGA (n=3)					
Case 3	31.51*	28.13*	51.03	54.63	41.33
Case 4	36.69	38.1	32.98	29.77*	34.39
Case 7	29.37*	25.93*	46.4	47.77	37.37
NC-AGA (n=1)					
Case 3	42.44	38.5	32.68	29.97*	35.9
NC-SGA (n=1)					
Case 4	38.40	39.26	37.14	30.11*	36.23

3.2.4 Mean methylation in umbilical cord blood

In two cases where hypomethylation was detected in the placenta, the umbilical cord blood DNA was available for analysis. A similar mean methylation value was detected in the cord blood from the case ICSI-SGA case 7 (28.41%) as compared to the placenta (37.37%). In this case, lower methylation was not restricted to the placenta and may be present in other somatic cells of the infant. In another case (IVF-AGA case 17), methylation in the blood was higher than in the placenta (38.7% vs. 37.23%). Methylation analysis of umbilical cord blood

obtained from five other ART cases was available. There was no significant correlation between mean methylation values in the umbilical cord blood samples compared to the mean methylation values in the respective placental tissues ($r=0.54$, $p=0.21$) in 7 samples.

3.2.5 Clinical relevance

In both ART and NC placentas, pregnancy complications were noted and recorded at the time of delivery (Table 3.2). Of the NC-AGA group, in which we recruited patients that did not have any known prenatal complications at the time of consent, one was eventually diagnosed with polyhydramnios (NC-AGA case 7, Table 3.2). In the NC-SGA group, in which all patients were diagnosed as IUGR through ultrasound, one pregnancy was also diagnosed with severe pre-eclampsia in the mother. Placental anomalies were present in two ICSI-SGA cases (case 6 and case 7) that had velamentous cord insertions. ICSI-SGA case 7 had hypomethylation in the placenta (Table 3.1), while case 6 did not. Two cases in the ICSI-SGA, that were twin pregnancies, were diagnosed with marginal placenta previa. Furthermore, karyotyping of cord blood lymphocytes revealed one mosaic trisomy 21(46,XY/ 47, XY+21; 9/20) conception that was conceived by ICSI with a birth weight appropriate for gestational age. The infant was also diagnosed with a heart malformation post-delivery. The mean DNA methylation value at ICR1 in the placenta of this case was 43.28% which was similar to the mean of the ICSI-AGA group.

Table 3.2 Pregnancy complications and placental abnormalities in the study and control cases.

Type of Conception	Pregnancy / Placental Complication
ICSI-AGA case 8	47,XY +21; heart malformation
ICSI-AGA case 9	maternal bleeding at 6 months
ICSI-AGA case 10	transverse arrest
ICSI-AGA case 16	maternal cholestasis
ICSI-SGA case 4 and 5	marginal placental previa
ICSI-SGA case 6, case 7	velamentous cord insertion
IVF-AGA case 13	spontaneous rupture of the membranes
IVF-SGA case 2	asymmetrical IUGR
IVF-SGA case 19	cardiac anomaly
ICSI-AGA case 17	postpartum haemorrhage
ICSI-AGA case 23	diet-controlled gestational diabetes
NC-AGA case7	polyhydramnios
NC-SGA case 1	severe pre-eclampsia, severe asymmetrical IUGR

4. Conclusion

The results from chapter 3 suggest that IVF and ICSI pregnancies are not at a greater risk of confined placental mosaicism (CPM) or aberrant DNA methylation at the *H19* ICR1. The conclusion to each objective will be discussed here with a relevant account of the literature. The strengths and limitations of this thesis project are discussed with recommendations for future regarding pregnancies conceived by assisted reproductive technologies.

4.1 Conclusions of Objective 1

In this study, the prevalence of CPM in IVF and ICSI pregnancies (3.7%) was not significantly higher than that of published reports on natural conception pregnancies (2.22%) (p=0.23). The reference studies were selected based on their use of comparative genomic hybridization to ascertain CPM. CGH analysis provides a comprehensive investigation of the term placenta at multiple sites. Whereas in conventional CVS, where only one placental site is analyzed, it may not detect abnormalities confined to a small region of the placenta. Furthermore, CVS is an invasive procedure where only selected pregnancies, for reasons such as advanced maternal age, are involved. This may allow for selection bias towards pregnancies that are likely to have prenatal complications and chromosome abnormalities. In CPM detected by CVS of IVF and ICSI placentas, Jacod *et al.* reported no significant difference compared to natural conceptions (Jacod et al. 2008). The rate of CPM in IVF and ICSI pregnancies was 1.3% in 235 cases compared to a rate of 1.8% in 20,650 natural conceptions (OR 0.69, 95% CI 0.22-2.16) (Jacod et al. 2008). However, in 80 IVF cases, In't Veld *et al.* reported a higher rate of 6.2% of CPM (In't Veld et al. 1995). This higher incidence is suggested to be likely due to selection bias as specific counselling for IVF and ICSI pregnancies were involved (Jacod et al.

2008). Furthermore, it is likely that the reported rate of 6.2% by In't Veld *et al.* is not significantly higher than the rate observed by Jacod *et al.*, since the latter suggested that the confidence intervals overlap (Jacod et al. 2008).

One unusual case of CPM was observed in this study. In the placenta, a mosaic double trisomy was detected 47,XXY / 48,XXY +13, while the infant had a non-mosaic 47,XXY karyotype. The origin of the extra X chromosome was determined to be maternal, and the results suggest that a meiosis II non-disjunction occurred. Klinefelter syndrome is prevalent in 1 in 1000 males (Thomas and Hassold 2003). In approximately 50% of cases, the extra X chromosome is of maternal origin (Thomas and Hassold 2003). The results of genotyping microsatellite markers in this study suggest that recombination occurred at each distal end of the X chromosome. Normal recombination patterns in the sex chromosomes have been found in maternal meiosis II errors (Thomas and Hassold 2003). In CPM, double trisomy has been reported and was associated with IUGR (Wilkins-Haug et al. 1995). The origin of trisomy 13 in the placenta was not investigated in this study, but it is likely to be due to a post-zygotic event. Mosaic trisomy 13 was reported in one IVF-ICSI placenta through CVS (Jacod et al. 2008). In one case of non-mosaic XXY in the placenta, the fetus was diagnosed with IUGR (Amiel et al. 2002). Despite the presence of non-mosaic XXY and mosaic trisomy 13 in the placenta, it appears that placental function was not impaired as the child had a birth weight that was appropriate for gestational age (3782g at 40 weeks gestation).

Although CPM has been consistently associated with fetal growth restriction, it was not observed in this study using small for gestational age as an indicator of growth restriction (0/20 cases). This was contrary to the findings of Robinson *et al.* and Amiel *et al.* who reported a rate of 9.3% and 50%, respectively (Amiel et al. 2002, Robinson et al. 2010). One of the reasons that

CPM was not observed may be due to a relatively lower sample size. However, Amiel *et al.* had only 16 IUGR samples but found CPM in 8 samples (Amiel et al. 2002). The prevalence of IUGR in CPM cases was 61%, which was much higher than a previously reported rate of 20% (Lestou and Kalousek, 1998). This discrepancy may be due to selection bias in the study by Amiel *et al.;* however, they did not describe how the placentas were ascertained in their study. The rate of 9.3% reported by Robinson *et al.* may be more representative of the prevalence of CPM in IUGR pregnancies (Robinson et al. 2010). In this study, a selection criteria of using birth weight corrected for gestational age (<3rd percentile), or birthweight <10th percentile plus the presence of uterine artery notching, absent or reversed end diastolic velocity on umbilical artery ultrasound, or oligohydramnios (Robinson et al. 2010). This definition was different from the one used in this thesis, as SGA was defined as a birthweight of <10th percentile corrected for gender and plurality. These varying definitions of IUGR and SGA, used in this study and in the reported studies, may account for the discrepancies in the rate of CPM in growth restricted pregnancies. The use of a unified definition of SGA or IUGR should be considered for future studies.

To detect polyploidy, which has been described to occur in CPM, flow cytometry was used in this study.. Tetraploidy and triploidy were not detected in the original sample in this study (0/41). In Dr. Sai Ma's lab, tetraploidy was previously detected by Minor *et al.* in 2 of 44 ICSI placentas (Minor et al. 2006). In one case, tetraploidy was present in low levels in stroma (5.9-9.2%) and in trophoblast (2.2-4.7%) (Minor et al. 2006). In the second case, tetraploidy was present in higher levels in stroma (30.0-37.2%) and trophoblast (49.6-89.6%) (Minor et al. 2006). Also Dr. Sai Ma's lab, four cases of tetraploidy were detected by flow cytometry, but subsequently confirmed to be false-positive error (type I error) by fluorescent in-situ hybridization analysis (unpublished data). Other studies have also reported false positive results

by flow cytometry (Jones et al. 1991, Konchuba et al. 1992, Zbieranowski et al. 1993). The high rate of a false-positive result may be due to inadequate sample preparation resulting in the incomplete separation of the cell suspension into single cells. Furthermore, the presence of a higher G2 peak was only used to determine tetraploidy. In the samples used in this study, in addition to a higher G2 peak (>15% of total cell count) the presence of an octaploid peak was also required. The octaploid peak is representative of tetraploid cells at the G2 phase of the cell cycle. Therefore, the higher stringency in this study may explain the lack of a false-positive detection of polyploidy. Inthis study, a G2 peak that was greater than 20% of the total cell count was not observed. In the low level tetraploid case by Minor *et al.*, a G2 peak of 28.4% was observed (Minor et al. 2006). In samples that had a G2 peak measuring 15-20% of the total cell count, if tetraploidy was present it would only be at extremely low levels that may not have any significant clinical effects. Low levels of tetraploidy (approximately 30%) in the trophoblast has been associated with IUGR (Kalousek et al. 1991). High levels of tetraploidy in the trophoblast may have clinical significance, as it was associated with intrauterine death and perinatal death (Kennerknecht et al. 1993, Leschot et al. 1996, Wilkins-Haug et al. 2006). Therefore, the presence of tetraploid cells in the placenta may indirectly lead to disruption of normal fetal growth.

Overall, in combining the data from this study and those from the rest of Dr. Ma's lab, there was no significant association of IVF and ICSI pregnancies with CPM. CPM was also not detected in SGA pregnancies. These results suggest that the risk of CPM may not be greater in assisted conceptions. Investigation of the term placenta may be the most accurate method to detect CPM. This is because the placenta can be sampled at multiple sites post-delivery, while CVS only allows for sampling of one placental site due the procedure's invasiveness and its risk to the pregnancy. Despite this, there are relatively few studies of this design, which warrant

more investigations. Furthermore, the causes of restricted growth in IVF and ICSI pregnancies still remain unclear. Further studies into non-genetic causes, such as aberrant DNA methylation and environmental exposure may provide greater insight.

4.2 Conclusions of Objective 2

In objective 2, the role of aberrant DNA methylation in ART pregnancies was investigated. DNA methylation at the *H19* ICR1 in the placentas from IVF, ICSI, and natural conception pregnancies were compared. Furthermore, pregnancies that resulted in small for gestational age was compared to those of appropriate gestational age. Due to the concerns that the processes involved in ART and infertility itself are associated with epigenetic alterations, we expected that ART may affect proper DNA methylation in the products of conception (Li et al. 2005, Mann et al. 2004, Marques et al. 2008, Marques et al. 2010, Poplinski et al. 2010, Van der Auwera and D'Hooghe 2001). It has been suggested that aberrant DNA methylation can persist in the resulting conception (Kanber et al. 2009, Marques et al. 2008). However, in this study there were no differences in DNA methylation at the ICR1 in ART-conceived placentas compared to those of natural conception.

No differences in DNA methylation in SGA pregnancies conceived by IVF or ICSI were observed when compared to natural conceptions. Altered imprinting was also not observed in ICSI SGA pregnancies compared to term-born AGA pregnancies conceived by natural conception, for which six imprinted loci was investigated, including the *H19/IGF2* region (Kanber et al. 2009). In contrast, in natural conceptions, the *H19/IGF2* ICR1 was reported to be significantly less methylated in IUGR in the absence of pre-eclampsia placentas when compared to normal birth weight placentas (Bourque et al. 2010). In a separate study, SGA or IUGR

placentas showed decreased expression of *IGF2* and altered expression of other imprinted genes compared to control placentas, all conceived by natural conception (McMinn et al. 2006). However, in fetal blood, no differences in DNA methylation at *IGF2, GNASAS, INSIGF,* and *LEP* were observed between SGA and AGA pregnancies that were born pre-term and conceived by natural conception (Tobi et al. 2011). Despite one study showing no apparent association of ART SGA pregnancies with aberrant DNA methylation, it remains inconclusive as to whether a subset of SGA pregnancies conceived by ART or natural conception are due to alterations at imprinted regions. The contradicting results may be due to differences in the criteria used for fetal growth restriction, sample size and population demographics, use of different quantification methods of DNA methylation, and analysis of different regions of an imprinted gene. The use of a homogeneous study group and a large sample size may provide further evidence of the role of abnormal DNA methylation in growth restricted pregnancies conceived by ART and by natural conception.

It is unknown whether the diagnosed pregnancy complications in the ART pregnancies had any effect on DNA methylation at the ICR1. Velamentous cord insertion was observed in two ICSI-SGA placentas. It occurs in approximately 0.2 – 1.8% of singleton pregnancies (Monteagudo et al. 2000). There is a higher prevalence of abnormal placental cord insertion in singleton pregnancies from ART (Daniel et al. 1999). In twin pregnancies from ART, abnormal placental cord insertion carries a significantly greater risk of IUGR (Cai et al. 2006). It is possible that velamentous cord insertion had an effect on fetal growth in the two ICSI-SGA cases. Marginal placenta previa was noted in two other ICSI-SGA cases that belonged to a single twin pregnancy. The incidence of placenta previa is higher in ART pregnancies than natural conception; however, its association with growth restriction is not clear (Jackson et al. 2004, Ogueh et al. 2003, Romundstad et al. 2006). In the ICSI-AGA pregnancy where trisomy

21 was detected in umbilical cord blood and the placenta, DNA methylation at ICR1 in the placenta was not affected. ICR1 is located on chromosome 11 and there are no known imprinted genes on chromosome 21.

Hypomethylation, detected in 8 placentas, was present at either one of the two sites sampled or one of the two CpG sites investigated. Although methylation analysis of the hypomethylated sites were not repeated in this study, it has been shown in using independent bisulfite conversions a significant correlation ($r=0.80$, $p<0.0001$, $n=93$) between replicates was observed using the same MS-SNuPE technique (Penaherrera et al. 2010). In this study, hypomethylation occurred more often at C12 (7 of the 8 hypomethylated cases) than at C10 (3 of 8 hypomethylated cases). This suggests that C12 may be more labile to hypomethylation than C10. However, it is unknown whether hypomethylation at a single CpG significantly affects expression of the imprinted allele. . Methylation of the CTCF binding site prevents insulator function, while non-methylated CpGs allow for the insulator activity of CTCF (Schoenherr et al. 2003). It is unknown whether ablation of methylation at C11 on the paternal allele results in activation of CTCF binding and subsequent suppression of *IGF2*. Nonetheless, it has been postulated that the placenta is susceptible to epigenetic changes as compared to the fetus (Mann et al. 2004). This may also be a mechanism of adapting placental physiology to changes in bioavailability of nutrients during pregnancy.

No differences in DNA methylation were detected between ART-conceived and naturally-conceived placentas. A similar finding was recently reported, in which 10 imprinted regions including the *H19/IGF2* ICR1 was investigated in different tissues (Tierling et al. 2010). However, at *MEST*, significantly higher methylation was observed in both maternal and umbilical cord blood in IVF pregnancies (Tierling et al. 2010). Hypomethylation at KvDMR1, a

maternally methylated imprinting control region, was observed in peripheral blood samples in both IVF and ICSI pregnancies (Gomes et al. 2009). Using bead-array technology, differences in DNA methylation at imprinted and non-imprinted loci were detected in IVF placentas and cord blood (Katari et al. 2009). In some of these genes, significant differences in expression were detected (*CEBPA* and *COPG2* in cord blood; *SERPINF1* and *MEST* in placenta) (Katari et al. 2009). However, the sample size was limited to n=10 for IVF and n=13 for natural conceptions (Katari et al. 2009). More recently, aberrant DNA methylation at the *H19/IGF2* ICR1 was observed more frequently in ART conceptions than in natural conceptions (Turan et al. 2010). Concurrently, significantly lower expression of *IGF2* and *H19* were observed in ART placentas (Turan et al. 2010). However, this difference in expression cannot be accounted for by the DNA methylation status of the ICR1 (Turan et al. 2010). Overall, these studies, including ours, suggest that the effect of ART or infertility on DNA methylation at imprinted genes is inconclusive. It is unknown whether such variation in findings is a result of differences in study design. However, it is important for future studies to relate epigenetic status of imprinted genes with transcriptional activity, as this may determine whether variations in the epigenome bear any clinical significance. Continuous monitoring of imprinting abnormalities that arise in children conceived by ARTs will also be important for future studies.

4.3 Strengths and Limitations

At the time of the design of this study, we set out to design a prospective study of the placenta from IVF and ICSI pregnancies. The placenta was chosen as the tissue of interest because it serves important function in fetal growth and development. Another reason is that the majority of the placenta's makeup arises from the trophoblast cells of the blastocyst, and they are the first line of cells that become exposed to the *in vitro* environment during embryo culturing. In

combination with the reported high levels of mosaicism in human pre-implantation embryos from ART, and the vulnerability of mouse embryos to epigenetic change *in vitro*, these factors put the placenta at a greater risk of abnormal function. Therefore, the placenta was the tissue of choice in this study.

As discussed in Chapter 1, confined placental mosaicism is primarily detected prenatally by CVS.. The majority of studies on mosaicism in the placenta were designed as retrospective studies. Results from CVS were the main source of data collection. Although these studies allow for a very large sample size, there has always been a concern of selection bias. CVS and amniocentesis are invasive procedures that are mainly offered to patients who are at higher risks of an abnormal pregnancy outcome. Indicators for these prenatal diagnostic tests include advanced maternal age (> 35 years), familial history of genetic disease such as Down syndrome, and when one of the partners carry a structural or numerical chromosome abnormality. Although CVS data are generally reliable and are routinely performed in hospitals, these biases in selection must be taken into account. Thus, one of the strengths of this study is that the only inclusion criteria were infertile couples conceived by IVF or ICSI. As this study did not involve any additional health risks to the mother and fetus, prospective study subjects would be more inclined to participate.

Despite the prospective design of this study, limitations were also experienced throughout its course. Firstly, the sample sizes in both objectives were limited, especially in the number of cases for pregnancies affected by SGA. Although some studies reported in the literature also had limited sample sizes that were comparable to this study, a larger sample size would provide greater statistical power to detect a significant difference. Although an investigation of the term placenta provided a comprehensive analysis of CPM in this study, it is not without technical

limitations. Although the clinical significance of low-level mosaicism is unknown, it is safe to assume that it also depends on which chromosome is affected. The use of CGH in detecting low-level mosaicism is limited to about 10.8% to 30% mosaicism (Barrett et al. 2001, Minor et al. 2006). Thus, CGH is unlikely to detect mosaicism below 10%. Therefore, in this study, low level mosaicism in the placenta would not be detected.

In objective 2, the imprinting status of the *H19* ICR1 was investigated in the placenta. Bisulfite conversion coupled with the use of the single nucleotide primer extension assay allowed for DNA methylation at an individual CpG to be measured. At the time of this study, MS-SNuPE was an efficient and reliable method to investigate this region of interest. The limitation of this technique was that high-throughput analysis of multiple CpGs in the *H19* ICR1 would be time and cost consuming. Therefore, two CpG sites were only assayed in this region. It is unknown whether these two CpG sites would accurately represent DNA methylation status of the entire imprinting control region. However, we found a significant positive correlation between DNA methylation at the two CpG loci in this study. Another technique, pyrosequencing became available during the term of this study. It allows for the analysis of multiple CpGs in one reaction. In Dr. Ma's lab, pyrosequencing is currently being used to assay the *H19* ICR1 at four CpG sites. Assays for other imprinted genes and for global assessment of DNA methylation are being used on placental, blood, and sperm samples. The effects of *in vitro* culturing and infertility are known to affect the epigenome at multiple genes. In this study, only the imprinting of *H19* and *IGF2* was investigated. Therefore, future investigations of multiple loci should be done to provide a comprehensive analysis of epigenetic changes in the placenta induced by ARTs or other factors of infertility.

4.4 Overall conclusions and future directions

In this thesis project, confined placental mosaicism and aberrant *H19* ICR1 methylation were not independently found to be associated with IVF and ICSI pregnancies. Additionally, no association was observed in pregnancies affected by SGA. Although the overall sample size was adequate in both objectives, the number of cases affected by SGA was relatively low, which may account for the insignificant findings. Another explanation may be that the definition of SGA used in this study may be too lenient, as some studies have used a birthweight of less than the third percentile to characterize growth restriction. Nonetheless, these results confirm some of the recently published articles in the literature. Jacod *et al.* did not find a higher prevalence of CPM in IVF and ICSI pregnancies when compared to natural conceptions (Jacod et al. 2008). Tierling *et al.* has shown that ARTs are not associated with aberrant DNA methylation at imprinted genes (Tierling et al. 2010). However, Gomes *et al.* reported abnormal methylation at *KvDMR1* in pregnancies conceived by ART (Gomes et al. 2009). There are still many questions that have not been answered regarding the genetic and epigenetic effects of ART. The number of children conceived by ART is currently over two million. Further research into the impact of ART on the health of these children should be an important research priority.

In future studies, it is important that some of the limitations in this study be addressed. In addition to a larger sample size, a more stringent definition for growth restriction must be defined and used. Although SGA using a birth weight below the 10^{th} percentile may yield a larger sample size, a lower cut-off such as using the 5^{th} or 3^{rd} percentile may allow significant differences. Comparative genomic hybridization has been a dependable technology to identify gains and deletions of a chromosome. With the advent of newer technologies, CGH can be performed on a micro-array platform. Array CGH allows resolutions of 200bp in copy number

changes to be detected, which is much higher than traditional CGH (Urban et al. 2006). Although it is not known whether small genomic variants involving the placenta are clinically relevant, array CGH allows higher throughput analyses. CPM, depending on which type, can be present in mosaic proportions in the placenta. To adequately detect CPM, multiple placental sites need to be analyzed, which can be done with greater efficiency using array CGH.

DNA methylation at imprinted genes has become a new field of interest in clinical research on reproductive health, cancer and nutrition. In reproductive health, it is suggested that that epigenetic variation in the placenta are adaptive responses to changes in the uterine environment. This variation may account for the lack of significant findings in this study and those by others. However, how the placenta alters its epigenome as a form of adaption is not clearly understood. Close monitoring of basic studies in animal models and other human samples may shed light on this possible theory. In addition to the investigation of more imprinted genes that are involved in fetal growth, such as those described in Table 1.3, expression of these genes should also be investigated. Ultimately, it is expected that any meaningful epigenetic variability will alter gene expression. Although epigenetic variation in the placenta may serve a physiological purpose, it is unknown whether these changes are passed on to the developing fetus. This question can be addressed by investigating fetal tissues such as umbilical cord blood collected at post-delivery.

Overall, the long-term implication of these future studies may lead to the discovery of new diagnostic tools to address the higher prevalence of adverse health outcomes associated with assisted conceptions.

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